The Development and Ultrastructure of Intergeneric Nuclear Transfer Embryos Using Ovine Ooplasm

by

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Abstract

This thesis encompasses work that aimed to further understand genomic reprogramming, an event crucial in obtaining development in cloned embryos produced by somatic cell nuclear transfer (SCNT). An increasing number of different mammalian species have been cloned using nuclear transfer technology since Dolly the cloned sheep was first successfully produced. However, the biological mechanisms involved in the process of nuclear reprogramming are yet to be fully described. At the centre of this study was an intergeneric SCNT model, which was implemented to determine whether reprogramming factors are conserved across genera. The interaction between donor nucleus and recipient ooplasm was characterised with regard to developmental potential, timing of genome activation, nucleolus formation, and expression of significant proteins.

In initial studies, fusion parameters of the intergeneric SCNT procedure were optimised for the ovine cytoplast and porcine donor granulosa cell. Cell fusion and lysis percentages were determined over a range of electrical pulse voltage, duration and repetition. The optimal electrofusion settings were a single DC pulse of 1.5 kV/cm for 20 μsec following a 2 sec 400 kHz alignment pulse. In addition, it was demonstrated that ovine oocytes were sensitive to electric stimulation to the extreme that oocyte activation would occur no matter how low the voltage. The practical significance was that it would not be possible to implement a fusion before activation protocol.

The ability of the ooplasm of one species to replicate chromosomes and support early embryo cleavage was determined in a preliminary experiment where intergeneric embryos were produced by SCNT using bovine and ovine foetal fibroblasts, and ovine ooplasm. After their construction, the embryos were allowed to develop for 7 days in vitro and the developmental stage determined by Hoechst staining and nuclei counting. In addition, chromosome spreads of the ovine and

bovine somatic foetal fibroblast cell lines used in SCNT, as well as the intra- and intergeneric SCNT embryos were prepared to determine whether the ovine ooplasm was replicating the chromosomes according to the karyotype of the donor nucleus. The somatic cells were karyotyped with 54 and 60 chromosomes counted for ovine and bovine cells respectively. Bovine-ovine embryos were characterised as having a bovine karyotype as distinct from an ovine karyotype, due to the presence of only two metacentric chromosomes as compared with six that are found in the latter. These preliminary results indicated that bovine nuclei obtained from foetal fibroblast cells could initiate early pre-implantation embryo development with the support of ovine oocyte cytoplasm. The development of a proportion (33%) of ovine-ovine intrageneric SCNT embryos beyond the 16-cell stage indicated that an extensive characterisation of an intergeneric model could be performed satisfactorily.

It was hypothesised that the ovine ooplasm would possess the ability to direct in vitro preimplantation embryo development after nuclear transfer using donor nuclei from a different genus, as has been demonstrated in studies using bovine and rabbit ooplasm. In this study, intergeneric SCNT embryos were constructed by the separate fusion of porcine and bovine cells with ovine cytoplasts (bovine-ovine and porcine-ovine respectively), cultured in vitro and the developmental characteristics compared with ovine-ovine SCNT embryos as well as ovine in vitro produced (IVP) embryos. These four groups of embryos were sampled to determine embryo cell numbers at 24, 36, 48, 72, 96, 120 and 168 h post-activation to compare development over time. Despite cleaving normally and undergoing the first three cleavage divisions at a rate comparable with ovine-ovine SCNT embryos, a major block in development occurred in the intergeneric embryos at the 8-16 cell stage. Consequently, no blastocyst formation was obtained as observed for the IVP and ovine-ovine SCNT controls. These results indicate that unlike the rabbit and bovine ooplasm, the ovine ooplasm is not suitable for intergeneric reprogramming of somatic nuclei from another genus, at least of porcine or bovine origin.

To determine the effect of a less differentiated donor nucleus on intergeneric developmental potential, embryonic cell nuclear transfer (ECNT) was conducted in a separate experiment by fusing pluripotent bovine and ovine donor cells (obtained from day-4 preimplantation embryos) to ovine cytoplasts. After 7 days of culture, the cell number of embryos was determined by Hoechst staining and fluorescent observation. Despite observing a single bovine-ovine blastocyst (4.8%), the developmental block remained at the 8-16 cell stage of development. This outcome indicates that a less differentiated nucleus does not increase intergeneric developmental capability.

It is well documented that the ooplasm supplies a large amount of mRNA and protein to the newly formed embryo, crucial for normal development leading up to the major activation of the embryonic genome. However, the interaction between the ooplasm as compared with the donor nucleus in SCNT embryos during this developmental period is poorly understood. This intergeneric SCNT model provided an opportunity to determine the role of the ooplasm on nucleolus formation, which is a marker for genome activation. Ultrastructural evidence was obtained that indicates the ovine ooplasm directs the initial assembly of the nucleolus independent of the species of the nuclear donor. Intergeneric porcine-ovine SCNT and intrageneric ovine-ovine SCNT embryos were constructed and the nucleolus ultrastructure and nucleolus associated rRNA synthesis examined in 1-, 2-, 4-, early 8-, late 8- and 16-cell embryos using transmission electron microscopy (TEM) and light microscopical autoradiography. Intergeneric porcine-ovine SCNT embryos exhibited nucleolar precursor bodies (NPBs) of an ovine (ruminant) ultrastructure, but no active rRNA producing fibrillogranular nucleoli at any of the stages. Unusually, cytoplasmic organelles were located inside the nucleus of two porcine-ovine SCNT embryos. The ovine-ovine SCNT embryos, on the other hand, revealed fibrillogranular nucleoli in 16-cell embryos. In parallel, autoradiographic labelling over the nucleoplasm and, in particular, the nulcleoli was detected. Bovine-ovine SCNT embryos at the 8-

cell stage were examined for nucleolar morphology and exhibited ruminant-type NPBs as well as structures that appeared to comprise of broken down fibrillar material, perhaps formerly of nucleolar origin from the donor cell. These observations indicate that factors within the ovine ooplasm are playing a role in the initial assembly of the embryonic nucleolus in intrageneric SCNT embryos.

To further characterise nucleolus formation, immunocytochemical localisation by confocal microscopy of nucleolin, fibrillarin and RNA polymerase, three key proteins involved in processing rRNA transcripts, was performed on early 8-, late 8- and 16-cell embryos for ovine-ovine and porcine-ovine SCNT embryos. Nucleolin was localised throughout the nucleoplasm for all developmental stages examined in porcine-ovine and ovine-ovine SCNT embryos and, in particular, intensity around the presumptive nucleolar compartments in the later developmental stages. Fibrillarin and RNA polymerase I, on the other hand, were not detected in any ovine-ovine or porcine-ovine SCNT embryos or ovine IVP controls, although both proteins were detected in control bovine IVP blastocysts. This result indicates that the antifibrillarin and anti-RNA polymerase I were not compatible with the ovine form of these respective proteins. As nucleolin is not present in porcine in vivo embryos before the major activation of the embryonic genome, its presence in porcine-ovine SCNT embryo nucleus indicates that nucleolin is derived from the abundant protein and mRNA stored in the ovine ooplasm.

The intergeneric SCNT model established in this thesis demonstrates that the ovine ooplasm lacks the ability to support embryonic development beyond the 16-cell stage. The TEM and autoradiographical studies, in combination with the protein immunocytochemistry study, confirmed that these embryos are unable to undergo the major activation of the embryonic genome, and that the ooplasm influences the initial nucleolar assembly in these embryos.

Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Hamish Hamilton

23rd January 2004

Acknowledgements

Definitions

Oocyte(s) – A female gamete.

Zygote(s) – The cell formed by the union of two gametes; a fertilised ovum containing a male and female pronucleus.

Embryo(s) – The zygote after the first cell division; contains two or more blastomeres.

Cytoplast(s) – An ovum from which nuclear chromatin located in the first polar body and metaphase plate have been removed.

Couplet(s) – The non-fused combination of a cytoplast and a donor cell.

Cybrid(s) – Fused couplets before activation.

Pseudozygote(s) – The cell formed by the union of a diploid nucleus and enucleated ovum after activation.

Abbreviations

A. Chemicals and Reagents

SCNT	somatic cell nuclear transfer
ECNT	embryonic cell nuclear transfer
BSA	bovine serum albumin
CO_2	carbon dioxide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
N_2	nitrogen
O_2	oxygen
6-DMAP	6-dimethylaminopurine
PBS	phosphate buffered saline
SOF	synthetic oviduct fluid
$SOF-HCO_3$	bicarbonate buffered synthetic oviduct fluid
TCM-199	tissue culture medium-199
BoOv	cloned embryo constructed with bovine donor nucleus and ovine cytoplast
NT	nuclear transfer
TEM	transmission electron microscopy
DFC	dense fibrillar component
DNA	deoxyribonucleic acid
FC	fibrillar centre
GC	granular component
IVC	*in vitro* culture
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
IVP	*in vitro* produced
NOR	nucleolar organiser region
NPB	nucleolar precursor body
FC	fibrillar centre
DFC	dense fibrillar component
GC	granular component
COC	cumulus-oocyte complex (es)
Ovaa	ovine oviductal fluid concentrations of amino acids
OvOv	cloned embryo constructed with ovine donor nucleus and cytoplast
PrOv	cloned embryo constructed with porcine donor nucleus and ovine cytoplast
DNA	deoxyribonucleic acid
mtDNA	mitochondrial DNA
RNA	ribonucleic acid
rRNA	ribosomal RNA
HIFCS	heat inactivated fetal calf serum
HISS	heat inactivated sheep serum
B	blastocyst
EB	early blastocyst
XB	expanded blastocyst
HB	hatched blastocyst
CM	compact morulae
LH	luteinising hormone
FSH	follicle stimulating hormone

B. Symbols and units

C	centigrade
g	gram (s)
h	hour (s)
i.u.	international units (s)
mg	milligram (s)
min	minute (s)
ml	millilitre (s)
mM	millimolar
mm	millimetre (s)
mOsm	milliosmolar
µg	microgram (s)
µl	microlitre (s)
µm	micrometre (s)
l	litre (s)
kV	kilovolt (s)
UV	ultraviolet

Preface

Aspects of the work presented in this thesis have been reported elsewhere:

Abstracts:

Hamilton, H.M., Walker, S.K., Webb, G.C., Maddocks, S. and Peura, T.T. 2000. Development of bovine-ovine interspecies clones: preliminary studies. Proceedings of the 17th Annual Conference of the Australian Society for Reproductive Biology, Canberra, ACT, Australia.

Hamilton, H.M., Kleeman, D.O., Rudiger, S., Maddocks, S., Walker, S.K., and Peura, T.T. 2003. In vitro development of cross-species nuclear transfer embryos constructed with ovine, bovine and porcine donor cells and ovine cytoplasts. Theriogenology 59:255 (Abst.).

Papers:

Hamilton, H.M., Walker, S.K., Maddocks, S.M., Maddox-Hyttel, P. and Peura, T.T. A developmental study on the capacity of ovine ooplasm to reprogram and support embryonic development after interspecies nuclear transfer. In preparation (to be submitted to *Cloning & Stem Cells*).

Hamilton, H.M., Peura, T.T., Laurincik, J., Walker, S.K., Maddocks, S. and Maddox-Hyttel, P. 2004. Ovine ooplasm directs initial nucleolar assembly in embryos cloned from ovine, bovine and porcine cells. *Molecular Reproduction and Development* **69(2):**117-125.

Peura, T.T., Hartwich, K.M., Hamilton, H.M. and Walker, S.K. 2003. No differences in sheep cell nuclear transfer outcomes using serum starved or actively growing donor granulosa cells. *Reproduction, Fertility and Development*. **15(3):**157-165.

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Chapter 1

Literature Review

Chapter 1. Literature Review

1.1 Introduction and background

In most mammalian species, oocytes are ovulated while at the metaphase II stage of meiosis, where they remain arrested until fertilisation. The oocyte completes meiosis upon fertilisation, resulting in the extrusion of the second polar body and the formation of paternal and maternal pronuclei consisting of sperm and oocyte derived chromatin, respectively. Embryonic development begins after oocyte activation with syngamy of the male and female pronuclei. Following this, a series of mitotic divisions take place before temporal and spatial differentiation into specialised cells that constitute the formation and organisation of the various tissues and organs of a new individual. The processes and mechanisms controlling this cell specialisation (differentiation) are not completely understood. Although cells of the early embryonic cleavage divisions remain totipotent (i.e. are capable of developing into a new individual), individual cells lose their totipotency beyond these stages due to epigenetic changes that have taken place in the DNA or its associated proteins, altering the patterns of gene expression during development. Reprogramming can be described as the ability of the DNA to revert from its differentiated program and regain its developmental totipotency. This occurs to the sperm and oocyte during normal fertilisation but can also be achieved using nuclear transfer whereby a differentiated nucleus is exposed to the cytoplasm of the ovum.

This chapter reviews the nuclear transfer approaches used for cellular reprogramming from the earliest work in the amphibian species through to more recent applications in mammals. Both embryonic and somatic cell cloning research will be covered. Potential applications in animal breeding, transgenesis, medicine, preservation of endangered species and basic research will also be addressed.

1.1.1 Reprogramming of embryonic nuclei

Early experiments in nuclear transfer were conducted with the intent to further understand the processes of differentiation or "nuclear equivalence". The first nuclear transfer experiment was proposed by Hans Spemann (1938) with the purpose of determining whether the information contained within the genetic material was lost or inactivated during cellular development and differentiation. The nuclear transfer procedure used in these early experiments involved activation of the ovum, microsurgical removal of the egg nucleus and introduction of a donor nucleus (reviewed in Di Berardino, 2001). An experiment such as this was performed in the leopard frog *Rana pipiens* by Briggs and King (1952). The developmental potential of embryonic nuclei was examined by transferring a single donor nucleus obtained from cells of blastula and early gastrula embryos consisting of 8,000 to 16,000 cells, into the previously prepared, activated and enucleated egg. These studies demonstrated that the reprogrammed DNA could support early embryonic development. However, the ability of these nuclei to direct embryonic development beyond the blastula stage was restricted (Briggs and King, 1952). In 1962, McKinnell and Gurdon independently provided the first examples of complete nuclear reprogramming by nuclear transfer. Fertile frogs were derived from blastula nuclei in *Rana pipiens* (McKinnell, 1962) and from blastula to hatched tadpole stage nuclei in the South African frog *Xenopus laevis* (Gurdon, 1962).

"Cloning" techniques were not adapted to mammals until relatively recently. In 1981, three live-born mice were reported, derived exclusively from transplanted inner cell mass (ICM) nuclei (Illmensee and Hoppe, 1981). The recipient cytoplasm was that of a zygote which was enucleated after the introduction of an ICM nucleus. This study was controversial as the results could not be repeated. McGrath and Solter (1983) obtained fertile mice after introducing pronuclear karyoplasts into enucleated zygotes. However, when nuclei from more advanced embryonic stages were used, no development beyond the blastocyst stage was

obtained (McGrath and Solter, 1984a). On the other hand, Willadsen (1986) showed that genetic totipotency existed in mammalian embryos when three live lambs were produced from reconstructed embryos. In this experiment single blastomeres from 8-cell stage embryos were fused to enucleated unfertilised oocytes. Since those times, live offspring, derived from embryonic cell nuclear transfer, have also been achieved in cattle (Prather et al., 1987), rabbits (Stice and Roble, 1988), pigs (Prather et al., 1989), goats (Zhang et al., 1991), mice (Kono et al., 1991) and Rhesus monkey (Meng et al., 1997).

1.1.2 Reprogramming of somatic nuclei

Besides embryonic pluripotent nuclei, nuclear transfer experiments have been conducted using nuclei from differentiated cells of particular tissues in larvae and adults in amphibian species (reviewed by DiBerardino and Hoffner, 1970). In all cases, it proved to be more difficult to reprogram differentiated nuclei. Nuclear transfer experiments using cells from various advanced embryonic stages, germ layers and primitive organs of amphibians revealed a progressive decrease in the percentage of normal nuclear transfer embryos.

When differentiated intestinal epithelial cells of tadpoles of the species *Xenopus laevis* were transferred to enucleated eggs, a number of fertile adult frogs were obtained (Gurdon, 1962; Gurdon and Uehlinger, 1966). However, since then studies using nuclei from differentiated keratinocytes (Gurdon et al., 1975) and non-cycling erythrocytes (Brun, 1978) have indicated that differentiated nuclei from adult cells could not direct development beyond post-neurula and larval stages. To explain this discrepancy, it has been suggested that the population of stem cells that exist in the intestine of the tadpole may have contributed to the production of adult frogs in the earlier successes (reviewed in Blau, 1989).

The first mammalian offspring produced following nuclear transfer using an established cell line was reported in 1996 (Campbell et al., 1996). In this experiment, cells derived from day-9 sheep embryos were cultured for 6-13 passages and induced to quiesce by serum starvation

before transferring their nuclei into enucleated oocytes. The following year, the birth of Dolly the sheep using the same procedure described above was reported, demonstrating that it was possible to produce live offspring using adult somatic cells as donor nuclei (Wilmut et al., 1997). This suggested that differentiation of that particular adult cell did not involve irreversible modification of genetic material required for development to term. The investigators started with 434 oocytes from which 277 were fused with cultured adult mammary gland cells induced into quiescence by serum starvation. Only 29 of the reconstructed embryos developed to the transferable stage and only one pregnancy was detected, which eventually resulted in the birth of Dolly. Since this groundbreaking experiment, cloned offspring derived from adult cells have been obtained in cattle (Kato et al., 1998; Wells et al., 1999), mice (Wakayama et al., 1998; Wakayama and Yanagamachi, 1999; Ogura et al., 2000), pigs (Polejaeva et al., 2000), cats (Shin et al., 2002), rabbits (Chesné et al., 2002) and horses (Galli et al., 2003).

1.1.3 Applications of cloning

The potential applications for cloning technology are far reaching and encompass benefits for animal breeding, transgenic animal production, medicine and research.

