EphA4 and ephrin-A interactions in avian neural crest cell segmentation

A thesis submitted for the degree of Doctor of Philosophy

Edwina Ashby, B.Sc. (Hons.)

School of Molecular and Biomedical Sciences, Genetics discipline
Centre for the Molecular Genetics of Development
The University of Adelaide

November, 2004

Table of Contents

Table of Contents i

List of Figures vi

List of Tables and Graphs ix

Abstract x

Declaration xi

Acknowledgements xii

Chapter 1 – Cellular and molecular mechanisms driving avian peripheral nervous system development 1

1.1 General Introduction 1

1.2 Regulation of the early phase of NC cell formation and initiation of their migration 5

1.2.1 Induction of the NC 6

1.2.2 Regulation of migration onset 12

1.3 Cell/Tissue interactions dictating NC cell migration through the somite 28

1.3.1 Role of the somitic mesoderm in PNS segmentation 28

1.3.2 Factors regulating the dorso-ventral positioning and orientation of NC cell migration/ DRG formation and motor axons 37

1.4 Segmentally expressed molecules and their putative functional roles 39

1.4.1 PNA-binding glycoproteins and T-cadherin: general inhibitory molecules with multiple roles in trunk development? 41

1.4.2 CSPG's and their role in directing NC cell migration through the rostral somite 43

1.4.3 ECM molecules also have dynamic expression patterns coincident with NC cell migration 51

1.4.4 Eph receptors and their ligands, ephrins 54

1.5 Positive influences of ECM molecules on NC cell migration- creating a permissive environment 56

1.5.1 fibronectin (FN) and laminin 56

1.5.2 Collagen 58

1.5.3 Thrombospondin-1 59

1.6 Cellular basis of NC cell migration: *in vitro* and *in vivo* time-lapse analyses 59

1.6.1 In vitro studies of the adhesion mechanisms underlying NC cell motility 60

1.6.2 In vivo time-lapse analyses suggest NC cell-cell contacts coordinate their migration 61

1.7 Mechanisms of Eph/ephrin-A signalling 62

1.7.1 "Forward" and "reverse" signalling pathways 62

1.7.2 EphA4 contributes to cell-cell adhesion and motility mechanisms required for tissue morphogenesis 69

1.7.3 EphA4 and Rho 75

1.8 Benefits of using the chicken embryo for the investigation of NC cell development.... 77
1.9 Aims of this research 83

Chapter 2 – Materials and Methods 85

2.1 Materials 85
2.1.1 Antibiotics 85
2.1.2 Antibodies 85
2.1.3 Buffers and Solutions 87
2.1.4 Reagents and Enzymes 92
2.1.5 Kits 93
2.1.6 Plasmids 94
2.1.7 Oligonucleotides 95
2.2 Methods 96
2.2.1 DNA manipulation 96
2.2.2 Preparation of 1Kb Plus Ladder 96
2.2.3 Agarose gel electrophoresis 96
2.2.4 Purification of DNA from agarose gels 96
2.2.5 De-phosphorylation of vector DNA 96
2.2.6 Ligation 96
2.2.6 Isolation of RNA from chick tissue 97
2.2.7 RT-PCR 97
2.2.8 Amplification of chick Eph/ephrin genes for expression constructs using Pfu DNA polymerase 97
2.2.9 Electrocompetent cell generation 97
2.2.10 Bacterial transformation 98
2.2.11 Colony cracking 98
2.2.12 Plasmid DNA amplification/ purification 98
2.2.13 Automated sequencing 98
2.2.14 Isopropanol precipitation of sequencing reactions 99
2.2.15 Ringer's solution protocol 99
2.2.16 Embryo preparation and collection 99
2.2.17 In ovo electroporation technque 100
2.2.18 Neural tube culture 101
2.2.19 Vibratome Sectioning 102
2.2.20 Whole-embryo in-situ hybridisation 102
2.2.21 Neural tube culture immuno-histochemistry 104
2.2.22 Eph and ephrin immuno-histochemistry 104
2.2.23 Immunostaining of electroporated embryos 105
2.2.24 TUNEL reaction 105
2.2.25 Confocal microscopy 105
2.2.25 Quantification of in vivo phenotypes using in vitro culture model 106

Chapter 3 – Spatiotemporal patterns of expression of EphA4 and ephrin-A's during avian trunk PNS patterning 107

3.1 Introduction 107

3.2 Results 109

3.2.1 Complementary and overlapping distribution of A-subclass Eph and ephrins in the developing peripheral nervous system 109

3.2.2 In Situ Hybridisation analyses of ephrin-A5 and EphA4 during NC cell development 119

3.2.3 Dynamic distribution of ephrin-A5 and EphA4 as NC cells invade the rostral half-somite 127

3.2.4 EphA4 and ephrin-A5 show complementary patterns of distribution within the rostral somite during dorsal root gangliogenesis 135

3.2.5 EphA4 closely surrounds sensory axon trajectories as they sprout from DRG towards the CNS an also to meet the spinal motor neurons 139

3.3 Discussion 143

3.3.1 EphA4 is co-distributed with ephrin-A's on migratory NC cells at the initial phases of migration.. 143

3.3.2 EphA4 expression in the somite and possible mechanisms for neural crest cell segmentation 144

3.3.3 EphA4 and its potential role(s) in DRG formation/axon guidance 147

3.4 Conclusion 149

Chapter 4 – Mis-expression of EphA4 in the dorsal neural tube perturbs normal NC cell development 150

4.1 Introduction 150

4.2 Results 151

4.2.1 EphA4 over-expression inhibits the segmental migration of NC cells through the somite 151

4.2.2 Does EphA4 over-expression cause apoptosis? 152

4.2.3 EphA4/EGFP-positive cells aggregate in the neural tube and emigrate from its lateral surface 161

4.2.4 EphA4 over-expression in the dorsal neuroepithelium inhibits NC cell delamination 165

4.2.5 KiEphA4/EGFP expression alters the migratory characteristics and morphology of NC cells 173

4.2.6 KiEphA4/EGFP expression enhances the progression of neuroepithelial cells through EMT 181

4.3 Discussion 189

4.3.1 Altered EphA4 expression in delaminating NC cells perturbs NC cell migration: axial level - dependent effects? 189

4.3.3 Possible insights into EphA4-mediated NC cell guidance through the rostral half-somite 193

4.3.4 Role for EphA4 in regulating cellular interactions contributing to EMT 199

4.3.5 A possible in vivo role for EphA4 in regulating delamination and/or early NC cell migration 203

4.4 Conclusions 209

Chapter 5 – Analysis of EphA4 effects on NC cell development using an *in vitro* culture assay 210

5.1 Introduction 210

5.2 Results 215

5.2.1 Electroporation and culture of the neural tube recapitulates in vivo phenotypes at NT^{m} levels 215
5.2.2 Electroporation and culture of the neural tube recapitulates in vivo phenotypes at NT^{pm} levels 221
5.2.3 EphA4/EGFP over-expression in NT^{m} cultures leads to a localised inhibition of EMT 229
5.2.4 kiEphA4/EGFP expression in NT^{m} cultures leads to a less dense outgrowth.....233
5.2.4 Morphological differences between EphA4/EGFP and kiEphA4/EGFP cultures in the epithelioid layer.....234
5.2.5 kiEphA4/EGFP-expressing cells in NT^{m} cultures exhibit morphologies characteristic of increased substrate adhesions.....239
5.2.6 EphA4 regulates cytoskeletal re-arrangement during NC cell emigration from NT^{m} explants 243
5.2.7 Do cytoskeletal differences between EphA4/EGFP and kiEphA4/EGFP cells coincide with changes in cadherin/catenin-mediated adhesion?.....247
5.3 Discussion 255
5.3.1 Putative mechanisms to explain the loss of cell density in kiEphA4/EGFP-expressing NT^{m} explants 255
5.3.2 EphA4 over-expression suggests a role in promoting cadherin-dependent cell-cell adhesion in neuroepithelial cells.....258
5.3.3 A possible role for EphA4 in cross-modulation of cell adhesive and cytoskeletal changes during EMT 263
5.3.4 EphA4/EGFP-expressing cells appear unable to form the epithelioid cell state: insights into EphA4-mediated EMT.....265
5.3.5 Possible intracellular mediators of EphA4 effects on stress fibre morphology during EMT 271
5.4 Conclusions 273

Chapter 6 – Concluding Remarks..... 274

6.1 Summary of results..... 274
6.2 Putative targets for EphA4 signalling activity during EMT/NC cell migration 276
6.2.1. A role for EphA4 in cross-modulating cytoskeletal and adhesive changes during EMT 277
6.3 Possible upstream regulators of EphA4 activity: a role for noggin/BMP4 signalling? 278
6.4 What can be learned from EphA4/ephrin-A transgenic mice studies? 287
6.5 Conclusions 288

Appendices..... 291

Appendix 1.1 Summary of control EGFP-expressing embryos stained with HNK1 291
Appendix 1.2 Summary of EphA4-EGFP phenotypes *in vivo*..... 292
1.2.A Analysis of whole-mount EphA4/EGFP-expressing embryoswith HNK1 292
1.2.B Delamination profiles in EphA4/EGFP and control EGFP-expressing embryos (transverse sections) 293
Appendix 1.3 – TUNEL-positive nuclei in EphA4/EGFP-expressing and control EGFP-expressing transverse sections..... 295
Appendix 1.4 Summary of kiEphA4/EGFP-expressing phenotypes 296
1.4.A Whole-mount embryos stained with HNK1 296

1.4.B Transverse sections 296
Appendix 1.5 *In vitro* cell counts 298
1.5.A Proportion of GFP cells in outgrowth of control, EphA4 and kiEphA4 NT^m cultures 298
1.5.B Number of cells in outgrowth of EphA4/EGFP- expressing NT^m cultures 299
1.5.C Number of SoxE nuclei in outgrowth of kiEphA4 and control NT^m cultures 300
Reference List 301

List of Figures

Figure 1.1- Schematic diagram illustrating the eventual dorso-ventral and segmental arrangement of the PNS. 2
Figure 1.2- Schematic diagram summarising the events occurring during neurulation..... 6
Figure 1.3- Schematic diagram summarising experiments carried out by Newgreen et al., (1982) that demonstrated a change in cell-cell adhesion coincident with the developmental program of NC cells along the rostro-caudal axis. 14
Figure 1.4- Schematic diagram summarising the expression patterns of N-cadherin, cad6B and cad7 during NC cell delamination and migration..... 18
Figure 1.5- Schematic diagram summarising the role of protein phosphorylation/de-phosphorylation and cross-modulation of cell-cell, cell-ECM, and cytoskeletal changes in EMT events. 27
Figure 1.6- NC cell delamination is dynamically regulated by noggin-derived signals from the developing somites. 32
Figure 1.7- Summary of the remodelling of ECM components during the course of ventromedial NC cell migration. 48
Figure 1.8- Factors influencing NC cell trajectories *in vitro*. 64
Figure 1.9- Schematic diagram illustrating the structure and critical residues involved in (a) Eph or (b) ephrin-A or ephrin-B signalling pathways..... 66
Figure 1.10- Schematic diagram summarising downstream interactors of EphA proteins and the subsequent effects on cell-substratum adhesion/cytoskeletal extension. 72
Figure 1.11- Schematic diagram illustrating targeted electroporation of the dorsal quadrant of the neural tube and subsequent expression of EGFP on delaminating NC cells..... 80
Figure 3.1- Whole-mount EphA3-Fc and ephrin-A5-Fc screen in stage 17- 20 embryos reveal varying distribution patterns for ephrin-A and EphA proteins, respectively, in PNS segmentation..... 112
Figure 3.2- Ephrin-A3-Fc surrounds the DRG in transverse sections from the top 12 somites of stage 20 embryos..... 114
Figure 3.3- EphA3-Fc stains the DRG in transverse sections through rostral half-somites taken from the top 12 somites of stage 20 embryos. 116
Figure 3.4- *Ephrin-A5 in situ* hybridisation on stage 19 embryos indicates a strictly rostral half-somite expression pattern for *ephrin-A5*..... 120

Figure 3.5- *EphA4 in situ* hybridisation on stage 19 embryos reveals a dynamic expression pattern for *EphA4* within the rostral half-somite with regards to NC cell development. 124
Figure 3.6. Ephrin-A5 and EphA4 proteins are distributed in complementary domains within the rostral half-somite in the mid-trunk region of stage 17 embryos. 128
Figure 3.7- EphA4 is closely associated with ventromedially migrating NC cells during the early phases of migration into the rostral half-somite. 132
Figure 3.8- EphA4 is distributed around the DRG whilst ephrin-A5 is expressed in the DRG in the rostral half-somite at stage 20. 136
Figure 3.9- EphA4-positive cells in the dorsolateral sclerotome closely surround DRG axons in the rostral half-somite of stage 20 embryos. 140
Figure 4.1- Ectopic expression of EphA4 after 24 hours incubation inhibits NC cell segmentation in the mid-trunk region of stage 18-20 embryos. 154
Figure 4.2- EphA4-mediated inhibition of NC cell segmentation reduces the number of NC cells at the dorsomedial neural tube. 156
Figure 4.3- EphA4-mediated inhibition of NC cell migration is not due to apoptosis in EphA4/EGFP-expressing cells. 158
Figure 4.4- EphA4/EGFP-expressing cells delaminate from the pial surface of the neural tube. 162
Figure 4.5- EphA4 inhibits the delamination of NC cells and reduces basement membrane breakdown at dorsal neural tube. 166
Figure 4.6- EphA4 causes a cell-autonomous inhibition of NC cell delamination. 170
Figure 4.7- Reduced kinase-dependant activity of EphA4 promotes uncoordinated NC cell outgrowth. 174
Figure 4.8- kiEphA4/EGFP-expressing cells migrate in a coordinated fashion through the rostral half-somite at more anterior levels. 178
Figure 4.9- kiEphA4 expression at caudal regions of the embryo enhances NC cell formation. 182
Figure 4.10- kiEphA4 enhances the formation of mesenchymal cells at the expense of the neuroepithelium. 186
Figure 4.11- Proposed model to explain the phenotypes observed in NC cells over-expressing EphA4 and in NC cells with inhibited EphA4 signalling due to the expression of kiEphA4. 196
Figure 4.12- Expression of a truncated EphA4 construct, EphA4Δcyto, does not perturb segmental migration of NC cells. 206

Figure 5.1- Schematic representation of the *in vitro* model for NC cell generation. 212
Figure 5.2- Electroporation of EphA4 and kiEphA4 in NT^{m} cultures recapitulates *in vivo* phenotypes. 216
Figure 5.3- Over-expression of EphA4 in NT^{pm} cultures inhibits the release of EphA4/EGFP-positive cells from the neural tube epithelium. 222
Figure 5.4- kiEphA4/EGFP expression in NT^{pm} cultures promotes the formation of mesenchymal cells. 226
Figure 5.5- EphA4 over-expression in neural $tube^{m}$ cultures results in a localised inhibition of EMT. 230
Figure 5.6- EphA4 and kiEphA4 NT^{m} cultures display distinct morphologies in the epithelioid layer. 236
Figure 5.7- kiEphA4/EGFP-expressing cells adopt abnormal square flattened morphologies indicative of enhanced cell-substrate adhesions. 240
Figure 5.8- Expression of EphA4/EGFP or kiEphA4/EGFP perturbs F-actin distribution during late-stage EMT. 244
Figure 5.9- β-catenin distribution is disrupted along with circumferential F-actin in the neuroepithelium of EphA4/EGFP-expressing NT^{m} cultures. 248
Figure 5.10- β-catenin distribution in the epithelioid layer of NT^{m} cultures is perturbed in cells over-expressing EphA4. 252
Figure 5.11- EphA4 over-expression has distinct effects on cell-cell adhesion. 260
Figure 5.12- Schematic diagram illustrating the possible intracellular mediators through which EphA4 might negatively regulate EMT in NC cells. 268
Figure 6.1. Schematic diagram summarising how EphA and EphB activation mediated by ephrin-A proteins provide a putative molecular switch that regulates NC cell EMT. ... 280

List of Tables and Graphs

Table 4.1- Summary of data collected from the caudal regions of stage 16-19 embryos stained with HNK1 and analysed for increased HNK1 distribution at the dorsal neuroepithelium on the electroporated side. 185

Graph 5.1- Proportion of EGFP cells in the outgrowth of NT^m cultures expressing EGFP (n = 8 explants), EphA4/EGFP (n = 7) or kiEphA4/EGFP (n = 8). Data presented as a pooled average of all counts for each experimental condition. Bars represent Standard Error of the Mean (EGFP: ± 2.5; EphA4/EGFP: ± 1.6; kiEphA4/EGFP: ± 4.7) 219

Table 5.1- Distribution of transfected (EGFP-positive) cells in neuroepithelial (NEp) versus Distal regions of NT^m cultures presented as a percentage of the total number of cells in any given region. 220

Graph 5.2- Mean number of cells (determined by phase contrast) in the outgrowth regions of EphA4 (n = 9) and control (n = 10) NT^m cultures. Error bars, EphA4: ±7.0; Control: ± 16.1 233

Graph 5.3- Mean number of Sox E nuclei in the outgrowth of kiEphA4 (n = 9) and control (n = 10) NT^m level cultures. The average level of expression in the neuroepithelium was 18.2% and 28.3% for controls and kiEphA4 respectively. Error bars, control: ± 17.7; kiEphA4: ± 7.5. 234

Abstract

The patterned migration of NC cells through the rostral half-somite contributes to the overall segmental arrangement of the peripheral nervous system. The Eph family of receptor tyrosine kinases and their interacting partners, the ephrins are involved in a vast array of morphogenetic processes during development including remodelling of the vasculature, axon guidance, and boundary formation. Functional blocking experiments have shown that Eph/ ephrin-B interactions are key mediators of neural crest cell segmentation. In addition, ephrin-A proteins have been described on the dorsal root ganglion and EphA7 in the caudal somite-half. Thus, the aim of this research was to further investigate the distribution of EphA/ ephrin-A proteins during peripheral nervous system segmentation in the trunk. Expression studies revealed that EphA4 and ephrin-A5 had a dynamic distribution with respect to neural crest cell migration, suggestive of a guidance role. Whilst ephrin-A5 was expressed on neural crest cells throughout their ventromedial migration, EphA4 was expressed both on and around neural crest cells during their intial migration into the rostral half-somite and then later defined neural crest avoidance zones in this tissue.

To test the possible role(s) of EphA4 in neural crest cell development, EphA4 was mis-expressed in the dorsal neural tube and subsequently on neural crest cells during their migration through the somite. Over-expression of EphA4 perturbed the segmental migration of neural crest cells, causing them to form aggregates at the dorso-medial neural tube. In the neural tube, EphA4 over-expression facilitated cell clustering and a non cell-autonomous inhibition of epithelio-mesenchymal-transition. Reduction of kinase-dependent EphA4 signalling enhanced neural crest cell motility leading to their premature emigration from the neural tube and migration into incorrect territories such as the caudal half-somite. Analysis of the cellular basis of EphA4 effects on NC cell EMT established that the cytoskeleton was a primary target for EphA4 activity during EMT. In addition, EphA4 over-expression produced a morphology indicative of enhanced cell-cell adhesions possibly through cadherin-mediated junctions. In summary, these data point to a novel role for EphA4 in negatively regulating the adhesive and/or cytoskeletal changes required for successful neural crest cell EMT. A model is suggested whereby the dynamic balance between ephrin-A-mediated signalling pathways, (that promote EMT) and EphA4-mediated regulation, (that inhibits EMT) provides a pivotal link between noggin/BMP4 activity and cellular changes required for delamination.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any other university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University library, being available for loan and photocopying.

Edwina Ashby
12th November 2004

Acknowledgements

So many people to thank as the last four years have been an amazing time in my life – both exhilarating and also very testing! I couldn't have done it without the support and guidance of so many people.

Firstly to the Genetics discipline (and the greater School for Molecular and Biomedical Sciences) and the Centre for Molecular Genetics of Development; in particular, thanks to the Richards and Saint labs, as well as Helli in the CMGD office, who have all provided a great support network and stimulated my learning during the course of my PhD. A special thanks to Tetyana for late night microscope advice and Cibo coffee! To members of the Koblar lab, past and present, especially to the chick girls: Saku, Agnes and Amanda, who have listened to all my hairbrain schemes and been great friends during my PhD.

To Velta – well I can honestly say I wouldn't be here today if it wasn't for you. Your continued friendship, support and ability to find the funnier side of life have kept me smiling. The long list of teaching incidents will be etched in my memory forever!

Joan, as my co-supervisor over the latter years of my PhD, you have added a well-needed strength and balance to all my decision-making processes (both science and non-science related)! Thanks also for being a great supporter and confidante. To my supervisor, Simon, thanks for encouraging me and giving me so much freedom. Also thanks for the opportunity to "discover" the wonder of chick embryos.

Three other key people who, over multiple phone calls, emails and lab visits, have enabled me to present the work in this thesis. Firstly, the late Toshiya Yamada who first demonstrated the technique of electroporation to me. His calm nature and wise approach to science was inspirational and will stay with me forever. Don Newgreen, who enabled me to work in his lab and provided critical feedback to this work. Finally to Cathy Krull, who has collaborated on this project from the outset and who has been a fantastic source of guidance, resources, and encouragement.

Thanks also to all the people who have housed me on my "science-related" travels including: Sinead and Chris in the US, Toschy in Cambridge, Alisa Poh in New York as well as Ben and Lisa and Kyrst and Dave.

To Amanda, thanks for being such a great friend and sounding board, I will miss all those afternoon "de-briefing" coffee sessions! Thanks for keeping me together in those moments when things were heading "pear-shaped".

A special thanks must go to Mum and Dad who at the end of the day, have believed in my passion for science and have been unerring in their support for my work. Also thanks to my sisters, extended family and friends who have all put up with me during my PhD!

Finally, to Dave, whom I love so much and, has been so patient with me and this "thesis" over the last year or so. Thanks for being my printing assistant and pushing me to finish. I know you thought you'd never see the back of it…well we did it!

Chapter 1 – Cellular and molecular mechanisms driving avian peripheral nervous system development

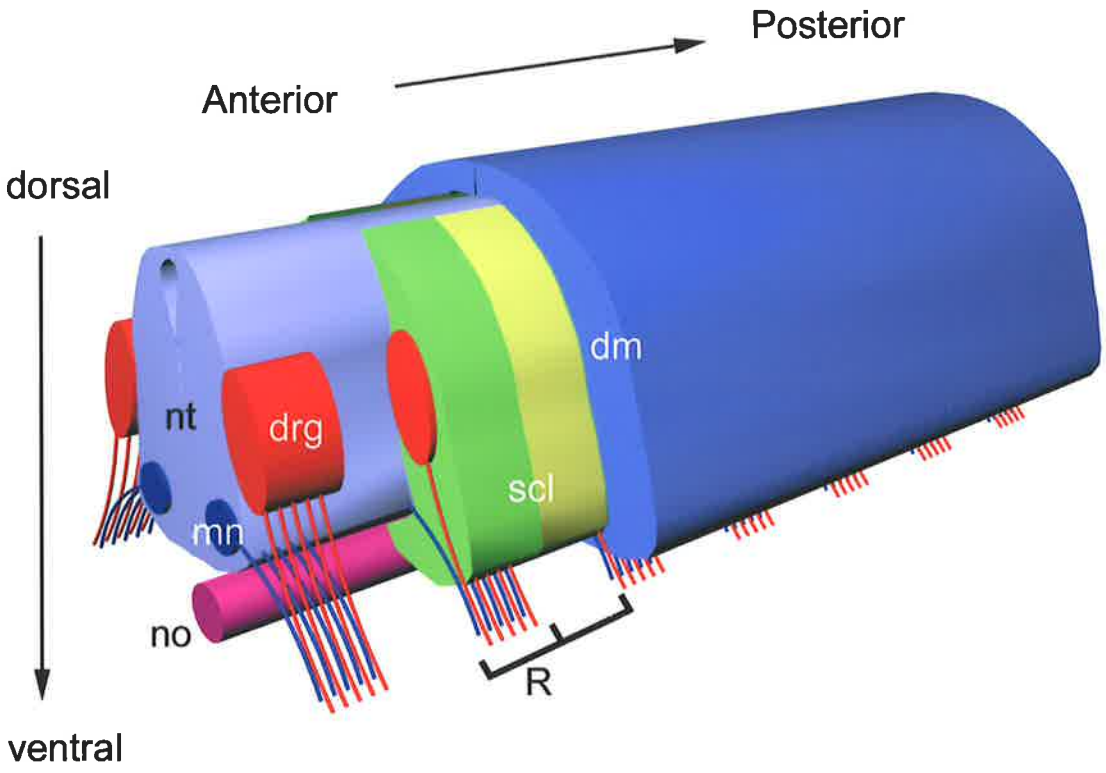
1.1 General Introduction

Patterning of the vertebrate embryo results in the formation of metameric structures, exemplified by the peripheral nervous system (PNS). PNS organisation is dictated by the metameric arrangement of mesodermal tissue or somites that lay adjacent to the neural tube (Tannahill et al., 2000). Somites bud off from the paraxial mesoderm in an antero-posterior wave along the embryo axis and exhibit an inherent rostro caudal polarity. Differences between the migration-supporting capacity of each of these halves then facilitates the selective growth of neural crest (NC) cells and spinal motor axons through the rostral half-somite and their exclusion from the caudal half-somite (Keynes and Stern, 1984; Loring and Erickson, 1987; Stern et al., 1991). Based on restricted expression patterns within the somite, many molecules have been implicated in guidance of cell/axon processes, thereby contributing to PNS segmentation (Krull, 1998). The aim of this review is to integrate current molecular information with the classical "cut and paste" approaches to embryology to gain a deeper insight into the known mechanisms driving PNS segmentation. Although the review encompasses both the motor and sensory components of the PNS, a more specific focus is given to the development of trunk NC cells from their origin in the dorsal neural tube to their ventral migration to form spinal sensory ganglia.

Precise spatiotemporal regulation of neural crest and motor axon migration in the somitic environment results in a dorsally located Dorsal Root Ganglion (DRG) and ventromedially extending processes of spinal motor neurons within the rostral half of the somite (Keynes and Stern, 1984; Loring and Erickson, 1987, Figure 1.1; Newgreen et al., 1986; Rickmann et al., 1985). NC cells, the first PNS precursors to emigrate from the neural tube, are induced in the dorsal neuroepithelium as a result of multiple inductive signals from the ectoderm and then undergo EMT, moving away from the neural tube (Christiansen et al., 2000). After delamination, NC cells travel along two major pathways: the ventromedial and dorsolateral and these specify their differentiation into different cell fates. NC cells on the ventromedial

Figure 1.1- Schematic diagram illustrating the eventual dorso-ventral and segmental arrangement of the PNS.

Cross-section of the chick neural tube illustrating the dorso-ventral and rostro-caudal arrangement of the dorsal root ganglion (drg, red), motoneurons (mn, blue), in the rostral half-somite (green) and avoiding the caudal half-somite (yellow) to create a metameric pattern of the PNS. nt, neural tube; dm, dermomyotome; no, notochord; R= rostral (rostral, green, caudal, yellow).


Posterior
Anterior
dorsal
dm
nt
drg
scl
mn
no
R
ventral

pathway form neurons, Schwann cells, glia and non-neural support cells whilst those on the dorsolateral become pigment cells (Le Douarin and Teillet, 1974; Weston, 1963). Following somite dissociation from a columnar epithelial ball to a multi-layered structure comprising dermomyotome (skin and muscle precursor) and sclerotome (cartilage and axial muscle precursor), NC cells travel ventrally through the rostral half-sclerotome avoiding the caudal-half (Bronner-Fraser, 1986a; Loring and Erickson, 1987; Rickmann et al., 1985). The exclusive ventral migration of NC cells through one half of the sclerotome generates a segmentally arranged dorsal root and sympathetic ganglia (Lallier and Bronner-Fraser, 1988; Teillet et al., 1987). Approximately 24 hours later, NC cells on the dorsolateral pathway travel non-segmentally between the ectoderm and somites (Le Douarin and Teillet, 1974). Motor axons exit the ventral neural tube and first invade the rostral half-sclerotome approximately 3-4 somite stages after neural crest invasion of the sclerotome, leading to segmentally arranged motor nerve branches (Koblar et al., 2000; Rickmann et al., 1985).

1.2 Regulation of the early phase of NC cell formation and initiation of their migration

NC cells arise from the lateral edge of the neural plate, contiguous with the epidermal ectoderm. At neurulation, the neural plate invaginates to form a cylindrical tube of neuroepithelium, creating a distinction between ectodermal and neural layers (Newgreen and Gibbins, 1982, Figure 1.2). Ultrastructural studies in the chick and mouse have established that NC cells can be distinguished from their neuroepithelial neighbours in the dorsal neural tube before their emergence along the dorsal cell surface (Sternberg and Kimber, 1986; Tosney, 1978). This can be discerned by the dorso-ventral re-orientation of neuroepithelial cells at the dorsal midline (where cells are preparing to exit the neuroepithelium), whilst those at the lateral edges remain oriented laterally, contiguous with the rest of the cells that comprise the columnar structure of the neural tube (Tosney, 1978). Coincident with their segregation from the surrounding neuroepithelium, NC cells lose specialised adherens junctions and their connections to the lumenal surface of the neural tube (Newgreen and Gibbins, 1982). Following this, cells become elongated, flat, and oriented tangentially to the neural tube, beginning their migration to multiple distant locations in the embryo (Le Douarin and Kalcheim, 1999). Since these initial observations were made, much work has been done to unravel the cellular and molecular basis underlying the induction of neuroepithelial cells to become NC cells, their subsequent transformation from an epithelial to mesenchymal cell

type, and the controlled dispersal of these cells to various locations. For the purposes of this section, recent advances have been divided into two distinct (and overlapping) components: firstly, mechanisms of induction i.e. a lineage specification event; and secondly, epithelio-mesenchymal transition (EMT) of neuroepithelial cells i.e. a morphogenetic event.

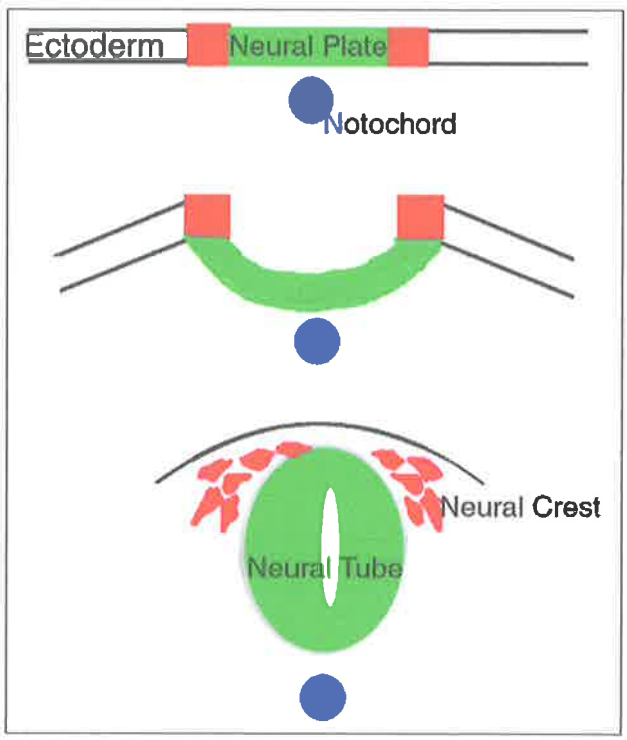


Figure 1.2- Schematic diagram summarising the events occurring during neurulation. The neural plate that is contiguous with the ectodermal layer invaginates and closes to form a cylindrical tube-like structure that is separate from the ectoderm. NC cells emigrate from the dorsal (red) portions of the neural tube. Image reproduced from CE Krull (personal communication).

1.2.1 Induction of the NC

Studies in both chick and *Xenopus* have established that NC cells arise in the dorsal neural tube as a result of interactions between the neural plate and non-neural ectoderm and that both of these cell types contribute to the NC population (Liem et al., 1995; Selleck and Bronner-Fraser, 1995). A number of families of growth factor molecules have been postulated to have a role in NC induction, including members of the bone morphogenetic protein (BMP) family, Wnt signalling factors, and fibroblast growth factor (FGF), in particular FGF-8 (Ikeya et al., 1997; LaBonne and Bronner-Fraser, 1998; Liem et al., 1995; Monsoro-Burq et al., 2003; Selleck and Bronner-Fraser, 1995). However, the mechanisms though which NC induction is

achieved appear to differ between *Xenopus* and chick based on recent studies suggesting that Wnt signalling molecules alone are sufficient to induce avian neural plate to form NC (Garcia-Castro et al., 2002). This is distinct from the two-step model proposed in *Xenopus* embryogenesis, whereby BMP inhibition is a precursor for FGF8 and Wnt-derived signals to induce the NC (LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2003).

More recently, a novel secreted glycoprotein, Noelin-1, has been postulated to have a role in regulating the competency of dorsal neural epithelial cells to become NC cells. It is expressed in the neural folds prior to *Slug* expression and in NC cells during their initial migration. (Slug is a transcription factor important in EMT, and is an important marker of NC induction; refer below). Prolonged expression of Noelin-1 by retroviral injection in chick embryos results in enhanced Slug expression and increased numbers of NC cells emigrating from the neural tube. This occurred independently of changes to expression of other known dorsal neuroepithelial markers, including Pax-3, Wnt-1 and BMP4, suggesting a role in modulating the competence of the neural tube to respond to inductive factors rather than patterning the neural epithelial tissue. This idea is further supported by experiments demonstrating that Noelin-1-expressing neural tube had an increased capacity to regenerate NC cells (in the absence of the neural folds) compared to controls, suggesting that its expression enhanced the temporal window of competence in the cranial neural tube (Barembaum et al., 2000). Thus, Noelin-1 is thought to be a potent regulator of NC cell formation in the cranial neural tube, acting to enhance the temporal window in which the neural tube can respond to inductive factors (Christiansen et al., 2000).

BMP4/Noggin-mediated control of the timing of NC induction/ EMT along the rostro-caudal axis

After neuroepithelial cells in the dorsal neural tube have been induced, they undergo an EMT and begin their migration. Studies in the avian trunk have demonstrated that control of the timing of EMT is determined by the coordinate expression of BMP4 and its antagonist, Noggin, in the dorsal neural tube. Both molecules are expressed in the dorsal neural tube, however BMP4 is expressed along the entire rostro-caudal axis whilst Noggin occurs in a high caudal to low rostral gradient; being down-regulated coincident with NC cell emigration (Sela-Donenfeld and Kalcheim, 1999). Given that BMPs in the ectoderm are capable of inducing NC cells and the expression of early NC cell differentiation markers, it was

hypothesised that the caudally increasing gradient of Noggin in the dorsal neural tube negatively regulates the action of BMP4 and thus, NC cell emigration.

Perturbation of the normal gradient of Noggin by ectopic application of *noggin*-expressing cells in the dorsal neural tube was sufficient to block crest emigration *in vivo*. *In vitro*, application of noggin and BMP4 proteins to neural tube explants from both the segmental plate and epithelial somite levels, had opposite effects on NC cell outgrowth (Sela-Donenfeld and Kalcheim, 1999). To correlate *in vitro* data with the expression patterns *in vivo*, Sela-Donenfield and Kalcheim (1999) showed that explants from epithelial somite levels, where noggin expression is low, were more sensitive to exogenous noggin protein than explants taken from segmental plate level (where noggin expression is high). Since follistatin, a known inhibitor of another BMP family member, BMP-7, was unable to reproduce similar effects to noggin, and BMP-2 is not expressed in the neural tube, it seems that interactions between BMP4 and its antagonist, noggin, are the key regulators of NC cell induction and/or EMT (Liem et al., 1995; Sela-Donenfeld and Kalcheim, 1999).

The role(s) of transcription factors in mediating NC cell induction

The likely targets for inductive signals include a range of transcription factors that initiate EMT via changes in dorsal neuroepithelial cell gene expression. The earliest marker known to be exclusively involved in NC cell specification is Slug or Snail, which are homologous zinc-finger transcription factors. In chick embryos, Slug inhibition by antisense oligonucleotides perturbs NC emigration in cranial regions (Nieto et al., 1994). Slug and Snail are implicated in many EMT-related processes both developmentally and in carcinoma progression (Nieto, 2002). Interestingly, in evolution, the two homologues seem to have swapped roles in NC generation, since in fish, amphibians and mammals, Snail seems to play the role that Slug takes in reptiles and birds (Nieto, 2002). Consistent with a role in NC cell specification, Slug is up-regulated in response to inductive signals such as BMP4 (Liu and Jessell, 1998) as well as FGF/Wnt signalling (Garcia-Castro et al., 2002). Interestingly, Snail has been shown to induce EMT in the primitive streak of gastrulating *Xenopus* embryos via repression of E-cadherin expression (Ciruna and Rossant, 2001), whilst Slug has been shown to act as a transcriptional repressor during NC formation in *Xenopus* (Nieto, 2002). Thus, it is possible that Slug and Snail might be acting through similar mechanisms of transcriptional repression to facilitate EMT.

Although the role of Slug in NC cell specification is well established at cranial and trunk levels, there are a number of lines of evidence suggesting that in the trunk, Slug may only be required for NC specification and not EMT. Firstly, expression analyses have shown that whilst *Slug* is expressed in both pre-migratory and migratory NC cells in cranial regions, it is only present in pre-migratory NC cells in the trunk (Sela-Donenfeld and Kalcheim, 1999). The lack of expression in migratory NC cells in the trunk implies that Slug activity is not required for EMT events in this population (Sela-Donenfeld and Kalcheim, 1999). To confirm these observations, over-expression of *Slug* in the neural tube caused precocious emigration of NC cells in cranial but not trunk regions (del Barrio and Nieto, 2002). It is a general phenomenon that cranial NC cells are more susceptible to inhibitors of NC cell-substrate interactions than the trunk (Bronner-Fraser, 1985; Bronner-Fraser, 1986b). Whether there is a causal link between these observations and possible differences in the downstream targets of Slug at cranial versus trunk regions is unknown. However, these data suggest that other transcription factors/ effector molecules in addition to Slug are required to induce EMT of the truncal NC cell population. This also points to NC induction and NC cell EMT being non-identical phenomena.

Other transcription factors that have been implicated in initiating the program of events required for NC cell specification include FoxD3 and Sox9 (Cheung and Briscoe, 2003; Dottori et al., 2001; Kos et al., 2001). *FoxD3* is expressed in pre-migratory and migratory NC cells, whilst Sox 9 is present exclusively in the precursor population in the dorsal neural tube (Cheung and Briscoe, 2003; Dottori et al., 2001; Kos et al., 2001). Over-expression of either of these genes in the neural tube causes neuroepithelial cells to express the migratory NC cell markers, HNK1 and cad7, indicative of these factors inducing a rapid and ubiquitous progression through EMT (Cheung and Briscoe, 2003; Kos et al., 2001). In the case of Sox9, the enhanced EMT appeared to occur through the normal pathway of induction (down stream of BMPs) as *Sox9* over-expression caused only a transient increase in the expression of pre-migratory crest markers, *Slug* and *cad6b*, at 6 hours post transfection (hpt) and this had disappeared completely by 24 hpt (Cheung and Briscoe, 2003). A possible explanation for the rapid down regulation is that Sox9 induced a ubiquitous progression of neuroepithelial cells through EMT, reducing the effective pool of NC cell precursors and subsequent pre-migratory crest (Cheung and Briscoe, 2003). Interestingly, Sox9 also enhanced Wnt 3a expression in the neuroepithelium at 6 and 12 hpt, suggesting that it may also (perhaps indirectly) facilitate proliferation of neuroepithelial cells, and reduce pre-migratory NC by inhibiting the further proliferative capacity of the surrounding neuroepithelium.

Although over-expression of either FoxD3 or Sox9 was able to facilitate NC cell induction, the pathways through which this occurred differed. Dottori et al., (2001) concluded that FoxD3 induced EMT via a Slug-independent pathway, as co-expression with Slug did not enhance the migratory phenotype (Dottori et al., 2001). However, given that the authors did not examine the effects of *FoxD3* expression alone on that of *Slug* at early time-points, (i.e. 6 and 12 hpt as per Cheung et al., 2003), it is possible that in a similar manner to Sox9, FoxD3 induced a transient up regulation of *Slug* that is rapidly turned off as cells proceed through EMT (and therefore not detected by 24 hpt). If this were the case *Slug* co-expression with *FoxD3* would not necessarily enhance the temporal onset of NC cell delamination, as predicted by Dottori et al., (2001). In addition, *Slug* expression is unable to induce EMT in the trunk (del Barrio and Nieto, 2002), confirming other reports suggesting that it is not involved in trunk NC cell EMT *per se* (Sela-Donenfeld and Kalcheim, 1999). Consistent with Slug acting downstream of Sox9 and FoxD3, the onset of their expression in the dorsal tips of the neural folds (in the trunk), precedes Slug (Cheung and Briscoe, 2003). Thus, despite earlier reports, it cannot be ruled out that Sox9 and FoxD3 are working through the same pathway to achieve NC cell induction, the first known detection of which is the expression of *Slug*. It is possible however that there may be a separate later role for FoxD3 that is Sox-9 independent, as *Sox9* over-expression did not induce *FoxD3* until 24 hpt (Cheung and Briscoe, 2003).

RhoB: A key effector molecule involved in NC cell EMT

To date, the one protein that has been shown to have a critical role in NC cell EMT is the GTP-binding protein, RhoB (Liu and Jessell, 1998). R*hoB* is expressed in the dorsal neural tube after neural tube closure and continues to be expressed in early migrating NC cells, and therefore has the correct spatio-temporal localisation for a role in NC cell EMT (Liu and Jessell, 1998). Given the role that Rho GTPases play in affecting cell movement, in particular through modulation of the actin cytoskeleton and stress fibre formation (Schmitz et al., 2000), it was hypothesised that RhoB was required for cytoskeletal extension during EMT. Consistent with such a role, inhibition of RhoB function *in vitro* using the C3 exotoxin, blocked NC cell EMT by inhibiting F-actin redistribution and subsequent mesenchymal morphology (Liu and Jessell, 1998). The actions of RhoB were specific to EMT, as inhibition did not effect specification (determined by *Slug* expression), or the motile properties of later migrating NC cells (Liu and Jessell, 1998). Furthermore, analysis of gene expression after BMP4-mediated induction, placed RhoB downstream of slug and cad6B in the temporal

hierarchy of gene expression required for NC cell EMT. Further support for RhoB-dependant EMT stems from Sela-Donenfield et al., (1999) who showed that over-expression of Noggin *in vitro* inhibited *RhoB* expression without affecting *Slug* (Sela-Donenfeld and Kalcheim, 1999). Thus, these data implicate RhoB exclusively in the EMT of NC cells, separate and downstream from BMP4-mediated induction.

Despite the central role that RhoB plays in NC cell EMT, there are a number of lines of evidence suggesting that NC cell EMT can occur in the absence of RhoB function. Firstly, though over-expression of *Sox9* largely induced EMT from the (RhoB-positive) dorsal neural tube, there was some emigration from the ventral and intermediate regions of the neural tube (Cheung and Briscoe, 2003). A similar phenomenon occurred in the case of *FoxD3* over-expression, leading the authors to conclude that FoxD3 induced RhoB-independent emigration of NC precursors (Dottori et al., 2001). These data, however are in contrast to that of Kos et al., (2001), who did not find any NC cell emigration from the lateral or ventral neural tube regions in response to FoxD3 over-expression but instead, found enhancement of HNK1 expression within the pseudo-stratified neuroepithelial structure, consistent with Sox9 (Kos et al., 2001). Finally, over-expression of Sox8, 9 and 10, all members of the SoxE group of HMG-box genes, has also been shown to elicit HNK-1 positive NC cell migration from all regions of the neural tube and therefore in the absence of rhoB up-regulation (McKeown et al., personal communication, 2003).

Thus, although discrepancies between the results yielded from transcription factor over-expression experiments could possibly be explained by differences in the methods of analysis (Cheung and Briscoe, 2003; Dottori et al., 2001; Kos et al., 2001), it cannot be ruled out that NC cell emigration might occur from neural tube regions where rhoB is not expressed. Possible mechanisms include via down-regulation of cad6B, or even N-cadherin; both of which are known to be important for maintaining epithelial junctions between cells of the neural tube (Nakagawa and Takeichi, 1998). Alternatively, other unidentified molecules might be involved; these might directly regulate Sox9/FoxD3 and therefore explain their effects, or alternatively could constitute an additional mechanism regulating NC cell EMT. Recently a screen for genes up regulated in NC induction has implicated multiple novel EMT effector molecules (Gammill and Bronner-Fraser, 2002), providing possible new insights into rhoB-independent mechanisms regulating EMT.

1.2.2 Regulation of migration onset

NC cell emigration correlates with the removal of a physical barrier, the basal lamina

Morphological analyses have suggested that at both cranial and trunk levels, emigration of NC cells correlates with the physical separation of basement membrane connecting the dorsal neural tube to the ectoderm (Newgreen et al., 1982). The neural tube is bounded by a basal lamina consisting of collagen type IV, fibronectin and laminin (Newgreen and Thiery, 1980; Sternberg and Kimber, 1986). TEM studies in both mouse and chick have shown that the basal lamina is discontinuous at the dorsal neural tube before NC cells have emerged, whilst at pre-migratory levels it remains fused with the epidermal ectoderm (Erickson and Weston, 1983; Newgreen and Gibbins, 1982; Tosney, 1978). Given that NC cells *in vivo* migrate away from the neural tube basal lamina (Ebendal, 1977) and the basal lamina is discontinuous in regions of extensive NC cell migration along the dorsal neural tube (Tosney, 1978), it was hypothesised that the breakdown of this structure might facilitate NC cell emigration (Newgreen et al., 1982). However, i*n vitro* analyses investigating the factors controlling the onset of NC cell emigration indicated that its precise timing was regulated independently than the presence or absence of basal lamina (Newgreen and Gibbins, 1982). Despite this, it remains discontinuous until the last NC cells have emerged, indicating that though it is not the stimulus for emigration, the absence of a continuous basement membrane is an integral component of NC cell EMT *in vivo* (Newgreen et al., 1982; Tosney, 1978).

The removal of the basement membrane appears to be more important for the emigration of cranial NC cells, whereby the appearance of NC cells coincides with basal lamina breakdown (Erickson and Perris, 1993). At this level, it has been suggested that Hyaluronic Acid (HA) synthesised by neural crest cells (and also the ectoderm) is responsible for creation of space between the neural and ectodermal layers (Greenberg and Pratt, 1977; Manasek and Cohen, 1977; Pratt et al., 1975), thereby facilitating NC cell emigration. *In vivo*, however, the contribution of HA to initial NC cell migration has not been validated as inhibition at cranial levels did not effect the emigration or patterned distribution of NC cells (Anderson and Meier, 1982). Interestingly, based on its expression patterns, it was postulated that the timed release/hydration of HA might facilitate the antero-posterior wave of NC cell emigration (Newgreen and Gibbins, 1982). Whilst it has since been shown that the gradient of effective BMP4 activity along the dorsal neural tube regulates the timing of NC cell EMT, the

molecular targets of this signalling pathway are as yet unknown (Sela-Donenfeld and Kalcheim, 1999). Perhaps the different mechanisms regulating cranial and truncal NC cell delamination events are underpinned by differences in the localisation/identity of downstream targets of BMP4/noggin signalling. Whether there is a direct interaction between HA and BMP4/noggin or a more indirect link remains to be tested; however, such a mechanism could provide a possible explanation for the lack of effect observed when HA activity was blocked (i.e. due to the persistence of upstream factors).

The role of cell-cell adhesion in regulating NC cell delamination

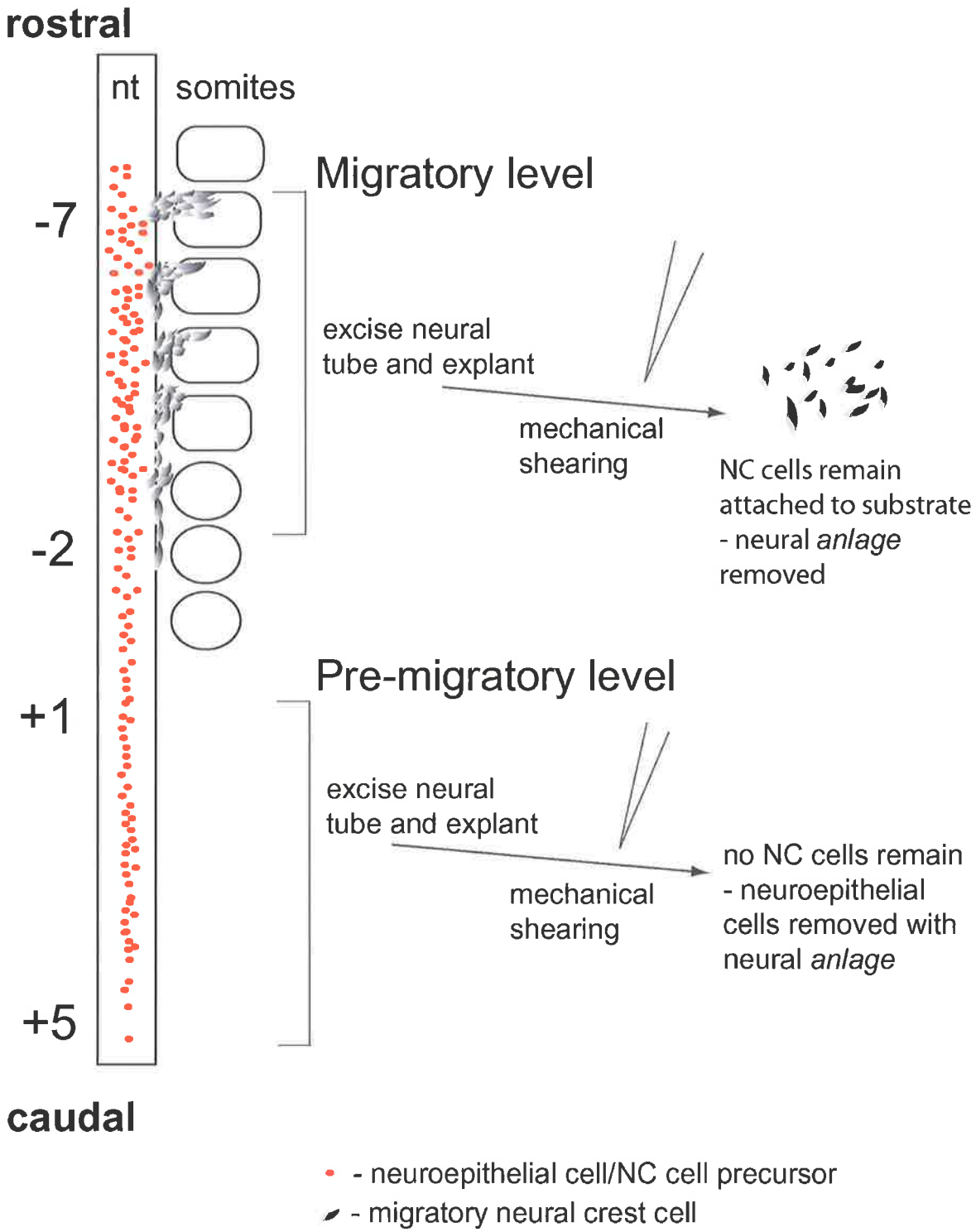
In vitro *studies on the role of calcium dependant cell-cell adhesion on the timing of NC cell emigration*

In vitro studies investigating the possible mechanisms controlling the timing of NC cell emigration from the neural tube have established that cell-cell adhesion within the neuroepithelium is a major component regulating the timing of EMT (Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985). Firstly, ultrastructural studies indicate that neuroepithelial cells are connected via specialised adherens junctions that are lost when cells begin to emigrate away from the neuroepithelium (Newgreen and Gibbins, 1982). Secondly, mechanical shearing assays *in vitro* indicate that development of NC cells from a pre-migratory to migratory cell type correlated with reduced cell-cell adhesion (Newgreen and Gibbins, 1982). This provided unequivocal evidence that a change in adhesiveness between NC cells coincided with their development into migratory cells, implicating a developmentally regulated adhesion system in controlling the timing of NC cell emigration *in vivo* (Figure 1.3), (Newgreen and Gibbins, 1982). Furthermore, exposure of neural explants from pre-migratory levels (and therefore high cell-cell adhesion) to calcium channel antagonists resulted in precocious emigration of NC cells (Newgreen and Gooday, 1985). Importantly, enhanced emigration was not due to cytoskeletal-based changes in cell spreading, as the neural anlagen began to disintegrate (indicative of a loss in cell-cell adhesion) when explants were subject to proteolysis in the absence of calcium (Newgreen and Gooday, 1985). Thus, a critical regulatory component of the onset of emigration stems from developmentally regulated changes in cell-cell adhesion between neuroepithelial cells, occurring in a calcium-dependant manner (Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985).

Figure 1.3- Schematic diagram summarising experiments carried out by Newgreen et al., (1982) that demonstrated a change in cell-cell adhesion coincident with the developmental program of NC cells along the rostro-caudal axis.

Neural tube indicated on left with the neuroepithelial precursors indicated in red and migratory NC cells in grey. Migratory level neural tube explants left a deposit of migratory cells on the plate after mechanical shearing whilst neuroepithelial cells from pre-migratory level explants were removed with the neural anlagen. This indicated that changes in the balance between cell-cell and cell-substrate attachment in neuroepithelial cells occurred coincident with their development to a migratory cell. Adapted from (Newgreen and Gooday, 1985).

St 13 (E2.5) embryo trunk



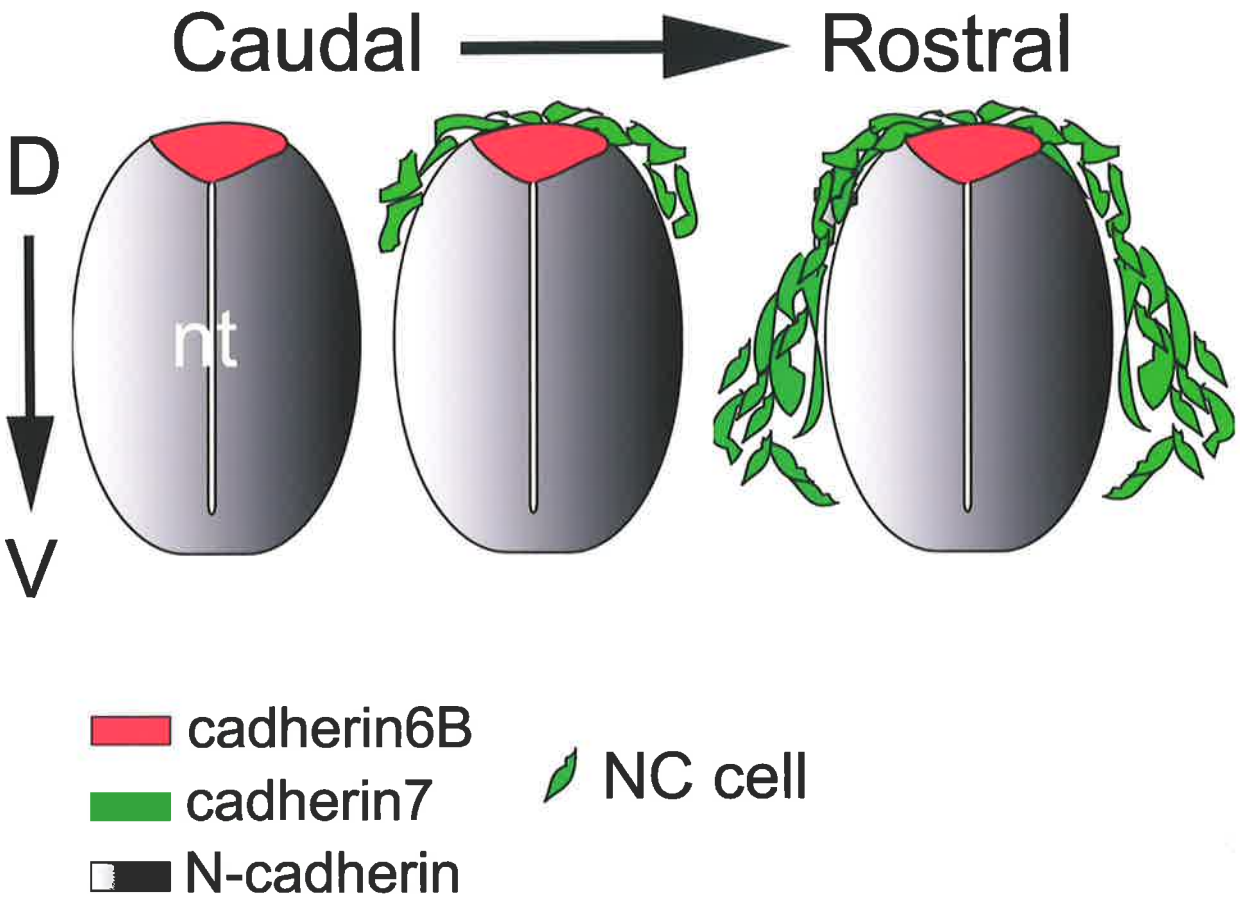
Distribution and function of cell-cell adhesion molecules during NC emigration

Based on expression and functional analyses *in vivo*, calcium-dependant cadherin and calcium-independent molecules have been implicated in controlling the onset of NC emigration. The cadherin family of cell-cell adhesion molecules have been shown to be involved in multiple adhesive events throughout tissue development, wound repair and tumourogenesis (Takeichi, 1988). Of the cadherin family, N-cadherin, cadherin6B (cad6B) and cadherin7 (cad7) have dynamic expression patterns in the dorsal neural tube and have been shown to directly regulate the loss of cell-cell adhesion required for NC emigration (Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998). In addition, the calcium-independent adhesion molecule, N-CAM is expressed in regions of the embryo relevant to epithelial morphogenesis including epithelialization of the somite and EMT in the dorsal neural tube (Duband et al., 1988; Thiery et al., 1982b).

N-cadherin is expressed on all cells of the ventral and lateral neural epithelium, being concentrated at the apical regions (Duband et al., 1988; Hatta et al., 1987; Hatta and Takeichi, 1986). Consistent with a role in regulating neuroepithelial cell adhesion, its expression is down regulated at the dorsal most aspect of the neural tube where NC cells originate, coincident with the loss of adherens junctions in neuroepithelial cells prior to migration (Newgreen and Gibbins, 1982; Tosney, 1978). In a similar manner, N-CAM is up regulated in the dorsal neuroepithelium; however, its expression is maintained on early migrating NC cells (Akitaya and Bronner-Fraser, 1992; Thiery et al., 1982b). N-cadherin and N-CAM is then re-expressed during condensation of the spinal ganglia, suggestive of a role in re-establishing strong cell-cell adhesive contacts at the end of migration (Akitaya and Bronner-Fraser, 1992). Thus, these two molecules have expression patterns that are intricately regulated with the initiation and cessation of NC cell migration (summarised in Figure 1.4).

Figure 1.4- Schematic diagram summarising the expression patterns of N-cadherin, cad6B and cad7 during NC cell delamination and migration.

Transverse view of the neural tube showing the distribution of cadherins during NC cell development (increasing from left to right). N-cadherin (grey) is expressed throughout the neural tube except at the dorsal midline where cadherin6B is expressed (pink). Cadherin7 (green) is up regulated coincident with NC cell emigration. Adapted from (Nakagawa and Takeichi, 1995).


Caudal
Rostral
D
V
nt
cadherin6B
cadherin7
N-cadherin
NC cell

In a similar way to N-cadherin, both cad6B and cad7 are expressed in a spatiotemporally regulated manner with regards to NC cell emigration (Nakagawa and Takeichi, 1995). *cad6B* is expressed in pre-migratory NC cells at the dorsal limit of the N-cadherin domain (Liu and Jessell, 1998). In contrast, *cad7* is expressed weakly at the apical surface of cells (those that have disrupted adherens junctions) in the dorsal neural tube and then strongly after NC cell detachment. Cad7 then remains on a sub-population of ventrally migrating NC cells (Liu and Jessell, 1998; Nakagawa and Takeichi, 1995).

Functional experiments *in vitro* have shown that disruption of cadherin-mediated adhesion acts to inhibit cell-cell contacts (Akitaya and Bronner-Fraser, 1992). This has been demonstrated *in vitro* in somite explants (Duband et al., 1987) with antibody blocking of N-cadherin and N-CAM (the latter phenotype being less penetrant), resulting in cellular detachment and dissociation of explants (Duband et al., 1987). On the other hand, over-expression of N-cadherin, cad7 and cad6B resulted in calcium-dependant adhesion and subsequent segregation of expressing cells into clusters of "like" molecules (Nakagawa and Takeichi, 1995). This hierarchy of adhesive contacts *in vitro* also translates to the *in vivo* situation, where developmentally distinct neuroepithelial cells are segregated based on their selective cadherin expression (Nakagawa and Takeichi, 1998).

Ectopic expression experiments *in vivo* have shown that N-cadherin and cad7 are both capable of inhibiting NC cell emigration. Over-expression in the dorsal neuroepithelium of stage 14 chick embryos resulted in the formation of ectopic aggregates of neuroepithelial cells at the dorsal neural tube and in some cases, an overflow into the lumen (Nakagawa and Takeichi, 1998). This effect was mediated by two possible mechanisms: firstly, elimination of the differential expression of cadherins in the neural tube could perturb their ability to change cell-cell adhesions or secondly, it could be through inhibition of mechanisms that would normally down-regulate cadherin-based adhesion (Nakagawa and Takeichi, 1998). Interestingly, the increased adhesion was found to be independent of ß-catenin, as expression of a construct that lacked the catenin-binding domain (but had a functional N-terminal domain) still caused a partial inhibition of NC cell emigration, whilst dominant-negative ß-catenin didn't have any effect (Nakagawa and Takeichi, 1998).

Despite the clear perturbation of NC cell emigration elicited by cadherin over-expression *in vivo*, blocking of N-CAM and N-cadherin with function-blocking antibodies did not elicit

precocious emigration from the neural tube in the trunk (though did do so in the cranial regions;(Bronner-Fraser et al., 1992)). There are a number of possible interpretations for this result. Firstly, it has been a general phenomenon *in vivo*, that NC cell emigration at trunk levels is less susceptible to inhibitors of cell-cell or cell-ECM interactions than cranial regions (Bronner-Fraser, 1993b). This could be explained by a reinforcement of cellular adhesion that occurs with neural tube maturity, particularly as in the head region, the levels of N-cadherin and N-CAM increase with age (Bronner-Fraser et al., 1992). Secondly, N-cadherin may be working in concert with cad7 and cad6b as well as other, as yet unidentified molecules, to coordinate neuroepithelial cell contact and their subsequent loosening (Bronner-Fraser, 1993a). Lastly, NC cell movement requires that the balance between cell-cell and cell-substrate adhesion is shifted towards the latter (Weston, 1970). Thus, environmental permissiveness and the competency of NC cells to acquire motility and adhere to the substrate are also likely to contribute to mechanisms controlling the timing of NC cell emigration.

Role of cell-ECM adhesions in NC emigration

Changes to the ECM coincident with the onset of NC cell migration

Temporal regulation of the onset of NC cell migration is therefore largely dependant on the intrinsic adhesive capabilities of pre-migratory crest cells, rather than a physical barrier to migration. Despite this, NC cells must be able to emigrate into an extracellular environment that is favourable for migration. Whether this involves changes in the migration-supporting potential of the matrix itself or alternatively in the ability of NC cells to interact with the matrix, it is a fundamental component of EMT.

Studies in the axolotl embryo have shown that NC cell emigration can be stimulated by matrix derived from more developed regions of the axis, and is therefore regulated by substrate permissiveness (Lofberg et al., 1985). The experiments utilised tissue from the epidermal ECM or solely the sub-epidermal ECM adsorbed onto microcarriers, grafted pieces from specific axial regions dorsally adjacent to the neural tube, and tested for migration-promoting capabilities. Grafting of either tissue resulted in a site-specific, contact-mediated stimulation of NC cell emigration, due to enhanced adhesion to the surrounding ECM (and not increased proliferation) (Lofberg et al., 1985). Though there was a correlation between increased quantities of ECM deposited and enhanced stimulatory behaviour of NC cells, Lofberg and co-workers (1985) were unable to pinpoint whether the effect was strictly

quantitative or determined by some qualitative change in the ECM that they were unable to detect.

Likely candidates mediating this effect might be inductive signals emanating from the epidermis, though the signals were not long-range as grafts from ventral or somite epidermis did not elicit a similarly precocious EMT (Lofberg et al., 1985). Later studies in the white mutant axolotl identified that a defective sub-epidermal matrix was the cause of reduced migration of pigment cell precursors (Lofberg et al., 1989). The nature of this defect has since been correlated to an altered biochemical composition and deposition of proteoglycans at the exact time when pigment cells require this substratum for migration (Perris, 1997). Thus, it was the combination of a delayed developmental program in the ECM and the acquisition of specific-interacting capacities of pigment cell precursors that resulted in their aberrant migration (Lofberg et al., 1989).

In comparison to the axolotl, TEM studies and antibody profiling in the trunk of the chick and mouse embryo have not found any such significant changes in the distribution of ECM components occurring coincident with the onset of NC cell emigration (Newgreen and Thiery, 1980). Despite this, there is an abundance of both highly adhesive ECM molecules such as fibronectin (FN), laminin, collagen I, IV and VI as well as non-adhesive molecules, such as proteoglycans, tenascin and hyaluronan that comprise the ECM adjacent to the neural tube at the time of NC cell emigration (Duband et al., 1986; Duband and Thiery, 1987; Krotoski et al., 1986; Mackie et al., 1988; Newgreen and Thiery, 1980; Newgreen et al., 1982; Perris et al., 1993; Pratt et al., 1975). Furthermore, *in vitro* studies analysing adhesion to extracellular materials at the onset of migration suggest that changing ECM molecules (or combinations thereof) does not affect the timing of NC emigration in either migratory or pre-migratory level explants (Delannet and Duband, 1992; Newgreen, 1982). Thus, although the ECM into which NC cells initially migrate is favourable for their migration due to the deposition of migration-promoting molecules, it is not thought to be the stimulus for migration (Newgreen and Gibbins, 1982).

Expression of substrate adhesion molecules on NC cells facilitates migration competency

An additional important consideration is that during EMT, NC cell precursors become competent to locomote via the acquisition of substrate adhesion receptors. The most direct evidence that enhanced substrate adhesion is an integral component of EMT comes from studies examining the adhesion profile of NC cells at the onset of their migration *in vitro* (Delannet and Duband, 1992). Taking advantage of the *in vitro* culture system established by Newgreen and colleagues, (1982), in which neural tubes taken from the last somites of an E2.5 embryo migrate within 2 hours of explantation (migratory levels), whilst those taken from the level of the segmental plate, experience some delay in their emigration (4-8 hours, pre-migratory levels; REFER Figure 1.3), Delannet and Duband, (1992) were able to assay for adhesive differences between cells from the two levels (Delannet and Duband, 1992; Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985). In cells of pre-migratory level neural tubes the transformation from a presumptive to migratory NC cell correlated strongly with an increase in substrate adhesion (Delannet and Duband, 1992). Given that no changes in the timing or order of NC cell emigration in either pre-migratory or migratory neural tubes occurred in the presence of different ECM substrates, the change in adhesive capacity was attributed to intrinsic changes occurring within pre-motile NC cells (Delannet and Duband, 1992).

Studies comparing the distribution of fibronectin (FN) receptors between stationary and motile NC cells have suggested that the switch from a stationary to migratory cell involves re-distribution of FN receptors from concentrated bundles that are tightly associated with microfilaments to a diffuse distribution around the membrane (maintaining loose connections with microfilaments) (Duband et al., 1986). The integrin β1 subunit has been shown to mimic the *in vivo* substratum in a similar manner to FN (Duband, 1991). Thus, a main focus in the search for receptors that might facilitate substratum adhesion of NC cells at the time of their emigration has been the integrin family of molecules (Erickson and Perris, 1993). Integrins are a large family of heterodimeric proteins, containing α and β sub-units that act as vital linking molecules between the extracellular matrix and intracellular proteins, and are essential in maintaining tissue integrity (Gumbiner, 1996).

Studies investigating the expression of various integrin components on migrating NC cells have revealed that there are at least 11 different heterodimers involved in NC cell migration

(Testaz et al., 1999). Furthermore, application of TGF-ß1 and TGF-ß2, known to facilitate up regulation of integrin molecules, elicited precocious emigration of NC cells from pre-migratory neural tubes (Delannet and Duband, 1992). Despite this, few integrin heterodimers have been implicated specifically in the process of NC cell delamination. For example, no differential distribution of the ß1 sub-unit of integrin was observed in the presence of the NC cell inducing molecules, TGF- ß1 and 2 (Delannet and Duband, 1992).

One integrin subunit that has been specifically involved in NC cell delamination is integrin α4 (Kil et al., 1998). Blocking of the α4 subunit in mouse neural tube explants inhibited NC cell emigration from the neural tube, suggesting a role for α4 specifically in this process, as well as in migration (Kil et al., 1998). Consistent with this, α4β1 integrin is up regulated in migratory NC cells compared to pre-migratory, making it an excellent candidate for the receptors up regulated in the presence of TGF-ß inducing molecules. Despite this, function-blocking antibodies did not prevent NC cell emigration *in vitro*, suggesting that it is not essential for delamination (Testaz and Duband, 2001). Further analysis of the individual contribution of specific heterodimers to NC cell emigration *in vivo* is complicated by the functional redundancy displayed by groups of integrin receptors (Delannet et al., 1994). Recently, however, conditional gene deletion of the β1 integrin sub-unit in NC cells lead to severe PNS segmentation defects suggesting an integral role for this sub-unit in PNS formation (Pietri et al., 2004). Thus, whilst *in vitro* studies have provided a functional link between NC cell induction and increased substrate adhesion (Delannet and Duband, 1992), the analysis of mice with particular integrin mutations specifically in NC cells will be the key to generating an integrative model for integrin function in EMT *in vivo*.

Regulation of NC cell EMT via protein phosphorylation

Studies utilising neural tube explant assays, that mimic the *in vivo* timetable of events leading to EMT, have shown that protein kinases (PKs) have an integral regulatory role (Minichiello et al., 1999; Newgreen and Minichiello, 1995; Newgreen and Minichiello, 1996). Inhibition of protein kinase activity by staurosporine resulted in the precocious emigration of NC precursors from the neural epithelium (Newgreen and Minichiello, 1995). Neural epithelial cells adopted a mesenchymal morphology and emigrated uniformly from both the dorsal and ventral neural tube regions after only 3 hours incubation with staurosporine. Utilising an antibody to N-cadherin (termed ACAM), cells still maintained strong ACAM-positive contacts suggesting that induced EMT primarily involved cytoskeletal changes leading to the

subsequent dispersion of cadherin complexes from adherens junctions (Newgreen and Minichiello, 1996). Initial tests using a process of elimination indicated that precocious EMT in the presence of staurosporine was mediated by protein kinase-C, a serine/threonine kinase (Newgreen and Minichiello, 1995). More recently, by testing various combinations of protein kinase modulators, it has been established that the atypical subclass of protein kinase-C is likely to be involved. Consistent with their having a regulatory role in protein phosphorylation during EMT, atypical isoforms λ and ζ are expressed in the dorsal neural tube at the apical borders of neuroepithelial cells prior to their emigration (Minichiello et al., 1999).

Given that the regulation of NC cell outgrowth *in vitro* occurs in the normal rostro-caudal gradient present *in vivo* (Newgreen and Minichiello, 1995), the observation that inhibition of protein phosphorylation caused a uniform, precocious emigration of NC cells suggests that staurosporine treatment was able to override the usual controls that regulate NC cell delamination. Likely possible targets of staurosporine include TGF-β-like molecules, as they are known to be serine/threonine kinases and have an essential function in NC cell induction (Delannet and Duband, 1992; Liem et al., 1995; Sela-Donenfeld and Kalcheim, 1999). The model underlying the effects of PK phosphorylation suggests that it principally affects cytoskeletal function, through such proteins as talin or vinculin, and the resultant balance of F-actin/G-actin in pre-migratory NC cells. Given the dependence of cadherin-based adhesion on cytoskeletal linkage at the adherens junctions, dispersion of F-actin would facilitate the dissolution of cadherin complexes, resulting in the observed loss of ACAM-positive junctions. Subsequent cytoskeletal extension and stress fibre formation would then enable the rapid transfer of ECM receptors to substratum adhesion sites at the cell periphery (Newgreen and Minichiello, 1996). In such a manner, whilst PKs act primarily on the cytoskeletal architecture, extensive cross-modulation between cell-cell, cell-ECM and cytoskeletal systems enables a precise, reversible control of EMT events, independent of genetic input (Figure 1.5), (Newgreen and Minichiello, 1996).

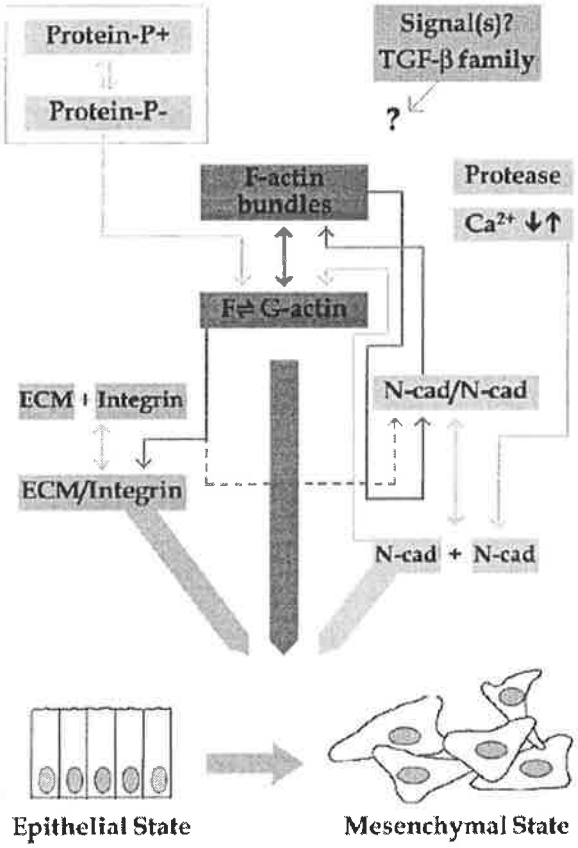


Figure 1.5- Schematic diagram summarising the role of protein phosphorylation/de-phosphorylation and cross-modulation of cell-cell, cell-ECM, and cytoskeletal changes in EMT events.

Image reproduced from (Newgreen and Minichiello, 1996).

Despite experiments to suggest that PKs act primarily on the cytoskeleton, a common consequence of reduced PK activity during either EMT or in motile cells is to enhance cellular adhesion, suggesting possible redundancies in the response to changes in PK activity. Similar studies have been conducted in motile cells investigating the influence of protein tyrosine kinases (PTKs)/phosphatases (PTPs) on NC cell motility (Brennan et al., 1999; Monier-Gavelle and Duband, 1995). Despite the slightly different cellular contexts, some interesting parallels can be made between the experiments in motile cells and those undergoing EMT that suggest similar functions in regulating the adhesive properties of NC cells. Firstly, in the staurosporine experiments, treatment elicited a general increase in cellular cohesiveness and speed of re-aggregation as well as ACAM stabilisation at cell junctions (Newgreen and Minichiello, 1996). Similarly, inhibition of PTK/PTP activity in motile cells enhanced the stability of ACAM-mediated junctions, causing a subsequent increase in cell-cell adhesion (Brennan et al., 1999; Monier-Gavelle and Duband, 1995). This increased

cohesion could be reversed by ACAM function-blocking antibodies in the PTK/PTP inhibition experiments but not for serine/threonine kinase-inhibited explants, suggesting that the primary mode of action for distinct kinase groups (and/or cell type) might differ (Monier-Gavelle and Duband, 1995). However, it is possible that a general response of NC cells (migratory or during EMT) to alteration in kinase activity is to enhance cellular cohesion between NC cells, suggesting a possible general mechanism for the action of PKs in NC cells (Brennan et al., 1999; Monier-Gavelle and Duband, 1995; Newgreen and Minichiello, 1996).