The ability to multiply individual animals indicates the potential of the technology in animal breeding. Reproducing identical animals with a special or unique genotype is an obvious application. Increasing the size of a gene pool offers the potential of and more cost-efficient animal production systems. However, the high cost of cloning technology and its low efficiencies make this an unrealistic option.

Utilisation of cloning in the production of transgenic animals would be beneficial in numerous ways. Modification of donor cell lines using gene targeting, followed by selection of successfully modified cells to be used for nuclear transfer, would ensure that all animals produced would carry an identical, well defined gene modification in their genome. This

would be a vast improvement from random and animal specific modifications resulting from traditional pronuclear microinjection techniques. Transgenic animal production by microinjection also has a low success rate with only 5% of sheep zygotes injected and transferred expressing the new gene in the resulting animal (Pursel and Rexroad, 1993). Despite current low efficiencies of the cloning procedures, all resulting offspring would be transgenic, thus reducing the cost and efforts associated with the production of transgenic animals. Transgenic animal production is desired for several reasons including the production of large quantities of valuable proteins in the milk and for the production of donor organs for xenotransplantation. It has also been suggested that transgenic animals carrying or being susceptible to particular human diseases could be used as animal models in the research of these diseases (e.g. cystic fibrosis).

An even more promising potential application of cloning technology in medicine is in human cell therapy, or what has now been termed therapeutic cloning (Colman and Kind, 2000). Embryonic stem (ES) cells are capable of differentiating into a wide range of differentiated cell in particular types of tissue and therefore may provide the basis for treatment of diseases associated with permanent damage to specific cell populations that lack the ability to repair or replace cells (Colman and Kind, 2000). In theory, people could provide their own cells as donor nuclei, and by using them to replace the genetic material of their own (if female) or a donor's ovum, embryonic stem cells in culture could be obtained. ES cells could subsequently be induced to differentiate into the desired cell type, providing individually tailored cell and tissue replacements without the current problems of tissue being rejected or the requirement of a strict regime of immunosuppressive drugs.

Another realistic extension of cloning technology is in the preservation and propagation of endangered animal breeds and species. If interspecies nuclear transfer proves to be biologically possible, the potential for species preservation is further advanced. There has

already been one report of nuclear transfer being used to preserve the female genetics of an endangered breed of cattle (Wells et al., 1998). As the efficiencies increase, the demand for the technology in this application will undoubtedly become greater.

In research, groups of genetically homogenous identical animals are desired in order to control genetic variation and to allow the transfer of cells between individual animals. Research on aging (telomeres, telomerase), the role of mitochondrial DNA and other biomedical areas where short generation intervals are advantageous requires the development of reliable methods of somatic cell cloning in mice. The fact that cumulus cells (Wakayama et al., 1998), fibroblasts (Wakayama and Yanagamachi, 1999) and Sertoli cells (Ogura et al., 2000) have been used to produce viable cloned mice is encouraging in this regard.

For commercial applications of this technology, the efficiency and safety of nuclear transfer using cultured cells must be increased. A further understanding on the reprogramming events (or lack there of) is crucial in order to improve success rates leading to the development of healthy cloned offspring.

1.2 Cell differentiation through development

1.2.1 Oogenesis and folliculogenesis

Primordial germ cells can first be seen outside the embryo proper very early in development, in the epithelium of the dorsal endoderm of the yolk sac near the developing allantois. They migrate by amoeboid movements along the hindgut of the embryo until they reach the tissue covering the ventral area of the primitive kidney (mesonephros) known as the genital ridge. At this stage, the germ cells are known as oogonia. During migration the number of oogonia increases rapidly by mitosis. The next phase is the transformation of the oogonia into primary oocytes by the commencement of meiosis, which only proceeds to the prophase I stage. The primary oocytes then become surrounded by a layer of flat epithelial cells to form the

primordial follicle. By the time of birth all oogonia have at least developed into primordial follicles, but the primary oocytes remain arrested in meiosis and do not finish their first meiotic divisions until puberty is reached. Figure 1.1 outlines the stages of germ cell differentiation in comparison with the developmental events that occur in the female reproductive life.

Follicle maturation is characterised by the primary oocyte beginning to increase in size with the surrounding follicle cells changing in shape to produce stratified layers of granulosa cells. At this stage the follicle is known as a primary follicle (Figure 1.2). The development of a primary follicle into a secondary follicle is characterised by the following events: i) granulosa cells and the oocyte secrete a layer of glycoproteins onto the surface of the oocyte forming the zona pellucida, ii) the fibrous cells around the follicle become vascularised to form the theca interna, which is in turn surrounded by theca externa and, iii) the follicle becomes fluid-filled to form an antrum. At the completion of these events, the secondary follicle enlarges further to form the tertiary (or Graafian) follicle, which then awaits the signal to ovulate (Figure 1.2). Whilst the follicle is undergoing this development, the oocyte remains arrested in prophase I of meiosis. However, the nucleus does increase in size, due mainly to the production of large amounts of 'nuclear sap', so that the nuclei of advanced oocytes appear to be bloated with fluid and are usually referred to as germinal vesicles (Baker, 1982).

The growth of the follicle occurs under the influence of the pituitary hormones follicle stimulating hormone (FSH) and luteinising hormone (LH). Not all follicles develop to the tertiary stage as the majority are eliminated by the degenerative process known as atresia. In ruminants, usually only one or two follicles per cycle is selected for ovulation. This number is greater in some species including mice and pigs. The number of follicles that develop and ovulate can be increased by increasing the concentration of FSH and LH in circulation, and

Figure 1.1

Life History of female germ cells (Baker, 1982).

Multiplication by mitosis

PRIMORDIAL
GERM CELLS

Migration to genital ridge

OOGONIA

> BIRTH — rabbit, ferret, mink, vole, hamster

Final interphase

DNA synthesis

Meiotic prophase begins

PRIMARY
OOCYTE

> BIRTH — most mammals

Growth of oocyte and follicle

> PUBERTY

Follicular maturation

> OVULATION — dog, fox

First meiotic division begins

Sperm penetration — dog, fox

First polar body emitted (may divide)

SECONDARY
OOCYTE

> OVULATION — most mammals

Sperm penetration — most mammals
Second meiotic division,
fertilization and emission
of second polar body

PRONUCLEATE
EGG (OOTID)

this is the basis of superovulation treatments that are routinely used in embryo transfer and nuclear transfer programs.

Oocyte meiosis resumes at the time of oestrus in response to the preovulatory surge of LH. Resumption is characterised by germinal vesicle breakdown (GVBD), chromosome condensation, formation of the meiotic spindle, completion of the first meiotic division, expulsion of the first polar body and arrest in metaphase of the second meiotic division.

Ovulation occurs under precisely controlled hormonal conditions. A pre-ovulatory surge in LH is thought to cause a final 'wave' of mitosis in granulosa cells so that an optimum number is reached for the tertiary follicle. The quantity of follicular fluid in the antrum also increases dramatically. At a precise time after this LH surge (25 h in the sheep) the oocyte is ovulated from the tertiary follicle.

1.2.2 Spermatogenesis

Spermatozoa are the male gametes that are produced in the testis by spermatogenesis. This is a complex process involving mitotic proliferation to produce large numbers of cells, meiotic division to generate genetic diversity and halve the chromosome number and extensive cell modelling to package the chromosomes for transport (reviewed in Setchell, 1982). Like oogenesis, the process begins in the developing foetus with the primordial germ cells. Mitotic proliferation recommences at puberty, at which time the germ cells are known as A0 spermatogonia. This occurs at the base of the seminiferous tubules, and these cells proliferate slowly and act as a reservoir from which A1 spermatogonia are produced. A1 spematogonia undergo a certain number of mitotic divisions to form primary spermatocytes. Meiotic divisions follow the initial proliferation resulting in the production of four haploid round spermatids. Spermiogenesis then occurs with the maturation of spermatids to spermatozoa,

Figure 1.2

Stages of follicular growth (Baker, 1982).

Antrum

Graffian
follicle

Granulosa
cells

Theca
cells

Oocyte

Early
corpus luteum

Primordial
follicle

Ovulated oocyte
in cumulus
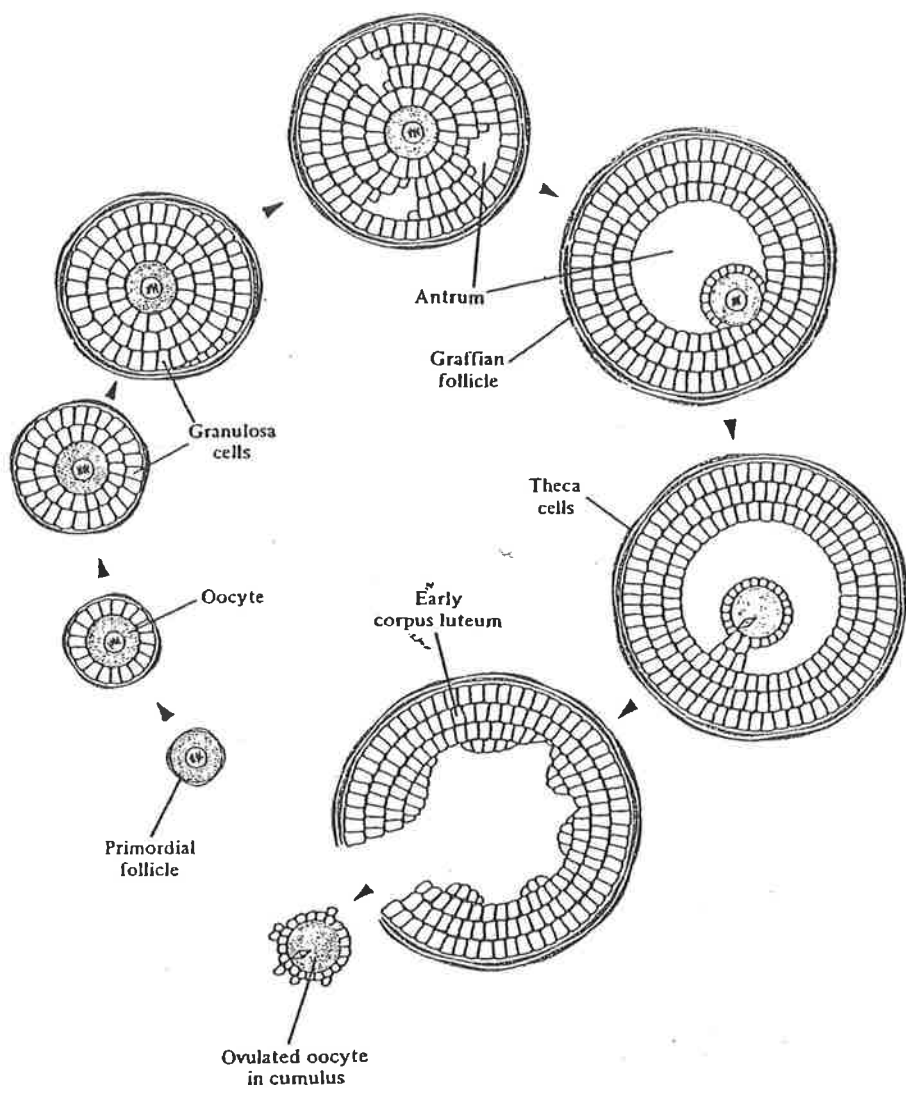
ready to be carried out of the testis. Fully mature spermatozoa are stored in the epididymis awaiting ejaculation. For a more extensive review on spermatogenesis see Setchell (1982).

1.2.3 Fertilisation

Fertilisation is the process by which the male and female gametes fuse, and it usually occurs in the oviduct. Before fertilisation, the sperm must undergo two essential processes; capacitation and acrosome reaction. Through the capacitation process spermatozoa gain the ability to fertilise and it involves stripping the coat of glycoprotein molecules, acquired from the epididymis and seminal fluid, from the membrane of the spermatozoa. The so called acrosomal cap is part of the nuclear envelope at the tip of the spermatozoa and the process by which it breaks down and releases its enzyme content is known as the acrosome reaction. The two main acrosomal enzymes that play important roles in fertilisation are hyularonidase, which digests hyularonic acid holding the cumulus cells together, and acrosin, which is trypsin-like and digests the zona pellucida (Longo, 1997).

After meeting the oocyte and proceeding through the cumulus cell layer, the spermatozoa binds to the zona pellucida and crosses it in a curved pathway with the assistance of the acrosome enzymes. Once the spermatozoon has penetrated the zona it binds to the plasma membrane (oolemma). Fusion of the sperm head with the oolemma results in the sperm nucleus, inner acrosomal membrane and the sperm tail (in most species) being incorporated into the oocyte cytoplasm (Longo, 1997).

Sperm fusion also results in a series of biochemical and morphological events that together constitute what is known as activation of the oocyte. Although this process is not completely understood, particular component events are recognised. The resting membrane potential shifts from −20mV to −40mV as a result of increasing potassium levels, and a release of calcium takes place as a series of waves beginning at the site of spermatozoan attachment.

This release in calcium is important for cortical granule release and polar body extrusion. Cortical granules are small cytoplasmic organelles located in the periphery of the cytoplasm. They contain hydrolytic enzymes, which are released into the perivitelline space after sperm fusion, causing the hardening of the zona and preventing polyspermy. Oocyte activation also induces changes in the oocyte cytoskeleton. The second meiotic division is completed and the second polar body extruded leaving the actual oocyte haploid (Longo, 1997).

Once the sperm nucleus is incorporated into the oocyte cytoplasm, its nuclear envelope disintegrates, disulphide bonds in the DNA are reduced, the chromatin decondenses and sperm specific protamines are replaced with histones. Both the maternal and paternal pronuclei are formed by the development of nuclear envelopes around the respective chromatin. Finally, the pronuclei migrate to the centre of the oocyte where syngamy occurs, consisting of the breakdown of pronuclear membranes and the assembly of chromosomes to the metaphase plate in preparation for the first cleavage division. This movement depends on cytoskeletal activity involving microtubal formation and actin filaments (Longo, 1997).

1.2.4 Developmental stages of the preimplantation embryo

A one-cell embryo that forms immediately after fertilisation, containing maternal and paternal pronuclei, is called a zygote. Once the chromosomes have aligned on the spindle and separated to opposite poles, a furrow appears on the surface of the zygote and the cytoplasm divides to form a diploid two-cell embryo. Each cell of the embryo is referred to as a blastomere (Longo, 1997).

Blastomeres of the embryo multiply mitotically, although the rate of division is not uniform. For example, in the sheep, the four-cell stage exists for approximately 10-12 hours, whereas the embryo remains at the 8-16 cell stage for 24 hours or longer. Importantly, there is evidence that blastomeres are predestined very early in development to differentiate into

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specific tissues. For example, the earliest dividing blastomere in the 2-cell embryo continues to divide early and eventually contributes preferentially to ICM (Moore and Kemler, 1997).

The morula stage generally refers to the 16- to 32-cell embryo in sheep. After this stage is reached, the blastomeres maximise their contact with each other by forming tight gap junctions. This process is known as compaction and the embryo is then referred to as a compact morula. At this stage of development, the outer blastomeres begin to develop polarity, leading to the development of a polar axis along which there is selective permeability to ions, an important feature in the formation of the blastocyst (Balinsky, 1981).

A blastocyst is characterised by the formation of a blastocoele, a fluid filled cavity that forms within the compacted blastomeres. The blastocoele develops as a consequence of fluid being released from polarised cells. The two groups of differentiated cells in the blastocyst are the trophectoderm cells and the ICM (see Figure 1.3). The trophectoderm cells develop from the polarised cells and form the periphery of the embryo and are involved in the formation of the placenta. The ICM cells develop from non-polar cells and are located on the inside of the embryo, becoming responsible for the formation of the foetus. Shortly after the formation of the blastocoele, the embryo sheds its zona pellucida in a process known as hatching. This occurs approximately seven days after fertilisation in sheep and the embryo remains free floating until the process of implantation commences.

1.2.5 Activation of the embryonic genome

The survival of the embryo before the embryonic genome is activated depends upon the synthesis and accumulation of mRNA and proteins in the cytoplasm during oogenesis and cytoplasmic maturation. Most of the RNA is ribosomal and provides the translational machinery to perform the first polypeptide synthesis, whereas proteins are involved in the general "housekeeping" of the early embryo as well as in gene expression. Some of these proteins are highly stable and can even be detected at the morula/blastocyst stage. However,

Figure 1.3

Diagram showing the fate of embryonic cells in the mouse (Moore and Kembler, 1997).

8-cell stage
embryo ──compaction──▶ mórula ──4th mitotic division──▶ 16 cell stage

inner cells ◀── outer cells

inner cell mass trophectoderm

germ cells ◀── embryonic
ectoderm   primitive
               endoderm

chorioallantoic
placenta

parietal
yolk sac

visceral
yolk sac

amnion

endoderm   ectoderm   mesoderm

extraembryonic components
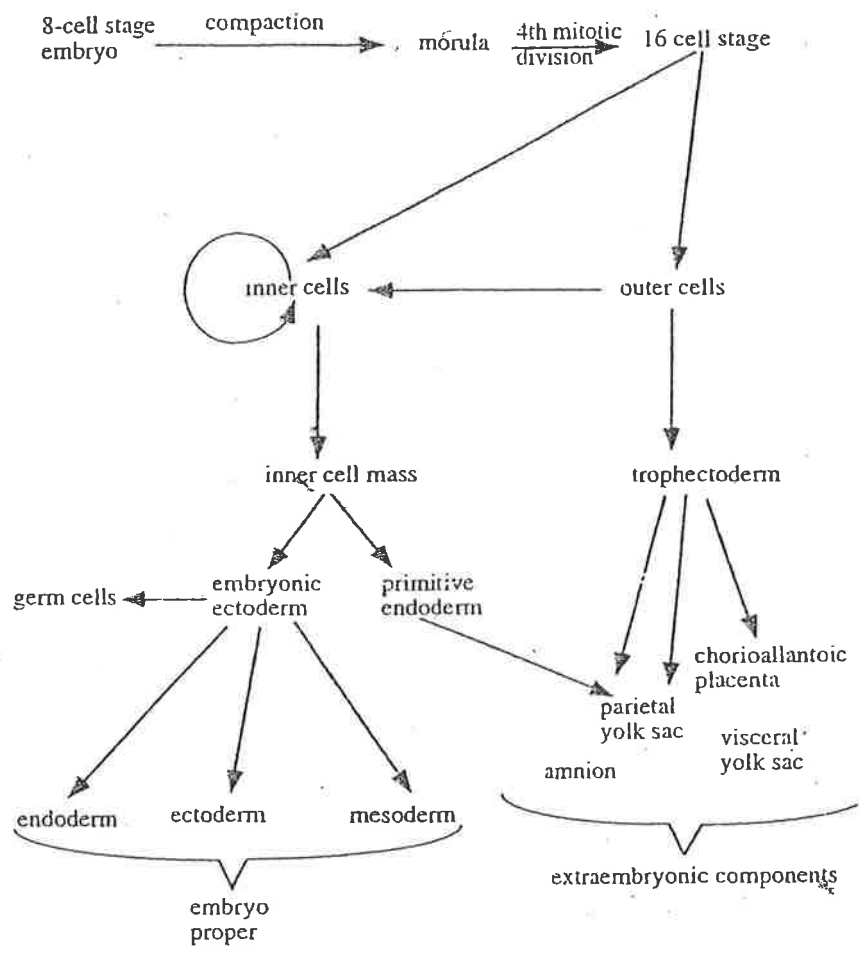
embryo
proper

the role of these proteins is poorly understood, but they are likely to be involved in the regulation of the newly formed genome.

The embryonic genome is not transcriptionally active at the zygote stage but becomes activated at a later stage that varies greatly between species. A major burst of embryo genomic activation occurs in the mouse at the late 2-cell stage (Telford et al., 1990) whereas in sheep it occurs at the 8-cell stage (Crosby et al., 1988). The timing of the genomic activation is not completely understood, although a biological event involving the depletion or accumulation of transcription activators or repressers is likely. Initial transcription involves the synthesis of 70kD proteins known as 'heat shock proteins', which protect genome activity against cellular stresses. Once the genome has been activated, the transcriptional activity of the embryo increases quickly to produce proteins that are important in compaction and blastocoele formation. Also, there are a number of growth factor genes transcribed early in the embryo, and there is evidence to suggest that endogenously produced growth factors such as transforming growth factor-β (TGFβ) may function as cell survival factors during preimplantation development (Brison and Shultz, 1997). A more detailed discussion of the molecular events of the activation of the embryonic genome is made in Section 1.6.

1.2.6 Metabolism of the early embryo

There is a considerable amount of inter- and intra-species variation in the metabolic requirements of the early embryo, which can be partially explained by variation in the activity of metabolic enzymes. Glucose is the key metabolite but is not required during the first two divisions. Lipids are required for the synthesis of new membranes during cell multiplication. Some lipids can be obtained by *de novo* synthesis; however, embryos are generally capable of also incorporating saturated and unsaturated long chain fatty acids taken up from secretions from the female reproductive tract. Amino acids play a pivotal role in energy metabolism,

particularly through the Krebs cycle, and are essential at the blastocyst and hatching blastocyst stages.

1.2.7 Differential expression of paternal and maternal genomes

Both paternal and maternal genomes are required for normal embryo development. However some genes are expressed differentially depending on whether the alleles are inherited from the mother or the father. This phenomenon is known as imprinting and is a consequence of the way in which the DNA is modified in the germ-line. One recognised determinant of imprinting is the degree of DNA methylation. Methylation in general is recognised as influencing gene expression so that hypo-methylation results in upregulated and hyper-methylation in downregulated expression of an allele. One example of an imprinted gene is that of insulin-like growth factor-2 (IGF-2). The IGF-2 allele from the mother is hyper-methylated and therefore minimally transcribed while the opposite is the case for the paternal IGF-2 allele. Imprinting contributes to epigenetic control of gene expression and can therefore influence phenotype. For example, the effect of the in vitro culture environment is thought to contribute to the occurrence of large offspring through changes in the imprinting process (Walker et al., 1992; Young and Fairburn, 2000).

1.2.8 Embryonic cell lineage differentiation

The first contact between the embryo and the endometrium of the uterus is established by the trophectoderm, which consists of two parts. The polar trophectoderm lies above the embryonic disc and, depending on the species, either disappears (ruminants, pigs and horses) allowing the embryonic disc to evert or continues to grow (mice and human) to play an important role in subsequent tissue formation. The parietal trophectoderm surrounds the blastocoele and, in ruminants, undergoes rapid elongation prior to implantation to form the elongated trophectoderm. In mice and humans, it stops dividing after hatching but plays an important role in implantation.

Soon after hatching, the ICM develops into the endoderm, mesoderm, and ectoderm layers. The primitive endoderm originates from the blastocoele side of the ICM and differentiates further into the visceral endoderm and parietal endoderm, which migrate under the trophectoderm. The visceral endoderm develops into the viscera, which is responsible for the formation of organs such as the intestines, lungs and liver. The primitive ectoderm develops from the outer layer of the ICM and subsequently differentiates into the ectoderm and the mesoderm. The ectoderm develops into the epidermis and the nervous system while the mesoderm develops into nonvisceral organs and muscle tissue. The development of the mesoderm indicates the end of histiotrophic support and the commencement of haemotrophic support of the foetus during pregnancy.

1.2.9 Implantation

Implantation patterns vary greatly between species and can be described as either invasive or non-invasive. Invasive implantation occurs when the embryo has a free-living phase of one to two days (e.g. the human). In this case, only a small number of trophectoderm cells make contact with the endometrium due to the small size of the embryo. The maternal response to the presence of a blastocyst occurs within hours and includes an increased vascular permeability in the stromal tissue underlying the endometrium that is in contact with the blastocyst. The stromal cells change in morphology and there is a multiplication and growth of capillaries. Collectively these changes are known as decidualisation, a process where the endometrial components of the placenta are prepared for implantation. The blastocyst signal that begins these changes is not known but it is thought that histamines and prostaglandins are implicated. Soon after decidualisation, the surface of the epithelium associated with the conceptus becomes eroded, resulting in glandular and decidual tissue being destroyed to release large quantities of metabolic substrates that serve to nourish the growing embryo.

Non-invasive implantation (e.g. in sheep, cows, pigs and horses) is characterised by a much later attachment of the conceptus compared with invasive implantation. Another characteristic of the non-invasive implantation is the rapid elongation of the trophectoderm, resulting in a vast surface area for nutrient exchange. In this case, there is no decidual response but some stromal changes do occur indicating that the mother recognises the presence of the embryo.

Following implantation the scene is set for the foetus to proceed in its growth, differentiation and development at a species-specific pace until the birth of a new individual. Throughout this development, foetal growth is characterised by differentiation of cells into complex, highly specialised tissues and organs in a carefully controlled spatial and temporal manner. Control and regulation of these events is outside the scope of this literature review, however at the completion of these differentiation processes, somatic cells of an individual have reached their final differentiated status. It was long though impossible for this status to revert to a non-differentiated status, but that was before the era of modern SCNT.

1.3 *In vitro* embryo production

Mammalian fertilisation and preimplantation embryo development occur within the female reproductive tract, which makes it difficult to study these events *in situ*. Therefore, much of what is known of these processes has been obtained from observations and experiments on embryos that are fertilised and cultured *in vitro,* using culture media and conditions that are able to sustain embryogenesis. The specific needs of embryos vary significantly between species and therefore culture media and conditions need to be suited to cater for these needs.

Production of embryos *in vitro* is desired for both research and commercial animal production systems, where a large number of offspring are desired from a particular sire or dam. It is also used for fertility treatments in human IVF clinics.

To produce embryos completely in a laboratory, immature oocytes can be obtained from ovaries (often from an abattoir), matured until completion of meiotic and cytoplasmic maturation, fertilised using capacitated sperm and cultured until the embryo reaches the preferred developmental stage. In addition, oocytes can be obtained by surgical retrieval.

1.3.1 *In vitro* maturation of oocytes

In vitro maturation of oocytes has become a frequently used technology in animal *in vitro* production (IVP) systems because of its low cost, relative to the cost of collecting mature oocytes from hormone stimulated superovulated female animals.

For the bovine and ovine, ovaries can be routinely collected from abattoirs (Cheng et al., 1986). The ovaries are transported to the lab in PBS at 30-37°C where follicles are aspirated. Immature oocytes, surrounded by thick layers of cumulus cells, are collected from the follicular fluid and transferred into final maturation medium after several washes in handling medium. Most mammals require follicle cell support (i.e. presence of cumulus and granulosa cells to aid cytoplasmic maturation; Staigmiller and Moor, 1984). A commonly employed medium in the maturation of bovine and ovine oocytes is Tissue Culture Medium 199 (TCM199), a commercially available complex medium. It is buffered with HCO_3^- to maintain correct pH in reduced CO_2 conditions within an incubator, and is usually supplemented with the gonadotropins FSH and LH, as well as with oestradiol and serum. Hormone supplementation has been shown to improve oocyte maturation in both ovine (Staigmiller and Moor, 1984) and bovine (Sirard et al., 1988) *in vitro* production systems. Handling medium used in oocyte collection and handling can be one of a variety of different media (TCM199, PBS, SOF), buffered with phosphate or Hepes to maintain correct pH in ambient laboratory conditions. Maturation is performed in a 38.5-39.0°C incubator in an atmosphere of 5% CO_2 in air.

1.3.2 Sperm preparation

For an extensive review of collection, dilution and freezing of ovine sperm, see Evans and Maxwell (1987). All sperm, whether epididymal or ejaculated, fresh or frozen-thawed, must undergo capacitation before they acquire the ability to fertilise ova. Sperm are first isolated from the seminal plasma or freezing diluent, by centrifugation in the case of bovine sperm, or simply by incubating in SOF in the case of ovine sperm. Capacitation of rodent sperm can be successfully achieved by a short incubation in a relatively simple synthetic medium containing bovine serum albumin (BSA). More complex media such as Ham's F10 are used for human sperm. In bovine *in vitro* fertilisation (IVF), highly motile sperm are isolated by a swim-up procedure, and capacitation achieved by exposure to heparin, penicillamine-hypotaurine-epinephrine (PHE) or caffeine treatment (Parrish et al., 1984). For ovine IVF, sperm are capacitated by a simple 60 minute swim-up in a basic medium (e.g. SOF), supplemented with oestrus sheep serum. In most species, high *in vitro* fertilisation rates are obtained although not as high when using *in vivo* capacitated sperm (Longo, 1997).

1.3.3 *In vitro* fertilisation

Mammalian *in vitro* fertilisation was first achieved in the rabbit in 1954 (Dauzier et al., 1954) in experiments where ovulated oocytes were inseminated with sperm capacitated *in utero*. Five years later the biological normality of the event was confirmed with the birth of live offspring. More recently, IVF has been mainly performed with *in vitro* capacitated sperm. Large numbers of sperm are usually used for *in vitro* insemination due to the unknown degree of capacitation of the motile population. This means that the number of spermatozoa near the oocytes *in vitro* is comparably larger than *in vivo*, and the rate of polyspermy can be considerably higher than under natural conditions. If sperm are not fully capacitated, fertilisation may be delayed for several hours. This is undesirable as oocyte aging prior to fertilisation can cause abnormalities, impairing further embryo development.

For IVF it is important to have culture conditions, which maintain sperm survival and motility for several hours. Commonly used media for domestic species include TALP-medium (Bavister and Yanagamachi, 1977; Parrish et al., 1986) for the bovine and SOF (Tervit et al., 1972) for the ovine. The mature ova are transferred into this medium after several washes to remove traces of maturation medium, and a pre-calculated number of sperm are added (usually between 1 and 5 million spermatozoa per millilitre; Parrish et al., 1986). The temperature is also of critical importance. The optimal temperature for humans and mice is 37°C, whereas 38-39°C is required for fertilisation in domestic species such as bovine and ovine. The gas atmosphere in the incubator usually consists of 5% CO_2 in air.

1.3.4 *In vitro* culture of embryos

The first culture medium specifically designed for mammalian embryos was described by Whitten (1956) using a CO_2-bicarbonate buffering system and BSA as a protein source. However, early cleavage stage embryos did not continue development in culture until it was realised that lactate was needed by 2-cell embryos as they are unable to utilise glucose. This demonstrated that early stage mammalian embryos are not like somatic cells, and a whole different approach to *in vitro* culture was required.

In vitro culture should aim to cater for the needs of embryos so that development correlates as closely as possible with *in vivo* development. Although the exact requirements of preimplantaion embryos are not yet fully known, it is recognised that an appropriate medium, a correct substrate for energy as well as sufficient amino acid, growth factor and vitamin supplementation must be provided in order to produce viable embryos (Bavister, 1995). There are three well known approaches to media design (reviewed by Bavister, 1995); i) simple media, which are derived from formulations originally developed for mouse embryos but are now being tailored to the metabolic and nutrient needs of other species, ii) media based on

composition of oviductal fluid, such as SOF (Tervit et al., 1972), and iii) complex media designed for somatic cell cultures (e.g. Ham's F10 and TCM199).

The culture environment used for preimplantation embryos can affect post-implantation events. For example, the production of abnormally large foetuses and offspring following transfer of preimplantation embryos that have spent 3-5 days in culture (Walker et al., 1992) indicates that even short-term exposure to in vitro culture conditions can have profound effects on outcomes. The optimal conditions for the culture of domestic species embryos is between 38 and 39°C in a gas mixture of 5% CO_2 : 5% O_2 : 90% N_2.

1.4 Cloning methods

1.4.1 Blastomere disaggregation and microsurgical bisection

These crude methods of cloning can only be applied to preimplantation embryos. Development to term of disaggregated individual blastomeres has been shown to be possible in the sheep and rabbit when disaggregation is performed up to the 8-cell stage (Willadsen, 1981; Moore et al., 1968). This method involves removal of the zona pellucida, separation of blastomeres by mechanical agitation and transfer of individual blastomeres to a surrogate zona pellucida followed by a period culture (Willadsen, 1981). Embryos at the blastocyst stage can be bisected with a razor blade or a micro-needle to produce monozygotic twins (Baker and Shea, 1985).

1.4.2 Nuclear transfer

The principle of cloning by nuclear transfer involves taking a mature oocyte, removing its chromosomes and replacing them with a diploid nucleus from another cell. This reconstructed embryo is then artificially activated and allowed to develop either *in vivo* or *in vitro*. The

steps required include recipient cytoplast and donor cell preparation, embryo reconstruction and activation.

1.4.2.1 Cytoplast preparation

Mature oocytes for nuclear transfer are obtained from either *in vivo* or *in vitro* sources. To maximise the number of *in vivo* oocytes per donor animal in ruminants, superovulation methods by gonadotrophin stimulation are frequently used (Walker et al., 1989).

Before the chromosomes are removed, oocytes require a pre-incubation in cytochalasin B and Hoechst 33342. Cytochalasin B blocks polymerisation of microfilaments within the oocyte leading to elasticity of the plasma membrane, enabling easy manipulation of the oocyte and resistance to lysis (Luchtel et al., 1976), whereas Hoechst 33342 is a DNA binding stain which facilitates visualisation of chromosomes under ultraviolet light. Oocytes are subsequently enucleated in a medium that also contains cytochalasin B. An oocyte is gently held in position with a holding pipette by aspiration and the zona pellucida penetrated with a bevelled, sharpened enucleation pipette. The polar body and some of the adjacent cytoplasm are aspirated into the pipette, which is then observed under ultraviolet light to ensure that both the polar body and the metaphase plate have been removed from the oocyte (Figure 1.4). Another method for chromosomal removal is oocyte bisection, where the polar body and part of the oocyte containing the metaphase plate are separated from the remaining cytoplasm physically by bisecting with a metal blade (Peura et al., 1998). Once the chromosomal material has been removed, what remains is termed the cytoplast and is ready for the introduction of the donor nucleus.

1.4.2.2 Donor nucleus preparation

Blastomeres at varying developmental stages are used as a source of donor nuclei in embryonic cloning. Blastomeres from uncompacted/early cleavage stage embryos can be isolated by cutting the zona pellucida with a fine glass needle and removing the desired

24

Figure 1.4

Schematic diagram illustrating the process of ovum enucleation.

1. Ovum denuded of cumulus cells.
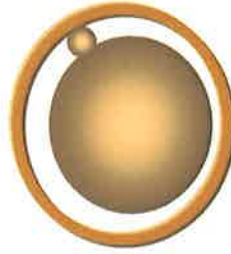
2. Polar body and adjacent cytoplasm removed using micromanipulation techniques.
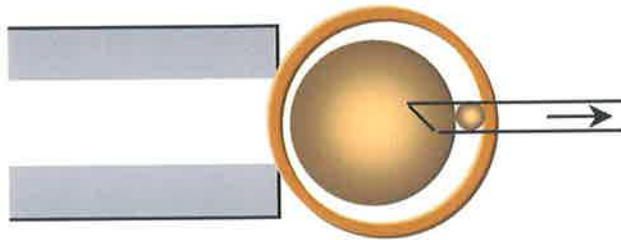
3. Removal of polar body and metaphase plate confirmed using UV fluorescence.
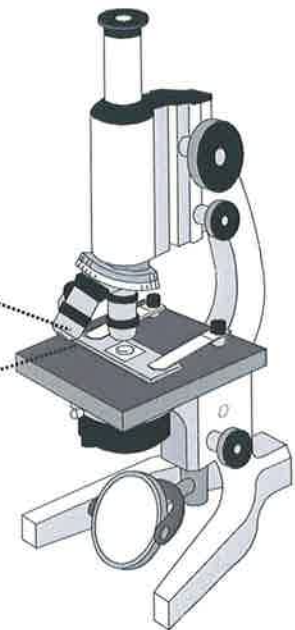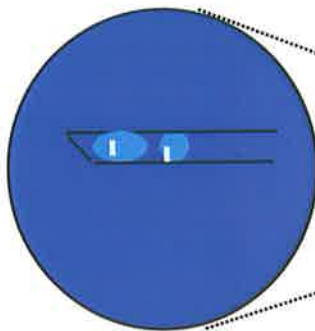
4. Encucleated cytoplasts.
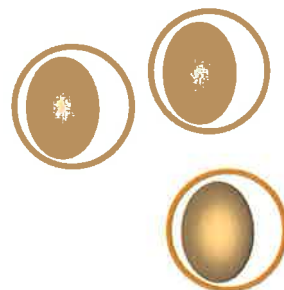
blastomere by aspiration (Campbell et al., 1994). Morulae can be mechanically disaggregated into individual blastomeres with a hand held fine glass pipette in Ca^{++}/Mg^{++}-free medium supplemented with cytochalasin B (Peura et al., 1998). However, isolating blastomeres from more compacted embryos, such as blastocysts, is not as simple as they have reduced dramatically in size and formed tight intercellular junctions. These blastocysts are first placed in either an acidic medium (pH 2) or a solution containing pronase to digest and remove the zona pellucida. To obtain either of the two differentiated cell types of a blastocyst (trophectoderm and ICM) the intercellular adhesions must be loosened. A trypsin/EDTA solution can be used for this purpose (Zakhartchenko et al., 1995). ICM cells can then be separated from trophectoderm cells with a fine glass pipette before final individual cell isolation by further agitation of the ICM and selection with a bevelled pipette.