1.3 Cell/Tissue interactions dictating NC cell migration through the somite

The earliest studies into NC cell development utilised tritiated thymidine (Weston, 1963), and quail-chick chimaeric embryos (Le Douarin and Teillet, 1974), as well as injection of vital dyes to map the migratory routes of neural crest cells (Bronner-Fraser et al., 1991). Using these approaches, there was some controversy regarding the exact pathway for NC cell migration; Weston (1963) noted that NC cell migration was enhanced in the somitic environment, whilst others believed the main migratory pathway to be between the somites (Thiery et al., 1982a; Vincent and Thiery, 1984). More accurate analyses have since been achieved using immunohistochemistry with the monoclonal antibody, HNK1 (Rickmann et al., 1985). Studies using HNK1 have determined that the primary migratory pathway for both NC cell and motor axons is through the rostral portion of each somite with some initial NC cell migration occurring in the inter-somitic clefts (Bronner-Fraser, 1986a; Loring and Erickson, 1987; Rickmann et al., 1985; Teillet et al., 1987). Classical embryological experiments have found that the somitic mesoderm is the key patterning tissue that determines the ultimate trajectory of PNS progenitors through the rostral half-somite; additional guidance tissues include the notochord and dermomyotome. The various contributions of the multiple different cell and tissue interactions are discussed below.

1.3.1 Role of the somitic mesoderm in PNS segmentation

NC cells emigrate uniformly from the dorsal neural tube, however migration becomes segmental when cells reach the sclerotome tissue (Bronner-Fraser, 1986a; Loring and Erickson, 1987; Rickmann et al., 1985; Teillet et al., 1987). Microsurgical techniques involving rotation of the somitic mesoderm and neural tube 180 degrees have established that

segmental growth of NC cells is not intrinsic but imparted by molecules present in the somitic mesoderm (Bronner-Fraser and Stern, 1991; Keynes and Stern, 1984). Given that the segmentally-arranged DRG are derived from NC cells arising opposite both caudal and rostral somites, the question arises as to whether a single DRG comprises NC cells arising opposite multiple somites or exclusively opposite the somite in which the DRG forms? To address this question, quail-chick chimaera studies showed that NC cells migrate along the length of the neural tube to colonise DRG structure up to two somites away from the neural tube region of origin; thus any one DRG is composed of NC cells arising opposite both rostral and caudal halves possibly located somites away from their original site of emigration (Teillet et al., 1987). Thus, the migration-supporting capacity of the rostral half-somite, and the inhibitory properties of the caudal half-somite, determine the segmental arrangement of the PNS (Lallier and Bronner-Fraser, 1988; Teillet et al., 1987).

The experiments previously discussed point to the rostral-half somitic mesoderm acting as a permissive but not essential migration substrate for NC cells (Keynes et al., 1996; Tosney, 1988). Interestingly, Stern and Keynes, (1987) observed that placing multiple rostral somite halves opposite the neural tube resulted not only in an unsegmented outgrowth but also widened the migratory pathway taken by NC cells through this tissue (Stern and Keynes, 1987). Later studies focussing on the differentiation of NC cells into DRG, found that in the presence of compound rostral-half somites, giant unsegmented "polyganglia" formed compared to small dorsally located ganglia with caudal somites (Kalcheim and Teillet, 1989). These data both suggested that the rostral somite not only supports the migration of NC cells but also regulates the proliferative expansion of the DRG precursor population. Direct tests of DRG precursor proliferation suggested that in compound rostral half-somites, the rate of proliferation was increased, thereby supporting the idea that the rostral half-somite has mitogenic properties (Goldstein et al., 1990). There are two mechanisms through which the rostral half-somite might facilitate proliferation: the first is that rostral half-somite cells directly influence DRG cell proliferation; the second, that it merely provides a scaffold for NC cell attachment and thereby enhances NC cell exposure to central nervous system (CNS)-derived growth factors (Goldstein et al., 1995; Goldstein et al., 1990; Gvirtzman et al., 1992). Whilst results from grafting experiments suggest that the latter might be the case (refer below), a further discussion of how cells of the somite might facilitate proliferation is beyond the scope of this review.

Are all mesodermal tissues generally conducive to NC cell migration/DRG formation?

Microsurgical studies investigating how the rostral half-somite influences the eventual shape and size of the DRG have revealed that the rostral half-mesoderm has a specific role in regulating DRG size (Goldstein et al., 1995; Goldstein et al., 1990). Despite its role in regulating size, the process of DRG condensation appears to be in large part determined by NC cell intrinsic mechanisms rather than the somitic environment. This is supported by two key observations. Firstly, in the presence of multiple caudal somite halves, though DRG were reduced in size, they still formed compact ganglionic structures (Bronner-Fraser and Stern, 1991; Goldstein et al., 1990; Gvirtzman et al., 1992; Kalcheim and Teillet, 1989); secondly, the cell-cell adhesion molecules N-CAM and N-cadherin are both up regulated in NC cells during DRG condensation, and these have been shown to mediate cellular adhesion suggesting that alterations in NC cell-cell adhesion might be the stimulus for condensation (Akitaya and Bronner-Fraser, 1992; Lallier and Bronner-Fraser, 1988; Thiery et al., 1982b). Thus, if the rostral half-somite is dispensable for DRG condensation, the question arises as to whether this tissue has a specific role in NC cell guidance or is just part of a more generalised mechanism for mesodermal tissues in regulating cell migration?

During PNS development, trunk NC cells migrate through multiple different types of mesodermal tissues, however their migration is coordinated in a precise spatiotemporal manner. Grafting experiments have been carried out to determine the migration-supporting capacity of various different mesodermal tissues as well as artificial substrates (Bronner-Fraser and Stern, 1991; Gvirtzman et al., 1992). Replacement of the somitic mesoderm with segmental plate, intermediate or an artificial three dimensional collagen lattice did not support NC cell migration (Bronner-Fraser and Stern, 1991; Gvirtzman et al., 1992). This result was not surprising given that none of these tissues support NC cell migration *in vivo*, and *in vitro* collagen matrices promote NC cell movement only weakly compared to FN (Newgreen, 1982). In contrast, grafting of the lateral plate mesoderm replicated both the migration-favourable character of the rostral half-somite (though obviously did not impose segmentation) as well as the proliferative influence of this tissue on DRG cells (Bronner-Fraser and Stern, 1991; Gvirtzman et al., 1992). The latter result implied that the proliferative properties of the rostral half-somite occurred secondarily to a migration-promoting environment (Gvirtzman et al., 1992). To extend these observations to proliferative mechanisms regulating DRG size, it could then be postulated that the somitic mesoderm does not have a direct proliferative role but rather facilitates access of DRG precursors to mitogens

secreted from the neural tube (Bronner-Fraser and Stern, 1991; Goldstein et al., 1995; Gvirtzman et al., 1992). The lateral plate mesoderm is a tissue that is invaded by NC cells during their migration to the limbs, however this is also under strict temporal control. These data suggest that both the lateral plate and somitic mesoderm permit NC cell migration whilst other mesodermal tissues in the developing embryo do not. This leaves open the possibility that common molecular mechanisms might underlie migration permissiveness and these are discussed below.

Molecular basis for migration "permissiveness" of mesodermal tissues

Correlating the results from grafting experiments (Bronner-Fraser and Stern, 1991; Gvirtzman et al., 1992) with more recent molecular data profiling the expression of noggin and BMP4 in mesodermal tissues (Sela-Donenfeld and Kalcheim, 2000), suggest a possible novel molecular mechanism driving migration-promoting qualities of the mesoderm. It is well established that the gradient of noggin along the rostro-caudal axis of the dorsal neural tube regulates the effective amount of BMP4, thereby regulating NC cell delamination (Sela-Donenfeld and Kalcheim, 1999), (refer Section 1.2). More recently, expression studies and later functional analyses, suggested that noggin in the dermomyotomal lip (DML) is responsible for down-regulating its expression in the dorsal neural tube, thereby coordinating somite maturation with NC cell emigration (Sela-Donenfeld and Kalcheim, 2000), (Figure 1.6). Furthermore, surgical removal of differentiated somites resulted in the maintenance of *noggin* expression in the dorsal neural tube, blocking NC cell emigration. Thus, this could provide a molecular basis for the observed lack of NC cell emigration when segmental plate mesoderm was placed adjacent to the dorsal neural tube in the grafting experiments carried out by Bronner-Fraser and colleagues (1991) (Bronner-Fraser and Stern, 1991).

An additional line of evidence supporting noggin as the molecular basis driving the permissiveness of mesodermal tissues to NC cell migration is that it is also expressed in the medial edge of the lateral plate mesoderm at caudal levels of the embryo (Sela-Donenfeld and Kalcheim, 2000). Its expression becomes more medially located coincident with somite

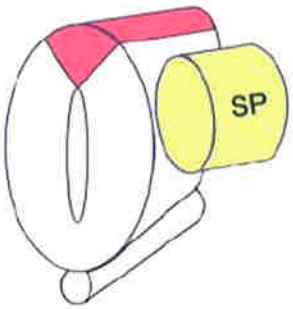
Figure 1.6- NC cell delamination is dynamically regulated by noggin-derived signals from the developing somites.

(A) Opposite segmental plate mesoderm, noggin expression is high in the dorsal neural tube, leading to inhibition of BMP4 activity and a subsequent inhibition of NC cell delamination.

(B) Opposite epithelial or differentiated somites, expression of noggin in the dorsomedial portion of the epithelial somite and subsequent dermomyotomal lip inhibits itself via an autoregulatory loop. Thus, noggin expression in the dorsal neural tube is decreased coincident with increasing somite maturity. A direct consequence of reduced noggin is increased BMP4 activity and subsequent NC cell delamination.

Adapted from (Sela-Donenfeld and Kalcheim, 2000).

A



High noggin in dorsal neural tube

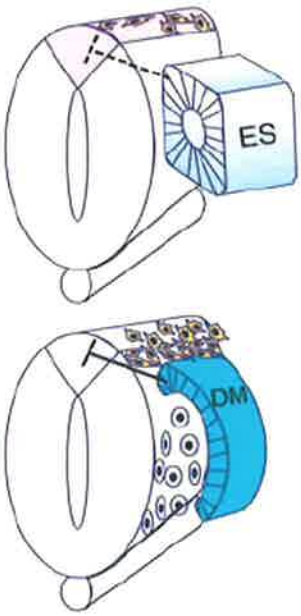
⊥

BMP4

⊥

NC cell delamination

B



low/no noggin in dorsal neural tube

↓

BMP4

↓

NC cell delamination

High noggin

Low noggin

no noggin

maturation and is directly regulated by BMP4 expressed initially in the intermediate mesoderm and then later in the neural tube of the developing embryo (Sela-Donenfeld and Kalcheim, 2002). Thus, if lateral plate mesoderm was transplanted adjacent to the neural tube as per (Bronner-Fraser and Stern, 1991), then it is possible that noggin-derived signals from the lateral plate may then down-regulate its expression in the neural tube and facilitate NC cell entry into the somitic environment. Similarly, the removal of somitic mesoderm and replacement with a collagen ECM would not stimulate significant NC cell migration (due to persistence of noggin signals in the dorsal neural tube in the absence of the DML) despite collagen acting as a known migration-promoting factor *in vitro* (Perris et al., 1991a). Thus, correlating results from early microsurgical experiments with more recent molecular data implicates a possible novel mechanism for coordination of NC cell development with somitogenesis mediated by noggin/BMP4 signalling in a more general mechanism for coordinating NC cell development with tissue morphogenesis.

Role of the dermomyotome in coordinating NC cell outgrowth

Studies involving the removal of the dorsal portion of the somite, the dermomyotome, have demonstrated that the ventral sclerotome is the key patterning tissue in determining the rostro-caudal, mediolateral and dorsoventral positioning of the PNS (Tosney, 1987). Despite this, Loring and Erickson (1987) observed that NC cells were in close association with the underneath surface of the dermomyotome (the myotomal basal lamina) during their initial migration, implicating this as an important migration substrate (Loring and Erickson, 1987). To determine the contribution of the myotome's basal lamina to the overall arrangement of NC migration along the ventromedial pathway, dermomyotome deletion experiments were carried out and the effect on NC cell trajectories analysed (Tosney et al., 1994). There were a number of key findings from these experiments that not only revealed a novel migration substrate for NC cells travelling ventromedially but also highlight the importance of detailed phenotypic analyses when determining subtle phenotypes. Firstly, in embryos where the myotome basal lamina was removed, no effects were observed on the gross organisation and positioning of the DRG within the rostral half-somite. However, comparison of the trajectories of NC cells at multiple precise levels of the trunk (with subsequent differing extents of NC cell migration) in the presence and absence of the basal lamina indicated that cells preferred this structure as a migration substrate than the sclerotome.

Further discussion of these results cannot take place without first considering the progression of somite development and how this is coordinated with NC cell migration. Breakdown of the epithelial somite and differentiation into sclerotome occurs in a lateral-medial direction such that at any one time, lateral sclerotome (near the notochord) is more mature than the medial regions (proximal to the neural tube). In addition, cells within the lateral sclerotome are quite loosely packed and therefore facilitate rapid NC cell migration, whilst the denser medial region is less favourable to invading NC cells (at least in the early phases) (Christ et al., 2000; Tosney et al., 1994). Contrary to the lateromedial direction of somite development, the dermomyotome (and subsequently, the myotome) differentiates in a mediolateral manner, such that at any one time the extent of basal lamina formation (underneath the developing dermomyotome) is most developed at the medial edge (Chen et al., 2004). Thus, when analysing the initial phases of migration, Tosney and colleagues (1994) found that NC cells entered the somite region only briefly, turned laterally upon contact with the medial sclerotome to contact the basal lamina and proceeded along this substrate until reaching the rapid migratory surface of the lateral sclerotome (Tosney et al., 1994). In the normal *in vivo* context, NC cells do not invade the medial sclerotome until moving as a mass of cells in more mature somites (Guillory and Bronner-Fraser, 1986). However, in the absence of the myotomal basal lamina, NC cells did not re-orient themselves to align laterally with this surface but instead entered directly into the medial portion of the sclerotome. These data suggested that whilst the myotomal basal lamina was not an essential migratory substrate, it was preferred over cells in the sclerotome (Tosney et al., 1994).

Whilst these experiments focussed on the single navigational trajectories of NC cells travelling through the ventral sclerotome, they can be re-interpreted in light of other experiments investigating the partitioning of NC cells into DRG and SG lineages (Gvirtzman et al., 1992; Tosney et al., 1994). Weston, (1963) observed that the first cells to exit the neural tube contributed to distal derivatives via a rapid migratory route through the lateral somitic mesoderm. Thus, the myotome may facilitate access of early migrating cells to the highly permissive lateral sclerotome and thereby influence the decision to contribute to DRG or SG lineages (Erickson and Perris, 1993; Tosney et al., 1994). In support of such a conclusion, data from compound somite experiments has suggested that an additional mechanism regulating DRG/SG partitioning is the medial sclerotome, such that in the presence of multiple rostral half-sclerotomes there was a bias towards NC cells contributing to DRG lineages when compared to SG. An explanation for these effects is that the medial portion of the rostral-half somite is unfavourable to NC cell migration and could act as a barrier to the

further ventral migration of NC cells. Thus, it would be interesting to re-examine the original dermomyotome/sclerotome ablation experiments conducted by (Tosney, 1987; Tosney, 1988) to determine if firstly, they influenced DRG/SG size (without altering gross morphology) and secondly, whether molecular cues expressed in the medial sclerotome could elicit such partitioning functions.

1.3.2 Factors regulating the dorso-ventral positioning and orientation of NC cell migration/ DRG formation and motor axons

Role of the neural tube

To determine the influence of the normal dorso-ventral orientation of the neural tube, on the organisation of NC cell migration into two distinct streams, the neural tube was removed and a donor neural tube inserted with the reversed dorso-ventral orientation (Stern et al., 1991; Weston, 1963). Despite their reversed dorsoventral (D-V) orientation, two migratory NC streams still formed; one towards the original dorsal side of the embryo (ventral in relation to the neural tube) and the other to ventral regions (dorsal with regards to neural tube orientation) (Weston, 1963). This suggested that the directionality and organisation of NC cell migration into two migratory streams was not under the control of the somitic environment but instead NC cells exploited the tissue spaces open to them. These results can be re-analysed in light of more recent molecular experiments describing the control of NC delamination and also directionality of migration and are discussed below.

Combining molecular data with those from neural tube inversion experiments some interesting comparisons can be drawn. Firstly, with regards to the timing of NC cell delamination from a re-oriented neural tube, it could be imagined that noggin diffusing from the DML would still be able to access the dorsal neural tube regions and facilitate NC cell exit, despite neuroepithelial cells being much further away from this structure than originally (Sela-Donenfeld and Kalcheim, 2000). Secondly, the data has interesting implications for the regulation of NC cell entry into the dorsolateral pathway as it has been postulated that their emigration is regulated by removal of proteoglycans from the region between the surface ectoderm and somite (Oakley et al., 1994). Given that cells on the dorsolateral path no longer directly face this migration pathway it implicates possible intrinsic mechanisms in coordinating the timing of dorsolaterally migrating NC cells.

In addition there are a number of key findings from these microsurgical experiments that have important implications for the mechanisms underlying the direction of NC cell migration. Firstly, the observation that NC cells formed two distinct streams despite the neural tube rotation implied that ventral migration is not driven by ventrally-derived chemo attractants (Weston, 1963). Secondly, in a closer examination of the effects of neural tube inversion on NC cell colonisation of DRG/SG lineages, Stern and colleagues, (1991), observed that NC cells formed segmentally arranged DRG with the correct D-V orientation for the inverted neural tube. This result implied that the orientation and segmental organisation of the DRG was dependent on the neural tube and somites, respectively (Stern et al., 1991). Consistent with the notochord having a role in SG development, NC cells that migrated in a sufficiently ventral direction to contribute to the SG only differentiated when in close proximity to the notochord or ventral neural tube (Stern et al., 1991). Once again it would be interesting to re-examine these experiments in light of the different guidance roles of medial versus lateral sclerotomes and whether the inversion of the neural tube affected NC migration through either of these somitic regions. One interesting possibility is that NC cells were able to pattern the somitic environment. It has been shown that NC cells, via secretion of such molecules as hyaluronan, are able to diffuse proteoglycans in the surrounding tissue, thereby creating space through which to migrate (Tosney, 1978). The possibility that migratory NC cells might alter the conformation of the somitic environment and subsequently, distribution of molecules expressed within this tissue, are discussed further in Section 1.4. Integral to these experiments, however is the role of the notochord in patterning the somitic environment and subsequent positioning of the PNS.

Role of the notochord in determining the overall D-V organisation of ventromedial NC cell migration/ DRG

An additional tissue that regulates NC cell migration/DRG positioning in the D-V axis is the notochord and surrounding perinotochordal mesenchyme (Newgreen et al., 1986; Pettway et al., 1990; Stern et al., 1991; Tosney and Oakley, 1990). *In vivo* NC cells migrating ventrally avoid this region, creating a cell-free space of 50-100 μm surrounding the notochord and subsequently the DRG forms dorsally adjacent to this region (Bronner-Fraser et al., 1991; Newgreen et al., 1986; Pettway et al., 1990; Stern et al., 1991; Tosney and Oakley, 1990). To test the role of this tissue as an inhibitory guidance tissue, NC cells were confronted with notochord tissue *in vitro*, resulting in a classic avoidance response that was independent of contact with the notochord but mediated by the extracellular ECM deposited around the

notochord (Newgreen et al., 1986). A similar phenomenon has been observed *in vivo*, such that placement of an ectopic notochord adjacent to the neural tube inhibits NC cell migration near the graft (Pettway et al., 1990; Stern et al., 1991). This avoidance behaviour was dependent on the presence of the notochord, suggesting that the notochord is an additional tissue sculpting the D-V positioning of the DRG within the rostral half-somite (Newgreen et al., 1986; Pettway et al., 1996; Stern et al., 1991).

Studies involving removal of motor axons *in vivo* have revealed that they may also contribute to the positioning of the DRG adjacent to the neural tube (Tosney and Oakley, 1990). *In vivo*, motor axons lie between the sensory ganglia and perinotochordal mesenchyme, and thereby prevent contact of migrating NC cells with the inhibitory mesenchyme. Loring and Erickson (1987) proposed that motor axons might act to inhibit the ventral migration of NC cells and facilitate their coalescence into the DRG (Loring and Erickson, 1987). In support of this hypothesis, selective removal of motor neurons from the neural tube enabled the DRG to become ventrally localised, so that it formed adjacent to the perinotochordal mesenchyme (Tosney and Oakley, 1990). Further evidence to suggest that motor axons influence the eventual dorso-ventral positioning of the DRG stems from experiments involving 90° rotation of the neural tube. In this situation, the DRG still formed dorsal to the motor axon branch despite its altered location with respect to the neural tube (Tosney and Oakley, 1990). Thus, there is sufficient evidence to suggest that ventrolaterally extending motor axons also influence D-V positioning of the DRG though the molecular basis of this guidance remains unknown.

1.4 Segmentally expressed molecules and their putative functional roles

The somitic mesoderm and perinotochordal matrix surrounding the notochord sculpt the eventual metameric organisation of the PNS in the rostro-caudal and dorso-ventral planes. However, what is the molecular basis of these guiding tissues? Many molecules have been implicated in PNS segmentation based on their exclusive distribution (mostly) in the caudal half-somite and also on functional studies *in vitro* and *in vivo*. These include chondroitin sulphate proteoglycans (CSPG), ECM molecules such as F-spondin and tenascin, T-cadherin, peanut agglutinin (PNA)-binding molecules, and EphB/ephrin-B interactions. Fewer molecules have been implicated in directing the trajectories of PNS precursors through the

rostral half-somite. The ECM molecules, fibronectin (FN) and laminin are distributed evenly throughout the somite, and are thought to contribute to creating a permissive migration substrate. In addition, thrombospondin-1 and a molecule described by Tanaka and colleagues (1989), termed the M7412 antigen, have been localised to the rostral half-somite during the time of NC cell migration (Tucker et al., 1999).

Whilst the inhibitory and positive guidance roles of somite-expressed molecules might have general implications in determining PNS segmentation, studies into the molecular basis of PNS segmentation as well as microsurgical experiments, indicate that distinct sets of guidance cues are responsible for regulating NC cell and motor axon migration through the somite (Koblar et al., 2000; Rickmann et al., 1985; Tosney, 1988). In somite ablation studies, motor axons showed a clear dependence on the ventral sclerotome tissue, becoming unsegmented upon removal of this tissue. In contrast, the DRG became unsegmented when the dorsomedial sclerotome was removed in a manner that correlated with the volume of sclerotome deleted (Tosney, 1988). Thus, not only do these two PNS precursors interact with distinct sclerotome regions, but also the eventual morphogenesis of the DRG appears also to depend more closely on the rostral half-somitic tissue (Gvirtzman et al., 1992; Tosney, 1988).

An additional key factor when considering candidates for regulating segmentation is the timing of their appearance in the somite. Thus the molecule would be required to be expressed exclusively in the rostral or caudal regions of the somite prior to PNS cell entry (Krull, 2001; Perris and Perissinotto, 2000). However, as more knowledge about the mechanics of NC cell migration *in vivo* is acquired through time-lapse analyses (Krull et al., 1995; Krull et al., 1997; Kulesa and Fraser, 2000) and NC cell migration studies *in vitro* (Duband et al., 1991; Newgreen, 1989; Rovasio et al., 1983), a more dynamic view of PNS segmentation must be considered. Thus, segmental NC cell migration appears to be controlled by a hierarchy of guidance cues within the somite, starting from caudally-derived repulsive molecules, whose expression is NC cell independent and followed by molecules localised to the rostral half-somite that appear to influence migration by restricting NC cells to particular migration-permissive regions within the rostral half-somite and whose expression is NC cell-dependent (Perris, 1997). Finally are the group of ECM molecules that are uniformly distributed across both somite halves and influence NC cell movement in a generally positive manner (Duband et al., 1986; Duband and Thiery, 1982; Duband and Thiery, 1987; Newgreen and Thiery, 1980; Thiery et al., 1982b). This section reviews the current molecules known to contribute to NC cell segmentation.

1.4.1 PNA-binding glycoproteins and T-cadherin: general inhibitory molecules with multiple roles in trunk development?

PNA-binding molecules

The first molecular marker found to differentiate between rostral and caudal somite halves is the plant lectin, peanut agglutinin (PNA), which binds exclusively to molecules in the caudal somite region during the time that PNS precursors are migrating through the rostral-half (Davies et al., 1990; Oakley and Tosney, 1991; Stern et al., 1986). In addition, PNA immunoreactivity is also found in the perinotochordal ECM along with chondroitin-6-sulphate (Davies et al., 1990; Krull et al., 1995; Oakley and Tosney, 1991; Tosney and Oakley, 1990). PNA-binding in the caudal and not rostral portion of the somite was suggestive of these molecules playing an inhibitory role in segmentation. To test this hypothesis, using an explant culture assay that mimics the normal environment for NC cell migration, Krull and colleagues (1995), showed that addition of exogenous PNA was sufficient to allow NC cell entry into the caudal half-somite *in vivo* (Krull et al., 1995). In addition, confrontation of DRG axons with somite material elicited growth cone collapse *in vitro,* and this effect was eliminated with immobilised PNA (Davies et al., 1990). Thus, PNA-binding molecules might represent a general mechanism to inhibit NC cell migration as well as motor and sensory axon outgrowth through the caudal somite.

These data place PNA-binding proteins as very strong candidates for mediating a general, contact-repulsive guidance mechanism in the caudal half-somite; however, the proteins responsible for this repellent activity are unknown (Vermeren et al., 2000). Studies utilising PNA affinity chromatography have identified two significant binding fractions at 48 and 55 K (Davies et al., 1990). Analysis of the binding of candidate molecules, known to be exclusively localised to the caudal half-somite revealed that PNA-agarose beads did not adsorb to ephrin-B1, Semaphorin-3A or F-spondin, implicating a novel repellent fraction (Vermeren et al., 2000). This is not surprising given that Semaphorin-3A is largely expressed in the dermomyotome, a non-essential tissue in mediating segmentation (Shepherd; Tosney, 1987 #508}. Furthermore, whilst ephrin-B1 is critical for maintaining the normal migration of NC cells through the rostral somite, it does not affect motor axon segmentation, implying a NC cell-specific function for these interactions (Koblar et al., 2000; Krull et al., 1997). Finally, a possible PNA-binding candidate might be the cytotactin binding proteoglycan (CTB), which is expressed in the caudal half-somite and has chondroitin sulphate moieties that are

recognised by PNA (Tan et al., 1987). Whilst the repellent properties of this protein have been shown *in vitro*, the individual contribution of this molecule to segmentation *in vivo* remains unknown (Tan et al., 1987).

PNA binds to multiple other axonal repellent regions in the embryo many of which are pre-chondrogenic tissues, including the perinotochordal mesenchyme and limb plexus, and suggests that PNA binding proteins might also be involved in chondrogenesis (Oakley et al., 1994). Support for an independent role for PNA binding proteins separate from their role in segmentation, stems from experiments involving unilateral neural tube deletion whereby the distribution of PNA-binding material remained unchanged in the absence of outgrowing PNS precursors (Oakley and Tosney, 1991). Whether the repellent functions of these proteins are related specifically to their role in chondrogenesis is currently unknown however in a similar manner to the proteins in caudal half-somite, pre-chondrogenic tissues also undergo extensive carbohydrate modifications (Christ et al., 2000). This leaves open the possibility that regulated carbohydrate attachment might underlie the contact-repulsive/chondrogenic capacity of PNA-binding proteins. Thus, in future, the search for specific PNA-binding proteins might be better targeted towards differentially expressed biochemical modification enzymes or carbohydrate moieties rather than distinct contact-repellent molecules.

T-cadherin

This cell-cell adhesion molecule belongs to the cadherin family of adhesion molecules and is expressed in the caudal half-somite from the onset of NC cell migration, approximately 3 somites rostral to the first formed somite (Ranscht and Bronner-Fraser, 1991). T-cadherin expression in the caudal half-somite begins in the third or fourth most newly formed somite of E2.5 embryos, coincident with the emigration of NC cells into the rostral half-somite, and becomes progressively stronger as somites mature (Ranscht and Bronner-Fraser, 1991). The selective distribution of this molecule in the caudal half-somite was suggestive of it acting as a repellent for migrating PNS precursors. *In vitro* studies have shown that T-cadherin substrata inhibit motor neurite outgrowth (Fredette et al., 1996) however deletion of T-cadherin in mice does not result in a PNS segmentation defect (C.E., Krull., personal communication).

Interestingly, ablation of the dorsal neural tube did not alter the pattern of T-cadherin expression (Ranscht and Bronner-Fraser, 1991) indicating that like PNA-binding molecules,

T-cadherin may have a more generalised function in morphogenesis of the vertebral column. Certainly, the caudal half-somite/chondrogenic tissues are packed more tightly and contain high levels of chondroitin sulphate proteoglycans, consistent with their later differentiation into cartilaginous tissue of the vertebral arches (Erickson and Perris, 1993; Tosney, 1988). It is possible that T-cadherin via its adhesive properties might mediate compaction of the caudal half-somitic mesoderm, creating an ECM conformation that is unable to be diffused by NC cells (Ranscht and Bronner-Fraser, 1991). Given that ablation of T-cadherin did not affect segmental NC cell migration (CE Krull personal communication), it would be interesting to determine whether it also caused any skeletal defects.

1.4.2 CSPG's and their role in directing NC cell migration through the rostral somite

Proteoglycans (PG), in particular those with chondroitin sulphate moieties (CSPG) are proteins that are bound to glycosaminoglycan (GAG) aggregates and are thought to influence PNS segmentation and more specifically, NC cell migration via inhibitory guidance mechanisms (Perris and Perissinotto, 2000). CSPG's are deposited in the ECM lining NC cell pathways through the rostral half-somite, as well as defining barrier tissues during PNS development such as the perinotochordal mesenchyme and caudal half-somite (Newgreen et al., 1982; Oakley and Tosney, 1991; Pintar, 1978). Early studies reported that NC cell migration was inhibited in the presence of CSPG's identified from cartilage tissue (Newgreen et al., 1982; Perris and Johansson, 1987). Furthermore, inhibition of CSPG function by enzymatic degradation results in the suppression of notochordal-derived repulsion *in vitro* and *in vivo* (Newgreen et al., 1986; Pettway et al., 1990). Thus, there is substantial evidence to suggest that proteins covalently bound to chondroitin sulphate moieties mediate repulsion of NC cells from the perinotochordal matrix as well as the caudal half-somite.

Whilst *in vitro* data points to an inhibitory guidance mechanism for these factors in NC cell migration, much of this data was based on proteins extracted from embryo regions not spatiotemporally specific to the inhibitory function of the notochord (Kerr and Newgreen, 1997; Newgreen et al., 1986; Perris et al., 1991b; Perris et al., 1996; Pettway et al., 1990). More recently, analyses using specific antibodies have identified the hyaluronan-binding proteoglycans aggrecan and versican (Perissinotto et al., 2000), and collagen IX (Ring et al., 1996) as important ECM constituents for directing NC cell migration. Their possible

mechanisms of action on PNS segmentation based on *in vitro* and some *in vivo* functional analyses are detailed below.

Aggrecan

Aggrecan is a CSPG originally isolated from adult cartilage and is thought to be the molecular basis of the repulsive activity of the notochord (Bundy et al., 1998; Kerr and Newgreen, 1997; Perissinotto et al., 2000). During early phases of NC cell development (E2.5 mid-trunk), aggrecan is expressed weakly in the notochord and then later as development proceeds, becomes localised to the perinotochordal ECM (Domowicz et al., 1995). Coincident with chondrogenesis in the ventral sclerotome, its expression in the perinotochordal mesenchyme expands dorsolaterally and ventrally in a metameric pattern that alternates with the formation of the peripheral ganglia (Bundy et al., 1998; Perissinotto et al., 2000; Pettway et al., 1996). This expansion of aggrecan expression continues until it surrounds the neural tube, defining the tissue destined to become the vertebral column (Perissinotto et al., 2000). Thus, in a similar manner to PNA-binding proteins, aggrecan distribution delineates chondrogenic regions, further supporting a common molecular mechanism underlying repulsive guidance functions and cartilage differentiation (Bundy et al., 1998).

There is a large body of evidence to support that aggrecan has an inhibitory effect on NC cell motility *in vitro* and is therefore likely to comprise the key inhibitory component of the notochordal ECM (Newgreen et al., 1982; Perris and Johansson, 1990; Perris et al., 1991b; Yamagata et al., 1989). Furthermore, the inhibitory functions can be attributed to the GAG side chains attached to this protein as chondroitinase treatment abolished any inhibitory effects (Newgreen et al., 1986; Pettway et al., 1996; Pettway et al., 1990). The exact GAG composition of the notochord-derived aggrecan has been the subject of much controversy, and has subsequently limited the interpretation of *in vitro* experiments that utilised non-notochordal derived aggrecan forms to investigate the mechanism of aggrecan-based NC cell guidance (Kerr and Newgreen, 1997). This discrepancy has since been resolved using an antibody specific for the chick cartilage aggrecan core protein, termed S103L (Domowicz et al., 1995; Kerr and Newgreen, 1997; Pettway et al., 1996). Further molecular and biochemical analysis of products isolated with the S103L antibody has revealed that the notochord aggrecan, expressed during E2- E4 is biochemically distinct from the cartilage-type, expressed from E10-16, though the core protein is the same in each case. This is therefore an example whereby regulated post-translational protein modification underlies different

identities and subsequent functions for aggrecan isoforms in cell guidance/tissue morphogenesis (Domowicz et al., 1995).

In a re-examination of earlier notochord implantation experiments, Pettway and colleagues (1996) found that the notochordal aggrecan was co-expressed with HNK1, and their combined expression correlated with repulsive function from the notochord (Pettway et al., 1996). HNK1-positive E2 and E3 notochords were the most potent in repelling NC cells, and this potency declined with age through E4 and E5, coincident with the loss of HNK1 reactivity and onset of chondrogenesis in the sclerotome (Pettway et al., 1996). Furthermore, pre-incubation of ectopic notochords with HNK1 blocked the repulsive activity of the notochord; whether this was directly related to the HNK1 reactive carbohydrate epitope attached to aggrecan or a conformational change in aggrecan side chains (induced by the HNK1 antibody) is unknown (Pettway et al., 1996). More recent experiments testing the function of aggrecan *in vivo* have uncovered a complex response to ectopic aggrecan placement wherein NC cells aggregated close to the implant. This result implied that the environmental context of aggrecan and/or presence of the HNK1 epitope could induce conformational changes/protein modification that regulates its activity (Perissinotto et al., 2000). In sum, the GAG side chains attached to the aggrecan core protein comprise the key inhibitory component of the perinotochordal ECM and this may be regulated in a complex manner by the presence of the HNK1 epitope (Domowicz et al., 1995; Perissinotto et al., 2000; Pettway et al., 1996).

PG-M/ versican

Versican belongs to the same group of aggregating proteoglycans (PG) as aggrecan, and has some overlapping expression domains including in the notochord (Perris et al., 1991b). Despite this, *in vitro* and *in vivo* studies have shown that versican influences NC cell migration in a quite different way than aggrecan (Perissinotto et al., 2000; Perris and Johansson, 1990). Versican is initially distributed diffusely in the epithelial somites, and is then rearranged to the caudal half-somite coincident with the infiltration of NC cells into its rostral half (Landolt et al., 1995; Perissinotto et al., 2000), (Figure 1.8). Given its distribution in the caudal half-somite, it was suggested that versican acted via a similar inhibitory mechanism as aggrecan, contributing to the exclusion of NC cells from the caudal half-somite (Tan et al., 1987). One of the key requirements for a molecule to be considered a barrier to cell migration/ axon advance is that its expression must occur independently of the invading

cells (Oakley and Tosney, 1991). Whilst initial studies suggested that versican staining preceded NC cell entry into the somite (Henderson et al., 1997; Landolt et al., 1995; Tan et al., 1987), a recent analysis of the effects of NC ablation on versican distribution demonstrated that its re-arrangement is dependent on NC cell invasion of the rostral half-somite (Perissinotto et al., 2000).

Thus, the question arises as to how versican might elicit guidance of NC cells. In addition to being distributed in the caudal half-sclerotome, versican also appears in an increasing dorso-ventral gradient in the rostral half-somite during ventromedial NC cell migration and surrounds NC cells once they have begun to form DRG (Landolt et al., 1995; Perris et al., 1991b). Thus, ventromedially migrating NC cells move in a haptotactic manner towards a versican-rich ECM during their colonisation of ventral structures (Perris, 1997). It has been hypothesised that the migration of NC cells along a gradient of PG's might occur via a "mechanical exclusion effect", whereby increased secretion of tenascin-C and hyaluronan (on NC cells) progressively displaces PG's in the ECM and diffuse the migration-unfavourable matrix components (Perris, 1997). More recent analyses have provided support for such a hypothesis whereby placement of ectopic versican-coated micromembranes resulted in a re-direction of NC cell trajectories towards the increasing versican gradient. Furthermore, this effect was only observed in the dorsolateral sclerotome where versican expression is normally low (Perissinotto et al., 2000).

Mechanistically, versican has been described to act in a similar manner to aggrecan, slowing the movement of NC cells on *in vitro* substrates such as FN and laminin, although aggrecan is the more potent of the two (Perris and Johansson, 1990; Perris et al., 1996; Yamagata et al., 1989). These proteins are thought to modulate cell-substrate adhesivity by directly competing with FN for substrate binding sites (Perris et al., 1996; Yamagata et al., 1989). Although thought initially to be a negative regulator of NC cell motility, studies have shown that versican, when distributed in low concentrations can increase the speed of movement via de-adhesion (Tucker and Erickson, 1984). This observation correlates well with the distribution of veriscan in the rostral half-somite whereby its co-expression with FN might be an important means of facilitating cell movement (Perris, 1997). This is supported by the observation that in Splotch mutant mice, that display severe NC cell defects, versican expression is up regulated in (normally) NC-positive regions, suggesting a causal link between increased concentrations of versican and aberrant NC migration (Henderson et al., 1997). In contrast, the refractory response of NC cells to both aggrecan and versican *in vitro*

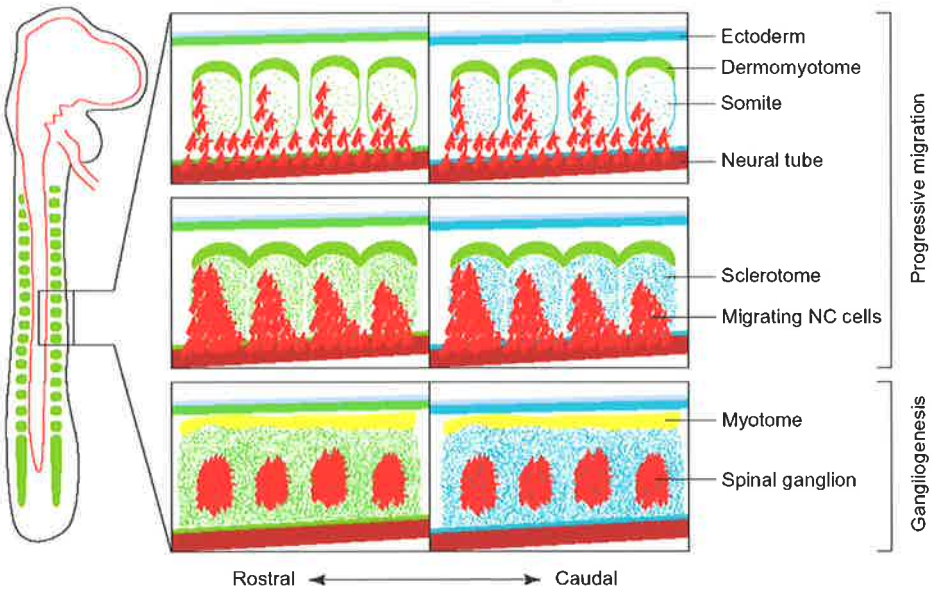
might more closely reflect their roles in NC avoidance zones, such as the perinotochordal matrix and caudal half-somite respectively, where they are distributed in high concentrations (Henderson et al., 1997; Perris et al., 1996).

Figure 1.7- Summary of the remodelling of ECM components during the course of ventromedial NC cell migration.

Molecules on the left represent those that are remodelled into the caudal half-somite upon NC cell invasion of the rostral half-somite and include, fibronectin, collagens I and III and versican. Those on the right side are interspersed amongst NC cells during their migration and remain associated with NC cells during DRG gangliogenesis. Adapted from (Perris, 1997).

Reorganised with NC migration
-Fibronectin
-Collagens I and III
-Versican

NC-associated
-Tenascin-C
-Laminins
-CollagenIV



Collagen IX

Collagen IX is a CSPG that is expressed exclusively in the caudal half-somite during NC cell migration and motor axon outgrowth (Perris et al., 1991a). *In vitro* both sensory and motor neurites from DRG and motor neurons respectively, avoid regions containing collagen IX. Furthermore, plating of neural tube explants on alternating stripes of collagen IX and fibronectin, resulted in a clear preference for fibronectin, whilst collagen IX almost completely inhibited NC cell emigration (Ring et al., 1996). The inhibitory effects of collagen IX were abolished by chondroitinase treatment, implicating the chondroitin sulphate side chain as the inhibitory component (Ring et al., 1996). This is consistent with the generally positive influences of collagens on NC cell migration (refer Section 1.5.2 below). Thus collagen IX could be an additional important contributor to the exclusion of migrating PNS precursors mediated by the caudal half-somite.

1.4.3 ECM molecules also have dynamic expression patterns coincident with NC cell migration

Multiple extracellular matrix (ECM) molecules have been implicated in coordinating the migration of NC cells through the rostral half-somite, largely through the creation of scaffold that is permissive for NC migration (Perris, 1997; Perris and Perissinotto, 2000). Whilst perturbation of single molecules *in vivo* may not effect the overall organisation of NC cell migration, their combined actions contribute to the precise spatiotemporal coordination of NC cell movement.

F-Spondin

F-spondin is an ECM molecule that has a dynamic distribution with respect to NC migration and consequently, somitogenesis. In epithelial somites, F-spondin is localised to the staging area (between neural tube and somite) as well as the inner ECM of the somitic epithelium. Following somite dissociation, F-spondin is rearranged to demarcate the ventral perinotochordal mesenchyme in rostral and caudal halves as well as the dermomyotome, though the strongest expression is in the caudal half-sclerotome. Thus, as NC cell migration proceeds, F-spondin is localised to regions of NC cell avoidance including the perinotochordal mesenchyme and caudal sclerotome (Debby-Brafman et al., 1999). Although F-spondin is distributed in NC cell-negative regions during their migration, post-

gangliogenesis, it is expressed on Schwann cells associated with the DRG (Debby-Brafman et al., 1999).

Function-blocking and over-expression approaches both *in vivo* and *in vitro* have revealed a key role for F-Spondin in defining permissive areas for migration within the somite (Debby-Brafman et al., 1999). Addition of exogenous F-spondin protein to whole trunk explants inhibited the emigration of NC cells into the somite, causing cells to remain at the dorsal neural tube or in the neural tube lumen. On the contrary, neutralisation of F-spondin signal in caudal half-somite preparations *in vitro* induced a mesenchymal NC cell morphology compared to the typical rounded morphology observed. Furthermore, *in vivo* neutralisation perturbed the normal dorsoventral organisation of NC cell migration through the rostral half-somite as well as partially removing inhibition from the caudal half-somite (Debby-Brafman et al., 1999). This suggests that F-spondin influences NC cell homing decisions during their ventromedial migration through the rostral half-somite, implementing a highly localised contact-dependent control of cell movement. In contrast, the influence of F-spondin on the eventual segmentation of DRG was less significant; suggesting that it is not required for the higher order metameric arrangement of the PNS (Debby-Brafman et al., 1999; Tzarfati-Majar et al., 2001).

Tenascin/ cytotactin

Tenascin/ cytotactin is an extracellular ECM glycoprotein involved in neuron-glia adhesion and is expressed in the basement membranes of ECM in tissues such as the neural tube, notochord and somitic boundaries as well as in the mesenchymal edge of the somite lumen. (Mackie et al., 1988; Stern et al., 1989; Tan et al., 1987). It has an expression pattern that is closely associated with NC cell migration. In epithelial somites, it is expressed diffusely. At intermediate phases of NC cell migration it is more strongly localised to the caudal half-somite (Stern et al., 1989), but becomes progressively localised to the rostral-half coincident with NC cell infiltration (Mackie et al., 1988; Tan et al., 1987). Interestingly, its expression in the rostral half-somite is restricted to the rostro-dorsal portion surrounding HNK1-positive NC cells during DRG formation (Mackie et al., 1988; Stern et al., 1989; Tan et al., 1987).

The dynamic distribution of tenascin with respect to NC cell migration posed two important questions: Firstly, is tenascin secreted by NC cells themselves or are somitic cells the source of the specific tenascin distribution? Secondly, is the reorganisation of tenascin due to the

invasion of NC cells into the rostral half-somite? The earliest studies describing tenascin distribution reported that medium conditioned by NC cells *in vitro* was tenascin-negative, whilst tenascin immunoreactivity was associated with medium conditioned by somite cells (Mackie et al., 1988; Tan et al., 1987). In addition, dorsal neural tube ablation experiments suggested that the re-organisation of tenascin distribution was not dependent on NC cells (Tan et al., 1987). However, when these experiments were repeated, paying close attention to the number of somites required to completely eliminate NC cells, it was found that tenascin no longer re-arranged to the rostral somite, remaining largely localised to the caudal half-somite (Stern et al., 1989). These findings were later complemented by *in situ* hybridisation studies using a *tenascin-C* probe, that indicated that tenascin was secreted by NC cells during their ventromedial migration (Tucker and McKay, 1991). This implicated a NC-derived factor (rather than derived from the caudal half-somite) in regulating the migratory trajectories of NC cells through the somite.

How might the anti-adhesive molecule, tenascin, secreted by NC cells into the rostral half-somite affect NC cell migration? Functional studies *in vitro* have indicated that when plated on FN, tenascin inhibits NC cell migration, causing excessive cell rounding, though not completely inhibiting migration on FN (Mackie et al., 1988; Tan et al., 1987). Given its inhibitory effect on substrate attachment *in vitro*, a possible mechanism explaining tenascin secretion by NC cells is that it reduces NC cell adhesion to FN, facilitating movement through FN-rich areas (Tan et al., 1987). There are a number of *in vivo* experiments supporting a key role for tenascin in mediating NC cell migration via an anti-adhesive mechanism (Bronner-Fraser, 1988; Mackie and Tucker, 1999; Tucker, 2001). Firstly, micro-injection of anti-tenascin antibodies prevented the emigration of cranial but not trunk NC cells (Bronner-Fraser, 1988). The apparently cranial-specific mechanism might be an artefact of the technical difficulty associated with achieving high local concentrations of antibody when injected in the trunk. In support of this putative explanation, more recently, electroporation of morpholino anti-sense oligonucleotides (to inhibit the production of tenascin protein) caused a build-up of NC cells at the dorsal edge of the neural tube, indicative of increased adhesion (Tucker, 2001). Secondly, although tenascin knock-out mice develop normally, they have an impaired response to trauma and stress, possibly due to compensatory mechanisms for the loss of the anti-adhesive tenascin protein (Mackie and Tucker, 1999). Finally, tenascin is the natural versican ligand whose secretion from NC cells is thought to mediate a "mechanical exclusion effect" and enable migration through a proteoglycan-rich matrix (Perris, 1997). Thus, tenascin

might aid NC cell migration through the rostral half-somite by modulating the adhesive properties of the ECM and thereby facilitating their movement through this tissue.

1.4.4 Eph receptors and their ligands, ephrins

The Eph family of Receptor Tyrosine Kinases (RTKs) and their interacting proteins, ephrins, are a family of guidance molecules that have previously been implicated in PNS segmentation. The family comprises 14 Eph molecules and 10 ephrins, including ephrin-A6, a novel ephrin-A ligand isolated in aves (Menzel et al., 2001). The Eph family spans vertebrate and invertebrate species and is expressed widely in the developing and adult nervous system (Wilkinson, 2000). In vertebrates, there are two clear subclasses of Eph/ephrin molecules, A and B, and these are based on binding affinities of Eph receptors to ephrins. Thus, EphA binds ephrin-A and EphB to ephrin-B (Kullander and Klein, 2002). However as more is learned about the molecular nature of Eph/ephrin interactions it is likely that many more will be added to this list. There are currently two known exceptions to this rule: the first is EphA4, which can bind ephrin-B2 and ephrin-B3 proteins as well as ephrin-A (Gale et al., 1996; Xu et al., 1999) and the second is EphB2 that has recently been shown to interact with ephrin-A5 (Himanen et al., 2004). Both Eph and ephrin molecules are membrane-bound and require membrane attachment for function (Davis et al., 1994); thus they have been largely implicated in contact-mediated interactions between cells and tissues during embryogenesis. Eph/ephrin interactions have been shown to play a role in multiple different examples of tissue morphogenesis including: formation of segment boundaries (Xu et al., 1999); axon guidance (O'Leary and Wilkinson, 1999); vasculogenesis (Adams et al., 1999); and in segmentation (Koblar et al., 2000; Krull et al., 1997; Wang and Anderson, 1997).

Previously, it has been demonstrated that EphB and ephrin-B proteins are distributed in a complementary manner in the somite, suggestive of a role in PNS segmentation. In avian embryos, EphB3 is distributed on neural crest cells and on cells in the rostral half-somite whilst ephrin-B1 is in the caudal-half somite, suggestive of a repulsive interaction (Koblar et al., 2000; Krull et al., 1997). In neural tube explants, application of exogenous blocking proteins to perturb endogenous EphB3/ephrin-B1 interactions *in vivo* resulted in NC cells traversing both the rostral and caudal somite halves (Krull et al., 1997). A similar situation has been described for EphB/ephrin-B interactions in rodents though the ligand utilised in this organism is ephrin-B2 instead of ephrin-B1 (Wang and Anderson, 1997). It has been shown *in*

vitro using rodent neural tube that both ephrin-B1 and ephrin-B2 can mediate repulsive guidance of NC cells (Wang and Anderson, 1997).

Despite the apparently critical effects for EphB/ephrin-B interactions in determining NC cell migration *in vitro*, no PNS segmentation defects are observed in ephrin-B2 knock-out mice (Adams et al., 1999; Wang et al., 1998). Interestingly, however, both ephrin-B1 and EphB2/B3 mutants exhibit skeletal defects, suggesting that ephrin-B1 signalling may be important for chondrogenesis in the trunk (refer 1.4.1 for discussion of other caudal half-somite factors with putative chondrogenic capacity; (Compagni et al., 2003; Davy et al., 2004). This suggests that although EphB/ephrin-B interactions are important contributors to NC cell exclusion from the caudal somite, they are just one component of multiple functionally redundant guidance mechanisms regulating PNS segmentation (Krull, 1998). This also leaves opens the possibility that there might be other, as yet unidentified Eph/ephrin guidance systems involved in PNS segmentation. To this end, EphA7 has been described in the caudal-half somite during PNS segmentation (Araujo and Nieto, 1997), whilst more recent evidence suggests that ephrin-A ligands are expressed in the dorsal root ganglia (Eberhart et al., 2000).

During the course of this research, ephrins-A2, -A5 and –A6 were found to be co-expressed along with EphA4 on recently delaminated NC cells and in the initial phases of their emigration into the somite (McLennan and Krull, 2002). Functional studies *in vitro* demonstrated that perturbation of EphA4/ephrin-A interactions disrupted the segmental migration of NC cells. In addition, growth of NC cells on a substrate coated with a combination of EphA4 and ephrin-A2 or ephrin-A5 Fc proteins (mimicking the receptor distribution on surrounding NC cells *in vivo*) acted to positively regulate NC migration, whilst ephrin-A proteins alone were refractory for migration (McLennan and Krull, 2002). Thus, the possible role of these molecules in the coordinated migration of NC cells in the somite must be considered.

1.5 Positive influences of ECM molecules on NC cell migration- creating a permissive environment

The final group of molecules that contribute to the segmental migration of NC cells through the rostral half-somite via positive cell adhesion interactions, includes fibronectin, laminin and collagens I, III and IV. In addition, thrombospondin-1 is more specifically localised to the rostral half-somite during NC cell invasion (Tucker et al., 1999) as is the M7412 antigen identified by Tanaka and colleagues, (1989) in a screen of rostral-specific molecules (Tanaka et al., 1989). The latter however is expressed following the initiation of NC cell migration and is unaffected by NC removal, suggesting perhaps a more critical role in motor axon segmentation (Tanaka et al., 1989). Whilst *in vivo* studies have revealed a non-essential role for these molecules in facilitating NC cell segmentation, i*n vitro* studies have suggested that their combined actions *in vivo* are important for the positive regulation of NC cell guidance (Perris, 1997).

1.5.1 fibronectin (FN) and laminin

Both FN and laminin are distributed along the pathways of NC cell migration, in the basement membranes of epithelial structures and also in the somite (Krotoski et al., 1986; Newgreen and Thiery, 1980; Newgreen et al., 1982; Thiery et al., 1982a). At the initial phase of NC cell migration, (at the level of epithelial somites) FN and laminin are present on the basement membranes of the neural tube, epithelial somites, and the ectoderm, and to a lesser extent FN is on the membrane lining the ventral neural tube and notochord (Newgreen and Thiery, 1980). Ultra-structural studies have indicated that as NC cells exit the dorsal neural tube they are surrounded by interstitial bodies anchored in the ECM that contain FN, only occasionally contacting the laminin-positive basement membrane of the neural tube (Ebendal, 1977; Mayer et al., 1981; Tosney, 1978). For this reason laminin is not seen as a primary migration substrate for NC cells, (Newgreen et al., 1982) though it does have migration-promoting effects *in vitro* (Perris et al., 1991a).

As NC cell migration proceeds further ventrally, FN remains distributed along key areas of ventral migration such as the basal lamina of the myotome and surrounding the aorta (Newgreen and Thiery, 1980). However, during the period of active cell migration through the rostral somite, there is no differential distribution of either FN or laminin, though FN is

diffusely expressed on sclerotome cells surrounding the NC (Krotoski et al., 1986). During condensation of NC cells into DRG, FN is removed from the the sclerotome surrounding NC cells, whilst laminin is up-regulated around the DRG (Duband et al., 1987; Duband et al., 1986; Newgreen and Thiery, 1980). NC cell adhesion to laminin increases with developmental age *in vitro* (Rovasio et al., 1983) suggesting that the timed expression of these molecules in the ECM contributes to NC cell morphogenesis. In addition to the developmentally regulated expression of adhesive ECM components, NC populations also have differential competencies to respond to FN. For example unlike the cranial neural crest, truncal NC cells don't synthesise FN, possibly enhancing their sensitivity to narrow FN-defined pathways during migration (Erickson et al., 1980; Newgreen and Thiery, 1980).

In vitro, plating of neural tube explants on FN (plasma form) or laminin, results in the rapid emigration of NC cells with a flattened morphology indicative of extensive substrate attachment (Newgreen et al., 1982; Rovasio et al., 1983). Studies have investigated the function of FN in NC cell morphogenesis both *in vitro* and *in vivo* using antibodies (CSAT/JG22) that recognise a 140-kD FN-receptor complex on the surface of NC cells, to block substratum adhesion of NC cells (Boucaut et al., 1984; Bronner-Fraser, 1985; Bronner-Fraser, 1986b; Duband et al., 1986; Krotoski et al., 1986; Rovasio et al., 1983). Application of the antibody to NC cell cultures *in vitro* inhibited adhesion, causing cell blebbing, rounding, and also blocked their emigration from the neural tube (Bronner-Fraser, 1985; Rovasio et al., 1983). Furthermore, analysis of the distribution of the 140 kD FN-receptor complex indicates that it co-distributes with NC cells *in vivo* and localises with similar structures to FN in motile cells *in vitro*, suggestive of it acting as an intermediary between substrate adhesion and the cytoskeleton (Duband et al., 1986). Thus given the prevalence of FN in NC cell migratory pathways, and its role in mediating NC cell attachment at least *in vitro*, it is likely to be a major contributor to the creation of a permissive migration substrate *in vivo* (Mayer et al., 1981; Newgreen and Thiery, 1980; Newgreen et al., 1982).

Though FN and laminin have clear motility-promoting properties *in vitro*, application of the same antibodies *in vivo* suggest that NC cell migration can occur in the absence of FN, at least in the trunk region (Boucaut et al., 1984; Bronner-Fraser, 1986b). In cranial regions, loss of cell adhesion to FN at the time of NC cell emigration results in the abnormal localisation of cells in the lumen of the neural tube and migration along aberrant pathways. In the trunk, no such effects were observed (Bronner-Fraser, 1986b). A similar phenomenon has been observed in a variety of other experiments blocking cell-ECM interactions, (for example,

tenascin-C) suggesting that these interactions have less relevance for trunk NC cell migration compared to cranial (Bronner-Fraser, 1993a). This may be in part due to molecular differences between the ECM receptors expressed on the two NC populations and that this then dictates their preferences for particular substrate types and conformations (Lallier et al., 1992). Additionally, the influence of inhibitory guidance tissues in the trunk such as the caudal half-somite and perinotochordal mesenchyme may be sufficient to channel NC migration, whilst molecules such as FN or laminin might play a less instructive role (Bronner-Fraser, 1993b). Lastly, as the experiments with tenascin-C demonstrated, (refer Section 1.4.3), the lack of effects observed in truncal regions might be due to problems in achieving high enough concentrations of blocking antibody in the trunk (Bronner-Fraser, 1993b).

1.5.2 Collagen

As NC cells emigrate from the neural tube, they enter into a cell-free space containing a meshwork of fibrillar structures that are collagenous in nature (Ebendal, 1977; Tosney, 1978). Subsequent studies have found that the predominant collagen isoforms expressed in these fibrils at the dorsomedial edge of the somite and also in the somite itself are collagens I and III (Perris et al., 1991b). In addition, collagen IV is expressed in basement membranes and is co-distributed with laminin in epithelial structures (Duband et al., 1987). Similar to other ECM molecules, versican and tenascin (Landolt et al., 1995; Stern et al., 1989) that undergo dynamic changes with NC cell migration, collagens I and III are displaced from the rostral somitic mesoderm during NC cell migration and into the caudal somite (Perris et al., 1991a). Interestingly, this remodelling is NC cell dependent, suggesting that NC cells themselves are capable of reorganising the distribution of collagenous material, in a similar way to that described for versican (Perissinotto et al., 2000). NC cells move with varied efficiency through collagen substrata *in vitro* depending on the concentration/density and conformation of collagen types (Davis and Trinkaus, 1981; Perris et al., 1991a; Tucker and Erickson, 1984). The migration efficacy of NC cells is greatly improved with the addition of FN suggesting that *in vivo*, collagens may act in concert with FN to facilitate NC cell motility (Newgreen et al., 1982; Rovasio et al., 1983).

1.5.3 Thrombospondin-1

Thrombospondin-1 is a human ECM molecule that is distributed along the pathways of NC cell migration; indicative of a molecule that promotes NC cell motility. Thrombospondin-1 is expressed along the ventral surface of the myotome, co-localising with NC cells migrating laterally along the myotome and in more rostral regions of the embryo trunk it is distributed in the dorsal sclerotome (Tucker et al., 1999). Co-localisation of the earliest Thrombospondin-1 expression with HNK1 indicated that the source of immunoreactivity was not NC cells, but instead the ECM underneath the myotome (Tucker et al., 1999; Tucker and McKay, 1991). A high rate of NC cell migration was achieved on Thrombospondin-1 substrata *in vitro* at low levels (equivalent to endogenous levels) whilst at higher concentrations it caused excessive cell attachment and increased proliferation. It is hard to extrapolate the latter effects to the *in vivo* environment, as this molecule is not distributed in such high concentrations in the somite. Thus, *in vivo*, Thrombospondin-1 is likely to facilitate movement of NC cells by promoting weak ECM attachment, thereby facilitating migration (Tucker et al., 1999).

1.6 Cellular basis of NC cell migration: *in vitro* and *in vivo* time-lapse analyses

The results of multiple function blocking experiments *in vitro* have suggested that during their migration, NC cells obey a hierarchy of cellular guidance cues arranged in order of increasing importance; from repulsion mediated by the caudal half-somite to inhibitory or anti-adhesive ECM molecules, to the classic ECM attachment glycoproteins that positively influence migration (Perris, 1997). The question remains however, as to how NC cells achieve directed migration through the somite? Although this decision may in part, be determined by regulated NC cell-ECM interactions, as the experiments implanting versican-coated micro-membranes suggest (Perissinotto et al., 2000), such interactions are likely to have a general sculpting influence rather than influencing local cellular interactions (Krull, 2001). More precise *in vitro* and *in ovo* time-lapse imaging techniques have suggested that communication between NC cells is important for the coordination of isolated NC cell movement into organised streams (Bronner-Fraser, 1993b; Krull, 1998). The specific contacts and consequences of NC cell interactions for orienting and directing NC migration are discussed below.

1.6.1 In vitro *studies of the adhesion mechanisms underlying NC cell motility*

The first hypothesis to explain the driving force for NC cell movement away from their source in the dorsal neural tube to ventral locations was based on extensive *in vitro* studies as well as ultra-structural studies of NC emigration *in vivo* and stated that NC cells moved according to the principles of contact inhibition of movement (Davis and Trinkaus, 1981; Ebendal, 1977; Newgreen, 1982; Newgreen, 1989; Newgreen et al., 1979; Rovasio et al., 1983; Tosney, 1978; Tucker and Erickson, 1984). This hypothesis implies that upon contact, cells experience a local membrane paralysis resulting in the movement of a population of cells away from regions of high density (at the neural tube) to low-density (away from the source) and *in vitro* the forming of a monolayer of "contact-inhibited" cells (Carter, 1965). Although this theory may have concurred with observations of NC cell movement at a population level, it was unable to explain control of NC movement at a cellular level for a number of reasons. Firstly, although NC cells exhibited the correct morphology for contact-inhibited cells *in vivo* (Ebendal, 1977; Newgreen et al., 1982; Tosney, 1978), time-lapse analysis of migration in a three-dimensional collagen lattice indicated that they migrated in a chain-like fashion, maintaining leading-tail edge cellular adhesions (Davis and Trinkaus, 1981). In addition, placement of ectopic NC cells in the midst of a stream of ventromedially migrating NC cells did not necessarily lead to ventral migration of these cells (towards the less dense region) alongside their endogenous counterparts (Erickson et al., 1980). Thus although it could be argued that cells obey a specialised form of contact inhibition of movement (Newgreen et al., 1982), these data suggested that NC cells might communicate with one another to regulate their movement in a precise, localised manner.

Despite contact inhibition of movement not being able to completely explain the local control of cell movement, *in vitro* studies have demonstrated that cell density is an important means of controlling NC cell directionality (Krull et al., 1995; Newgreen et al., 1979; Rovasio et al., 1983; Tosney, 1978). Analyses both *in vivo* and *in vitro* have indicated that NC cells show consistent cellular alignment when placed amongst a dense population of cells, whilst those in isolation exhibit more random movements (Rovasio et al., 1983; Tosney, 1978). Thus, when the trajectories of leading edge cells versus those behind the migratory front were compared, a clear persistence of movement was observed amongst those cells surrounded by neighbours (Figure 1.8) (Rovasio et al., 1983). The observation that movement persisted in cells behind the migratory front, implicates contact guidance between NC cells as a mediator of NC cell directionality, at least *in vitro* (Newgreen et al., 1979; Rovasio et al., 1983).

An additional factor when considering the directed movement of NC cells is how local changes in the distribution/availability of ECM receptors within the membrane contribute to the net direction of movement. Firstly however, this requires an understanding of the mechanics of ECM receptor/ECM interactions. In stationary cells FN-receptors are concentrated in particular sites within the cell membrane and attached to microfilaments to form stable cell-substrate adhesions (Figure 1.8B). In contrast, in motile cells FN-receptors are distributed diffusely throughout the membrane enabling rapid recruitment and subsequent disassembly of microfilaments to facilitate movement (Duband et al., 1986). Thus, it could be imagined that regulated changes in the distribution of FN-receptor complexes at particular sites within the membrane (in response to particular ECM substrates), will influence local adhesion and subsequent movement. Consequently, interactions between receptors on the cell surface and the ECM facilitate a highly localised regulation of cell directionality by directly influencing the retraction and/or consolidation of membrane protrusions extended by NC cells during migration (Newgreen et al., 1979).

1.6.2 In vivo *time-lapse analyses suggest NC cell-cell contacts coordinate their migration*

In vivo, time-lapse analyses have demonstrated that NC cells move as an increasingly dense population of cells in highly coordinated streams (Krull et al., 1995; Kulesa and Fraser, 2000). In addition, mitoses amongst migratory NC cells leads to small groups of clonally related cells travelling together, maintaining close contacts (Krull et al., 1995). Interestingly, in the presence of exogenous PNA or ephrin-B1-Fc, both of which alleviate caudal half-somite-derived repulsion, the trajectories of NC cells in this tissue lacked the clear organization of NC cell streams in the rostral half-somite (Krull et al., 1995; Krull et al., 1997). Whether the loss of PNA-based channelling mechanisms enabled the loosening of cell-cell contacts between NC cells, or that it was related to a subsequent loss of rostral half-somite cues (due to the blocking antibodies) remains unknown. However, this implicates contact guidance between NC cells, (via regulated cell adhesion) in combination with somite-localised factors, as key regulators of the defined navigational routes taken by NC cells through the rostral half-somite (Krull, 2001). Although the molecular basis of these NC cell-cell contacts is currently unknown, cad7 is a good candidate as it is expressed in a sub-population of NC cells and its over-expression causes cell aggregation, which is suggestive of adhesion defects (Nakagawa and Takeichi, 1998). In addition, the observation that NC cells travel in groups of dividing

cells (Krull, 1998) and that high cell densities are required for persistent movement (Rovasio et al., 1983), suggests that spatiotemporally regulated NC proliferation might be an important mechanism for regulating NC cell directionality.

1.7 Mechanisms of Eph/ephrin-A signalling

Recently the molecular dissection of Eph/ ephrin signalling via classical biochemical, gene deletion studies and *in vitro* cellular response assays have opened up a wide array of possible signalling mediators and mechanisms for this family of RTKs and their ligands (for review refer (Holmberg and Frisen, 2002)). For the purposes of this review, therefore, only a few relevant signalling topics have been addressed and these include: modes of Eph "forward" and ephrin "reverse" signalling; the role of EphA4 in regulating cell movement and adhesion during tissue remodelling events such as hindbrain segmentation (Mellitzer et al., 1999; Xu et al., 1995; Xu et al., 1999) and somite formation (Barrios et al., 2003; Durbin et al., 1998); and possible downstream mediators of EphA4 signalling that lead to alterations in the cytoskeleton (Kullander and Klein, 2002).

1.7.1 "Forward" and "reverse" signalling pathways

EphA4 is a unique member of the Eph family because it shows promiscuous binding across subclasses, binding both ephrin-A and ephrin-B2, -B3 proteins (Wilkinson, 2000). Given that ephrin-B proteins contained a cytoplasmic domain, it was not surprising to discover that activation of ephrin-B expressing cells facilitated signalling through "ligand-expressing" cells, implicating bi-directional signalling mechanisms in Eph-ephrin activation (Figure 1.9)(for review refer (Kullander and Klein, 2002). Analyses of EphA4-ephrin-B interactions in mouse knock-out studies (Henkemeyer et al., 1996; Kullander et al., 2001b) and zebrafish cell sorting experiments (Mellitzer et al., 1999; Xu et al., 1999) have been useful in the study of the mechanics of Eph ("forward") and ephrin ("reverse") signalling. On the other hand, ephrin-A proteins, that are anchored to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor (Figure 1.9), were initially not thought to be capable of intracellular signalling (Gale et al., 1996). However, mouse knock-out studies and *in vitro* cell adhesion assays have shown that ephrin-A proteins can also facilitate adhesion, thereby mediating "reverse" signalling (Davy et al., 1999; Davy and Robbins, 2000; Holmberg et al., 2000).

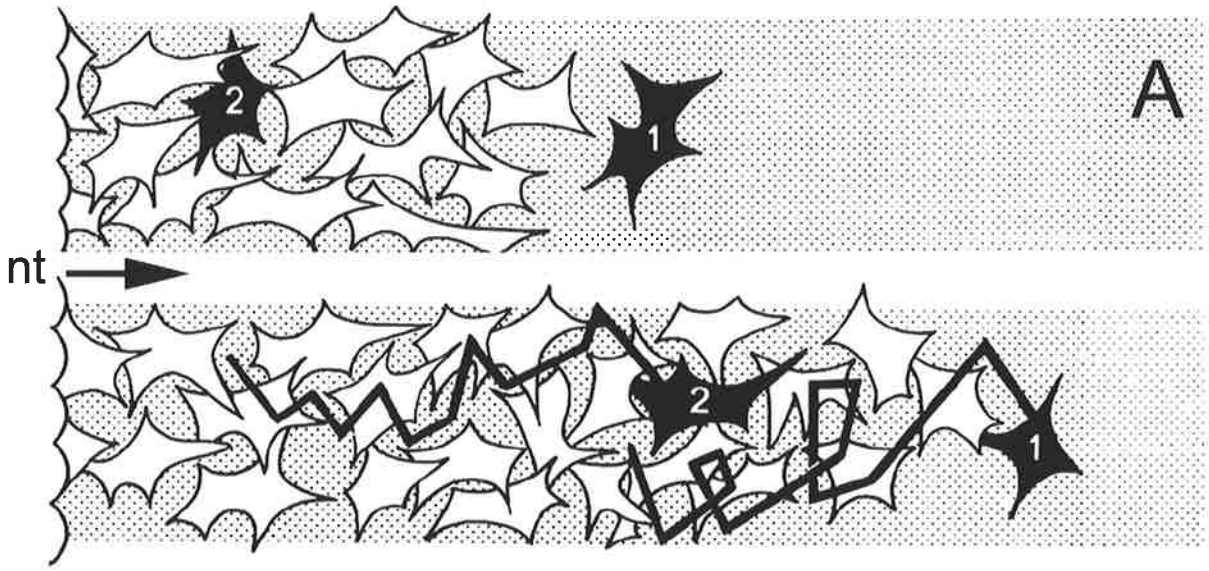
To test the molecular mechanisms driving Eph signalling, multiple different dominant negative approaches have been utilised (reviewed by (Kullander and Klein, 2002)). One approach that has been used successfully in amphibians and also higher vertebrates has been to introduce a C-terminally truncated Eph protein that competes with endogenous receptors for binding to ephrin ligands (Durbin et al., 1998; Henkemeyer et al., 1996; Xu et al., 1995). However, the discovery of possible signalling pathways mediated through ephrin-B (Henkemeyer et al., 1996; Holland et al., 1996) and more recently ephrin-A proteins (Davy et al., 1999; Davy and Robbins, 2000) has complicated the analysis of phenotypes generated using this dominant-negative strategy.

Biochemical analysis of specific residues in the Eph proteins has revealed that the juxtamembrane tyrosine residues on the C-terminal side of the membrane (Figure 1.9) play a critical role in regulating Eph catalytic function (Binns et al., 2000). Upon ephrin-binding, these residues are auto-phosphorylated, facilitating a conformational change that enables signal transmission through the catalytic domain (Binns et al., 2000; Kullander and Klein, 2002). Genetic dissection by mutation of these residues and others within specific Eph domains has revealed distinct kinase-dependent and kinase-independent functions for EphA4 in the formation of major axon tracts in mice (Kullander et al., 2001a). In addition, clustering of Eph receptors into oligomeric complexes appears to be an important means of generating context-specific cellular responses; an effect possibly mediated by the sterile α motif (SAM) located at the C-terminal end of Eph receptors and ephrin-B proteins (Stein et al., 1998). Furthermore, biochemical analyses of the downstream mediators of Eph signalling have implicated specific interacting proteins, leading to the identification of specific Eph signalling pathways with multiple cellular outcomes including cytoskeletal re-arrangement and adhesive functions during embryogenesis (Kullander and Klein, 2002). Thus, the elucidation of separate and distinct functions not only for Eph "forward" but also ephrin "reverse" signalling via mutational analysis has enabled a much more targeted approach to understanding the molecular mechanisms of Eph signalling.

Figure 1.8- Factors influencing NC cell trajectories *in vitro*.

(A) NC cell trajectories at the leading edge of the migratory front (cell 1) show a less persistent direction of movement than those in the middle of the migratory stream (cell2). nt = neural tube. Image taken from (Rovasio et al., 1983).

(B) Cell movement is regulated by interactions between the FN-receptor complex and the ECM such that localised changes in the distribution of these complexes within the membrane directly influence the direction of migration. In stationary cells, actin microfilaments are clustered with FN-receptors to form a stable adhesive complex. In motile cells, the distribution of FN is diffuse along the entire surface of the cell, enabling rapid recruitment and de-stabilisation of actin microfilaments.


A
nt
2
1
2
1

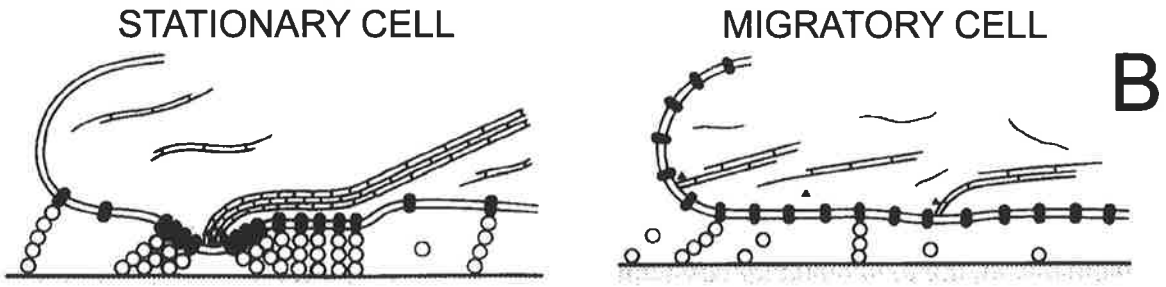
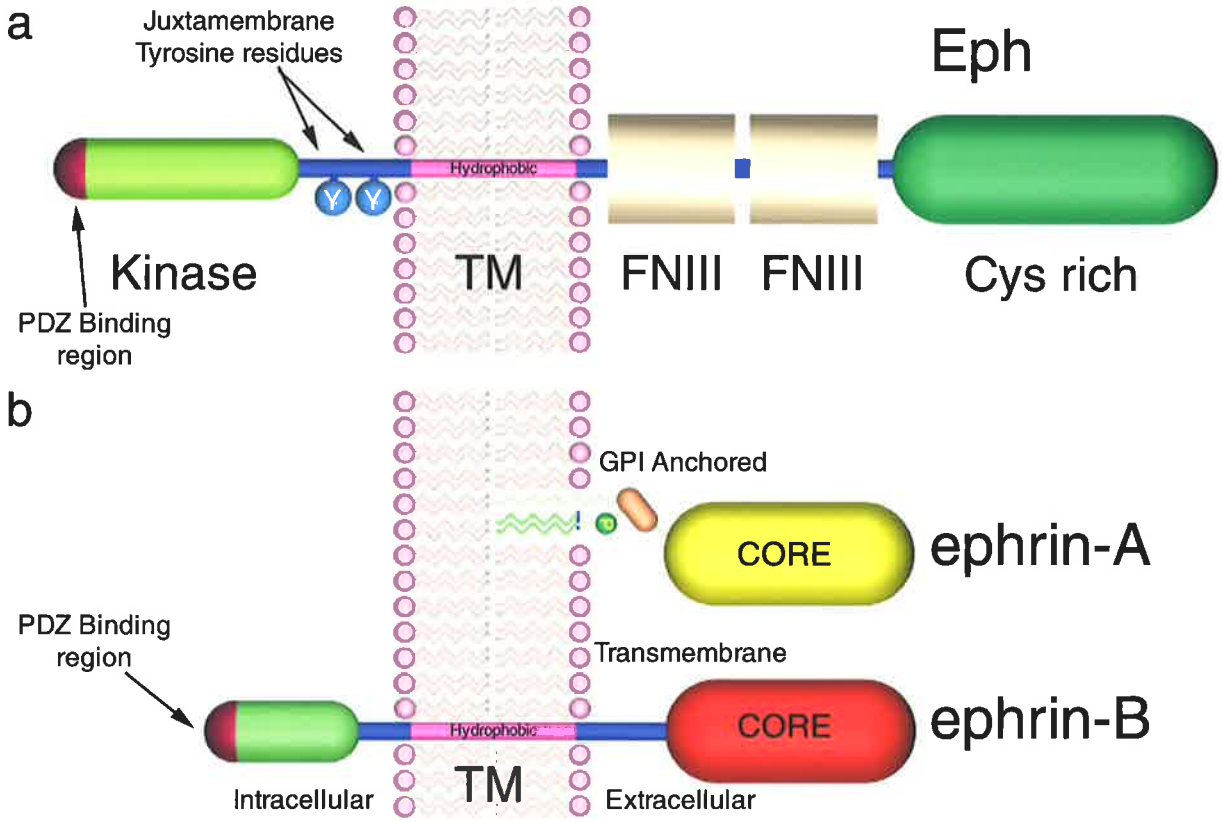

STATIONARY CELL
MIGRATORY CELL
B

Figure 1.9- Schematic diagram illustrating the structure and critical residues involved in (a) Eph or (b) ephrin-A or ephrin-B signalling pathways.