Somatic cells can be obtained *ex vivo* or from *in vitro* cultured cell lines. Cumulus cells (Wakayama et al., 1998) and skin cells (Wakayama and Yanagamachi, 1999) obtained from mice *ex vivo* have produced live offspring following nuclear transfer, whereas *ex vivo* neuronal and adult Sertoli cells were not successful. Cultured cell lines can be established from a wide range of different tissue types at different stages of development. Cultured fibroblasts derived from both adult (Zakhartchenko et al., 1999) and foetal tissue (Wilmut et al., 1997; Cibelli et al., 1998; Baguisi et al., 1999) have been used as donor nuclei, with development to term following reconstruction. Cultured cells derived from mammary gland (Wilmut et al., 1997), skin cells (Zakhartchenko et al., 1999; Hill et al., 2000), mural granulosa cells (Wells et al., 1999) and oviductal epithelial cells (Kato et al., 1998) have also been used to produce live offspring.

To establish a cell line, a fresh tissue sample (appropriate for the donor cell type) is cut into small pieces and incubated in a medium containing trypsin in order to separate individual cells from each other. Separated cells are then grown in culture flasks until the population

reaches the point where cells have multiplied sufficiently to occupy the whole surface of the flask (confluency) The cells can then be passaged, a process involving washing, removal from the culture flask enzymatically with trypsin, centrifugation and resuspension before being placed into fresh culture medium at a lower cell concentration. Cell growth is measured in population doubling time (ie. the time it takes a population of cells to double). A cell population can be passaged several times until it reaches senescence and dies, which for foetal fibroblasts is usually between 50 and 70 population doublings (Lanza et al., 2000). Cells can be frozen in culture medium supplemented with 10% dimethyl sulfoxide (DMSO) and thawed as required (Hill et al., 2000). Fresh or frozen-thawed cells can then be serum starved by culturing in a medium containing only 0.5% serum instead of the normal 10% for 5-10 days before nuclear transfer to render them quiescent (i.e. G_0 stage; Wilmut et al., 1997). However, a report of live offspring obtained from non-starved foetal fibroblasts indicating that this cell cycle stage is not required for cloning (Cibelli et al., 1998).

1.4.2.3 Embryo reconstruction

For embryo reconstruction, enucleated oocytes (cytoplasts) are held firmly in position with the at the end of a holding pipette with suction and the donor cell introduced into the perivitelline space with an injecting pipette utilising the hole already present in the zona pellucida (Figure 1.5). At this point, the cytoplast and donor cell are adjacent and touching each other, ready for fusion.

Cell fusion was originally accomplished using the Sendai virus (McGrath and Solter, 1983), but today it is most commonly achieved using electrical pulses (Willadsen, 1986; Campbell et al., 1996; Wilmut et al., 1997; Wells et al., 1999). When fusing electrically, the couplet is placed between two parallel metal wires in a medium containing mannitol (Willadsen, 1986; Campbell et al., 1996) and the donor cell and cytoplast are aligned perpendicularly to the metal wires (Figure 1.6). The couplet is then aligned with an AC electric current, transmitted

Figure 1.5

Schematic diagram illustrating the process of subzonal cell injection.

1. 'Empty' cytoplasts after enucleation.
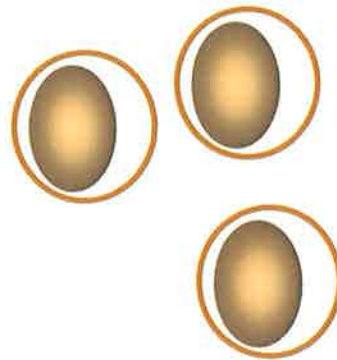
2. Introduction of somatic cell into an enucleated ovum using micromanipulation techniques.
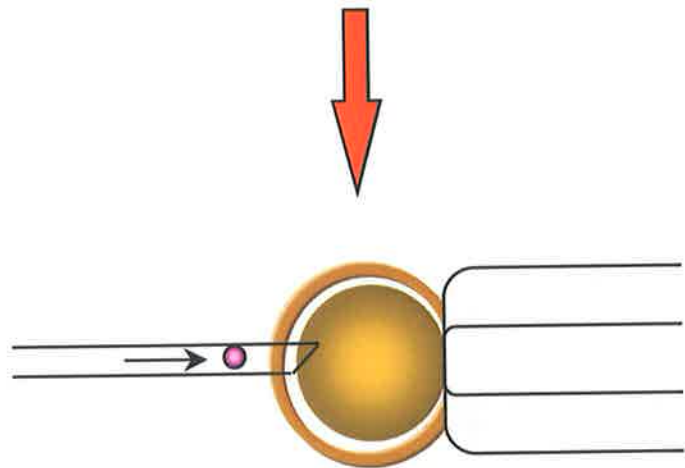
3. Couplets ready for fusion.
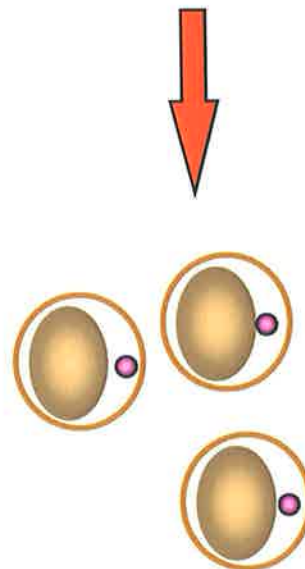
Figure 1.6

Schematic diagram illustrating the electrofusion of a couplet between two parallel plates.

1. Unfused couplets after subzonal injection.
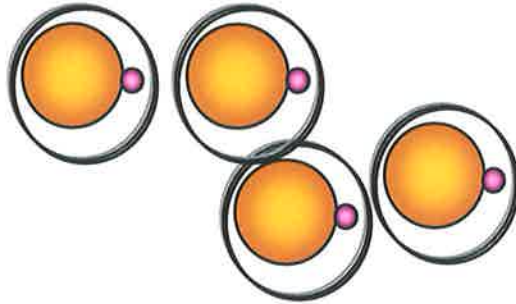
2. Electrofusion of couplet.

Electrodes

Couplet to be fused

Direction of pulse
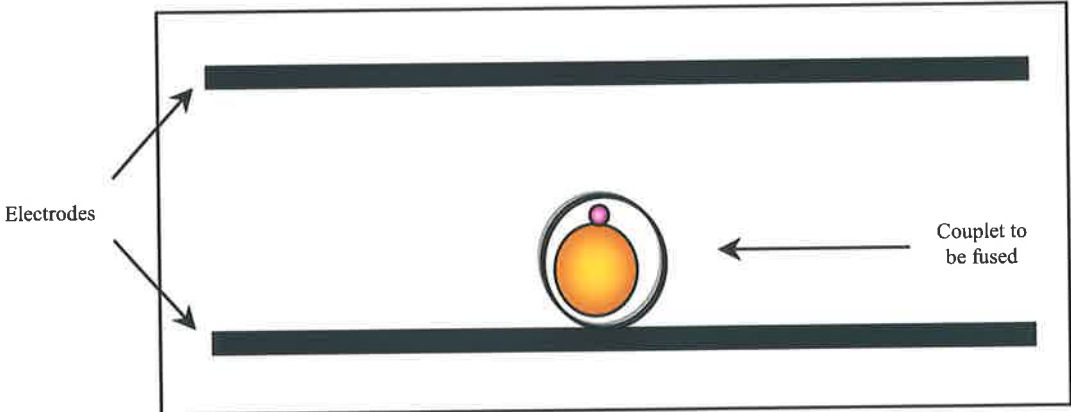
3. Fused couplets, or 'cybrids'.
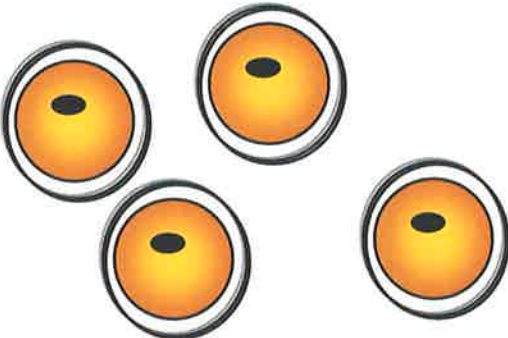
for a few seconds, before the DC fusion pulses are applied. The number, duration and strength of these pulses can vary depending on cell size and species. For example, parameters used to reconstruct the sheep embryo that became Dolly were 3 DC pulses of 1.25 kVcm^{-1} each applied for 80 μsec each (Wilmut et al., 1997).

An alternative method for nucleus delivery, the injection method, involves isolating the donor nucleus from the rest of the cell by breaking the cell plasma membrane, before introducing it directly into the oocyte cytoplasm. To achieve this the oocyte is held with a holding pipette as with sub-zonal injection. However, instead of placing the whole cell under the zona adjacent to the cytoplast, the injection pipette pierces the plasma membrane and the nucleus is deposited in the cytoplasm. This method has been used successfully to produce live offspring in mice (Wakayama et al., 1998; Wakayama and Yanagamachi, 1999). In these studies, the injection procedure occurred with the aid of a piezo-micromanipulator drill and an injection pipette, which is not sharpened but has a flat tip. The piezo drill is attached to the injection pipette and produces very precise, controlled and intense vibrations through the glass pipette, enabling it to pass through the zona pellucida and plasma membrane without excessive force and minimal deformation of the cytoplast (Yanagida et al., 1998). This method of embryo reconstitution makes the fusion step unnecessary and therefore is advantageous when time is a limiting factor.

1.4.2.4 Activation of the cytoplasm after fusion

The aim of artificial activation is to imitate the stimulation to the ovum that occurs naturally sperm penetration. In some cases, electric pulses are adequate to provide the necessary stimulus (Campbell et al., 1996; Wilmut et al., 1997), although, reconstructed embryos can also be activated chemically. This can be accomplished using the combined Ca^{2+}-ionophore (or ionomycin) and 6-dimethylaminopurine (6-DMAP) protocol (Susko-Parrish et al., 1994), where ionophore causes enough calcium transients to activate the cytoplasm, followed by

inhibition of the maturation promoting factor (MPF) activity by 6-DMAP. After the activation, newly reconstructed embryo has the potential to develop as a normal newly fertilised embryo, subject to the success of nuclear reprogramming.

1.5 Factors affecting nuclear transfer

Although it is possible to produce offspring by cloning in a range of mammalian species, there are many developmental problems associated with the technology. Blastocyst rates are often lower compared with conventional *in vitro* production systems, ranging extensively between species and studies. High rates of foetal loss throughout pregnancy, as well as high birth weights, perinatal deaths and poor adaptation to extra-uterine life have been reported (Hill et al., 2000; Wilmut et al., 2002; Peura et al., 2003).

Some of the factors likely to influence the success of cloning are cell cycle stage, mitochondrial DNA status, telomere length and genomic imprinting. A lack of understanding of how these factors and their influence on subsequent embryonic and foetal development after nuclear transfer limits success of the technology. Collectively or singularly, abnormal events brought about by nuclear transfer may lead to inappropriate patterns of gene expression at specific key stages during embryonic, foetal or placental development, thereby contributing to pregnancy losses (Wells et al., 1999).

1.5.1 Cell Cycle

A typical cell cycle consists of four distinct phases: G_1 (gap 1), S (DNA synthesis), G_2 (gap 2), and M (mitosis). Mature oocytes used as recipients in somatic cell nuclear transfer are arrested in meiotic metaphase II (MII), a stage characterised by high levels of active maturation promoting factor (MPF). In normal mammalian sexual reproduction, the transcriptionally quiescent sperm cell enters the MII arrested oocyte at fertilisation, triggering a series of Ca^{++} spikes resulting in ovum activation. Among other effects, Ca^{2+} transients

inhibit MPF activity causing chromatin decondensation, nuclear membrane reassembly, formation of pronuclei and subsequent DNA synthesis in preparation for the first mitotic cell division. However, if an interphase nucleus (G_1, S, G_2) is introduced into the MII arrested cytoplasm in the absence of oocyte activation, MPF activity causes nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC), leading to subsequent DNA synthesis irrespective of the cell cycle stage of the nucleus. As a result, S- and G_2-phase nuclei will not be able to maintain the correct ploidy in a reconstructed embryo because they will re-replicate their DNA and become mixo- or tetraploid (Fulka et al., 1998). During embryo reconstruction, correct ploidy can nevertheless be maintained either by transferring nuclei at G_0 or G_1 stage (equivalent to sperm nucleus stage at fertilisation) into enucleated metaphase II oocytes, or by activating the recipient cytoplast and transferring the donor nucleus irrespective of the cell cycle stage after the disappearance of MPF activity (Campbell et al., 1993).

In the experiments conducted by Wilmut et al. (1997), donor cells were induced into the G_0 stage by serum starvation before use. The authors hypothesised that the degree of gene silencing and inactivation occurring in G_0 nuclei makes those cells resemble sperm cells (also transcriptionally inactive) and therefore facilitates better nuclear reprogramming. However, no direct evidence exists to indicate that nuclei from G_0 stage somatic cells are more readily reprogrammed than those from other cell cycle phases (Fulka et al., 1998). It has since been argued that G_0 stage may not be a crucial factor in the success of somatic cell nuclear transfer after live offspring were produced using G_1 stage cells as donor nuclei (Cibelli et al., 1998). However, whatever the reprogramming factors or mechanisms are, it seems that direct contact of chromatin with the cytoplasm is beneficial for nuclear reprogramming. Recent experiments have suggested that longer exposure of the donor nuclei to the oocyte cytoplasmic factors increases the rate of successful development of reconstructed embryos (Wakayama et al., 1998; Wells et al., 1999).

1.5.2 Mitochondrial DNA

"After the genomic DNA is removed from an oocyte, are the remaining cytoplasmic contents including mitochondria and their DNA compatible with any incoming nucleus, or do they remain species specific?" This is an intriguing question arising from the transfer of nuclei between different species. Eukaryotic cells contain two distinct types of genomes, one located in the nucleus (nDNA) and the other in the mitochondria (mtDNA). Mitochondria are semi-autonomous organelles found in all mammalian eukaryotic cells except mature red blood cells. They are responsible for supplying cells with energy in the form of ATP. Both nuclear and mitochondrial genomes contain genes responsible for transcribing proteins involved in mitochondrial function, and these genes must work in synergy for the mitochondria to function normally. Mitochondrial DNA is of particular interest to reproductive biologists due to its asymmetric inheritance – it is almost exclusively maternal (for a complete review of the role of mtDNA in reproduction see Cummins, 1998). During fertilisation, the mitochondria maintained in the mid-piece of the sperm are introduced into the oocyte cytoplasm (Ankel-Simons and Cummins, 1996). The sperm mitochondrial contribution is, however, thought to be eliminated or inactivated, as it cannot be detected in subsequent offspring. How this happens is not thoroughly understood, although it is known that in rodents and cattle the sperm mitochondria are eliminated by proteolysis during early embryogenesis (Cummins, 1998). Sutovsky et al. (1996) proposed that the mitochondrial sheath in cattle may be tagged with ubiquitin for proteolytic destruction, and there is now evidence confirming this (Sutovsky et al., 1999).

The fate of paternally derived mitochondrial DNA is relevant to cloning as it has implications for the synergistic control of mitochondrial function by both nuclear and mitochondrial encoded genes. Like mammalian fertilisation, cloning involves two sources of mtDNA – from the cytoplast and the donor cell. A study conducted to determine the composition of parental

mtDNA in cloned bovine embryos found that in embryos produced by intraspecific cytoplast-blastomere fusion, mixing and co-existence of paternal mtDNA was observed (Steinborn et al., 1998a). The mixing was non-balanced and the ratio of parental mtDNA was in accordance with the estimated quantitative participation of mtDNA from the fusion partners. A further study (Steinborn et al., 1998b) investigated transmission of parental mtDNA to cloned offspring was investigated. The population of mtDNA was also heteroplasmic with the amount of donor blastomere mtDNA much lower in cloned animals than that of the cytoplast mtDNA. It was also observed that the fewer the cell divisions that had occurred in the donor embryo prior to nuclear transfer, the higher the amount of donor blastomere mtDNA in the resulting cloned offspring.

In a more recent study, the origin of the mtDNA was determined in Dolly, the first mammal cloned from an established adult cell line, as well as in nine other nuclear transfer-derived sheep generated from foetal cells (Evans et al., 1999). From each of these ten sheep it was found that the mtDNA was derived exclusively from the recipient cytoplasts with no detectable contribution from the respective somatic donor cells. It is not known whether this is due to the effect of dilution of the donor cell mtDNA or whether there is an active elimination mechanism involved.