Eph protein structure consists of a cysteine rich N-terminal domain followed by two fibornectin typeIII (FNIII) repeats, the hydrophobic transmembrane domain. Two juxtamembrane tyrosine residues lie on the cytoplasmic side of the membrane. The kinase domain represented in green and followed by the C-terminal PDZ binding region.
Ephrin-A proteins are anchored in the membrane via a glycosyl-phosphatidylinositol (GPI) anchor.
Ephrin-B proteins are transmembrane proteins containing N-terminal core domain, hydrophobic mebrane-spanning region and intracellular kinase domain. Similar to Eph receptors, ephrin-B proteins have a PDZ-binding region at their C terminus.

Image taken from (Tosch, 2002).


a
Juxtamembrane
Tyrosine residues
Eph
Hydrophobic
Y
Y
Kinase
TM
FNIII
FNIII
Cys rich
PDZ Binding
region
b
GPI Anchored
CORE
ephrin-A
PDZ Binding
region
Transmembrane
Hydrophobic
CORE
ephrin-B
Intracellular
TM
Extracellular

Ephrin-A proteins have largely been implicated in mediating repulsion of cells upon contact with an Eph receptor (O'Leary and Wilkinson, 1999). *In vitro* experiments have shown that cellular repulsion can be mediated by regulated cleavage of the ephrin-A molecule from the membrane upon binding to an EphA receptor, leading to movement of the ephrin-A-expressing cell away from the EphA-expressing cell (Hattori et al., 2000). However, analysis of neural tube closure defects in ephrin-A5 mutant mice, have suggested that in addition to contact repulsion, ephrin-A5 signalling might also be capable of mediating adhesion (Holmberg et al., 2000; Holmberg and Frisen, 2002). Given that the ephrin-A5 receptor, EphA7 was also expressed in the dorsal neural tube, along with three other splice variants, it was hypothesised that the defects arose through an adhesive interaction between EphA7 and ephrin-A5. Over-expression of a truncated form of EphA7 altered the balance between adhesive and de-adhesive (forward signalling) signalling pathways resulting in a switch from repulsion (in the presence of full-length EphA7) to adhesion (with the truncated Eph receptor and subsequent repression of Eph-mediated signalling) (Holmberg et al., 2000). Thus, ephrin-A5 activation can lead to adhesion (in the cell on which ephrin-A5 is expressed) and not just repulsion of the Eph-expressing cell (Holmberg and Frisen, 2002). Later characterisation of ephrin-A5 signalling pathways *in vitro* has demonstrated that these proteins are organised into lipid rafts along the cell membrane and facilitate integrin-mediated adhesion in neuronal cells, via Fyn tyrosine kinase (Davy et al., 1999; Davy and Robbins, 2000).

1.7.2 EphA4 contributes to cell-cell adhesion and motility mechanisms required for tissue morphogenesis

Two major examples of EphA4-mediated regulation of cell-cell adhesion in the developing embryo include the establishment of segmental restriction and the subsequent separation of adjacent cell populations in the vertebrate hindbrain (Mellitzer et al., 1999; Xu et al., 1999) and also in somite epithelialization (Barrios et al., 2003). An additional site of Eph-mediated cell-cell adhesion during embryogenesis is in the gastrulating *Xenopus* embryo, possibly acting through C-cadherin (Winning et al., 1996). The cellular and molecular mechanisms of EphA4 actions on cell-cell adhesion are discussed in detail below.

In the vertebrate hindbrain, EphA4 is expressed in rhombomeres (r) 3 and 5 whilst ephrin-B proteins are distributed in r2, r4 and r6 (Nieto et al., 1992). Utilising a carboxy-terminal truncated EphA4 receptor to compete with endogenous receptors for binding, Xu and colleagues (1995) showed that EphA4 is important for the maintenance of cellular boundaries

between rhombomere segments (Xu et al., 1995). Mosaic activation of Eph receptors in r3 and r5 by injection of ephrin-B2 messenger RNA, resulted in ephrin-expressing cells sorting to the boundaries of the Eph-receptor containing segments, whilst their behaviour in ephrin-expressing segments was uniform. This indicated that the expression of receptor on one cell and ligand on the other at the interface between two boundaries was necessary to prevent cells intermixing between boundaries (Xu et al., 1999). Furthermore in an *in vitro* cell-intermingling assay, it was established that bi-directional signalling between adjacent cells expressing EphA4 or EphB2 receptor and ephrin-B2 was sufficient to prevent cell mixing, whilst unidirectional signalling through ephrin-B2 prevented cell communication through gap junctions (Mellitzer et al., 1999). Thus, bi-directional signalling between Eph receptors and ephrins in the hindbrain is required to maintain a de-adhesive response between cells of adjacent rhombomere populations preventing cells from mixing with one another, whilst uni-directional signalling between populations restricts cell-cell communication (Mellitzer et al., 1999).

Similar to hindbrain segmentation, EphA4/ephrin-B2 interactions have also been shown to play a key role in boundary formation during segmentation of the vertebrate paraxial mesoderm (Barrios et al., 2003; Durbin et al., 1998). EphA4 and ephrin-B2 define the presumptive anterior and posterior segments respectively, in the pre-somitic mesoderm (PSM) in chick, mouse and fish. Disruption of Eph signalling by injection of dominant negative Eph or ephrin constructs perturbs the final stages of somite formation (Durbin et al., 1998). More recently, the mechanisms through which EphA4 might regulate somite boundary formation has been investigated using the zebrafish segmentation mutant, *fused somites (fss)* (Barrios et al., 2003). Zebrafish embryos lacking *fss* (normally expressed in the PSM) fail to undergo the mesenchymal-to-epithelial transformation required to form distinct somite boundaries (Barrios et al., 2003). Preliminary analysis suggested that Eph/ephrin expression was disrupted in the presumptive somites of *fss* -/- mutants, making Eph/ephrin signalling a good candidate as a possible cell surface mediator of the effects observed (Barrios et al., 2003). To test this hypothesis a number of different experiments were conducted, and the results are summarised as follows: Firstly, restoration of EphA4 signalling in wildtype cells led to the formation of a boundary between EphA4-expressing and endogenous *fss* -/- cells; and secondly, EphA4 expression in *fss* -/- cells activated an epithelial morphology, including apical redistribution of β-catenin and the adoption of a columnar cell shape (Barrios et al., 2003). Thus, EphA4 appears to be a key regulator of the morphological and cell-cell adhesive

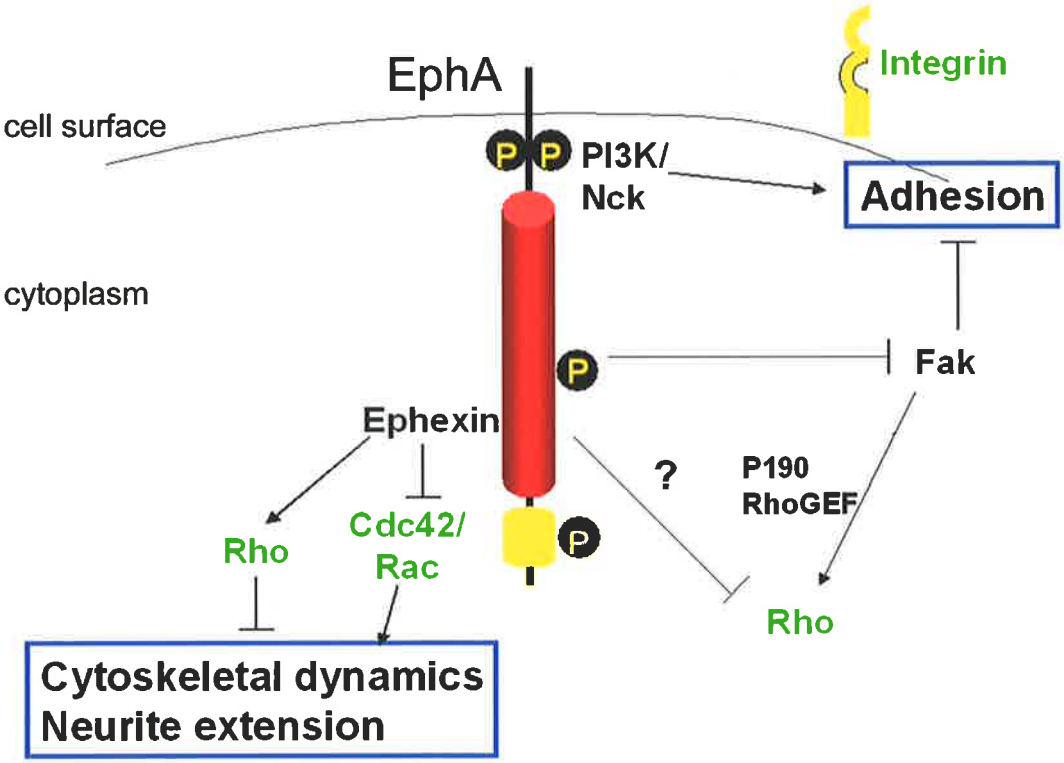
changes required for somite formation (Barrios et al., 2003; Durbin et al., 1998; Durbin et al., 2000).

In the *Xenopus* embryo, perturbation of EphA4 signalling using a chimeric receptor containing the extracellular domain of human Epidermal Growth Factor Receptor (EGFR) attached to the intracellular domain of EphA4 (termed EPP), have provided valuable insights into the possible signalling mechanisms of EphA4 (Park et al., 2004; Winning et al., 1996; Winning et al., 2002; Winning et al., 2001). In addition to a restricted expression pattern in the hindbrain and other sites of nervous system development, *EphA4* mRNA is expressed at low levels in the mesoderm during gastrulation (Winning and Sargent, 1994). Injection of *EPP* mRNA into fertilised frog eggs resulted in significant cell-cell dissociation prior to gastrulation, in a manner that was dependent on the presence of functional intracellular signalling through EphA4 (Winning et al., 1996). Co-injection of *C-cadherin* messenger RNA, a molecule that is a known key regulator of cell-cell adhesion during gastrulation, rescued the EPP phenotype. Thus, C-cadherin might be a downstream target of EphA4, acting as an additional local regulator of cell-cell adhesion in concert with the more ubiquitous cadherin-based adhesion system (Winning et al., 1996).

Molecular analysis of putative downstream targets of EphA signalling that might mediate changes to cellular adhesion has revealed multiple distinct signalling systems (Kullander and Klein, 2002). One of these is via Focal Adhesion Kinase (FAK), which acts as an intermediary between the cell surface and signalling through integrins bound to the ECM (Kullander and Klein, 2002). In prostrate carcinoma cells, activation of endogenous EphA2 receptors inhibited integrin function and subsequently perturbed cell spreading, adhesion and migration. In addition, activation of EphA2 by ephrin-A1 induced dephosphorylation of FAK and as a consequence, further inhibited cell-substrate attachment (Miao et al., 2000). Providing a possible link between the action of EphA proteins on cellular adhesion (both cell-substrate and cell-cell) and effects on the cytoskeleton, it has recently been demonstrated that FAK can activate RhoA via a specific p190RhoGEF pathway in mouse neuroblastoma cells (Zhai et al., 2003). Thus, negative regulation of FAK activity by EphA receptors could indirectly inhibit RhoA activation in neuronal cells. Alternatively, EphA4 could directly interact with the p190RhoGEF and inhibit RhoA activity specifically through this protein. In sum, this provides just one example whereby EphA interactions with a specific cytoplasmic kinase, can elicit changes in cellular adhesion and cytoskeletal architecture, thereby influencing multiple cellular processes during development (summarised in Figure 1.10).

Figure 1.10- Schematic diagram summarising downstream interactors of EphA proteins and the subsequent effects on cell-substratum adhesion/cytoskeletal extension.

EphA receptors interact with Focal adhesion Kinase (Fak) to negatively regulate cell-substratum adhesion. In addition, via p190RhoGEF, EphA receptors influence cytoskeleetal dynamics. In neurons, EphA4 interacts with ephexin to stimulate Rho activity simultaneously inhibiting Rac/Cdc42 and thereby influencing the cytoskeleton and also neurite extension. In non-neuronal cells, EphA4 inhbits RhoA activity, via an ephexin-independent-pathway. Schematic adapted from (Kullander and Klein, 2002).


EphA
Integrin
cell surface
P P
PI3K/
Nck
Adhesion
cytoplasm
P
Fak
Ephexin
?
P190
RhoGEF
Rho
Cdc42/
Rac
P
Rho
Cytoskeletal dynamics
Neurite extension

1.7.3 EphA4 and Rho

EphA4 has been shown to interact with the Rho family of GTPases, known to be involved in actin reorganisation in response to extracellular signals (Braga, 2000). The outcome of EphA4 interactions with Rho proteins and subsequently on the cytoskeleton, are dependent on the cellular context in which it is acting and are summarised in Figure 1.10. EphA4 signalling in growth cones activates RhoA through the RhoGEF, Ephexin, leading to growth cone collapse (Shamah et al., 2001). In contrast, in non-neural cells, EphA4 activation appears to have a negative effect on RhoA activity, leading to cell-cell dissociation (Kullander and Klein, 2002). The actions of EphA4 on Rho proteins (and subsequently on the cytoskeleton) within different cell types are compared and contrasted below.

The most well documented scenario in which EphA4 interacts with Rho proteins has been in retinal ganglion cell (RGC) axons, where it activates RhoA, causing subsequent growth cone collapse. Addition of the RhoA inhibitor, C3 transferase, abolished ephrin-A5 induced growth cone collapse, suggesting that RhoA was a critical component of the action of ephrin-A5 (and resultant EphA activation) on RGC's. Furthermore, immuno-precipitation and western analysis of growth cone extracts grown on ephrin-A5 indicated that the activity of RhoA increased whilst Rac activity decreased in ephrin-A5 stimulated growth cones, providing a direct link between EphA activation and RhoA activity (Wahl et al., 2000). These studies suggested that Rho proteins are likely possible mediators of ephrin-induced growth cone collapse, a response that *in vivo*, results in the correct targeting of retinal axons to the nasal and not temporal retina (Wilkinson, 2000).

In a screen aimed at identifying possible mediators between EphA signalling pathways and Rho proteins in axon growth cones, a novel RhoGEF was identified, termed Ephexin (Shamah et al., 2001). Ephexin belongs to the Dbl family of guanine nucleotide exchange factors (GEF's), and facilitates the exchange of bound GDP for GTP, thereby activating Rho GTPases (Shamah et al., 2001). GEF's act in concert with negative regulators of Rho, the GTPase activating proteins (GAP's), to achieve regulated changes in the actin cytoskeleton that are coordinated with local environmental cues (Schmitz et al., 2000). In retinal ganglion cell axons, EphA4 interacts with Ephexin through specific residues in the kinase domain and promotes RhoA activity in RGC axons, causing growth cone collapse (Shamah et al., 2001). Furthermore, the activated EphA4/ephexin complex bound preferentially to RhoA, only weakly activating Rac1 and Cdc42 (Shamah et al., 2001). Studies in fibroblasts have

established that activation of each of the three main subsets of the Rho protein family (i.e. RhoA, Cdc42 and Rac1) has a distinct characteristic effect on cell morphology (Nobes et al., 1998). RhoA promotes stress fibre formation, Rac activity promotes lamella formation whilst Cdc42 activity is associated with membrane ruffling (Schmitz et al., 2000). Thus, EphA4 inhibiting RhoA concurs with its role in promoting growth cone collapse in neuronal cells. Similar effects have been found in vascular smooth muscle cells, wherein EphA4 promotes actin stress fibre formation/ contractility through RhoA activation, and this effect is mediated by the Ephexin homologue, Vsm RhoGEF (Ogita et al., 2003). These data implicate Ephexin as an important mediator of EphA signalling in axon guidance and also vasculature remodelling (Kullander and Klein, 2002; Ogita et al., 2003).

In contrast to the positive influence of EphA4 on RhoA activity in axonal growth cones, EphA4 inhibits the function of Rho in the gastrulating *Xenopus* embryo, causing cell-cell dissociation and significant disruption to embryo morphology (Winning et al., 2001) (refer Section 1.7.2). An interdependence between EphA4 signalling and RhoA pathways in the blastula of *Xenopus* embryos was demonstrated in two ways: firstly, addition of the Rho inhibitors, C3 transferase and Toxin A (a general Rho function inhibitor) reproduced the EPP phenotype; and secondly, co-injection of constitutively active RhoA prevented EPP-induced cell dissociation. Thus, this implicated EphA4 as a negative regulator of RhoA activity (Winning et al., 2002). To determine if the inhibitory activity of EphA4 on Rho was also mediated by ephexin, (the RhoGEF interacting with EphA4 in retinal growth cones), Winning and colleagues (2002) co-expressed ephexin with the over-active EphA4 construct, EPP, and found that co-injection of these two molecules rescued the dissociation phenotype. On the basis of this "rescue" phenotype and the assumption that ephexin was activating RhoA in this cellular context (rather than inhibiting it), the authors concluded that EphA4 was acting through an ephexin-independent pathway to inhibit RhoA during *Xenopus* gastrulation (Figure 1.10)(Winning et al., 2002). However, no biochemical analysis accompanied this data, making it difficult to draw any precise conclusions about the role of ephexin in these EphA4-mediated effects. Furthermore, the exact protein distribution of EphA4, RhoA or ephexin in these cells at the time of regulated changes in cell-cell adhesion during gastrulation has not been detailed, therefore putting into some doubt the possible *in vivo* role of EphA4. In sum, characterisation of this phenotype has yielded interesting insights into the modes of EphA4 action on the cytoskeleton in an *in vivo* environment.

1.8 Benefits of using the chicken embryo for the investigation of NC cell development

The chick was chosen as the vertebrate model in which to focus the analyses in this work for a number of reasons. Firstly, owing to its external gestation, the chicken embryo is a highly accessible organism for experimental manipulation. Thus, operations can be carried out and the embryo re-incubated for later analysis. Secondly, with the advent of *in ovo* electroporation, both gain-of-function and loss-of-function analyses can be carried out in this species in a spatiotemporally specific manner (Krull, 2004). Thirdly, the use of quail-chick chimaeras have provided a reliable means of cell labelling and thus, an extensive body of knowledge, detailing the exact timetable of NC cell development in chicken embryogenesis, is available for use in experimental analyses (Le Douarin and Kalcheim, 1999). Finally, the short gestation period and relative affordability of avian embryos make this a highly suitable organism for the study of avian NC cell segmentation.

Prior to *in ovo* electroporation, gain and loss-of-function experiments in the chick were limited to exogenous blocking proteins/bead experiments or more descriptive studies on particular cell and tissue interactions during development (Bronner-Fraser, 1985; Bronner-Fraser, 1993b; Koblar et al., 2000; Krull et al., 1995). *In ovo* electroporation has enabled a wave of targeted genetic approaches to now be utilised in the avian embryo (Itasaki et al., 1999). Electroporation is a procedure that utilises an electric current to burst open cell membranes and thereby allow entry of negatively charged plasmid DNA into a cell. *In ovo* electroporation applies this basic principle of DNA transfection to the chicken embryo and involves injection of plasmid DNA into an embryonic cavity and the subsequent application of a current to drive DNA expression into particular regions of the embryo (Krull, 2004). As the main key requirement for successful electroporation is the presence of a cavity in which to inject plasmid DNA, this technique has been applied to multiple different tissue regions during both embryogenesis and also in the adult (Itasaki et al., 1999; Krull, 2004; Momose et al., 1999; Swartz et al., 2001a). Some of these areas include in the eye and somite (Chen and Ruley, 1998; Swartz et al., 2001a; Swartz et al., 2001b); whilst the neural tube has been exploited as an electroporation target, partly because of its fundamental role in embryogenesis and also the ease of injection into this tissue (Swartz et al., 2001a). As well as the analysis of specific gene function, electroporation also offers potential for studies into promoter

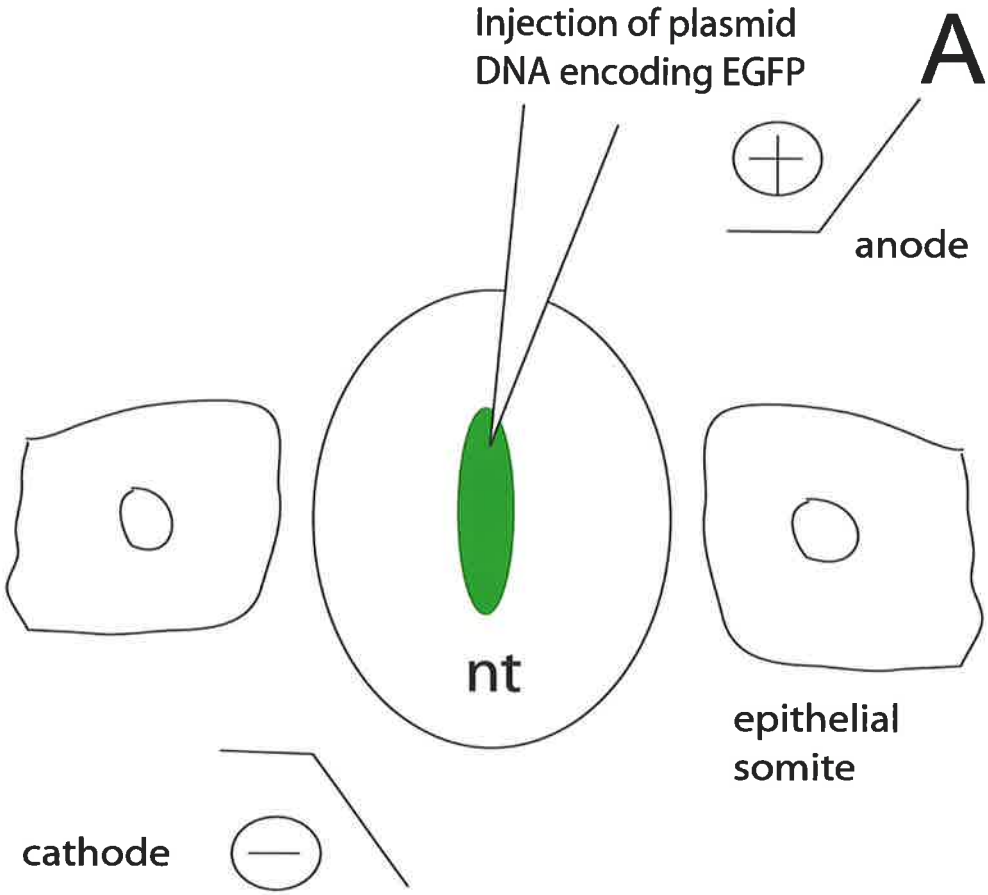
regulation and has been used to successfully dissect critical Hox promoter regions during axial specification (Itasaki et al., 1999).

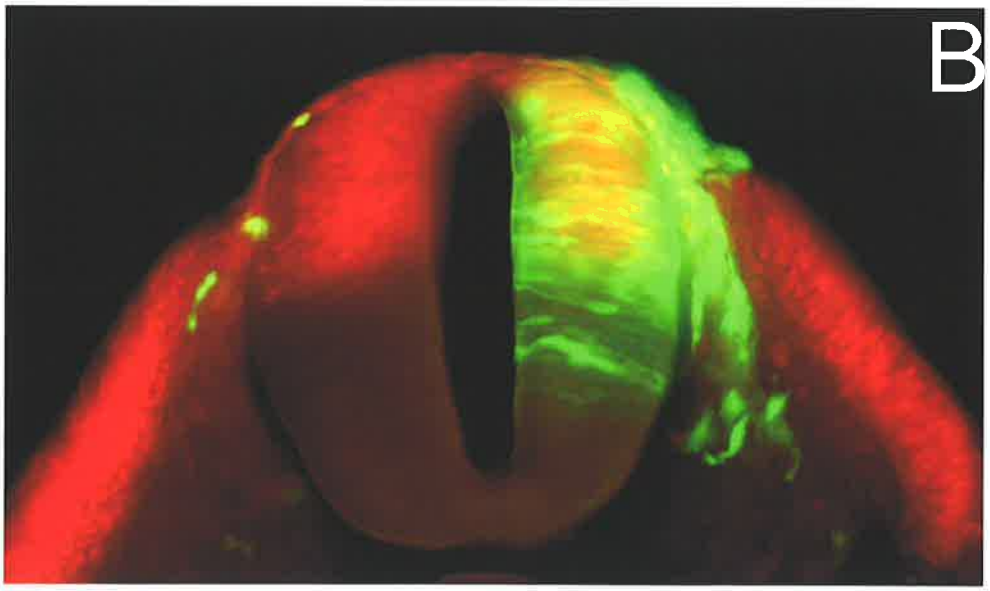
After injection of plasmid DNA into the region of interest, precise spatio-temporal targeting of particular tissues can be achieved by specific placement of electrodes or microelectrodes (Momose et al., 1999; Swartz et al., 2001a). In the neural tube, placement of electrodes in a dorsoventral orientation enables the direct targeting of a specific quadrant of the neural tube (Figure 1.11). Thus, electroporation of the dorsal quadrant can lead to expression in NC cells delaminating from this region (as in Figure 1.11) as well as in specific neuron populations and has been used extensively to test the role of various proteins in both NC cell development and regulation of CNS neuron specification (Cheung and Briscoe, 2003; Dottori et al., 2001; Poh et al., 2002). By reversal of the electrode polarity, electroporation can also be used to target ventral regions of the neural tube (Eberhart et al., 2004; Eberhart et al., 2002).

Previously *in ovo* electroporation studies have been limited to gain-of-function approaches and loss-of-function analyses using dominant negative constructs (Cheung and Briscoe, 2003; Dottori et al., 2001; Eberhart et al., 2004; Eberhart et al., 2002; Kos et al., 2001). More recently however, specific knockdown approaches have been applied to chicken embryos including the use of dsRNA, RNAi (Pekarik et al., 2003) and also morpholinos (Kos et al., 2001; Kos et al., 2003). Although these techniques still require some optimisation to achieve a similar degree of reliability as standard dominant negative approaches, they promise to add to the growing list of gene manipulation techniques available for use in the chick embryo (Krull, 2004).

Figure 1.11- Schematic diagram illustrating targeted electroporation of the dorsal quadrant of the neural tube and subsequent expression of EGFP on delaminating NC cells.

(A) Schematic diagram of neural tube in transverese section showing injection of plasmid DNA into the neural tube lumen and dorso-ventral orientation of electrodes to target the dorsal quadrant of the neural tube.
(B) Transverse section of stage 20 embryo expressing EGFP (green) and stained with Pax7 to highlight the dorsal neural tube and developing somite (red).


Injection of plasmid
DNA encoding EGFP
A
anode
nt
epithelial
somite
cathode


B

1.9 Aims of this research

As described in the above review, the coordinate regulation of NC cell migration by multiple environmental cues within the somite and between PNS precursor cells creates the metameric patterning of the PNS. Given the role previously described for Eph/ephrin-B interactions (Koblar et al., 2000; Krull et al., 1997; Wang and Anderson, 1997) in dictating trunk NC cell segmentation, and the absence of a phenotype in ephrin-B or EphB2/B3 mouse mutants (Birgbauer et al., 2000; Compagni et al., 2003; Davy et al., 2004; Henkemeyer et al., 1996; Orioli et al., 1996), it is clear that there is still much more to learn about the mechanisms driving PNS segmentation and how Eph/ephrin interactions are involved. In addition to the described role for Eph/ephrin-B interactions, the expression of EphA7 in the caudal half-somite during the migration of PNS precursors through the rostral-half (Araujo and Nieto, 1997) and reports of ephrin-A2 and –A5 distribution on the DRG and their co-expression along with EphA4 on motor axons in the hindlimb region (Eberhart et al., 2000), suggests that interactions between Eph/ephrin-A might contribute to the segmental migration of NC cells. The overall aim of this research was therefore to determine whether interactions between Eph/ephrin-A might contribute to the segmental migration of NC cells.

The initial specific aim of this research was to further characterise the expression of Eph/ephrin-A proteins during segmentation of the PNS, taking advantage of receptor-F and ligand-Fc proteins that enable a global view of the distribution of ephrin-A and EphA proteins in the embryo respectively. Having established possible candidate Eph/ephrin-A molecules, a more detailed analysis of their expression patterns was carried out. Based on the outcome of the experiments that addressed the first aim, the second aim was to address the role of Eph/ephrin-A candidate genes in PNS segmentation utilising *in ovo* electroporation. This technique is a transient, transgenic system for gene expression that enables gain and loss-of-function approaches to be conducted *in vivo* (Swartz et al., 2001a). Pending the outcome of these functional analyses, the final aim was to further dissect the results from *in vivo* experiments using a well-established *in vitro* culture system for quail neural tube explants (Newgreen and Gibbins, 1982). Quail embryos have been used as a reliable alternative to chicken embryos as they have a similar program of NC cell development but are more amenable to culture conditions (Newgreen, 1999). The culturing technique involves electroporation and subsequent plating of electroporated neural tube explants on substrate-bound FN. Following processing for various markers of EMT, analysis of these explants

would then enable the identification of specific roles for candidate Eph molecules in NC cell EMT (Newgreen and Minichiello, 1995; Newgreen and Minichiello, 1996).

Chapter 2 – Materials and Methods

2.1 Materials

2.1.1 Antibiotics

Antibiotic	Concentration	Source
ampicillin	25μg/ml	Sigma
kanamycin	30μg/ml	Sigma

2.1.2 Antibodies

Primary Antibodies

Antibody	Host	Concentration	Dilution	Source
anti-β-catenin	mouse	250μg/ml	1:100	Transduction Laboratories
anti-βIII Tubulin monoclonal (TuJ1)	mouse	1mg/ml	1:1000	Promega
anti-EphA4	rabbit	antiserum	1:3000	David Wilkinson
anti-ephrin-A5	rabbit	0.52μg/ml		Elena Pasquale
anti-ephrin-B1	rabbit		1:500	Elena Pasquale
anti-GFP	goat		1:400	Rockland
anti-laminin	rabbit		1:200	Sigma
anti-SC-1	rabbit		1:2000	Murray Hargraves
anti-Sox 9/ 10 (Sox E)	rabbit		1:6000	Don Newgreen
HNK1	mouse		1:2	Cathy Krull
HNK1	mouse		1:20	Don Newgreen
N-Cadherin (anti-A cell adhesion molecule monoclonal)	mouse	mouse ascites fluid	1:100	Sigma
quail Pax-3 monoclonal	mouse		1:200	C. Ordahl
Terminal deoxynucleotidyl transferase (TdT) (TUNEL	bovine		1:10	In Situ Cell Death Detection Kit, TMR

reaction)			red, Roche

Secondary Antibodies

Antibody	Concentration	Dilution	Source
Alexa488 anti-sheep		1:400	Molecular Probes, OR
Alexa488 goat anti-rabbit IgG	1mg/ml	1:500	Molecular Probes, OR
biotinylated anti-mouse IgM		1:100	Jackson ImmunoResearch, PA
Cy3 donkey anti-mouse IgG	0.7mg/ml	1:200-1:500	Jackson ImmunoResearch, PA
Cy5 donkey anti-mouse IgG	0.7mg/ml	1:200-1:500	Jackson ImmunoResearch, PA
Cy5 donkey anti-rabbit IgG	0.75mg/ml	1:200-1:500	Jackson ImmunoResearch, PA
Cy5 goat anti-rabbit IgG		1:150	Cathy Krull
donkey anti-rabbit Alexa594		1:1500	Molecular Probes, OR
RITC goat anti-mouse IgM		1:150	Cathy Krull
Streptavidin AMCA		1:250	Jackson ImmunoResearch, PA
TMR red labelled nucleotides (TUNEL reaction)			In Situ Cell Death Detection Kit, TMR red, Roche
TRITC-Phalloidin	1mg/ml	1:2000	Sigma
alkaline phosphatase (AP) anti-digoxigenin Fab		1:2000	Boehringer Mannheim

2.1.3 Buffers and Solutions

Buffer/solution	Ingredients
Agarose gel loading buffer (10x)	2.5ml glycerol 2.4ml 0.5M Na_2EDTA 0.024g bromophenol blue (SIGMA)
Blocking solution (BS)	PBT +10% horse serum (horse serum heat inactivated for 10 min at 37°C)
Colony cracking solution	125µl 0.4M NaOH 50µl 10% SDS 10µl 0.5M EDTA 125µl 80% Glycerol 715µl dH_2O touch bromophenol blue
PBT	PBS + 0.01% TritonX
Phenol/ chloroform	50% Phenol 48% chloroform 2% isomyl alcohol stored under TE
Phosphate buffered saline (PBS)	7.5mM Na_2HPO_4 2.5mM NaH_2PO_4 145mM NaCl
Ringer's solution	4000ml of ddH_2O 28.8g NaCl 0.68g $CaCl_2$ or 0.90g $CaCl_2 \cdot 2H_2O$ 1.48g KCl 0.460g Na_2HPO_4

Buffer/solution	Ingredients
TAE	40mM Tris-acetate 20mM sodium acetate 1mM EDTA pH 8.2
TE	10mM Tris-HCL pH 7.4 1mM EDTA
RIPA (0.2μm filter sterilised) (for 100ml, RNAse free)	89.8 ml H_2O 150mM NaCl (3ml of 5M stock) 1% Nonidet-P-40 (1 ml) 0.5% w/w sodium deoxycholate (500mg, allowing that the 100ml weigh 100 g) 0.1% SDS (1 ml of 10% SDS stock, pH 7.2) 1mM EDTA (200 μL of 0.5M stock, pH 8.0) 50mM Tris (pH 8.0) (5ml of 1 M stock, pH 8.0)
4% para-formaldehyde (PFA) (for 50ml, make fresh, RNAse free)	42.5ml H_20 at 67-70°C (use temperature stirrer in hood, heat level No. 3) 2g PFA (can use the non-RNAse free, but be careful with it) 12.5μl 4M NaOH (soln. should now turn clear) 1.25 ml 5M NaCl 5 ml 0.1 M PO_4 (actually a mix of NaH_2PO_4 and Na_2HPO_4), pH 7.4 (from stocks) → pH 7.52 add 2.5μl conc. HCl → pH 7.3 let cool in hood to room temp., then filter (0.2 μm), and chill at 4°C
PG-PBT (post-fixative) (for 10 ml, make fresh, RNAse free)	4% PFA 0.2% EM-grade glutaraldehyde in PBT {80 μL of 25% glutaraldehyde stock made in PBT

Buffer/solution	Ingredients
	(stored at -20°C) into 10ml 4% PFA}
Hybridisation buffer (HB) (For 50 ml, make fresh, RNAse free)	12.4ml H_2O 25ml 50% ultrapure formamide (stored at -20°C) 5x SSC, pH4.5 (use a 20x SSC stock solution.* pH4.05, acidified with citric acid). The acidified stock may be made ahead of time (500ml) {12.5ml} 50 μg/ml heparin (25μl of 100 mg/ml stock) 0.1% Tween-20 (50μl) *NB: It's easier to make a non-RNAse-free 20x SSC stock solution. and then DEPC-treat the final solution. Use 130μl (= 0.26 μL/ml) DEPC, stir overnight, and then autoclave x45 minutes at 120°C
SSC (20x) (for 500ml, RNAse free)	400ml H_2O 87.7g NaCl 31.5g (44.1g per Maniatis) citric acid monohydrate (NOT trisodium dihydrate) {pH ~ 1.0} adjust to pH 4.05 w/ NaOH pellets (about 12.4g) and adjust volume to 500 ml
SSC-FT (post-HB, more stringent than HB) (for 50 ml, make fresh)	20ml H_2O 5ml 2x SSC, pH 4.5 (OK to use the 20x stock) 25ml 50% formamide 50μl 0.1% Tween-20
TBST (10x)	5ml H_2O (to start; obviously you need

Buffer/solution	Ingredients
Dilute (100ml for washes) to 1x from a stock. (for 100ml)	eventually to bring up the volume to 100ml) 8g NaCl 0.2g KCl 25ml 1M Tris pH 7.5 10ml 10% Tween (= 1% Tween; use 1ml Tween-20 in 9 ml H_2O)
Tris pH 7.5 (for 100ml)	22.3ml 1M Tris 77.7ml 1M Tris HCl Adjust pH as necessary
Alkaline phosphate buffer (APB) (make fresh daily from stocks, 100 ml) (2μm filter sterilised)	100mM NaCl (2ml of 5M stock) 50mM $MgCl_2$ (5ml of 1M stock; 20.33g $MgCl_2$ (s) into 100ml H_2O = 1M) 0.1% Tween-20 (100μl) 100mM Tris, pH 9.5 (10ml of 1M stock; 94.83ml base + 5.17ml Tris HCl → pH 9.5, 100ml) H_2O, 83ml 2mM Levamisole (48mg)
NBT ** (75mg/ml in 70% DMF)	3.5ml DMF 1.5 ml H_2O 0.375g NBT in APB Store at -20°C and dilute as needed
BCIP**	50mg/ml in 100% DMF Store at -20°C and dilute as needed
	** **These are made together, as one solution, with aliquots of NBT and BCIP in APB/ 2mM Levamisole

Buffer/solution	Ingredients
NBT/ BCIP/ APB (for 10ml)	45µl NBT 35µl BCIP 9.92ml APB
PBT/ 1% acetic acid/ 1mM EDTA (to stop in situ colour reaction)	500ml PBS (13ml 5M NaCl, 50ml 0.1M PO_4, 431ml H_2O) 5ml glacial acetic acid 0.5ml Tween-20 1ml 0.5M EDTA
TBST/ 2mM Levamisole	100ml 1x TBST 48mg Levamisole

2.1.4 Reagents and Enzymes

Reagent/enzyme	Details	Source
1Kb Plus Ladder		Gibco
Agarose	LE, analytical grade	Promega
Avidin	Avidin/ Biotin Blocking Kit	Vector Laboratories
BigDye Reaction Mix		Applied Biosystems
Biotin	Avidin/ Biotin Blocking Kit	Vector Laboratories
Calf Intestinal Alkaline Phosphatase (CIP)		Boehringer Mannheim
ethidium bromide		Sigma
Low melting point agarose	ultra low gelling temperature	Roche
Pfu DNA polymerase, Pfu reaction buffer (10x)		Stratagene
T4 DNA ligase, ligase buffer (2x)		Promega
TritonX-100		Sigma
Trizol		Sigma
Tween-20		Sigma

Reagent/enzyme	Details	Source
Fast Green		
Indian Ink	Calligraphy Solution	

2.1.5 Kits

Kit	Source
Avidin/ Biotin Blocking Kit	Vector
In situ Cell Death Detection Kit, TMR Red	Roche
Plasmid Midi Kit	QIAGEN
PGEMT-easy Sub-cloning system	Promega
Plasmid Mini Kit	QIAGEN
QIAquick gel extraction kits	QIAGEN

2.1.6 Plasmids

Electroporation constructs:

pMES- Cathy Krull

EphA4- *Gallus gallus* EphA4 cDNA sequence inserted via a *Pst1* site into pMES (obtained from Cathy Krull)

KiEphA4- *Gallus gallus* EphA4 cDNA sequence containing point mutation in kinase domain (obtained from Cathy Krull)

EphA4Δ cyto- N-terminal region of EphA4 including trnasmembrane domain and truncated prior to the Jx1 region of EphA4 (constructed by Rena Hirani)

Ephrin-A5- Made by Edwina Ashby (full length ephrin-A5 fragment encoding ephrin-A5 isoform inserted into pMES)

pCAX- (obtained from Cathy Krull)

Subcloning vectors:

PBS (KS), Stratagene

PGEMT-easy, Promega

2.1.7 Oligonucleotides

Name	Sequence (5' to 3')	T_m (°C)
Sequencing primers		
pUC/M13 Primer, Forward (-40)	GTT TTC CCA GTC ACG AC	
pUC/M13 Primer, Reverse	CAG GAA ACA GCT ATG AC	
GFPN1-5'	GAG GTC TAT ATA AGC AGA GCT G	53
GFPN1-3'	GAC TTG AAG AAG TCG TGC TG	56
GPFN1-3'R	CAG CAC GAC TTC TTC AAG TC	56
GFP4705	CTG TGG ATA ACC GTA TTA CCG C	53
EGFP-N	CGT CGC CGT CCA GCT CGA CCA	67
b-act963r (chick)	CAC GAT TAC CAT AAA AGG C	
b-act1265r (chick)	AAC ATG GTT AGC AGA GGC	
pMESseqfwd	CTT CTC CAT CTC CAG CC	49
pMESseqrev	TAG ACA AAC GCA CAC CGG	50
EA4seqprimer	CTG ACA GTT CGA AAC CTG GC	62
B-actF	ATC ATG TTT GAG ACC TTC AA	46
B-actR	CAT CTC TTG CTC GAA GTC CA	52
EphB2 and ephrin-A5 constructs		
EphB2DNF	GGA ATT CCT TCT GAT GCC CG	62
EphB2DNR	GGG GTA CCC CGT TCA AAG CC	66
DNB2ires	GGA ATT CCT TCT GAT GCC CGG CCC G	64
DNB2iresR	CGC GGA TCC TTA AGC ACG TTC AAA GCC CCG TC	68
CA5ires	GGA ATT CCC CGC GAT GCC GCA CGT G	66
Ca5IRESr	CGC GGA TCC CTA TAA TAT CAA AAG CAT TGC CAG	63

2.2 Methods

2.2.1 DNA manipulation

Standard molecular genetic techniques were performed as described in (Sambrook J., 1989).

2.2.2 Preparation of 1Kb Plus Ladder

20µl of 1Kb Plus ladder stock (1µg/ml) was added to 15µl of loading buffer and 165µl of dH_2O for a final concentration of 100ng/µl.

2.2.3 Agarose gel electrophoresis

Molten 1% agarose dissolved in 1x TAE was poured onto a glass plate and set with loading well combs in place. The gels were placed in an electrophoresis tank and submerged in 1x TAE. The samples were mixed with loading buffer and loaded alongside a suitable size marker and then separated by applying 40-100V to the tank. The gel was stained with ethidium bromide to visualise the DNA under UV light.

2.2.4 Purification of DNA from agarose gels

QIAquick gel extraction kits from QIAGEN were used to purify DNA bands cut from agarose gels following the manufacturer's instructions.

2.2.5 De-phosphorylation of vector DNA

Re-circularisation of vectors with compatible ends was prevented by de-phosphorylation. During or after restriction enzyme digestion, 1-2 units CIP was added to the reaction and incubated for at least 1 hour at 37°C.

2.2.6 Ligation

A reaction mix of purified insert and vector DNA fragments (typically in a ratio of 3:1, insert:vector), 1unit of T4 DNA ligase and 1x ligation buffer was incubated at 18°C overnight. For transformation by electroporation, the ligation was phenol/ chloroform

extracted and precipitated with 1/10 volume 3M Sodium acetate (pH 5.2) and 2.5 volumes ethanol. The pellet was washed in 70% ethanol and resuspended in 10µl of dH_2O.

Ligation of PCR products generated by standard *Taq* polymerase reactions into pGEMT-Easy was carried out essentially as described, Promega. Amplicons generated using *Pfu Taq* polymerase into pGEMT-easy was A-tailed and then inserted into pGEMT-Easy as described, Promega.

2.2.6 Isolation of RNA from chick tissue

RNA was isolated from chick tissue using the TRIZOL reagent, (Sigma), according to the manufacturers instructions.

2.2.7 RT-PCR

Reverse transcription reactions were carried out using M-MLV reverse transcriptase, Promega.

2.2.8 Amplification of chick Eph/ephrin genes for expression constructs using Pfu DNA polymerase

Pfu polymerase was used all reactions according to the manufacturers instructions. PCR conditions were using 0.5U *Pfu* polmymerase, 1 ng template DNA, 0.1ng primers and 0.2 mM dNTPs in *Pfu* PCR buffer. Reactions were performed using a MJ Research, PTC-200 peltier, thermal cycler with the following conditions: 34 cycles at 95˚C for 90 secs, 60˚C for 60 1 min, 72˚C for 3 mins, followed by a 72˚C extension step for 10 mins.

2.2.9 Electrocompetent cell generation

Fresh DH5α cells were inoculated in 5ml of LB and incubated overnight at 37°C. They were then diluted into 500ml of pre-warmed LB and left to incubate at 37°C with agitation for approximately 2.5 hours or until the cells had reached an optical density at A_{600} of 0.4-0.5. Once they had reached this value, cells were cooled down on ice for fifteen minutes and kept cool throughout the following protocol. Cells were harvested by centrifugation at 3000rpm in cold 250ml ocheridge tubes at 4°C for fifteen minutes. The supernatant was removed and cell

pellets were gently re-suspended in 500ml of cold ddH_20. The above centrifugation step was repeated, the supernatant removed and pellet re-suspended in 250ml of cold ddH_20. Cells were then centrifuged at 4000rpm for 15 minutes at 4°C, the supernatant was removed and pellet gently re-suspended in 10ml of 10% cold glycerol. Cells were harvested by centrifugation at 5000rpm at 4°C for fifteen minutes, and finally resuspend in 1ml of 10% cold glycerol. Cells were snap-frozen in liquid nitrogen in 50µl aliquots and stored at -80°C.

2.2.10 Bacterial transformation

Ligations were electroporated into the electrocompetent cells using BioRad electroporation cuvettes with a 2mm gap. The ligation was incubated with freshly thawed cells for 1min on ice, transferred into a chilled cuvette and electroporated with the BioRad Gene Pulser set at 2.5 V, 200 Ω, 25 µFD and the Capacitance Extender set to 500µFD. Immediately after electroporation, cells were revived by incubation in 1ml SOC at 37°C for 1 hour before plating on the appropriate selective antibiotic plate.

2.2.11 Colony cracking

Individual colonies were picked from a stock plate with a pipette tip. These were first used to inoculate a master plate then placed into 19µl of freshly made cracking solution, with the tip left in the tube. They were then incubated for 15mins at 65°C. The samples were then dry-loaded into the wells of a 1% agarose gel and run at 40V for 10mins. Enough TAE was then added to submerge the gel and the voltage was increased to complete eletrophoresis of the plasmid DNA.

2.2.12 Plasmid DNA amplification/ purification

Plasmid DNA was prepared using QIAGEN Plasmid Purification Midi and Maxi Kits following the manufacturers instructions.

2.2.13 Automated sequencing

DNA templates were prepared for sequencing using a Qiagen purification kit. BigDye chemistry was used to generate sequence fragments as per the manufacturer's instructions. Reactions were cycled through 25 cycles of: 95°C for 10sec, 50°C for 5sec, and 60°C for

5min in a PTC-200 DNA engine (MJ research). The 20µl reactions were then isopropanol precipitated before sequencing. Automated sequencing equipment at the Department of Molecular Pathology, IMVS, Adelaide was used to analyse the sequence reactions.

2.2.14 Isopropanol precipitation of sequencing reactions

80µl of freshly made 75% isopropanol was added to the 20µl sequencing reaction mix and precipitated for 15mins at RT. The sample was then centrifuged for 20mins at 14,000rpm and then washed with 250µl 75% isopropanol. It was then centrifuged for 10mins at 14,000rpm and the pellet was air-dried.

2.2.15 Ringer's solution protocol

In a 4litre flask, the ingredients were added, in order and the solution was continuously stirred until each ingredient had dissolved. The solution was adjusted to pH 7.4 and then filter sterilised.

2.2.16 Embryo preparation and collection

White leghorn cross New Hampshire Red chicken (*Gallus gallus)* or quail (*Coturnix coturnix japonica*) fertilised eggs from HiChick P/L, Gawler or Lago Game, Melbourne, respectively, were incubated at approx 38°C in a humidified incubator until the embryos had reached the required developmental stage (according to Hamburger and Hamilton).

For *in ovo* electroporations the eggs were incubated lying sideways. A syringe with an 18½gauge needle was used to remove 5ml of eggwhite from the egg after piercing the eggshell at one end. The eggs were then windowed and Pelikan calligraphy ink, diluted 1/10 in ringer's solution, was injected underneath the vitelline membrane for easier visualisation of the embryo.

To harvest embryos for analysis, the embryos were removed from the egg, placed into ice-cold PBS and dissected under the microscope as required.

2.2.17 In ovo *electroporation technque*

Preparation of DNA for injection

DNA was dissolved in PBS to a final concentration of 2-3 μg and picolitre volumes were placed with a drop of Fast Green (small end of pipette tip dissolved into PBS to yield a dark mouldy colour) on a piece of parafilm and then transferred to a glass capillary needle using a Hamilton Syringe (or variations thereof). Glass capillaries, 1.0mm OD x 0.78mm ID thin wall with inner filament, were sourced from Clark Electromedical Instruments c/- SDR Clinical Technology, 213 Eastern Valley Way Middle Cove NSW 2068. Capillaries were pulled using Sutter needle pulling instrument.

Injection apparatus

The loaded capillary was mounted into an MPPI-/PH needle holder attached to a MPPI-2 Milli-Pulse pressure injector (ASI). For the control of needle movement, the capillary/needle holder unit was inserted into a MM-3 Micromanipulator (Narishige group, Olympus), attached to the workbench using a retort stand. Finally, to enable "hands-free" injections, an MPPI-/FS foot pump regulated the MPPI-2 pressure injector. The needle was tipped and pressure adjusted to approximately 20 psi with 2 and 3/4 turns anti-clockwise for back-pressure, to facilitate a steady small flow of solution out of the capillary. Alternatively, for quail electroporations, DNA was mouth-pipetted into the lumen of the neural tube.

Embryo preparation

Stages 12- 14 (E2.5) embryos were staged according to (Hamburger and Hamilton, 1951)), windowed and injected with Indian Ink (diluted 1:10 in PBS) to directly visualise the embryo structure. Immediately prior to electroporation, a hole was inserted into the vitelline membrane directly above the region of injection using a fine tungsten needle. This was followed by a drop of ringer's solution to tease away the membrane from the site of injection (dorsal neural tube) as well as preventing dehydration and providing a good source of electrolytes for electroporation. Tungsten needles were prepared using a basic electrolysis approach. The negative electrode was placed in 10M sodium hydroxide solution and positive electrode clamped onto blunt tungsten wire. Using standard DC voltmeter, with voltage set to approximately 6.5V, the tungsten needle was dipped into the solution several times, with bubbles indicating good circulation. Check the tip of the needle under a stereomicroscope and

continue (long dipping makes a finer tip) until a satisfactory level of fineness is achieved. Bend the fine end 90° so that it lies at right angles with the rest of the needle (and is approximately 7-8 mm long).

Electroporation parameters

All electroporations were carried out using an ECM 830 square wave electroporator (BTX). Electroporation parameters depended on the age of embryo and particular region targeted.

Type of electroporation	Parameters (volts, pulse duration, number of pulses)
Standard (stages 12-14 chick embryos)	15-25V, 3x, 50ms
Japanese Quail embryos	25V, 5x, 50ms
Somites	50V, 3x, 50ms

Electrodes also varied depending on the type and exact region of electroporation. To electroporate the entire dorso-ventral extent of the neural tube, BTX Genetrodes with a 3mm plate were used in a BTX Genetrode holder. To target the dorsal neural tube specifically, platinum electrodes were constructed with a 3mm plate and the anode inserted underneath the embryo surface and cathode at the dorsal surface to facilitate movement of DNA into the dorsal right hand side of the neural tube. The orientation of the anode and cathode was reversed to electroporate the ventral neural tube regions. In quail embryos, two consecutive electroporations were delivered to each embryo (swapping orientation of electrodes) to achieve expression on both sides of the dorsal neural tube.

2.2.18 Neural tube culture

Quail embryonic day2 embryos were electroporated with the same parameters as above then left to recover for 1-2 hours in the humidified incubator. Embryo dissection and plating of explants onto FN was carried out essentially as described in Chapter 21, Developmental Biology Protocols, volume III, p 201 (Newgreen, 1999). For each embryo, two pieces of neural tube were dissected; neural tube at the axial level of migrating neural crest cells, neural tube migratory (NT^{m}), and neural tube at the axial level prior to the outgrowth of neural crest cells, neural tube pre-migratory (NT^{pm}).

2.2.19 Vibratome Sectioning

7% low melting point agarose in PBS was melted in the microwave for 10-20sec or until just melted. Fixed embryos were placed onto parafilm and the excess fluid removed with a kimwipe. A small amount of melted agarose was placed into the base of a Tissue-Tek® cryomold and allowed to begin to set. The embryo was then laid on the agarose, care taken to align the embryo correctly, and the mould filled to the top with more melted agarose. After the agarose had well set the blocks were removed from the moulds and trimmed of excess agarose to ~ $5mm^3$. The trimmed block was then attached to the Vibratome 1000Plus Sectioning System chuck with superglue, the vibratome interior vessel filled with cold PBS and ice-filled 10ml tubes and the dial adjusted to the desired section depth. Sections were transferred into PBS before further treatment.

2.2.20 *Whole-embryo* in-situ *hybridisation*

Dissection and fixation

All solutions and glassware from the dissection/ fixation step (excluding the actual dissection itself) through to the hybridisation, inclusive, were RNAse free. Water was used that was diethyl pyrocarbonate (DEPC)-treated (0.1% DEPC for 12 hours, stirring) followed by autoclaving and glassware was baked. Reagents, stir bars, spatulas, *etc.*, used were RNAse-free and all solutions were mixed wearing gloves. Care was taken not to contaminate solutions with pipettes. The pH meter was not used to check the pH of the entire volume of solution. Instead, either pH strips were used or 10ml of solution was taken and adjusted to correct pH, then the volume of acid/ base needed was calculated to adjust the pH for the bulk of the solution.

Embryos were dissected in a petri dish with sterile cold PBS. Needles were used to dissect away all of the extra-embryonic membranes and the amnion. Embryos were then gently transferred with a transfer pipette into a small jar with freshly prepared 4% PFA and fixed overnight at 4 °C on a neutator. Embryos were washed 2x 5min with PBT. For all rinses except as noted, 4mls of the appropriate solution was used.

In the need for storage of embryos, they were dehydrated through serial changes of 25%, 50%, 75% methanol-PBS and 2x 100% methanol for 5min per wash, on ice and stored at -20°C in methanol. To resume the protocol embryos were re-hydrated through a methanol-PBS series.

Pre-treatments, Pre-hybridisation and Hybridisation

Embryos were washed 3x 30min with RIPA at RT, postfixed for 20min in PG-PBT at RT on a neutator then washed 5x 5min in PBT at RT. Embryos were washed for 15min with a 1:1 mix of HB and PBT (2ml each) at RT on a neutator then for 5-10min with 1ml HB at RT on a shaker. They were then pre-hybridised in 1ml HB + 100 µg/ml tRNA and 100 µg/ml sheared denatured salmon sperm DNA (both which had been phenol extracted) for 1-5 hours at 70°C with agitation. The digoxigenin-labelled riboprobe was denatured at 95°C for 3min with 1 ml HB/ tRNA/ DNA solution then equilibrated at 70°C. 1ml HB/ vial was added containing the tRNA/ DNA mixture and the denatured riboprobe then mixed well. They were then hybridised overnight with agitation at 70°C in a chamber humidified with 50% formamide in water. The embryos were washed in pre-warmed HB for 2x 30min at 70°C, 2x 5min in SSC-FT at RT, then 3x 30min with pre-warmed SSC-FT at 65°C. The embryos were cooled to RT then washed for 3x 5min at RT in 1x TBST.

Antibody binding

Embryos were blocked by incubating for 1-3 hours at RT in TBST + 10% heat-inactivated sheep serum (~300µl of solution per vial).

To heat-inactivate the mouse powder 2mg mouse powder and 1ml TBST each was added to 2 centrifuge tubes. They were vortexed and then placed in a 70°C water bath for 30min. The tubes were then centrifuged, the supernatant discarded and the tubes vortexed to break up the pellets. 1ml TBST + 10µl heat-inactivated serum was added to each tube then 0.5 µl of anti-dig antibody (1:2000 dilution).

The anti-dig antibody was pre-adsorbed by incubating the antibody (in TBST + 1% heat-inactivated sheep serum and heat-inactivated mouse powder as prepared above) with agitation for 1 hour at 4°C. The tubes were centrifuged for 5min at 4°C, the supernatant transferred to fresh tubes and the mouse powder discarded.

The embryos were incubated in the antibody conjugate solution (600µl/ vial) overnight at 4°C.

Washing and colour development

The antibody conjugate was removed and the embryos washed with TBST at RT for 3x 5min. The embryos were transferred to fresh vials, washed in TBST/ 2mM Levamisole for 5x 1-1.5

hours then washed for 3x 10min at RT in APB. For the colour reaction 3ml/ vial of NBT/ BCIP/ APB was added to the embryos and incubated on a shaker, well covered from light. The progress of the reaction was observed with a dissecting microscope at brief intervals. As appropriate according to extent/ darkness of staining, the embryos were removed from vials and transferred to a plastic tissue culture well for rinsing. The colour reactions were stopped by rinsing embryos at RT for 3x 5mins in the dark in PBT/ 1mM EDTA/ 1% glacial acetic acid. The embryos were post-fixed for at least 1 hour in 4% PFA in PBT/ 1% acetic acid, washed for 3x 15min in PBT/ 1mM EDTA/ 1% acetic then cleared with serial 10min incubations of 20%, 40%, 60% and 80% glycerol/ PBT /1% acetic and stored in the dark.

2.2.21 Neural tube culture immuno-histochemistry

After fixation in 4% PFA for 15min at RT, the neural tube cultures were washed for 3x 5min in PBS, blocked and permeabilised in 1ml PBS/ 1% BSA/ 0.1% TritonX for 5 mins then washed well for 3x 15min in PBS. The area of the culture dish around the explant was carefully dried, 100µl of 1° antibody in PBS/ 1% BSA was applied as a drop onto the explant, which was then incubated in a humidified box overnight at 4°C. The cultures were then washed for 3x 15min in PBS and the area of the culture dish around the explant carefully dried again. The 2° antibody was applied as above, incubated at RT for 2 hours then washed for 3x 15min in PBS. The cultures were then covered with a coverslip with anti-fade for imaging.

2.2.22 Eph and ephrin immuno-histochemistry

(Combined protocol for wholemounts and ***sections*** of chick embryos).

After fixation in 4% PFA either O/N at 4°C or 2 hours at RT, the samples were washed 3x 15mins in PBS. They were then blocked in BS + 4 drops of avidin per ml of BS at RT for 1 hour incubating on a neutator ***(with gentle agitation).***

The 1° antibodies were added in BS + 4 drops of biotin per ml BS then incubated at 4°C overnight on a neutator ***(no agitation).*** The samples were then washed for 3x 10-15mins at RT with PBT on a neutator ***(with gentle agitation).*** The 2° antibodies (for the antibody to be amplified) were then added in BS and incubated for 5-6 hours ***(2 hours at RT).*** They were then washed for 3x 10-15mins at RT on a neutator ***(with gentle agitation).***

The 2° antibodies were added for the monoclonal antibody and 3° antibodies for the amplified antibody in BS. They were incubated at 4°C overnight on a neutator ***(RT for 1-2 hours)*** then

serially incubated for 10-15mins at RT in 10%, 30%, 50% and 80% glycerol before mounting. ***(No incubation in glycerol. Sections were placed in dH_20 before mounting, slightly dried then covered with a coverslip).***

The edges of the coverslip were sealed with nail polish for imaging.

2.2.23 Immunostaining of electroporated embryos

After fixation and dissection of electroporated embryos, embryos were placed in blocking solution containing PBT (PBS, 0.1% TritonX 100) + 10% HIHS for half hour. Incubate in primary antibody in blocking solution. Embryos were viewed using Olympus AX70 upright microscope and processed using Adobe Photoshop 6.

2.2.24 TUNEL reaction

As per *In Situ* Cell Death Detection Kit, TMR red (Roche).

2.2.25 Confocal microscopy

Images were produced using the BioRad Radiance 2100 confocal microscope (at Detmold Imaging Core Facility, Hanson Institute) equipped with three lasers, Argon ion 488nm (14mw); Green HeNe 543nm (1.5mw); Red Diode 637nm (5 mw) outputs and Olympus IX70 inverted microscope. The objective used was a 10x or 20x UPLAPO with NA water. The dual or triple labelled tissues were imaged with three separate channels (PMT tubes) in a sequential setting. The Green fluorescence (Alexa 488 or GFP) was excited with Ar 488 nm laser line and the emission was viewed through a HQ515/30nm narrow band barrier filter in PMT1. The red fluorescence (Cy3) was excited with Green HeNe 543nm laser line and the emission was viewed through a long pass barrier filter (570LP) to allow only red light wavelengths longer than 570nm to pass through PMT2. The far-red fluorescence (Cy5) was excited with Red Diode 637nm laser line and the emission was viewed through a long pass barrier filter (660LP). Automatically all signals from PMTs 1, 2 and 3 were merged. The image data were stored on a CD for further analysis using a Confocal Assistant software program for the Microsoft® Windows™ (Todd Clark Brelje. USA). All images were processed using Adobe Photoshop 6.

2.2.25 *Quantification of* in vivo *phenotypes using* in vitro *culture model*

Using the *in vitro* model, the observations regarding EphA4 and kiEphA4 phenotypes *in vivo* were quantified. A total of eight separate experiments were conducted, each one consisting of a combination of EphA4/kiEphA4 and control constructs. For the quantification of the phenotypes, neural tubes pooled from 4 individual experiments were stained with GFP, SoxE and HNK1. For all counts, a column of a defined width (170 pixels) was drawn from the neural tube (in the region of highest expression along the neural tube) directly out towards the edge of the outgrowth. The neural tube was lined horizontally along the bottom of the page so that the direction of the outgrowth went from the bottom to top. For estimation of cell density and/or the efficiency of EMT, counts were presented as the total number of cells in the mesenchymal region. For quantification of how cells expressing different constructs progressed through EMT, the number of GFP cells in the proximal (neuroepithelium) and distal (mesenchymal) regions of the cultures was counted and presented as a percentage of the total cells present.

Chapter 3: Spatiotemporal patterns of expression of EphA4 and ephrin-A's during avian trunk PNS patterning

3.1 Introduction

NC cells and motor axons originate from distinct regions of the neural tube and migrate into the periphery in a precise spatiotemporal manner (Rickmann et al., 1985). NC cells migrate on two stereotypical pathways in the trunk and these specify their differentiation into multiple cell types (Weston, 1963). The first is a ventromedial route through the somitic mesoderm, involving segmental migration through the rostral but not caudal half-sclerotome; cells travelling along this pathway colonise the DRG, SG, and glial derivatives (Le Douarin and Kalcheim, 1999). Approximately 24 hours later, NC cells navigate the dorsolateral pathway in a non-segmental manner between the overlying ectoderm and somites (Erickson et al., 1989). Shortly after NC cell migration has begun, motor neurons (originating in the ventral neural tube) extend their axons segmentally through the rostral half-sclerotome (Loring and Erickson, 1987).

Classical microsurgical experiments have demonstrated that the caudal half-somite mediates repulsion of PNS precursors, facilitating a dominant influence on PNS segmentation (Bronner-Fraser and Stern, 1991; Keynes and Stern, 1984). Many molecules have been ascribed a role in repulsion of PNS precursors from the caudal half-somite, one family of which includes the Eph/ ephrin-B subclass of receptors (reviewed by (Krull, 1998)). In both rodents and chick, EphB3 is expressed on NC cells during their migration through the rostral somite-half and ephrin-B1 in the caudal somite-half (ephrin-B2 in rats), generating a complementary expression pattern that is suggestive of a guidance role. Functional blocking experiments *in vivo* and *in vitro* have shown that this interaction contributes to the repulsion of NC cells from the caudal somite-half (Krull et al., 1997; Wang and Anderson, 1997). Motor axons also express EphB receptors, however, perturbation of these interactions is not sufficient to elicit axon entry into the caudal somite (Koblar et al., 2000). Despite these apparently critical roles for Eph/ ephrin-B interactions, EphB or ephrin-B knock-out mice do not display any segmentation defects (Adams et al., 1999; Compagni et al., 2003; Davy et al.,

2004; Orioli et al., 1996), suggesting a combinatorial role for factors localised to the caudal half-somite in mediating the overall arrangement of the PNS (Vermeren et al., 2000).

Much less is known about cellular interactions between NC cells/ motor axons and the rostral somite (Krull, 1998). The migration of NC cells through the rostral half-somite is highly organised and spatiotemporally regulated with regards to somite morphogenesis (Krull, 1998; Perris and Perissinotto, 2000; Sela-Donenfeld and Kalcheim, 2000; Tosney et al., 1994). Many ECM molecules, known to promote NC cell migration, are expressed in the rostral half-somite and multiple *in vitro* and *in vivo* studies have indicated a positive guidance role for these molecules, however gene deletion studies point to functionally redundant roles in determining the overall pattern of PNS segmentation *in vivo* (reviewed by (Perris and Perissinotto, 2000)). In addition, time-lapse analyses have revealed that NC cells migrate in a chain-like fashion *in vivo*, making extensive contacts with one another during their migration (Krull et al., 1995; Kulesa and Fraser, 2000).

Eph/ ephrin-A molecules have been described in a spatiotemporally correct manner for a possible role in PNS segmentation. EphA7 is expressed in the caudal portion of the somite throughout trunk development (Araujo and Nieto, 1997), whilst ephrin-A proteins are expressed in the DRG (Eberhart et al., 2000). The aim of the work in this chapter is to describe the precise distribution of Eph/ephrin-A's in the somitic tissue during the course of PNS segmentation, with the intention of further understanding PNS cell guidance mechanisms mediated by the rostral half-somite. More recently and during the course of this research, EphA4 along with ephrin-A2, -A5 and –A6 were found on NC cells during their initial emigration into the somite (McLennan and Krull, 2002). Furthermore, *in vitro* data suggested that these interactions contributed to the coordinated migration of NC cells through the somitic environment (McLennan and Krull, 2002).

3.2 Results

To gain a broad understanding of Eph/ ephrin distribution in avian embryos during trunk segmentation, embryos aged between stages 17- 20 as described by Hamburger and Hamilton, 1951, were analysed (Hamburger and Hamilton, 1951). Because trunk PNS development is tightly co-ordinated with somitogenesis, occurring in antero-posterior wave along the embryo axis, any single embryo displays a differing extent of NC cell and motor axon outgrowth depending on the axial level analysed. Thus throughout the analyses, the mid-trunk region (between forelimb and hindlimb) was focussed on in embryos ranging from stages 17-20; at this level somites have differentiated into dermomyotome and sclerotome and both NC cells and motor axons have begun to migrate segmentally into the rostral half-somite.

3.2.1 Complementary and overlapping distribution of A-subclass Eph and ephrins in the developing peripheral nervous system

As a general indicator of the distribution of EphA/ ephrin-A proteins during trunk segmentation, fusion proteins comprised of the extracellular region of ephrin-A5 or EphA3 fused to the Fc portion of human Immunoglobulin1 (Fc) were used to stain whole chicken embryos (Krull et al., 1997). Both ephrin-A5-Fc and ephrin-A3-Fc proteins bind promiscuously to a variety of EphA receptors, including EphA3, A4, A5 and A7, but each of these has a different affinity profile for each of the receptor subtypes (Gale et al., 1996). EphA3-Fc binds ephrin-A2 and ephrin-A5 with high affinity (Flanagan and Vanderhaeghen, 1998; Lackmann et al., 1997). The use of these Fc proteins enabled an overall perspective of EphA and ephrin-A distribution during avian trunk segmentation without identifying the specific members of the sub-class involved.

Ephrin-A5-Fc localised strongly to cells in the caudal-half somite at Stage 17 (Figure 3.1A), and was distributed in a manner that was coincident with somite morphogenesis, such that at caudal regions no ephrin-A5-Fc binding was detected (compare arrow with arrowhead, Figure 3.1A). This caudal half-somite localisation was also found in stage 20 embryos (data not shown) and was consistent with that previously reported for EphA7 in the caudal half-sclerotome during PNS segmentation (Araujo and Nieto, 1997). *In vitro* studies indicate that ephin-A5 binds EphA7 with highest affinity, providing an explanation for the strong staining of ephrin-A5-Fc to the caudal half-somite in these embryos (Holmberg et al., 2000). Ephrin-

A5-Fc also weakly stained the rostral portion of the anterior-most somites in the region dorsomedially adjacent to the neural tube; however the staining was quite diffuse (asterisks, Figure 3.1A). Given the unique binding affinities of particular ephrins to their respective partners, and the possible distribution of EphA proteins in the rostral half-somite, the staining pattern of ephrin-A3-Fc was also analysed in stage 20 embryos. Ephrin-A3-Fc staining was found at a very low level in the caudal half-somite, (asterisk, Figure 3.1B), and much more intensely in the dermomyotome (arrow, 3.1B). In the fore- and hindlimb regions, ephrin-A3-Fc stained a defined cell population present dorsomedially adjacent to the neural tube at the boundary between rostral and caudal somites (arrowheads, Figure 3.1B).

To detect ephrin-A ligands, the EphA3-Fc fusion protein was used to stain whole embryos and revealed a strong localisation of ephrin-A proteins in the rostral region of the somite in stage 20 embryos (Figure 3.1C). A strong line of EphA3-Fc staining demarcated the dorsal neural tube (arrow, Figure 3.1C), whilst intense staining was also found in the lateral plate mesoderm. To more closely analyse EphA3-Fc binding in the rostral half-somite, whole-mount embryos were viewed longitudinally in high magnification (Figure 3.1D). This confirmed that EphA3-Fc protein bound to cells in the dorsal region of the rostral half-somite in a position spatiotemporally consistent with ventromedially migrating NC cells (asterisks, Figure 3.1D). Ephrin-A proteins did not appear to be expressed on NC cells of the dorsolateral pathway at any stage.

To localize ephrin-A3-Fc and EphA3-Fc labelling at the cellular level in the rostral half-somite, the top 12 somites of stage 20 embryos were cut into 100 μm transverse sections after Fc immuno-histochemistry and stained with the NC cell marker, HNK1 (Bronner-Fraser, 1986a; Loring and Erickson, 1987; Newgreen et al., 1990). Transverse sections revealed a compartmentalised expression pattern for EphA and ephrin-A proteins in the rostral somite. Ephrin-A3-Fc strongly stained the dermomyotomal lip (DML) of the dermomyotome (asterisk, Figure 3.2A) and sclerotome cells around the DRG, leaving a region devoid of any ephrin-A3-Fc staining in the teardrop shape of the DRG (Figure 3.2A and D). Overlay of the ephrin-A3-Fc image (Figure 3.2A) with HNK1 (Figure 3.2B) to indicate the distribution of EphA proteins with respect to NC cells of the DRG anlage, showed that ephrin-A3-Fc demarcated the dorsolateral border of the DRG (ventral to the DML) (Figure 3.1C). High magnification view suggested that there was some overlap between HNK1 cells and cells stained with EphA3-Fc protein (arrows, Figure 3.2D&F). The tight configuration of EphA proteins around the DRG was no longer observed in sections taken through the caudal edge of

the DRG (asterisk indicates the absence of HNK1 cells, Figure 3.2H), suggesting a role specific to the rostral somite-half (arrows, Figure 3.1G).

Transverse sections of the top somites of stage 20 EphA3-Fc stained embryos and co-labelling with HNK1 indicated that ephrin-A proteins localised within the DRG structure in the rostral half-somite (Figure 3.3). EphA3-Fc strongly co-localised with neural crest cells during their ventromedial migration and also with NC cells that had condensed into DRG (data not shown and Figure 3.3). EphA3-Fc cells were present in the core of the DRG (arrows, Figure 3.3A and C), as outlined by HNK1 staining (Figure 3.3B and C). In addition to the positive staining within the DRG, EphA3-Fc stained the dorsal neural tube intensely (asterisk, Figure 3.3A). High magnification view of the same section indicated that EphA3-Fc bound to a subset of cells within the DRG that were largely HNK1-negative, suggestive of NC cells that had differentiated into sensory neurons. EphA3-Fc cells were not observed in association with NC cells of the sympathetic lineage at any of the stages analysed (Figure 3.3 and data not shown).

Figure 3.1- Whole-mount EphA3-Fc and ephrin-A5-Fc screen in stage 17- 20 embryos reveal varying distribution patterns for ephrin-A and EphA proteins, respectively, in PNS segmentation.

(A) Stage 17 embryo trunk stained with ephrin-A5-Fc to detect EphA proteins. Asterisks indicate weak diffuse staining in the rostral somite. Arrow indicates strong staining in the caudal somite. Arrowhead indicates lack of ephrin-A5-Fc staining in the caudal regions of the trunk.
(B) Stage 20 embryo trunk stained with ephrin-A3-Fc, asterisk indicates weak staining of caudal half-somite, arrow indicates dermamyotomal staining, and arrowheads highlight strong staining at forelimb and hindlimb regions dorsomedially adjacent to the neural tube.
(C) Stage 20 embryo stained with EphA3-Fc to detect ephrin-A proteins. Arrow indicates staining in dorsal neural tube. (D) High-magnification view of mid-trunk region of embryo stained with EphA3-Fc. Asterisks show rostral half-somite.
R = rostral; nt = neural tube; lpm = lateral plate mesoderm.
Scale bar: 200 μm (A, D); 500 μm (B, C).

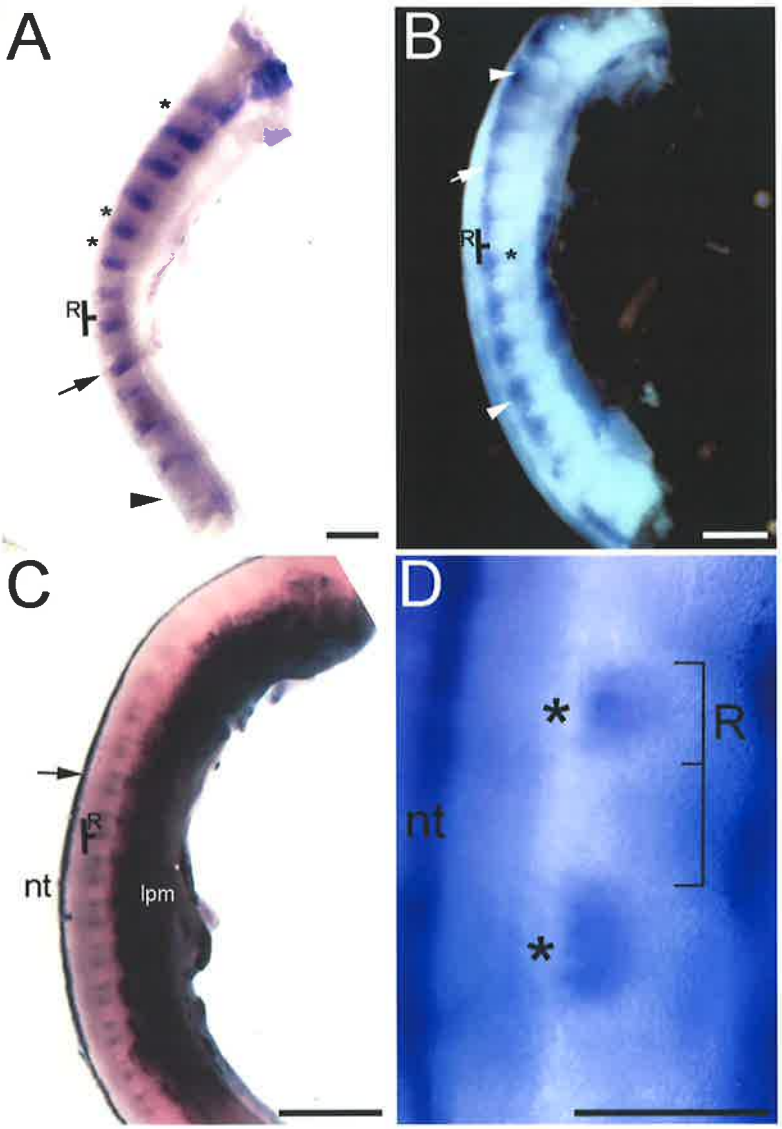

A
B
C
D
R
nt
lpm

Figure 3.2- Ephrin-A3-Fc surrounds the DRG in transverse sections from the top 12 somites of stage 20 embryos.