1.5.3 Telomere length

Telomeres are specific DNA sequences at the chromosome ends in all eukaryotic cells, assumed to protect chromosomes from fusion, recombination and degradation (Greider, 1996). It is known that with each DNA replication and subsequent cell division, telomeres are slightly eroded so that, as a general rule, cells from older animals have shorter telomeres than their younger counterparts. This is thought to be important in the aging process (Harley et al., 1990). In a study by Shiels et al. (1999), the telomere lengths in cells obtained from three cloned sheep derived from adult, embryonic and foetal donor cells were shorter relative to

those of age-matched control animals. The authors suggested that full restoration of telomere length did not occur because these animals were produced without germline involvement. It was also suggested that the shorter telomeres in a sheep derived from an adult cell (Dolly) were consistent with the time the donor cells spent in culture before nuclear transfer. It is important to note, however, that a veterinary examination of these cloned animals confirmed that they were healthy and typical for sheep of their respective breeds and ages (Brem and Kuhholzer, 2002) despite having shorter telomeres. Furthermore, Dolly underwent three normal pregnancies and has successfully delivered healthy lambs, indicating no disturbances in her reproductive health.

In a recent study using cattle, not only was it demonstrated that viable offspring could be produced from senescent somatic cells (cells with zero to four population doublings remaining), but that nuclear transfer could extend the telomere length of these animals beyond that of newborn and age-matched control animals (Lanza et al., 2000). Nuclear transfer also extended the replicative lifespan of senescent cells to greater than 90 population doublings. It is speculated that differences between this study and that reported by Shiels et al. (1999) could be species specific. Differences in nuclear transfer techniques or in donor cell types and cell cycle stages are also possible explanations. Dolly was produced using quiescent cultured adult mammary cells whereas in the study by Lanza and colleagues the cells were senescent bovine foetal fibroblasts. This ability of nuclear transfer to restore somatic cells to a phenotypically youthful state is poorly understood and the mechanisms involved unknown at this stage.

1.5.4 Genomic imprinting

Higher than normal levels of embryonic and foetal losses are experienced as a consequence of most embryo technologies, including nuclear transfer (reviewed in Young and Fairburn,

2000). It is thought that this loss, as well as the so-called Large Offspring Syndrome associated with *in vitro* culture of embryos, may be a result of a disrupted imprinting process.

Imprinting is a parentally dependent trait in which both paternal and maternal alleles are present, but gene expression is restricted to only one allele (Barlow, 1995). Genomic imprinting was first realised after failure in development of mouse parthenogenotes and androgenotes, which contained only maternal or paternal chromosome pairs respectively (McGrath and Solter, 1984b; Surani et al., 1984). These studies revealed that both paternal and maternal genomes are required for development to term.

The process of differentially marking/imprinting parental alleles is known to be initiated or reinforced during gametogenesis and early embryogenesis in the mouse (Latham, 1999). Very little work has been carried out with other species, although in vitro culture and micromanipulation commonly used in nuclear transfer at the time when imprints are established or maintained would provide a mechanism for the induction of imprinting errors (Young and Fairburn, 2000). It has been suggested that genomic imprinting and the associated changes in chromatin configuration are likely candidates for controlling reprogramming after nuclear transplantation (Rideout et al., 2001). At least 40 imprinted genes have been identified (http://www.mgu.har.mrc.ac.uk), with estimates of between 100 and 1000 existing in the mammalian genome (Reik and Surani, 1997). Among the identified imprinted genes are several that have roles in foetal growth and development, including IGF-2, H19 and IGF-2r (Young and Fairburn, 2000).

The most likely mechanism responsible for imprinting is DNA methylation. In mammals, it involves attachment of a methyl group to a CpG dinucleotide (Bird, 1986). For a more detailed description on the process of DNA methylation see Barlow (1995).

In nuclear transfer, imprinting errors are likely to be induced during the culture of the donor somatic cells or in the failure of recipient cytoplasm to restore imprinting patterns of early

embryos in the somatic cell genome. It has been suggested that the successful use of nuclei for nuclear transfer may sometimes depend on the incidence of intrinsic methylation errors that are found in imprinted genes in the donor cell (Jaenisch, 1997). It is not expected that these errors would be erased by demethylation and *de novo* methylation events that occur during early embryogenesis (Young and Fairburn, 2000).

1.6 Activation of rRNA genes in pre-implantation embryos

1.6.1 Introduction

The importance of the cytoplasm's ability to reprogram the donor nucleus to obtain successful cloning outcomes has been discussed. The ability of the newly formed embryo to activate rRNA genes is an important event in the major activation of the embryonic genome. The nucleolus is an organelle found within the nuclei of eukaryotic cells. It plays a crucial role in the synthesis of ribosomal RNA (rRNA), which is then exported from the nucleus into the cytoplasm and used for translation/synthesis of cell proteins (Wachtler and Stahl, 1993). A fully functional rRNA synthesising nucleolus is known as a fibrillo-granullar nucleolus, and it is comprised of entities that represent a specific functional state. It has been mentioned already that major transcriptional activity in an embryo includes the activation of the rRNA genes (Kopecny et al., 1989; Tomanek et al., 1989). The rRNA genes are located at the nucleolus organizer regions (NOR) of the chromosomes, and the transcription of these genes is necessary for the formation of a fully functional fibrillo-granular nucleolus. A fully functional nucleolus appears in an embryo only after the major activation of the embryonic genome. In addition, the detail of nucleolus ultrastructure can serve as a marker of successful embryonic genome activation.

1.6.2 Ultrastructure and rRNA gene activation

An active ribosome-synthesising nucleolus contains three main ultrastructural components. They are the fibrillar centres (FCs) representing the enzymic machinery for synthesis of ribosomal subunits are, a dense fibrillar component (DFC) which carries the unprocessed ribosomal transcripts and a granular component (GC) that represents the location of the processed transcripts associated with protein in the form of pre-ribosomal particles (Wachtler and Stahl, 1993). These structures are easily recognised in cells that have been processed using transmission electron microscopy (TEM) techniques. Figure 1.7 presents a light and electron micrograph of a nucleolus and the afore-mentioned associated structures.

1.6.2.1 Cattle

Active fibrillo-granular nucleoli appear in bovine embryos during the fourth cell cycle, and ultrastructural events associated with formation of active nucleoli are described by Laurincik et al. (2000). During the first three cell cycles however, large electron-dense spheres, known as nucleolar precursor bodies (NPBs) are the most prominent nuclear structures (Figure 1.8). NPBs develop one or several vacuoles as the cell cycles progress. A central primary vacuole develops first, followed by several peripheral secondary vacuoles (Figure 1.8).

At the beginning of the fourth cell cycle, NPBs are found in the nucleoplasm. As the cell cycle progresses, a primary vacuole appears, followed later by several peripheral secondary vacuoles. Towards the end of the cell cycle, a DFC and small FCs develop in the peripheral zone of vacuolated NPBs. The FCs are associated with heterochromatin, to perhaps allow for rRNA genes to be located at the FCs. Thereafter, FCs, a DFC and a GC consisting of pre-ribosomal particles emerge and occupy the remaining portion of the fibrillar sphere, resulting in a functioning fibrillo-granular nucleolus. Functional fibrillogranular nucleoli appear at the beginning of the fifth cell cycle indicating that the meiotically induced inactivation of the rRNA genes has ceased. Although the DFC and FC were initially thought to form at the rim

Figure 1.7

Transmission electron micrograph of a ribosome producing nucleolus from a bovine blastocyst showing the location and ultrastructure of the granular component (GC), the fibrillar centres (FC) and the dense fibrillar component (DFC). The green line represents the possible site of transcription by the rRNA genes (photo courtesy of Poul Maddox-Hyttel of the Royal Veterinary and Agricultural University, Copenhagen, Denmark).
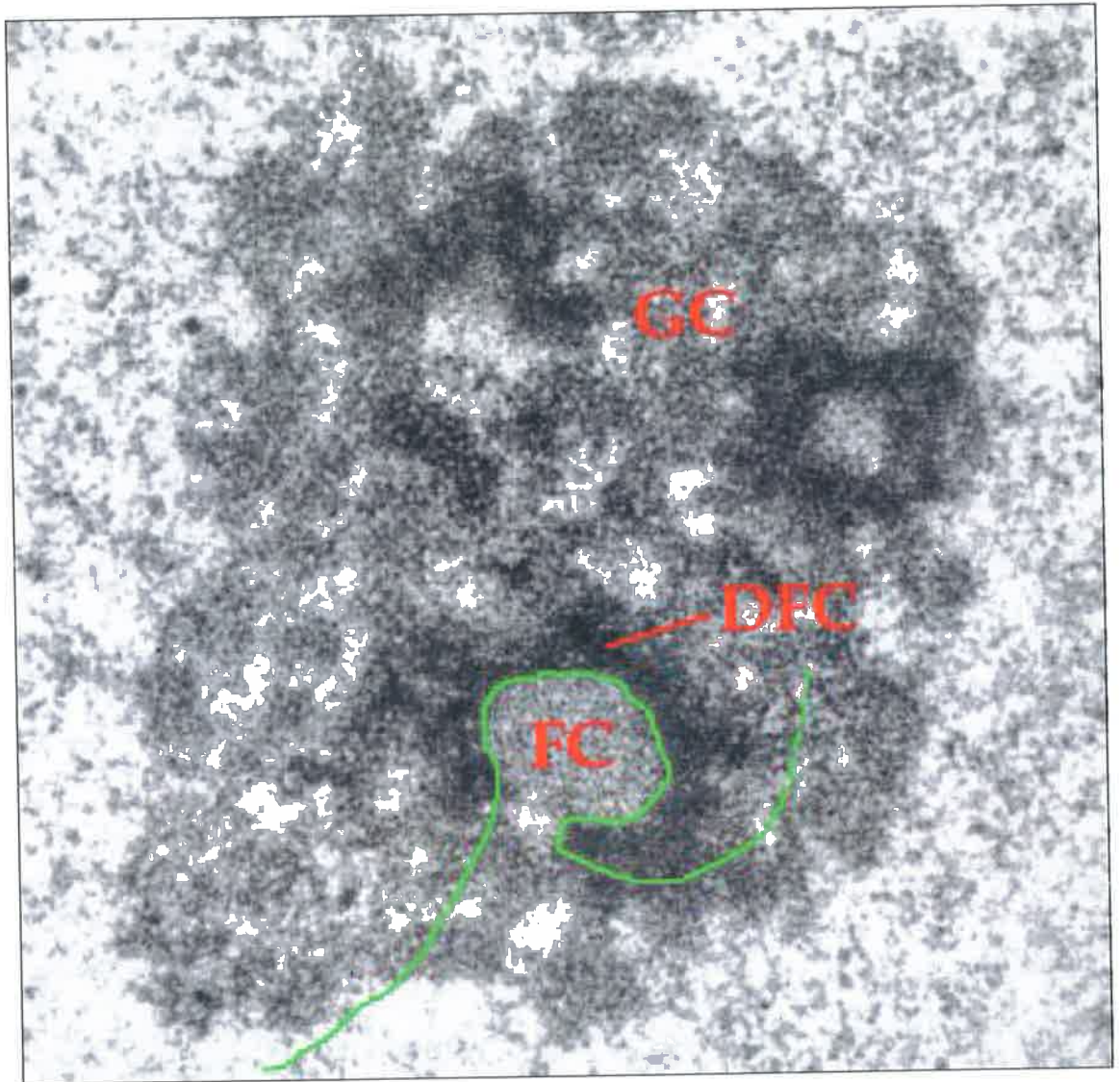
Figure 1.8

Schematic diagram illustrating models of a) bovine b) swine nucleogenesis during embryo development. The embryo stage where the formation of the FCs (red), DFC (blue) and GC (granular) occurs are shown (Hyttel et al., 2000b)
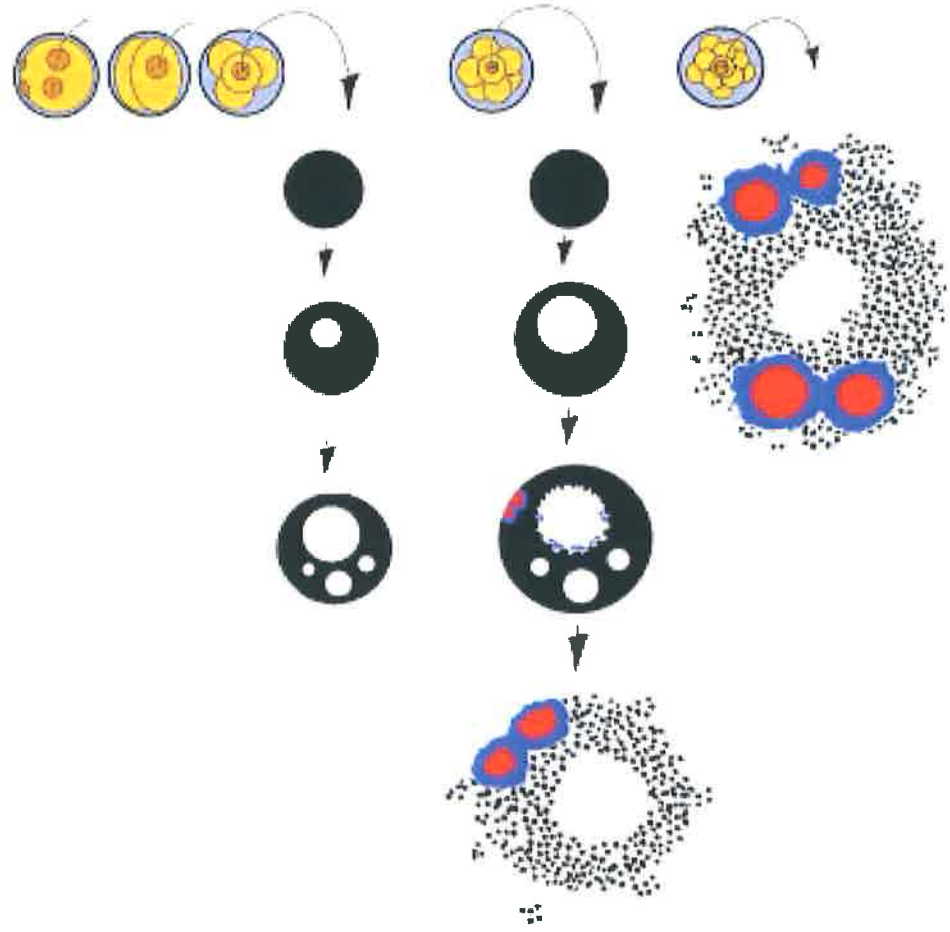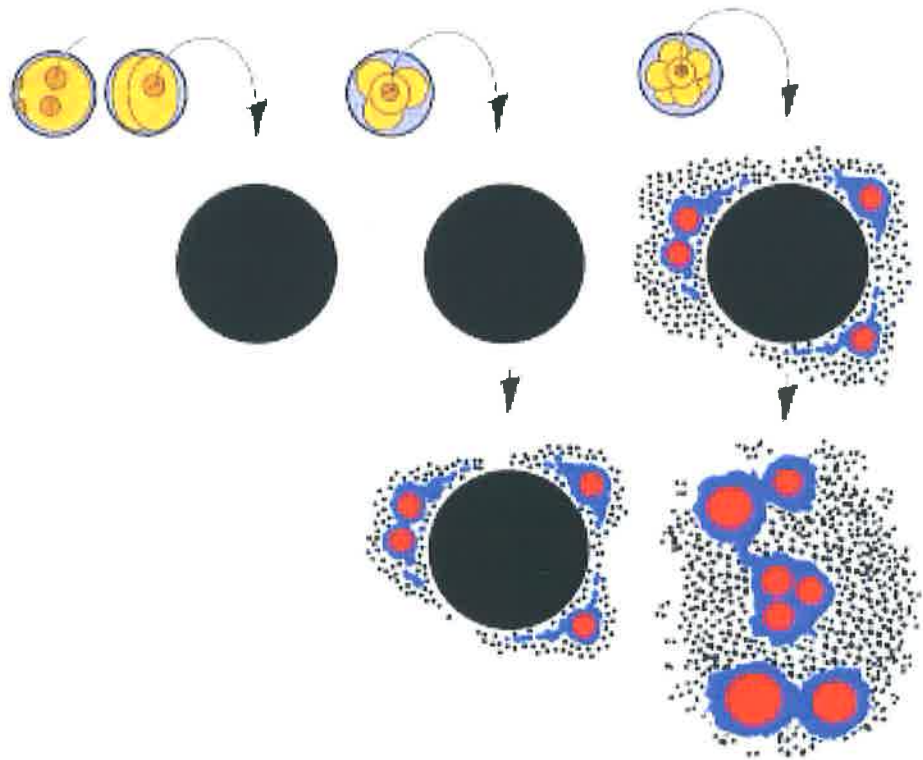
of the primary vacuole inside the NPB (Kopecny et al., 1989), it is now thought that they are formed in the periphery of the NPB (Hyttel et al., 2000b).

1.6.2.2 Swine

Fibrillar centres first appear in porcine in vivo derived embryos at the end of the third cell cycle (Tomanek et al., 1989). NPBs also exist in the embryo during the first and second cell cycles but they are significantly larger compared with bovine embryos. In addition, the NPBs do not undergo vacuolisation as happens with ruminant embryos. At the beginning of the third cell cycle, NPBs are present in the nuclei (Figure 1.8; Hyttel et al., 2000b). At around the midpoint of this cell cycle, there are nuceoli at a variety of different stages of nucleolar formation co-existing in the nucleoplasm. These include inactive NPBs and active fibrillo-granular nucleoli. Nucleolus formation is initiated by the formation of DFC and GC, and later FCs on the surface but not in the NPB (Hyttel et al., 2000b). As this process continues, the structures form semilunar fibrillo-granular nucleoli that encapsulate the fibrillar sphere. By the fifth cell cycle, fibrillogranular nucleoli are formed at the beginning of the cell cycle indicating that the meiotically induced inactivation of rRNA genes has ceased. However, inactive NPBs still co-exist with the active nucleoli (Hyttel et al., 2000a).