(A-F) 100 μm transverse section taken through the rostral portion of somite 10 from a stage 20 embryo stained with ephrin-A3-Fc and co-stained with HNK1. Images presented as ephrin-A3-Fc alone (A,D,G), HNK1 (red, B,EH,) and merged (C,F). Asterisk in (A) indicates dermomyotome.

(D-F) A high magnification view of (A), presented in the same order as (A-C). Arrows in (D) and (F) highlight ephrin-A5-Fc binding at the edge of the DRG.

(G) Transverse section of the caudal limits of the DRG (asterisk); section also stained with HNK1 (red, H). Arrows in (G) and (H) indicate diffuse staining at the lateral somite region.

dm = dermomyotome; drg = dorsal root ganglion; nt = neural tube; scl = sclerotome.

Scale bar: 100 μm (A-C), (D-F), (G-H).

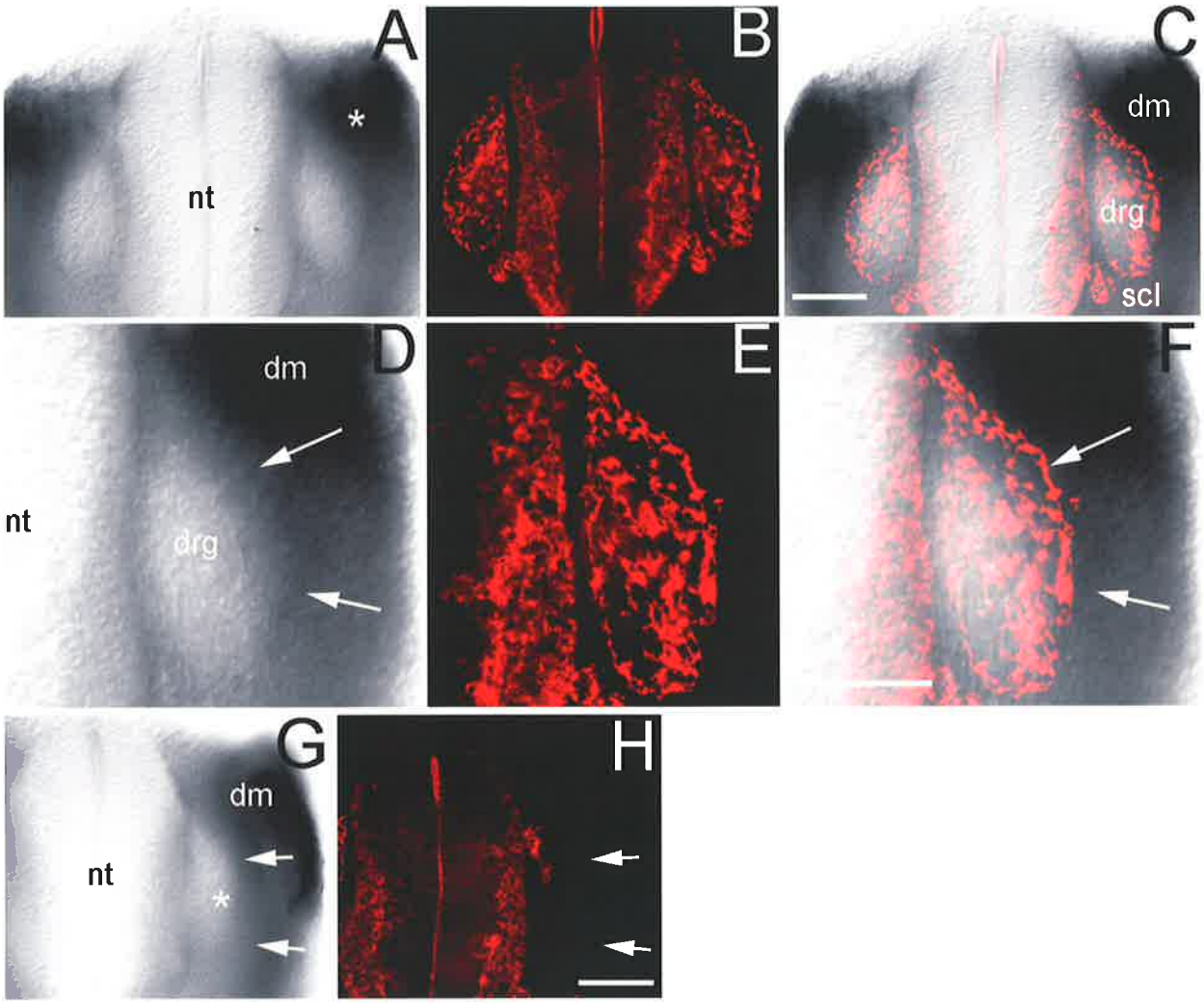

A
B
C
nt
*
dm
drg
scl
D
E
F
dm
nt
drg
G
H
dm
nt
*

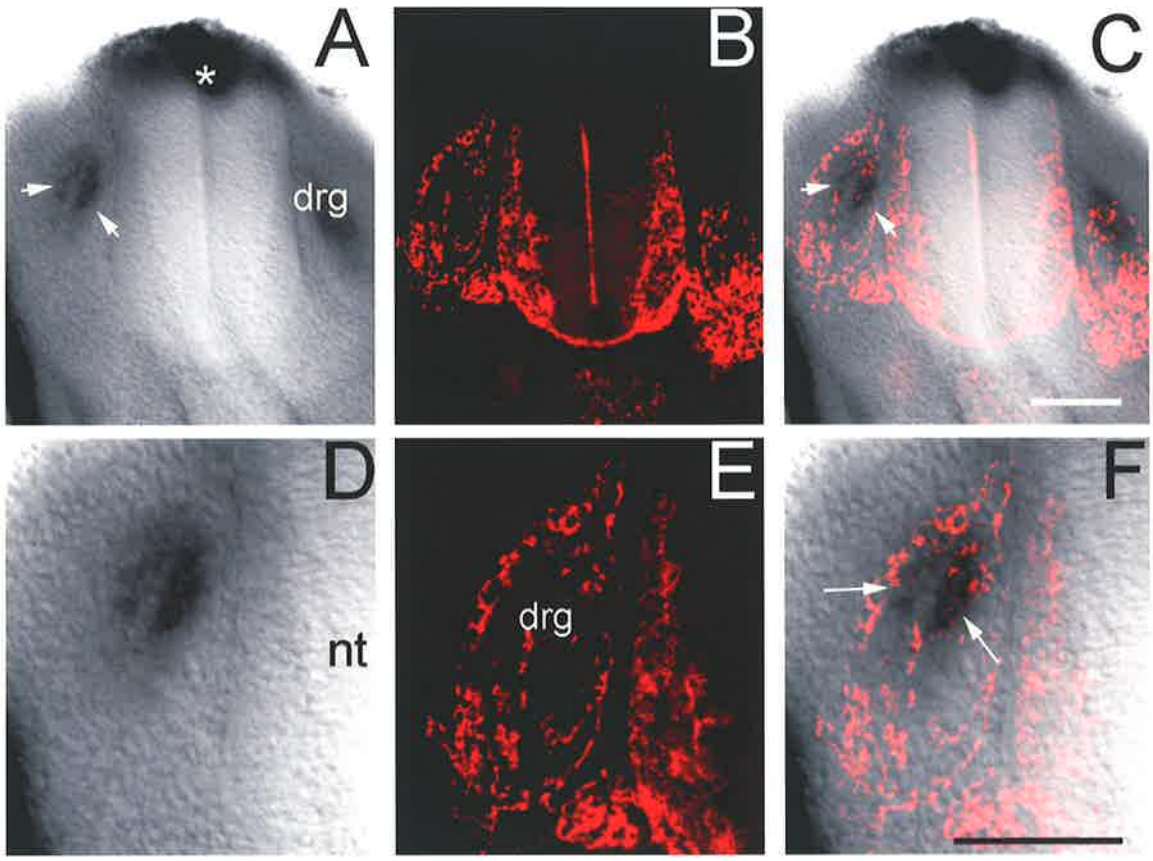
Figure 3.3- EphA3-Fc stains the DRG in transverse sections through rostral half-somites taken from the top 12 somites of stage 20 embryos.

(A-F) 100 μm transverse section taken through the rostral portion of somite 10 from a stage 20 embryo stained with ephrin-A3-Fc and co-stained with HNK1. Images presented as ephrin-A3-Fc alone (A,D), HNK1 (red, B,E) and merged (C,F). Arrows in (A&C) shows EphA3-Fc positive regions in the dorsal root ganglion; (A) asterisk shows strong dorsal neural tube staining.

(D-F) High magnification view of left-hand side DRG from (A-C); (F) merged image of (D) and (E). (F) Arrows point to compartmentalised staining of EphA3-Fc in the dorsal root ganglion.

drg = dorsal root ganglion; nt = neural tube.

Scale bar: 100 μm (A-C), (D-F).


A
B
C
D
E
F
drg
nt
drg

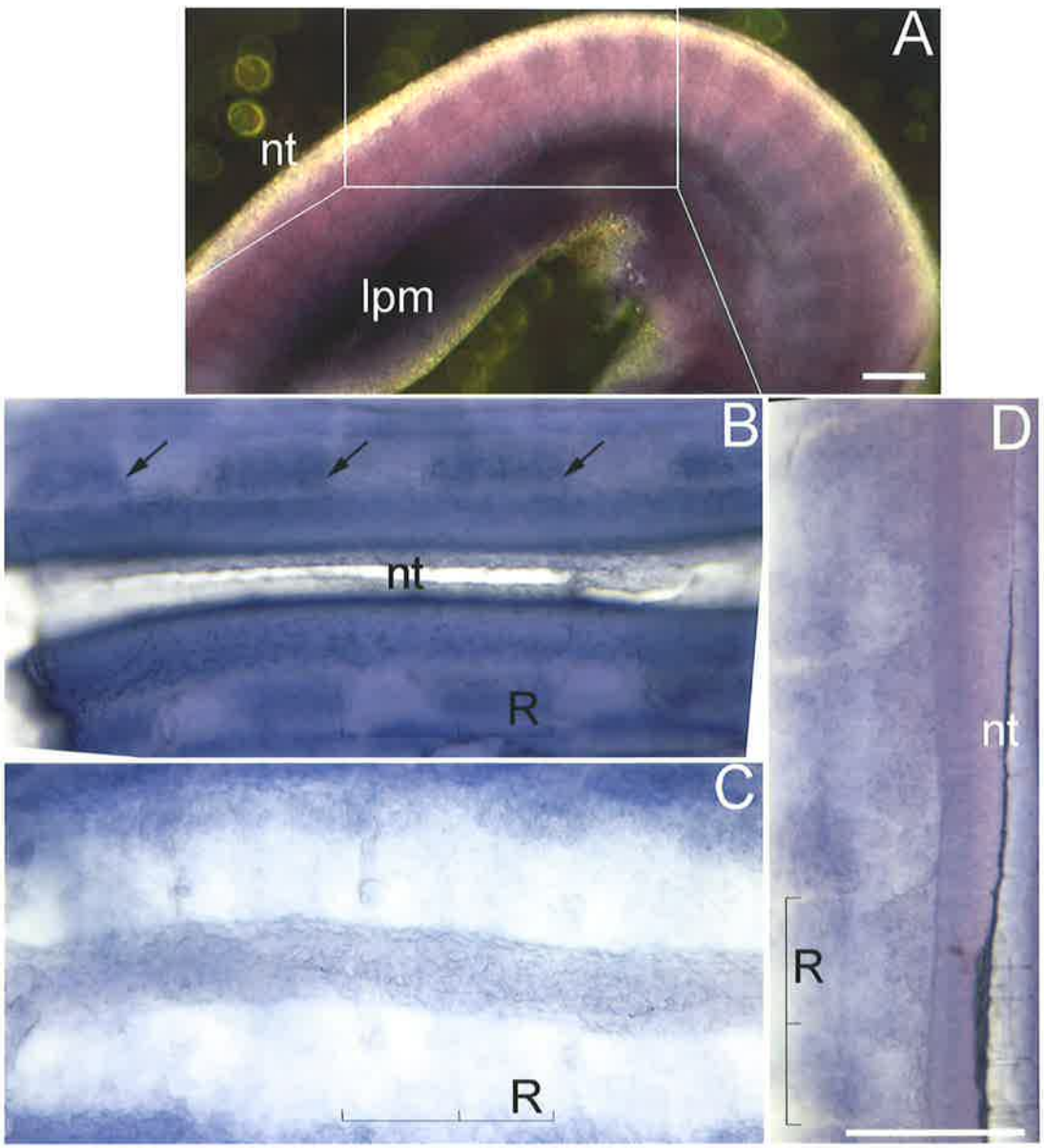
3.2.2 In Situ *Hybridisation analyses of ephrin-A5 and EphA4 during NC cell development*

The complementary expression patterns of EphA and ephrin-A proteins in the rostral half-somite were suggestive of a role for these proteins in contributing to NC cell guidance during their ventromedial migration to form DRG. Possible candidate receptors and ligands were then decided upon based on previous studies profiling the expression of EphA receptors during trunk segmentation. EphA7 was ruled out as a candidate for the rostral-localised expression of EphA receptors as its expression had already been described in the caudal half-somite (Araujo and Nieto, 1997). EphA4 was chosen for further investigation for a number of reasons. Firstly, it was a likely candidate based on previous reports describing expression in the dermomyotome of the hindlimb region and not in the DRG (Eberhart et al., 2000; Patel et al., 1996). Secondly, the specific distribution of ephrin-A3-Fc in the limb mesenchyme (data not shown) matched previous reports describing EphA4 expression in this tissue (Patel et al., 1996). Finally, EphA4 is a unique member of the EphA subclass as it interacts with both ephrin-B and ephrin-A proteins (Gale et al., 1996; Xu et al., 1999). Ephrin-B proteins are involved in PNS segmentation (Koblar et al., 2000; Krull et al., 1997; Wang and Anderson, 1997), whilst multiple different ephrin-A proteins, including ephrin-A2 and ephrin-A5, are expressed on DRG sensory neurons in the hindlimb (Eberhart et al., 2000). Thus, EphA4 was a good candidate for further investigation and ephrin-A5 was chosen as a candidate ligand expressed. The expression of *EphA4* and *ephrin-A5* was initially examined in whole-mount embryos and then later in transverse and longitudinal sections, and in some cases, co-localised with HNK1. All embryos were analysed in the mid-trunk at stage 19, during the period of active NC cell segmentation through the rostral half-somite.

Ephrin-A5 was expressed in a repeating metameric pattern in the rostral half-somites in stage 19 embryos and along the dorsal midline of the neural tube, mimicking the pattern of EphA3-Fc staining observed in Figure 3.1C (Figure 3.4A). 100 μm longitudinal vibratome sections of *ephrin-A5* stained embryos in the dorsal (Figure 3.4B) and ventral (Figure 3.4C) regions, indicated that *ephrin-A5* transcript was restricted to the dorsal regions of the embryo, expressed in the rostral half-somite and dorsal neural tube. This was a similar pattern to what would be predicted for NC cell precursors in the dorsal neural tube and also during their emigration into the somite (arrows, Figure 3.4B). Sense controls indicated that staining patterns in Figure 3.4B were specific to *ephrin-A5* transcript and not caused by background staining (Figure 3.4D).

Figure 3.4- *Ephrin-A5 in situ* hybridisation on stage 19 embryos indicates a strictly rostral half-somite expression pattern for *ephrin-A5*.

(A) Sagittal view of whole-mount embryo trunk stained for *ephrin-A5*. Note high staining in lateral plate mesoderm (lpm).
(B) and (C) 100 μm longitudinal sections of the boxed region in (A) demonstrating staining patterns at dorsal (B) and ventral (C) positions of the embryo trunk. (B) Arrows indicate rostral half-somite staining.
(D) 100 μm longitudinal section stained with sense probe for *ephrin-A5*.
R = rostral; nt = neural tube; lpm = lateral plate mesoderm.
Scale bar: 200 μm (A); 50 μm (B-D).


A
nt
lpm
B
nt
R
C
R
D
nt
R

EphA4 was expressed more widely throughout the embryo, including the rostral portion of the two newly formed somites, in the hindbrain and also in the distal tip of the limb bud (arrows, Fig 3.5A), (Nieto et al., 1992; Patel et al., 1996). In the somite, *EphA4* was expressed in the dorsal region, demarcating the caudo-rostral boundary of each somitic structure, (asterisks, Figure 3.5B). This staining pattern replicated that of ephrin-A3-Fc in the rostral somite, suggesting that the EphA receptor that was observed is likely to be EphA4 (Figures 3.1 and 3.2). In longitudinal sections stained with HNK1, regions of *EphA4* expression surrounded NC cells as they were coalescing into DRG, creating a "cup-like" effect (Figure 3.5C-E). Transverse sections of the mid-trunk region of *EphA4*-stained embryos showed *EphA4*-positive cells in somitic regions normally devoid of NC cells (asterisks, Figure 3.5F). However, high magnification view of this region indicated a small number of EphA4-positive cells in a position indicative of early migrating NC cells (arrows, Figure 3.5G; (McLennan and Krull, 2002)). In ventral regions, *EphA4* was expressed in ventrolateral cells of the neural tube and the ventral motor root (arrows, Figure 3.5H) as well as in the floorplate (asterisk, Figure 3.5H; (Eberhart et al., 2000).

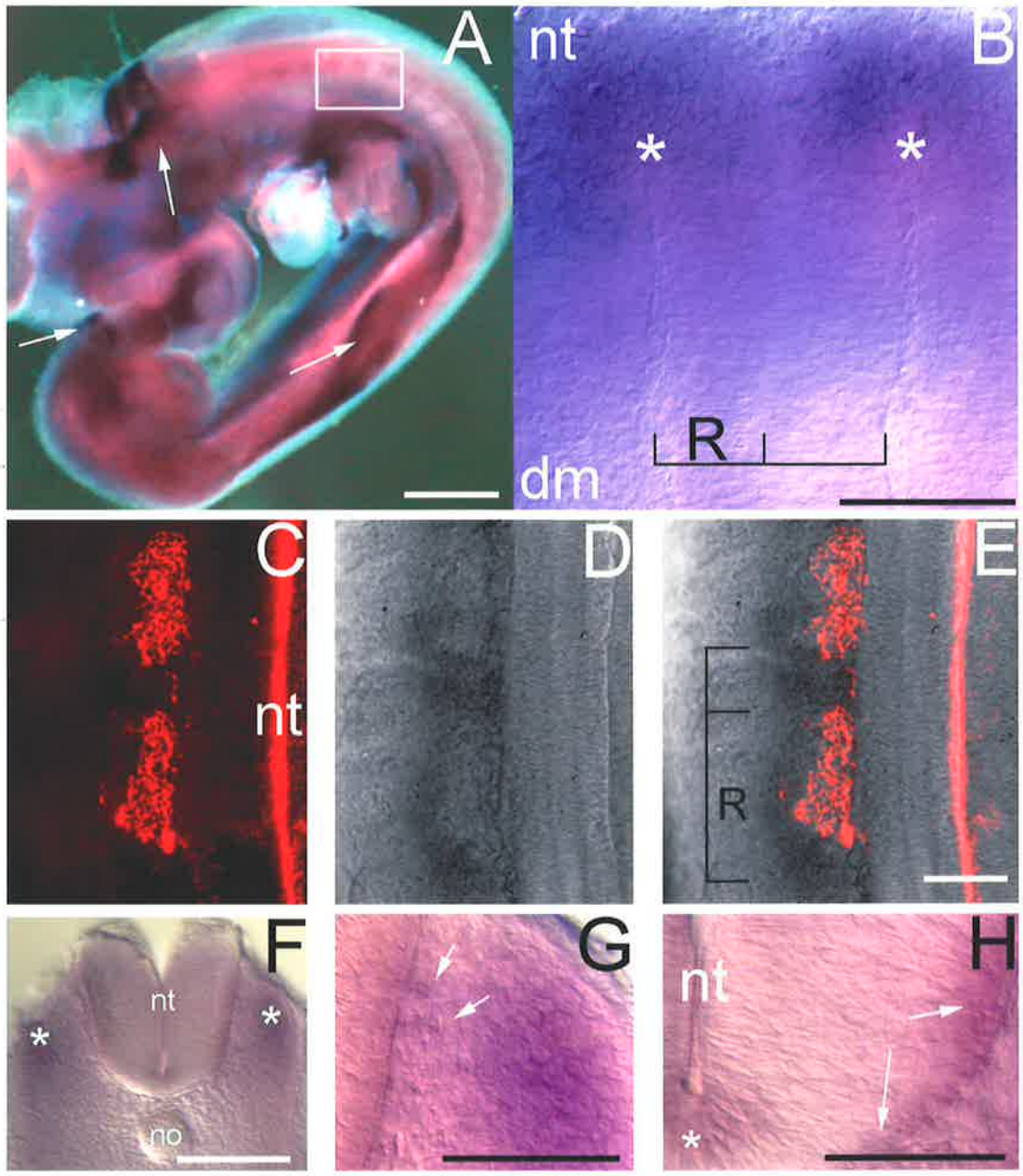
Figure 3.5- *EphA4 in situ* hybridisation on stage 19 embryos reveals a dynamic expression pattern for *EphA4* within the rostral half-somite with regards to NC cell development.

(A) Sagittal view of whole-mount embryo hybridised with *EphA4* probe. Arrows point to regions of intense staining in the embryo including rhombomeres 3 and 5, distal tip of the limb bud and in the anterior half of the presumptive somites. Whole-mount embryos processed for EphA4 *in situ* hybridisation by C.Jayasena, PhD student, 2003.

(B) Sagittal view of boxed region in (A) showing staining in the rostro-dorsal quadrant of successive somites and highlighted by asterisks.

(C-E) 100 μm longitudinal sections of an *EphA4* stained embryo taken from the mid-trunk region of a stage 19 embryo and presented as Hnk1 alone (red, C), EphA4 (D) and co-localised in (E).

(F-H) Transverse sections of *EphA4* stained embryo taken from the mid-trunk region of a Stage 19 embryo. (F) Transverse section through the rostral half-somite. Asterisks indicate staining in NC avoidance zones. (G) High magnification view of (F) focussing on the somitic region through which ventromedially migrating NC cells travel (denoted by arrows). (H) High magnification view of (F) focussing on the ventral region of the neural tube. Arrows shows *EphA4* positive ventrolateral neural tube; asterisk indicates floor plate staining. dm = dermomyotome; fp = floorplate; no = notochord; nt = neural tube; R = rostral . Scale bar: 500 μm (A, F); 200 μm (B); 100 μm (C-E), (G); 50 μm (H).

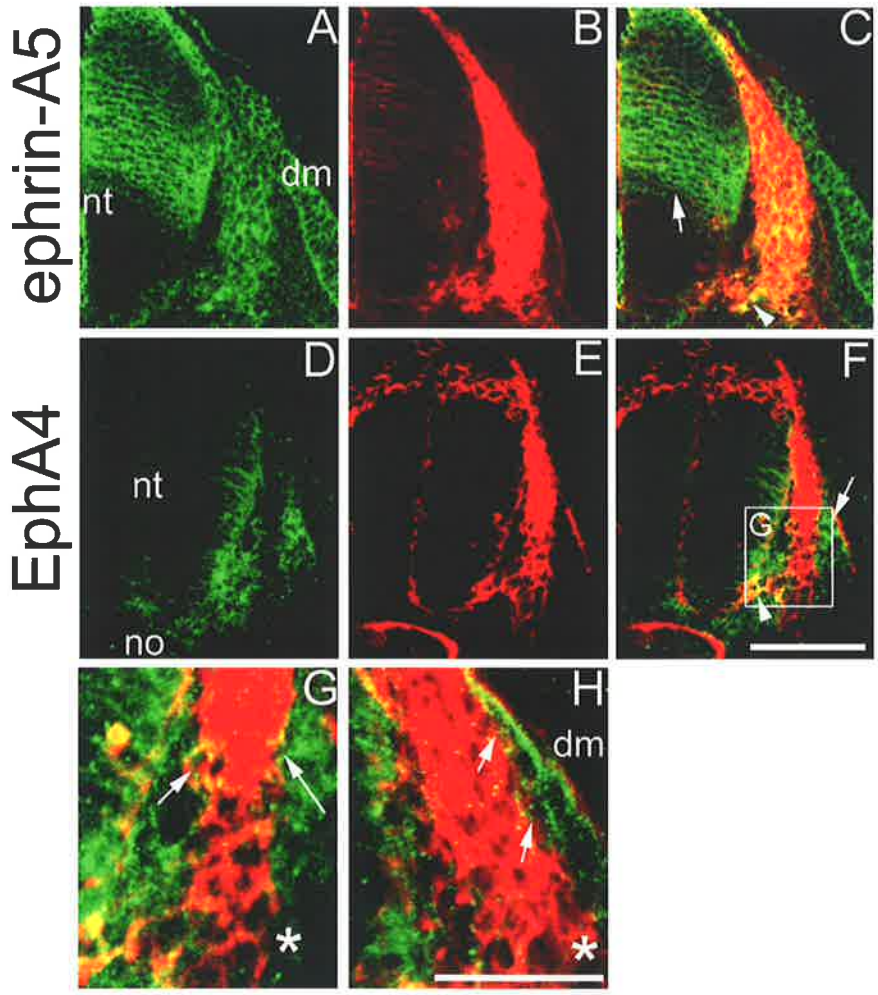

A
nt
B
*
*
R
dm
C
nt
D
E
R
F
nt
*
*
no
G
nt
H
*

3.2.3 Dynamic distribution of ephrin-A5 and EphA4 as NC cells invade the rostral half-somite

The dynamic expression of *ephrin-A5* and *EphA4* within the rostral half-somite suggested that these two proteins were good candidates for mediating NC cell guidance. To determine the protein distribution of these cells with regards to the exact spatiotemporal localisation of NC cells in the somite, avian-specific antibodies to EphA4 and ephrin-A5 (Eberhart et al., 2000) were used on transverse sections in conjunction with the HNK1 antibody. To determine the distribution of these molecules during the infiltration of NC cells into the rostral somite, transverse sections were taken from the mid-trunk region of Stage 17 embryos.

Consistent with the previously described data, ephrin-A5 protein clearly demarcated the truncal neural crest population, from the point of delamination from the neural tube to their migration into the ventral regions of the rostral half-sclerotome (Figure 3.6A-C). As NC cells flooded into the somite, ephrin-A5 protein was maintained on NC cells, and expression was slightly up regulated at the ventral motor exit point, where motor axons exit the neural tube and begin to navigate their way through the somite (Figure 3.6C, arrowhead). In addition to this, ephrin-A5 positive staining defined an intermediate region of the neural tube (arrow, Figure 3.6C).

Figure 3.6. Ephrin-A5 and EphA4 proteins are distributed in complementary domains within the rostral half-somite in the mid-trunk region of stage 17 embryos.
(A-C) 10 μm stack of a 100 μm transverse section labelled with avian-specific anti-ephrin-A5 antibody (green; A) and anti-HNK1 (red; B) and co-localised in (yellow, C). (C) Arrow indicates staining in intermediate neural tube; arrowhead highlights ephrin-A5 distribution at the ventral motor exit point. Ephrin-A5 image provided by R. McLennan, PhD student, 2002. (D-H) Transverse sections labelled with avian-specific anti-EphA4 antibody (green, D, F, G, H) and HNK1 (red, E, F, G, H) presented as single optical slices. (F) Arrow highlights staining at lateral edge of HNK1 stream; arrowhead shows co-localisation at the ventral motor root exit point. (G) High magnification view of boxed region in (F); arrows highlight edge of NC stream. (H) High magnification view of different section also for EphA4 and HNK1, but approximately 2-5 somites anterior to section in (F). Arrows highlight lateral edge of NC stream. (G, H) Asterisks indicate ventral extent of NC migration. dm = dermomyotome; nt = neural tube; no = notochord. Scale bar: 100 μm (A-F); 50 μm (G, H).


ephrin-A5
EphA4
A
B
C
D
E
F
G
H
nt
dm
nt
no
G
dm
*
*

In contrast to ephrin-A5, EphA4 at Stage 17 demonstrated a complementary distribution to NC cells within the rostral half-somite. EphA4-positive regions seemed to compartmentalise the rostral somite region, creating a "channel" in which NC cells were able to migrate (Figure 3.6D-F). In addition, EphA4-positive cells were distributed at the ventral motor root exit zone, co-localised with NC cells (arrowhead) and also lining the ventrolateral edge of the neural tube (Figure 3.6F). A common feature of the distribution of EphA4 in the somite was that EphA4 appeared to delineate the region between the basal lamina of the myotome and NC cells (arrow, Figure 3.6F), and the extent of this staining depended on the size of the NC cell mass. Comparison of EphA4 distribution at the point where NC cells enter into the ventral sclerotome region, (adjacent to the ventral motor root exit point), illustrated the extent of co-localisation in two sections with slightly differing extents of NC migration (though both still in the mid-trunk stage 17). In the younger section (Figure 3.6G, boxed region from 3.6F), EphA4 contacted the edge of the NC stream on both lateral and medial sides (arrows, Figure 3.6G). Approximately 2-5 somites anterior to this, where NC cell migration had progressed further ventrally (compare asterisks, Figure 3.6G and 3.6H), EphA4 still appeared in contact with NC cells on the lateral side but no longer on the medial edge of the migratory stream (arrows, Figure 3.6H).

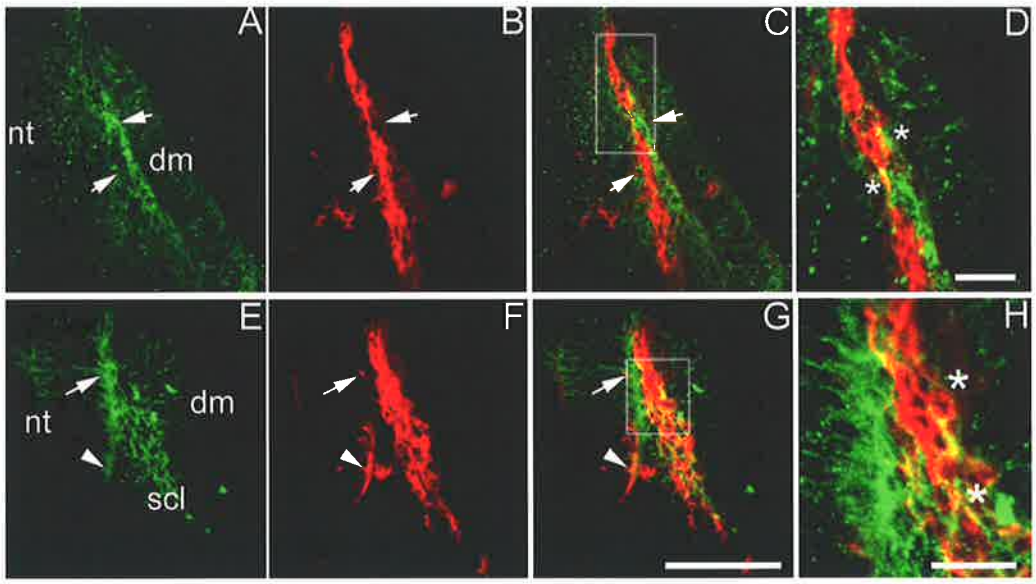
To determine when EphA4 expression was first present in the somite and at an early stage of NC migration, EphA4 immunohistochemistry was performed on transverse sections and whole-mount embryos at stage 14 and 15. Apart from expression in the rostral portion of the two most newly formed somites, (seen in Figure 3.5A), the first detectable level of EphA4 staining in the somite was in the recently formed somites of Stage 15 embryos (Figure 3.7). EphA4 was restricted to a line of expressing cells underneath the forming myotome, but dorsal to NC cells migrating along the edge of this tissue, creating a high degree of complementarity between EphA4 and HNK1 stains (Figure 3.7A-C). High magnification of the region of EphA4/HNK1 complementarity indicated that there was extensive co-localisation possibly reflecting EphA4 expression on NC cells (asterisks, Figure 3.7D). In a somite approximately two somites anterior, the distribution of EphA4 in the rostral somite appeared different again (Figure 3.7E-H). EphA4 was localised to the lateral edge of the neural tube, in the region adjacent to migratory NC cells (arrows) and also at the ventral motor root exit point where NC cells were contacting the ventrolateral neural tube (arrowheads, Figure 3.7E-G). High magnification of the region where NC cells were entering into the sclerotome (boxed in G), suggested that the extent of co-localisation between EphA4 and HNK1 increased as cells progressed ventrally (compare asterisks, Figure 3.7H).

Figure 3.7- EphA4 is closely associated with ventromedially migrating NC cells during the early phases of migration into the rostral half-somite.

(A-F) 100 μm transverse sections taken through the mid-trunk region of a stage 15 embryo through the rostral somite stained with anti-EphA4 (green; A, E) and HNK1 (red, B, F), presented as single images and co-localised in (C, D) and (G, H). (A-D) Section is approximately two somites posterior to image in (E-H). Arrows in (A-C) indicate extent of co-distribution between EphA4 and HNK1. (D) High magnification view of boxed region in (C); asterisks indicate co-distribution between EphA4 and HNK1.

(E-G) Arrow shows staining at the lateral edge of neural tube; arrowheads point to ventrolateral staining. (H) High magnification view of boxed region in (G); asterisks highlight difference in co-localisation between EphA4 and HNK1 in dorsal and ventral regions. dm = dermomyotome; nt = neural tube; scl = sclerotome. Scale bar: 100 μm (A-C), (E-G); 25 μm (G), (H).

Images in (C-L) provided by A. Stokowski, 2001.


A
B
C
D
nt
dm
E
F
G
H
nt
dm
scl

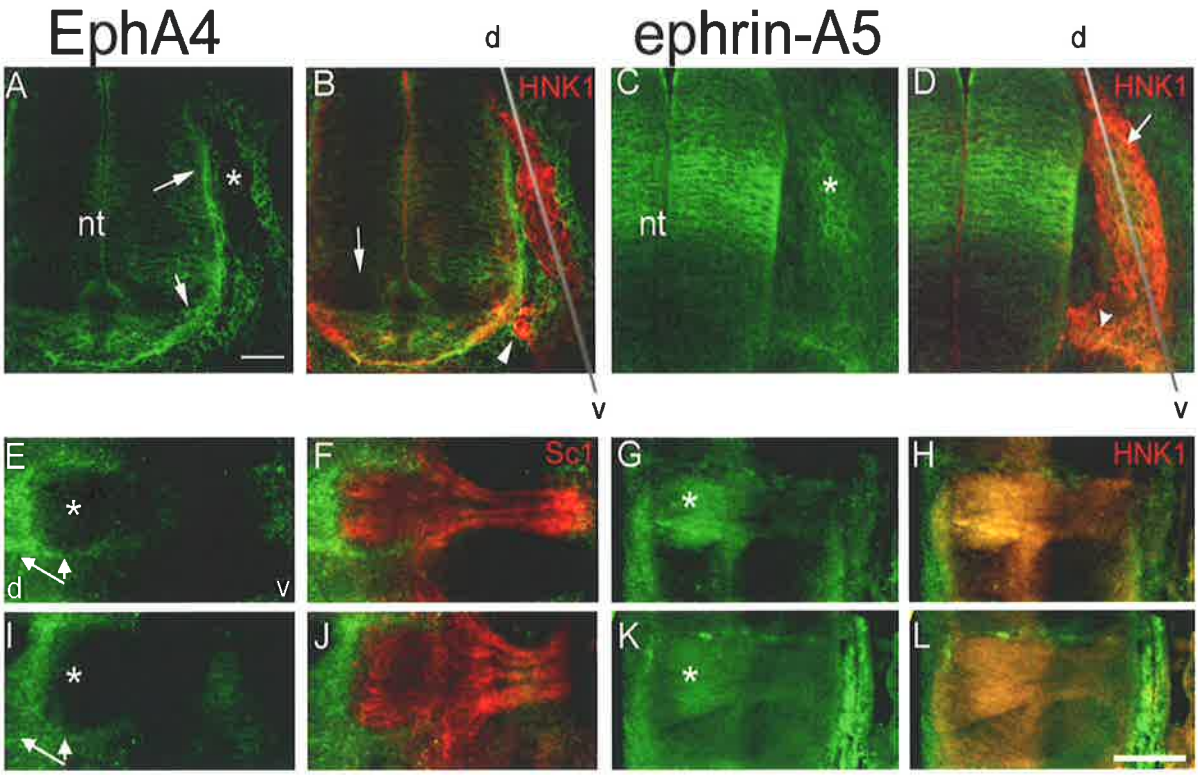
3.2.4 EphA4 and ephrin-A5 show complementary patterns of distribution within the rostral somite during dorsal root gangliogenesis

To gain a three-dimensional perspective on EphA4 and ephrin-A5 distribution during dorsal root gangliogenesis, transverse vibratome sectioning and whole mount immunohistochemical analyses of the mid-trunk region of stage 20 embryos was performed. At stage 20, EphA4 demarcated the dorso-medial boundary of the DRG, and to a lesser extent in the ventral regions (Figure 3.8A). Similar to the distribution at stage 17 (Figure 3.7), EphA4 was present at the lateral and ventrolateral neural tube in the region where spinal motor neurons lie (arrows, 3.8A) as well as on NC cells at the ventral motor root exit zone (arrowhead, Figure 3.8B). Conversely, ephrin-A5 strongly co-localised with cells in the central portion of the DRG (arrow, Figure 3.8D) and was distributed in a line extending ventrolaterally through the sclerotome, indicative of expression on the axons of spinal motor neurons (arrowhead, Figure 3.8D). In addition, EphA4 and ephrin-A5 defined distinct regions of the neural tube; EphA4 was characterised by an absence of staining in the region dorsal to the floorplate (arrow, Figure 3.8B) whilst ephrin-A5 was present as a band of expression in the intermediate neural tube (Figure 3.8C and D). Analysis of whole embryos stained with EphA4 and viewed in the sagittal plane, provided an interesting insight into further possible roles for EphA4 in DRG axon guidance (Figure 3.8E-J). EphA4 cells lined the edge of this structure at both the rostro-caudal and dorsolateral boundaries, creating a "cup-like" structure (arrows, Figure 3.8E). The extent of EphA4 staining around the DRG and ephrin-A5 in the DRG was more pronounced in the brachial ganglia (Figure 3.8E-H) than those located approximately four somites further posterior in the mid-trunk region (3.8I-L).

Figure 3.8- EphA4 is distributed around the DRG whilst ephrin-A5 is expressed in the DRG in the rostral half-somite at stage 20.

(A-D) 100 μm transverse sections of the mid-trunk region stained for EphA4 (green; A, B, E, F, I, J) or ephrin-A5 (green; C, D, G, H, K, L) and HNK1 (red; B, D, F, H, J, L) and presented as single optical slices. Asterisks indicate the dorsal root ganglion in (A, C, E, G, I, K). (A) Arrows point to EphA4 staining at the lateral and ventral neural tube edges. (B) Arrow highlights EphA4-negative region in the ventral neural tube; arrowhead indicates co-localisation between NC cells at the ventral motor root exit zone and EphA4. (C) The DRG is stained for ephrin-A5 (asterisk). (D) Arrow indicates the extent of co-localisation between ephrin-A5 and HNK1 in the DRG; arrowhead points to intense staining along the spinal motor axons. (B, D) The grey dorso-ventral lines illustrate plane of sections in (E –L).

(E-L) Sagittal oblique images are all presented with neural tube on the left and dermomyotome on the right side of the image. (E-L) Images of whole-mount trunks (forelimb region) at the level of somite 9 (E-H) and somite 11 (I-L) stained with EphA4 (green, left panels) or ephrin-A5 (green, right panels) and co-stained with Sc1 (red, F and J) or HNK1 (red, H and L). (E, I) Arrows indicate EphA4 staining around the DRG. d = dorsal; v = ventral; nt = neural tube. Scale bar: 50 μm (A-D); 50 μm (E-L).


EphA4
ephrin-A5
d
d
A
B
HNK1
C
D
HNK1
nt
nt
v
v
E
F
Sc1
G
H
HNK1
d
v
I
J
K
L

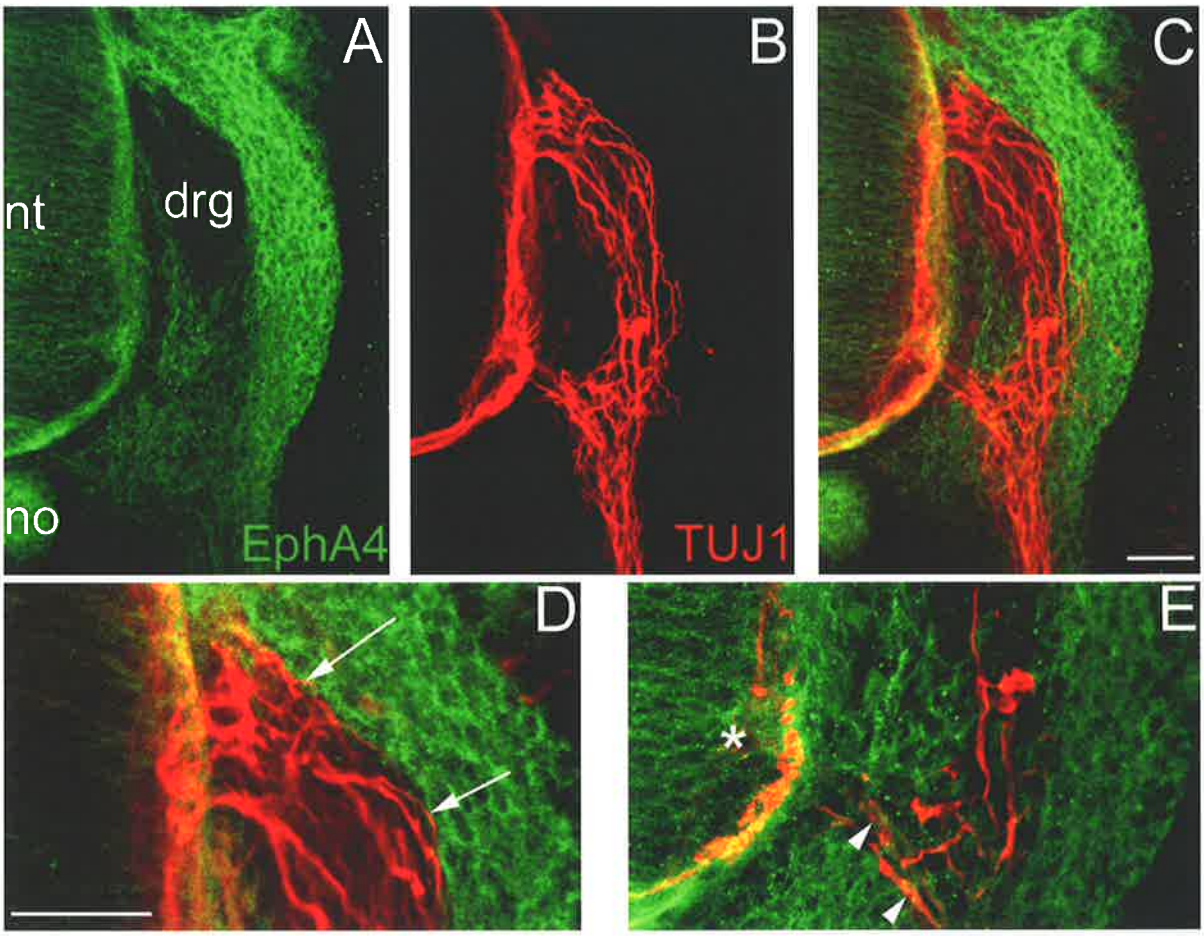
3.2.5 EphA4 closely surrounds sensory axon trajectories as they sprout from DRG towards the CNS an also to meet the spinal motor neurons

To further investigate the relationship between EphA4 on the lateral sclerotome and the axons of the recently formed DRG, EphA4 distribution was analysed in stage 20 embryos in the mid-trunk region with the axonal marker, TUJ1. As previously observed, EphA4 defined the dorso-medial and to a lesser extent, ventrolateral boundaries of sensory axonal trajectories (Figure 3.9A-C). Consistent with the ephrin-A3-Fc data (Figure 1B), EphA4 was also present on the dorso-medial lip of the dermomyotome at stage 20 (Figure 3.9A). High magnification of the dorsolateral sclerotome region suggested that EphA4-presenting cells in this tissue abutted DRG axons extending back into the neural tube (arrows, Figure 3.9D). At the ventral horn, EphA4 was expressed on the cell bodies of spinal motor neurons (asterisk, Figure 3.9E) and also to a lesser extent on spinal motor axons (arrowheads, Figure 3.9E).

Figure 3.9- EphA4-positive cells in the dorsolateral sclerotome closely surround DRG axons in the rostral half-somite of stage 20 embryos.

(A-C) 100 μm transverse section from the mid-trunk region of a stage 20 embryo and presented as a 5 μm optical stack. Sections were stained with anti-EphA4 (green) and the microtubule marker, TUJ1 (red), to label axonal processes. (D) and (E) high magnification view of dorsal (D) and ventral (E) neural tube regions in C. Arrows in (D) indicate degree of co-localisation between DRG axons extending back into the CNS and EphA4 in the sclerotome. Arrowheads in (E) indicate degree of co-localisation between EphA4 and spinal motor axons; asterisk shows EphA4 on spinal motor neurons. drg = dorsal root ganglion; nt = neural tube; no = notochord. Scale bar: 50 μm (A-C), (D-E).

Section stained by A. Stokowski, 2001.


A
nt
drg
no
EphA4
B
TUJ1
C
D
E
*

3.3 Discussion

The expression analyses in this chapter show a highly dynamic expression pattern for EphA and ephrin-A proteins during ventromedial NC migration into the DRG. Utilising a whole-mount Fc screen, EphA and ephrin-A molecules were found in complementary domains within the rostral somitic mesoderm during PNS segmentation. Further analyses explored the precise spatiotemporal distribution of EphA4 and ephrin-A5 during three main phases of ventromedial NC cell migration; during delamination and the emigration of NC cells into the somite; infiltration into rostral half-somites and subsequent condensation into DRG; and during DRG formation/axon outgrowth. These data revealed that ephrin-A5 was expressed on NC cells from their delamination at the dorsal neural tube to their emigration into the somite and condensation into DRG. In comparison to ephrin-A5, EphA4 had a highly dynamic distribution during the three phases of NC cell guidance. The expression of EphA4 on and around NC cells appeared to be bi-phasic. Initially, it was associated with NC and somite cells along the ventromedial NC cell pathway and then later defined the sclerotome regions surrounding NC cells. Finally, during differentiation and axonal extension of DRG precursors, EphA4 continued to surround the DRG, particularly in the dorsolateral sclerotome adjacent to DRG axons extending back towards the central nervous system (CNS). The implications of these dynamic expression patterns are discussed in the context of early NC cell migration into the somite; condensation of NC cells into DRG; and DRG differentiation and axon guidance. The expression of EphA4/ ephrin-A5 in ventral NC cells is also discussed with reference to the maintenance of CNS:PNS boundaries.

3.3.1 EphA4 is co-distributed with ephrin-A's on migratory NC cells at the initial phases of migration

Time-lapse analyses have shown that NC cells migrate in highly organised streams through the rostral somite, maintaining leading-to-tail edge contacts (Krull et al., 1995; Rovasio et al., 1983). These data suggest that cell-cell communication between NC cells might be important for coordinating their highly organised migration through the somite (Krull et al., 1995; Krull et al., 1997). A common observation in both the EphA3-Fc and ephrin-A5 analyses was that ephrin-A ligands (and in particular ephrin-A5) were strongly expressed on NC cells during their delamination and subsequent ventromedial migration to form DRG. This data was in accordance with previous studies demonstrating expression of ephrins –A2, -A5 and –A6 on

NC cells during delamination and their migration into the somite (McLennan and Krull, 2002), and also expression of ephrin-A5 and –A2 on the DRG at hindlimb levels (Eberhart et al., 2000). Given the extensive distribution of ephrin-A proteins on NC cells throughout the various migratory phases of NC cell migration, it is likely that spatiotemporally specific expression patterns for each of these underlies defined functions throughout NC development. Interestingly, ephrin-A5 has recently been shown to interact with EphB3 (Himanen et al., 2004), also co-expressed on NC cells (Krull et al., 1997), and therefore may be acting cooperatively with ephrin-A5 to facilitate cell-cell communication between NC cells.

In addition to ephrin-A molecules, McLennan and colleagues (2002) found that *EphA4* was expressed at a very low level on NC cells during delamination and their initial emigration into the somite (McLennan and Krull, 2002). Consistent with these observations, the ISH analyses presented in Figure 3.5 indicated that *EphA4* was expressed in regions indicative of recently delaminated NC cells. The low expression of EphA4 in these regions might explain why previous studies did not detect EphA4 on NC cells or in the rostral half-somite at the earliest phases of NC cell migration (Santiago and Erickson, 2002). Furthermore, *in vitro* studies suggest that interactions between ephrin-A proteins and EphA4 on migratory NC cells are "migration-promoting"(McLennan and Krull, 2002). Thus, reciprocal interactions between EphA4 and ephrin-A proteins could act as a recognition system on the cell surface of migrating NC cells, thereby coordinating NC cell movement during delamination. Given that the focus of the studies presented in this chapter was on the distribution of EphA4 in the somite, and thereby at later phases of NC migration than McLennan and colleagues (McLennan and Krull, 2002), the exact spatiotemporal distribution of EphA4 with NC cells would have to be re-analysed. However this opens up interesting possibilities with regards to Eph-ephrin-A mediated control of coordinated NC cell movement during delamination and the early entry of NC cells into the somitic environment.

3.3.2 EphA4 expression in the somite and possible mechanisms for neural crest cell segmentation

Throughout the ventromedial migration of NC cells through the rostral half-somite, EphA4 was expressed on cells in contact with NC cells in the sclerotome, indicative of a role in NC cell guidance. In general, guidance cues regulating the segmental migration of NC cells through the somitic environment have fallen into three broad classes based on restricted expression patterns within the somite and whether this precedes NC emigration into this tissue

(Krull, 1998; Perris, 1997). The first of these are factors that are localised to the caudal half-somite, known to mediate repulsion at least *in vitro* (Davies et al., 1990; Krull et al., 1997; Ranscht and Bronner-Fraser, 1991; Ring et al., 1996) and are unchanged by NC cell deletion, often being associated with regions of cartilage formation in the embryo ((Oakley and Tosney, 1991) refer Section 1.4.1.). The second are ECM molecules that are re-arranged as a result of NC invasion into the somite. This group includes versican (Landolt et al., 1995) and F-spondin (Debby-Brafman et al., 1999), that are re-arranged to the caudal portion of the somite and therefore negatively influence NC migration; as well as migration-positive molecules re-arranged into the rostral half-somite coincident with NC migration, such as tenascin-C and TSP-1 (Tucker, 2001; Tucker et al., 1999). The third and final group of molecules are migration-promoting ECM components that do not exhibit any differential distribution in the somitic tissue but are known to facilitate NC movement (Perris and Perissinotto, 2000). Given that EphA4 was expressed exclusively in the rostral half-somite and its expression in the somite occurred coincident with NC migration, EphA4-ephrin-A interactions may constitute a local guidance system that acts in concert with known ECM molecules to guide NC migration and subsequent differentiation through the somites. The possible consequences and similarities between EphA4 distribution and other known migration-promoting factors are discussed in more detail below.

Interactions between NC cells and ventral sclerotome cells are important for the sorting of NC cells between DRG and SG lineages (Goldstein and Kalcheim, 1991). During the initial entry of NC cells into the rostral-half sclerotome regions, NC cells are often found interspersed amongst sclerotome cells in the somitic milieu, suggestive of contact guidance (Bronner-Fraser and Stern, 1991; Krull et al., 1995; Newgreen et al., 1990). These interactions are thought to be important for NC lineage restriction as cells that remain dorsally localised end up colonising the DRG, whilst those that continue migrating ventrally will contribute to SG lineages (Goldstein and Kalcheim, 1991). In the ventral sclerotome regions, EphA4 cells appeared to be mixing with (and also possibly expressed on) NC cells in ventral sclerotome regions (Figure 3.7). Though it was impossible to determine whether or not EphA4 was expressed on NC or sclerotome cells at this time-point, a possible interpretation is that this reflects expression on sclerotome cells and a subsequent guidance role. Interestingly, comparison of EphA4 distribution in the two sections from stage 15 embryos in Figure 3.7, suggests that the ventral localisation of EphA4 occurred coincident with a slight compaction of the NC stream at dorsal regions. Thus, EphA4 on sclerotome cells (distinct from its co-expression with ephrin-A ligands on NC cells) might provide an adhesive scaffold for ephrin-

A presenting NC cells, sufficient to "hold" cells in a dorsal location. Consistent with this possibility, ephrin-A5 expressing cells were never found localised in the SG regions but instead only contributed to the DRG lineages.

After their initial emigration from the neural tube and entry into the somite, NC cells infiltrate the rostral half-somite in increasingly large numbers, populating the more medial regions (Tosney et al., 1994). Coincident with the large-scale entry of NC cells into the somite, EphA4 appeared to enter into a different phase of expression, clearly defining NC avoidance regions within this migration environment (Figure 3.6). An integral part of this phase of expression was that EphA4-positive regions appeared to be progressively displaced from the sclerotome by ventrally migrating NC cells, moving into medial and lateral regions around NC cells. A possible interpretation for the re-arrangement of EphA4 distribution is that this reflects a change in its guidance function for NC cells, from an active guidance role to a more general role in channelling NC precursors through specific regions of the rostral half-somite (compare Figure 3.6G with H). The re-arrangement of molecules coincident with NC cell invasion into the rostral half-somite has been reported for multiple ECM molecules (Perris and Perissinotto, 2000). However, little is known about whether their re-arrangement is a cause or consequence of NC cell entry into the somite and therefore has any functional significance for NC guidance (Krull, 1998).

Two functional blocking studies *in vivo* have been able to shed some light on the possible guidance role of the ECM molecules, versican and F-spondin, that are re-distributed into NC avoidance zones within the rostral half-somite coincident with NC cell emigration (Debby-Brafman et al., 1999; Henderson et al., 1997). Analysis of the distribution of versican in Pax3-deficient mice (that exhibit severe NC cell migration defects) indicated that versican expression domains had expanded into normally NC cell-negative regions. Given that the altered versican distribution was not related to a general somite defect, it was suggested that the expansion of versican into NC migratory pathways could explain the inability of Pax3 -/- NC cells to migrate correctly through the somite (Henderson et al., 1997). Additionally, F-Spondin demarcates tissue barriers for both dorsolateral and ventromedial NC migratory pathways. Using *in vivo* neutralisation studies, it has been shown to determine migration-permissive regions within the rostral-half somitic tissue (Debby-Brafman et al., 1999). Thus there is some evidence to suggest that *in vivo*, the creation of permissive migratory environments by ECM molecules (and possibly EphA4) within the rostral half-somite have important consequences for NC migration.

3.3.3 EphA4 and its potential role(s) in DRG formation/axon guidance

Coalescence of NC cells into the DRG is thought to be mediated by increased expression of cell-cell adhesion molecules such as N-cadherin and N-CAM, as well as in part determined by ventrally-located cues in the somitic mesoderm (Akitaya and Bronner-Fraser, 1992; Bronner-Fraser, 1993a). Multiple ECM molecules, including CSPGs (Perris et al., 1991b), versican (Landolt et al., 1995), hyaluronectin (Perris et al., 1991b) and PNA-binding proteins (Oakley and Tosney, 1991) have been described surrounding the DRG on the rostro-caudal and dorso-ventral axes. The exact role of these proteins in determining the eventual positioning and formation of the DRG is unknown, though they do demarcate pre-chondrogenic areas, suggesting that they might simply provide a general scaffold in which the DRG can form (Christ et al., 2000; Oakley and Tosney, 1991). EphA4 has been shown to demarcate areas of mesenchyme condensation in the limb (Patel et al., 1996), whilst its distribution around the DRG is maintained until at least stage 26 when chondrogenesis begins (data not shown; (Christ et al., 2000; Eberhart et al., 2000; Iwamasa et al., 1999; Santiago and Erickson, 2002)). Thus, in a similar manner to other ECM molecules expressed surrounding the DRG, EphA4 expression at this time might be primarily important for vertebral column development, only influencing DRG positioning indirectly.

In addition, the rostral half-somite has been shown to be necessary for facilitating the proliferation of DRG precursors before their differentiation into sensory neurons (Goldstein et al., 1990). A possible mechanism through which the rostral half-somitic mesoderm could facilitate proliferation is by creating a local pool of CNS-derived mitogenic factors, such as FGF (Le Douarin and Kalcheim, 1999). In *Xenopus* embryos, defects caused by perturbation of EphA4 signalling were minimised by co-injection of FGF-8, suggesting a possible interaction between these two molecules in this embryological system (Park et al., 2004). In addition EphA4 expression in the apical ridge of the wing bud is regulated by FGF signalling, suggesting that these two molecules might be interacting in similar pathways (Patel et al., 1996). Thus, it is quite possible that EphA4 might also be interacting with FGF in the rostral half-somite to maintain it in a high concentration, subsequently promoting proliferation of DRG precursors.

The axons of DRG sensory neurons orient and then extend their growth cones in highly specific bipolar trajectories. On the dorsal side, DRG axons target the CNS and on the ventral side, fasciculate along spinal motor axons, targeting the periphery (Keynes et al., 1997). The

distribution of EphA4 on sclerotome cells at the border of DRG axon trajectories suggests a contact guidance role. It has been established that the caudal-half sclerotome maintains the direction of sensory neuron trajectories within the rostral half-somite via contact repulsive cues (Davies et al., 1990). Less is known about guidance in the dorso-ventral plane. More recent experiments have indicated that the dermamyotome, surface ectoderm and notochord are potent sources of chemorepellent molecules and mediate guidance via "surround repulsion" (Tannahill et al., 2000). Thus, EphA4 might represent an additional level of control in this axis through contact between DRG axons and EphA4-presenting cells.

As functional redundancy is a common feature of the Eph family of RTKs, it is possible that there are other EphA receptors and/or ligands (not detected by EphA3-Fc) contributing to neural crest/ DRG axon guidance. For example, Iwamasa and colleagues, (1999) have shown that EphA3 is distributed on the dermomyotome and on the sclerotome around the DRG (Iwamasa et al., 1999). Interestingly, ephrin-A6 is distributed in the same tissues as EphA4 on NC cells at early phases of their migration and also on the sclerotome during DRG axon outgrowth (McLennan and Krull, 2002; A. Stokowski, unpublished data). Thus, in a similar fashion to the visual system, ephrin-A6 might be required to modulate EphA4 function (Hindges et al., 2002). In support of a functionally redundant role for EphA4 in NC cell guidance/DRG formation, EphA4 knock-out mice do not display any gross PNS defects (Kullander and Klein, 2002). However further analysis of these mice at the correct spatiotemporal location during NC migration would be required to ascertain if there were any transient effects on NC cell movement.

3.4 Conclusion

In conclusion, the expression analyses presented here suggest multiple possible roles for EphA4/ephrin-A interactions in ventromedial NC cell migration and subsequent DRG formation/axon guidance. The implications of these have been divided into three main developmental outcomes: crest-crest interactions during early development, crest-somite interactions during condensation to form DRG and lastly, roles for EphA4 in DRG formation and contact guidance between DRG axons and the sclerotome. Given the dynamic nature of the expression pattern of EphA4, both on NC cells and also in the rostral half-somite during ventromedial NC migration, this protein was selected as a candidate for further analysis using *in ovo* electroporation.

Chapter 4 - Mis-expression of EphA4 in the dorsal neural tube perturbs normal NC cell development

4.1 Introduction

The expression data indicated that EphA4 was distributed in a dynamic manner with respect to NC cell development. Initially, EphA4 was expressed on and around NC cells migrating through the rostral somitic tissue. Later, once NC cells began to condense into DRG, EphA4 defined NC avoidance zones with the rostral half-sclerotome, becoming restricted to the dorsolateral and ventromedial areas surrounding the region of condensation. This pattern was maintained during axonogenesis; however, EphA4 expression demarcated the rostro-caudal boundary of the somite, at the dermomyotomal lip (DML) and on the dorsolateral side of the growing axonal processes (refer Chapter 3).

The close proximity of EphA4-expressing cells to NC cells during their migratory period and their subsequent withdrawal from NC cells to surround the DRG at the onset of gangliogenesis is similar to such migration-promoting molecules as fibronectin (Thiery et al., 1982b), tenascin (Mackie et al., 1988), or even hyalouronectin (Perris and Perissinotto, 2000). Recently it has been shown that EphA4 is expressed on NC cells during delamination and their early emigration into the rostral half-somite (albeit at low levels) ((McLennan and Krull, 2002); Chapter 3), suggesting some role in the coordination of NC cell migration by facilitating cell-cell communication. Thus, to determine if EphA4 has a role in NC cell guidance either through maintaining cell-cell contacts, or mediating NC cell interactions with the rostral half-somite, EphA4 was over-expressed in NC cells at the onset of their migration, and also EphA4 function was blocked using a kinase-inactive EphA4 construct. The effects were analysed using a marker for migratory NC cells, the HNK1 antibody (Tucker et al., 1988).

In ovo electroporation was utilised to perturb EphA4 expression. This technique is a transient, transgenic system that enables both gain-of-function and loss-of-function approaches to be utilised in the avian embryo (REFER Section 1.8). Plasmid DNA, encoding EGFP and the gene of interest is injected into the lumen of the neural tube. Platinum electrodes are then placed in a ventro-dorsal orientation either side of the embryo to facilitate expression of the

genes in the dorsal quadrant (cathode side) of the neural tube and subsequently, on NC cells that delaminate from this region. Embryos transfected via electroporation are viewed 24 hours later using fluorescence microscopy either longitudinally (from the dorsal aspect) as a whole-mount embryo or in transverse sections. This technique has been used extensively in the field of NC cell development to analyse the role of various transcription factors/ cell adhesion molecules in NC cell development (Cheung and Briscoe, 2003; Dottori et al., 2001; Kos et al., 2001).

4.2 Results

4.2.1 EphA4 over-expression inhibits the segmental migration of NC cells through the somite

For the gain-of-function approach, *Gallus gallus* EphA4 cDNA was inserted into the pMES vector, which contains a chicken β-Actin promoter and CMV-IE enhancer driving expression of a bi-cistronic messenger RNA in neuronal tissues (Krull, 2004). Due to the insertion of an internal ribosome entry site (IRES) between the gene of interest and EGFP, each protein is translated independently, yielding two distinct protein products (Swartz et al., 2001a). All electroporations were carried out on stages 12-14 embryos, staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). The over-expression construct, EphA4/EGFP, was injected into the neural tube lumen at the level of epithelial somites in order to target NC cells prior to their emergence from the neural tube. Embryos were incubated for 24 hours (stages 17-20) and analysed in the mid-trunk regions for the effects of EphA4 over-expression on NC cell migration, utilising the NC cell marker, HNK1, and ephrin-B1 to indicate the caudal half-somite.

In control embryos, EGFP-expressing cells exited the neural tube and migrated into the rostral half-somite in an organised fashion, integrating efficiently into the population of endogenous NC cells (Figure 4.1A-C; n =16). The emigration of EGFP-positive cells occurred contiguously with endogenous NC cells and produced an equivalent degree of ventral migration into the somite on both sides of the embryo (data not shown). In comparison, EphA4/EGFP-expressing cells that had budded off from the neuroepithelium remained dorsally adjacent to the neural tube and adopted a round morphology, distinct from the endogenous NC cell population (Figure 4.1D and E). In regions with the highest number of

EphA4/EGFP-expressing cells outside the neural tube, NC cells did not enter the adjacent somite, instead forming a wedge of tightly associated cells at the dorsomedial edge of the neural tube (n = 8/13; arrows, Figure 4.1D and F). The most severe loss of NC cells in the somite occurred ventrally, adjacent to regions where multiple EphA4/EGFP-positive cells were located (arrows, Figure 4.1D and F; data not shown). In control embryos, a similar NC cell aggregation was observed in 1/14 of all embryos analysed (REFER Appendix 1.2).

Due to the orientation of the embryo in Fig 4.1D-F, it was impossible to determine if the loss of cells in the somite occurred in the dorsal portion of the somite alone. Examination of the entire dorso-ventral extent of the embryo indicated that the majority of transfected NC cells were clumped at the dorsomedial neural tube, leaving the somite devoid of NC cells (arrowhead, Figure 4.1G). The virtual absence of NC cells in these somites did not affect their rostro-caudal polarity as indicated by ephrin-B1, though the electroporated and un-electroporated sides did appear slightly out of phase (Figure 4.1G; compare asterisks between electroporated and un-electroporated sides). To summarise the effect of EphA4 over-expression in the transverse plane, a schematic diagram (Figure 4.1 H) illustrates the localisation of EphA4/EGFP-positive cells (green) at the dorsomedial edge of the somite, causing a reduction in the number of endogenous NC cells on the transfected side of the neural tube (grey; Figure 4.1H).

A possible mechanism for the reduction of NC cells in the somites observed in embryos expressing EphA4/EGFP was that EphA4 was affecting the number of cells emanating from the dorsal neural tube. Examination of the dorsal aspect of embryos with a clumping phenotype (Figure 4.2A and B) indicated that the number of NC cells on the electroporated side of the dorsal neural tube was significantly reduced (arrows, Figure 4.2B). This seemed to correlate with decreased progress of NC cell migration through the somite on the transfected side (arrowheads, Figure 4.2D).

4.2.2 Does EphA4 over-expression cause apoptosis?

One possible explanation for the rounded morphology and reduced number of NC cells at the dorsal neural tube was that cells over-expressing EphA4 were undergoing apoptosis. To examine the degree of apoptosis in EphA4 and control electroporated embryos, transfected embryos (stages 16-18) were sectioned transversely in the mid-trunk regions and analysed for the distribution of TUNEL-positive nuclei. In both control and EphA4-expressing embryos,

TUNEL-positive nuclei were localised at the dorsal edge of the neural tube and also in the floorplate region (Figure 4.3B and E). In the dorsal region, however, EphA4-expressing sections demonstrated a strong localisation of TUNEL-positive nuclei on the expressing side compared to a more even distribution in controls (compare Figure 4.3A-C with D-F). Despite an increased distribution of TUNEL-positive nuclei on the EphA4/EGFP-expressing side, in most cases this did not correlate with increased co-localisation between EphA4/EGFP-positive cells and TUNEL-positive nuclei. As demonstrated in high magnification in Figure 4.3G-I, EphA4-expressing cells were often associated with TUNEL nuclei but were not themselves undergoing apoptosis (arrowheads, Figure 4.3G-I).

To confirm the extent of apoptosis on the EphA4-expressing side of the neural tube, transverse sections from EphA4 and control embryos were pooled (EphA4, n = 4 and Control, n = 3) and the number of TUNEL-positive nuclei counted in the dorsal quadrant of the expressing and non-expressing sides for each (REFER Appendix 1.3 for summary). This indicated that whilst there was a trend towards increased apoptosis on the EphA4/EGFP-expressing side (not observed in controls), this was not statistically significant ($p = 0.057$; Figure 4.3J).

Figure 4.1- Ectopic expression of EphA4 after 24 hours incubation inhibits NC cell segmentation in the mid-trunk region of stage 18-20 embryos.

Control (A-C) and EphA4/EGFP (D-F) transfected whole-mount embryos, viewed dorsally and presented as 20 μm stacks of optical sections. Transfected embryos were stained with HNK1 (red) to indicate migratory NC cells and ephrin-B1 (blue) to label the caudal half-somite. EGFP distribution in this image and all subsequent images indicated in green. Asterisks in (E) point to EphA4/EGFP-expressing cells at the dorsomedial neural tube; arrows in (D) and (F) indicate NC cell distribution in the somite.
(G) Image taken from entire dorso-ventral extent (76 μm stack) of EphA4/EGFP-expressing embryo in (D-F), focussing on the three rostral-most somites in (D-F). Asterisks indicate spacing of somites between electroporated and un-electroporated sides; arrowhead points to migratory NC cells unaffected by EphA4 expression. (H) Schematic reconstruction in the transverse plane summarising the EphA4 phenotype. Green cells represent EphA4/EGFP-expressing cells, grey are NC cells. Brackets indicate somite outline R= rostral. nt = neural tube; dm = dermomyotome. Scale bar: 100 μm (A-G).

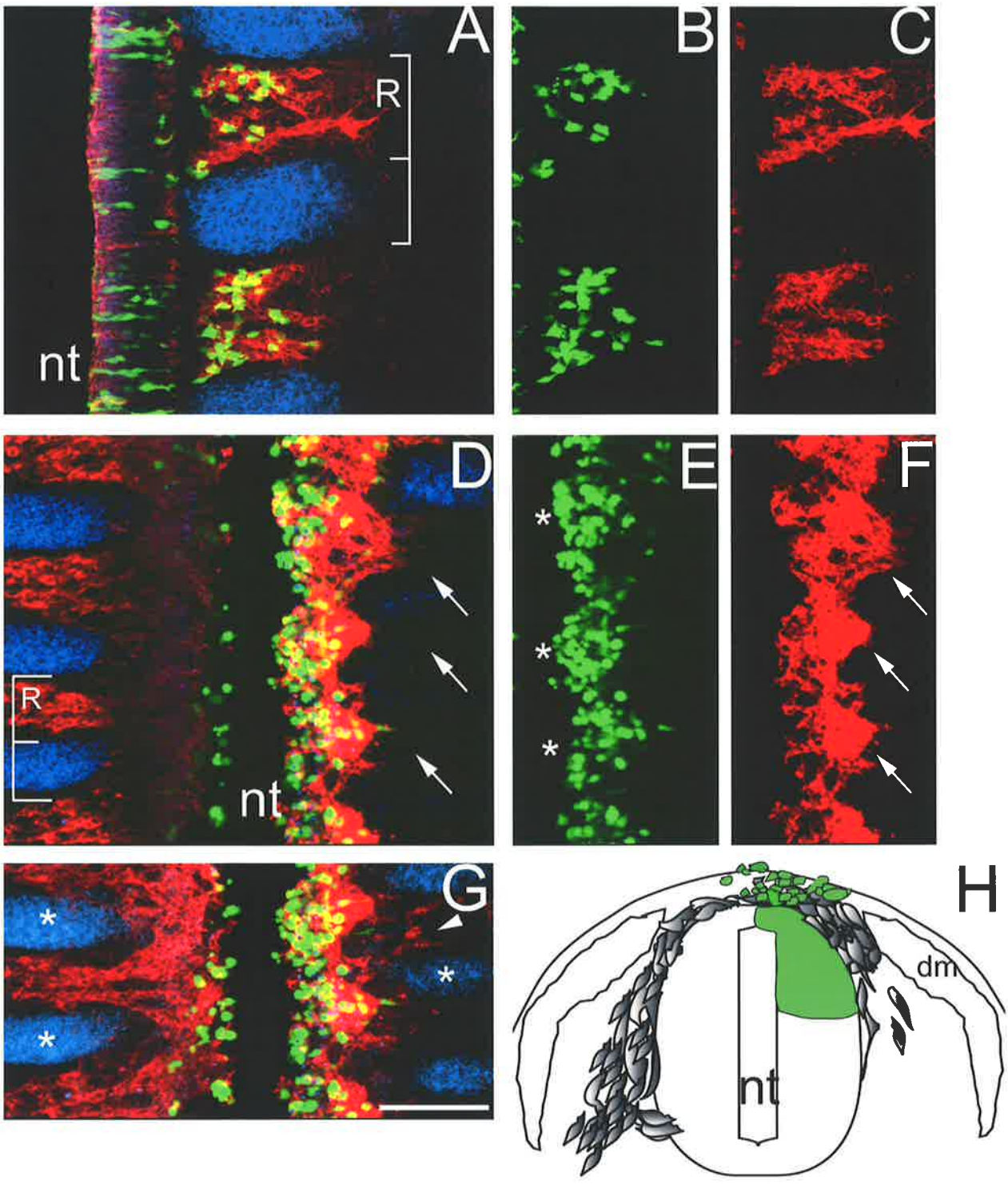

A
R
nt
B
C
D
R
nt
E
F
G
H
dm
nt

Figure 4.2- EphA4-mediated inhibition of NC cell segmentation reduces the number of NC cells at the dorsomedial neural tube.

Schematic diagram of a transverse section of the neural tube illustrates the plane of section in the longitudinal views in (A and B) and (C and D).
(A and C) 4 μm stack of optical sections taken from a whole-mount stage 18 embryo in the mid-trunk region, expressing EphA4/EGFP (green), and co-stained with HNK1 (red) and HNK1 alone (B and D). (A) and (B) are taken from the dorsal regions (of the series of optical sections) and (C) and (D) from the ventral regions. Arrows in (B) indicate reduced numbers of HNK1 at dorsal levels. Arrowheads indicate reduced delamination on the electroporated side. R = rostral, bracket indicates somite outline. Scale bar: 100 μm (A-D).

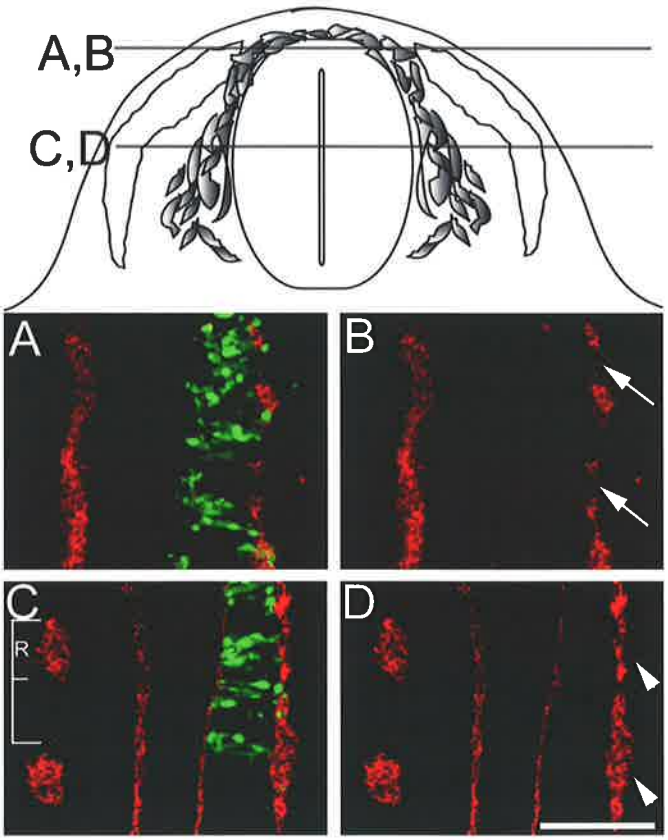
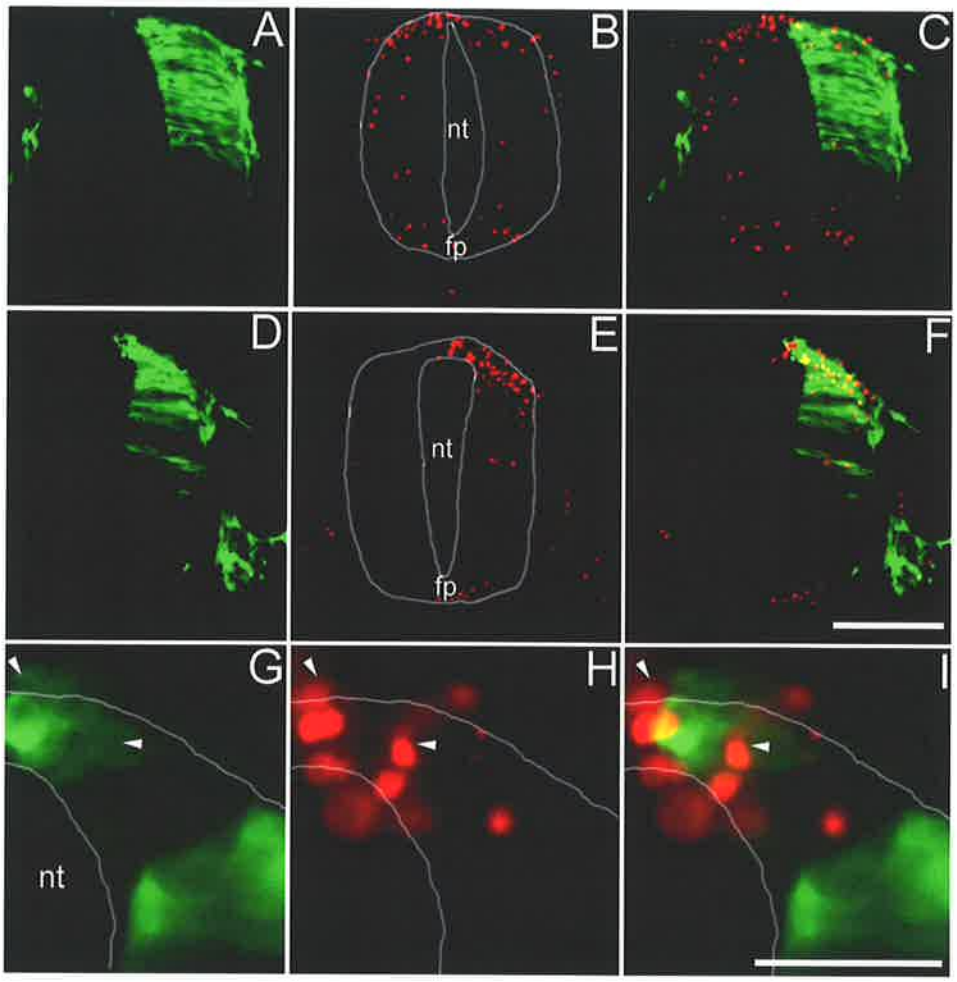

A,B
C,D
A
B
C
R
L
D

Figure 4.3- EphA4-mediated inhibition of NC cell migration is not due to apoptosis in EphA4/EGFP-expressing cells.

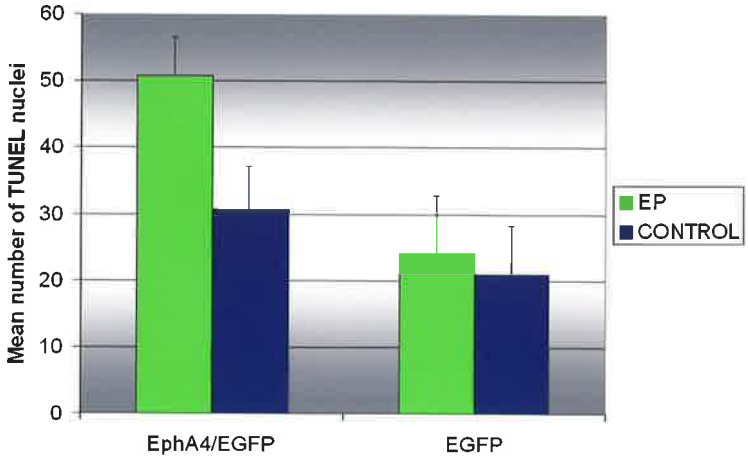
Control (A-C), and EphA4/EGFP (D-F), electroporated embryos, sectioned transversally and screened for TUNEL-positive nuclei (red). Sections were taken from the mid-trunk region of stage 18 embryos and presented as a 10 μm stack of optical sections with EGFP alone (green, A and D), TUNEL nuclei (red, B and E), and the EGFP/TUNEL overlay in (C and F). (G) Fluorescent micrograph of the dorsal neural tube of an EphA4/EGFP transverse section viewed in high magnification and presented as EphA4/EGFP alone (green, G), TUNEL nuclei (red, H) and co-localised (I). Arrowheads in (G-I) highlight the position of TUNEL nuclei with regards to EphA4/EGFP-expressing cells. nt = neural tube; fp = floorplate. Scale bar: 50 μm (A-F); 100 μm (G-I). White line in (G-I) denotes neural tube outline.

(J) Distribution of TUNEL nuclei in the dorsal neural tube in EphA4/EGFP (n = 4) and EGFP-expressing embryos (n = 3). Counting of TUNEL nuclei was performed on transverse sections taken from the mid-trunk region of embryos sectioned 24 hours after electroporation and the data pooled for EphA4/EGFP and EGFP sections. Mean number of TUNEL nuclei for electroporated (green) and control (blue) sides are compared for each construct. No significant differences were found between electroporated and control sides for either EphA4/EGFP or EGFP-expressing embryos, EphA4 ($p = 0.057$) or controls ($p = 0.977$). Error bars for EphA4/EGFP, EP: ± 5.8, Control: ± 6.3 and EGFP, EP: ± 8.5, Control: ± 7.3. EP = electroporated side; Control = contralateral side



Distribution of TUNEL nuclei in the dorsal neural tube of EphA4/EGFP and EGFP embryos

J



4.2.3 EphA4/EGFP-positive cells aggregate in the neural tube and emigrate from its lateral surface

A common feature found in embryos electroporated with EphA4/EGFP when viewed in dorsal longitudinal view was that EphA4/EGFP-positive cells were distributed in isolated clusters within the neuroepithelium. To further examine this effect, embryos were viewed in transverse sections and the number of sections with clumped EphA4/EGFP-positive cells compared to controls (Figure 4.4; REFER Appendix 1.2B). In control embryos (n = 6), EGFP-expressing cells were oriented laterally and aligned with neuroepithelial cells, forming a columnar structure contiguous with endogenous cells (Figure 4.4A). External to the neural tube, EGFP-expressing cells were aligned at positions coincident with the normal progression of cells undergoing delamination and subsequent emigration into the somite (arrows, Figure 4.4A and B). In all but one embryo analysed (n = 8/9), EphA4/EGFP-expressing cells in the neural tube were not laterally aligned contiguous with neuroepithelial cells but formed a clump of cells that extended out of the plane of the neural tube surface (Figure 4.4C-G). Exterior to the neural tube, EphA4/EGFP-positive cells were often found adjacent to these clumps, appearing to have exited the neural tube from the lateral regions (arrow, Fig 4.4C and D). Interestingly, these cells had a normal migratory morphology and some had acquired HNK1 immunoreactivity indicative of cells that had successfully delaminated (arrowheads, Figure 4.4C and D).

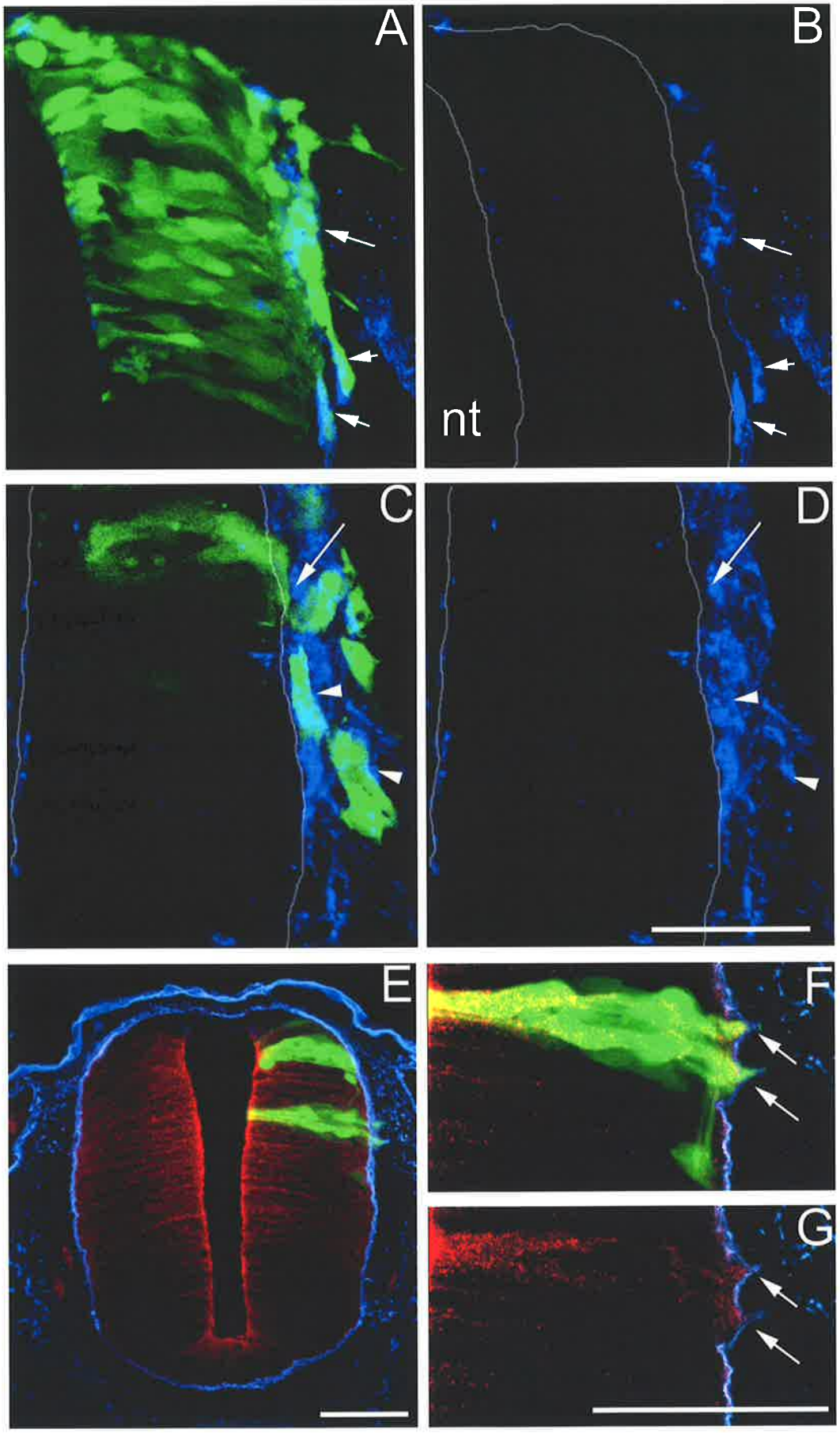
To better examine the arrangement and possible consequences of EphA4/EGFP-expressing clumps within the neural tube, transverse sections were analysed using antibodies against the basal lamina protein, laminin and N-cadherin (Figure 4.4E-G). EphA4/EGFP cells extending from the lateral edge of the neural tube were bounded by basal lamina, indicating that their conformation had distended the lateral edges of the neural tube (arrows, Figure 4.4F and G). Furthermore, the band of EphA4/EGFP-positive cells within the intermediate neural tube correlated with a concentration of N-cadherin protein; this included EphA4-expressing cells at the lateral edge (Figure 4.4F).

Figure 4.4- EphA4/EGFP-expressing cells delaminate from the pial surface of the neural tube.

Transverse sections of control (A and B), and EphA4/EGFP-expressing embryo (C-G) taken from the mid-trunk region of stage 18-20 embryos and presented as 10 μm stacks of optical sections. A-D are co-stained with HNK1 (blue). (A-D) arrows indicate extent of co-expression of HNK1 in control and EphA4/EGFP cells at intermediate neural tube. Arrowheads in (C- D) indicate HNK1 and EphA4/EGFP co-expression. White line in (B-D) denotes neural tube outline.

(E-G) 4 μm stack of transverse EphA4/EGFP (green) section stained with N-cadherin (red) and laminin (blue) in (E). (F) and (G), high magnification view focussing on EphA4/EGFP-positive band in intermediate neural tube. Arrows in (F) and (G) indicate EphA4/EGFP-expressing cells protruding out of neural tube.

Scale bar: 50 μm (A-D), (E), (F-G).


A
B
nt
C
D
E
F
G

4.2.4 EphA4 over-expression in the dorsal neuroepithelium inhibits NC cell delamination

Given the inhibitory effects of EphA4 on NC cell migration through the somite, the effect of EphA4 over-expression on EMT was examined more closely. Transverse sections of embryos transfected with EphA4/EGFP were analysed 24 hours post-electroporation in the mid-trunk region, utilising HNK1 as a marker for the successful completion of EMT, N-cadherin to indicate the distribution of neuroepithelial precursors, and laminin to indicate basal lamina breakdown at the dorsal neural tube (Figure 4.5). On the EphA4/EGFP-positive side of the neuroepithelium, HNK1 distribution was significantly reduced at the dorsal neural tube (arrowheads, Figure 4.5A and B; EphA4, n = 4/5; control n = 0/3). Comparison of the number of HNK1-positive cells at the dorsal neural tube in EphA4/EGFP-expressing neuroepithelium with controls indicated that delamination was inhibited at the dorsal neural tube in 80% of EphA4 embryos (n = 4/5) compared to not at all in control embryos (n= 0/3). This often coincided with a reduction in the extent and number of NC cells at the dorsomedial edge of the somite on the electroporated side (arrow, Figure 4.5B). Furthermore, co-localisation of the image (in Figure 4.5B) with laminin immunoreactivity indicated that the dorsal edge of the EphA4/EGFP transfected side was characterised by a thickening of the basal lamina, such that the lateral extent of membrane breakdown (indicated by laminin antibody) was more advanced on the un-transfected side (compare arrowheads, Figure 4.5C).

Breakdown of the basement membrane above the neural tube is an integral component of EMT (Newgreen and Gibbins, 1982). An additional requirement for correct neuropeithelial cell exit from the neural tube is the loss of N-cadherin mediated epithelial cell-cell junctions (Nakagawa and Takeichi, 1998; Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985). Thus, these two key requirements for EMT were examined in more detail in EphA4/EGFP-expressing and control embryos using laminin and N-cadherin antibodies (Figure 4.5D-G).

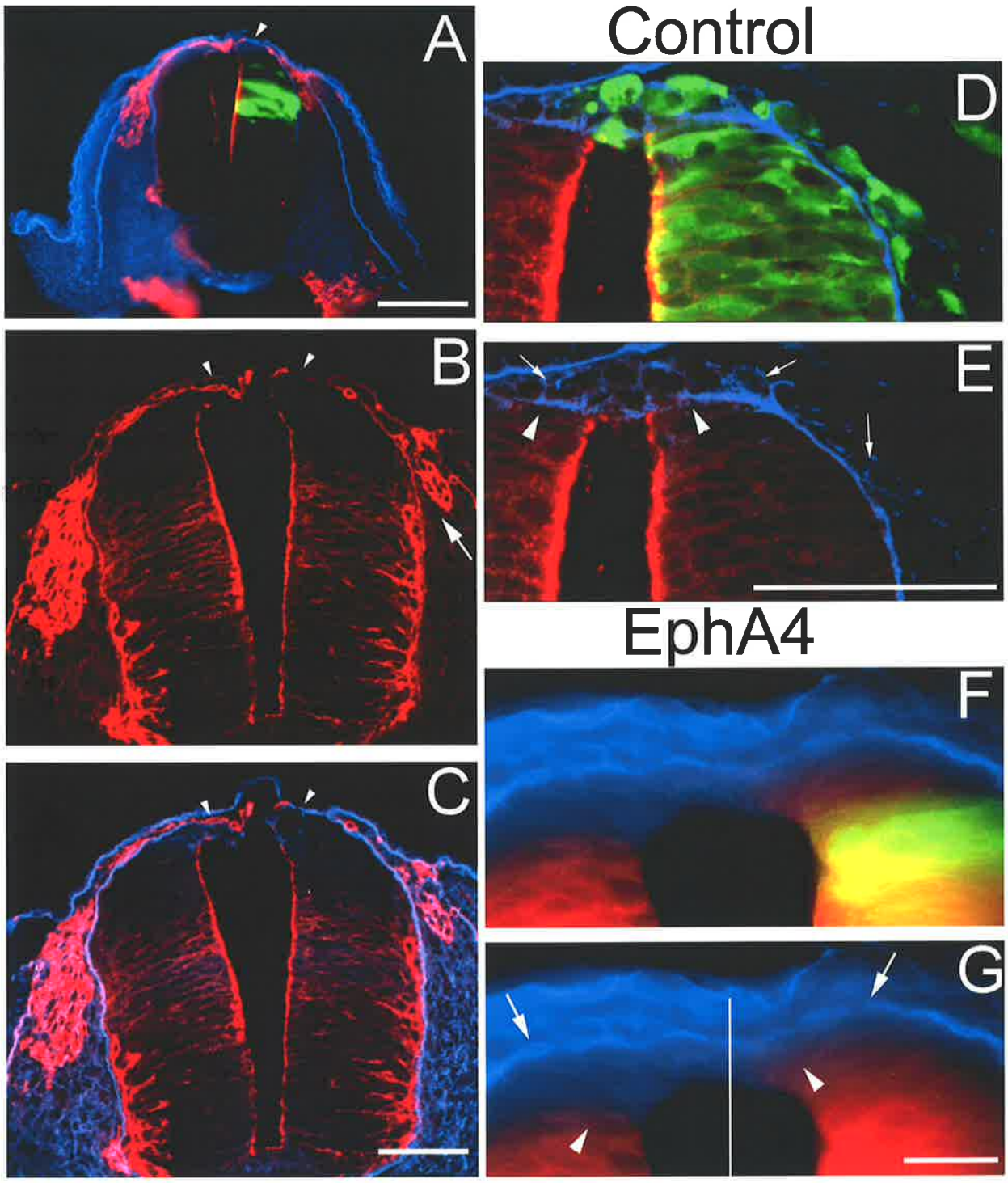
Figure 4.5- EphA4 inhibits the delamination of NC cells and reduces basement membrane breakdown at dorsal neural tube.

Transverse sections of EphA4/EGFP-expressing embryos (A-C and F, G) and control embryos (D, E) taken from the mid-trunk of stage 18-20 embryos.

(A) EphA4/EGFP (green) transverse section stained with HNK1 (red) and laminin (blue). (B) and (C) show the same section presented as a single optical section; HNK1 alone (B) and HNK1/Laminin (C). Arrowheads in (A-C) indicate NC cells at dorsal neural tube above EphA4/EGFP region; arrow in B indicates reduced HNK1 on electroporated side.

(D-G) Single optical sections of control EGFP-expressing (D, E) and EphA4/EGFP-expressing (F, G) embryos stained with N-cadherin and laminin; EGFP, N-cadherin (red) and laminin (blue) co-localisation in (D and F); N-cadherin/laminin co-localisation in (E and G). Arrowheads in (E and G) indicate dorsal extent of N-cadherin; arrows in (E) point to laminin distribution on and around recently delaminated cells. Arrows in (G) point to basement membrane breakdown on transfected and un-transfected sides; dorsal midline indicated by line.

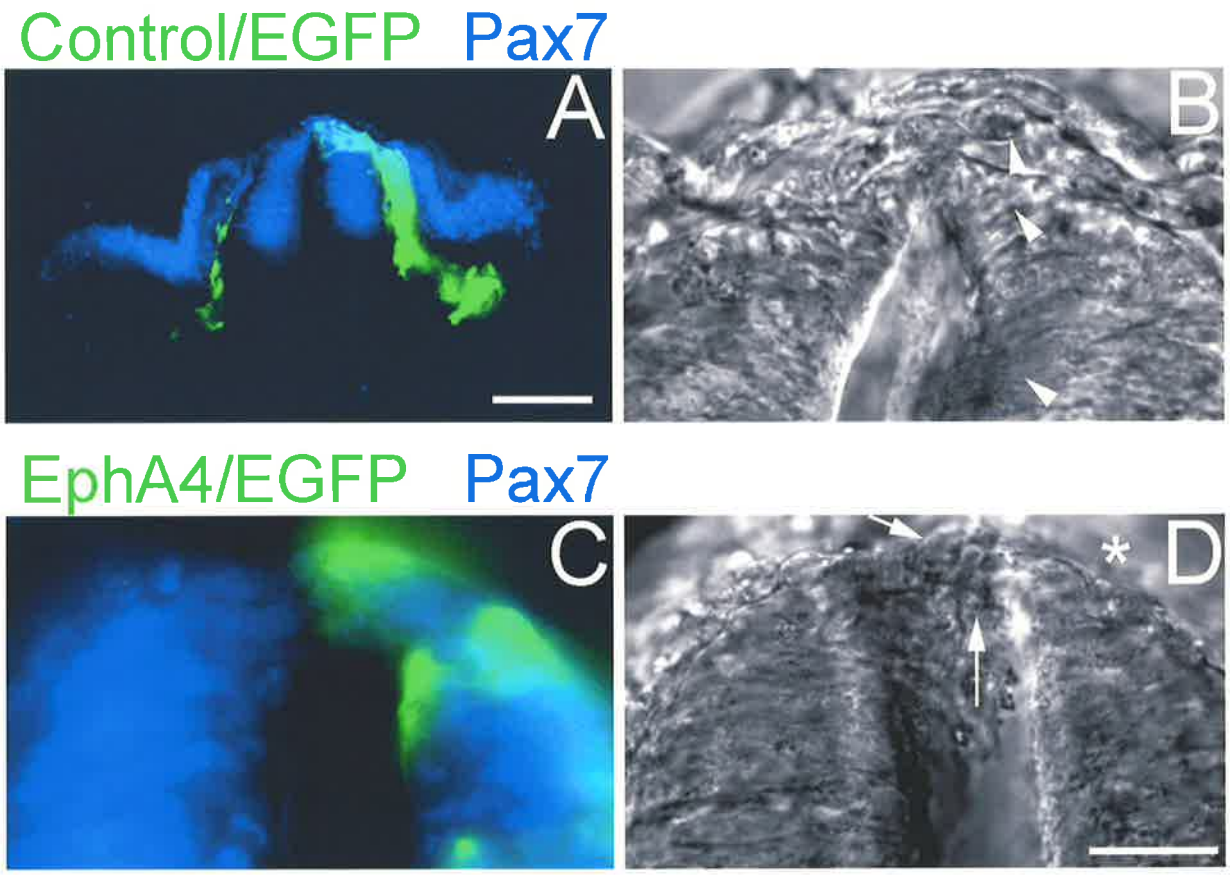
Scale bars: 100 μm (A); 50 μm (B, C), (D-G).


Control
A
D
B
E
EphA4
F
C
G

In control embryos, NC cell precursors expressing EGFP were observed at the dorsal midline in the N-cadherin negative region of the neural tube, and located above a discontinuous basement membrane (Figure 4.5D). Furthermore, neuroepithelial cells on the transfected side appeared to lose N-cadherin immunoreactivity at approximately the same distance from the dorsal edge of the neural tube as on the unaffected side (arrowheads, Figure 4.5E). The breakdown of the basement membrane at the dorsal midline was also consistent with EGFP having no effect on the normal process of EMT, generating cells with laminin-positive membrane around their exterior surfaces and in the surrounding interstitial matrix to a similar extent on both the transfected and un-transfected sides (arrows, Figure 4.5E). In contrast, EphA4/EGFP-expressing cells formed a thick wedge in the N-cadherin positive region at the dorsal midline (Figure 4.5F). Compared to controls, the dorsal limit of N-cadherin staining was extended dorsally, above the region of EphA4/EGFP-expressing cells, creating an asymmetrical distribution between transfected and un-transfected sides (arrowheads, Figure 4.5G). Overlay of N-cadherin and laminin staining indicated that, in a similar way to Figure 4.5C, the edge of continuous basement membrane on the EphA4 side was much closer to the dorsal midline than the contralateral, indicative of reduced EMT (compare arrows, Figure 4.5G).

To further examine a possible inhibition of EMT from the dorsal neural tube of EphA4/EGFP-expressing neural tubes, the morphology of EphA4/EGFP-positive neuroepithelial cells was examined (Figure 4.6). Utilising the Pax7 antibody to indicate the dorsal neural tube, expression of EGFP alone in this region and on subsequently emigrating NC cells didn't affect the progression of cells through EMT (Figure 4.6A and B). Focusing on cellular morphology at the dorsal midline, there was a clear progression of cell shape and orientation from laterally connected neuroepithelial cells, to the more dorso-ventrally aligned cells at the dorsal midline of the neural tube and to a recently detached cell, (following arrowheads ventro-dorsally, Figure 4.6B). In contrast, EphA4/EGFP-expressing cells at the neural tube midline were arranged in a cluster, still attached to the neural epithelium (Figure 4.6C). The localisation of these cells at the midline perturbed the normal neuroepithelial structure of the dorsal edge of the neural tube (arrows, Figure 4.6D), whilst the region above the midline was characteristically devoid of cells (asterisk, Figure 4.6D).

Figure 4.6- EphA4 causes a cell-autonomous inhibition of NC cell delamination.
Control (A-B; top panel) and EphA4/EGFP-expressing (C-D; bottom panel) transverse sections taken from the mid-trunk regions of stage 18 (A-B) and Stage 16 embryos (C-D). (A, C) Electroporated transverse sections (EGFP in green) were co-stained with Pax7 (blue) to highlight dorsal neural tube and developing demomyotome.
(B and D) Bright field images of (A) and (C) respectively. (B) Arrowheads indicate progressive changes in cell shape and orientation of neuroepithelial cells during NC cell delamination from neuroepithelium to dorsal midline and those recently emigrated from the neural tube. (D) Arrows point to EphA4/EGFP-positive cell clump in neural tube lumen, and asterisk indicates cell-free region above dorsal neural tube. Scale bar: 100 μm (A); 50 μm (B-D).


Control/EGFP Pax7
A
B
EphA4/EGFP Pax7
C
*
D

4.2.5 KiEphA4/EGFP expression alters the migratory characteristics and morphology of NC cells

Over-expression of EphA4 in the dorsal neural tube perturbed the normal development of NC cells during their migration and also their exit from the neural tube. However, in the normal situation, levels of EphA4 expression on NC cells are low and therefore it is unknown how accurately these results might reflect a possible role for EphA4 on NC cells *in vivo*. Thus, to further examine the function of EphA4 in NC cell migration/EMT, kinase signalling through EphA4 was inhibited using a loss-of-function construct. This involved insertion of a *Gallus gallus* EphA4 cDNA sequence containing a point mutation in the codon for a critical lysine residue (in the kinase domain) into the pMES vector (Eberhart et al., 2004). The kiEphA4 construct has been shown to abolish signalling through the kinase domain of endogenous EphA4, acting as a dominant negative (Ethell, 2001). To maintain consistency with EphA4 over-expression analyses, the kiEphA4/EGFP construct was injected into the neural tube lumen at the level of epithelial somites in stage 12-14 embryos (Hamburger and Hamilton, 1951) and these were analysed 24 hours after incubation (stages 18-20) with HNK1 and ephrin-B1.

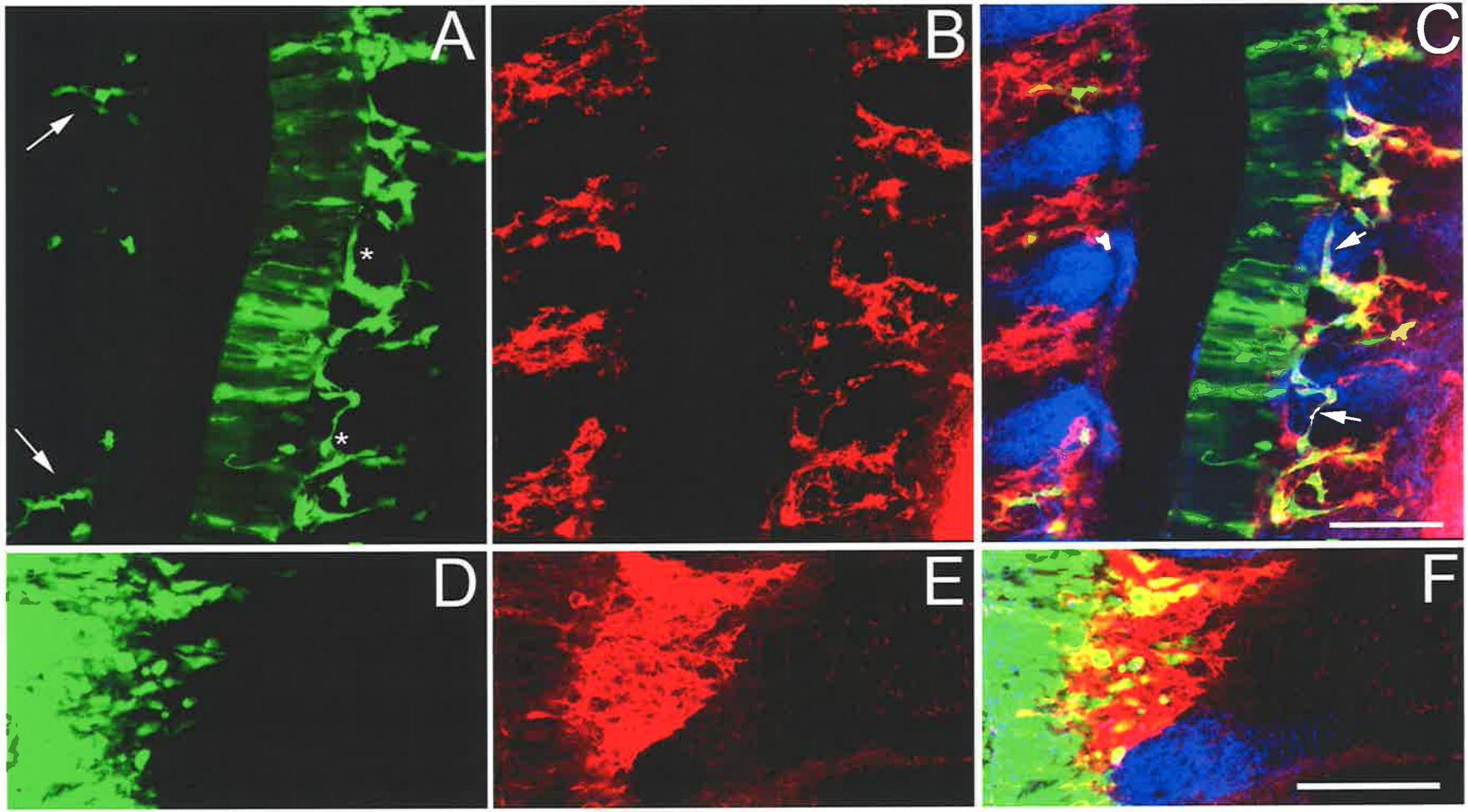
Contrary to the inhibitory effects of ectopic EphA4 treatment on NC cell migration, kiEphA4/EGFP-expressing cells readily exited the neural tube and migrated ventrally into the somites on both sides of the neural tube in a similar manner to controls (Figure 4.7; n = 14; Control: n = 15). Despite their motile behaviour, kiEphA4/EGFP-expressing cells migrated in two distinctly different ways and this appeared to be dependent on the exact developmental stage at which the construct was expressed in the neuroepithelium (REFER Appendix 1.4 for summary of phenotypes).

Figure 4.7- Reduced kinase-dependant activity of EphA4 promotes uncoordinated NC cell outgrowth.

(A-C) 34 μm stack of optical sections of the mid-trunk region of a kiEphA4/EGFP-expressing embryo showing distribution of EGFP-positive cells (green, A), HNK1 (red, B and C) and ephrin-B1 (blue), presented as a co-localised image (C). (A) Arrows point to EGFP cells expressing kiEphA4 on the un-transfected side of the neural tube; asterisks highlight orientation and filopodial extensions of kiEphA4/EGFP-expressing cells. (C) Arrows indicate same cells highlighted (asterisks in A) crossing caudal half-somite region. Embryo electroporated by R. McLennan, PhD student, 2002.

(D-F) 34 μm stack of optical sections from control EGFP-expressing embryo showing EGFP distribution in (green, D), and co-stained with HNK1 (red, E) and ephrin-B1 (blue, F) presented as a co-localised image in (F).

Scale bar: 100 μm (A-C), (D-F).


A
B
C
D
E
F

The most severe phenotype occurred when kiEphA4/EGFP-positive cells were at the leading edge of the NC cell outgrowth (24 hours after electroporation) (Figure 4.7). kiEphA4/EGFP-expressing cells appeared to have an elongated morphology and migrate in a disoriented manner with their long axes aligned tangentially to the neural tube as opposed to the normal dorso-ventral orientation of a ventrally migrating NC cell (asterisks, Figure 4.7A; n = 3). Despite their morphology, kiEphA4/EGFP-expressing cells remained HNK1-positive, creating a disorganised NC cell outgrowth (compare electroporated and un-electroporated sides, Figure 4.7B and C). Some kiEphA4/EGFP-expressing NC cells crossed the boundary between rostral and caudal somite-halves and entered into the caudal somite-half, a region normally avoided by migratory NC cells (arrows, Figure 4.7C). In control embryos, electroporation of EGFP in the same regions did not ever result in EGFP-expressing cells entering into the caudal half-somite; instead EGFP-expressing cells migrated through the rostral half-somite in a coordinated group (Figure 4.7D-F; n = 15; refer Appendix 1.1). Comparison between the level of HNK1 expression in kiEphA4/EGFP-expressing cells located adjacent to the neural tube edge (and crossing inhibitory territories; arrows, Figure 4.7C) and in control EGFP-expressing cells (Figure 4.7D-F) indicated that cells expressing kiEphA4/EGFP had a significantly lower level of HNK1 immunoreactivity.

When kiEphA4/EGFP was expressed in slightly more anterior levels of the neural tube, the disorientated migratory behaviour of kiEphA4/EGFP-expressing cells appeared to be "rescued" (n = 7; Figure 4.8). Viewing NC cell migration in the presence of kiEphA4/EGFP, through dorsal intermediate and ventral levels of a whole-mount embryo, indicated that these cells exited the neural tube at the appropriate time and as a cohesive mass with endogenous NC cells, avoiding the caudal half-somite regions (arrows, Figure 4.8A-C). Despite their coordinated migration, kiEphA4/EGFP-expressing cells had proceeded further into the ventral regions of the rostral half-somite than their counterparts on the un-electroporated side, creating an asymmetrical distribution of HNK1 immunoreactivity at dorsal levels (compare arrows, Figure 4.8A). This also translated to intermediate and ventral levels, where the front of NC cell migration appeared more ventrally located on the electroporated side (arrows Figure 4.8B and C; n = 5; refer Appendix 1.5A for summary). To better analyse the ventral extent of migration in the presence of kiEphA4/EGFP-positive cells, transverse sections were examined (n = 2; Figure 4.8D-F). As was the case for the longitudinal view, kiEphA4/EGFP-expressing cells were located amongst the ventral-most group of cells near the dorsal aorta, creating a more advanced NC stream on the electroporated side (arrow, Figure 4.8D and F).

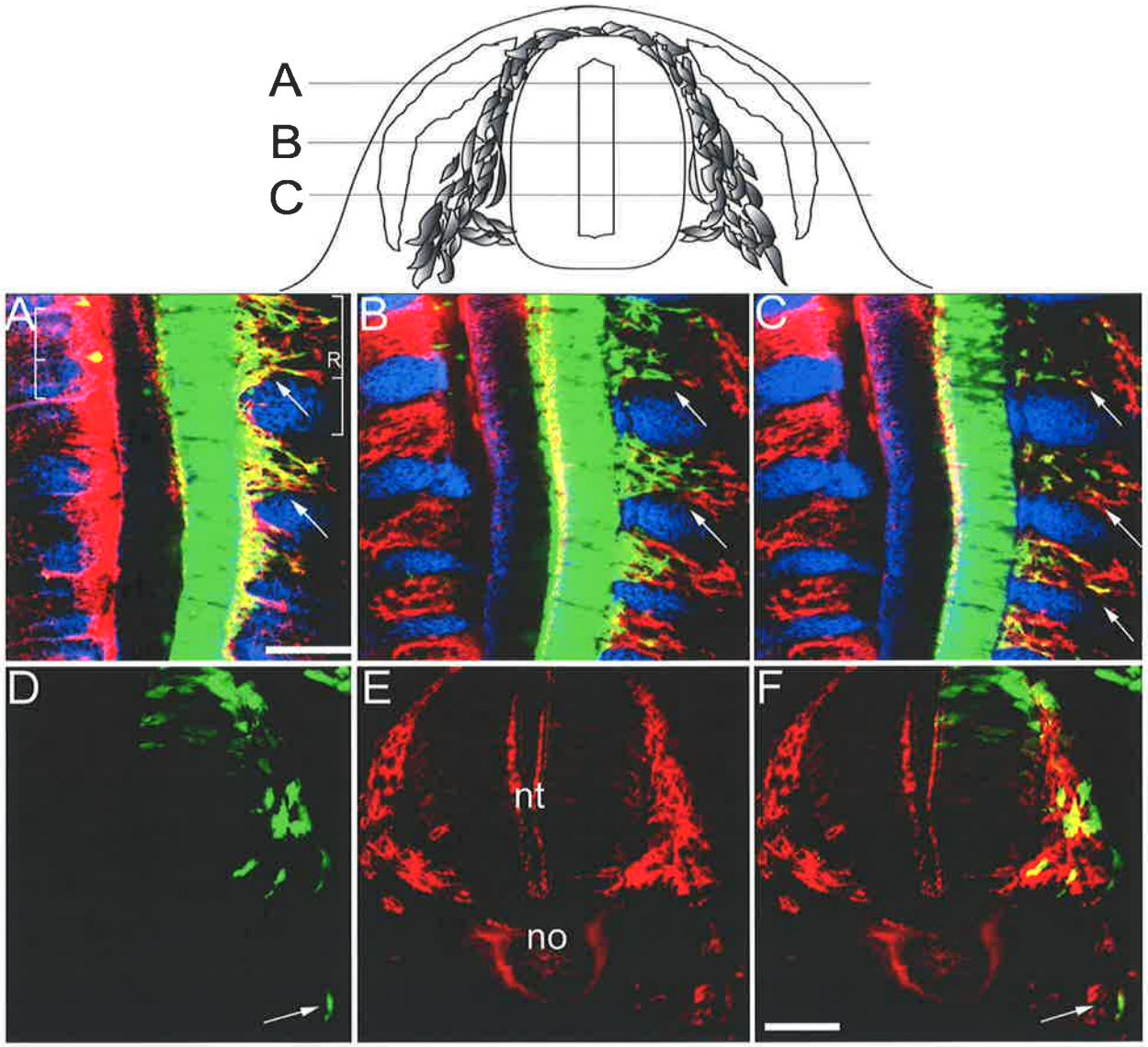
Figure 4.8- kiEphA4/EGFP-expressing cells migrate in a coordinated fashion through the rostral half-somite at more anterior levels.

Schematic diagram summarising planes of section of the embryo viewed longitudinally in (A-C).

(A-C) 10 μm stacks of optical sections taken at the dorsal (A), intermediate (B) and ventral (C) levels of the mid-trunk region of a stage 20 embryo presented in dorsal longitudinal view expressing kiEphA4/EGFP (green); co-stained with HNK1 (red) and ephrin-B1 (blue). Arrows in (A-C) indicate the localisation of kiEphA4/EGFP-expressing cells in migratory NC cell streams.

(D-F) Transverse section of mid-trunk region of stage 18 kiEphA4 embryo, presented as 18 μm z-stack and showing EGFP distribution (green, D), HNK1 (red, E) and co-localisation in (F). Arrow in (D) and (F) highlights ventrally migrating kiEphA4/EGFP cell located near dorsal aorta.

Bracket indicates somite outline and R = rostral; nt = neural tube; no = notochord. Scale bar: 100 μm (A-C); 200 μm (D-F).


A
B
C
A
L
R
B
C
D
E
nt
no
F

4.2.6 KiEphA4/EGFP expression enhances the progression of neuroepithelial cells through EMT

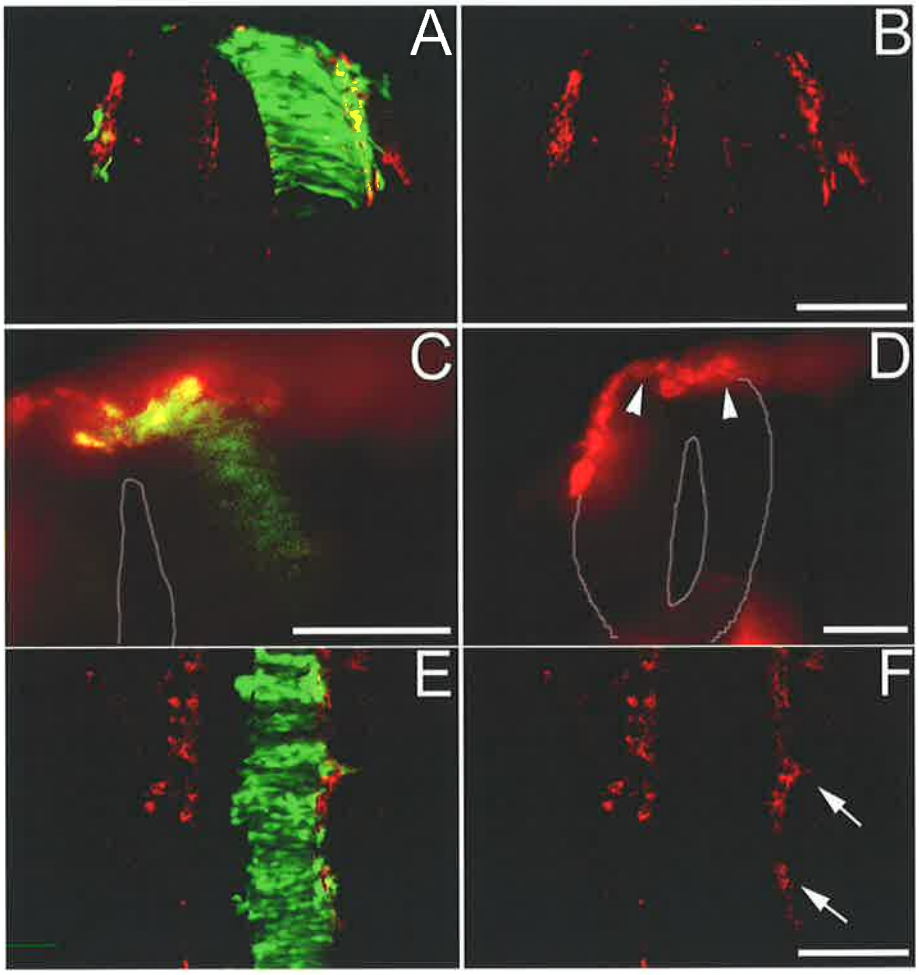
In contrast to EphA4 over-expression, kiEphA4/EGFP expression facilitated NC cell motility at mid-trunk levels. To determine the influence of kiEphA4/EGFP expression on the generation of NC cells during EMT, electroporations were carried out on the caudal-most regions of stages 12-14 embryos, opposite segmental plate mesoderm, and analysed 18-24 hours after electroporation. Electroporation at this stage enables neuroepithelial cells that are still in the pre-migratory phase of their development to be targeted with the expression construct (Delannet and Duband, 1992; Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985).

Embryos transfected with kiEphA4/EGFP in the caudal regions demonstrated a clear bias towards NC cell formation on the electroporated side (Figure 4.9, Table 4.1). This suggested that kiEph4/EGFP expression altered the timing of NC cell development, enhancing their progression through EMT. Transverse sections were analysed with HNK1 to determine the precise location along the dorsal neural tube at which cells became mesenchymal (Figure 4.9). In control sections, HNK1 immunoreactivity was acquired at a similar point on the lateral surface of the neural tube on both the electroporated and contralateral sides (Figure 4.9A and B, Table 4.1 n = 2/7). In contrast, kiEphA4/EGFP expression in the dorsal neural epithelium stimulated HNK1 in a region normally negative for mesenchymal markers at the dorsal midline (Figure 4.9C and arrowheads, Figure 4.9D, n = 6/6). In longitudinal view, the presence of kiEphA4/EGFP was frequently associated with an asymmetrical distribution of HNK1 favouring the electroporated side (Figure 4.9E and arrows, Figure 4.9F).

Figure 4.9- kiEphA4 expression at caudal regions of the embryo enhances NC cell formation.

(A-B) 10 μm stack of optical sections taken of a control EGFP-expressing transverse section from the lower mid-trunk of a stage 18 embryo, and (C-D) kiEphA4/EGFP-expressing transverse section from hindlimb level of a stage 18 embryo, co-stained with HNK1 (red). (C) Co-localisation between cells expressing kiEphA4/EGFP and HNK1 in the dorsal neural tube. (D) Low magnification image of (C) showing HNK1 alone; arrowheads indicate dorsal edge of HNK1 cell expression at dorsal neural tube.

White outline in (C) denotes neural tube lumen; outline in (D) denotes neural tube structure. (E-F) Dorsal longitudinal view of stage 16 kiEphA4/EGFP expressing embryo in the caudal region (-4 to –7 somites) co-localised with HNK1 in (E) and presented as HNK1 alone in (F). Image (E-F) is 10 μm stack of optical sections. Arrows (F) indicates increased HNK1 distribution at the dorsomedial neural tube. Scale bar: 50 μm (A-B), (C-D); 100 μm (E-F)


A
B
C
D
E
F

	WM	Trans	TOTAL frequency
kiEphA4	n = 5/5	n = 1/1	6/6 (100%)
Control	n = 2/6	n = 0/1	2/7 (29%)

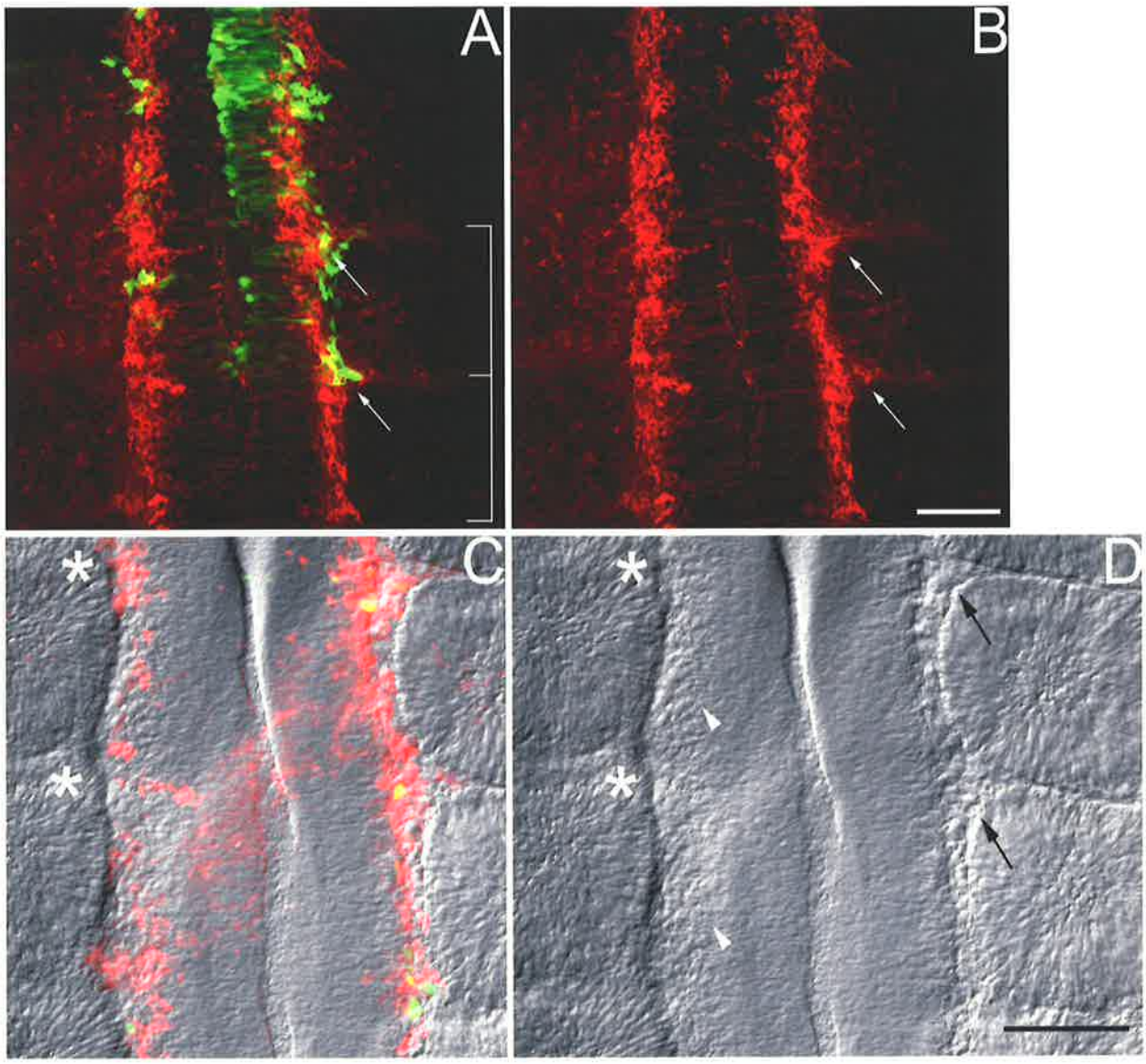
Table 4.1- Summary of data collected from the caudal regions of stage 16-19 embryos stained with HNK1 and analysed for increased HNK1 distribution at the dorsal neuroepithelium on the electroporated side.

Ultrastructural studies have demonstrated that an important component in the final stages of EMT are morphological changes to NC cell precursors with regards to their orientation and organization at the lateral edges of the neural tube (Newgreen et al., 1982; Tosney, 1978). One such change that is morphologically recognisable in dorsal longitudinal view is a flattening of cells at the lateral edges of the neural tube prior to their final exit from this structure (Figure 4.10 and data not shown). To determine if such morphological changes occurred normally in kiEphA4/EGFP-expressing embryos, the transfected and un-transfected sides were examined longitudinally using Nomarski optics. As previously demonstrated with epiflourescence (Figure 4.9), the emergence of HNK1-positive cells from the neural tube was slightly more developed on the kiEphA4/EGFP-expressing side than control, creating an asymmetrical distribution of HNK1 on the transfected side of the neural tube (arrows, Figure 4.10A and B). Overlaying the epiflourescent image (bracketed region in 4.10A/B) with the same image taken using Nomarski optics, indicated that kiEphA4/EGFP-positive NC cell clusters exhibited a flattened morphology and were located in the dorsomedial somite (staging area), exterior to the lateral edge of the neural tube (arrows, Figure 4.10D). In comparison, no cells were observed out of the neural tube boundary and in the somite staging area on the un-transfected side (asterisks, Figure 4.10C and D). Instead, on the un-transfected side, flattened neuroepithelial cells were observed within the confines of the neural tube structure indicative of cells that had begun, but not yet completed EMT/exited the neuroepithelium (arrowheads, Figure 4.10D). Coincident with an increase in NC cell number on the kiEphA4/EGFP-transfected side; the width of the neural tube on the kiEphA4/EGFP-positive side was reduced in 77% of transverse sections (10/13 sections; n = 3) analysed, compared to 33% in controls (3/9 sections; n = 3), suggesting that kiEphA4/EGFP was promoting NC cell formation at the expense of neuroepithelium (Figure 4.10D, REFER Appendix 1.4 for summary).

Figure 4.10- kiEphA4 enhances the formation of mesenchymal cells at the expense of the neuroepithelium.

(A-B) Dorsal longitudinal view of stage 19 kiEphA4/EGFP-expressing embryo (GFP in green) in the caudal region (somites –3 to –5) stained with HNK1 (red) and co-localised (A). Image is presented as 10 μm stack of optical sections; HNK1 alone is in (B). Arrows highlight the increased developmental progress of kiEphA4/EGFP-expressing NC cells, brackets indicate region equivalent to two somite lengths; embryo is oriented so that rostral is towards top of page.

(C-D) Higher magnification view of bracketed region in A, showing the co-distribution of EGFP cells with HNK1 and overlayed with Nomarski image. Asterisks in (C) indicate reduced number of NC cells on un-electroporated side compared with equivalent somites on electroporated side. Arrows in (D) highlight extensive cell flattening in the somite staging area (lateral exterior of neural tube) on the kiEphA4/EGFP electroporated side. Arrowheads in (D) highlight the flattened morphology of neuroepithelial cells within the lateral neural tube on the un-electroporated side. Scale bar: 100 μm (A-B), (C-D).


A
B
C
D

4.3 Discussion

The findings in this chapter point to a novel role for EphA4 in NC cell development. Firstly, over-expression of EphA4 at the point of NC cell delamination caused cell rounding and inhibited the emigration of NC cells from the neural tube, perturbing NC segmentation. Secondly, EphA4/EGFP expression appeared to have general over-adhesive effects as expression in the neural tube resulted in cell clumping leading to reduced efficacy of EMT in dorsal neuroepithelial cells on the EphA4/EGFP-positive side. Thirdly, blocking of EphA4 function using kiEphA4/EGFP facilitated NC cell motility although this has distinct effects depending on the exact axial level analysed. When expressed at the onset of delamination, kiEphA4/EGFP-expressing cells migrated in a disoriented fashion through the somite, entering both rostral and caudal somite-halves. An abnormally elongate morphology with multiple cellular processes and tangential alignment accompanied this migratory behaviour in kiEphA4/EGFP-positive cells. Finally, expression of kiEphA4/EGFP in the caudal regions of the embryo trunk promoted NC cell development, facilitating the formation of HNK1-positive NC cells and their subsequent emigration from the neural tube, in some cases at the expense of the neuroepithelium. These data are discussed in the context of possible cellular and molecular mechanisms governing both NC cell migration and also EMT.

4.3.1 Altered EphA4 expression in delaminating NC cells perturbs NC cell migration: axial level - dependent effects?

NC cell development occurs in a wave from the rostral to caudal end of the embryo such that in any one embryo, NC cell migration will be most advanced at the rostral end of the embryo and least advanced caudally (Bronner-Fraser, 1993a). Thus to target NC cells as they were emigrating from the neural tube and beginning their segmental migration through the rostral half-somite, embryos were electroporated at stages 12-14 in the mid-trunk regions. Despite this targeted approach, embryos varied with phenotypic severity and this appeared to coincide with the exact level of the neural tube targeted. In both gain-of-function and loss-of-function EphA4 experiments the most sensitive developmental window was in the mid-trunk region, where EphA4 over-expression yielded an almost directly opposite response to kiEphA4/EGFP expression in NC cells (i.e. cell rounding/inhibited migration compared with cellular extension/enhanced motile capacity). In contrast, at more anterior levels (approximately forelimb level of the neuraxis) significantly milder phenotypes were observed. EphA4/EGFP

expression at this level perturbed coordinated NC migration through the rostral half-somite though didn't induce the rounded cell morphology observed in EphA4/EGFP-positive cells at mid-trunk levels (data not shown; EphA4/EGFP n = 2; refer Appendix 1.2A). Similarly, kiEphA4/EGFP expression in the anterior mid-trunk region did not yield the same disoriented migratory phenotype (compare Figure 4.7 and 4.8). These data imply that EphA4 effects were intricately related to the level of the neural tube and subsequently, developmental age of the NC in which it was expressed. To extrapolate the effects in the mid-trunk back to a particular developmental event (when the construct was first expressed 24 hours earlier), the developmental stage of NC cells affected by altered EphA4 activity would be approximately at the onset of delamination. The migratory effects of EphA4 gain-of-function and loss-of-function analyses might therefore be explained by a role for EphA4 in regulating NC cell delamination. The possible mechanisms through which EphA4 might elicit axial level-dependent effects are discussed below.

Can cytoskeletal-based effects provide a possible mechanism to explain developmental dependence?

NC cell delamination involves re-arrangement of the F-actin cytoskeleton, dissolution of cadherin-rich adhesive complexes and the recruitment of cell-ECM substrate attachment receptors to faclitate motility (Delannet and Duband, 1992; Newgreen and Minichiello, 1995; Newgreen and Minichiello, 1996). The Rho family of GTPases have integral functions in mediating cytoskeletal re-arrangement and are therefore excellent candidates to facilitate actin re-arrangement during NC cell delamination (Braga, 2000; Schmitz et al., 2000). Utilising neural tube explant cultures to assay EMT in the presence of the rho GTPase inhibitor, C3 exotoxin, it was found that inhibition of Rho function in NC cell precursors at the edge of the neuroepithelium, perturbed cytoskeletal organisation and the subsequent delamination of NC cells (Liu and Jessell, 1998). Furthermore, in explants from more anterior regions of the neural tube, where NC cell emigration had already commenced, cells were no longer sensitive to C3-mediated inhibition, suggesting that Rho activity was only required in a restricted developmental window. These data, combined with expression analyses suggesting that RhoB was expressed (in response to BMP4-mediated induction) in the dorsal neural tube and early emigrating NC cells, lead the authors to propose that this protein has a critical role in facilitating stress fibre formation in neuroepithelial cells during EMT (Liu and Jessell, 1998). There is much evidence to support EphA4 mediating cytoskeletal change through Rho proteins in a cell context-dependent manner during vertebrate embryogenesis (Lawrenson et

al., 2002; Shamah et al., 2001; Winning and Sargent, 1994; Winning et al., 1996; Winning et al., 2002; Winning et al., 2001). In delaminating NC cells, EphA4 over-expression was synonymous with RhoB inhibition *in vitro* (Liu and Jessell, 1998). In addition, RhoB inhibition *in vitro* also exhibited a degree of developmental dependency (Liu and Jessell, 1998), providing an additional putative link between RhoB activity and that of EphA4.

On the contrary, although RhoB over-expression experiments have not been conducted either *in vivo* or *in vitro*, kiEphA4/EGFP expression in the neuroepithelium produced a migratory phenotype that mimicked what might be expected for de-regulated (or enhanced) RhoB activity during EMT. Time-lapse analyses of NC cell migration *in vitro* have demonstrated that NC cells extend and retract multiple projections during migration and that the frequency of such projections might directly impact on NC cell directionality *in vivo* (Newgreen et al., 1979). Thus, if reduced kinase-dependent EphA4 activity directly enhanced mobilisation of cytoskeletal elements, a possible consequence might be a loss of responsiveness to the normal regulatory cues controlling NC cell migration. Whether the kiEphA4 phenotype at mid-trunk levels was cytoskeletal-based is unknown, though given the effect of kiEphA4 expression on NC cell EMT, it is tempting to suggest that the enhanced motility might be related to increased RhoB activity. Interestingly, the expression patterns for RhoB and EphA4 during delamination and early migration correlate quite closely (refer Chapter 3;(Liu and Jessell, 1998; McLennan and Krull, 2002)), creating a possible *in vivo* context for their combined actions on NC cells. Alternatively, the migratory phenotype of cells lacking EphA4 might reflect a more general role for this protein in mediating cytoskeletal change in NC cells. In sum, EphA4 acting on cytoskeletal motility, possibly through RhoB might provide a putative explanation for the axial level-dependent effects observed in EphA4 perturbation experiments. Furthermore, these data imply that effects on the timing of EMT, rather than specifically on NC cell guidance mechanisms might mediate the aberrant migration of NC cells in the absence of EphA4.

Does analysis of HNK1 expression in kiEphA4/EGFP-expressing cells explain disoriented migration?

An additional observation that might underpin the effects of kiEphA4 on NC cell motility is that HNK1-immunoreactivity appeared to inversely correlate with the extent of uncoordinated migration in kiEphA4/EGFP-expressing cells. For example, kiEphA4/EGFP-expressing cells that were oriented tangentially to the neural tube (arrows, Figure 4.7) only expressed a low

level of the HNK1 epitope whilst those cells migrating amongst a stream of endogenous NC cells had high levels of HNK1 co-staining (arrows, Figure 4.8A). These data suggest that there might be a causative relationship between the apparent loss of HNK1 reactivity and disoriented NC cell migration. An additional possible interpretation of these data is that at the mid-trunk region of the neural tube, kiEphA4/EGFP-expressing cells were delaminating (possibly via increased motility or enhanced cell-substrate adhesions) before completely differentiating into fully mature migratory NC cells providing an explanation for their inability to migrate in a coordinated fashion. The appearance of the HNK1 epitope has been previously implicated in mediating NC cell guidance in the ventral regions of the embryo (Newgreen et al., 1986). In the perinotochordal matrix, the wave of expression of HNK1 during vertebral development correlates with repulsive activity of this tissue (Domowicz et al., 2003). Thus, it could be imagined that the absence or presence of HNK1 might also contribute in some way to coordinating the movement of NC cells through the rostral half-somite. Whether this relates to a specific Eph-mediated mechanism for NC cell-cell guidance might be an interesting avenue for further study.

In addition, at caudal levels of the embryo, kiEphA4/EGFP expression appeared to have distinct effects on the migratory profile of NC cells and this coincided with the pattern of HNK1 immunoreactivity in kiEphA4/EGFP-expressing cells (Figure 4.9 and 4.10). At these levels, whilst kiEphA4/EGFP expression promoted premature emigration, this was usually accompanied by an upregulation of HNK1 expression. In addition, and in support of the hypothesis that HNK1 might be important for NC cell guidance, kiEphA4/EGFP-expressing cells at this region did not exhibit the clear disoriented phenotype observed at mid-trunk levels (compare Figure 4.7 with 4.10). Whilst it is possible that the more ordered appearance of kiEphA4/EGFP-expressing cells at this level might simply reflect their behaviour at an earlier stage of NC migration, analyses of embryos with kiEphA4/EGFP expression in the mid-trunk regions but with slightly reduced incubation times suggest that this was not the case (data not shown). In sum, these data have two key implications in understanding the role of EphA4 in NC cell EMT (and subsequent migration profiles of cells with inhibited EphA4 signalling through the kinase domain). Firstly, that the disoriented migratory morphology of kiEphA4/EGFP-expressing cells at mid-trunk levels might be a consequence of their rapid exit from the neural tube (possibly via cytoskeletal effects) and subsequent entry into the somite prior to completing EMT (as determined by HNK1 expression). Secondly, comparison between kiEphA4/EGFP effects at mid-trunk and caudal levels of the embryo suggest that EphA4 may be affecting NC cell EMT via distinct mechanisms at particular levels of the axis.

Noggin/BMP4 interactions have recently been implicated in regulating NC cell delamination via distinct mechanisms opposite epithelial somites compared with the segmental plate (Burstyn-Cohen et al., 2004). Thus, EphA4 might be acting in this pathway to regulate EMT; providing a putative explanation for the described developmentally stage-dependent effects of reduced EphA4 on NC migration (refer Section 6.3 for complete discussion).

4.3.3 Possible insights into EphA4-mediated NC cell guidance through the rostral half-somite

KiEphA4/EGFP-expressing cells lacked the ability to migrate through the rostral half-somite in an organised fashion, leading to a loss of segmental NC migration. This effect may be directly related to the intracellular consequences of reduced EphA4 (discussed above) and therefore may not reflect any changes to the interactions between NC cells and the somitic environment during migration per se. However it cannot be ruled out that the behaviour of kiEphA4/EGFP-expressing cells reflected a role for EphA4/ephrin-A interactions in NC cell guidance through the rostral-half somite. A number of possible scenarios can be imagined. Firstly, that EphA4 expression in the rostral half-somite is an important guidance cue for ephrin-A expressing NC cells. Subsequently, kiEphA4/EGFP expression might perturb the normal balance of EphA4/ephrin-A interactions (on somite and NC cells respectively), thereby indirectly affecting migration. Secondly, the aberrant entry of kiEphA4/EGFP-expressing cells into the caudal half-somite might reflect a role for EphA4 on NC cells in mediating the exclusion of these cells from the caudal half-somite. Thirdly, EphA4 might have a role in mediating intercellular adhesion between NC cells during their migration through the rostral half-somite. These possibilities are discussed below giving consideration to the range of kiEphA4 phenotypes observed.

A possible cause for the aberrant migration of kiEphA4/EGFP-expressing cells could be that its expression affects the normal interactions between NC cells and the rostral half-somite. Given that EphA4 is distributed along the ventral pathway of NC cells (refer Chapter 3; McLennan, 2002 }, it is possible that kiEphA4/EGFP expression could alter the balance between ephrin-A signals (on NC cells) and EphA4 in the sclerotome leading to a loss of coordinated migration. NC cell migration in the presence of EphA and ephrin-A exogenous blocking proteins produced a similar migratory profile to kiEphA4/EGFP-positive cells (McLennan and Krull, 2002), lending support to the idea that EphA/ephrin-A interactions (on somite and NC cells, respectively) play a direct role in NC cell guidance. Further experiments

analysing the migration of kiEphA4/EGFP-expressing cells on different substrates *in vitro*, including various EphA/ephrin-A combinations, would be required to completely understand how kiEphA4/EGFP expression might affect NC cell interactions with the rostral half-somite and ultimately, whether EphA4 in the rostral half-somite is important for NC cell guidance.

The loss of segmental migration in NC cells expressing kiEphA4/EGFP could also reflect a role for EphA4 (on NC cells) in mediating repulsive signals from the caudal somite-half. A key requirement when attributing a particular molecule in mediating segmental migration of NC cells is that perturbation of its function leads to a loss of segmental NC migration (Krull, 2001). In this case, however, EphA4 on NC cells would have to be interacting with a molecule expressed in the caudal half-somite. A number of Eph/ephrin molecules are expressed in the caudal half-somite including ephrin-B1 (Koblar et al., 2000; Krull et al., 1997), and EphA7 (Araujo and Nieto, 1997) and perturbation of Eph/ephrin-B interactions disrupts segmental migration of NC cells (Krull et al., 1997; Wang and Anderson, 1997). However, EphA4 has not yet been found to interact with either ephrin-B1 (Gale et al., 1996; Mellitzer et al., 1999) or EphA7 (Araujo and Nieto, 1997), making it unlikely that reduced EphA4 signalling on NC cells would directly affect NC cell interactions with caudal half-somite. Alternatively, EphB3 is expressed on NC cells as well as the rostral half-somite during migration (Krull et al., 1997) and EphB receptors have recently been shown to interact with ephrin-A5 (Himanen et al., 2004), adding an additional level of complexity (i.e. EphB/ephrin-A interactions) to the putative Eph/ephrin interactions regulating NC cell migration. Thus, it is possible that by expressing kiEphA4/EGFP on NC cells and shifting the balance between EphA4 and ephrin-A-mediated signalling towards ephrin-A "reverse" signalling (Figure 4.11) EphB3 interactions with ephrin-B1 in the caudal half-somite might also be affected leading to NC cells no longer responding to ephrin-B1-mediated cell repulsion.

The final possible explanation for the loss of coordinated migration in kiEphA4/EGFP-expressing cells is that EphA4 provides an intercellular adhesion system between NC cells to coordinate their migration through the rostral half-somite. Time-lapse analyses *in vivo* have demonstrated that NC cells make dynamic contacts with one another, leading to their migration in highly organised streams through the rostral half-somite (Krull et al., 1995; Krull et al., 1997). This observation is supported by two other key observations: firstly, that NC cells migrate in a chain-like fashion through three dimensional collagen matrices *in vitro* and secondly, ultrastructural studies analysing NC cell emigration at the dorsal neural tube have

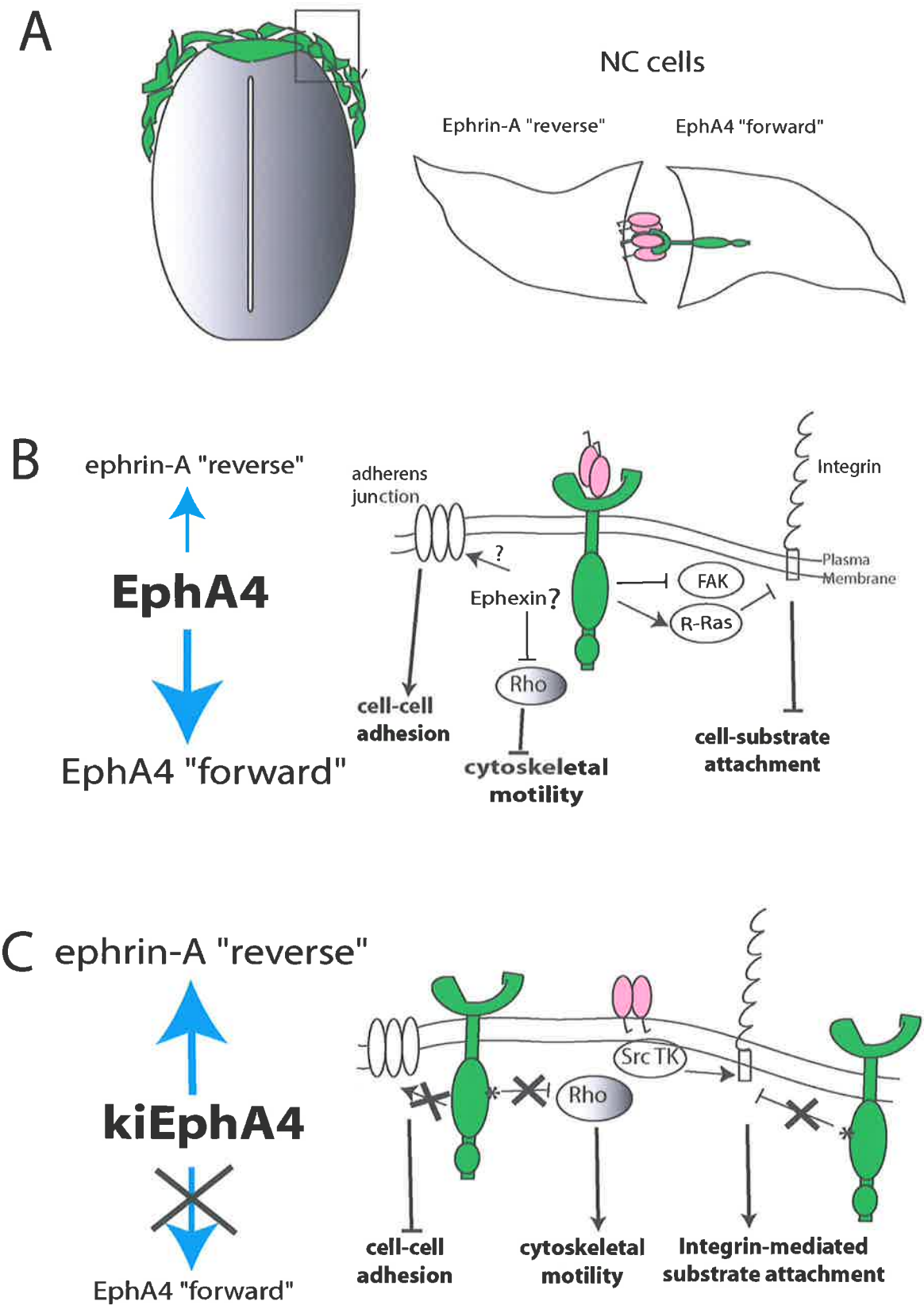
shown that cells maintain leading-to-tail edge adhesions, and make numerous contacts with neighbouring cells on all sides (refer section 1.6 for summary; (Davis and Trinkaus, 1981; Krull et al., 1995; Tosney, 1978)). These data point to differential NC cell adhesion as having a pivotal role in NC cell migration. To date there are no molecular correlates that have been directly implicated in such a role though possible candidates include cad7 and thrombospondin-1 (Krull, 2001). If there is a NC cell intrinsic role for EphA4 signalling in coordination of NC cell migration, this might explain the apparently contradictory interactions that kiEphA4/EGFP-positive cells displayed when migrating through the somite. Thus, when kiEphA4/EGFP was expressed at the onset of delamination, generating mostly kiEphA4/EGFP-positive cells in the somite, these cells would be unable to communicate effectively leading to a loss of intercellular adhesion and subsequent entry into normally inhibitory territories. Conversely, when expressed after the onset of delamination, such that kiEphA4/EGFP-positive cells entered the somite alongside or behind endogenous NC cells, interactions with endogenous NC cells would be sufficient to re-establish coordinated migration through the rostral half-somite. If this were the case, it would implicate EphA4 as a novel candidate in mediating a NC cell intrinsic mechanism to regulate NC migration through the rostral half-somite.

Figure 4.11- Proposed model to explain the phenotypes observed in NC cells over-expressing EphA4 and in NC cells with inhibited EphA4 signalling due to the expression of kiEphA4.

(A) The normal expression of EphA4 on NC cells is low compared to the expression of ephrin-A5, so that the balance of EphA4/ephrin-A interactions is driven towards ephrin-A "reverse" signalling pathways in endogenous NC cells.

(B) In NC cells over-expressing EphA4, the balance between EphA4 and ephrin-A activity is switched towards EphA4 "forward" signalling. Activation of EphA4 signalling pathways inhibits the progression of NC cells through EMT and their subsequent migration through the rostral half-somite. EphA4 might elicit these effects via inhibition of Rho activity, thereby inhibiting cytoskeletal motility and/or reduced cell-substrate attachment, possibly via inhibition of FAK activity or stimulation of R-Ras. EphA4 may also influence cell-cell adhesion in dorsal neuroepithelial cells by positively regulating N-cadherin-mediated adherens junctions.

(C) In kiEphA4/EGFP-expressing NC cells, signalling through the kinase domain of EphA4 is inhibited, shifting the balance of signalling towards ephrin-A "reverse" signalling. The combined inhibition of EphA4 "forward" signalling and promotion of ephrin-A "reverse" signalling leads to enhanced motility in NC cells, and in some cases their aberrant entry into otherwise inhibitory zones, as well as promoting EMT in the dorsal neural tube.


A
NC cells
Ephrin-A "reverse"
EphA4 "forward"
B
ephrin-A "reverse"
EphA4
EphA4 "forward"
adherens junction
?
Ephexin?
Rho
FAK
R-Ras
Integrin
Plasma Membrane
cell-cell adhesion
cytoskeletal motility
cell-substrate attachment
C
ephrin-A "reverse"
kiEphA4
EphA4 "forward"
Src TK
Rho
cell-cell adhesion
cytoskeletal motility
Integrin-mediated substrate attachment

4.3.4 Role for EphA4 in regulating cellular interactions contributing to EMT

EMT is a highly dynamic event involving coordinated changes in intercellular adhesion, adhesion to the substrate and also the cytoskeleton, to facilitate detachment of cells from the neuroepithelium and subsequent migration away into the periphery (Newgreen and Minichiello, 1995; Newgreen and Minichiello, 1996). *In vitro* experiments have demonstrated that a reduction in cell-cell adhesion and the acquisition of receptors to facilitate substrate attachment are critical in stimulating NC cell EMT from the dorsal neuroepithelium (Delannet and Duband, 1992; Newgreen and Gibbins, 1982). In addition to changes in adhesion, Newgreen and Gibbins stated that there was two other main requirements for NC cell EMT: changes in NC cell motility and the removal of a physical barrier to migration (Newgreen and Gibbins, 1982). EphA4 over-expression and reduction of EphA4 activity with kiEphA4 suggested a role for this molecule in EMT. These phenotypes are discussed in light of the four criterion required for successful EMT.

Changes in cell-cell and cell-substrate adhesion

A consistent effect observed in both the gain-of-function and loss-of-function EphA4 analyses, was that perturbation of EphA4 function in the neuroepithelium produced cell behavioural responses indicative of an altered adhesive profile. Thus, EphA4/EGFP-expressing cells displayed morphologies and behaviours indicating increased cell-cell association and/or reduced cell-substrate adhesions (required to facilitate movement). In addition, a possible explanation for the premature exit of kiEphA4/EGFp-expressing cells during EMT might be that these cells have reduced cell-cell and increased cell-substrate adhesions. Thus EphA4 might contribute to adhesive mechanisms regulating EMT.