1.6.2.3 Comparitive aspects

For cattle and swine embryos, fibrillo-granular ribosome-synthesising nucleoli become structurally recognisable towards the end of the fourth and third cell cycles respectively. In cattle embryos, the NPBs are vacuolised, and significantly smaller than those found in swine embryos. In addition, the formation of DFC and FCs occur in the periphery of pre-existing NPBs in cattle, whereas in the swine, the DFC and FCs appear on the surface of the NPBs (Hyttel et al., 2000b).

1.6.3 Molecular aspects of rRNA gene activation

1.6.3.1 Nucleolar proteins and their role in rRNA gene processing

Nucleolar proteins that have been described and characterised in relation to rRNA gene transcription and processing include topoisomerase I, RNA polymerase I, upstream binding factor (UBF), nucleolin, fibrillarin and nucleolphosmin. These nucleolar proteins can generally be categorised into either 1) proteins such as topoisomerase I, RNA polymerase I and UBF with a well-defined role in transcription and processing of rRNA genes or 2) those such as nucleolin, fibrilarin and nucleophosmin with well-characterised spacial localisation within the nucleolus (Hyttel et al., 2000).

Before rRNA genes can be transcribed, the supercoiled DNA must be uncoiled. This process is mediated by topoisomerase I (Muller et al., 1985) which has been localised to the DFC and FCs (Wachtler and Stahl, 1993). Actual transcription of rRNA genes relies on RNA polymerase I, which has been localised to FCs and to a lesser degree to the DFC (Raska et al., 1989). RNA polymerase is bound to the DNA by several transcription factors, one of which is UBF (Schnapp et al., 1994). UBF is thought to bind to the promoter and recruit another transcription factor, the promoter selectivity factor (SL1; Maeda et al., 1992), thus forming a pre-initiating complex to which RNA polymerase can bind and initiate transcription (Jordan et al., 1996).

The roles for nucleolin, fibrillarin and nucleophosmin are more putative and they are primarily characterised by their nucleolar location. Nucleolin is a phosphorylated protein present in large amounts in nucleoli with active ribosomal biogenesis (Lapeyere et al., 1987). It has RNA-binding properties (Ghisolfi-Nieto et al., 1996) and is identical to human DNA helicase IV, which unwinds RNA-RNA, DNA-DNA and RNA-DNA duplexes (Teteja et al., 1995). Nucleolin may act in promoting the functional secondary structure of the rRNA that is

necessary for the assembly of the pre-ribosomal particles, although it is not part of the final product. Nucleolin has been localised to the DFC and the GC (Biggiogera et al., 1990).

Fibrillarin was first identified by human autoimmune sera from patients with schleroderma and subsequently localised to the DFC and FCs (Ochs et al., 1985; Raska et al., 1989). It is a small nucleolar ribonucleoprotein (snoRNP) associated with U3 small nucleolar RNA (snoRNA; Lishwe et al., 1985; Lapeyere et al., 1990) and with U8 and U13 snoRNAs (Tyc and Steitz, 1989; Baserga et al., 1991). SnoRNAs are believed to be involved in processing of primary rRNA transcripts (Jansen et al., 1991; Aris and Bloberg, 1991; Tollervey et al., 1991, 1993).

Nucleophosmin may be involved in shuttling nucleolin and other proteins such as the nucleolar protein p120 (Valdez et al., 1994) into the nucleolus. Nucleophosmin also has DNA and RNA binding properties (Wang et al., 1994), ribonuclease activity (Herrera et al., 1995) and associates with the most mature nucleolar pre-ribosomal RNP (Dumbar et al, 1989). Therefore, it has been proposed that nucleophosmin, together with nucleolin functions in the assembly of pre-ribosomal particles. Nucleophosmin has been localised to the DFC and the GC (Biggiogera et al., 1990).

1.6.3.2 Characterisation of bovine embryos

During the first cell cycle, topoisomerase I, RNA polymerase I, UBF, fibrillarin, nucleolin and nucleophosmin have been localised to discrete foci in the pronuclei. It has been suggested that the foci are identical with the NPBs observed by TEM. During the second cell cycle, only fibrillarin and UBF are localised to such foci (Laurincik et al., 2000), indicating that the proteins observed in the first cell cycle were of maternal origin. During the third cell cycle, fibrillarin and UBF display the same pattern of expression as with the second and, in addition, nucleolin and nucleophosmin are localised to foci or NPBs presenting a central vacuole.

During the fourth cell cycle, fibrillarin is initially localised to spherical bodies that later in the cell cycle develop a central vacuole. Towards the end of this cell cycle, this protein is localised to more complex bodies showing several distinct intensely labelled foci embedded in a less intensely labelled matrix. During this cell cycle, UBF is initially localised to small discrete foci, which later in the cycle appear to cluster. Near the end of the cycle, UBF can be localised to complexes similar to those described for fibrillarin. Nucleolin and nucleophosmin are initially localised to small discrete foci that, during the cell cycle, develop a central vacuole (Laurincik et al., 2000). Towards the end of the cell cycle, these entities develop into large shell-like structures as the vacuole expands in size. Topoisomerase can be detected for the first time at around the midpoint of this cell cycle. It is localised to discrete foci that appear to cluster towards the end of the cell cycle (Laurincik et al., 2000). RNA polymerase is also detected at the end of this cell cycle in clusters of discrete foci. The localisation of this range of nucleolar proteins to more complex entities is associated with the formation of fibrillo-granular nucleoli within the NPBs as observed by TEM (discussed above) towards the end of the fourth cell cycle. Therefore, topoisomerase I, UBF, RNA polymerase I and fibrillarin are thought to be mainly located in the fibrillar components and nucleolin and nucleophosmin to the GC (Hyttel et al., 2000b). During the fifth cell cycle, the same labelling pattern described in the fourth cell cycle is observed throughout the cell cycle.

1.6.3.3 Molecular characterisation of swine embryos

In the first cell cycle, labelling of nucleophosmin is localised to large shell-like bodies. The remaining proteins are not localised to any nuclear entities (Hyttel et al., 2000a). During the second and early third cell cycles, none of the proteins are localised to any nuclear entities. This indicates that the nucleophosmin present in the first cell cycle is of maternal origin. Towards the end of the cell cycle, RNA polymerase I can be localised to discrete foci arranged in a shell like pattern (Hyttel et al., 2000a). None of the other proteins have been localised to the nucleus during this cell cycle. This fact parallels the observation by TEM of

the first fibrillo-granular nucleoli forming on the surface of the NPBs towards the end of this cycle (see above).

Early in the fourth cell cycle, topoisomerase I, RNA polymerase I, UBF and fibrillarin, are localised to small foci arranged in a shell-like pattern, whereas nucleolin and nucleophosmin are found in shell-like entities (Hyttel et al., 2000a). This pattern of labelling is compatible with the presence of fibrillo-granular nucleoli.

1.7 Interspecies nuclear transfer

Interspecies nuclear transfer is a phrase used to describe nuclear transfer experiments where the donor nucleus and recipient cytoplasm are from different species. The basic procedures are the same as in other intraspecies nuclear transfer experiments, although whether the culture conditions of reconstructed embryos should be designed to suit those of the donor or recipient species, is yet to be determined.

Brun (1973) showed that nuclei of some mouse and human cell types were capable of replication within the cytoplasm of enucleated frog eggs. This indicated that the possibility existed for an enucleated cytoplasm from one species to successfully promote development of a nucleus from a different species.

1.7.1 Embryonic interspecies nuclear transfer

No further interspecies studies were carried out until after mammalian nuclear transfer technology was used to produce live offspring in the domestic ruminants (Willadsen, 1986; Prather et al., 1987). The first study examining the possibility of mammalian interspecies nuclear transfer was done using embryonic blastomere as a source of donor nuclei (Wolfe, 1993). Bovine donor nuclei from morula stage embryos were transferred to bison, goat, sheep and hamster bisection-enucleated metaphase oocytes. Following in vivo culture in female sheep oviducts, blastocyst development rates observed were 1.9%, 1.7%, 0% and 0%

respectively, compared with 17.7% obtained in bovine-bovine intraspecies control embryos. The development of reconstructed embryos after transfer of caprine morula stage nuclei to bovine and ovine bisection enucleated metaphase oocytes resulted in 1.8% and 0% developing to the blastocyst stage. This study showed that in the species represented, reconstituted embryos produced by interspecies nuclear transfer did undergo one to three nuclear divisions but did not complete normal preimplantation development.
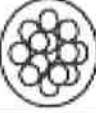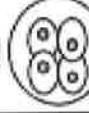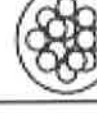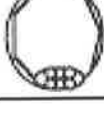
1.7.2 Somatic cell interspecies nuclear transfer

In the most successful interspecies nuclear transfer study carried out to date, the ability of bovine metaphase oocyte cytoplasm to support early embryonic development after the transfer of cultured skin fibroblasts from cattle, sheep, pigs, monkeys and rats was assessed (Dominko et al., 1999). Figure 1.9 summarises the subsequent embryonic development observed for the nuclear donor species. Blastocyst development was achieved in all species except for the rat, for which data was not available due to the fact that all embryos were transferred to recipients after cleavage. An interesting observation was that formation of the blastocoele occurred at a time appropriate for the donor fibroblast species and did not uniformly correlate with bovine embryonic development (Figure 1.9). Reconstructed embryos were transferred to respective recipients in all species except in Rhesus monkey. However, pregnancies were established only in the bovine-bovine intraspecies embryos. Development of interspecies reconstructed embryos to the blastocyst stage has also been achieved using somatic cells of the giant panda (*Ailuropoda melanoleuca*) transplanted to enucleated rabbit metaphase ova (Chen et al., 2002).

Wells et al. (1998) were successful in cloning the last surviving Enderby Island cow using oocytes of a different breed of cattle from slaughterhouse ovaries in what is so far the only successful application (in terms of offspring produced) of nuclear transfer technology to save an endangered breed. Also, ovine (*Ovis aries*) oocyte cytoplasm has supported early

Figure 1.9

Diagram illustrating the average timing of early embryonic development in intra- and interspecies nuclear transfer embryos (Dominko et al., 1999). Skin fibroblasts of each species were transferred to bovine metaphase ovum cytoplasm.

Donor Nucleus Species	Time of In Vitro Culture - Hours After Activation								
	24	48	72	96	120	144	170	194	218
Bos taurus (cow)									
Ovis aries (sheep)									
Sus scrofa (pig)									
Macaca fascic. (monkey)									
Rattus rattus (rat)									

embryonic development under the direction of nuclei from an established cell line of a closely related endangered wild sheep (*Ovis ammon*) after nuclear transplantation (White et al., 1999). One pregnancy was achieved although this did not carry to term. More recently, there was a report of a live offspring obtained using ovine ova (*Ovis aries*) to support development, after transfer, of a closely related wild mouflon sheep (*Ovis orientalis musimon*; Loi et al., 2001).

The difference in developmental success between recent somatic cell interspecies nuclear transfer (Dominko et al., 1999) and that of the embryonic cell nuclear transfer experiments (Wolfe, 1993) is large. Dominko and colleagues suggest that the reason for such high proportions of reconstructed embryos progressing beyond the normal time of transcription is due to the efficient reprogramming of the donor nucleus that occurs regardless of the species. They also suggested the existence of incompatibilities between the new components synthesised by the donor nucleus and the components remaining in the recipient cytoplasm. In such cases, the introduced fibroblast nucleus could be directing simple cell proliferation, and the resulting multicellular structure would have few or no embryonic characteristics. The authors did, in fact, report the occurrence of a number of embryo-like structures with large numbers of cells that would not undergo compaction thus supporting the latter scenario.

1.8 Project Aim

The aim of this project was to develop an intergeneric SCNT model to investigate the interaction between the ovum cytoplasm and donor nucleus, and subsequently examine and characterise this interaction. The additional aim was to study feasibility of ovine cytoplasts to serve as universal recipient cytoplasts for donor cells of other species.

The first objective was to determine suitable experimental parameters that would enable the successful production of intergeneric SCNT embryos, and determine whether embryo

development can be initiated by the ovine ooplasm when combined with somatic nuclei from other genera.

The second objective was to characterise the developmental potential of intergeneric SCNT embryos over the seven-day preimplantation period as compared with intrageneric SCNT embryos and in vitro fertilised embryos.

The third objective was to examine the intergeneric model using transmission electron microscopy and autoradiography to determine the timing of genome activation in intergeneric SCNT embryos (if this did occur) as well assessing the influence of the ooplasm versus the donor nucleus on nucleolus formation.

Finally, the fourth objective was to determine the presence or absence of key proteins involved with either nucleolus formation or genome activation using protein immunohistochemistry techniques.

Chapter 2

Materials and Methods

Chapter 2. Materials and Methods

2.1 Introduction

This chapter provides detailed descriptions of the core materials and methods used in these studies. Procedures used include *in vitro* embryo production, somatic cell culture, nuclear transfer, transmission electron microscopy, autoradiography, immunofluorescence labelling and laser confocal microscopy. Where appropriate, due reference to this chapter and detailed descriptions of other non-core methods are provided in the relevant chapters.

2.2 Chemicals and reagents

The following chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA (catalogue numbers in brackets): agarose (A-4718), alanine (L-form; A-3534), arginine (L-form; A-3784), aspartic acid (L-form; A-4534), BME essential amino acid solution (50x; B-6766), caffeine (C-0750), calcium chloride ($CaCl_2.H_2O$; C-7902), calcium ionophore (C-7522), cytochalasin-B (C-6762), 6-dimethylaminopurine (6-DMAP; D-2629), cystine (L-form; C-8786), dimethyl sulfoxide (DMSO; D-2650), EDTA (ethylenediaminetetraacetic acid; E-6758), EGTA (E-3889), estradiol (E-2758), ethylene glycol (E-9129), gentamycin (G-1264), Glasgows minimal essential medium (G-5154), glucose (D-form; G-7021), glutamic acid (L-form; G-5638), glutamine (L-form; G-5763), glutathione (G-4251), glycine (L-form; G-6388), HEPES (acid; H-6147), HEPES (sodium salt; H-3784), histidine (L-form; H-9386), Hoechst 33342 (bisbenzimide; B-2261), hyularonidase (H-4272), isoleucine (L-form; I-7383), lactic acid (DL-form; sodium salt; L-4263), leucine (L-form; L-1512), lysine (L-form; L-1137), magnesium sulfate ($MgSO_4$; M-2643), mannitol (M-9546), MEM non-essential amino acid solution (100x; M-7145), methionine (L-form; M-2893), mineral oil (M-8410), myo-inositol (I-7508), nocodazadole (N-1404)

ornithine (L-form; O-6503), paraformaldehyde (P-6148), penicillin G (P-3032), phenol red (P-5530), phenylalanine (L-form; P-5030), potassium chloride (KCl; P-5405), potassium dihydrogen phosphate (KH_2PO_4; P-5655), proline (L-form; P-4655), pronase (P-8811), PVA (P-8136), pyruvic acid (P-5280), serine (L-form; S-5511), sodium bicarbonate ($NaHCO_3$; S-5761), streptomycin sulfate (S-9137), sodium pyruvate (P-3662), sodium chloride (NaCl; S-5886), sodium phosphate (Na_2HPO_4; S-5011), sucrose (S-1888), taurine (L-form; T-7146), tissue culture medium 199 (TCM-199 10 x stock; M-0650), threonine (L-form; T-1645), Tris (Trizma; T-1410), trypsin (T-4665), tyrosine (L-form; T-1020), valine (L-form; V-6504).

Bovine serum albumin (BSA; 30036-487), DMEM-powder (1300-021), L-glutamine (25030-081) were obtained from GibcoBRL, Invitrogen Corporation, Carlsbad, CA, USA. Fetal calf serum (FCS; 09702301) was obtained from CSL, Parkville, Vic, Australia. Lutenising hormone (LH; Lutropin-V) and follicle stimulating hormone (FSH; Folltropin) were obtained from Vetrepharm, Belleville, Ontario, Canada. 7X detergent (76-671-25), and phytohemagglutinin (PHA; 151-885) were obtained from ICN Pharmaceuticals Seven Hills, NSW, Australia. Heparin (25000 IU in 5 ml) was obtained from Pharmacia & Upjohn, Bently, Western Australia, Australia. Percoll was obtained from Amersham Biosciences, Uppsala, Sweden. Gluteraldehyde was obtained from ProSciTech, Thuringowa Central, Qld., Australia.

Heat-inactivated sheep serum (HISS) was obtained by collecting approximately 300-400 ml blood from healthy oestrous adult animals into a glass beaker. Blood was allowed to clot for 1 h at room temperature and clot was ringed with 1 ml pipette, and then kept at + 4°C overnight. The serum was decanted and centrifuged at 400 *g* for 30 min. The supernatant was removed and again centrifuged at 400 *g* for 10 min, followed by heat treatment in a water bath set to +56°C for 30 min. The serum was allowed to cool and was then filtered through a 0.22-μm filter, aliquoted and stored at -20°C.

2.3 Consumables, tools and equipment

2.3.1 Consumables

50 ml Falcon conical tubes (352070), 15 ml Falcon conical tubes (352099), 1 ml disposable pipettes (357521), 2 ml disposable pipettes (357507) 5 ml disposable pipettes (357543), 10 ml disposable pipettes (357551), 25 ml disposable pipettes (357525), 14 ml Falcon round bottom tubes (352057), 5 ml Falcon round bottom tubes (352058) were obtained from Becton Dikinson, Franklin Lakes, NJ, USA. T25 tissue culture flasks (163371), T80 tissue culture flasks (153732), four-well culture dishes (176742), and cryovials were obtained from Nunc A/S, Roskilde, Denmark. Petri dishes (36 mm and 90 mm) were obtained from Sarsdtet, Technology Park, SA, Australia. Glass Pasteur pipettes (PP900) were from Maple Leaf Brand, Canada. Thick-wall glass capillaries (GC100-15) and thin-walled glass capillaries (GC100T-15) were obtained from Clark Electromedical Instruments, Pangbourne, Reading, UK. Semen insemination straws were obtained from IMV, France. Glass thaw tubes (13 x 100 mm) were obtained from Borex. 0,22 µm filters were obtained from Millipore Corporation, Bedford, MA, USA).

2.3.2 Tools and equipment

A mouth controlled glass pipette holder was used to move embryos during all aspects of in vitro embryo production except when mentioned otherwise. This was made by attaching a 70 cm length of synthetic silastic tubing to a 0.22 µm Millipore filter with a 5 cm piece of synthetic silastic tubing attached to the other end that held the glass pipette.

Olympus SZ40, SZ60, and SZ11 stereomicroscopes with SZ-ILA trans-illuminator base, Olympus CK40 inverted (cell culture microscope) and Olympus IX-70 inverted (micromanipulation) microscope with an attached U-RFL-T fluorescence illumination were obtained from Olympus Optical Co. Ltd, Tokyo, Japan.

Micromanipulation set up: Narishige IM-55 micromanipulator and Narishige IM-16 microinjector from Narishige International, Tokyo, Japan. Microinjector tubings were filled with Fluorinert (Sigma F-4758).

Electrofusion machine: GenAust fusion machine from Genetics Australia, Bacchus Marsh, Vic 3340, Australia.

Follicle aspiration pump: Cook Medical Technology (V-MAR-5000) from Cook IVF, Eight Mile Plains, Qld., Australia.

Embryo CO_2 culture incubator (MCO-15A) was obtained from Sanyo, Leicester, UK.

Micro-pipettes: P20 for 2-20 µl, P200 for 20-200 µl and P1000 for 200-1000 µl from Gilson Inc., Middleton, WI, USA.

Microscope warm stages: from LEC Instruments, Scoresby, Vic., Australia.

Sutter P87 micropipette puller was obtained from Sutter, Novato, CA, USA.

Microforge was a de Fonbrune-type microforge from Technical Products International Inc., St. Louis, MO, USA.

Capillary tip grinder was from Bachofer GmbH, Reutlingen, Germany.

2.4 Ovine *in vitro* embryo production

2.4.1 Media

Tables detailing all components of stock solutions, base media and final culture media are provided in Appendix I. Medium was prepared in a laminar flow hood observing sterile techniques and filtered into sterile containers using a syringe and a 0.22 µm Millipore filter.

Oocyte aspiration medium comprised of Hepes-buffered tissue culture media 199 (TCM199) (ovine aspiration base medium) supplemented with 2% (v/v) heat inactivated sheep serum (HISS).

Oocyte maturation medium (ovine) comprised of bicarbonate-buffered TCM199 (ovine maturation base medium) supplemented with 20% (v/v) HISS, 5 µg/ml luteinising hormone (LH) and 5 µg/ml follicle stimulating hormone (FSH). After filtration 1 µg/ml of oestradiol was added.

In vitro fertilisation medium (ovine) consisted of bicarbonate-buffered synthetic oviduct fluid (SOF-HCO$_3$) base medium supplemented with 2% (v/v) HISS.

In vitro culture medium (ovine) comprised of SOF-HCO$_3$ base medium supplemented with ovine oviductal fluid amino acids concentrations (OVaa; see Appendix I), and 0.4 g/l BSA.

2.4.2 Ovary collection and *in vitro* maturation

Ovine ovaries collected from the abattoir were placed immediately into phosphate buffered saline (PBS) warmed to 33°C and subsequently transported to the laboratory in a thermosflask within 2 h of collection. Ovaries were placed in a 1000 ml glass beaker and washed three times in PBS prewarmed to 33°C.

Cumulus-oocyte complexes (COCs) were recovered by aspirating 2-4 mm follicles into 2 ml of aspiration medium in a 14 ml Falcon tube. This was done through an 18-gauge needle connected to a follicle aspiration vacuum pump (Cook Australia, Eight Mile Plains, Queensland, Australia) set at a negative pressure of 25-30 mm Hg (see Figure 2.1). COCs with a compact, nonatretic cumulus corona radiata, and a homogenous ooplasm were selected under a stereomicroscope, and washed three times in 3 ml of aspiration medium held in 35 mm petri dishes. Selected COCs were washed twice in 3 ml of maturation medium and placed into 4-well culture dishes. COCs were placed in each well (n = 30-35) containing 0.6ml of maturation medium covered with

0.38ml of mineral oil. Culture dishes were incubated at 38.8°C in 5% CO_2 in humidified air for 24 h.

2.4.3 *In vitro* fertilisation

2.4.3.1 Oocyte preparation

Mature oocytes were treated with ovine aspiration media supplemented with 400 IU of hyularonidase for 20-30 sec to remove excess granulosa cells. Oocytes were subsequently washed three times in 3 ml of fertilisation medium, and placed in groups of 30-35 in 4-well culture dishes containing 0.45 ml of fertilisation medium covered with 0.38 ml of mineral oil.

2.4.3.2 Sperm preparation and gamete incubation

Three pellets of frozen diluted ram semen (100 µl per pellet) were taken from liquid nitrogen storage and placed in a 5 ml glass test tube, and thawed by immersing the tube in a water bath set to 35-36°C. The thawed semen was placed in a 200 µl portion underneath 1 ml of fertilisation medium in a 14 ml Falcon tube and incubated at 38.5°C in 5% CO_2 in humidified air for 1 h. The top 120 µl was subsequently removed and sperm viability and motility was visually assessed and concentration calculated by diluting 1 in 10 in water and completing a haemocytometer count. Sperm were then added to each well containing oocytes at a concentration equivalent to 1×10^6 sperm/ml. Wells of oocytes and sperm were co-cultured at 38.8°C in 5% CO_2 in humidified air for 24 h.

2.4.3.3 In vitro culture

Remnant cumulus-corona cells were completely cleaned from presumptive zygotes using a glass pipette with a bore diameter only slightly larger than the zygote diameter. Presumptive zygotes were washed three times in 3 ml of culture medium in 36 mm petri dishes, before 30-35 zygotes

per well were placed in 4-well culture dishes. The wells contained 0.6 ml of culture medium overlain with 0.38 ml of mineral oil. Embryos were incubated in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C. After 48 h, embryos were assessed for cleavage and uncleaved ova were discarded. Embryos were cultured in this same medium and assessed for blastocyst formation at day 7.

2.5 Bovine *in vitro* embryo production

Tables detailing all components of stock solutions, base media and final culture media are found in Appendix I. Media were prepared in a laminar flow hood observing sterile techniques and filtered into sterile containers using a syringe and a 0.22 μm Millipore filter.

2.5.1 Media

Oocyte maturation medium (bovine) comprised of bicarbonate-buffered TCM199 (bovine maturation base medium) supplemented with 20 % heat-inactivated foetal calf serum (HIFCS), 5 μg/ml LH and 5 μg/ml FSH. After filtration, 1 μg/ml of estradiol was added.

Sperm preparation medium comprised of Hepes-buffered SOF medium (bovine SOF-Hepes base) supplemented with 0.5 g/l BSA, 50 μg/ml caffeine and 30 μg/ml glutathione. After filtration 20 μg/ml of heparin was added.

In vitro fertilisation medium (bovine) consisted of bicarbonate-buffered SOF medium (bovine SOF-HCO_3 base medium) supplemented with 0.6 g/l of BSA.

In vitro culture medium (bovine) comprised of bicarbonate-buffered SOF supplemented with 0.6 g/l BSA, Eagle's BME essential amino acids (1:100 of 50x stock), Eagle's MEM non-essential amino acids (1:100 of 100x stock), 0.2 mM glutamine and 50 mg/ml myo-inositol.

A discontinuous Percoll gradient was prepared by first measuring fractions of 50%, 70% and 90% Percoll in separate 5 ml Falcon tubes using bovine sperm preparation medium as the diluent. Using a glass pipette, the 1 ml Percoll fractions were layered together in a 15 ml Falcon centrifuge tube. This discontinuous Percoll gradient was placed in the incubator until the sperm were thawed.

2.5.2 Ovary collection and *in vitro* maturation

Ovary collection was performed as described in Section 2.4.2. COCs were recovered by aspirating the contents of 2-8 mm follicles using a 10 ml syringe and an 18-gauge needle. Follicular fluid containing the COCs was collected into a 14 ml Falcon tube containing 2 ml of PBS supplemented with 5% (v/v) HIFCS. The fluid was allowed to settle and the sediment containing COCs was transferred to a 90 mm petridish and observed under a stereomicroscope. COCs with compact, nonatretic cumulus corona radiata, and a homogenous ooplasm were selected, and washed three times in 3 ml PBS containing 5% (v/v) HIFCS in 36 mm petri dishes. COCs were further washed twice more in 3 ml of bovine maturation medium, and placed into 4-well culture dishes with 30-35 COCs per well, each containing 0.6 ml of bovine maturation medium covered with 0.38 ml of mineral oil. Culture dishes were incubated at 38.5°C in 5% CO_2 in air (humidified) for 24 h.

2.5.3 *In vitro* fertilisation

2.5.3.1 Oocyte preparation

Matured oocytes were washed three times in 3 ml of bovine fertilisation medium and were physically 'teased' apart from each other using a glass pipette during the washes. Oocytes were placed in groups of 30-35 in 4-well culture dishes containing 0.5 ml of fertilisation medium covered with 0.38 ml of mineral oil.

2.5.3.2 Sperm preparation and gamete incubation

A straw of diluted frozen bull semen was taken from liquid nitrogen storage and thawed by immersing in a water bath set to 35-36°C. After 10 sec, the thawed semen was emptied into a sterilised glass tube. The semen was layered on the surface of a previously prepared (and warmed) Percoll gradient (see Section 2.5.1). The semen was centrifuged for 20 min at 750 g, before removing 120 µl of the pellet and diluting it in 200 µl of sperm preparation medium. Sperm motility was assessed visually and concentration calculated by diluting 1 in 10 in water and completing a haemocytometer count. Sperm were then added to each well containing ova at a concentration equivalent to 1 x 10^6 motile sperm/ml. Inseminated ova were cultured at 38.5°C in 5% CO_2 in humidified air for 24 h.

2.5.4 *In vitro* culture

Presumptive zygotes were denuded (using a glass pipette with a bore diameter only slightly larger than that of a single embryo), after which they were washed three times in bovine culture medium. Presumptive zygotes (30-35 per well) were placed in a 4-well culture dish containing 0.6 ml of culture medium covered with 0.38 ml of mineral oil. Embryos were incubated in an atmosphere of humidified 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C. After 48 h, embryos were visually assessed and the non-cleaved discarded.

2.6 Somatic cell culture

All handling and preparation of somatic cell cultures were performed in a laminar flow hood whilst observing sterile techniques.

2.6.1 Media

Glasgows medium consisted of Glasgows MEM, supplemented with 10% (v/v) HIFCS, 1.2 mM sodium pyruvate, 2mM L-glutamine, 0.15 mg/ml Dulbecco's modified Eagle medium (DMEM), 0.01 mg/ml penicillin G and 0.01 mg/ml streptomycin sulphate. For primary cell cultures, 0.005 mg/ml of gentamycin was also added. This medium was stored at +4°C for 2 weeks.

Starvation medium was made as above for Glasgows medium but with 0.5% (v/v) HIFCS.

Trypsin-EGTA solution consisted of 0.63 g NaCl, 0.012 g Na_2HPO_4, 0.022 g KH_2PO_4, 0.034 g KCl, 0.09 g glucose, 0.27 g Tris, 0.04 g EGTA, 0.01 g PVA and 0.25 g trypsin dissolved in 100 ml of MilliQ water. Aliquots (1 ml) were stored at -80°C.

Freezing medium was prepared by mixing 3 ml of freshly prepared Glasgows culture medium with an additional 20% (v/v) HIFCS and 20% (v/v) DMSO. It was prepared on the day of use.

Calcium and magnesium-free PBS consisted of 0.2 g KCL, 0.2 g KH2PO4, 8.0 g NaCl and 1.15 g Na_2HPO_4 dissolved in 1 l of MilliQ water. It was stored at +4°C for up to one month.

2.6.2 Establishing fibroblast cell line

In the case of foetal tissue, the desired tissue was excised in the laminar flow and placed immediately into a sterile petri dish containing PBS. In the adult animal, the area of skin was swabbed with 70% ethanol, and afterwards the biopsy placed in a sterile Falcon tube containing PBS. The tissue was washed twice in PBS, and once in culture medium. The tissue was then placed in a dish containing 2 ml of HIFCS and cut into fine pieces (approximately $1mm^3$). Using a sterile pipette, the finely cut tissue was placed evenly in a T25 culture flask. The flask(s) was kept at 38.5°C in an humidified atmosphere of 5% CO_2 in air for 30 min, before removing the excess fluid and carefully adding 5 ml of Glasgows culture medium supplemented with 0.005

mg/ml gentamycin and 10% (v/v) HIFCS to the bottom of the flask. The flasks were gently laid on their sides ensuring that the tissue pieces were not disturbed.

2.6.3 Establishing a granulosa cell line

Mural granulosa cells were collected by aspirating antral follicles from an ovary using an 18 gauge needle and 10 ml syringe. The follicular fluid was centrifuged and resuspended in Glasgows medium supplemented with 0.005 mg/ml of gentamycin and 10% (v/v) HIFCS. The cells were washed twice by centrifugation in Glasgows medium supplemented with 10% (v/v) HIFCS, 1.2 mM sodium pyruvate, 2 mM L-glutamine, Dulbecco's modified Eagle medium, penicillin and streptomycin, before seeding into a T25 culture flask containing 5 ml of culture medium. Once a confluent monolayer had been established in approximately 7 days time, the cell culture line was passaged. Cells were cultured for two to three passages in Glasgows medium and small aliquots of passage-2 cells were frozen.

2.6.4 Passaging somatic cells

Somatic cells were cultured in either T25 or T80 culture flasks. The following details are for the T25 flasks and, in brackets, for the T80 flasks. The culture medium was removed from the flask using a sterile pipette, and the monolayer rinsed with 10 ml (20 ml) of calcium and magnesium-free PBS, before the addition of 1 ml trypsin-EGTA solution. The flask was then incubated at 38.5°C for 2 min to detach cells from the flask's surface. A 7 ml (12 ml) portion of Glasgow's culture medium containing 10% (v/v) HIFCS was added to the flask to inactivate the trypsin. The cell suspension was moved with a sterile pipette into a 15 ml Falcon tube and centrifuged at 750 g for 5 min. The pellet was resuspended in Glasgow's culture medium and the cells ready to be split or frozen. In a routine passage, cells would be split 1:4 and seeded in a new flask.

2.6.5 Freezing of somatic cells

Freezing medium was freshly prepared, filtered and placed on ice at least 1 h before freezing. Cryovials were labelled and placed in -20°C freezer at least 1 h before freezing. Cells for freezing were prepared in T80 culture flasks and were trypsinised as previously described (see Section 2.6.4), the pellet was resuspended in 50% of the final volume of culture medium intended to be frozen and placed on ice. The cryovials were taken from the freezer and the lids unscrewed. An equal amount of freezing medium was carefully added dropwise to the cell suspension whilst shaking the tube. Working quickly and using a sterile pipette, the suspension was mixed before dispensing 0.5 ml into each cryovial. The cryovials were immediately placed in -80°C freezer, kept there overnight and subsequently transferred to liquid nitrogen.

2.6.6 Serum starvation

Serum starvation treatment of somatic cells was performed as described by Polejaeva and colleagues (2000). For nuclear transfer we plated cells at 1-5 x 10^4 cells per 35 mm Petri dish in Glasgows medium. Serum starvation was performed when the monolayer reached 100% confluency, which was approximately five days after the cells wereinitially seeded into the plate. The culture medium was removed, and the monolayer rinsed five times with pre-warmed calcium and magnesium-free PBS. Starvation medium was then added into the flask, and cells cultured for an additional two days.

2.7 Open-pulled straw vitrification and warming of donor embryos

2.7.1 Media

During vitrification, all embryos were handled in bench medium, which consisted of ovine Hepes-buffered SOF base medium supplemented with 20% (v/v) HIFCS. Media for embryo freezing included VS 1 medium that consisted of bench medium containing 7.5%

dimethylsulfoxide (v/v) (DMSO) and 7.5% ethylene glycol (v/v), as well as VS 2 medium consisting of bench medium containing 0.6 mol sucrose l^{-1}, 16.5% DMSO (v/v) and 16.5% ethylene glycol (v/v). Media for embryo warming included WS 1 that consisted of bench medium supplemented with 0.3 mol sucrose l^{-1} as well as WS 2 medium comprising of bench medium containing 0.15 mol sucrose l^{-1}.

2.7.2 Preparation

The open pulled straws were prepared from 0.25 ml insemination straws by heating above a heating plate and pulling gently to form long narrow straws. These were cut with a razor blade to yield straws approximately 9 cm long with an outer diameter, at the narrow end, of 0.8 mm. Straws containing frozen embryos were stored in 15 ml conical tubes in liquid nitrogen.

2.7.3 Vitrification

Vitrification of ovine and bovine embryos was performed according to the method of Vajta et al. (1998) with slight modifications. Throughout the vitrification process, all embryos and media were kept at a temperature of 38°C. Morula stage embryos that appeared healthy and with minimal fragmentation were selected and washed twice in bench medium, followed by a 3 min incubation in VS 1 medium. Finally, the embryos were incubated for 20-25 sec in VS 2 medium. During this time they were loaded into the pulled straws by capillary action in a 2 µl volume, followed by immediate immersion into liquid nitrogen.

2.7.4 Warming

On the day before nuclear transfer, the open pulled straw containing donor embryos was taken from liquid nitrogen and warmed in air for 2-3 sec. The narrow end of the straw containing the embryos was then submerged in WS 1 medium. The embryos were immediately expelled into this medium by covering the other open end of the straw with a fingertip. After 1 min, the

embryos were transferred to WS 2 and incubated for 5 min, followed by two x 5 min incubation periods in bench medium alone. Embryos were then cultured in ovine or bovine culture medium, as appropriate, in an atmosphere of humidified 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C until required for NT the following day.