Over-expression studies *in vivo* have been particularly informative in determining the role of candidate adhesion molecules in mediating changes to the adhesive interactions between neuroepithelial cells during EMT (Nakagawa and Takeichi, 1995; Nakagawa and Takeichi, 1998). One such family of molecules are the cadherins; these provide a cell-cell adhesion system critical for the maintenance of epithelial junctions and the loss of which is a key stimulus for EMT (Braga, 2000; Newgreen and Gooday, 1985). Three cadherin molecules that have been implicated in mediating adhesive changes required for neuroepithelial cell EMT include N-cadherin, cadherin-6b (cad6b) and cadherin-7 (cad7)(Nakagawa and Takeichi, 1995). Over-expression of either N-cadherin or cad7 inhibited the detachment of

neuroepithelial cells from the neural tube in a similar manner to EphA4 over-expression, causing cell rounding and a build up of cells in clumps at the dorsal midline ((Nakagawa and Takeichi, 1998); Figures 4.4 to 4.6). Interestingly, regions of EphA4/EGFP expression in the neuroepithelium were associated with an upregulation of N-cadherin expression (Figure 4.4), whilst the dorsal extent of N-cadherin was increased on the EphA4/EGFP-expressing side of the neural tube (Figure 4.5). Conversely, the behaviour of kiEphA4/EGFP-expressing cells in the neural tube was synonymous with reduced cell-cell adhesion (Figures 4.9 and 4.10) although this cannot be confirmed, as the distribution of N-cadherin was not analysed in these embryos. Whether the enhanced N-cadherin representation in EphA4/EGFP-positive regions of the neuroepithelium reflects a direct interaction between EphA4 and N-cadherin or an indirect effect of excess EphA4 activity, is unknown. In sum, these data suggest a convergence between EphA4 and cadherin signalling pathways, providing a possible mechanism to explain the behaviour of cells in the presence of excess EphA4 and also when kinase-dependent EphA4 signalling was inhibited during EMT.

Studies investigating the role for EphA receptors in regulating cellular adhesion mechanisms during oncogenesis and tissue morphogenesis point to a common role for EphA signalling in positively regulating cell-cell adhesion whilst negatively regulating cellular attachment to the substratum (Klein, 2004). EphA receptor activation has been implicated previously with multiple other possible signalling pathways to affect cellular responses to its environment, however two specific mediators relevant to the changes occurring during EMT can be discussed: firstly, EphA receptors and cadherins and secondly, a possible link between EphA and FAK activity. In embryonic stem cells and breast cancer cell lines, EphA expression is dependant on E-cadherin, which in turn is required for the maintenance of an epithelial phenotype (Orsulic and Kemler, 2000; Zantek et al., 1999). Thus, EphA activity correlates strongly with increased cell-cell adhesion mediated through E-cadherin and reduced cell-substratum attachment (Lawrenson et al., 2002; Miao et al., 2000; Orsulic and Kemler, 2000). In addition, EphA activation in both prostrate and melanoma cell lines has been shown to reduce substratum adhesion by inhibiting the integrin linking protein Focal Adhesion Kinase (FAK) in a kinase-dependent manner (Lawrenson et al., 2002; Miao et al., 2000). Activation of FAK-dependent signalling pathways has been shown in turn to directly modulate the function of Rho GTPases, thereby mediating cytoskeletal responses to changes in adhesion (Zhai et al., 2003). Thus interactions with integrin-signalling mediators, such as FAK, might provide a kinase-dependent mechanism through which EphA4 could influence substratum attachment and subsequently negatively regulate NC cell progression through EMT.

Changes in motility

A role for EphA4 in mediating cytoskeletal motility has previously been discussed in the context of developmental stage-dependent effects of EphA4 over-expression and migratory NC cell phenotypes when EphA4 signalling through the kinase domain was inhibited (refer Section 4.3.2). In addition however, EphA4/EGFP expression in the intermediate regions of the neural tube lead not only to enhanced N-cadherin expression but also to the abnormal exit of EphA4/EGFP-expressing cells from the pial surface of the neural tube (Figure 4.4). Based on the expression patterns of EphA4 in the dorsal neuroepithelium (at the dorsal edge of the neural tube where cells are beginning to undergo EMT), the direct relevance of the effect of EphA4 over-expression in the intermediate neural tube, to a role for EphA4 in NC cell EMT is questionable. However, ephrin-A5 is expressed in a band in the intermediate neural tube (refer Chapter 3; (McLennan and Krull, 2002)), providing a possible means of EphA4 activation in this region. Thus, these observations might provide an insight into the consequences of excess catalytic activity of EphA4 within an epithelium.

At first glance, the abnormal exit of EphA4/EGFP-expressing cells from the neural tube does not correlate well with the previously described effects of EphA4 over-expression (refer Section 4.3.1). However, upon closer analysis, EphA4/EGFP-positive cells did not appear contiguous with endogenous neuroepithelial cells; instead appearing to extend out of the plane of the neural tube structure (Figure 4.4). Maintenance of apico-basal polarity is a key requirement for cells to form a polarised epithelium such as the neural tube (Braga, 2000). Thus, a possible explanation for the "exclusion" effect mediated by EphA4/EGFP in the neuroepithelium is that its expression perturbed apico-basal polarity and this (indirectly) caused their entry into a default EMT pathway. In the pre-somitic mesoderm of zebrafish, where EphA4 has been shown to play a critical role in the reverse of EMT, mesenchymal-to-epithelial transition, loss of cell polarity has been proposed to be a common intracellular consequence of enhanced EphA4 signalling (Barrios et al., 2003). Furthermore, taking into account that apico-basal polarity is driven by re-arrangement of microtubules and the actin-based cytoskeleton (Barrios et al., 2003; Braga, 2000; Newgreen, 2005); the described effects might provide an additional line of evidence to suggest that the cytoskeleton is a principal target of EphA4 signalling in EMT.

Recent analyses investigating the role of transcription factors in EMT, including members of the SoxE group and FoxD3, have provided some evidence to suggest that NC cell EMT can occur in the absence of RhoB (Cheung and Briscoe, 2003(SJM, unpublished) . The ectopic

emigration of NC cells from the intermediate neural tube that was observed in EphA4/EGFP-expressing regions has two important implications for NC cell motility. Firstly, it supports the data suggesting that NC cell EMT can occur in the absence of RhoB {Newgreen, 2005 ; Dottori et al., 2001). Secondly, this result could be interpreted as further evidence to support that the inhibitory effects of EphA4 on NC cell motility during EMT are dependent on RhoB. In support of EphA4 having a specific role in inhibiting RhoB during delamination; EphA4 has been shown to interact with Ephexin in a kinase-dependent manner, thereby promoting Rho activity whilst inhibiting that of Rac and Cdc42 (Shamah et al., 2001). Given that RhoB would not be expected to be expressed in these cells and the observation that EphA4 also influences Rac/Cdc42 activity (Shamah et al., 2001; Tanaka et al., 2004); it is possible that in this situation, EphA4 could be promoting Rac or Cdc42 activity and thereby influencing epithelial characteristics (i.e apico-basal polarity) in intermediate neuroepithelial cells. In sum, it is possible that the different cellular contexts (absence versus presence of RhoB) in the neuroepithelium could explain these apparently disparate effects of excess EphA4 activity.

Removal of a physical barrier to migration

Ultrastructural studies have revealed that morphological changes associated with cell exit from the neural tube correlates with the loss of a continuous basement membrane over the dorsal midline regions (Newgreen et al., 1982; Tosney, 1978). Despite these observations, the loss of basement membrane does not appear to be a critical stimulus for EMT as the timing of neuroepithelial cell EMT *in vitro* was still regulated along the normal *in vivo* timetable of events in the absence of basement membrane (Newgreen and Gibbins, 1982). Consistent with the reduced EMT of neuroepithelial cells expressing EphA4/EGFP, indicated by HNK1 and N-cadherin staining, a thickening of the basal lamina surrounding the dorsal edge of the neural tube was also observed in EphA4/EGFP-expressing regions (Figure 4.5). It would be interesting to determine if, in the converse situation, kiEphA4/EGFP expression lead to an increase in the lateral extent of basement membrane breakdown, as would be expected for the previously described effects of kiEphA4 on EMT.

4.3.5 A possible in vivo *role for EphA4 in regulating delamination and/or early NC cell migration*

The behaviour of NC cells when EphA4 signalling through the kinase domain was blocked implied that EphA4 has a role in NC cell development, during both EMT and also NC

migration into the somite. The key question that remains, however; is how a putative role for EphA4 might fit with the observed expression patterns for EphA4 and ephrin-A proteins in NC cells, and thus relate to an *in vivo* model of Eph/ephrin interactions during NC development. Analyses in this research and also from McLennan and Krull, (2002), showed that EphA4 is expressed only weakly on NC cells during the early phases of their migration, whilst multiple different ephrin-A's, including ephrin-A5 are present (Chapter 3) and (McLennan and Krull, 2002). Thus, ephrin-A proteins are distributed in excess of EphA4 in endogenous NC cells, suggesting that limited availability of EphA4 might have important consequences for the regulation of NC cell interactions. In addition to EphA4, EphB3 is also expressed on NC cells during their migration through the rostral half-somite (Krull et al., 1997) and EphB proteins have recently been shown to interact with ephrin-A5 (Gauthier and Robbins, 2003). Thus, EphB receptors might be an additional possible source of ephrin-A activation in NC cells. In sum, a number of complex possible interactions are likely to contribute to the ultimate regulation of NC cell EMT and their subsequent migration, however given that perturbation of EphA4 kinase-dependent signalling disrupted NC cell development, this implicates EphA4 as a key contributor to Eph/ephrin interactions in NC cells.

To directly test a role for EphA4 in NC cell EMT and/or migration, the kinase-inactive EphA4 construct was expressed in dorsal neuroepithelial cells and subsequently on NC cells during their migration through the somite. This construct contained a point mutation in a critical lysine residue required for activation of the kinase domain of EphA4, whilst all other signalling regions including the juxtamembrane tyrosine residues, SAM and PDZ domains remained intact (Binns et al., 2000; Kullander et al., 2001b). Thus, the kiEphA4 construct inhibited signalling specifically through the EphA4 kinase domain, via a dominant negative mechanism (Eberhart et al., 2004). In NC cells, kiEphA4 would be expected to bind to and activate ephrin-A signalling whilst simultaneously inhibiting signalling through the kinase domain of EphA4. This would ultimately lead to a shift towards an ephrin-A mediated response in kiEphA4/EGFP-expressing cells (Figure 4.11). Thus a possible explanation for the response of NC cells to kiEphA4 expression could be excess activation of ephrin-A-dependent signalling pathways. Ephrin-A proteins have been implicated in facilitating integrin-based adhesion through Fyn, a protein tyrosine kinase belonging to the Src family (Davy et al., 1999; Davy and Robbins, 2000; Huai and Drescher, 2001). Perhaps the combination of a loss of EphA4-mediated adhesive mechanisms and enhanced ephrin-A signalling in NC cells could therefore lead to the cellular responses observed in kiEphA4/EGFP-expressing NC cells (Figure 4.11).

Further evidence to support that EphA4 has a critical regulatory role during EMT is provided by the preliminary analysis of the effects of a truncated EphA4 construct (EphA4Δcyto). This construct is designed to test the role of ephrin-A "reverse" signalling (kinase-independent functions of EphA4) by activating ephrin-A signalling pathways and thereby shifting the balance between EphA4 and ephrin-A-mediated signalling towards that of ephrin-A (Henkemeyer et al., 1996; Holmberg et al., 2000; Kullander and Klein, 2002; Orioli et al., 1996)(Figure 4.12A). Electroporation of EphA4Δcyto in st12-14 embryos (as per EphA4 and kiEphA4) did not perturb the segmental migration of NC cells (n = 10; Figure 4.12B and C). Truncated Eph receptor constructs have been shown to dimerise with endogenous Eph receptors, and in some instances, potentiate Eph-mediated signalling (Koblar et al., 2000). Thus, there may be some residual activation of EphA4 "forward" signalling pathways in addition to the activation of ephrin-A-dependent pathways in NC cells expressing EphA4Δcyto. Furthermore, the lack of any clear effect on NC cell migration in EphA4Δcyto experiments implies that inhibition of EphA4-mediated "forward" signalling and not simply, activation of ephrin-A "reverse" signalling is responsible for the effects of kiEphA4. The effect of EphA4Δcyto on the timing of EMT in the dorsal neural tube was not analysed in this data. However, taking into account the distinct effects of EphA4Δcyto to kiEphA4 on NC cell migration and the hypothesis that EphA4 is required to negatively regulate the timing of EMT, an effect (at least with regard to the timing of neuroepithelial cell exit) would not be expected to be observed. In sum, these data support that a basal level of EphA4 activation provides a key negative regulatory role in NC cell EMT and possibly also regulates NC cell-cell contacts during their migration through the rostral half-somite (Figure 4.11).

Whilst expression of EphA4Δcyto did not perturb segmental migration of NC cells through the somite, some residual aggregation was observed (Figure 4.12) suggesting that increased ephrin-A activity might have detrimental effects for NC cell motility. Ephrin-A activation has been shown to promote integrin-based attachment through the Src family of tyrosine kinases (Davy et al., 1999; Davy and Robbins, 2000). Thus, it could be imagined that excess activation of ephrin-A signalling and subsequent increases in the adhesion of NC cells to the ECM might reduce motile capacity (Delannet and Duband, 1992; Delannet et al., 1994; Duband et al., 1991). Interestingly, in EphA4/EGFP-expressing embryos, non-cell autonomous adhesion of HNK1-positive cells to EphA4/EGFP-expressing cells was frequently observed (Figure 4.1 and data not shown). In these NC cells, similar to those expressing EphA4Δcyto, both ephrin-A "reverse" signalling was activated as well as

"forward" signalling through EphA4 (although EphA4 signal was much stronger in NC cells over-expressing EphA4; Figure 4.11). Thus, activation of ephrin-A-mediated substrate attachment mechanisms could also explain the reduced migration of endogenous HNK1 cells in contact with those NC cells expressing EphA4/EGFP.

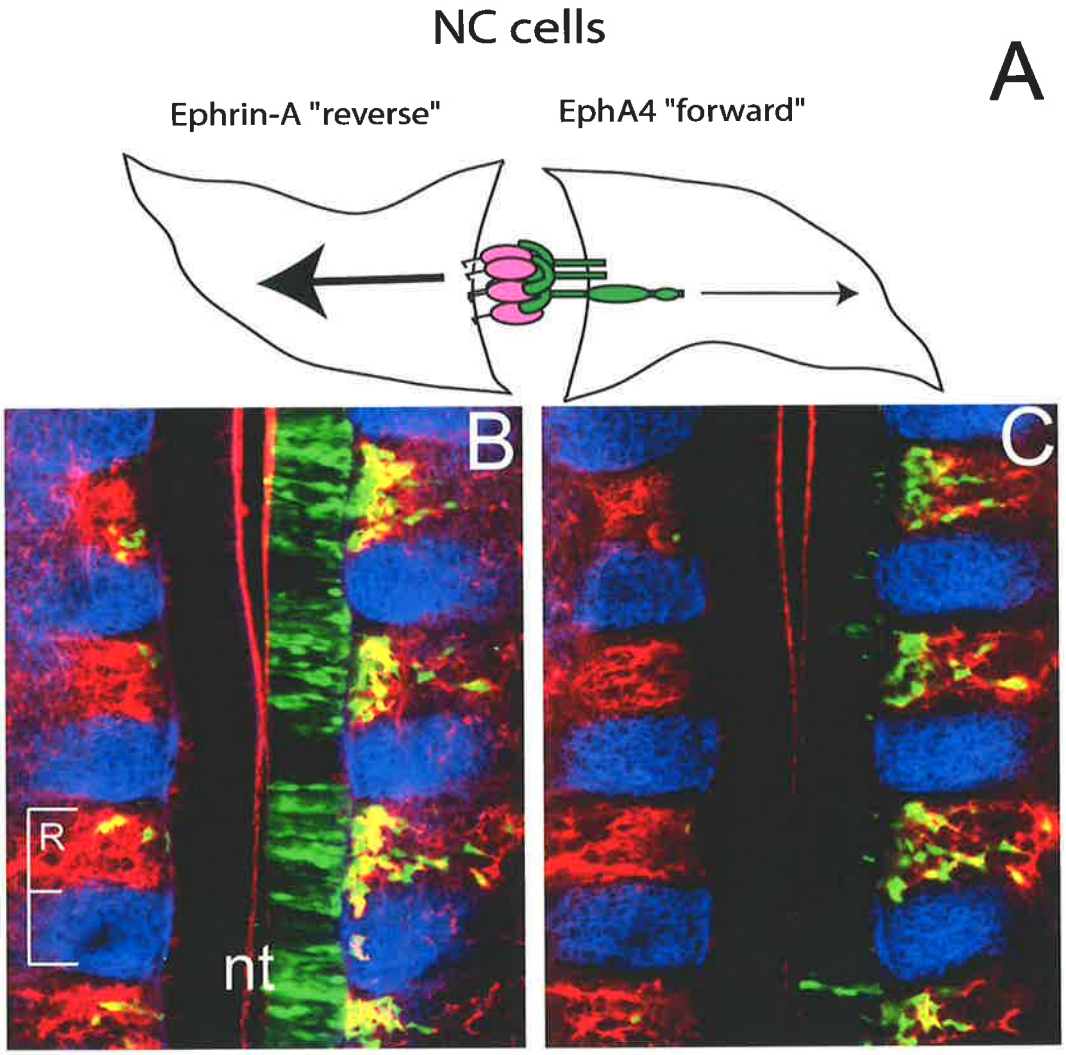
Figure 4.12- Expression of a truncated EphA4 construct, EphA4Δcyto, does not perturb segmental migration of NC cells.

(A) Schematic diagram illustrating the effect of EphA4Δcyto on endogenous EphA4/ephrin-A signalling. Expression of this construct would promote ephrin-A "reverse" signalling without inhibiting EphA4-mediated "forward" signalling. Embryo electroporated by R. McLennan, 2002, PhD student.

(B and C) Dorsal longitudinal views of whole-mount embryos expressing EphA4Δcyto taken through the dorsal (B) and ventral (C) regions of the embryo. Embryos were stained with HNK1 (red) and ephrin-B1 (blue). (B) is presented as 10 μm stack of optical sections and (C) is a 30 μm stack.

Brackets indicate single somite length; nt = neural tube; R = rostral.

Scale bar: 100 μm.


NC cells
A
Ephrin-A "reverse"
EphA4 "forward"
B
C
R
nt

4.4 Conclusions

The gain and loss-of-function analyses presented here suggest a novel role for EphA4 in mediating the exit of neuroepithelial cells from the neural tube and their subsequent ventromedial migration into the somite. Whilst the expression of these factors *in vivo* enabled the analysis of EphA4 over-expression and also inhibition of EphA4 function using kiEphA4/EGFP in the normal context of NC cell development; a similar analysis however, in an *in vitro* environment would be useful for a number of reasons. Firstly, given that NC development occurs in a rostro-caudal wave along the antero-posterior axis of the embryo, and the effects of EphA4 functional analyses were dependent on the exact developmental time-point in which the construct was expressed, it was difficult to discern the primary mode of EphA4 function *in vivo*. Examination of transfected neural crest cells *in vitro* from migratory and pre-migratory levels of the neuraxis would provide an isolated system in which to study the effects of perturbation of EphA4 signalling in a controlled environment. Secondly, neural tube explant cultures have provided a wealth of knowledge on the key cellular mechanisms regulating EMT (Delannet and Duband, 1992; Newgreen and Gibbins, 1982; Newgreen and Gooday, 1985; Newgreen and Minichiello, 1995; Newgreen and Minichiello, 1996). Mis-expression of EphA4 in this explant system therefore provides a robust tool to analyse the possible cellular basis of EphA4 function in NC cell EMT.

Chapter 5 – Analysis of EphA4 effects on NC cell development using an *in vitro* culture assay

5.1 Introduction

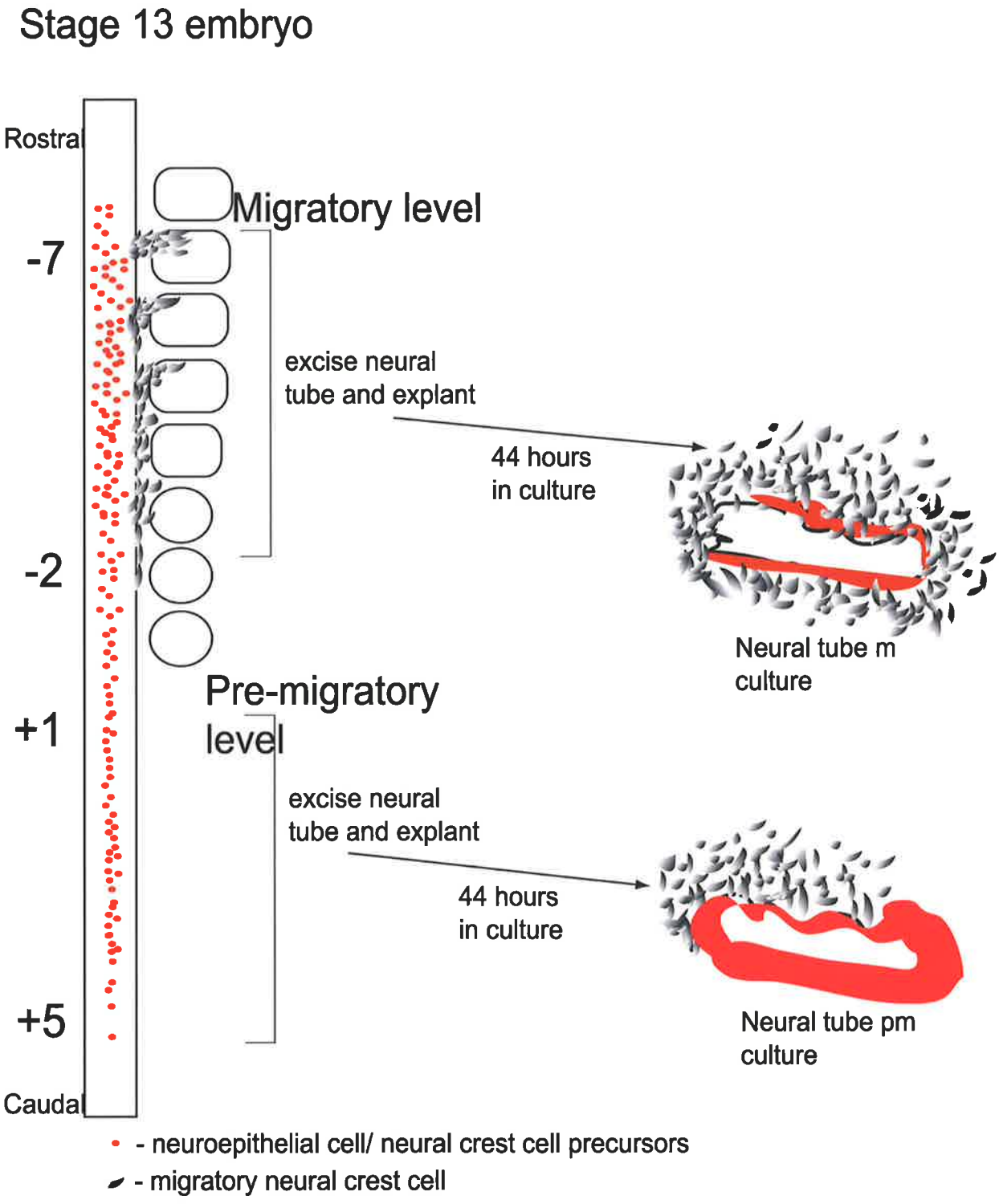
There were two clear phenotypes in embryos that were ectopically expressing EphA4/EGFP or kiEphA4/EGFP in the dorsal neural tube. Over-expression of EphA4/EGFP in NC cells at the initial phases of ventral migration resulted in the formation of cellular aggregates at the dorso-medial neural tube and a concomitant reduction in the number of HNK1-positive cells migrating through the rostral half-somite. In contrast, kiEphA4/EGFP expression in delaminating NC cells promoted motility and uncoordinated NC cell outgrowth, occasionally leading to entry into otherwise inhibitory territories. At caudal levels, regions of kiEphA4/EGFP expression in the dorsal neuroepithelium facilitated premature NC cell emigration (refer Chapter 4).

To examine these phenotypes in more detail, an *in vitro* model of neural crest migration was employed. The culturing of neural primordia on FN substrates has been utilised previously to examine the mechanisms of NC cell migration/attachment on various ECM substrates (Newgreen et al., 1979). In addition, these cultures have provided a wealth of information on critical factors involved in EMT (Newgreen and Gibbins, 1982). Given that NC cell EMT occurs in a rostro-caudal wave along the neural tube axis, cell emigration from any given neural tube culture is dependant on embryo age and the rostro-caudal level from which it was excised. Thus to maintain consistency with *in vivo* analyses, Japanese quail embryos were electroporated at E2.5 (HH stage13-early 14) and neural tubes excised from two different levels along the rostro-caudal axis corresponding to the key regions where EphA4 mis-expression appeared to effect NC cell development (Figure 5.1). To analyse the effect of EphA4 perturbation at the onset of delamination (equivalent to mid-trunk region 24 hours post-electroporation *in vivo*), neural tubes were taken from regions adjacent to somites –2 to –7, where the negative prefix denotes the number of somites rostral to the first formed somite (or –1; Figure 5.1). These cultures will be referred to in all subsequent analyses as migratory level neural tube (NT^m) explants, as the outgrowth generated from this type of explant yields mostly migratory cells (Newgreen, 1999). To assay the effect of EphA4 perturbation in

neuroepithelial cells before they had begun the process of EMT, (corresponding to caudal levels *in vivo*) neural tube regions were excised adjacent to somites -1 to +5, where the positive prefix is assigned to blocks of somitic precursor tissue in the unsegmented mesoderm and +1 is the region closest to the first formed somite (Figure 5.1). These cultures will be referred to in all subsequent analyses as pre-migratory neural tube (NT^{pm}) explants, as cells in the outgrowth generated from these cultures have not fully completed EMT but adopt an epithelioid (NC cell precursor) morphology (Newgreen and Minichiello, 1995).

The morphology of NC cell outgrowths from both NT^{m} and NT^{pm} cultures have previously been described (Newgreen and Gibbins, 1982; Newgreen et al., 1979). Briefly, NT^{m} cultures display an initial mesenchymal outgrowth, which forms rapidly over a few hours as a wave along the rostro-caudal extent of the explanted neural tube. After 44 hours in culture, three distinct layers can be identified in an NT^{m} culture (Figure 5.1). The remainder of the neural primordium appears as a multi-layered epithelium. Proximal to the neural primordium is the epithelioid layer; this is composed of flattened pavement-style cells that do not display HNK1 or SoxE immunoreactivity. The bulk of the outgrowth in an NT^{m} culture is mesenchymal, located at a distance from the neural primordium; cells in this layer exhibit an extended multipolar migratory morphology and stain positively for HNK1/SoxE (Figure 5.1). Occasionally, however, mesenchymal cells can be found in alcoves in the neural tube; these cells are likely to be generated by exposure to high levels of inducing factors (Newgreen and Minichiello, 1995). In contrast, the outgrowth of NT^{pm} cultures is largely epithelioid, with a few mesenchymal cells at the distal edge. Given that NC cell EMT occurs in a wave from the rostral to caudal end of the embryo, NC cell development is always the most developed at the rostral (ie. developmentally most mature) end and therefore the number of mesenchymal cells at one end of the outgrowth will differ from the other. In addition, the exact age of the neural tube at the time of excision also affects the extent of mesenchymalisation observed in the outgrowth (Newgreen and Minichiello, 1995).

Figure 5.1. Schematic representation of the *in vitro* model for NC cell generation. Neural tube pieces taken from migratory (m) and pre-migratory (pm) regions of the neural tube of a stage 13-14 embryo (E 2.5). After excision, neural tube pieces grown in culture for 18 or 44 hours on fibronectin; NC cell differentiation and their subsequent migration occurs in a rostro-caudal wave along the axis of the explanted neural tube. Migratory level (NT^{m}) cultures have a characteristically mesenchymal outgrowth (grey cells); Pre-migratory level (NT^{pm}) cultures have a small mesenchymal layer at the most mature end and a wide band of epithelioid cells comprising the epithelioid layer (red) surrounding the explanted neural tube.


Stage 13 embryo
Rostral
-7
-2
+1
+5
Caudal
Migratory level
excise neural
tube and explant
44 hours
in culture
Neural tube m
culture
Pre-migratory
level
excise neural
tube and explant
44 hours
in culture
Neural tube pm
culture
- neuroepithelial cell/ neural crest cell precursors
- migratory neural crest cell

5.2 Results

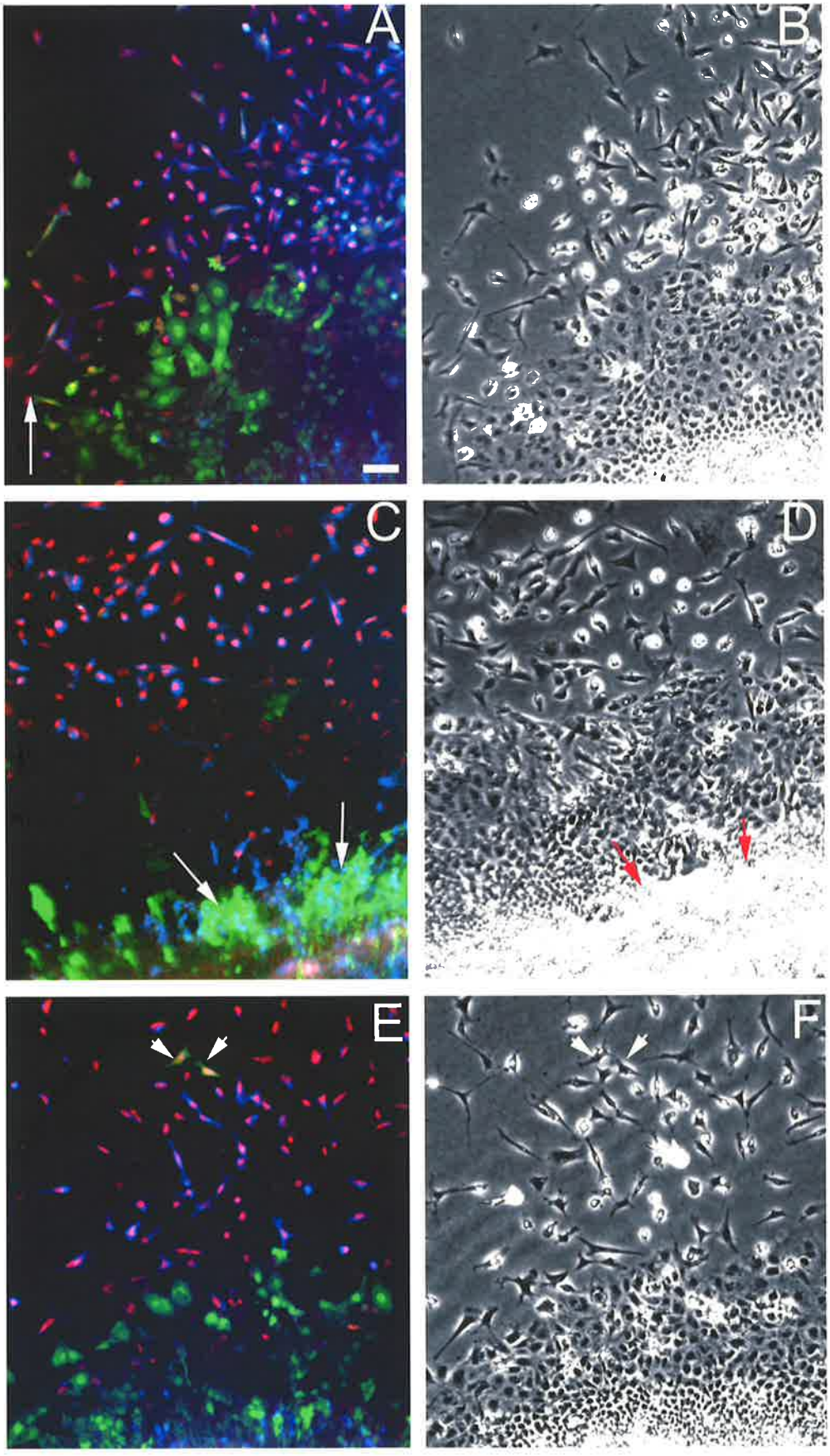
5.2.1 Electroporation and culture of the neural tube recapitulates in vivo *phenotypes at NT^m levels*

Given the phenotypes *in vivo*, the first objective was to clarify that these occurred independently of the somitic/ ectodermal environment. In control cultures expressing EGFP, the NC cell outgrowth was similar in morphology and size to un-electroporated neural tubes. After 44 hours in culture, EGFP-expressing cells were distributed in both the epithelioid and migratory regions of the outgrowth (Figure 5.2A). The first EGFP-positive cells to migrate away were at the anterior (to the left in the figures) end of the neural tube and these cells stained positively for both HNK1 and SoxE (blue and red respectively, Figure 5.2A; n = 18). Proximal to the neural tube, EGFP-expressing cells were located in the epithelioid layer and also some cells remained attached to the neural primordium, indicative of a normal progression through EMT (Figure 5.2A, B).

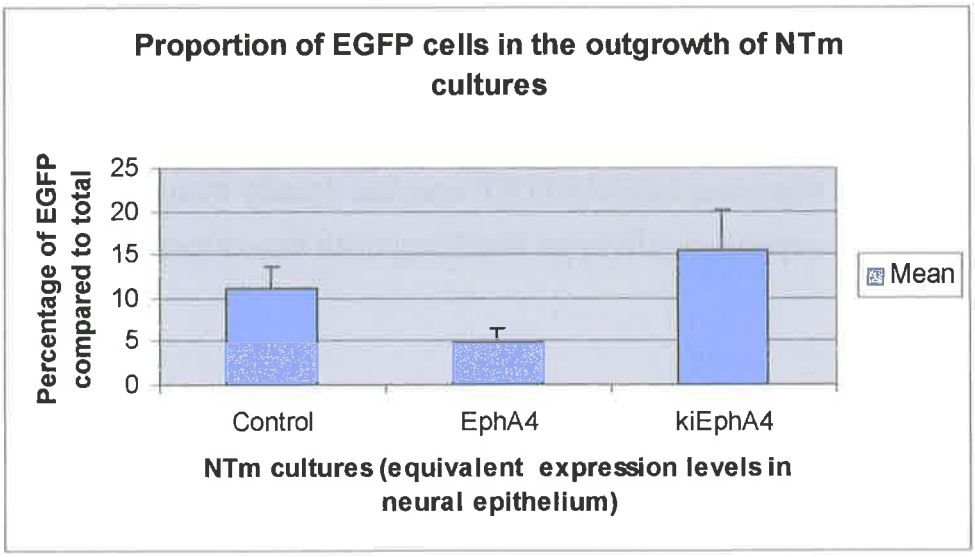
In contrast, neural tubes over-expressing EphA4/EGFP were characterised by clumps of EGFP-positive cells at the edge of the neuroepithelium with few EGFP cells in the outgrowth (Figure 5.2C; n = 18). Interestingly, EphA4/EGFP-expressing cells in the neural primordium appeared to be HNK1-positive (arrows, Figure 5.2C) indicating that they were still capable of acquiring the avian migratory mesenchymal marker HNK1. The clumping of EphA4/EGFP cells in NT^m cultures affected the morphology of the neural primordium, causing it to appear thickened and compacted in EphA4/EGFP-positive regions (arrows, Figure 5.2D). In contrast to EphA4/EGFP explants, kiEphA4/EGFP-expressing explants had a morphology and EGFP distribution that closely resembled controls (Figure 5.2E and F; n = 14). However, kiEphA4/EGFP-expressing cells appeared to preferentially localise to the epithelioid/mesenchymal layers rather than the neural primordium (arrows, Figure 5.2E&F). In addition, cells in the outgrowth of kiEphA4/EGFP-expressing explants appeared more dispersed, creating a characteristically less dense outgrowth in NT^m cultures (Figure 5.2F).

Figure 5.2. Electroporation of EphA4 and kiEphA4 in NT^m cultures recapitulates *in vivo* phenotypes.

EGFP (A, B), EphA4 (C, D) and kiEphA4 (E, F) constructs were electroporated (EGFP-expressing cells in green and for all subsequent images) and co-stained with Sox E antibody (red) and HNK-1 (blue). Arrow in A indicates direction of outgrowth for all cultures in (A-F). B, D and F are corresponding phase contrast images of A, C and E, respectively. In (C and D), arrows indicate HNK1-positive EphA4 clumps in the neuroepithelium; and in (D) indicate the morphology of the edge of the neuroepithelium in EphA4/EGFP-expressing regions. Arrows in (E and F) indicate kiEphA4/EGFP-expressing cells at the edge of the outgrowth. Scale bar: 100 μm.


A
B
C
D
E
F

A consistent observation in the EphA4/EGFP NT^m cultures was that transfected cells exited the neural tube with reduced efficiency compared to cells expressing kiEphA4/EGFP or EGFP alone. To determine the proportion of EGFP cells that successfully exited the neural tube in each of the experimental scenarios, EGFP-expressing cells in the epithelioid and mesenchymal regions of the outgrowth of NT^m cultures were counted and presented as a percentage of the total number of cells. To perform this quantitative analysis, the rostro-caudal axes of neural tube explants were aligned so that the direction of outgrowth was the same in all explants analysed. Along the rostro-caudal extent of the neural tube, the neural primordium was divided into columns approximately 210 μm in width. Each counting column (3-4 per explant) was positioned based on the level of EGFP expression in the neural epithelium, such that only those containing 40-60% EGFP-expressing cells (out of the total cells in that particular region of the neural epithelium) were used. Thus, the level of transfection in the neural epithelium was normalised. The number of EGFP-positive cells in the outgrowth (for each designated column) was counted and this value converted to a percentage of the total number of cells in the outgrowth/per column. For the comparison, percentages were pooled (3-4 values per explant and combined across explants) and averaged to calculate the mean proportion of EGFP cells in the outgrowth for EGFP, EphA4/EGFP and kiEphA4/EGFP NT^m cultures (Graph 5.1; refer Appendix 1.5A for summary).



Graph 5.1- Proportion of EGFP cells in the outgrowth of NT^m cultures expressing EGFP (n = 8 explants), EphA4/EGFP (n = 7) or kiEphA4/EGFP (n = 8). Data presented as a pooled average of all counts for each experimental condition. Bars represent Standard Error of the Mean (EGFP: ± 2.5; EphA4/EGFP: ± 1.6; kiEphA4/EGFP: ± 4.7)

These data demonstrate that despite equivalent expression levels in the neural epithelium, EphA4/EGFP-expressing cells were the least likely to migrate away from the explant. Calculating the ratio between the percentage of cells expressing in the neuroepithelium compared to that in the distal regions, demonstrated that EphA4/EGFP-expressing cells were less than half as likely as EGFP-expressing and less than one third as likely as kiEphA4/EGFP-expressing cells to migrate away from the explant (Table 5.1).

	% EGFP-expressing cells in the neuroepithelium	% EGFP-expressing cells in the distal outgrowth	Ratio NEp: Distal
Control (n=8)	36	11	3:1
EphA4 (n=7)	32	5	7:1
KiEphA4 (n=8)	34	15.5	2:1

Table 5.1- Distribution of transfected (EGFP-positive) cells in neuroepithelial (NEp) versus Distal regions of NT^m cultures presented as a percentage of the total number of cells in any given region.

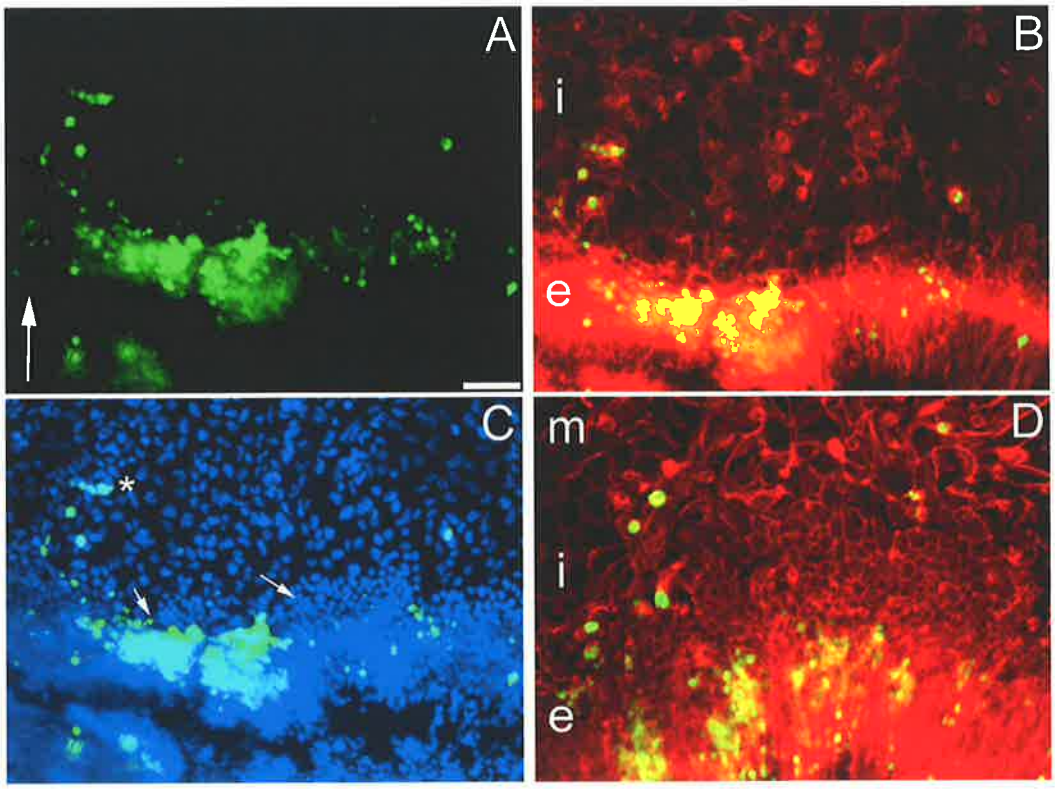
The phenotypes observed *in vitro* therefore mimicked that observed *in vivo*. NT^m cultures over-expressing EphA4/EGFP were characterised by clumps of electroporated cells at the neuroepithelium whilst those expressing kiEphA4/EGFP were less densely arranged and had a greater proportion of EGFP-expressing cells in the distal outgrowth than controls.

5.2.2 Electroporation and culture of the neural tube recapitulates in vivo phenotypes at NT^{pm} levels

In vivo, electroporation of either EphA4/EGFP or kiEphA4/EGFP in the dorsal neural tube at caudal levels of the embryo resulted in the inhibition or enhancement of EMT, respectively (refer Chapter 4). To correlate these results *in vivo* with the *in vitro* explant assay, the effects of EphA4/EGFP or kiEphA4/EGFP expression were examined in NT^{pm} cultures. Explanting of neural tube pieces from these levels (somites +1 to –5) enables the direct visualisation of neuropeithelial cell progression through EMT with a similar timetable to what would be expected *in vivo* (Newgreen and Minichiello, 1995). In NT^{pm} cultures expressing EphA4/EGFP, EphA4/EGFP-positive cells lined the edge of the neuroepithelium (indicated by F-actin staining), remaining distinct from the few cells beginning to emigrate from the edge of the neural tube (Figure 5.3A-C). Using DAPI staining to determine the pattern of cell emigration from the neural tube in the presence of EphA4/EGFP, the region above EphA4/EGFP expression in the neural epithelium was consistently accompanied by a reduction in the number of cells directly above this structure implicating an inhibition of EMT (compare expressing to non-expressing regions, arrows, Figure 5.3C). To determine if the distribution of EphA4/EGFP-positive cells also expanded into the epithelial layer in NT^{pm} cultures after 44 hours incubation, F-actin staining was utilised to highlight the proliferation and expansion of neuroepithelial cells into the epithelioid layer (Figures 5.3D). Despite the formation of a clear epithelioid layer after 44 hours incubation, EphA4/EGFP-positive cells remained tightly aggregated in the neuroepithelium (Figure 5.3D). In the epithelioid layer, non-expressing cells displayed a strong cortical F-actin and flattened epithelioid morphology. In contrast, the few EphA4/EGFP-positive cells that localised to this region retained a rounded cell shape surrounded by a tight ring of cortical F-actin and were clustered together in small aggregates (asterisk, Figure 5.3C and Figure 5.3D).

Figure 5.3. Over-expression of EphA4 in NT^{pm} cultures inhibits the release of EphA4/EGFP-positive cells from the neural tube epithelium.

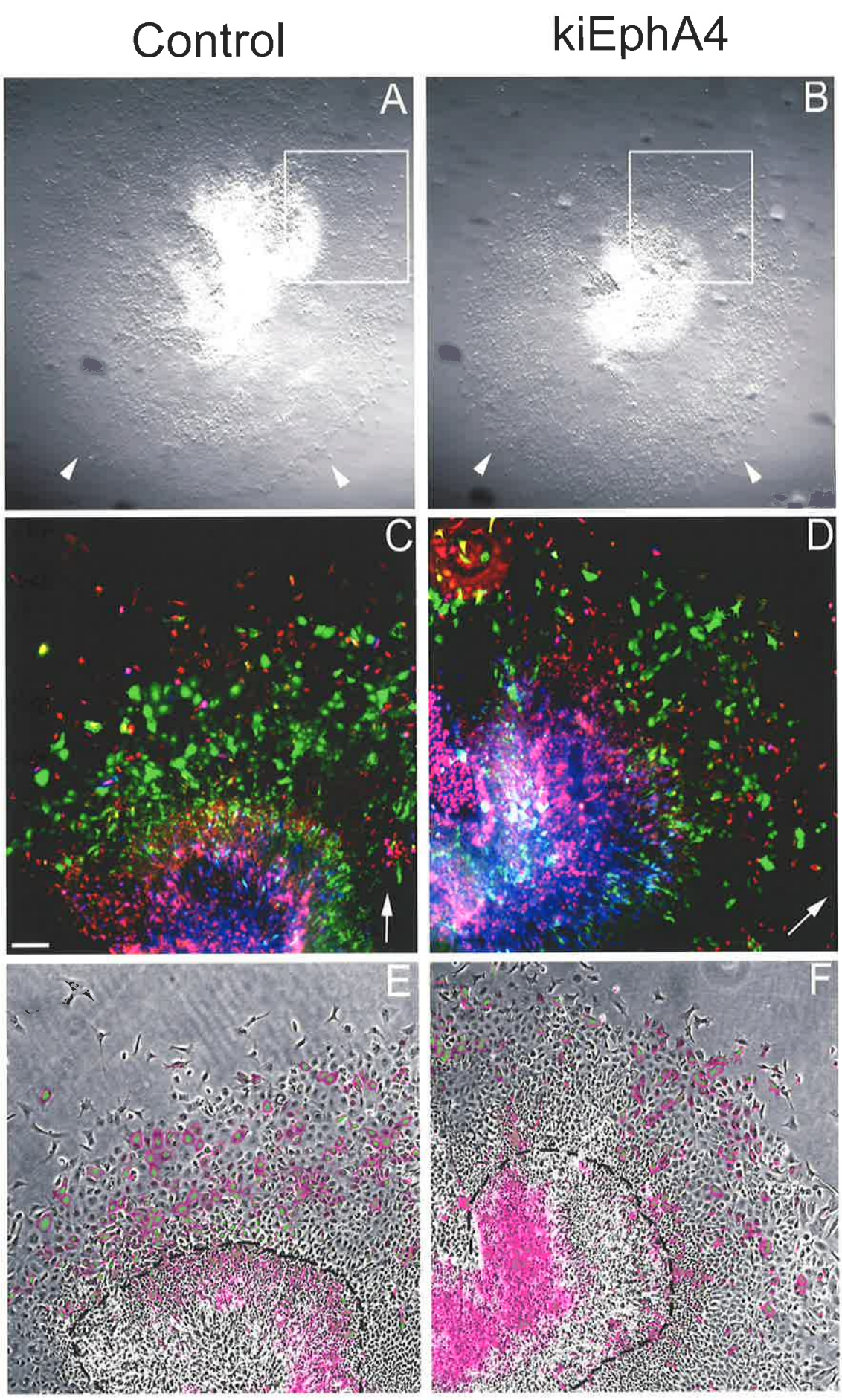
(A-C) EphA4/EGFP-expressing NT^{pm} culture after 18 hours incubation, represented as EGFP alone in (A) and co-stained for F-actin with phalloidin (red, B) and DAPI (blue, C). (A) Arrow indicates direction of outgrowth. (C) Asterisk indicates EphA4/EGFP cells in outgrowth; arrows highlight differences between DAPI distribution in expressing and non-expressing regions. (D) EphA4/EGFP-expressing NT^{pm} culture after 44 hours (green) and co-stained with phalloidin (red). e, multilayered neural tube epithelium; i, intermediate flat epithelioid layer; m, mesenchymal regions. Scale bar: 50μm.


A
B
i
e
C
*
m
D
i
e

In kiEphA4/EGFP-expressing NT^{pm} cultures, the general distribution of EGFP-positive cells was similar to controls with EGFP-positive cells occurring in all three layers of the outgrowth (Figure 5.4). Similar to their localisation in NT^{m} cultures and in contrast to controls, kiEphA4/EGFP-expressing cells were rarely found in the neuroepithelium and preferentially localised to the epithelioid/mesenchymal layers (compare Figure 5.4D, F). In addition, kiEphA4/EGFP-expressing NT^{pm} cultures appeared to have a more extensive and dispersed mesenchymal layer at the distal edge compared to the densely packed epithelioid layer observed in controls (compare arrowheads, Figure 5.4A and B). These results correlated with the increased formation of HNK1-positive processes identified at caudal levels *in vivo* (refer Figure 4.9). To quantify whether kiEphA4 expression lead to a more extensive mesenchymal cell outgrowth *in vitro*, the number of mesenchymal cells in the outgrowth was counted (as previously described) for both kiEphA4 and control NT^{pm} cultures. This revealed that 3 out of 4 kiEphA4/EGFP-expressing explants exhibited a higher proportion of mesenchymal cells in the outgrowth compared to controls (kiEphA4, n = 4 explants; Control, n = 3 explants). Thus, these data provide preliminary evidence to suggest that kiEphA4/EGFP expression in pre-migratory neuroepithelial cells enhanced their progression through EMT, leading to a more developed mesenchymal cell layer.

Figure 5.4. kiEphA4/EGFP expression in NT^{pm} cultures promotes the formation of mesenchymal cells.

Controls (left panel; A, C, E) and kiEphA4 (right panel; B, D, F) NT^{pm} cultures. (A) and (B) are low magnification images of control and kiEphA4 cultures, respectively. Arrowheads in (A) and (B) indicate cell morphology at distal edge of outgrowth. (C, E) and (D, F) are higher magnification images of the boxed regions in (A and B). (C, D) Stained with SoxE (red), HNK1 (blue) and EGFP (green). Arrows indicate direction of outgrowth. (E, F) illustrate the distribution of EGFP cells with respect to the overall NC cell outgrowth by overlaying with the corresponding phase contrast image. Dashed line indicates remaining neural primordium. Scale bar: 100 μm, (C - F).

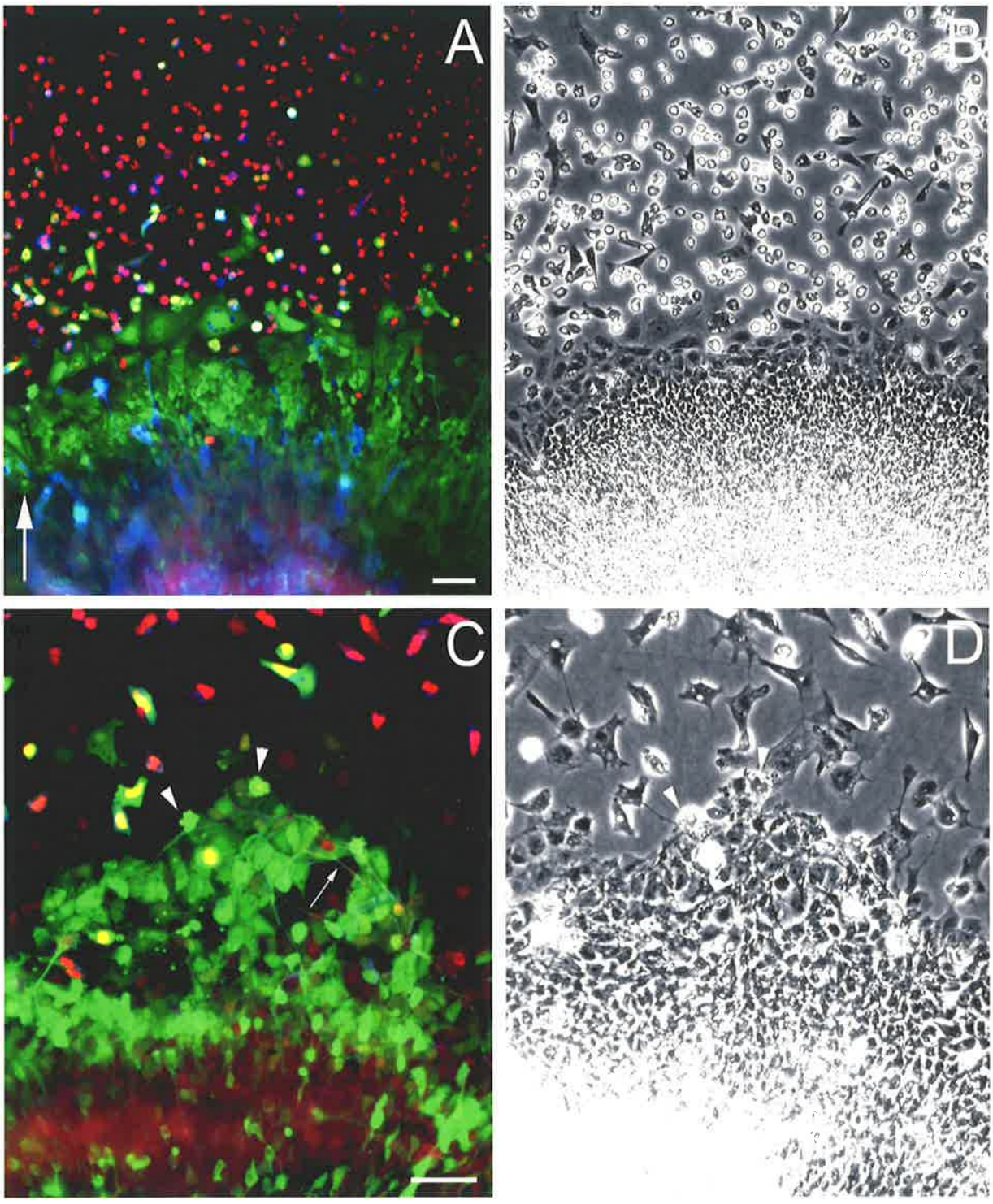

Control
kiEphA4
A
B
C
D
E
F

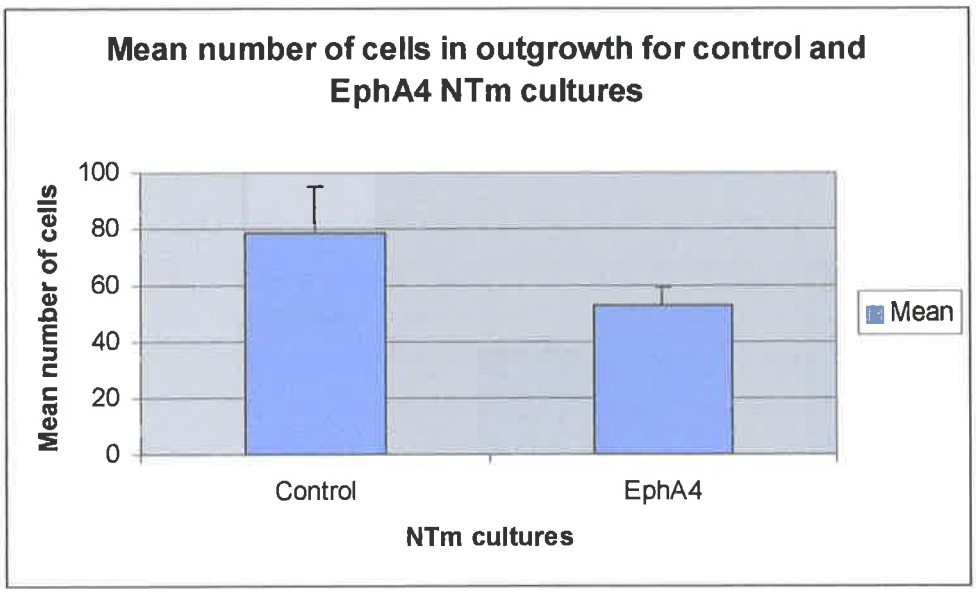
5.2.3 EphA4/EGFP over-expression in NT^m cultures leads to a localised inhibition of EMT

Similar to the effects of EphA4/EGFP expression in the mid-trunk of the neural tube *in vivo*, EphA4/EGFP expression in NT^m cultures resulted in the formation of EGFP-positive cellular aggregates and a subsequent reduction in the number of mesenchymal cells in the outgrowth compared with controls (Figure 5.5). Comparison between the outgrowths of EphA4/EGFP-expressing and control EGFP-expressing neural tubes indicated that there was a clear localised reduction in EMT above EphA4/EGFP-positive regions (compare Figure 5.5A, B and C, D). To determine whether EphA4-mediated clumping reduced the efficacy of EMT along the length of neural tube (indicated by mesenchymal cells in the outgrowth), outgrowth cells were counted in phase contrast images according to the method described previously. These data indicated that there was some reduction in the number of cells generated from EphA4/EGFP-expressing neuroepithelium when compared to controls though this was not to a level of statistical significance (EphA4: n=9 and Control: n=10; Graph 5.2; $p = 0.08$, one-tailed t-test; Appendix 1.5B). Thus, EphA4/EGFP expression appeared to elicit a dramatic but highly localised reduction in EMT. Analysis of the EphA4/EGFP-positive cellular aggregates in higher magnification also yielded some interesting insights into the possible cellular effects of EphA4 over-expression. Firstly, many of the EphA4/EGFP-expressing cells within the aggregates displayed long neurite-like extensions that were enmeshed with other cells within the group (arrow, Figure 5.5C). In addition, and consistent with *in vivo* data, some EphA4/EGFP-expressing cells showed surface blebbing (arrowheads, Figure 5.5C and D), which were often found in cells with extensive membrane ruffling (in preparation for migration) or in apoptotic nuclei (Newgreen et al., 1979).

Figure 5.5. EphA4 over-expression in neural tube^{m} cultures results in a localised inhibition of EMT.

Control EGFP-expressing culture (A, B), and EphA4/EGFP-expressing culture viewed in high magnification (C, D) stained with Sox E antibody (red) and HNK1 (blue) in A and C. Arrow in A indicates direction of outgrowth. (B, D) Corresponding phase contrast images of control and EphA4 cultures, respectively. In C and D, arrowheads indicate apoptotic cells and arrow in (C) indicates neurite-like processes. Scale Bar: 50 μm (A, B); 100 μm (C, D).

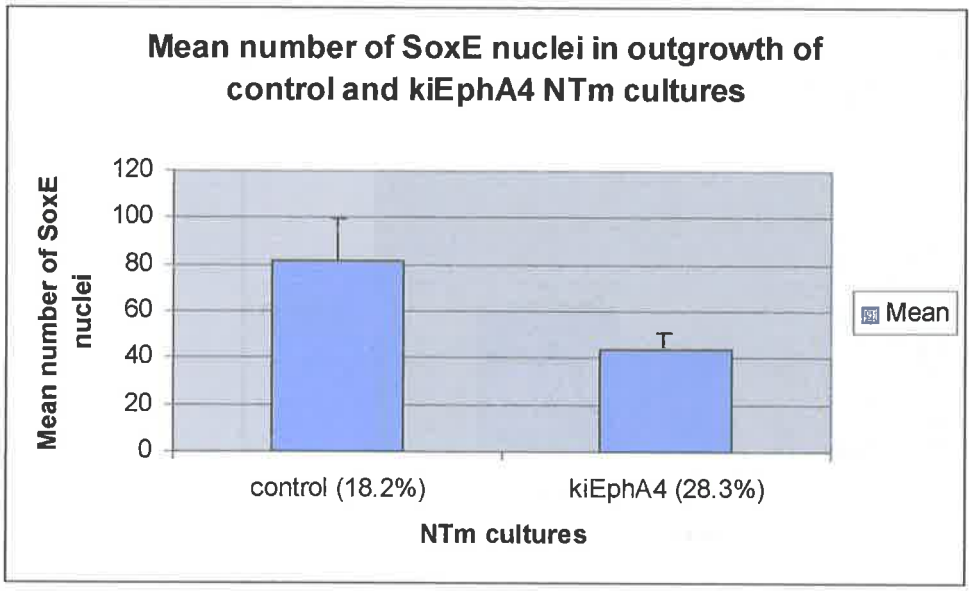

A
B
C
D



Graph 5.2- Mean number of cells (determined by phase contrast) in the outgrowth regions of EphA4 (n = 9) and control (n = 10) NT^{m} cultures. Error bars, EphA4: ±7.0; Control: ± 16.1

5.2.4 kiEphA4/EGFP expression in NT^{m} cultures leads to a less dense outgrowth

In contrast to the apparent inhibitory effects of ectopic EphA4 on EMT, kiEphA4/EGFP-expressing cells readily acquired mesenchymal characteristics in both NT^{m} and NT^{pm} cultures, although in NT^{m} cultures this consistently lead to a less dense outgrowth than in controls (Figure 5.2). To quantify this observation, the number of Sox E nuclei in the distal outgrowth was counted for kiEphA4/EGFP and control EGFP-expressing NT^{m} cultures, as previously described in Section 5.2.1. The data obtained from these analyses indicated that there was a significant reduction of Sox E-positive nuclei in kiEphA4/EGFP-expressing explants (kiEphA4= 43; control= 82; p = 0.03, one-tailed t-test; Graph 5.3 below, Appendix 1.5C), despite demonstrating a higher average level of expression in the neuroepithelium than controls (kiEphA4: 28.3%, n=9; control: 18.2%, n=10).



Graph 5.3- Mean number of Sox E nuclei in the outgrowth of kiEphA4 (n = 9) and control (n = 10) NT^m level cultures. The average level of expression in the neuroepithelium was 18.2% and 28.3% for controls and kiEphA4 respectively. Error bars, control: ± 17.7; kiEphA4: ± 7.5.

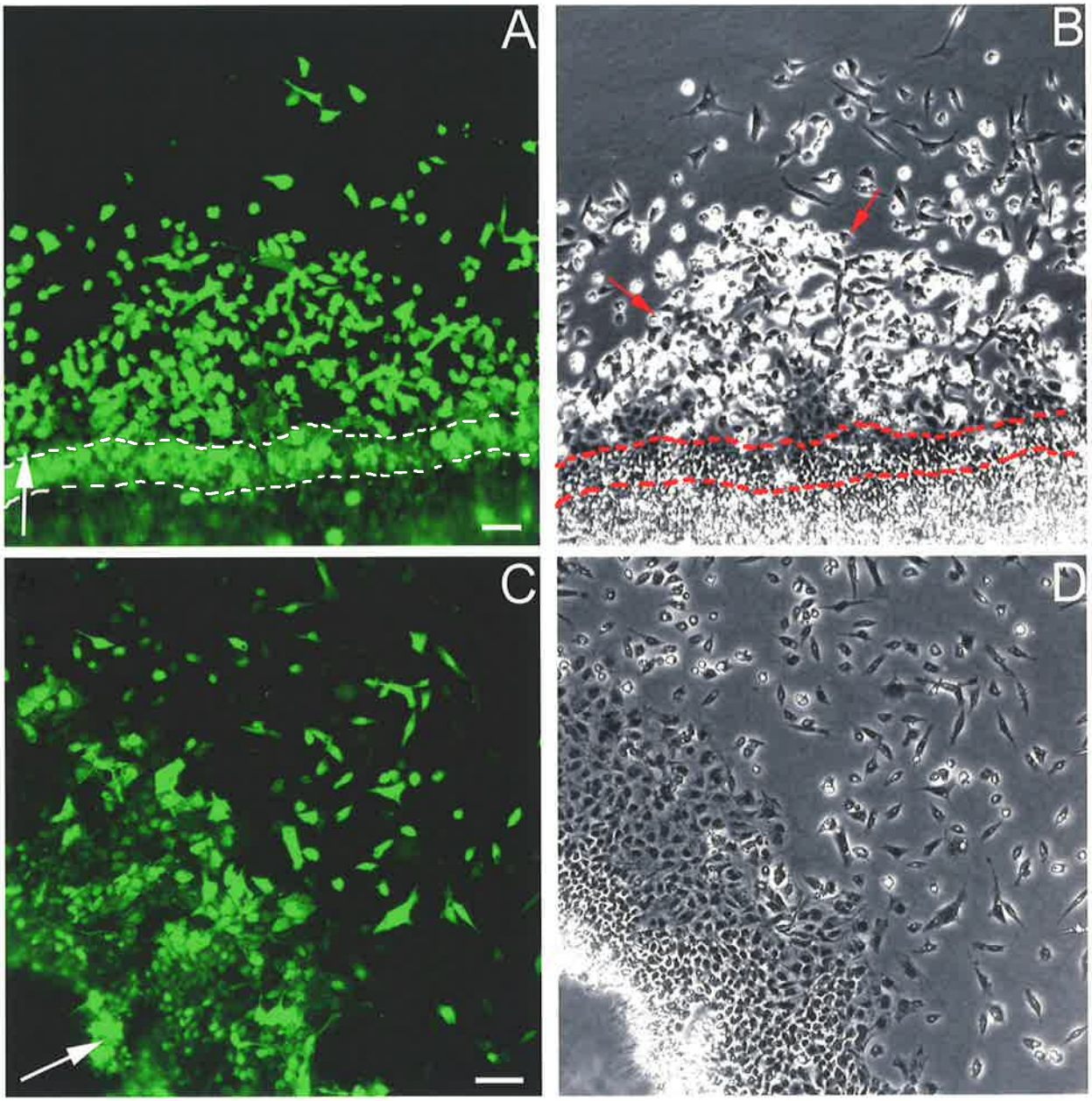
5.2.4 Morphological differences between EphA4/EGFP and kiEphA4/EGFP cultures in the epithelioid layer

Given the multiple roles previously described for EphA4 in affecting cell adhesion and morphology changes during tissue morphogenesis, it was postulated that EphA4 might be acting on the cytoskeleton during EMT. Prior to this analysis however, the epithelioid layer of EphA4/EGFP and kiEphA4/EGFP-expressing NT^m cultures was compared (Figure 5.6). EphA4/EGFP-expressing cells were arranged at the edge of the neuroepithelium, where NC cell EMT is occurring, in a highly compacted line of cells (dashed line, Figure 5.6A). This line of cells caused the neuroepithelium to appear densely packed, and occurred at the expense of a clear epithelioid layer (dashed line, Figure 5.6B). EphA4/EGFP-positive cells that did leave the neural primordium appeared to regress directly into a mesenchymal cell state, though they did not spread like normal mesenchymal cells and instead formed a close group, separated from the rest of the migratory NC cell population (arrows delineate edge of this group in Figure 5.6B). In stark contrast to NT^m cultures over-expressing EphA4, kiEphA4/EGFP-expressing neural primordia were characterised by a reduced neuroepithelial layer, with the majority of the EGFP-positive cells contributing to the epithelioid and mesenchymal layers (Figure 5.6C and D). The localisation of kiEphA4/EGFP-expressing cells in the epithelioid layer created a well-spaced network of flattened cells bordered by sparsely

distributed mesenchymal cells (Figure 5.6D). In this particular culture the neural anlage had been dislodged from the plate, though this was not a common feature of kiEphA4 cultures. Thus, there were clear differences in the distribution and subsequent morphology of the epithelioid layers in NT^{m} cultures over-expressing EphA4 compared to those expressing kiEphA4/EGFP.

Figure 5.6. EphA4 and kiEphA4 NT^m cultures display distinct morphologies in the epithelioid layer.

(A-D) Images show distribution of EphA4/EGFP-expressing cells (A), and kiEphA4/EGFP-expressing cells (C) in the epithelioid layer of NT^m cultures with corresponding bright field images in (C) and (D) respectively. (A, C) White arrow indicates direction of outgrowth. Dashed lines in (A) and (B) indicate cluster of EphA4/EGFP-expressing cells at the edge of the neuroepithelium. (B) Red arrows indicate edge of group of EphA4/EGFP cells.
Scale bar: 100 μm (A, B): 50 μm (C, D).


A
B
C
D

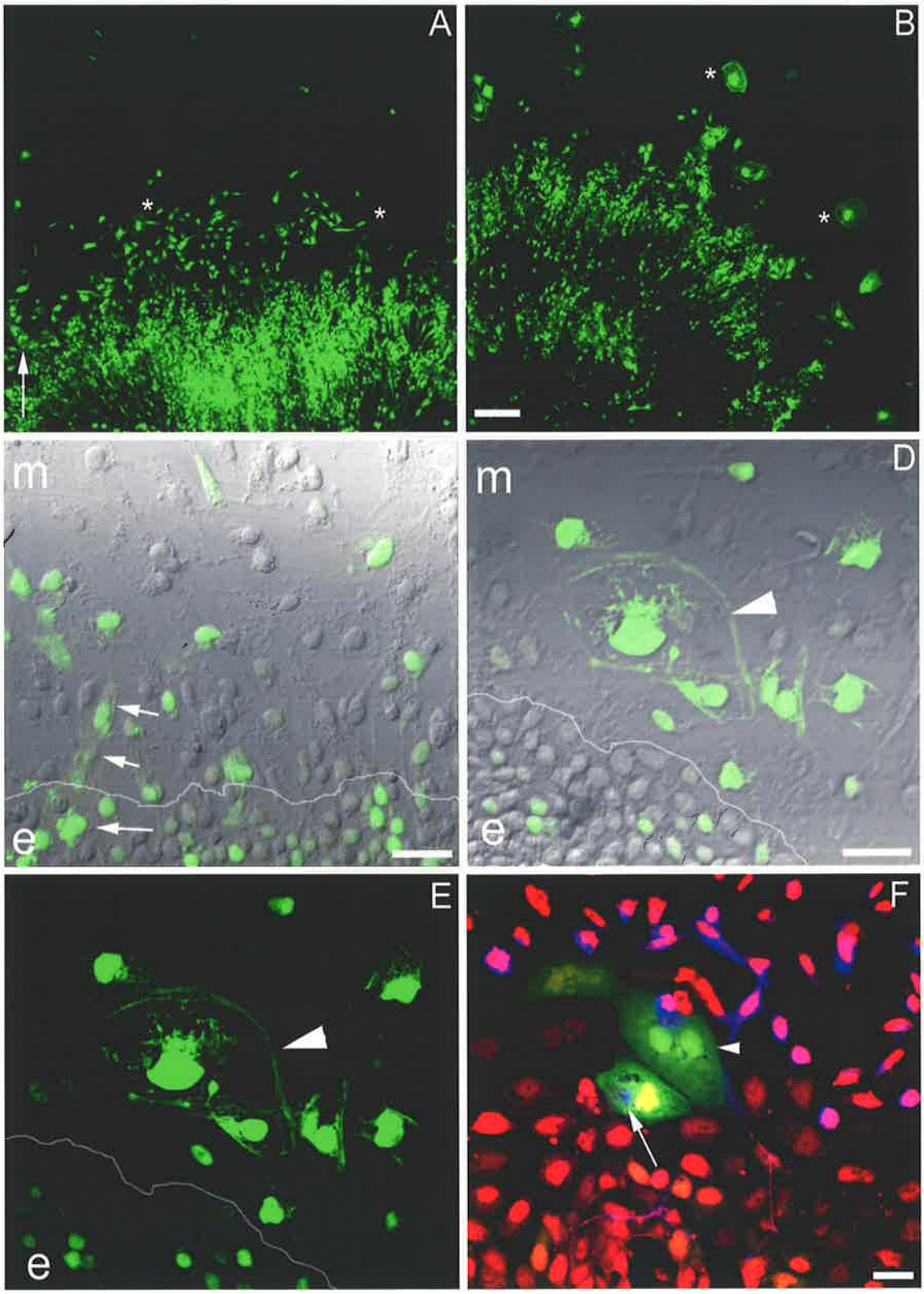
5.2.5 kiEphA4/EGFP-expressing cells in NT^m cultures exhibit morphologies characteristic of increased substrate adhesions

Although kiEphA4/EGFP-expressing cells had a similar distribution to EGFP-expressing cells in NT^m cultures, kiEphA4/EGFP-positive cells often exhibited square, flattened morphologies indicative of increased substrate attachment (Figure 5.7). Whilst EGFP-expressing cells only occasionally exhibited this sort of morphology, enlarged kiEphA4/EGFP-expressing cells were found in both the mesenchymal layer (compare asterisks, Figure 5.7A and B), and in some cases, directly abutting the remaining neural primordium (Figure 5.7D and E). In addition, the density of EGFP-expressing cells gradually decreased with increasing localisation away from the densely packed neural tube structure (arrows, Figure 5.7C). In contrast, there was a distinct drop in cell density at the edge of the kiEphA4/EGFP-expressing neuroepithelium (Figure 5.7D and E). KiEphA4/EGFP-positive cells adjacent to the remaining neural epithelium were often isolated and abnormally shaped, appearing disoriented and showing no clear alignment with the direction of outgrowth away from the neural tube (arrowhead, 5.7D and E). In addition, isolated kiEphA4/EGFP-expressing cells exhibited an increased frequency of multinucleation (arrowhead, Figure 5.7F). Despite their shape being atypical of NC cells, kiEphA4/EGFP-expressing cells adjacent to the neural tube were also HNK1-positive, suggesting that NC cells with disrupted EphA4 signalling differentiated into a migratory cell type (arrow, Figure 5.7F).

Figure 5.7. kiEphA4/EGFP-expressing cells adopt abnormal square flattened morphologies indicative of enhanced cell-substrate adhesions.

(A-B) Control (A) and kiEphA4 (B) NT^{m} cultures indicating EGFP distribution. (A) Arrow indicates direction of outgrowth and this applies to all images. (A, B) Asterisks indicate morphology of cells in outgrowth of control and kiEphA4 cultures, respectively. (C-D) High magnification view of control (A) and kiEphA4 (B) transfected cells emanating from the neural tube (green) and overlayed with the corresponding Nomarski image. (C) Arrows highlight changes in cell density moving away from the neural primordium. (D, E) Arrowhead indicates the outline of a kiEphA4/EGFP-expressing cell next to the neural tube. (E) Same image as (D) presented as EGFP alone. White line in C, D and E indicates edge of neural tube. (F) Multinucleated kiEphA4/EGFP-expressing cell co-stained with Sox E (red) and HNK1 (blue). Arrowhead indicates multinucleated cell, arrow highlights HNK1 co-staining. m = mesenchymal; e = epithelium.

Scale Bar: 100 μm (A, B); 25 μm (C-F)


A
B
m
D
m
e
e
E
F
e

5.2.6 EphA4 regulates cytoskeletal re-arrangement during NC cell emigration from NT^m explants

In the absence of a direct test for cell-cell or cell-ECM adhesion, an additional key indicator of EMT progression is the re-arrangement of circumferential F-actin fibres into a stress fibre morphology to facilitate cell motility (Newgreen and Minichiello, 1996). In neuroepithelial cells, high levels of cortical actin facilitate stable cell-cell adhesion. During EMT and coincident with the reduction of N-cadherin based cell-cell adhesion, cortical F-actin fibres form stress fibre bundles that cross the length of the cell and are required for tensile strength. In the final stages of EMT, F-actin is reduced to a fine layer around the cell perimeter whilst G-actin is increased (Newgreen and Minichiello, 1996). In control EGFP-expressing cells, cells in the epithelioid layer exhibited a typical stress fibre morphology with fibres extended across the length of the cell and strong circumferential bundles of F-actin (arrowheads, Figure 5.8A, B). Compared to cells expressing EGFP-alone, EphA4/EGFP-positive cells had a clearly disrupted F-actin organisation, charaterised by the lack of any clear cell outlines or specific F-actin structures (Figure 5.8C, D and also 5.8H). In place of a characteristic epithelioid state, EphA4/EGFP-expressing cells appeared to revert directly into a stress fibre morphology despite their continued attachment to neighbouring cells (arrowheads, Figure 5.8C and D).

In contrast, kiEphA4/EGFP-expressing cells in the epithelioid layer formed a network of flattened cells each surrounded by bands of thick circumferential F-actin at cell-cell junctions (arrowheads, Figure 5.8E, F). In addition, kiEphA4/EGFP-expressign cells often displayed a thickening of F-actin fibres at the cell edge in contact with the substrate (arrows, Figure 5.8G). Comparison between the epithelioid layers of EphA4 (Figure 5.8H) and kiEphA4 (Figure 5.8I) NT^m cultures highlighted the distinct stress fibre morphology in cells with reduced EphA4 signalling compared to the lack of any clear F-actin network in EphA4/EGFP-expressing explants (Figure 5.8 H and I). The re-establishment of circumferential F-actin distribution in kiEphA4/EGFP-expressing cells compared to cells over-expressing EphA4 was accompanied by a significant flattening of kiEphA4/EGFP-positive cells (arrows, Figure 5.8J, K), to the extent that expressing cells extended out of the epithelial layer (arrowheads, Figure 5.8J, K).

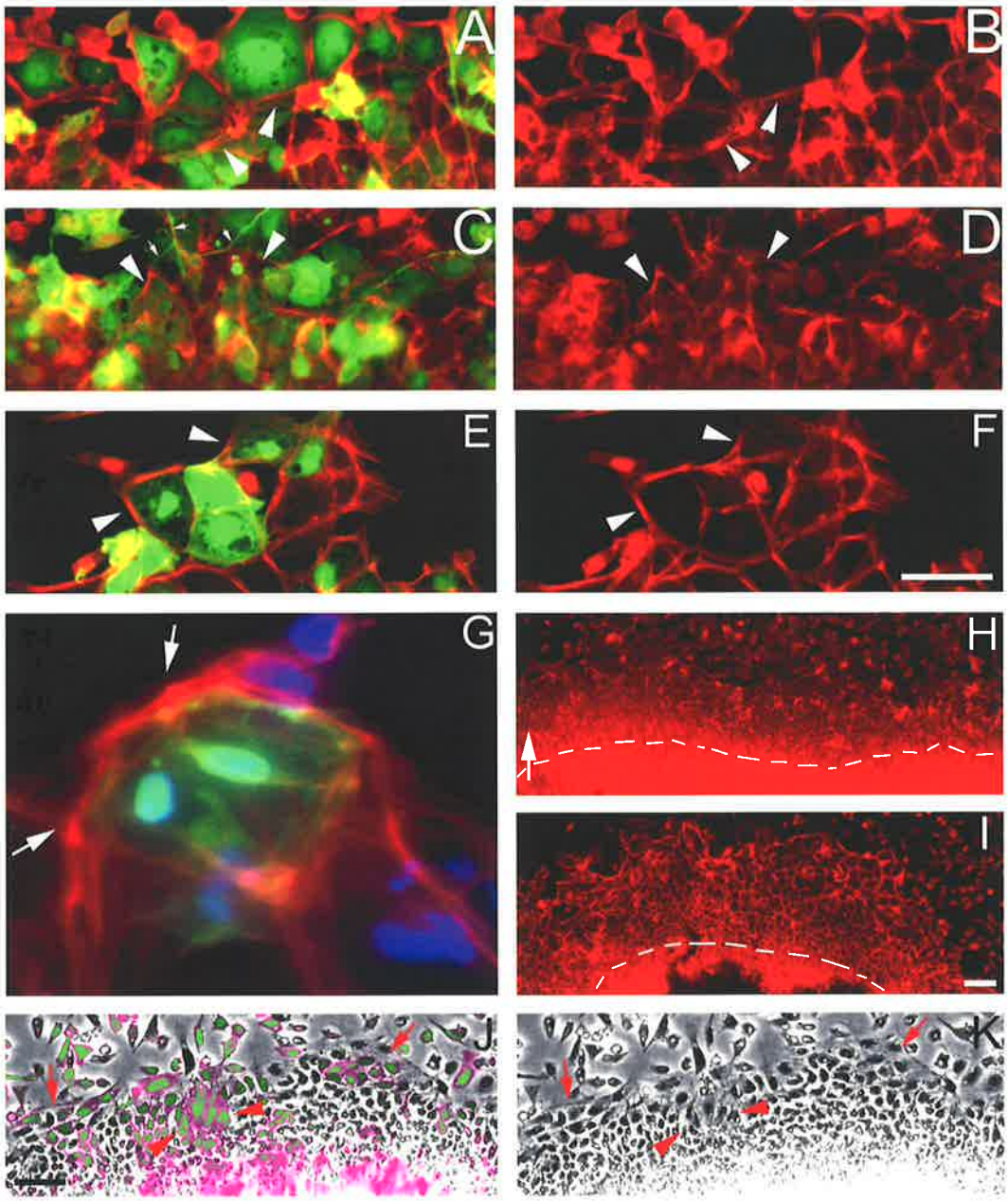
Figure 5.8. Expression of EphA4/EGFP or kiEphA4/EGFP perturbs F-actin distribution during late-stage EMT.

(A-F) Control (A, B), EphA4 (C, D) and kiEphA4 (E, F) NT^m cultures stained with phalloidin (red) to highlight F-actin morphology, and EGFP (green). Images presented as phalloidin/EGFP co-stain on in left panels (A, C, E), and Phalloidin only in right panels (B, D, F). White arrowheads highlight stress fibre morpohologies. Arrows in (C) indicate EphA4/EGFP-positive processes.

(G) kiEphA4/EGFP-expressing cell stained with phalloidin (red) and DAPI (blue). Arrows highlight intense F-actin deposits at regions in contact with substratum.

(H, I) Low magnification images illustrating F-actin morphology in the epithelioid layer of highly expressing EphA4 (H) and kiEphA4 (I) NT^m cultures. Dashed lines indicate edge of neural primordium.

(J, K) Low magnification image of kiEphA4/EGFP-expressing NT^m cultures expressing kiEphA4/EGFP and presented as EGFP overlayed with phase contrast in (J) and phase image alone (K). Arrows highlight morphology of kiEphA4/EGFP-expressing cells in the neural primordium; arrowheads indicate kiEphA4/EGFP-expressing cells adjacent to the epithelium. Scale bar (A-G): 100 μm; (H-K): 50 μm.


A
B
C
D
E
F
G
H
I
J
K

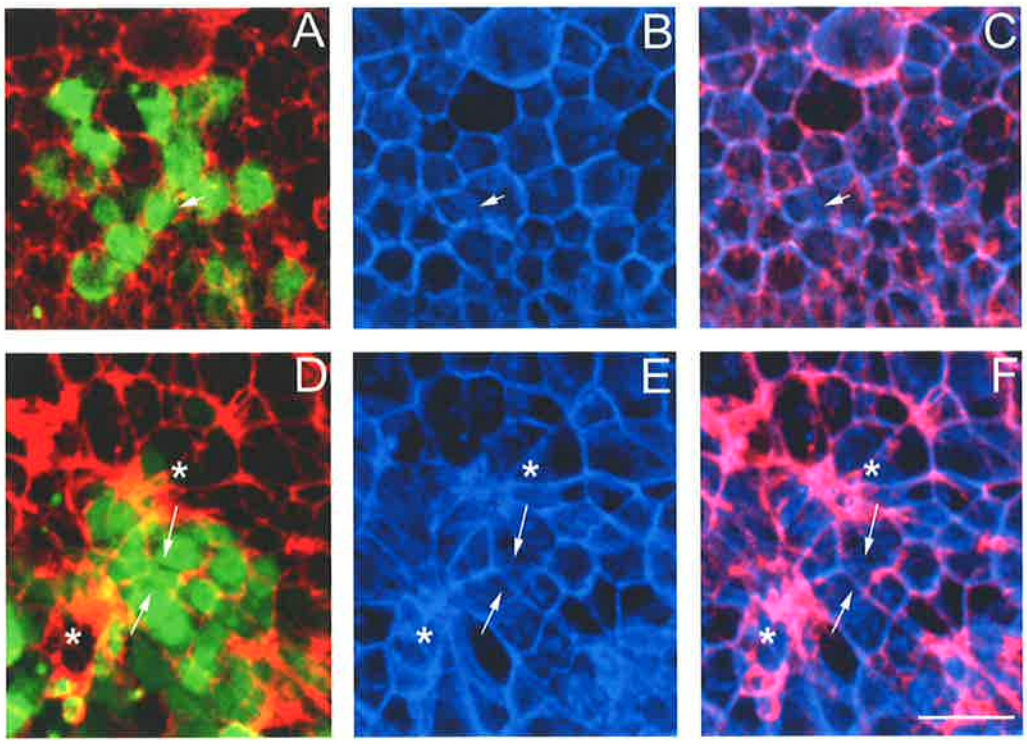
5.2.7 Do cytoskeletal differences between EphA4/EGFP and kiEphA4/EGFP cells coincide with changes in cadherin/catenin-mediated adhesion?

To determine if the altered actin distribution in EphA4/EGFP-expressing cells also coincided with a re-distribution of the cadherin linking molecule, ß-catenin, its distribution was examined in both EphA4 and kiEphA4 NT^{m} cultures. In the neuroepithelium, ß-catenin binds to the cytoplasmic domain of cadherins at one end and then links these with the cytoskeleton, acting as an intermediary protein linking cadherins with the F-actin cytoskeleton (de Melker et al., 2004). Coincident with changes to cellular adhesion associated with EMT, ß-catenin distribution becomes restricted to sites of cell-cell contact as cells enter into the epithelioid layer (Newgreen and Minichiello, 1996). In addition to its role as a cadherin linking protein, ß-catenin also functions as a transcription factor during EMT, complexing with Tcf/LEF transcriptional activation complex (in the canonical Wnt signalling pathway) to promote expression of genes associated with the mesenchymal state, for example Slug (Newgreen, 2005). Thus, for a brief period during EMT, ß-catenin becomes focussed in the cytoplasm/nuclear region and eventually disappears from both the membrane and cytoplasm in the final stages of EMT (de Melker et al., 2004).

In the neuroepithelium, ß-catenin followed the arrangement of circumferential actin in both control EGFP and EphA4/EGFP-expressing cells (Figure 5.9). Whilst control EGFP-expressing cells contributed normally to the paved arrangement of F-actin and ß-catenin at cell-cell junctions in the epithelium, (Figure 5.9A and B), the presence of a clump of EphA4/EGFP-positive cells clearly disrupted the normal distribution of both of these proteins (Figure 5.9D-F). Distinct from controls, ß-catenin accumulated along with F-actin at the exterior edge of EphA4/EGFP-positive cell clumps, indicating a tight cross-linking between F-actin bundles and cadherins at regions of cell-cell junctions (asterisks, Figure 5.9D and E). In addition, compared to controls, there appeared to be a general increase in the distribution of ß-catenin both in the cytoplasm and also along the perimeters of EphA4/EGFP-expressing cells (compare Figure 5.9B with E). In EphA4/EGFP-expressing cells, ß-catenin at cell-cell junctions often remained in the absence of F-actin fibres (arrows, Figure 5.9D-F), whilst in EGFP-expressing cells, the distribution of ß-catenin and F-actin at cell-cell junctions correlated more closely (arrow, Figure 5.9A-C).

Figure 5.9. β-catenin distribution is disrupted along with circumferential F-actin in the neuroepithelium of EphA4/EGFP-expressing NT^m cultures.

(A-C) Control, EGFP and (D-F) EphA4/EGFP-expressing cells co-stained with phalloidin to indicate F-actin (red), β-catenin (blue), and EGFP (green) and presented as EGFP/F-actin in (A) and (D), β-catenin only in (B) and (E), and β-catenin/F-actin in (C) and (F). (A-C) Arrow indicates occasional loss of cell-cell junctions between control EGFP-expressing cells. (D-F) Asterisks indicate β-catenin/F-actin foci; arrows indicate loss of cell-cell junctions. Scale bar: 50 μm.


A
B
C
D
E
F

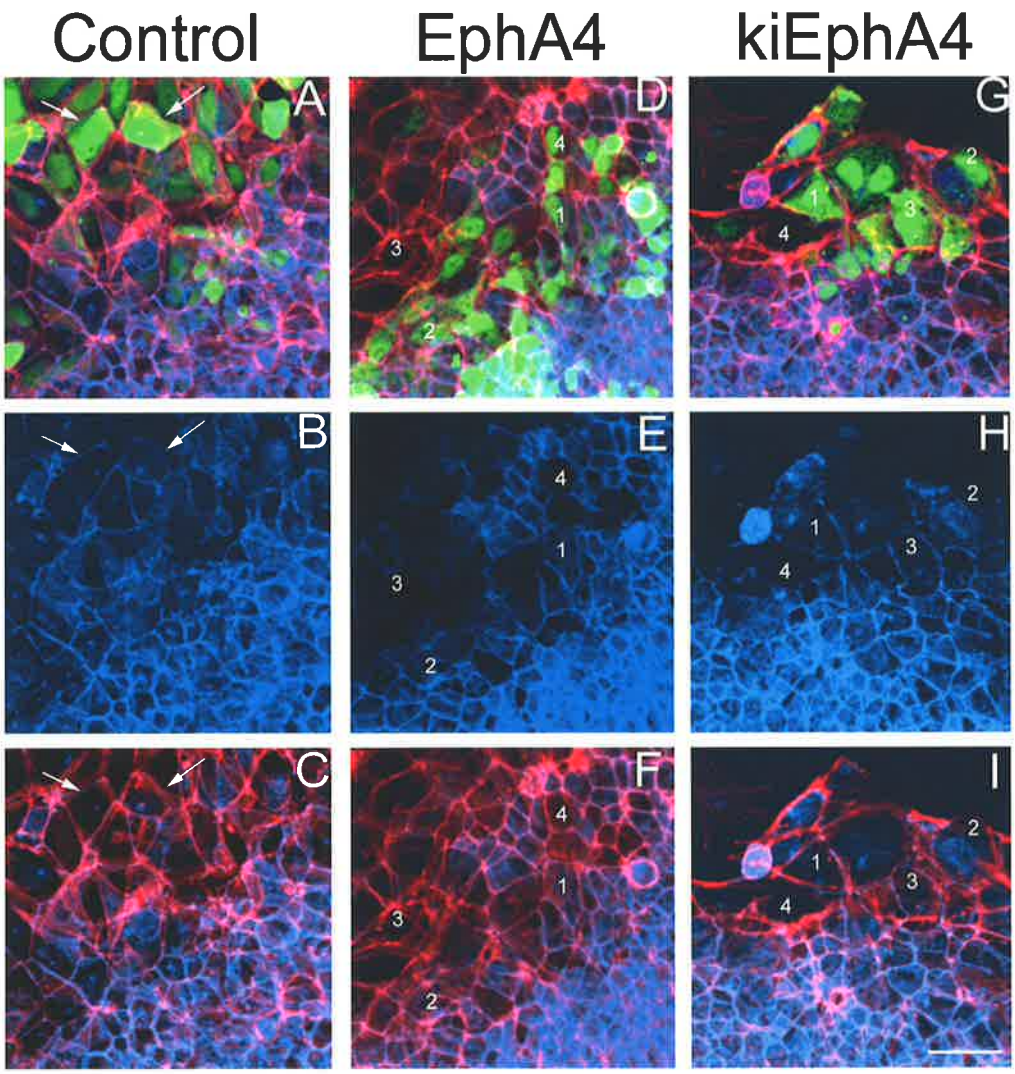
A common theme observed in NT^m cultures over-expressing EphA4 was that cells were consistently localised in tight aggregates in the epithelioid layer (Figures 5.5 and 5.7). Given the integral role of ß-catenin in both facilitating cross-talk between cadherins and the cytoskeleton as well as its role downstream of Wnt signalling during EMT (de Melker et al., 2004), the distribution of this molecule was examined specifically in EGFP, EphA4/EGFP and kiEphA4/EGFP-expressing cells in the epithelioid layer of NT^m cultures (Figure 5.10; Control: n = 3; kiEphA4: n = 3; EphA4: n = 5). In control EGFP and kiEphA4/EGFP-expressing NT^m cultures, ß-catenin expression became restricted to regions of cell-cell contact and was deposited in the nuclear regions of cells in the epithelioid layer (arrows Figure 5.10A-C and in cells 1 and 3, Figure 5.10 G-I). In both cases, localisation of ß-catenin to cell-cell contacts occurred coincidently with the re-distribution of F-actin fibres into thickened circumferential bands, indicative of coordinated changes in cell-cell and cytoskeletal adhesion systems (compare Figures 5.10 B, C and H, I, respectively). In addition, kiEphA4/EGFP-expressing cells appeared to retain ß-catenin distribution at cell-cell junctions between kiEphA4/GFP-expressing cells but not adjacent to non-expressing cells attached (compare cell 4 with cells 1 and 3 in Figure 5.10H).

In contrast, in EphA4/EGFP-expressing cells in the epithelioid layer, ß-catenin remained diffuse with intermittent distribution at regions of cell-cell contact (cells 1, 2 and 4, Figure 5.10 D-F). This occurred concordantly with an inability of EphA4/EGFP-expressing cells to support thick bands of circumferential F-actin, occasionally reverting to an elongated morphology with fine F-actin fibres at cell-cell junctions (for example, cell 1 in Figure 5.10D-F). In all of the EphA4/EGFP-expressing NT^m cultures analysed, there was also an increase in cytoplasmic ß-catenin, so that it tended to remain diffuse in the cytoplasm instead of becoming downregulated as with other epithelioid cells (compare regions 2 and 3 in Figure 5.10F).

Figure 5.10. β-catenin distribution in the epithelioid layer of NT^m cultures is perturbed in cells over-expressing EphA4.

Control (A-C), EphA4 (D-F) and kiEphA4 (G- I) NT^m cultures co-stained with EGFP (green, A, D, G), phalloidin to indicate F-actin (red, A, C, D, F, G, I), and β-catenin (blue, C, F, I). (A-C) Arrows show control EGFP-expressing cells with typical F-actin and β-catenin distribution for cells distributed in the epithelioid layer. Cells 1, 2 and 4 in (D-F) and cells 1, 2 and 3 in (G-I) highlight specific morphologies of particular EphA4/EGFP-expressing and kiEphA4/EGFP-expressing cells, respectively. Cell 3 in (D-F) and cell 4 in (G-I) indicate the morphology of non-expressing cells for comparison.

Scale bar: 50 μm.


Control
EphA4
kiEphA4
A
B
C
D
E
F
G
H
I
1
2
3
4

5.3 Discussion

The effects of EphA4 over-expression or blocking kinase-dependent EphA4 signalling utilising an *in vitro* assay for EMT paralleled the *in vivo* results (refer Chapter 4) suggesting that EphA4 is involved in NC cell EMT. Similar to *in vivo*, over-expression of EphA4 in culture lead to a significant inhibition of EMT, promoting the formation of cellular aggregates in both NT^{m} and NT^{pm} cultures (Figures 5.3 and 5.5). Furthermore, EphA4 over-expression lead to morphological and molecular changes indicative of increased cell-cell adhesion, possibly even influencing cellular differentiation (Figure 5.3 and 5.11). In addition, in the neuroepithelium, F-actin and β-catenin were found in intense deposits at cell-cell junctions, possibly indicating EphA4 involvement in regulating cadherin-dependent cell-cell adhesion (Figure 5.10). Conversely, loss of EphA4 activity promoted the acquisition of motile characteristics causing cells to adopt a polygonal, flattened morphology and to localise to the distal outgrowth (Figure 5.6). A possible interpretation for these effects on NC cell morphology is that in the absence of EphA4 signalling, cell-substrate interactions were enhanced. Similar to the *in vivo* analyses, expression of kiEphA4/EGFP had distinct effects on the overall EMT outcome depending on whether it was expressed at the onset of EMT (NT^{pm}) or in the advanced stages (NT^{m}). In NT^{m} cultures expressing kiEphA4/EGFP, a reduction in outgrowth density was observed (Figure 5.2 and 5.7); in NT^{pm} cultures, the loss of EphA4 activity increased the EMT outcome, as measured by the number of mesenchymal cells (Figure 5.4). Finally, analysis of F-actin distribution in delaminating cells in the presence of excess EphA4 suggested that EphA4 negatively regulated stress fibre formation in delaminating NC cells (Figure 5.8 and 5.10). In sum, these data suggest that EphA4 negatively regulates NC cell EMT. The possible mechanisms through which EphA4 might be acting in EMT are discussed below.

5.3.1 Putative mechanisms to explain the loss of cell density in kiEphA4/EGFP-expressing NT^{m} explants

A possible mechanism to explain why reduced EphA4 activity produced a sparser NC cell outgrowth than controls is that EphA4 positively regulates proliferation of migratory NC cells. Analyses both *in vitro* and *in vivo* have demonstrated that recently delaminated migratory NC cells undergo a series of mitoses and this leads to groups of clonally related daughter cells travelling together *in vivo* (Krull et al., 1995; Newgreen and Minichiello,

1995). If EphA4 was required for the positive regulation of proliferation in recently delaminated cells, inhibition of EphA4 signalling with kiEphA4 might directly inhibit mitoses amongst recently emigrated NC cells. Whilst this hypothesis would explain the loss of density in kiEphA4/EGFP-expressing explants as well as the increased frequency of multinucleate cells (Figure 5.7), there are a number of possible reasons why this hypothesis is not particularly feasible when considering an *in vivo* role for EphA4. Firstly, EphA4 is expressed at a low level on NC cells and therefore would be expected to have a negative regulatory role in events required for NC cell migration. For example, if EphA4 was specifically required for mitoses in migratory NC cells it would not be expected to be expressed at such low levels. Secondly, EphA receptors have been shown to negatively regulate the ERK/MAPK pathway in prostatic epithelial cells and cancer, having a largely non-mitogenic effect (Kullander and Klein, 2002). Although the downstream effects of Eph activation in any particular cell type is likely to be specific, a similar situation was described for EphB2 in neuronal cells (Kullander and Klein, 2002), suggesting that EphA4 would be unlikely to positively regulate cell proliferation during NC cell migration.

If EphA4 is unlikely to be positively regulating mitoses in NC cells during the early phases of migration, the question remains as to how reduced EphA4 activity might decrease the density of emigrating NC cells. Analyses of kiEphA4 in NT^{pm} cultures suggested that NC cell emigration in the absence of EphA4 occurred on a more rapid timetable. A possible explanation that would encompass the delamination profiles of kiEphA4/EGFP-expressing cells and the concomitant reduction in NC cell outgrowth density is that EphA4 might negatively regulate entry of neuroepithelial cells into S phase; a critical requirement for successful delamination (Burstyn-Cohen and Kalcheim, 2002). EphA receptors are considered negative regulators of proliferation and act to suppress the MAPK pathway in multiple different cell types (Kullander and Klein, 2002). Thus, there is circumstantial evidence to support that EphA4 might negatively regulate G1/S transition and subsequent delamination. Furthermore, a recent paper has demonstrated that this particular pathway is axial level-dependent providing further support for the distinct effects of kiEphA4 at NT^{m} and NT^{pm} levels. In the case of kiEphA4/EGFP-expressing cells, reduced EphA4 would enhance G1/S transition and subsequently, NC cell delamination, whilst over-expression would inhibit this process. Despite this possibility, it is difficult to then link increased proliferative capacity with the observed reduction in cell density in kiEphA4 NT^{m} explants. A possible explanation could be that cells expressing kiEphA4 might be forced prematurely into S phase without acquiring the correct machinery for completion of replication. Interestingly, inhibition of DNA

replication in cells that had already entered into S phase did not affect delamination but produced enlarged cells and a sparser outgrowth (Burstyn-Cohen and Kalcheim, 2002). Thus, it is possible that kiEphA4 could promote G1/S transition (and delamination) whilst inhibiting subsequent mitoses in NC cells in the outgrowth. To further confirm if this was the case, the pattern of S phase entry would have to be compared in delaminating cells expressing kiEphA4/EGFP, EphA4/EGFP and EGFP alone. In addition, it would be useful to analyse earlier time-points in kiEphA4 NT^{pm} cultures to ensure that kiEphA4 does actually increase the rate of EMT in NC cell precursors.

Aberrant migratory morphologies of kiEphA4/EGFP-expressing cells in NT^{m} explants: implications for NC cell migration?

Studies investigating the mechanisms of migration of NC cells *in vitro* have indicated that NC cells exhibit density-dependant orientation and directionality, and that in the absence of many cell contacts, they have an increased tendency to migrate in a random manner (Newgreen et al., 1979; Rovasio et al., 1983). This was also the case for kiEphA4/EGFP-expressing cells; at reduced density they adopted an abnormal cell shape and were disoriented, however once in a mass of NC cells, they assumed a more uniform orientation and morphology. A similar phenomenon was noted *in vivo*; kiEphA4/EGFP-expressing cells at the leading edge of a migratory NC stream were often tangentially aligned and entered into inappropriate territories, whilst expression amongst a stream of non-transfected cells resulted in their correct orientation and subsequent migration. Thus, a possible explanation for the migratory morphologies adopted by kiEphA4/EGFP-expressing cells *in vivo* and in NT^{m} explants could be that these cells lacked sufficient cell-cell contacts to orient and direct their trajectories correctly. In support of this idea, recently delaminated kiEphA4/EGFP-expressing cells often lacked any clear cell-cell contacts (Figure 5.7). Furthermore, the extent of disorientation was dose-dependent both *in vivo* and *in vitro*. Thus, when kiEphA4/EGFP-expressing cells were in the majority of cells in an outgrowth, density was significantly reduced and the reverse was true when there were only a few kiEphA4/EGFP-positive cells present. In sum, these data lend support to the idea that the aberrant migration of kiEphA4/EGFP-expressing cells might be directly related to reductions in cell density amongst the migratory NC cell population.

One of the possible mechanisms to explain the aberrant migration of kiEphA4/EGFP-expressing cells *in vivo* was that these cells had reduced HNK1 expression (refer Figure 4.7). Whilst *in vitro*, there was no significant correlation between disoriented NC cell morphology

and low HNK1 immunoreactivity (Figure 5.7), the level of HNK1 expression in the outgrowth of kiEphA4/EGFP-expressing NT^m explants was reduced compared to that produced from EphA4/EGFP-expressing explants (Figure 5.2). Thus, further analyses would have to be conducted to detemine whether in fact disoriented migratory morphologies correlated with reduced HNK1 staining in kiEphA4/EGFP-expressing cells. Given the high levels of HNK1 expression in EphA4/EGFP-expressing cells it could be speculated that EphA4 positively regulates the presentation of the HNK1 epitope at the cell surface, although how this explanation might fit with the observed inhibitory effects of EphA4 activity on EMT is unknown. A more likely mechanism that might explain these effects is that increased HNK1 expression in cells of the epithelioid layer was a downstream consequence of EphA4-mediated inhibition of F-actin (discussed further below). In comparison, kiEphA4/EGFP-expressing cells expressed SoxE more frequently than EphA4/EGFP-expressing cells. This observation, along with the increased neuronal-like morphologies associated with EphA4/EGFP-expressing cells might implicate EphA4 more directly in regulating neuronal differentiation, although the *in vivo* relevance of this possibility remains unknown (discussed in section 5.3.2).

5.3.2 EphA4 over-expression suggests a role in promoting cadherin-dependent cell-cell adhesion in neuroepithelial cells

One of the key effects of EphA4 over-expression *in vivo* was thought to be changes in cell-cell adhesion leading to the preferential association of EphA4/EGFP-expressing cells with one another and to endogenous NC cells (refer Figures 4.5 and 4.6). In both the neural tube *in vivo* and in the neuroepithelium *in vitro*, EphA4/EGFP-expressing cells were associated in clusters of "like" cells, culminating in a striped arrangement of EGFP-positive cells (Figure 5.11A and B). Whether this effect was due to the preferred attachment of EphA4/EGFP-expressing cells to one another or their exclusion from neighbouring, non-expressing neuroepithelial cells is unknown. However, in the few examples where EphA4/EGFP-positive cells could be found in the outgrowth, they exhibited a rounded morphology and often were found attached to one another in aggregates, implicating an active role for this protein in promoting cell-cell over cell-substrate adhesions in NC cells (Weston, 1970).

Previously, it was hypothesised that changes to cell-cell adhesion in EphA4/EGFP-expressing cells during EMT might be through interactions with N-cadherin (refer Section 4.3.4). Analysis of the distribution of the cadherin-linking molecule, β-catenin, in EphA4/EGFP-expressing cells in the neuroepithelium showed that it was distributed in excessive amounts

along with F-actin at cell-cell junctions (Figure 5.9). The intense deposits of F-actin and β-catenin at cell-cell junctions suggest that EphA4 promoted cadherin-mediated junctions in the epithelium. In epithelia, cadherin molecules expressed on the cell surface provide critical tight adhesions between neighbouring epithelial cells and comprise a specialised type of epithelial junction or adherens junction (Gumbiner, 1996). Adherens junctions facilitate extensive adhesion between epithelial cells and are required for maintenance of the integrity of an epithelial layer particularly in response to mechanical stress (Takeichi, 1988). Thus, these data support the idea that EphA4 might promote epithelial junctions, possibly through a cadherin-dependent mechanism. In support of such a notion, restoration of EphA4 signalling in cells of the pre-somitic mesoderm induced boundary formation and also the apical re-distribution of β-catenin; both of which are involved in the acquisition of an epithelial phenotype (Barrios et al., 2003). Thus, in the neuroepithelium *in vivo*, EphA4 might promote adherens junctions and thereby negatively regulate neuroepithelial cell progression during EMT. As EGFP does not necessarily indicate EphA4 distribution, a critical test to assert that EphA4 directly regulates epithelial junctions would be to confirm its co-localisation with β-catenin and F-actin at cell-cell junctions.

In the epithelioid layer, aggregated EphA4/EGFP-expressing cells appeared to differentiate into neuron-like cells (Figure 5.5). In addition to extending spiny EGFP-positive processes (arrowheads Figure 5.11C), in some cases the distal tips of EphA4/EGFP-positive processes displayed lamellipodia typical of a neuronal growth cone (arrowheads, Figure 5.11D). Furthermore, EphA4/EGFP-expressing cells displaying this morphology rarely co-expressed SoxE despite continued levels of HNK1 expression (arrows, Figure 5.11C and Figure 5.5), supporting the observation that clustered EphA4/EGFP-expressing cells had acquired a neuronal cell fate (Kim et al., 2003). To clarify their differentiation status, HuN or TUJ1 could be utilised to stain EphA4/EGFP-expressing NT^{m} explants. Interestingly, re-examination of the anterior neural tube of EphA4/EGFP-expressing embryos suggested that a similar phenomenon might occur *in vivo* (refer Appendix 1.2A). This effect was only seen in some of the EphA4/EGFP-positive cell clumps. A possible explanation for the observed variation might be that explants taken from slightly more anterior regions would be likely to display neuronal differentiation more readily than more immature (posterior) explants.

Figure 5.11. EphA4 over-expression has distinct effects on cell-cell adhesion.

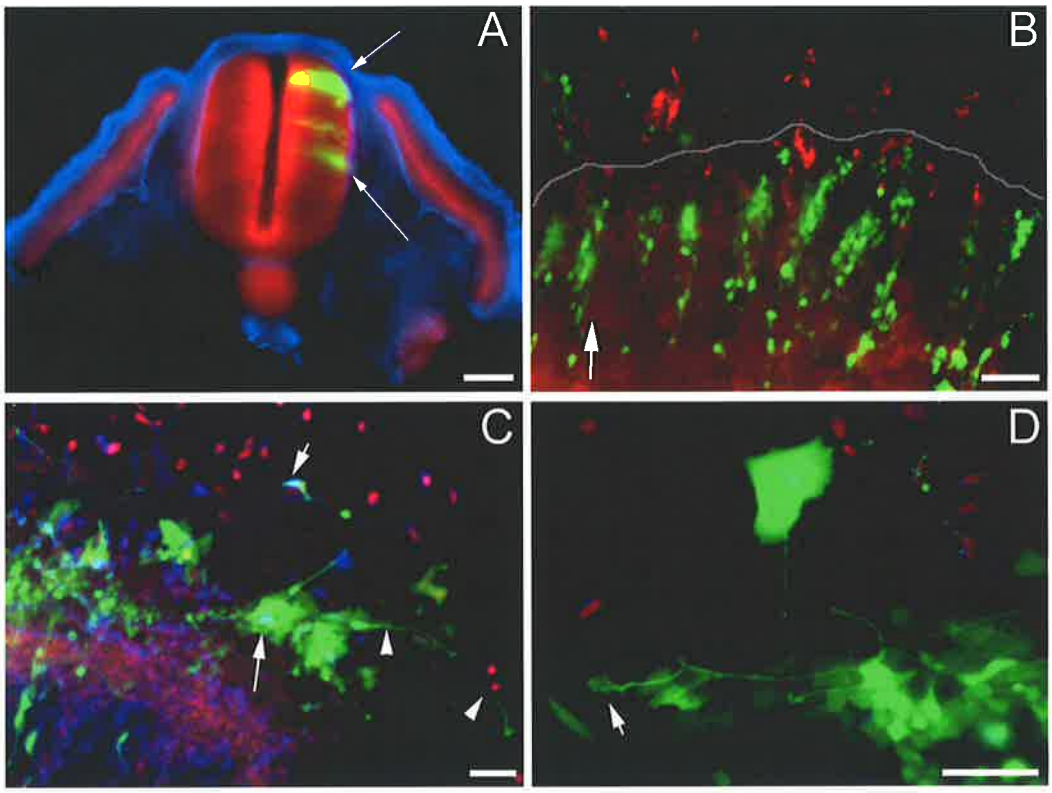
(A) Transverse section of EphA4/EGFP transfected embryo (EGFP in green), co-stained with N-cadherin (red) and Laminin (blue), arrows point to EphA4/EGFP-positive clusters in the neural tube. (B) EphA4/EGFP-expressing neural tube^{m} culture stained with HNK1 (red); arrow indicates direction of outgrowth, line denotes neural tube outline.

(C and D) Prolonged cellular aggregation promotes the formation of cellular processes typical of a growth cone in EphA4/EGFP-expressing NT^{m} explants.

(C) NT^{m} culture expressing EphA4/EGFP and stained with HNK1 (blue) and SoxE (red). Arrows points to clumped EphA4/EGFP-expressing cells at the edge of the neuroepithelium, arrowheads highlight spiny processes that are also EGFP-positive.

(D) Higher magnification image of same EphA4/EGFP-expressing explant as in (C). Arrow indicates growth cone-like structure at tip of nascent EphA4/EGFP-positive axon.

Scale bars: 50 μm.


A
B
C
D

Earlier studies documenting the different phases of NC cell emigration/differentiation *in vitro* have revealed that after 2 days in culture, NC cells at the distal tip of a NC cell outgrowth (the developmentally oldest) begin to form tight cellular groups, occasionally producing nascent axons (Newgreen et al., 1979). This process of re-aggregation could be likened to DRG/SG formation *in vivo*, whereby NC cells, having migrated away from the neural tube, increase their cell-cell adhesions to facilitate coalescence and differentiation (Newgreen et al., 1979). Possible mediators of increased cell-cell adhesions are N-cadherin and N-CAM; both of which are up regulated at the time of DRG condensation (Akitaya and Bronner-Fraser, 1992; Duband et al., 1988; Nakagawa and Takeichi, 1995; Thiery et al., 1982b). Thus, in a similar manner, prolonged aggregation of cells expressing EphA4/EGFP might facilitate neuronal differentiation despite the localisation of these cells at the edge of the neural tube. Apart from the observation that aggregation might indirectly stimulate differentiation (Newgreen et al., 1979), an additional possibility is that the prolonged exposure of these cells to neural tube-derived trophic factors might also promote neuronal differentiation, mimicking the *in vivo* environment. Thus, the combination of persistent tight cell-cell adhesion facilitated by EphA4 and the proximity of EphA4/EGFP-expressing clumps to neural tube-derived trophic factors might lead to differentiation (Gumbiner, 1996; Le Douarin and Kalcheim, 1999). In sum, these data lend support to the hypothesis that in the neuroepithelium and possibly the epithelioid layers, EphA4 might be acting in concert with cadherin-based cell-cell adhesion signals to negatively regulate EMT.

5.3.3 A possible role for EphA4 in cross-modulation of cell adhesive and cytoskeletal changes during EMT

In kiEphA4/EGFP-expressing NT^{pm} cultures, a more extensive mesenchymal layer was observed than in control cultures, suggesting that in the absence of kinase-dependent EphA4 signalling, EMT occurred at a more rapid rate. These data concurred with what was observed *in vivo* (refer Chapter 4). Combining these observations with the enhanced flattening and spread appearance of kiEphA4/EGFP-expressing cells, a likely possible interpretation is that cell-substratum contacts were enhanced in these cells. A key stimulus regulating the timing of neuroepithelial cell EMT is the acquisition of integrin-mediated substrate attachment mechanisms (Delannet and Duband, 1992). There are a number of possible mechanisms whereby EphA4 might directly influence the process of integrin-mediated adhesion in NC cell precursors. As previously described (refer Section 4.3.5), kiEphA4/EGFP expression acts to directly inhibit kinase-dependent EphA4-mediated signalling whilst stimulating signalling

pathways downstream of ephrin-A5. EphA receptors negatively regulate substrate attachment through the integrin-linking kinase, FAK (Kullander and Klein, 2002; Miao et al., 2000), whilst ephrin-A signalling promotes integrin-mediated adhesion through the Src family of tyrosine kinases (Gauthier and Robbins, 2003)(Figure 5.12). Thus, it could be imagined that a dynamic balance between EphA4 and ephrin-A activity in neuroepithelial cells during EMT, might directly regulate the maturity of integrin-receptor sites and the subsequent exit of NC cell precursors from the neural tube. In cells expressing kiEphA4/EGFP, this balance would be pushed towards ephrin-A signalling pathways and subsequently strong integrin-mediated attachment. It has been shown that expression of ECM receptors is directly regulated by TGF-β family members, which are key inducing factors during EMT (Delannet and Duband, 1992). It would therefore be interesting to determine if ephrin-A expression was also regulated by TGF- β; if this were the case, it would implicate ephrin-A activation in a novel mechanism to facilitate substrate competency in neuroepithelial cells during EMT. To test the requirement of ephrin-A activation in this pathway, ephrin-A knockdown studies using anti-sense morpholinos or RNAi would have to be conducted *in vivo* (Krull, 2004; Pekarik et al., 2003).

An additional observation that might shed light on the mechanism of EphA4 activity during EMT is that kiEphA4/EGFP-positive cells displayed intense bundles of circumferential F-actin and in the epithelioid zone; this was often strongest at the cell edge in contact with the substratum (Figure 5.10). In addition to increased F-actin deposits at the site of substratum attachment, EGFP was also deposited at points of contact along the cell surface. Whilst this doesn't necessarily represent the distribution of EphA4 (due to the IRES sequence), this might reflect a push towards the localisation of signalling mediators at the cell surface near sites of active engagement of ECM receptors. A similar phenomenon of enhanced actin at the cortical zone in contact with the substrate has been reported in NT^{pm} cultures treated with the protein kinase inhibitor, staurosporine; and was thought to be a means of acquiring tensile strength (Newgreen and Minichiello, 1996). Whilst the morphological effects and also enhancement of EMT in NT^{pm} cultures in kiEphA4 explants was also reported for staurosporine-treated explants, direct tests of the ECM adhesivity of staurosporine-treated cells demonstrated that these effects were not caused by increased cell-ECM attachment. Without a direct test for cell-ECM attachment in kiEphA4/EGFP-expressing cells, it cannot be asserted whether this is the case. In the case of staurosporine treatment the primary mode of action was thought to be on the actin cytoskeleton and cytoskeletal associated proteins such as vinculin, paxillin and talin (Newgreen and Minichiello, 1996). Thus, an alternative explanation for the morphology

and rapid acquisition of motile characteristics in kiEphA4/EGFP-expressing cells might be that EphA4 is directly influencing redistribution of the cytoskeleton during EMT.

During EMT, a consequence of changes in the F-actin cytoskeleton is that N-cadherin complexes in adherens adhesions at the cell perimeter disperse and a subsequent reduction in cell-cell adhesion is observed, leading to a tight cross-regulation of EMT (Newgreen and Minichiello, 1996). In EMT induced by protein kinase inhibitors, changes in cell morphology precede reduction in cell-cell adhesions, (and the reverse occurs in spontaneous EMT) (Brennan et al., 1999; Monier-Gavelle and Duband, 1995; Newgreen and Minichiello, 1996). A common feature of kiEphA4/EGFP-expressing cells in the epithelioid layer was that they formed organised networks of flattened cells indicative of increased cohesion (Figure 5.8 and 5.10). In addition, a preliminary analysis of β-catenin distribution in kiEphA4/EGFP-mediated EMT suggested that its localisation at cell-cell junctions might also have been retained in kiEphA4/EGFP-expressing cells in the epithelioid layer (Figure 5.10). Thus, these data suggest that the normal sequence of events leading to EMT might have been altered in kiEphA4/EGFP-expressing cells to mimic that induced by staurosporine treatment. If kiEphA4/EGFP expression was acting in a similar way to staurosporine, it might promote EMT by stimulating cytoskeletal change rather than by directly and specifically affecting cell-cell or cell-substratum adhesion mechanisms.

5.3.4 EphA4/EGFP-expressing cells appear unable to form the epithelioid cell state: insights into EphA4-mediated EMT

An additional consequence of EphA4 over-expression (to increased cell-cell adhesion as discussed in Section 5.3.2) was that EphA4/EGFP-positive cells in the epithelioid layer appeared unable to form the characteristic epithelioid cell type. Although these cells had abnormal distribution of F-actin at cell-cell junctions at all levels of the NT^m cultures (Figure 5.8, 5.9 and 5.10), the virtual lack of a discernible epithelioid layer was a consistent effect. Instead of forming an epithelioid state, cells adopted a mesenchymal morphology, displaying fine F-actin fibres at their cell perimeters. In addition, cells showed a reduction in both cytoplasmic β-catenin and also distribution at cell-cell junctions consistent with the conversion to a mesenchymal cell type (Newgreen and Minichiello, 1995). Despite their apparent loss of cell-cell adhesion complexes at the cell surface, these cells still remained deep in the layer normally occupied by epithelioid cells, indicating that whilst they had acquired some mesenchymal characteristics, they had not successfully progressed through

EMT. In addition, a common phenomenon observed at the edge of the neuroepithelium of EphA4/EGFP-expressing cultures was that EphA4/EGFP-expressing cells had high HNK1 immunoreactivity (Figure 5.2), providing further evidence to suggest that these cells had completed EMT. A possible explanation for these effects is that prolonged inhibition of F-actin (mediated by EphA4) might promote entry of EphA4/EGFP-expressing cells into a default low F-actin, high G-actin state, diffusing N-cadherin/ β-catenin complexes at the cell surface and subsequently promoting the change to a mesenchymal state (Newgreen and Minichiello, 1996). It leaves open for further examination why these cells could not then migrate away from the neural tube, although perhaps their physical location in the dense epithelioid layer might have impeded further movement.

An additional observation in EphA4/EGFP-expressing cells in the epithelioid layer was that they were characterised by a diffuse "halo" of β-catenin immunoreactivity. In contrast, in both control and kiEphA4/EGFP-expressing cells in the epithelioid layer, there was a general reduction of cytoplasmic β-catenin coincident with their localisation away from the neural tube and a focussing of staining into the nuclear region (Figure 5.10). It has been proposed that nuclear localisation of β-catenin for a brief period during EMT is critical to NC cell delamination (de Melker et al., 2004). Certainly, in the canonical Wnt signalling pathway, a key regulatory target of the Tcf/LEF pathway is Slug, one of the first known markers for EMT in NC cells (Nieto et al., 1994). Thus, activation of the β-catenin-dependent pathway is associated with EMT in NC cells and also in promoting tumourigenesis (Savagner, 2001; Yanfeng et al., 2003). Whether the lack of nuclear-localised β-catenin in the epithelioid layer of EphA4/EGFP-expressing cultures reflected that these cells did not undergo successful F-actin re-arrangement to become "epithelioid" or that this lack of β-catenin signalling has some functional significance for the molecular mechanisms regulating EphA4 signalling is unknown. However, given that activation of the Tcf/LEF-responsive gene Slug requires nuclear-localised β-catenin and Slug directly represses E-cadherin expression (Conacci-Sorrell et al., 2003)(Figure 5.12), it could be imagined that inhibition of this pathway in NC cells during delamination would prohibit EMT. In sum, these data provide further evidence that EphA4 over-expression in neuroepithelial cells inhibited the formation of F-actin stress fibre bundles, possibly also affecting the nuclear localisation of β-catenin required for successful EMT.

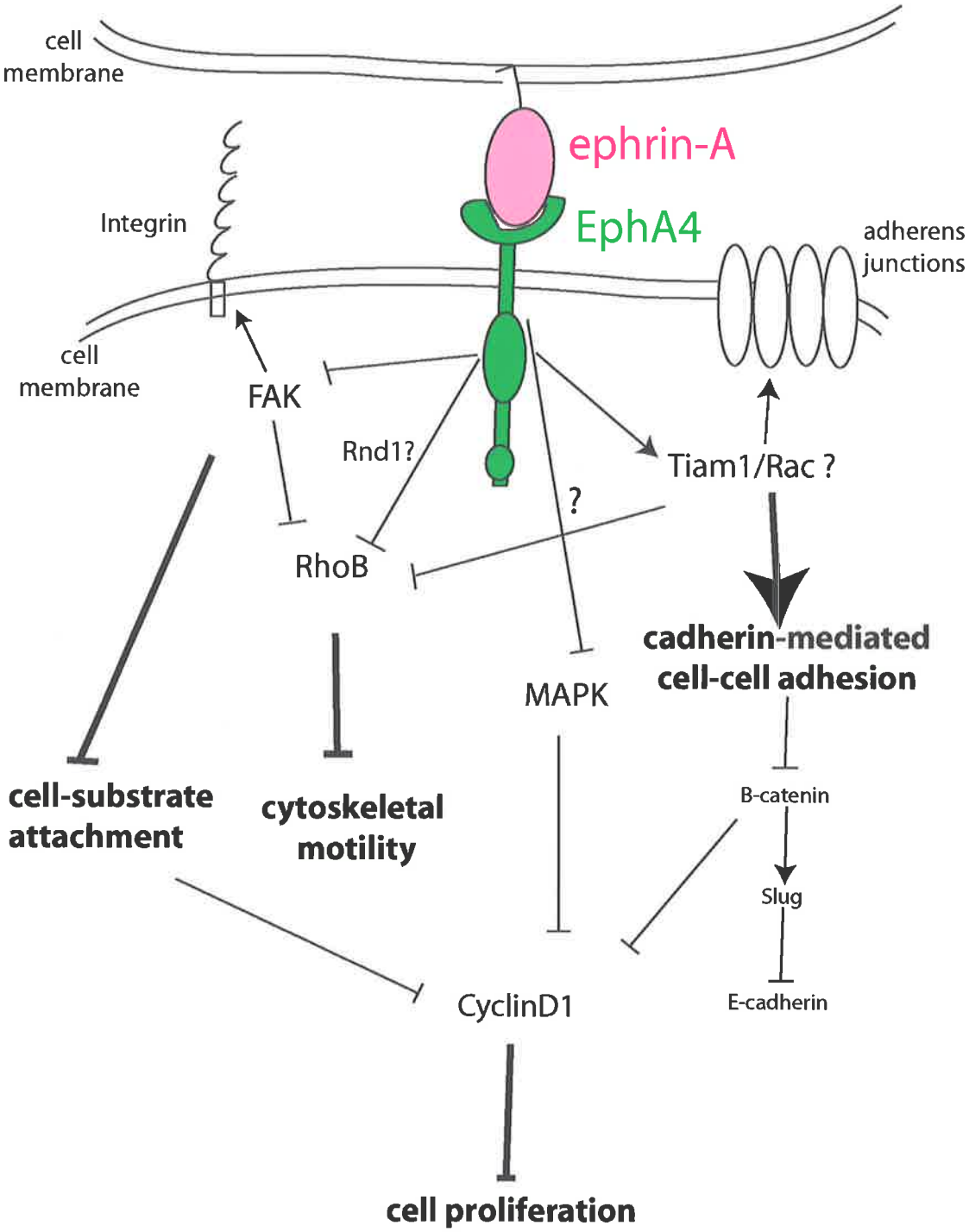
Cell density has been shown to be a negative regulator of β-catenin localisation in the nucleus and subsequent EMT (Conacci-Sorrell et al., 2003). Given that EphA4 mis-expression also

affected cell density and EMT; these observations might provide an additional possible clue to the cellular basis of EphA4-mediated effects. It was shown that when SW480 cells were plated at high cell density, there was an increased localisation of E-cadherin/β-catenin complexes at cell-cell junctions and a concomitant reduction in nuclear localised β-catenin. This reduction lead to reduced Slug expression and also increased E-cadherin expression (summarised in Figure 5.12). Conversely, cultures plated at low density exhibited a strong deposition of nuclear-localised β-catenin and a subsequent increase in Slug expression/activation of ERK, both of which lead to reduced E-cadherin expression (Conacci-Sorrell et al., 2003). Without analysing the expression of Slug and N-cadherin in EphA4/EGFP-expressing cells, it is impossible to determine whether alterations in EphA4 activity caused similar effects. However, EphA4 over-expression yielded both an increase in cell density at the edge of the neuroepithelium as well as reduced nuclear localisation of β-catenin during EMT, suggesting that excess activation might inhibit β-catenin signalling via activation of E-cadherin-mediated junctions. Alternatively, EphA receptors have been shown to negatively regulate integrin-dependent substrate attachment through FAK (Miao et al., 2000). In colon cancer cells a direct consequence of reduced substrate attachment is the inhibition of β-catenin signalling and subsequent reduction of Slug expression/enhancement of E-cadherin (Tan et al., 2001). Finally, in densely packed cells, where β-catenin signalling was reduced, a significant reduction in the number of cells in S phase was observed (Conacci-Sorrell et al., 2003). These effects are consistent with the putative effects of kiEphA4 on promoting entry into S phase (discussed in Section 5.3.1), a step that is critical for NC cell delamination. Further tests would be required to consolidate this hypothesis such as analysing the expression of Slug and N-cadherin, as well as the number of cells in S phase analysed in both EphA4/EGFP and kiEphA4/EGFP-expressing cells.

Figure 5.12. Schematic diagram illustrating the possible intracellular mediators through which EphA4 might negatively regulate EMT in NC cells.

EphA4 (green) interacts with ephrin-A proteins (pink) on the cell surface of delaminating NC cells. EphA4, via interactions with FAK, could negatively regulate cell-substratum attachment, which might also indirectly inhibit RhoB activity. In addition, through interactions with Rnd1 or Tiam1/Rac, EphA4 could also inhibit RhoB-mediated stress fibre formation (Malliri and Collard, 2003; Nobes et al., 1998). More specifically, EphA4 interactions with Tiam1 could promote epithelial characteristics including cadherin-mediated adherens junctions (Malliri et al., 2004). Based on studies correlating increased cadherin-mediated cell-cell adhesions and changes in β-catenin/Slug activity/proliferative activity (Conacci-Sorrell et al., 2003); EphA4 might then indirectly inhibit β-catenin localisation to the nucleus and consequently the expression of Slug as well as cyclinD1 (Burstyn-Cohen et al., 2004; Conacci-Sorrell et al., 2003). An additional downstream consequence of reduced Slug expression is that E-cadherin (or N-cadherin in the case of delaminating NC cells) is no longer inhibited and its expression/activity maintained. Thus, the ultimate cellular response to EphA4 over-expression (and the reverse is true for kiEphA4) could be inhibition of cell-substrate attachment mechanisms, increased cell-cell adhesion and perhaps reduced RhoB activity, leading to a negative regulation of the cellular changes required for EMT. In addition, EphA4, indirectly or through MAPK inhibition, might also negate EMT by inhibiting the entry of delaminating NC cells into S phase.

Model adapted from (Conacci-Sorrell et al., 2003).


cell
membrane
ephrin-A
EphA4
Integrin
adherens
junctions
cell
membrane
FAK
Rnd1?
?
Tiam1/Rac ?
RhoB
cadherin-mediated
cell-cell adhesion
MAPK
cell-substrate
attachment
cytoskeletal
motility
B-catenin
Slug
E-cadherin
CyclinD1
cell proliferation

5.3.5 Possible intracellular mediators of EphA4 effects on stress fibre morphology during EMT

One of the possible mechanisms to explain increased motility and cell rounding mediated by kiEphA4 and EphA4, respectively *in vivo* was that EphA4 might be inhibiting RhoB during EMT (refer Section 4.3.2). The results from both EphA4 over-expression and kiEphA4 analyses *in vitro* concur with this hypothesis on a number of levels. Firstly, apart from the observed inhibition of delamination, the cellular effects of EphA4 over-expression mimicked that of Rho inhibition *in vitro*. In the presence of the Rho inhibitor, C3 transferase, NC cell precursors exhibited a F-actin morphology similar to EphA4/EGFP-expressing cells; both leading to an inability of NC cell precursors to generate the F-actin stress fibres required for successful EMT (Liu and Jessell, 1998). In contrast, in the absence of EphA4 kinase-dependent signalling, kiEphA4/EGFP-expressing cells displayed thickened F-actin stress fibre bundles, indicative of enhanced recruitment of F-actin at the cell perimeter. Whether this reflects enhanced ECM interactions in kiEphA4/EGFP-expressing cells has been previously discussed (refer Section 5.3.3).

If the net effect of EphA4 over-expression was to inhibit RhoB function, the question remains as to how EphA4 might mediate such an effect on RhoB activity. In retinal ganglion cell axons, EphA4 has been shown to stimulate RhoA through the RhoGEF, ephexin, leading to growth cone collapse (Shamah et al., 2001). However, in an example analogous to the effects of EphA4 during EMT, where EphA4 inhibits Rho activity, EphA4 is thought to act through an Ephexin-independent pathway (Winning et al., 2002). In this paper, the authors suggested that EphA4 might act to inhibit Rho signalling by promoting the activity of Rnd1, an additional member of the Rho GTPase family (Nobes et al., 1998). In cell culture, Rnd1 over-expression inhibits stress fibre formation and substrate adhesion leading to cell rounding. Furthermore, in epithelial cells, Rnd1 localises to adherens junctions and although the effect on cadherins junctions was not analysed directly, cell-cell junctions were resistant to Rnd1-mediated promotion of actin disassembly (Nobes et al., 1998). Interestingly, in the gastrulating *Xenopus* embryo, over-expression of XRnd1 phenocopied the cell dissociation effects associated with EphA4 over-expression (Wunnenberg-Stapleton et al., 1999). Furthermore, Rnd1 is a RhoA antagonist, suggesting that Rnd1 might be a critical link between EphA4 an inhibition of Rho activity (Winning et al., 2002) (Figure 5.12)

An alternative possible mediator of the effects of EphA4 on NC cell EMT is the RacGEF, Tiam1 that has been shown to promote cadherin-dependent cell-cell adhesion and cell motility in epithelial cells (Malliri and Collard, 2003; Malliri et al., 2004; Uhlenbrock et al., 2004). Although Tiam1 has not previously been implicated in NC cell EMT, over-expression of this protein (leading to constitutive Rac activity) induces an epithelial morphology in melanoma cells whilst mice lacking Tiam1 showed increased malignancy, suggesting that in the absence of Tiam1 malignant conversion was enhanced (Malliri and Collard, 2003; Uhlenbrock et al., 2004). In epithelial cell lines, loss of Tiam1 activity resulted in a significant flattening of cells and promotion of mesenchymal morphology. Moreover, this effect was shown to be dependent on cadherin-based cell-cell junctions, directly implicating cadherins along with Tiam1 and subsequent Rac activation in the formation of cadherin-based epithelial cell junctions (Malliri et al., 2004). Finally, Tiam1/Rac and Rho regulate EMT by exerting opposite effects on cellular processes. Thus it could be imagined that EphA4 positively influencing Rac activity might lead to inhibition of Rho and a subsequent inhibition of EMT. Evidence for a specific interaction between EphA4 and Tiam1 stems from studies showing that EphA2 can promote neurite extension by activation of Tiam1 (Tanaka et al., 2004). In addition, EphA2-dependent stimulation of Tiam1/Rac1 promoted neurite extension and also formation of lamellipodia providing a possible explanation for the observed neuronal-like processes in EphA4/EGFP-expressing cells (Tanaka et al., 2004). Thus, although neither Tiam1 nor Rac have been previously specifically implicated in NC cell EMT, it could be speculated that EphA4 might regulate the balance between epithelial and mesenchymal states by promoting Rac1 activity and subsequently inhibiting Rho.

5.4 Conclusions

This data presents the first evidence implicating an Eph molecule specifically in EMT of NC cells, in addition to their known critical functions in other fundamental processes of tissue morphogenesis (reviewed by (Klein, 2004)). Furthermore, the actions of EphA4 in neuroepithelial cells appears to promote cell-cell adhesion and the formation of cadherin-based complexes. A possible mediator of the adhesive effects of EphA4 is Tiam1, a Rac-specific GEF, as this has been shown to interact with EphA receptors in other systems (Tanaka et al., 2004). Alternatively, EphA4 might directly inhibit stress fibre formation and subsequent EMT, by activating the RhoA antagonist, Rnd1 (Nobes et al., 1998). As neither of these molecules has been specifically implicated in avian NC cell EMT, their expression patterns in delaminating NC cells would have to be analysed concurrently with EphA4. In addition, to confirm that the cytoskeleton is the principal target of EphA4-based inhibition of EMT, the Rho protein inhibitor, C3 transferase, could be used to "rescue" the effects of kiEphA4/EGFP on NC cell motility. More information about how EphA4 signalling influences the cellular machinery during EMT might be gleaned from the analysis of the distribution and phosphorylation of focal adhesion-related proteins (eg. integrins, paxillin, vinculin, FAK, ILK) as well as the phosphorylation and localisation of cell-cell adhesion-related intracellular mediators such as catenins in conjunction with N-cadherin. Finally, an alternative (and possibly indirect function of EphA4) might also be to regulate the transition of neuroepithelial cells into S phase; an event known to be critical for EMT (Burstyn-Cohen and Kalcheim, 2002; Burstyn-Cohen et al., 2004). In sum, these data suggest that EphA4 plays a negative regulatory role in the progression of cells from a neuroepithelial to mesenchymal state, possibly explaining its low expression level on NC cells at the time of delamination.

Chapter 6 – Concluding Remarks

The Eph/ephrin family of molecules have been shown to contribute to multiple morphogenetic processes during development including boundary formation, axon guidance and cell migration, and angiogenesis/ vasculogenesis (Holder et al., 2000; Kullander and Klein, 2002; Wilkinson, 2000). A patterning event that is fundamental for the metameric arrangement of the PNS is the exclusive migration of NC cells through the rostral half-somite and their avoidance of the caudal-half (Keynes and Stern, 1984; Rickmann et al., 1985; Stern et al., 1991). In particular, interactions between EphB receptors, expressed on NC cells and ephrin-B ligands, expressed in the caudal half-somite, have been implicated in restricting NC cell migration to the rostral half-somite (Krull, 2001; Krull et al., 1995; Wang and Anderson, 1997). The role of the rostral half-somite in coordinating the outgrowth of NC cells to the periphery is less well documented, although a number of ECM molecules have a dynamic expression with respect to NC cell/somite morphogenesis and are likely to contribute to NC cell guidance through this tissue (Perris, 1997). At the outset of this research the only known EphA molecule expressed in the somite during PNS segmentation was EphA7, present in the caudal half-somite (Araujo and Nieto, 1997). So the initial aim of this research was to further characterise the distribution of Eph/ephrin-A molecules during trunk PNS development.

6.1 Summary of results

Using ligand-Fc and receptor-Fc fusion proteins to identify the overall distribution of EphA and ephrin-A proteins, respectively, during PNS segmentation, EphA/ephrin-A proteins appeared to compartmentalise specific regions within the rostral half-somite. Further analyses using *in situ* hybridisation and chick-specific antibodies to EphA4 and ephrin-A5, revealed that whilst ephrin-A5 was expressed on NC cells from delamination through to their colonisation of the DRG, EphA4 exhibited a highly dynamic expression pattern coincident with NC cell morphogenesis. Initially, during the early stages of the ventral migration of NC cells, EphA4 was associated with NC cells as well as cells of the rostral half-somite in close proximity to these traversing NC. Later, once the DRG began to coalesce, EphA4 became restricted to NC cell avoidance zones within the rostral half-sclerotome.

To test the significance of the dynamic expression patterns of EphA4 in regulating NC cell guidance, EphA4 was over-expressed using *in ovo* electroporation in dorsal neuroepithelial cells and subsequently, on NC cells during their migration through the rostral half-sclerotome. Over-expression of EphA4 yielded a marked reduction in NC cell migration and a subsequent inhibition of NC cell segmentation. Analysis of the effects of EphA4 over-expression in the dorsal neural tube, suggested that increased EphA4 representation on NC cells also inhibited NC cell delamination as measured by HNK1 staining. In addition, EphA4/EGFP-expressing regions in the neuroepithelium coincided with increased N-cadherin staining and reduced basal lamina breakdown suggesting that EphA4 promoted cell-cell adhesion in the neural tube. Whilst over-expression studies are useful tools to determine if a protein is sufficient for a particular developmental event, a kinase-inactive EphA4/EGFP construct (kiEphA4/EGFP) was utilised to determine if EphA4 was required for NC cell EMT/migration. Expression of this construct promoted uncoordinated NC cell migration through both rostral and caudal sclerotome-halves in the mid-trunk regions of the neural tube and premature NC cell emigration at caudal levels. Analysis of neural tube morphology and HNK1 distribution suggested that kiEphA4 led to premature NC emigration possibly through enhanced motility or substrate attachment mechanisms.

Based on these functional analyses and the low level of EphA4 expression on NC cells compared to that for ephrin-A proteins, a model was proposed to explain a putative *in vivo* role for EphA4 in NC cell development. The model was underpinned by the observation that EphA4 is expressed on NC cells at very low levels, whilst ephrin-A proteins are distributed in high concentrations. The low expression level of EphA4 correlated well with its negative influence on EMT and implied that *in vivo*, these negative effects might be counteracted by ephrin-A-dependent signalling pathways. Thus, in the normal situation, the balance between EphA4 and ephrin-A-mediated signalling would be shifted towards ephrin-A in NC cell precursors whilst EphA4 would provide a critical "brake" to prevent their premature entry into EMT.

To explore the cellular basis of EphA4 function in NC cell EMT, an *in vitro* model was employed that involved *in ovo* electroporation and subsequent seeding of neural tube explants on FN. These experiments were carried out at two different levels (NT^{m} and NT^{pm}) to enable a better assessment of the axial level-dependent effects of EphA4 *in vivo*. As per *in vivo*, EphA4 had inhibitory effects on NC cell delamination at both levels of the axis. In contrast, expression of kiEphA4 had distinct effects depending on the axial level analysed. In NT^{m}

level explants, the loss of EphA4 activity reduced the density of NC cell outgrowths, promoting uncoordinated migration. At NT^{pm} levels, preliminary evidence suggested that kiEphA4/EGFP expression increased the number of mesenchymal cells generated during EMT. A possible explanation for these effects might be that EphA4 regulates NC cell mitoses during delamination. In terms of the possible cellular basis of EphA4 activity during EMT, three key observations were made: (1) EphA4 affected the formation of F-actin stress fibres during EMT. Thus, in the absence of EphA4 activity, cells were characterised by thickened F-actin bundles. In cells with excess EphA4, circumferential F-actin was clearly disrupted; (2) EphA4 may act to negatively regulate cell-substratum adhesion during EMT as cells lacking EphA4 activity showed a morphology indicative of enhanced substratum adhesions; and (3) in the over-expression experiments, cells expressing EphA4/EGFP appeared unable to detach from the neuroepithelium and were characterised by intense deposits of F-actin and β-catenin at cell-cell junctions, suggestive of tight cell-cell adhesion. Given the distribution of β-catenin in these junctions, combined with evidence showing increased N-cadherin distribution in EphA4/EGFP-expressing regions in the neural tube *in vivo*, these data suggested that EphA4 might positively regulate the formation of adherens junctions, the specialised cadherin-mediated junction required for maintenance of epithelial cell contacts. These data are discussed with regards to how EphA4 might fit into the currently known cascade of events regulating NC cell EMT.

6.2 Putative targets for EphA4 signalling activity during EMT/NC cell migration

EMT occurs as a result of inductive interactions between the neural plate and epidermis, a process that is regulated by three main families of growth factors, namely BMPs, Wnts, and FGFs (Garcia-Castro et al., 2002; Ikeya et al., 1997; LaBonne and Bronner-Fraser, 1998; Liem et al., 1995; Monsoro-Burq et al., 2003; Selleck and Bronner-Fraser, 1995). Following growth factor-mediated induction, neuroepithelial cells undergo changes in gene expression mediated by a number of transcription factors, the first of which is thought to be Slug (Nieto et al., 1994). This is followed by the appearance of a number of other transcription factors including FoxD3 (Dottori et al., 2001; Kos et al., 2001), and the SoxE subgroup of HMG-box genes (Cheung and Briscoe, 2003)(McKeown et al., personal communication, 2003) as well as the adhesion molecule, cad6b, and the cytoskeletal mediator RhoB (Cheung and Briscoe, 2003; Liu and Jessell, 1998). In addition, the growth factor BMP4 is also expressed in dorsal

neuroepithelial cells and is thought to facilitate NC cell EMT as well as NC cell induction at the boundary between neural plate and epidermis (Sela-Donenfeld and Kalcheim, 1999). Slug expression is maintained in emigrating NC cells at cranial but not trunk levels (del Barrio and Nieto, 2002). Following emigration cad7 is expressed in a specific population of migrating NC cells and migratory avian NC cells express the HNK1 epitope, signifying their successful progression through EMT (Nakagawa and Takeichi, 1995; Newgreen et al., 1990).

6.2.1. A role for EphA4 in cross-modulating cytoskeletal and adhesive changes during EMT

The analyses investigating EphA4 function during EMT *in vitro*, focussed on the cell and behavioural consequences of altered EphA4 activity in delaminating NC cell precursors. This makes it difficult to determine where exactly EphA4 might fit in the molecular cascade for EMT. Further analyses would have to be conducted to profile the expression of such molecular markers as SoxE, cad6b, RhoB, and Pax3, in the presence of excess EphA4 and then conversely, in NC cells with reduced EphA4 signalling. However, unlike the effects of over-expression of EMT-promoting transcription factors such as Sox9/10 (McKeown S, personal communication, 2003), reduced EphA4 activity promoted cell motility without always leading to a direct increase in HNK1 immunoreactivity. This observation along with the analyses on circumferential F-actin distribution during EMT, implied that a primary consequence of reduced EphA4 activity was to stimulate the cytoskeletal/adhesive changes required for EMT, whilst only indirectly influencing differentiation. The suggested mechanism through which EphA4 could exert a negative regulation of cytoskeletal change during EMT was through inhibition of RhoB, the only known Rho GTPase previously implicated in NC cell EMT (Liu and Jessell, 1998). Thus, in the absence of EphA4, regulated changes in the actin network would be de-regulated, due to a relief on the EphA4-mediated inhibition of Rho, resulting in prominent stress fibre formation and the premature entry of neuroepithelial cells into EMT. Possible signalling mediators of such an effect include Tiam1, the RacGEF that has been shown to interact specifically with EphA4 in neurons (Tanaka et al., 2004), as well as Rnd1, the RhoA antagonist (Nobes et al., 1998)(Figure 5.12). In the analyses investigating the effects of over-expression of SoxE transcription factors, ectopic EMT occurred with a concomitant decrease in RhoB expression (Cheung and Briscoe, 2003; Dottori et al., 2001) (McKeown et al., personal communication, 2003). Thus, it would be interesting to determine RhoB expression in kiEphA4/EGFP-expressing cells. To test that

EphA4 effects during EMT were mediated specifically by interactions with RhoB, a constitutively active form of RhoB could be co-expressed with EphA4 in the neuroepithelium to "rescue" the EphA4 phenotype. Alternatively pre-migratory neuroepithelial cells could be treated with C3 transferase, the Rho protein inhibitor, to test for a reduction in the abnormally thick stress fibres and premature NC cell emigration associated with kiEphA4 expression in NT^{pm} cultures.

An additional morphology that was consistently observed in kiEphA4/EGFP-expressing cells was increased cell spreading and significant flattening, indicative of increased cell-substratum contacts. Previously, it was postulated that the shift towards ephrin-A-mediated signalling might facilitate neuroepithelial cell exit by increased ECM attachment. Whether this occurred indirectly via the enhanced re-organisation of circumferential F-actin and a subsequent recruitment of cytoskeletal associated proteins or more directly through increased ephrin-A-mediated activation of integrin receptors, or both, is unknown. However to further understand how the ECM responsiveness of these cells changed with reduced EphA4 activity, the distribution of focal adhesion complexes via vinculin, or integrin-cytoskeletal mediators such as integrin-linked kinase or FAK, must be analysed in kiEphA4/EGFP-expressing cells. In sum, these data suggest that the activation of ephrin-A-mediated signalling pathways during NC cell EMT orchestrate cytoskeletal and adhesive changes in NC cell precursors (Figure 6.1). It would be interesting to determine if ephrin-A-dependent signalling was directly regulated by NC inductive factors such as TGF-β, that also directly stimulate EMT (Nawshad et al., 2004) and in a similar manner to kiEphA4 expression, elicit premature NC cell emigration (Delannet and Duband, 1992).

6.3 Possible upstream regulators of EphA4 activity: a role for noggin/BMP4 signalling?

Whilst it is unknown exactly where EphA4 might fit into the genetic and molecular cascade leading to EMT, the observations implicating EphA4 in cytoskeletal re-arrangement and adhesive processes suggest that it is acting downstream of NC inductive factors during EMT. Of particular interest is the BMP family of proteins, as these have been implicated not only in inductive events but also in coordinating the timing of NC cell emigration along the rostro-caudal axis (Newgreen, 2005). The activity of BMP4 is regulated by the graded distribution of its inhibitor noggin that is expressed in a high-caudal to low-rostral gradient in the dorsal

neural tube, and their combined actions control NC delamination in a wave from rostral to caudal levels of the axis (refer 1.2.1; (Sela-Donenfeld and Kalcheim, 1999)). In addition, transplantation studies have suggested that noggin is expressed in the dermomyotomal lip of differentiated somites and, via a negative feedback loop, inhibits its own expression in the dorsal neural tube. Thus the expression of noggin in the dorsal region of differentiated somites provides a molecular correlate to explain the coordinate regulation of NC cell development with that of the somites (refer Section 1.2.1;(Sela-Donenfeld and Kalcheim, 2000)).

Evidence to support a role for EphA4 as a noggin effector molecule in the dorsal neural tube stems from the functional analyses examining a role for EphA4 in NC cell EMT/migration both *in vivo* and *in vitro*. EphA4 over-expression was functionally equivalent to the effect of noggin treatment on pre-migratory NC cells, inhibiting NC delamination (Sela-Donenfeld and Kalcheim, 1999). More specifically, the hypothesis that EphA4/ephrin-A interactions are specific downstream regulators of noggin signalling might also explain the axial level-dependent effects of EphA4 perturbation (refer Chapters 4 and 5). If EphA4 was expressed at neural tube levels adjacent to epithelial or dissociating somites, noggin expression in the dorsal neural tube would normally be starting to be downregulated and the amount of effective BMP4 protein increased (refer Figure 1.6). Thus, the persistent expression of EphA4 in regions where noggin was normally downregulated and BMP4 activity high would inhibit the normal process of NC delamination. Interestingly, over-expression of EphA4 in more anterior levels of the axis did not yield cell rounding, suggesting that excess EphA4 did not inhibit NC cell motility. RhoB, in addition to cad6b, have both been implicated as downstream effectors of BMP4 signalling and were both inhibited in the presence of ectopic noggin-producing cells (Sela-Donenfeld and Kalcheim, 1999). Thus, a possible explanation for the observed axial level-dependent effects of EphA4 on NC cell motility is that by the time EphA4 protein was produced in these cells, RhoB had already been activated by BMP4 and was therefore no longer responsive to EphA4-mediated inhibition. These data place EphA4 downstream of noggin-mediated signals but upstream of RhoB and also possibly cad6b in the molecular cascade leading to EMT.

Figure 6.1. Schematic diagram summarising how EphA and EphB activation mediated by ephrin-A proteins provide a putative molecular switch that regulates NC cell EMT.

BMP4-mediated effector pathway

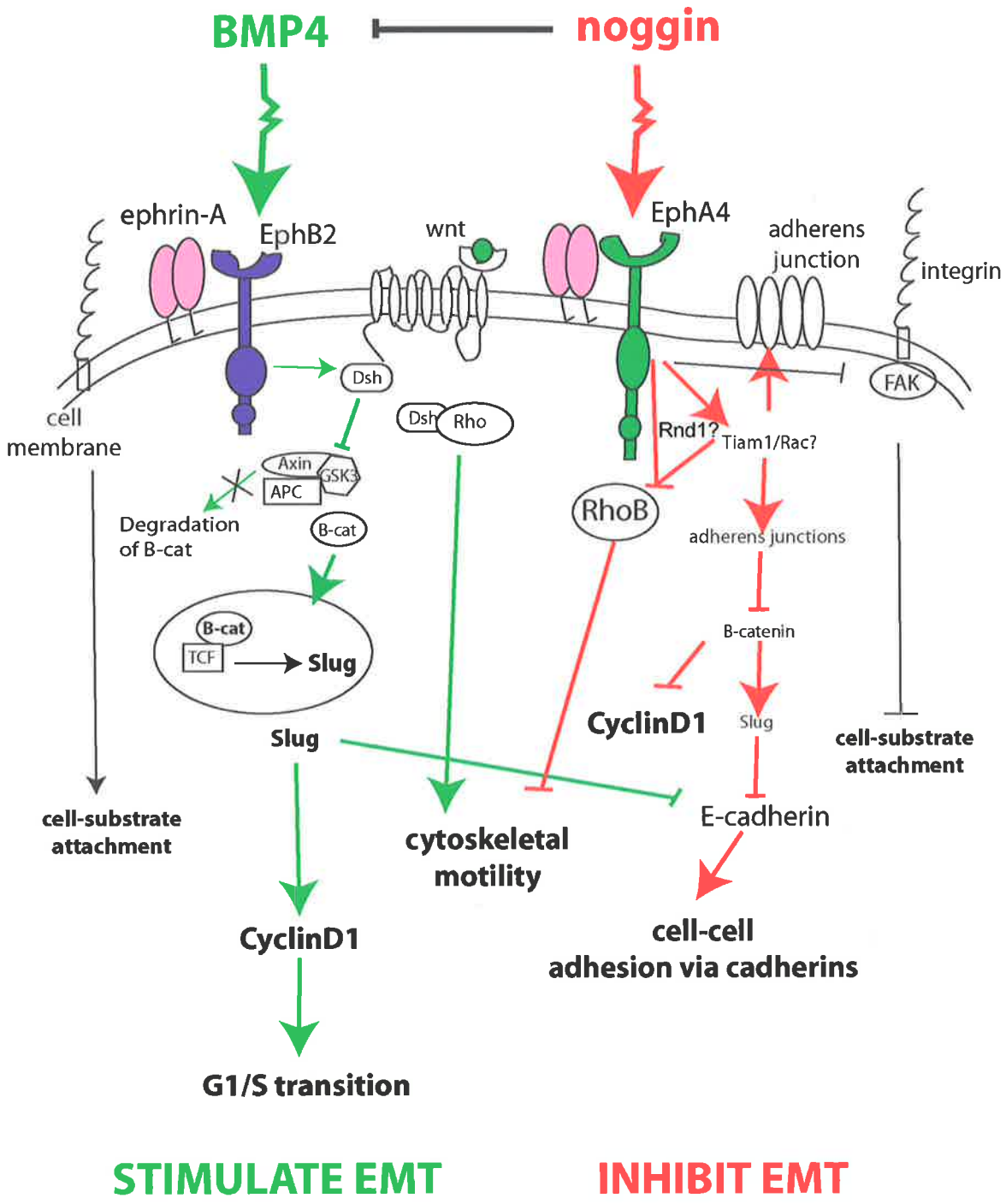
Activation of EphB2-mediated signalling pathways might directly promote EMT, acting downstream of BMP4-mediated signalling. EphB2 binds to Dishevelled (Dsh) that leads to an inhibition of the Axin/GSK3β/APC complex that is responsible for degradation of β-catenin. Thus activation of Dsh enables β-catenin to enter into the nucleus and promote expression of Slug, among other Tcf/LEF-responsive gene targets. This leads to increased cyclinD1 activity; G1/S transition and subsequent NC cell delamination. In addition, via Dsh interactions with Rho, EphB2-mediated activity might stimulate cytoskeletal motility. Activation of ephrin-A proteins in this pathway might also lead to increased substratum attachment.

Noggin-mediated effector pathway

Activation of EphA4 has effects that are functionally equivalent to activation of noggin-mediated pathways. Via interactions with Rho protein mediators, possibly Tiam1 or Rnd1, EphA4 inhibits Rho protein activity, whilst simultaneously activating cadherin-mediated adherens junctions. Based on cell studies examining the effect of cadherin-mediated junctions on EMT, EphA4 activation could then lead to reduced β-catenin, a loss of Slug stimulation, reduced cyclinD1 expression and increased E-cadherin leading to an overall increase in cell-cell adhesions with a concomitant reduction of Rho activity. In addition, EphA4 interactions with FAK, would reduce cell-substrate attachment in NC cells.

Schematic diagram adapted from (Burstyn-Cohen et al., 2004; Conacci-Sorrell et al., 2003; Malliri and Collard, 2003).

In NC cells...


BMP4
noggin
ephrin-A
EphB2
wnt
EphA4
adherens junction
integrin
Dsh
FAK
cell membrane
Dsh
Rho
Rnd1?
Tiam1/Rac?
Axin
GSK3
APC
RhoB
Degradation of B-cat
B-cat
adherens junctions
B-cat
TCF
Slug
B-catenin
CyclinD1
Slug
cell-substrate attachment
Slug
cell-substrate attachment
cytoskeletal motility
E-cadherin
CyclinD1
cell-cell adhesion via cadherins
G1/S transition
STIMULATE EMT
INHIBIT EMT

If excess EphA4 activity produced a comparable effect to that of ectopic noggin on NC delamination, then reduction of EphA4-mediated signalling would be expected to have an effect analogous to BMP4 treatment of NC cell precursors. Certainly, kiEphA4 expression at caudal levels led to premature NC cell emigration both *in vivo* and *in vitro*, similar to the effect of BMP4 on pre-migratory NC cells *in vitro* (Sela-Donenfeld and Kalcheim, 1999)(refer Chapters 4 and 5). Moreover, as with EphA4 over-expression, the effects of kiEphA4 on NC cell motility were axial level-dependent (refer Section 4.3.1). Thus, at mid-trunk levels, inhibition of EphA4 signalling in the dorsal neural tube promoted both motility and uncoordinated NC cell migration, whilst at anterior levels no significant disruption to NC cell migration patterns was observed. A possible explanation for the developmental window of effect is that at the time of kiEphA4 expression, noggin and BMP4 were switching from high to low and low to high expression levels, respectively. Thus, reducing EphA4 signalling in delaminating NC cells might have heightened their response to BMP4, so that they no longer responded to noggin/EphA4-mediated "brake" signals. This could lead to a rapid de-regulation of RhoB activity and enhanced substratum contact, as well as a decrease in cad6b expression.

At caudal levels, kiEphA4 expression in the dorsal neural tube led to premature NC cell emigration, similar to what was observed in BMP4-treated explants (Sela-Donenfeld and Kalcheim, 1999). Interestingly, at these levels, the increased delamination of NC cells was also accompanied by increased somite formation, suggesting that at this level the increased emigration of NC cells had an overall growth-promoting effect on somite development (Figures 4.9 and 4.10). Noggin and BMP4 are thought to coordinate somite development with NC cell emigration, though the source of positive regulation has been proposed to be generated by increased noggin expression in the somite (Sela-Donenfeld and Kalcheim, 2000). The idea that enhanced emigration of NC cells might promote somite formation has not previously been analysed (Sela-Donenfeld and Kalcheim, 2000). Perhaps at this level, reduction of effective noggin activity in the dorsal neural tube by expression of kiEphA4 might via a feedback mechanism, increase expression of noggin in the somites, and ultimately stimulate somite development. Noggin expression in the somites at this level would have to be examined to confirm this possibility. Alternatively, this morphogenetic change could be mediated by kiEphA4 on NC cells interacting with ephrin-B2 expressed on blood vessels lining the somitic furrows (Krull et al., 1997). EphA4 has been implicated in non cell-autonomously affecting somitic boundary formation in the pre-somitic mesoderm, making

this a feasible explanation for the effects (Barrios et al., 2003). In sum, these data suggest a putative novel mechanism whereby NC cells, either mediated by noggin/BMP4 signals or perhaps specifically reduced EphA4 signalling, might promote somite development.

Eph/ephrin-A interactions and Wnt signalling might coordinate to regulate NC cell emigration downstream of noggin/BMP4

Recently, it has been shown that the timed entry of NC cells into S phase is specifically regulated by BMP4-derived signals, and that this mitotic phase progression is crucial for delamination ability (Burstyn-Cohen et al., 2004). Moreover, by over-expression and dominant negative studies, a direct pathway between noggin/BMP4 activity and Wnt signalling has been established that regulates G1/S transition in delaminating NC cells in an axial level-dependent manner. Thus at mid-trunk regions, noggin in the developing somites downregulates its own expression, leading to increased BMP4 activity, activation of the canonical Wnt signalling pathway through Wnt 1 and a subsequent increase in cyclinD1, a critical mediator of G1/S transition (Burstyn-Cohen et al., 2004). Interestingly, inhibition of canonical Wnt signalling lead to a reduction in the expression of the dorsal neural tube markers, *Cad6B*, *Pax3*, and *Msx1* but not *RhoB*. These data implied that RhoB might occur in a separate, albeit BMP-dependent pathway for NC cell delamination (Burstyn-Cohen et al., 2004)(Figure 6.1). Thus, EphA4 might be acting in a parallel pathway to Wnt1-mediated signals to link noggin activity with that of RhoB. EphA4 might also indirectly inhibit canonical Wnt signalling through its effects on Rho proteins or alternatively via inhibition of MAPK pathways that directly regulate cyclinD1 (Kullander and Klein, 2002; Malliri and Collard, 2003).

An additional factor that must be considered in the Eph/ephrin-dependent regulation of NC cell development is that EphB3 is expressed on NC cells and, via interactions with ephrin-B1, assists in excluding NC cells from the caudal half-somite (Krull et al., 1997). More recently, EphB3 has been demonstrated to interact with ephrin-A5, providing an additional possible regulatory interaction in NC cells (Himanen et al., 2004). Thus, perhaps EphB3 also interacts with ephrin-A proteins on the surface of NC cells, possibly contributing to the stimulatory effects observed in NC cells when EphA4 signalling was reduced. In addition, EphB3 might provide a more specific link between the noggin/BMP4/Wnt-mediated pathway for NC cell delamination as it interacts with Dishevelled (Dsh), a key regulator of the canonical Wnt

pathway (Tanaka et al., 2003; Yanfeng et al., 2003). In EMT, Dsh inhibits the formation of the GSK3β degradation complex, which is responsible for degrading β-catenin in the cytoplasm. Thus, in the presence of Dsh, β-catenin accumulates in the cytoplasm and is able to enter into the nucleus (Malliri and Collard, 2003)(Figure 6.1). Furthermore, the association of EphB2 with Dsh also correlated with enhanced Rho and Rho Kinase activation, leading to significant cellular repulsion (Tanaka et al., 2003)(Figure 6.1). Thus it is possible that EphB3 expressed on NC cells might interact with Dsh to stimulate the canonical Wnt pathway and subsequent G1/S transition. Although β-catenin appeared to readily localise to the nuclear region during EMT in kiEphA4/EGFP-expressing cells, owing to the brief amount of time that this protein can be observed in the nuclear region (de Melker et al., 2004), it is difficult to gauge whether its activity was increased in these cells. A more critical test to determine whether EphB-mediated activation of canonical Wnt signalling was a key regulator of EMT, would be to co-express the dominant negative Dsh construct, Xdd1, with kiEphA4. Given that expression of Xdd1 in neural tube explants inhibited NC delamination in a cell-autonomous manner (Burstyn-Cohen et al., 2004), if EphB-mediated interactions with Dsh were important for kiEphA4 effects, Xdd1 co-expression with kiEphA4/EGFP would be expected to rescue premature emigration for kiEphA4/EGFP-expressing NT^{pm} explants. Alternatively, co-expression of β-catenin with kiEphA4 might be expected to have a synergistic effect on NC cell emigration.

An additional line of evidence supporting a putative role for EphB receptors in positively mediating NC cell motility and/or G1/S transition in NC delamination stems from time-lapse analyses describing the migratory profile of NC cells in the presence of ephrin-B1-Fc (an exogenous blocker of EphB/ephrin-B1 interactions). In these experiments, a distinct inhibitory effect on NC cell orientation and directionality was observed when NC cells were migrating on ephrin-B1-Fc compared to ephrin-B1-myc (Krull et al., 1997). Analyses of EphB receptor phosphorylation in cells exposed to ephrin-B1-Fc revealed that these exogenous proteins have agonist properties, whilst ephrin-B1-myc did not induce receptor phosphorylation (Krull et al., 1997). These data imply that EphB receptor phosphorylation might contribute specifically to the de-regulated migratory profile observed in kiEphA4/EGFP-expressing cells. To explain this in a mechanistic sense, in kiEphA4/EGFP-expressing cells, the activation of EphB receptors by ephrin-A ligands (and in the absence of EphA4 signalling) suggest that perhaps the crucial molecular switch might be between EphB3 and EphA4 receptor activation. Thus, in NC cells *in vivo*, rather than the regulation of NC cell delamination by EphA4/ephrin-A activity; the balance of Eph signalling activities might be

between EphB-mediated activation of Dsh and Rho pathways and EphA4-mediated inhibition of Rho (Figure 6.1).

To establish that EphA4 and ephrin-A/EphB proteins regulate the timed delamination of NC cells, possibly through activation of the canonical Wnt signalling pathway, a number of key issues would need to be resolved. Firstly, how does the expression of EphA4 in the dorsal neural tube relate to that of noggin/BMP4? EphA4 is expressed in a number of mesodermal tissues with a similar spatiotemporal distribution to noggin including in the dermomyotomal lip, medial edge of the lateral plate mesoderm and dorsal neural tube (McLennan and Krull, 2002; Nieto et al., 1992; Patel et al., 1996; Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000; Sela-Donenfeld and Kalcheim, 2002). It has been shown that EphA4 is expressed in the dorsal neuroepithelium during the early phases of NC cell migration, (Chapter 3), (McLennan and Krull, 2002); whether this is graded in a similar manner to noggin with increasing rostral locations requires further investigation. If EphA4 was acting downstream of noggin, its expression might mimic the switching of high noggin transcription in the dorsal neural tube to high expression in the dermomoyotomal lip. Thus, EphA4 would be expected to also be reduced in the dorsal neural tube coincident with its upregulation in the dorsal somitic regions (Eberhart et al., 2000; Iwamasa et al., 1999; Sela-Donenfeld and Kalcheim, 2000).

To confirm a functional synergy between EphA4 and noggin in regulating NC delamination, exogenous noggin could be applied to kiEphA4/EGFP-expressing NT^{pm} cultures and these cultures analysed for a reduction in precocious EMT. Conversely, the effects of EphA4 over-expression might be rescued by application of exogenous BMP4 to the culture medium. Finally, the effects of EphA4 on G1/S transition would have to be analysed; if EphA4 was directly involved in regulating G1/S transition, kiEphA4/EGFP-expressing cells in the neuroepithelium would be expected to promote the entry of cells into S phase in a cell autonomous manner, detectable by an increase in BrdU incorporation in these cells as they pass through S phase.

6.4 What can be learned from EphA4/ephrin-A transgenic mice studies?

To tease out the indiviual contributions of EphA4, ephrin-A and EphB3 signalling pathways in regulating NC cell development *in vivo*, genetic dissection studies in the mouse would be particularly useful. Mutational analyses and gene deletion studies have been highly informative in determining the specific contribution of EphA4 signalling to axon guidance systems (Dottori et al., 1998; Henkemeyer et al., 1996; Kullander et al., 2001a; Kullander et al., 2001b). To date no PNS segmentation defects have been reported for EphA4, ephrin-A5 or EphB2/B3 knockout mice (Dottori et al., 1998; Frisen et al., 1998; Henkemeyer et al., 1996; Kullander et al., 2001a; Compagni et al., 2003; Davy et al., 2004), though the ability of NC cells to replenish the migratory population and thus, recover from injury, make it easy to overlook any transient migratory NC defects (Boucaut et al., 1984). Furthermore, analysis of kiEphA4/EGFP-expressing avian embryos after 48 hours incubation indicated that these cells were able to colonise DRG structures, suggesting that aberrant migratory patterns of kiEphA4/EGFP-expressing cells are likely to produce a transient defect (data not shown). An additional factor to consider when analysing specific defects in mouse knockouts is that chick and mice often use different Eph/ephrin combinations to achieve developmentally similar effects. For example, in NC cell segmentation, ephrin-B1 is expressed in the caudal half-somite of the chick whilst ephrin-B2 appears to play the role of ephrin-B1 in rats (Krull et al., 1997; Wang and Anderson, 1997). Due to the wide expression patterns of Eph/ephrin proteins throughout embryogenesis, a common problem faced in analyses of Eph knockout mice is that these molecules display a high degree of functional redundancy (Adams et al., 1999). Recently a similar problem within the integrin family of adhesion molecules has been overcome using a conditional gene deletion of the β1 integrin sub-unit in migratory NC cells (Pietri et al., 2004). Thus, spatiotemporally specific elimination of particular genes combined with specific mutations would be a highly useful tool to dissect the key molecular contributions of EphA4, ephrin-A proteins and EphB proteins to NC cell development.

6.5 Conclusions

This thesis has involved the analysis of Eph/ephrin-A gene expression with respect to NC cell segmentation, identified EphA4 as a putative regulator of cell-cell communication during NC cell migration and more specifically, established that this protein plays a critical negative regulatory role in EMT. EphA4 appears to elicit its effects on NC cell EMT by promoting cell-cell adhesion and negatively regulating cytoskeletal conformations favouring motility. In addition, via an axial level-dependent mechanism, EphA4 might also regulate the timed entry of neuroepithelial cell precursors into S phase. The model presented in this chapter suggests that EphA4 and ephrin-A, possibly interacting with EphB receptors, comprise a molecular switch that regulates NC cell delamination. More specifically, it was hypothesised that EphA4 might be a downstream effector of noggin signalling, acting in the noggin/BMP4/Wnt pathways to regulate NC cell delamination. In sum, these data suggest that EphA4 interactions with ephrin-A proteins might provide a novel additional molecular control of cytoskeletal, cell-cell and cell-substratum adhesion changes during EMT. Multiple further analyses must be carried out to confirm the downstream targets and putative upstream regulators of EphA4 activity in NC cell EMT.

Appendices

Appendix 1.1 Summary of control EGFP-expressing embryos stained with HNK1

Phenotypic groups (EGFP summary):	
More advanced on EP'ed side	n = 4/ 14 (ventral; 3 cell autonomous)
Entry into caudal somite	n = 0/ 15
reduced HNK1	n = 1/14
rounded morphology	n = 0/16
Sc1 increased on EP'ed side	n = 0/2 (1 decrease)
DRG incorporation	n = 3/6 (inc 2 x 111103 48 hour embryo)
Somites?	n = 0/2 (can look further if need)

Appendix 1.2 Summary of EphA4-EGFP phenotypes *in vivo*

1.2.A Analysis of whole-mount EphA4/EGFP-expressing embryoswith HNK1

	Phenotypic analysis	**numbers**
m levels	HNK1 ball-up	n = 8/13 (including trans sections, n = 2)
	Rounded morphology	n =13/16
	A4 dorsal clumps	n = 13/16
	nt banding	n = 16/16
	DRG inc	n = 0/6
pm levels	reduced HNK1	n = 6/11
	delayed?	n = 7/11

1.2.B Delamination profiles in EphA4/EGFP and control EGFP-expressing embryos (transverse sections)

A4 Transverse sections					
	section name	Reduced delam (morph)	pial surface protrusions	clumped nt	reduced somite
6303	section a	yes		yes	
	section b	yes	yes	yes	
	section c	no	yes	yes	
27203	section a		yes	yes	
	section b		no	yes	
	section c	yes		yes	
	section d			yes	
24303/ HNK1	section a	no	yes	yes	
	section b	no	yes	yes	
	section d	yes	yes	yes	
	section u	no	no	yes	
	Section h	no	no	yes	
ACAM 61103	A4a20T	yes		n/a	
19203	section 1a	n/a		yes	yes
	section 1b	n/a		yes	yes
	section 1c	n/a		yes	n/a
	section 2c	n/a		yes	yes
	section 2d	n/a		yes	no
24303/Pax7	section a			yes	yes
	section c			yes	yes
	section e			yes	no
	section f			yes	no
14203	section b			n/a	yes
	section d			n/a	no
	section ua (upper)	no		yes	n/a
	section uc (upper)	no		yes	n/a
41203-ACAM	section b	n/a		yes	more
	section c	n/a		yes	more
	section d	yes		yes	yes
	section e	yes		yes	more
	section f	yes		yes	more
	section g	no-heaped up tho'		yes	equal
	section h	n/a		yes	yes
HNK1	A	yes		yes	more
	C	yes		yes	more
	D	yes		mild	equal

MES transverse sections					
	section name	Reduced delam	pial surface	clumped nt	reduced somite
6303	section a	no	no	no	
	section b	no	no	no	
	section c	no	no	no	
	section d	mild	no	no	
19203	Caxaco	no	no	no	
24303	section 1a	n/a	n/a	n/a	yes
	section 1b	n/a	n/a	n/a	more
	section 1c	n/a	no	no	yes
	section 1d	n/a	n/a	no	yes
	section 1e	n/a	n/a	n/a	
24303 #2	section 2a		no	n/a	more
	section 2b		no	n/a	equal
	section 2c		no	no	equal
	section 2d		no	no	equal
ACAM 61103	mesa20	mild	no	no	n/a
	mes20	n/a	possibly?	no	yes
41203	sectionA	no	no	no	less
	sectionB	no	no	no	less
	sectionC	no	no	no	equal

SUMMARY:

	delamination	reduced somite formation	clumped in nt
MES	n = 2/9 sections (22%)	n = 4/12 sections (33.3%)	n = 0/6 embryos
A4	n = 11/19 sections (58%)	n = 8/20 sections (40%)	n = 8/9 embryos

Appendix 1.3 – TUNEL-positive nuclei in EphA4/EGFP-expressing and control EGFP-expressing transverse sections

	EP	CONTROL
A4#1	52	43
A4#2	63	40
A4#3	35	19
A4#4	53	21
SUM	203	123
AVERAGE	**50.75**	**30.75**
StDEV	**11.61536**	**12.5**
SEM	**5.807682**	**6.25**
MES#2	31	27
MES#3	9	12
MES#4	37	37
SUM	77	76
AVERAGE	**25.66667**	**25.33333333**
StDEV	**14.74223**	**12.58305739**
SEM	**8.51143**	**7.264831573**

Averages		
	EP	CONTROL
EphA4/EGFP	50.75	30.75
EGFP	24.19551	20.91667
SEM		
	EP	CONTROL
EphA4/EGFP	5.807682	6.25
EGFP	8.51143	7.264832

t-test (A4)	0.057742
t-test (control)	0.977697

Appendix 1.4 Summary of kiEphA4/EGFP-expressing phenotypes

1.4.A Whole-mount embryos stained with HNK1

Phenotypic analysis:	
HNK1 normal-advanced	n = 7/10
Entry into caudal somite	n = 3/10
Increased MA's	n =4/6

Frequency of advanced ncc migration at caudal levels in kiEphA4 and control embryos			
	WM	**Trans**	**TOTAL frequency**
kiEphA4	n = 5	n = 1	6/6 (100%)
Controls	n = 1	n = 0	1/7 (14%)

1.4.B Transverse sections

kiEphA4 transverse sections				
		increased somite	nt width	width-unEP'ed
91203	section a	yes	1 1/4	1 1/2
	section b	yes	1 1/4	1 2/3
	section c	mild	1 1/2	slight inc
	section d	mild	1 1/4	1 3/4
	section e	yes	1 2/3	2 1/2
	HNKa	mild		slight inc
	HNKb	equal	slight inc	
	HNKc	equal		slight inc
	HNKd	mild		slight inc
	HNKe	equal	equal	
161103	Kda	n/a		more
	KDb	n/a	n/a	
	KDc	n/a		more
	KDd	n/a	equal	
	n = 3	**increased somite**	**nt width**	
		n = 7/10	**n = 10/13**	

MES transverse sections				
	section name	somite difference?	nt width	width unEP
24303	section 1a	yes		more
	section 1b	more	n/a	
	section 1c	yes	n/a	
	section 1d	yes	n/a	
	section 1e	yes	slight inc	
24303 #2	section 2a	more	equal	
	section 2b	equal	equal	
	section 2c	equal	equal	
	section 2d	equal	slight inc	
41203	sectionA	more		slight inc
	sectionB	more		slight inc
	sectionC	equal	equal	
		increased somite		**nt width**
	n = 3	**n = 4/12**		**n = 3/9**

Appendix 1.5 *In vitro* cell counts

1.5.A Proportion of GFP cells in outgrowth of control, EphA4 and kiEphA4 NT^{m} cultures

GFP cells in distal outgrowth (expression between 10-60% in Nep)			
	Control	A4	kiEphA4
	10.52632	0	20.89552
	14	4.444444	39.02439
	9.803922	3.636364	8.333333
	3.448276	5.940594	16.66667
	12.90323	8.333333	7.272727
	2.061856	0	0
	24.61538	11.5942	4.285714
	11.82796		27.5
SUM	89.18694	33.94894	123.9784
MEAN	**11.14837**	**4.849848**	**15.49729**
STDEV	6.939373	4.232605	13.16342
SEM	**2.453439**	**1.599774**	**4.653971**
		Mean	
	Control	11.14837	
	EphA4	4.849848	
	kiEphA4	15.49729	
Percentage expression in Nep			
	Control	A4	kiEphA4
	13.33333	13.33333	10
	16.07143	13.33333	13.33333
	21.66667	22.5	28
	26.66667	25	38.70968
	46.66667	31.66667	40
	50	60	41.66667
	53.33333	60	50
	56.66667		50
Sum	284.4048	225.8333	271.7097
Average	**35.5506**	**32.2619**	**33.96371**

1.5.B Number of cells in outgrowth of EphA4/EGFP-expressing NT^{m} cultures

Number of cells in distal outgrowth for EphA4			
	% Nep expression	Soxe	Phase
4cM2a	13.33333333	34	40
4aM2a	13.33333333	41	45
9cM2a (a)	22.5	44	55
4bM2a	25	89	101
4dM2a	31.66666667	48	48
9aM2a	60	26	36
9bM2c (a)	60	62	69
9eM2b (a)	80	37	37
9dM2 (a)	100	38	42
SUM	**405.8333333**		**473**
AVERAGE	**45.09259259**		**52.55556**
ST DEV			**20.87529**
SEM			**6.95843**
Number of cells in distal outgrowth for Controls			
	% Nep expression	Soxe	Phase
2dM2a	13.33333333	30	38
3cM2a	16.07142857	46	50
3bM2a	21.66666667	51	51
3dM2a	26.66666667	22	29
2bgrb2	46.66666667	29	31
12dM2	50	95	97
13aM2	53.33333333	192	195
13bM2	56.66666667	88	93
12aM2a	66.66666667	111	113
12cM2a	70	91	92
SUM	**421.0714286**		**789**
AVERAGE	**42.10714286**		**78.9**
St DEV			**51.00207**
SEM			**16.12827**

Value of 2-tailed t-test				
0.159119				
However, expect to find less cells therefore directional hypothesis, use 1-tailed				
(value of 1-tailed = 0.08)				

1.5.C Number of SoxE nuclei in outgrowth of kiEphA4 and control NT^{m} cultures

kiEphA4 nt cultures		
	TOTAL %	Sox e TOTAL
10bM2	15.068493	65
1aM2a(a)	15.384615	18
11dM2	16.190476	76
5cM2	19.642857	36
10cM2	31	67
1bM2a (a)	33.333333	8
11bM2	35.59322	40
5bM2b	41.269841	40
11cM2a	46.875	40
SUM	254.35784	390
AVERAGE	**28.261982**	**43.33333333**
STDEV		22.56656819
SEM		**7.522189398**
	CORREL	-0.245830222

Control cultures		
	TOTAL %	TOTAL Sox
12aM2a	7.3529412	122
12dM2	10.25641	97
3dM2a	11.363636	23
2dM2a	14.583333	32
3cM2a	16.666667	50
2bgrb2	18.75	34
13bM2	20.869565	99
3bM2a	23.4375	57
12cM2a	25.225225	95
13aM2	33.476395	208
SUM	181.98167	817
AVERAGE	**18.198167**	**81.7**
STDEV		55.86899359
SEM		**17.66732703**
	CORREL	0.500087918

Reference List

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W. and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* **13**, 295-306 http://www.genesdev.org.

Akitaya, T. and Bronner-Fraser, M. (1992). Expression of cell adhesion molecules during initiation and cessation of neural crest cell migration. *Dev Dyn* **194**, 12-20.

Anderson, C. B. and Meier, S. (1982). Effect of hyaluronidase treatment on the distribution of cranial neural crest cells in the chick embryo. *J Exp Zool* **221**, 329-35.

Araujo, M. and Nieto, M. A. (1997). The expression of chick EphA7 during segmentation of the central and peripheral nervous system. *Mech Dev* **68**, 173-7.

Barembaum, M., Moreno, T. A., LaBonne, C., Sechrist, J. and Bronner-Fraser, M. (2000). Noelin-1 is a secreted glycoprotein involved in generation of the neural crest. *Nat Cell Biol* **2**, 219-25.

Barrios, A., Poole, R. J., Durbin, L., Brennan, C., Holder, N. and Wilson, S. W. (2003). Eph/Ephrin signaling regulates the mesenchymal-to-epithelial transition of the paraxial mesoderm during somite morphogenesis. *Curr Biol* **13**, 1571-82.

Binns, K. L., Taylor, P. P., Sicheri, F., Pawson, T. and Holland, S. J. (2000). Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors. *Mol Cell Biol* **20**, 4791-805.

Birgbauer, E., Cowan, C. A., Sretavan, D. W. and Henkemeyer, M. (2000). Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. *Development* **127**, 1231-41.

Boucaut, J. C., Darribere, T., Poole, T. J., Aoyama, H., Yamada, K. M. and Thiery, J. P. (1984). Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest cell migration in avian embryos. *J Cell Biol* **99**, 1822-30.

Braga, V. (2000). Epithelial cell shape: cadherins and small GTPases. *Exp Cell Res* **261**, 83-90.

Brennan, H., Smith, S. and Stoker, A. (1999). Phosphotyrosine signalling as a regulator of neural crest cell adhesion and motility. *Cell Motil Cytoskeleton* **42**, 101-13.

Bronner-Fraser, M. (1985). Alterations in neural crest migration by a monoclonal antibody that affects cell adhesion. *J Cell Biol* **101**, 610-7.

Bronner-Fraser, M. (1986a). Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. *Dev Biol* **115**, 44-55.

Bronner-Fraser, M. (1986b). An antibody to a receptor for fibronectin and laminin perturbs cranial neural crest development in vivo. *Dev Biol* **117**, 528-36.

Bronner-Fraser, M. (1988). Distribution and function of tenascin during cranial neural crest development in the chick. *J Neurosci Res* **21**, 135-47.

Bronner-Fraser, M. (1993a). Environmental influences on neural crest cell migration. *J Neurobiol* **24**, 233-47.

Bronner-Fraser, M. (1993b). Mechanisms of neural crest cell migration. *Bioessays* **15**, 221-30.
Bronner-Fraser, M. and Stern, C. (1991). Effects of mesodermal tissues on avian neural crest cell migration. *Dev Biol* **143**, 213-7.
Bronner-Fraser, M., Stern, C. D. and Fraser, S. (1991). Analysis of neural crest cell lineage and migration. *J Craniofac Genet Dev Biol* **11**, 214-22.
Bronner-Fraser, M., Wolf, J. J. and Murray, B. A. (1992). Effects of antibodies against N-cadherin and N-CAM on the cranial neural crest and neural tube. *Dev Biol* **153**, 291-301.
Bundy, J., Rogers, R., Hoffman, S. and Conway, S. J. (1998). Segmental expression of aggrecan in the non-segmented perinotochordal sheath underlies normal segmentation of the vertebral column. *Mech Dev* **79**, 213-7.
Burstyn-Cohen, T. and Kalcheim, C. (2002). Association between the cell cycle and neural crest delamination through specific regulation of G1/S transition. *Dev Cell* **3**, 383-95.
Burstyn-Cohen, T., Stanleigh, J., Sela-Donenfeld, D. and Kalcheim, C. (2004). Canonical Wnt activity regulates trunk neural crest delamination linking BMP/noggin signaling with G1/S transition. *Development* **131**, 5327-39.
Carter, S. B. (1965). Principles of cell motility: the direction of cell movement and cancer invasion. *Nature* **208**, 1183-7.
Chen, J. and Ruley, H. E. (1998). An enhancer element in the EphA2 (Eck) gene sufficient for rhombomere-specific expression is activated by HOXA1 and HOXB1 homeobox proteins. *J Biol Chem* **273**, 24670-5 http://www.jbc.org/ http://highwire.stanford.edu/jbc/$ gopher://exogene.genethon.fr:/70/11/Biblio/Journaux/Journal%20of%Biological%20Chemistry$.
Chen, Y., Gutmann, D. H., Haipek, C. A., Martinsen, B. J., Bronner-Fraser, M. and Krull, C. E. (2004). Characterization of chicken Nf2/merlin indicates regulatory roles in cell proliferation and migration. *Dev Dyn* **229**, 541-54.
Cheung, M. and Briscoe, J. (2003). Neural crest development is regulated by the transcription factor Sox9. *Development* **130**, 5681-93.
Christ, B., Huang, R. and Wilting, J. (2000). The development of the avian vertebral column. *Anat Embryol (Berl)* **202**, 179-94.
Christiansen, J. H., Coles, E. G. and Wilkinson, D. G. (2000). Molecular control of neural crest formation, migration and differentiation. *Curr Opin Cell Biol* **12**, 719-24.
Ciruna, B. and Rossant, J. (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev Cell* **1**, 37-49.
Compagni, A., Logan, M., Klein, R. and Adams, R. H. (2003). Control of skeletal patterning by ephrinB1-EphB interactions. *Dev Cell* **5**, 217-30.
Conacci-Sorrell, M., Simcha, I., Ben-Yedidia, T., Blechman, J., Savagner, P. and Ben-Ze'ev, A. (2003). Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. *J Cell Biol* **163**, 847-57.
Davies, J. A., Cook, G. M., Stern, C. D. and Keynes, R. J. (1990). Isolation from chick somites of a glycoprotein fraction that causes collapse of dorsal root ganglion growth cones. *Neuron* **4**, 11-20.
Davis, E. M. and Trinkaus, J. P. (1981). Significance of cell-to cell contacts for the directional movement of neural crest cells within a hydrated collagen lattice. *J Embryol Exp Morphol* **63**, 29-51.

Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotak, V., Pawson, T., Goldfarb, M. and Yancopoulos, G. D. (1994). Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. *Science* **266**, 816-9.
Davy, A., Aubin, J. and Soriano, P. (2004). Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev* **18**, 572-83.
Davy, A., Gale, N. W., Murray, E. W., Klinghoffer, R. A., Soriano, P., Feuerstein, C. and Robbins, S. M. (1999). Compartmentalized signaling by GPI-anchored ephrin-A5 requires the Fyn tyrosine kinase to regulate cellular adhesion. *Genes Dev* **13**, 3125-35 http://www.genesdev.org.
Davy, A. and Robbins, S. M. (2000). Ephrin-A5 modulates cell adhesion and morphology in an integrin-dependent manner. *EMBO J* **19**, 5396-405.
de Melker, A. A., Desban, N. and Duband, J. L. (2004). Cellular localization and signaling activity of beta-catenin in migrating neural crest cells. *Dev Dyn* **230**, 708-26.
Debby-Brafman, A., Burstyn-Cohen, T., Klar, A. and Kalcheim, C. (1999). F-Spondin, expressed in somite regions avoided by neural crest cells, mediates inhibition of distinct somite domains to neural crest migration. *Neuron* **22**, 475-88.
del Barrio, M. G. and Nieto, M. A. (2002). Overexpression of Snail family members highlights their ability to promote chick neural crest formation. *Development* **129**, 1583-93.
Delannet, M. and Duband, J. L. (1992). Transforming growth factor-beta control of cell-substratum adhesion during avian neural crest cell emigration in vitro. *Development* **116**, 275-87.
Delannet, M., Martin, F., Bossy, B., Cheresh, D. A., Reichardt, L. F. and Duband, J. L. (1994). Specific roles of the alpha V beta 1, alpha V beta 3 and alpha V beta 5 integrins in avian neural crest cell adhesion and migration on vitronectin. *Development* **120**, 2687-702.
Domowicz, M., Li, H., Hennig, A., Henry, J., Vertel, B. M. and Schwartz, N. B. (1995). The biochemically and immunologically distinct CSPG of notochord is a product of the aggrecan gene. *Dev Biol* **171**, 655-64.
Domowicz, M. S., Mueller, M. M., Novak, T. E., Schwartz, L. E. and Schwartz, N. B. (2003). Developmental expression of the HNK-1 carbohydrate epitope on aggrecan during chondrogenesis. *Dev Dyn* **226**, 42-50.
Dottori, M., Gross, M. K., Labosky, P. and Goulding, M. (2001). The winged-helix transcription factor Foxd3 suppresses interneuron differentiation and promotes neural crest cell fate. *Development* **128**, 4127-38.
Dottori, M., Hartley, L., Galea, M., Paxinos, G., Polizzotto, M., Kilpatrick, T., Bartlett, P. F., Murphy, M., Kontgen, F. and Boyd, A. W. (1998). EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. *Proc Natl Acad Sci U S A* **95**, 13248-53.
Duband, J. L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G. M. and Thiery, J. P. (1987). Adhesion molecules during somitogenesis in the avian embryo. *J Cell Biol* **104**, 1361-74.
Duband, J. L., Dufour, S., Yamada, S. S., Yamada, K. M. and Thiery, J. P. (1991). Neural crest cell locomotion induced by antibodies to beta 1 integrins. A tool for studying the roles of substratum molecular avidity and density in migration. *J Cell Sci* **98 (Pt 4)**, 517-32.

Duband, J. L., Rocher, S., Chen, W. T., Yamada, K. M. and Thiery, J. P. (1986). Cell adhesion and migration in the early vertebrate embryo: location and possible role of the putative fibronectin receptor complex. *J Cell Biol* **102**, 160-78.
Duband, J. L. and Thiery, J. P. (1982). Distribution of fibronectin in the early phase of avian cephalic neural crest cell migration. *Dev Biol* **93**, 308-23.
Duband, J. L. and Thiery, J. P. (1987). Distribution of laminin and collagens during avian neural crest development. *Development* **101**, 461-78.
Duband, J. L., Volberg, T., Sabanay, I., Thiery, J. P. and Geiger, B. (1988). Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *Development* **103**, 325-44.
Durbin, L., Brennan, C., Shiomi, K., Cooke, J., Barrios, A., Shanmugalingam, S., Guthrie, B., Lindberg, R. and Holder, N. (1998). Eph signaling is required for segmentation and differentiation of the somites. *Genes Dev* **12**, 3096-109 http://www.genesdev.org.
Durbin, L., Sordino, P., Barrios, A., Gering, M., Thisse, C., Thisse, B., Brennan, C., Green, A., Wilson, S. and Holder, N. (2000). Anteroposterior patterning is required within segments for somite boundary formation in developing zebrafish. *Development* **127**, 1703-13.
Ebendal, T. (1977). Extracellular matrix fibrils and cell contacts in the chick embryo. Possible roles in orientation of cell migration and axon extension. *Cell Tissue Res* **175**, 439-58.
Eberhart, J., Barr, J., O'Connell, S., Flagg, A., Swartz, M. E., Cramer, K. S., Tosney, K. W., Pasquale, E. B. and Krull, C. E. (2004). Ephrin-A5 exerts positive or inhibitory effects on distinct subsets of EphA4-positive motor neurons. *J Neurosci* **24**, 1070-8.
Eberhart, J., Swartz, M., Koblar, S. A., Pasquale, E. B., Tanaka, H. and Krull, C. E. (2000). Expression of EphA4, ephrin-A2 and ephrin-A5 during axon outgrowth to the hindlimb indicates potential roles in pathfinding. *Dev Neurosci* **22**, 237-50.
Eberhart, J., Swartz, M. E., Koblar, S. A., Pasquale, E. B. and Krull, C. E. (2002). EphA4 constitutes a population-specific guidance cue for motor neurons. *Dev Biol* **247**, 89-101.
Erickson, C. A., Loring, J. F. and Lester, S. M. (1989). Migratory pathways of HNK-1-immunoreactive neural crest cells in the rat embryo. *Dev Biol* **134**, 112-8.
Erickson, C. A. and Perris, R. (1993). The role of cell-cell and cell-matrix interactions in the morphogenesis of the neural crest. *Dev Biol* **159**, 60-74.
Erickson, C. A., Tosney, K. W. and Weston, J. A. (1980). Analysis of migratory behavior of neural crest and fibroblastic cells in embryonic tissues. *Dev Biol* **77**, 142-56.
Erickson, C. A. and Weston, J. A. (1983). An SEM analysis of neural crest migration in the mouse. *J Embryol Exp Morphol* **74**, 97-118.
Flanagan, J. G. and Vanderhaeghen, P. (1998). The ephrins and Eph receptors in neural development. *Annu Rev Neurosci* **21**, 309-45.
Fredette, B. J., Miller, J. and Ranscht, B. (1996). Inhibition of motor axon growth by T-cadherin substrata. *Development* **122**, 3163-71.
Frisen, J., Yates, P. A., McLaughlin, T., Friedman, G. C., O'Leary, D. D. and Barbacid, M. (1998). Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. *Neuron* **20**, 235-43.

Gale, N. W., Holland, S. J., Valenzuela, D. M., Flenniken, A., Pan, L., Ryan, T. E., Henkemeyer, M., Strebhardt, K., Hirai, H., Wilkinson, D. G. et al. (1996). Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron* **17**, 9-19.
Gammill, L. S. and Bronner-Fraser, M. (2002). Genomic analysis of neural crest induction. *Development* **129**, 5731-41.
Garcia-Castro, M. I., Marcelle, C. and Bronner-Fraser, M. (2002). Ectodermal Wnt function as a neural crest inducer. *Science* **297**, 848-51.
Gauthier, L. R. and Robbins, S. M. (2003). Ephrin signaling: One raft to rule them all? One raft to sort them? One raft to spread their call and in signaling bind them? *Life Sci* **74**, 207-16.
Goldstein, R. S., Avivi, C. and Geffen, R. (1995). Initial axial level-dependent differences in size of avian dorsal root ganglia are imposed by the sclerotome. *Dev Biol* **168**, 214-22.
Goldstein, R. S. and Kalcheim, C. (1991). Normal segmentation and size of the primary sympathetic ganglia depend upon the alternation of rostrocaudal properties of the somites. *Development* **112**, 327-34.
Goldstein, R. S., Teillet, M. A. and Kalcheim, C. (1990). The microenvironment created by grafting rostral half-somites is mitogenic for neural crest cells. *Proc Natl Acad Sci U S A* **87**, 4476-80.
Greenberg, J. H. and Pratt, R. M. (1977). Glycosaminoglycan and glycoprotein synthesis by cranial neural crest cells in vitro. *Cell Differ* **6**, 119-32.
Guillory, G. and Bronner-Fraser, M. (1986). An in vitro assay for neural crest cell migration through the somites. *J Embryol Exp Morphol* **98**, 85-97.
Gumbiner, B. M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* **84**, 345-57.
Gvirtzman, G., Goldstein, R. S. and Kalcheim, C. (1992). A positive correlation between permissiveness of mesoderm to neural crest migration and early DRG growth. *J Neurobiol* **23**, 205-16.
Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *Journal of Embryology and Experimental Morphology* **88**, 49-92.
Hatta, K., Takagi, S., Fujisawa, H. and Takeichi, M. (1987). Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Dev Biol* **120**, 215-27.
Hatta, K. and Takeichi, M. (1986). Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature* **320**, 447-9.
Hattori, M., Osterfield, M. and Flanagan, J. G. (2000). Regulated cleavage of a contact-mediated axon repellent [see comments] [comment]. *Science* **289**, 1360-5.
Henderson, D. J., Ybot-Gonzalez, P. and Copp, A. J. (1997). Over-expression of the chondroitin sulphate proteoglycan versican is associated with defective neural crest migration in the Pax3 mutant mouse (splotch). *Mech Dev* **69**, 39-51.
Henkemeyer, M., Orioli, D., Henderson, J. T., Saxton, T. M., Roder, J., Pawson, T. and Klein, R. (1996). Nuk controls pathfinding of commissural axons in the mammalian central nervous system. *Cell* **86**, 35-46.
Himanen, J. P., Chumley, M. J., Lackmann, M., Li, C., Barton, W. A., Jeffrey, P. D., Vearing, C., Geleick, D., Feldheim, D. A., Boyd, A. W. et al. (2004). Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. *Nat Neurosci* **7**, 501-9.

Hindges, R., McLaughlin, T., Genoud, N., Henkemeyer, M. and O'Leary, D. D. (2002). EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. *Neuron* **35**, 475-87.
Holder, N., Durbin, L. and Cooke, J. (2000). Eph receptors and ephrins are key regulators of morphogenesis. *Ernst Schering Res Found Workshop* **29**, 123-47.
Holland, S. J., Gale, N. W., Mbamalu, G., Yancopoulos, G. D., Henkemeyer, M. and Pawson, T. (1996). Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature* **383**, 722-5.
Holmberg, J., Clarke, D. L. and Frisen, J. (2000). Regulation of repulsion versus adhesion by different splice forms of an Eph receptor. *Nature* **408**, 203-6.
Holmberg, J. and Frisen, J. (2002). Ephrins are not only unattractive. *Trends Neurosci* **25**, 239-43.
Huai, J. and Drescher, U. (2001). An ephrin-A-dependent signaling pathway controls integrin function and is linked to the tyrosine phosphorylation of a 120-kDa protein. *J Biol Chem* **276**, 6689-94.
Ikeya, M., Lee, S. M., Johnson, J. E., McMahon, A. P. and Takada, S. (1997). Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* **389**, 966-70.
Itasaki, N., Bel-Vialar, S. and Krumlauf, R. (1999). 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. *Nat Cell Biol* **1**, E203-7.
Iwamasa, H., Ohta, K., Yamada, T., Ushijima, K., Terasaki, H. and Tanaka, H. (1999). Expression of Eph receptor tyrosine kinases and their ligands in chick embryonic motor neurons and hindlimb muscles. *Dev Growth Differ* **41**, 685-98.
Kalcheim, C. and Teillet, M. A. (1989). Consequences of somite manipulation on the pattern of dorsal root ganglion development. *Development* **106**, 85-93.
Kerr, R. S. and Newgreen, D. F. (1997). Isolation and characterization of chondroitin sulfate proteoglycans from embryonic quail that influence neural crest cell behavior. *Dev Biol* **192**, 108-24.
Keynes, R., Tannahill, D., Morgenstern, D. A., Johnson, A. R., Cook, G. M. and Pini, A. (1997). Surround repulsion of spinal sensory axons in higher vertebrate embryos. *Neuron* **18**, 889-97.
Keynes, R. J., Johnson, A. R., Pini, A., Tannahill, D. and Cook, G. (1996). Spinal nerve segmentation in higher vertebrates: axon guidance by repulsion and attraction. *Seminars in The Neurosciences* **8**, 339-345.
Keynes, R. J. and Stern, C. D. (1984). Segmentation in the vertebrate nervous system. *Nature* **310**, 786-9.
Kil, S. H., Krull, C. E., Cann, G., Clegg, D. and Bronner-Fraser, M. (1998). The alpha4 subunit of integrin is important for neural crest cell migration. *Dev Biol* **202**, 29-42.
Kim, J., Lo, L., Dormand, E. and Anderson, D. J. (2003). SOX10 maintains multipotency and inhibits neuronal differentiation of neural crest stem cells. *Neuron* **38**, 17-31.
Klein, R. (2004). Eph/ephrin signaling in morphogenesis, neural development and plasticity. *Curr Opin Cell Biol* **16**, 580-9.
Koblar, S. A., Krull, C. E., Pasquale, E. B., McLennan, R., Peale, F. D., Cerretti, D. P. and Bothwell, M. (2000). Spinal motor axons and neural crest cells use different molecular guides for segmental migration through the rostral half-somite. *J Neurobiol* **42**, 437-47.

Kos, R., Reedy, M. V., Johnson, R. L. and Erickson, C. A. (2001). The winged-helix transcription factor FoxD3 is important for establishing the neural crest lineage and repressing melanogenesis in avian embryos. *Development* **128**, 1467-79.
Kos, R., Tucker, R. P., Hall, R., Duong, T. D. and Erickson, C. A. (2003). Methods for introducing morpholinos into the chicken embryo. *Dev Dyn* **226**, 470-7.
Krotoski, D. M., Domingo, C. and Bronner-Fraser, M. (1986). Distribution of a putative cell surface receptor for fibronectin and laminin in the avian embryo. *J Cell Biol* **103**, 1061-71.
Krull, C. E. (1998). Inhibitory interactions in the patterning of trunk neural crest migration. *Ann N Y Acad Sci* **857**, 13-22.
Krull, C. E. (2001). Segmental organization of neural crest migration. *Mech Dev* **105**, 37-45.
Krull, C. E. (2004). A primer on using in ovo electroporation to analyze gene function. *Dev Dyn* **229**, 433-9.
Krull, C. E., Collazo, A., Fraser, S. E. and Bronner-Fraser, M. (1995). Segmental migration of trunk neural crest: time-lapse analysis reveals a role for PNA-binding molecules. *Development* **121**, 3733-43.
Krull, C. E., Lansford, R., Gale, N. W., Collazo, A., Marcelle, C., Yancopoulos, G. D., Fraser, S. E. and Bronner Fraser, M. (1997). Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr Biol* **7**, 571-80.
Kulesa, P. M. and Fraser, S. E. (2000). In ovo time-lapse analysis of chick hindbrain neural crest cell migration shows cell interactions during migration to the branchial arches. *Development* **127**, 1161-72.
Kullander, K., Croll, S. D., Zimmer, M., Pan, L., McClain, J., Hughes, V., Zabski, S., DeChiara, T. M., Klein, R., Yancopoulos, G. D. et al. (2001a). Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. *Genes Dev* **15**, 877-88.
Kullander, K. and Klein, R. (2002). Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* **3**, 475-86.
Kullander, K., Mather, N. K., Diella, F., Dottori, M., Boyd, A. W. and Klein, R. (2001b). Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. *Neuron* **29**, 73-84.
LaBonne, C. and Bronner-Fraser, M. (1998). Neural crest induction in Xenopus: evidence for a two-signal model. *Development* **125**, 2403-14.
Lackmann, M., Mann, R. J., Kravets, L., Smith, F. M., Bucci, T. A., Maxwell, K. F., Howlett, G. J., Olsson, J. E., Vanden Bos, T., Cerretti, D. P. et al. (1997). Ligand for EPH-related kinase (LERK) 7 is the preferred high affinity ligand for the HEK receptor. *J Biol Chem* **272**, 16521-30. Order.
Lallier, T., Leblanc, G., Artinger, K. B. and Bronner-Fraser, M. (1992). Cranial and trunk neural crest cells use different mechanisms for attachment to extracellular matrices. *Development* **116**, 531-41.
Lallier, T. E. and Bronner-Fraser, M. (1988). A spatial and temporal analysis of dorsal root and sympathetic ganglion formation in the avian embryo. *Dev Biol* **127**, 99-112.
Landolt, R. M., Vaughan, L., Winterhalter, K. H. and Zimmermann, D. R. (1995). Versican is selectively expressed in embryonic tissues that act as barriers to neural crest cell migration and axon outgrowth. *Development* **121**, 2303-12.

Lawrenson, I. D., Wimmer-Kleikamp, S. H., Lock, P., Schoenwaelder, S. M., Down, M., Boyd, A. W., Alewood, P. F. and Lackmann, M. (2002). Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3-expressing 293T and melanoma cells by CrkII and Rho-mediated signalling. *J Cell Sci* **115**, 1059-72.
Le Douarin, N. M. and Kalcheim, C. (1999). The Neural Crest, Second Edition. London: Cambridge University Press.
Le Douarin, N. M. and Teillet, M. A. (1974). Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. *Dev Biol* **41**, 162-84.
Liem, K. F., Jr., Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-79.
Liu, J. P. and Jessell, T. M. (1998). A role for rhoB in the delamination of neural crest cells from the dorsal neural tube. *Development* **125**, 5055-67.
Lofberg, J., Nynas-McCoy, A., Olsson, C., Jonsson, L. and Perris, R. (1985). Stimulation of initial neural crest cell migration in the axolotl embryo by tissue grafts and extracellular matrix transplanted on microcarriers. *Dev Biol* **107**, 442-59.
Lofberg, J., Perris, R. and Epperlein, H. H. (1989). Timing in the regulation of neural crest cell migration: retarded "maturation" of regional extracellular matrix inhibits pigment cell migration in embryos of the white axolotl mutant. *Dev Biol* **131**, 168-81.
Loring, J. F. and Erickson, C. A. (1987). Neural crest cell migratory pathways in the trunk of the chick embryo. *Dev Biol* **121**, 220-36.
Mackie, E. J. and Tucker, R. P. (1999). The tenascin-C knockout revisited. *J Cell Sci* **112 (Pt 22)**, 3847-53.
Mackie, E. J., Tucker, R. P., Halfter, W., Chiquet-Ehrismann, R. and Epperlein, H. H. (1988). The distribution of tenascin coincides with pathways of neural crest cell migration. *Development* **102**, 237-50.
Malliri, A. and Collard, J. G. (2003). Role of Rho-family proteins in cell adhesion and cancer. *Curr Opin Cell Biol* **15**, 583-9.
Malliri, A., van Es, S., Huveneers, S. and Collard, J. G. (2004). The Rac exchange factor Tiam1 is required for the establishment and maintenance of cadherin-based adhesions. *J Biol Chem* **279**, 30092-8.
Manasek, F. J. and Cohen, A. M. (1977). Anionic glycopeptides and glycosaminoglycans synthesized by embryonic neural tube and neural crest. *Proc Natl Acad Sci U S A* **74**, 1057-61.
Mayer, B. W., Jr., Hay, E. D. and Hynes, R. O. (1981). Immunocytochemical localization of fibronectin in embryonic chick trunk and area vasculosa. *Dev Biol* **82**, 267-86.
McLennan, R. and Krull, C. E. (2002). Ephrin-as cooperate with EphA4 to promote trunk neural crest migration. *Gene Expr* **10**, 295-305.
Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.
Menzel, P., Valencia, F., Godement, P., Dodelet, V. C. and Pasquale, E. B. (2001). Ephrin-A6, a new ligand for EphA receptors in the developing visual system. *Dev Biol* **230**, 74-88.
Miao, H., Burnett, E., Kinch, M., Simon, E. and Wang, B. (2000). Activation of EphA2 kinase suppresses integrin function and causes focal-adhesion-kinase dephosphorylation. *Nat Cell Biol* **2**, 62-9.

Minichiello, J., Ben-Ya'acov, A., Hearn, C. J., Needham, B. and Newgreen, D. F. (1999). Induction of epithelio-mesenchymal transformation of quail embryonic neural cells by inhibition of atypical protein kinase-C. *Cell Tissue Res* **295**, 195-206.
Momose, T., Tonegawa, A., Takeuchi, J., Ogawa, H., Umesono, K. and Yasuda, K. (1999). Efficient targeting of gene expression in chick embryos by microelectroporation. *Dev Growth Differ* **41**, 335-44.
Monier-Gavelle, F. and Duband, J. L. (1995). Control of N-cadherin-mediated intercellular adhesion in migrating neural crest cells in vitro. *J Cell Sci* **108 (Pt 12)**, 3839-53.
Monsoro-Burq, A. H., Fletcher, R. B. and Harland, R. M. (2003). Neural crest induction by paraxial mesoderm in Xenopus embryos requires FGF signals. *Development* **130**, 3111-24.
Nakagawa, S. and Takeichi, M. (1995). Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* **121**, 1321-32.
Nakagawa, S. and Takeichi, M. (1998). Neural crest emigration from the neural tube depends on regulated cadherin expression. *Development* **125**, 2963-71.
Nawshad, A., LaGamba, D. and Hay, E. D. (2004). Transforming growth factor B (TGFB) signalling in palatal growth, apoptosis and epithelial mesenchymal transformation (EMT). *Archives of Oral Biology* **49**, 675-689.
Newgreen, D. and Gibbins, I. (1982). Factors controlling the time of onset of the migration of neural crest cells in the fowl embryo. *Cell Tissue Res* **224**, 145-60.
Newgreen, D. and Thiery, J. P. (1980). Fibronectin in early avian embryos: synthesis and distribution along the migration pathways of neural crest cells. *Cell Tissue Res* **211**, 269-91.
Newgreen, D. a. M. M. (1999). Neural crest cell outgrowth cultures and the analysis of cell migration. In *Developmental Biology Protocols*, vol. III (ed. R. S. T. a. C. W. Lo.), pp. 201-211. Totowa, New Jersey: Humana Press.
Newgreen, D. F. (1982). Adhesion to extracellular materials by neural crest cells at the stage of initial migration. *Cell Tissue Res* **227**, 297-317.
Newgreen, D. F. (1989). Physical influences on neural crest cell migration in avian embryos: contact guidance and spatial restriction. *Dev Biol* **131**, 136-48.
Newgreen, D. F., Gibbins, I. L., Sauter, J., Wallenfels, B. and Wutz, R. (1982). Ultrastructural and tissue-culture studies on the role of fibronectin, collagen and glycosaminoglycans in the migration of neural crest cells in the fowl embryo. *Cell Tissue Res* **221**, 521-49.
Newgreen, D. F. and Gooday, D. (1985). Control of the onset of migration of neural crest cells in avian embryos. Role of Ca++-dependent cell adhesions. *Cell Tissue Res* **239**, 329-36.
Newgreen, D. F. and Minichiello, J. (1995). Control of epitheliomesenchymal transformation. I. Events in the onset of neural crest cell migration are separable and inducible by protein kinase inhibitors. *Dev Biol* **170**, 91-101.
Newgreen, D. F. and Minichiello, J. (1996). Control of epitheliomesenchymal transformation. II. Cross-modulation of cell adhesion and cytoskeletal systems in embryonic neural cells. *Dev Biol* **176**, 300-12.
Newgreen, D. F., Powell, M. E. and Moser, B. (1990). Spatiotemporal changes in HNK-1/L2 glycoconjugates on avian embryo somite and neural crest cells. *Dev Biol* **139**, 100-20.
Newgreen, D. F., Ritterman, M. and Peters, E. A. (1979). Morphology and behaviour of neural crest cells of chick embryo in vitro. *Cell Tissue Res* **203**, 115-40.

Newgreen, D. F., Scheel, M. and Kastner, V. (1986). Morphogenesis of sclerotome and neural crest in avian embryos. In vivo and in vitro studies on the role of notochordal extracellular material. *Cell Tissue Res* **244**, 299-313.
Newgreen, D. F. a. M., S.J. (2005). The neural crest: a model developmental EMT. In *Rise and Fall of Epithelial Phenotype*, (ed. P. Savagner), pp. pp.1-11: ©2004 Eurekah.com and Kluwer Academic / Plenum Publishers.
Nieto, M. A. (2002). The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* **3**, 155-66.
Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116**, 1137-50.
Nieto, M. A., Sargent, M. G., Wilkinson, D. G. and Cooke, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science* **264**, 835-9.
Nobes, C. D., Lauritzen, I., Mattei, M. G., Paris, S., Hall, A. and Chardin, P. (1998). A new member of the Rho family, Rnd1, promotes disassembly of actin filament structures and loss of cell adhesion. *J Cell Biol* **141**, 187-97.
Oakley, R. A., Lasky, C. J., Erickson, C. A. and Tosney, K. W. (1994). Glycoconjugates mark a transient barrier to neural crest migration in the chicken embryo. *Development* **120**, 103-14.
Oakley, R. A. and Tosney, K. W. (1991). Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. *Dev Biol* **147**, 187-206.
Ogita, H., Kunimoto, S., Kamioka, Y., Sawa, H., Masuda, M. and Mochizuki, N. (2003). EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. *Circ Res* **93**, 23-31.
O'Leary, D. D. and Wilkinson, D. G. (1999). Eph receptors and ephrins in neural development. *Curr Opin Neurobiol* **9**, 65-73.
Orioli, D., Henkemeyer, M., Lemke, G., Klein, R. and Pawson, T. (1996). Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. *EMBO J* **15**, 6035-49.
Orsulic, S. and Kemler, R. (2000). Expression of Eph receptors and ephrins is differentially regulated by E-cadherin. *J Cell Sci* **113**, 1793-802.
Park, E. K., Warner, N., Bong, Y. S., Stapleton, D., Maeda, R., Pawson, T. and Daar, I. O. (2004). Ectopic EphA4 receptor induces posterior protrusions via FGF signaling in Xenopus embryos. *Mol Biol Cell* **15**, 1647-55.
Patel, K., Nittenberg, R., D'Souza, D., Irving, C., Burt, D., Wilkinson, D. G. and Tickle, C. (1996). Expression and regulation of Cek-8, a cell to cell signalling receptor in developing chick limb buds. *Development* **122**, 1147-55. Order.
Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S. and Stoeckli, E. T. (2003). Screening for gene function in chicken embryo using RNAi and electroporation. *Nat Biotechnol* **21**, 93-6.
Perissinotto, D., Iacopetti, P., Bellina, I., Doliana, R., Colombatti, A., Pettway, Z., Bronner-Fraser, M., Shinomura, T., Kimata, K., Morgelin, M. et al. (2000). Avian neural crest cell migration is diversely regulated by the two major hyaluronan-binding proteoglycans PG-M/versican and aggrecan. *Development* **127**, 2823-42.
Perris, R. (1997). The extracellular matrix in neural crest-cell migration. *Trends Neurosci* **20**, 23-31.

Perris, R. and Johansson, S. (1987). Amphibian neural crest cell migration on purified extracellular matrix components: a chondroitin sulfate proteoglycan inhibits locomotion on fibronectin substrates. *J Cell Biol* **105**, 2511-21.
Perris, R. and Johansson, S. (1990). Inhibition of neural crest cell migration by aggregating chondroitin sulfate proteoglycans is mediated by their hyaluronan-binding region. *Dev Biol* **137**, 1-12.
Perris, R., Krotoski, D. and Bronner-Fraser, M. (1991a). Collagens in avian neural crest development: distribution in vivo and migration-promoting ability in vitro. *Development* **113**, 969-84.
Perris, R., Krotoski, D., Lallier, T., Domingo, C., Sorrell, J. M. and Bronner-Fraser, M. (1991b). Spatial and temporal changes in the distribution of proteoglycans during avian neural crest development. *Development* **111**, 583-99.
Perris, R., Kuo, H. J., Glanville, R. W. and Bronner-Fraser, M. (1993). Collagen type VI in neural crest development: distribution in situ and interaction with cells in vitro. *Dev Dyn* **198**, 135-49.
Perris, R. and Perissinotto, D. (2000). Role of the extracellular matrix during neural crest cell migration. *Mech Dev* **95**, 3-21.
Perris, R., Perissinotto, D., Pettway, Z., Bronner-Fraser, M., Morgelin, M. and Kimata, K. (1996). Inhibitory effects of PG-H/aggrecan and PG-M/versican on avian neural crest cell migration. *FASEB J* **10**, 293-301.
Pettway, Z., Domowicz, M., Schwartz, N. B. and Bronner-Fraser, M. (1996). Age-dependent inhibition of neural crest migration by the notochord correlates with alterations in the S103L chondroitin sulfate proteoglycan. *Exp Cell Res* **225**, 195-206.
Pettway, Z., Guillory, G. and Bronner-Fraser, M. (1990). Absence of neural crest cells from the region surrounding implanted notochords in situ. *Dev Biol* **142**, 335-45.
Pietri, T., Eder, O., Breau, M. A., Topilko, P., Blanche, M., Brakebusch, C., Fassler, R., Thiery, J. P. and Dufour, S. (2004). Conditional beta1-integrin gene deletion in neural crest cells causes severe developmental alterations of the peripheral nervous system. *Development* **131**, 3871-83.
Pintar, J. E. (1978). Distribution and synthesis of glycosaminoglycans during quail neural crest morphogenesis. *Dev Biol* **67**, 444-64.
Poh, A., Karunaratne, A., Kolle, G., Huang, N., Smith, E., Starkey, J., Wen, D., Wilson, I., Yamada, T. and Hargrave, M. (2002). Patterning of the vertebrate ventral spinal cord. *Int J Dev Biol* **46**, 597-608.
Pratt, R. M., Larsen, M. A. and Johnston, M. C. (1975). Migration of cranial neural crest cells in a cell-free hyaluronate-rich matrix. *Dev Biol* **44**, 298-305.
Ranscht, B. and Bronner-Fraser, M. (1991). T-cadherin expression alternates with migrating neural crest cells in the trunk of the avian embryo. *Development* **111**, 15-22.
Rickmann, M., Fawcett, J. W. and Keynes, R. J. (1985). The migration of neural crest cells and the growth of motor axons through the rostral half of the chick somite. *J Embryol Exp Morphol* **90**, 437-55.
Ring, C., Hassell, J. and Halfter, W. (1996). Expression pattern of collagen IX and potential role in the segmentation of the peripheral nervous system. *Dev Biol* **180**, 41-53.
Rovasio, R. A., Delouvee, A., Yamada, K. M., Timpl, R. and Thiery, J. P. (1983). Neural crest cell migration: requirements for exogenous fibronectin and high cell density. *J Cell Biol* **96**, 462-73.
Sambrook J., F., E.F., and Maniatis, T. (1989). Molecular Cloning: A laboratory manual, 2nd Edition: Cold Spring Harbour Laboratory Press.

Santiago, A. and Erickson, C. A. (2002). Ephrin-B ligands play a dual role in the control of neural crest cell migration. *Development* **129**, 3621-32.
Savagner, P. (2001). Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays* **23**, 912-23.
Schmitz, A. A., Govek, E. E., Bottner, B. and Van Aelst, L. (2000). Rho GTPases: signaling, migration, and invasion. *Exp Cell Res* **261**, 1-12.
Sela-Donenfeld, D. and Kalcheim, C. (1999). Regulation of the onset of neural crest migration by coordinated activity of BMP4 and Noggin in the dorsal neural tube. *Development* **126**, 4749-62.
Sela-Donenfeld, D. and Kalcheim, C. (2000). Inhibition of noggin expression in the dorsal neural tube by somitogenesis: a mechanism for coordinating the timing of neural crest emigration. *Development* **127**, 4845-54.
Sela-Donenfeld, D. and Kalcheim, C. (2002). Localized BMP4-noggin interactions generate the dynamic patterning of noggin expression in somites. *Dev Biol* **246**, 311-28.
Selleck, M. A. and Bronner-Fraser, M. (1995). Origins of the avian neural crest: the role of neural plate-epidermal interactions. *Development* **121**, 525-38.
Shamah, S. M., Lin, M. Z., Goldberg, J. L., Estrach, S., Sahin, M., Hu, L., Bazalakova, M., Neve, R. L., Corfas, G., Debant, A. et al. (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell* **105**, 233-44.
Stein, E., Lane, A. A., Cerretti, D. P., Schoecklmann, H. O., Schroff, A. D., Van Etten, R. L. and Daniel, T. O. (1998). Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. *Genes Dev* **12**, 667-78 http://www.genesdev.org.
Stern, C. D., Artinger, K. B. and Bronner-Fraser, M. (1991). Tissue interactions affecting the migration and differentiation of neural crest cells in the chick embryo. *Development* **113**, 207-16.
Stern, C. D. and Keynes, R. J. (1987). Interactions between somite cells: the formation and maintenance of segment boundaries in the chick embryo. *Development* **99**, 261-72.
Stern, C. D., Norris, W. E., Bronner-Fraser, M., Carlson, G. J., Faissner, A., Keynes, R. J. and Schachner, M. (1989). J1/tenascin-related molecules are not responsible for the segmented pattern of neural crest cells or motor axons in the chick embryo. *Development* **107**, 309-19.
Stern, C. D., Sisodiya, S. M. and Keynes, R. J. (1986). Interactions between neurites and somite cells: inhibition and stimulation of nerve growth in the chick embryo. *J Embryol Exp Morphol* **91**, 209-26.
Sternberg, J. and Kimber, S. J. (1986). The relationship between emerging neural crest cells and basement membranes in the trunk of the mouse embryo: a TEM and immunocytochemical study. *J Embryol Exp Morphol* **98**, 251-68.
Swartz, M., Eberhart, J., Mastick, G. S. and Krull, C. E. (2001a). Sparking new frontiers: using in vivo electroporation for genetic manipulations. *Dev Biol* **233**, 13-21. Order.
Swartz, M. E., Eberhart, J., Pasquale, E. B. and Krull, C. E. (2001b). EphA4/ephrin-A5 interactions in muscle precursor cell migration in the avian forelimb. *Development* **128**, 4669-80.
Takeichi, M. (1988). The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* **102**, 639-55.

Tan, C., Costello, P., Sanghera, J., Dominguez, D., Baulida, J., de Herreros, A. G. and Dedhar, S. (2001). Inhibition of integrin linked kinase (ILK) suppresses beta-catenin-Lef/Tcf-dependent transcription and expression of the E-cadherin repressor, snail, in APC-/- human colon carcinoma cells. *Oncogene* **20**, 133-40.
Tan, S. S., Crossin, K. L., Hoffman, S. and Edelman, G. M. (1987). Asymmetric expression in somites of cytotactin and its proteoglycan ligand is correlated with neural crest cell distribution. *Proc Natl Acad Sci U S A* **84**, 7977-81.
Tanaka, H., Agata, A. and Obata, K. (1989). A new membrane antigen revealed by monoclonal antibodies is associated with motoneuron axonal pathways. *Dev Biol* **132**, 419-35.
Tanaka, M., Kamo, T., Ota, S. and Sugimura, H. (2003). Association of Dishevelled with Eph tyrosine kinase receptor and ephrin mediates cell repulsion. *EMBO J* **22**, 847-58.
Tanaka, M., Ohashi, R., Nakamura, R., Shinmura, K., Kamo, T., Sakai, R. and Sugimura, H. (2004). Tiam1 mediates neurite outgrowth induced by ephrin-B1 and EphA2. *EMBO J* **23**, 1075-88.
Tannahill, D., Britto, J. M., Vermeren, M. M., Ohta, K., Cook, G. M. and Keynes, R. J. (2000). Orienting axon growth: spinal nerve segmentation and surround-repulsion. *Int J Dev Biol* **44**, 119-27.
Teillet, M. A., Kalcheim, C. and Le Douarin, N. M. (1987). Formation of the dorsal root ganglia in the avian embryo: segmental origin and migratory behavior of neural crest progenitor cells. *Dev Biol* **120**, 329-47.
Testaz, S., Delannet, M. and Duband, J. (1999). Adhesion and migration of avian neural crest cells on fibronectin require the cooperating activities of multiple integrins of the (beta)1 and (beta)3 families. *J Cell Sci* **112 (Pt 24)**, 4715-28.
Testaz, S. and Duband, J. L. (2001). Central role of the alpha4beta1 integrin in the coordination of avian truncal neural crest cell adhesion, migration, and survival. *Dev Dyn* **222**, 127-40.
Thiery, J. P., Duband, J. L. and Delouvee, A. (1982a). Pathways and mechanisms of avian trunk neural crest cell migration and localization. *Dev Biol* **93**, 324-43.
Thiery, J. P., Duband, J. L., Rutishauser, U. and Edelman, G. M. (1982b). Cell adhesion molecules in early chicken embryogenesis. *Proc Natl Acad Sci U S A* **79**, 6737-41.
Tosch, P. (2002). Investigations of ephrin ligands during development. In *Department of Molecular Biosciences*, (ed., pp. 174. Adelaide: University of Adelaide.
Tosney, K. W. (1978). The early migration of neural crest cells in the trunk region of the avian embryo: an electron microscopic study. *Dev Biol* **62**, 317-33.
Tosney, K. W. (1987). Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: deletion of the dermamyotome. *Dev Biol* **122**, 540-58.
Tosney, K. W. (1988). Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: partial and complete deletion of the somite. *Dev Biol* **127**, 266-86.
Tosney, K. W., Dehnbostel, D. B. and Erickson, C. A. (1994). Neural crest cells prefer the myotome's basal lamina over the sclerotome as a substratum. *Dev Biol* **163**, 389-406.
Tosney, K. W. and Oakley, R. A. (1990). The perinotochordal mesenchyme acts as a barrier to axon advance in the chick embryo: implications for a general mechanism of axonal guidance. *Exp Neurol* **109**, 75-89.

Tucker, G. C., Delarue, M., Zada, S., Boucaut, J. C. and Thiery, J. P. (1988). Expression of the HNK-1/NC-1 epitope in early vertebrate neurogenesis. *Cell Tissue Res* **251**, 457-65.
Tucker, R. P. (2001). Abnormal neural crest cell migration after the in vivo knockdown of tenascin-C expression with morpholino antisense oligonucleotides. *Dev Dyn* **222**, 115-9.
Tucker, R. P. and Erickson, C. A. (1984). Morphology and behavior of quail neural crest cells in artificial three-dimensional extracellular matrices. *Dev Biol* **104**, 390-405.
Tucker, R. P., Hagios, C., Chiquet-Ehrismann, R., Lawler, J., Hall, R. J. and Erickson, C. A. (1999). Thrombospondin-1 and neural crest cell migration. *Dev Dyn* **214**, 312-22.
Tucker, R. P. and McKay, S. E. (1991). The expression of tenascin by neural crest cells and glia. *Development* **112**, 1031-9.
Tzarfati-Majar, V., Burstyn-Cohen, T. and Klar, A. (2001). F-spondin is a contact-repellent molecule for embryonic motor neurons. *Proc Natl Acad Sci U S A* **98**, 4722-7.
Uhlenbrock, K., Eberth, A., Herbrand, U., Daryab, N., Stege, P., Meier, F., Friedl, P., Collard, J. G. and Ahmadian, M. R. (2004). The RacGEF Tiam1 inhibits migration and invasion of metastatic melanoma via a novel adhesive mechanism. *J Cell Sci* **117**, 4863-71.
Vermeren, M. M., Cook, G. M., Johnson, A. R., Keynes, R. J. and Tannahill, D. (2000). Spinal nerve segmentation in the chick embryo: analysis of distinct axon-repulsive systems. *Dev Biol* **225**, 241-52.
Vincent, M. and Thiery, J. P. (1984). A cell surface marker for neural crest and placodal cells: further evolution in peripheral and central nervous system. *Dev Biol* **103**, 468-81.
Wahl, S., Barth, H., Ciossek, T., Aktories, K. and Mueller, B. K. (2000). Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J Cell Biol* **149**, 263-70.
Wang, H. U. and Anderson, D. J. (1997). Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron* **18**, 383-96.
Wang, H. U., Chen, Z. F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4 [see comments]. *Cell* **93**, 741-53.
Weston, J. A. (1963). A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. *Dev Biol* **6**, 279-310.
Weston, J. A. (1970). The migration and differentiation of neural crest cells. *Adv Morphog* **8**, 41-114.
Wilkinson, D. G. (2000). Eph receptors and ephrins: regulators of guidance and assembly. *Int Rev Cytol* **196**, 177-244.
Winning, R. S. and Sargent, T. D. (1994). Pagliaccio, a member of the Eph family of receptor tyrosine kinase genes, has localized expression in a subset of neural crest and neural tissues in Xenopus laevis embryos. *Mech Dev* **46**, 219-29.
Winning, R. S., Scales, J. B. and Sargent, T. D. (1996). Disruption of cell adhesion in Xenopus embryos by Pagliaccio, an Eph-class receptor tyrosine kinase. *Dev Biol* **179**, 309-19.

Winning, R. S., Ward, E. K., Scales, J. B. and Walker, G. K. (2002). EphA4 catalytic activity causes inhibition of RhoA GTPase in Xenopus laevis embryos. *Differentiation* **70**, 46-55.
Winning, R. S., Wyman, T. L. and Walker, G. K. (2001). EphA4 activity causes cell shape change and a loss of cell polarity in Xenopus laevis embryos. *Differentiation* **68**, 126-32.
Wunnenberg-Stapleton, K., Blitz, I. L., Hashimoto, C. and Cho, K. W. (1999). Involvement of the small GTPases XRhoA and XRnd1 in cell adhesion and head formation in early Xenopus development. *Development* **126**, 5339-51.
Xu, Q., Alldus, G., Holder, N. and Wilkinson, D. G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. *Development* **121**, 4005-16.
Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-71.
Yamagata, M., Suzuki, S., Akiyama, S. K., Yamada, K. M. and Kimata, K. (1989). Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrates. *J Biol Chem* **264**, 8012-8.
Yanfeng, W., Saint-Jeannet, J. P. and Klein, P. S. (2003). Wnt-frizzled signaling in the induction and differentiation of the neural crest. *Bioessays* **25**, 317-25.
Zantek, N. D., Azimi, M., Fedor Chaiken, M., Wang, B., Brackenbury, R. and Kinch, M. S. (1999). E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. *Cell Growth Differ* **10**, 629-38.
Zhai, J., Lin, H., Nie, Z., Wu, J., Canete-Soler, R., Schlaepfer, W. W. and Schlaepfer, D. D. (2003). Direct interaction of focal adhesion kinase with p190RhoGEF. *J Biol Chem* **278**, 24865-73.

Addendum

List of Abbreviations

RTK	Receptor Tyrosine Kinase	DEPC	diethyl pyrocarbonate	SAM	Sterile Alpha Motif
kb	kilobases	µg	microgram	WM	Whole-mount
EDTA		µl	microlitre	Trans	Transverse
UV	Ultra violet	µm	micrometres	EMT	Epithelial-to-Mesenchymal Transition
V	Volts	IMVS	Institute of Medical and Veterinary Science	NC	Neural Crest
Ω	Ohm	mins	minutes	PDZ	PSD-95, DlgA, ZO-1
OD^{600}	Optical Density 600	mM	microMolar	Jx1	Juxtamembrane region 1
ID	Inner diameter	ng	nanogram	NBT	4-nitroblue tetrazolium chloride
OD	Outer diameter	rpm	revolutions per minute	BCIP	5-bromo-4-chloro-3-indoyl-phosphate
BSA	bovine serum albumin	RT	room temperature	1°	primary
pen	penicillin	sec	seconds	2°	secondary
strep	streptomycin	DMF	N,N-dimethyl formamide	3°	tertiary
NaOH	Sodium Hydroxide	PFA	paraformaldehyde	PCR	Polymerase Chain Reaction
EDTA	Disodium Salt	PBS	Phosphate Buffered Saline	DIG	Digoxigenin
SDS	Sodium Dodecyl Sulphate	PBT	PBS + 0.1% Tween-20 or 0.01% TritonX-100	LB	Luria Bertani medium

Title 3.2.1, page
The title
"Complementary and overlapping distribution of A-subclass Eph and ephrins in the developing peripheral nervous system"
should be changed to
"Complementary and overlapping distribution of A-subclass Eph and ephrins relevant to the developing peripheral nervous system"

Section 4.2.4, page 165
The sentence
"Furthermore…characterised by a thickening of the basal lamina"
should be changed to
"Furthermore… that at the dorsal edge of the EphA4/EGFP transfected side, the lateral extent of membrane breakdown (indicated by laminin antibody) was more advanced on the un-transfected side…".

Chapter 4, page 191
The sentence
"cells lacking EphA4…"
should be changed to
"cells lacking EphA4 kinase activity…"

Chapter 4, page 193
Reference mark number 447 removed

Chapter 5, page 199
The sentence
"Newgreen and Gibbins stated that there was two requirements…"
should be changed to
"there were two requirements"

Chapter 4, Page 201
SJM
should be changed to
Sonja J McKeown

Chapter 4, Page 202
Reference mark number 907 removed

Chapter 4, Page 204
EphA4Δcyto is defined in Section 2.1.6, Electroporation constructs