2.8 Micromanipulation tools

2.8.1 Holding pipettes

Thick-walled glass capillaries without filament (1.0 mm outer diameter and 0.58 mm inner diameter) were flame-pulled by hand over an ethanol burner. Immediately, whilst still warm, they were broken at an appropriate diameter, ensuring that there was no jagged edge. A microforge with a glass bead anvil on a 0.1 mm platinum wire was set to a high temperature and the broken capillary moved close to the bead, melting the edges of the capillary leaving a rounded opening of approximately 30 μm (Figure 2.1). The holding pipette was not bent.

2.8.2 Enucleation pipettes

Enucleation pipettes were designed to remove the polar body and adjacent metaphase plate from matured ova. They were made from thin-walled glass capillaries without filament (outer diameter of 1.0 mm and inner diameter 0.78 mm). Capillaries were pulled using a pipette puller (settings: P = 100, heat = 317 (or ramp value ± 10), pull = 255, vel = 80, time = 200), producing two microcapillaries of equal dimensions. Each microcapillary was then cut to a diameter of approximately 20 μm by holding it horizontally and lowering so that it touched the top surface of the glass bead. The bead was then heated momentarily causing the capillary to break at the point of contact. A 30° bevel was created on the tip of the capillary by lowering the end of the pipette against a micropipette grinder in motion. Finally, a spike was made at the tip of the micropipette using the microforge by touching the bevelled capillary tip against the heated glass bead and

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Figure 2.1

The making of holding pipettes for micromanipulation. A glass capillary is flame pulled and broken by hand (A), before the end is rounded using a heating element to the dimensions shown (B).

A

B

1 mm

|←—— 4 cm ——→|←— 1-2 cm —→|

C

100-120 μm
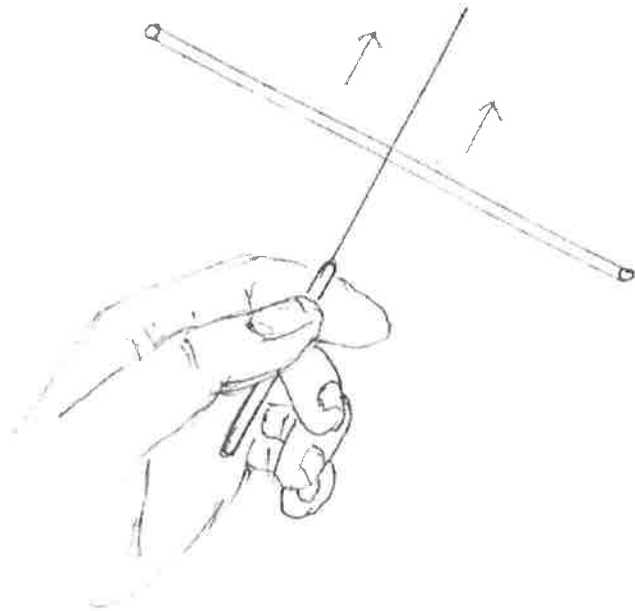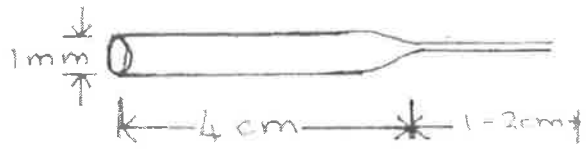
withdrawing it immediately, resulting in a pipette with a sharp spike to facilitate the piercing of the zona pellucida (Figure 2.2).

2.8.3 Injection pipettes

Injection pipettes were designed for picking up and placing a somatic cell into the periviteline space of an enucleated ovum. These were prepared as for enucleation pipettes with slight modifications. Instead of a microcapillary diameter of 20 μm, they were cut with the microforge to a diameter of approximately 25 μm. Also, they did not require a spike on the capillary tip (Figure 2.2).

2.8.4 Micromanipulation chamber

Two-part epoxy glue was used to fix together six glass objective slides to form a rectangular frame (Figure 2.3). When performing NT, two coverslips (50 x 25 mm) were attached to the frame with glycerol-wax adhesive, sandwiching a drop of micromanipulation medium. The frame was cleaned after each experiment and used repeatedly, whilst the coverslips were discarded.

2.8.5 Fusion chamber

A drop of Sigmacote was placed on a glass objective slide and immediately wiped away with a tissue in the area where embryos would be placed (this would contain the drop of fusion medium). Two platinum wires (0.1 mm in diameter) of approximately 20 mm in length were aligned parallel in the centre of the slide, 0.2 mm apart and glued to the slide at the ends with epoxy glue. Each platinum wire was soldered to a stainless steel metal solder tag previously glued to the slide at opposite ends of the wires (Figure 2.3).

Figure 2.2

The making of enucleation and injection pipettes. After the glass capillary has been pulled using a pipette puller, the tip is broken using a glass bead attached to a heating element (A). The capillary tip is then ground to an angle of 45° using a pipette grinder (B), and in the case of enucleation pipettes, a spike made at the tip using the glass bead as illustrated.

A

Heating filament.

Glass bead.

B

45°

Grinding surface

C

Bevelled capillary.

Heating filament.

Glass bead.

D

20 μm — Sharp enucleation pipette

25 μm — Blunt injection pipette
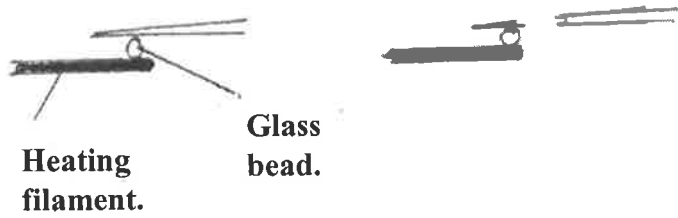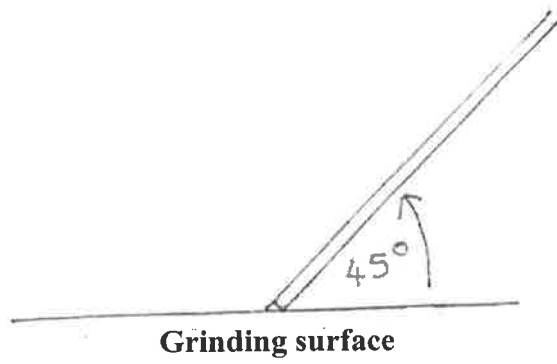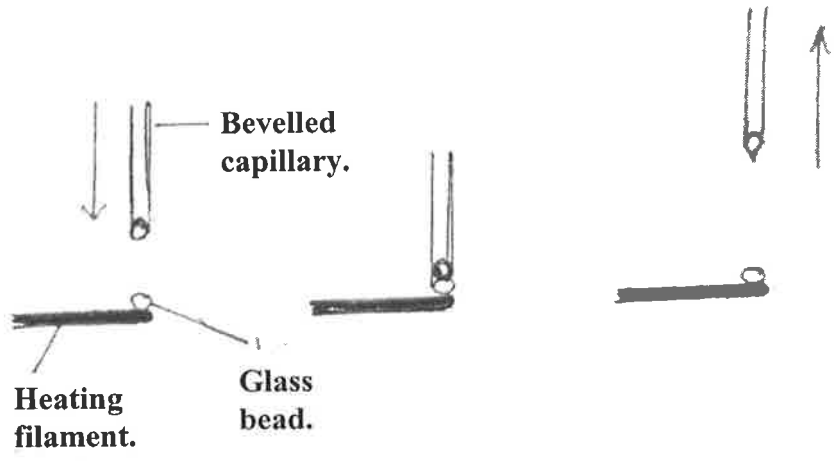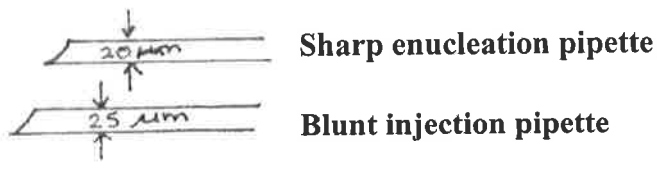
Figure 2.3

The micromanipulation chamber (a) fusion chamber (b) used in nuclear transfer experiments.

A
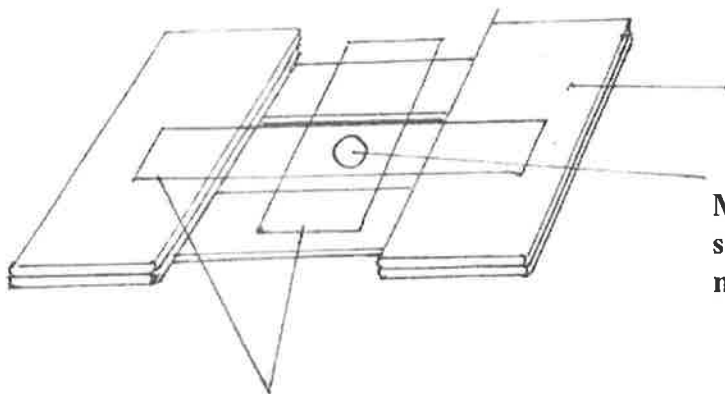
Chamber frame.

Medium drop
surrounded by
mineral oil.

Upper and lower
coverslips.
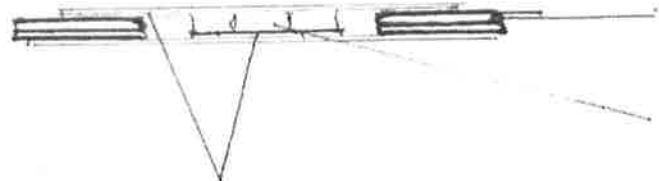
Upper and lower
coverslips.

Medium drop
surrounded by
mineral oil.

B
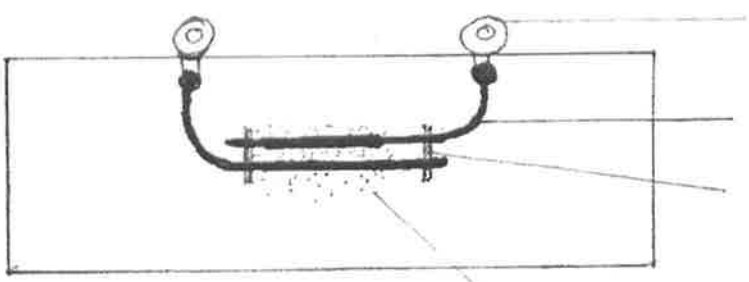
Solder tag to
attach electrodes.

Platinum wire.

Epoxy glue.

Sigmacote.

2.9 Embryonic cell nuclear transfer

2.9.1 Media

Media for holding and manipulating matured ova and cytoplasts was ovine Hepes-buffered (handling medium) or ovine bicarbonate-buffered (culture medium) SOF as appropriate, containing 4 mg/ml BSA and Ovaa amino acids. After the zona pellucida was removed, cytoplasts and reconstructed embryos were held or manipulated in ovine Hepes-buffered (modified handling medium) or ovine bicarbonate-buffered (modified culture medium) SOF supplemented with 20 mg/ml BSA and Ovaa amino acids. An increased concentration of BSA was required to prevent zona-free embryos from sticking onto plastic dish surfaces. Calcium and magnesium free handling medium was prepared using a Hepes-buffered SOF medium made with stocks containing no calcium or magnesium, and supplemented with 0.4 g/l BSA and Ovaa amino acids.

Fusion medium was prepared with 1 ml of 10 mM $MgSO_4$ stock solution, 1 ml of 5 mM $CaCl_2$ stock solution and 8.199 g of mannitol dissolved in 50 ml of MilliQ water. Once dissolved, the total volume was made up to 100 ml with MilliQ water. Osmolarity was adjusted to 280 mOsm with water or mannitol, and pH adjusted with 0.1 M Trizma base to 7.4-7.8. This medium was stored at +4°C for up to 4 weeks, and the pH readjusted before use.

2.9.2 *In vitro* maturation of ovine recipient oocytes

Ovine COCs were obtained and matured as described in Section 2.4, with the exception that the maturation period was 18-20 h. Subsequently cumulus cells were removed, and ova selected for enucleation.

2.9.3 Ovine cytoplast preparation

On the day of nuclear transfer, presumptive metaphase II arrested oocytes were incubated in 400 IU of hyularonidase for 1 min and the cumulus-corona radiata cells completely removed by pipetting. Intact ova with visible polar bodies were selected and placed in ovine culture medium and kept at 38.5°C in 5% CO_2 in humidified air until enucleation.

Enucleation was performed using Narishige micromanipulators (Narishige International, Tokyo, Japan) attached to an Olympus IX70 inverted microscope (Olympus Optical Co. Ltd, Tokyo, Japan). A manipulation chamber was prepared by pipetting 50 µl of handling medium supplemented with 7.5 µg/ml cytochalasin B onto the cover slip which was then covered with a second coverslip (see Figure 2.3). The drop was sealed by pipetting 1 ml of mineral oil between the coverslips. In batches of 15-20, oocytes were incubated in handling medium containing 10 µg/ml Hoechst 33422 and 7.5 µg/ml cytochalasin B for 5 min, washed in handling medium and moved to a drop of handling medium supplemented with 7.5 µg/ml cytochalasin B. Enucleation was performed using a 20x objective and 10x eyepieces. The exact location of the metaphase plate within an oocyte was established with a <1 sec exposure to UV illumination, after which it was removed by aspiration using an enucleation pipette (Figure 2.4). In embryonic nuclear transfer, no attention was paid to the removal of the first polar body, but the successful removal of the metaphase plate was confirmed by viewing the contents of the pipette under UV (Figure 2.4).

2.9.4 Cytoplast activation

After enucleation, at 20-22 hours post maturation (hpm), cytoplasts were placed in protein free ovine Hepes-buffered SOF supplemented with 10 µM calcium ionophore for 10 minutes. They were then washed twice in culture medium before being cultured in culture medium

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Figure 2.4

Oocyte enucleation as seen through an inverted microscope. An ovum is held with a holding pipette so that the polar body is positioned at 2 o'clock (a). The zona pellucida is then pierced and the polar body and adjacent portion of ooplasm aspirated into the enucleation pipette (b). Removal of the Hoechst stained polar body and metaphase plate is confirmed under UV fluorescence exposure (c).

Polar body

Enucleation pipette

Holding pipette

Ovum

Zona pelucida

supplemented with 2 mM 6-dimethylaminopurine (6-DMAP) at 38.5°C in 5% CO_2 in humidified air for 2 h.

2.9.5 Zona-free electrofusion

2.9.5.1 Zona removal

Recently made cytoplasts (22-24 hpm) and donor embryos, were incubated in protein-free ovine Hepes-buffered SOF containing 0.5% pronase for 5 min, washed three times in handling medium and placed in culture medium for 30 min to recover. Immediately before electrofusion, the cytoplasts were cleaned of polar bodies and all remnant zona pellucidae using a glass pipette with a diameter only slightly larger than a zona-free single cytoplast.

2.9.5.2 Donor embryo disaggregation

Donor embryos were incubated in calcium and magnesium free handling medium containing 7.5 µg/ml cytochalasin B for 10 min, and mechanically disaggregated in this medium using a fine-bore glass pipette. The groups of blastomeres were subsequently moved into drops of modified handling medium.

2.9.5.3 Electrofusion

Blastomeres and cytoplasts were first equilibrated in fusion medium for approximately 10 sec, before being transferred into the electrofusion chamber. Electrofusion pulses were produced using a GenAust fusion machine connected to the fusion chamber. The initial couplet alignment was achieved with an AC pulse of 8V and 500 kHz for 5-10 sec, followed by the fusion pulse consisting of a single DC pulse of 100 µsec and 500 V/cm. Couplets were checked 20-40 min later for successful fusion before being transferred to culture medium.

2.9.6 Culture of zona-free embryos

2.9.6.1 Well of the well culture system

All reconstructed embryos were cultured using modified culture medium. Embryos were cultured as a group using the well of the well system (WOW; Vajta et al., 2000) or individually in 20 µl drops to prevent aggregation of zona-free embryos. WOW culture dishes were made by melting 20 small wells into the surface of a well of a polystyrene 4-well culture dish using a darning needle heated to a high temperature under a gas flame for 3-6 sec. Individual wells were filled with SOF supplemented with 20% HIFCS (v/v) and rigorously flushed several times by pipetting to remove air bubbles and any toxic residue that may have arisen from the melting process. The medium was changed and incubated overnight at 38.5°C. The following day the medium was again flushed and replaced with 0.5 ml of modified culture medium, covered with 0.38 ml mineral oil, equilibrated overnight in 5% CO_2 in humidified air at 38.5°C, after which each well was ready to receive an individual zona-free embryo.

2.9.6.2 Microdrop culture

For microdrop culture, eighteen individual 20 µl drops of equilibrated modified culture medium were pipetted into 35 mm culture dishes standing on a cold surface. Drops were immediately overlain with 2 ml of pre-warmed mineral oil and placed in the incubator.

2.9.6.3 Agarose well culture

A 1% agarose solution was prepared by heating 0.02 g of high-resolution agarose in 2 ml of embryo culture medium (without BSA, serum or amino acids) to boiling point 2-3 times. Working rapidly, 200 µl was pipetted onto the bottom of the wells of a 4-well dish, which was then left in the refrigerator for exactly 4 min before 700 µl of complete culture medium was added on top of the agarose layer, followed by 380 µl of mineral oil. A flame-pulled Pasteur

pipette with a bore diameter of 50-100 μm was attached to a mouth hose, and delicately pushed through the agarose layer. The agarose plug was removed by aspiration, and expelled into the well. Once all the agarose-wells had been prepared (up to 35 per well), the dish was swirled to concentrate the agarose plugs into the middle of the well. They were then removed along with 200 μl of medium using a P1000 micropipette leaving a total of 500 μl of media in each well. The dish was then placed in the incubator to equilibrate overnight.

2.10 Somatic cell nuclear transfer

SCNT was performed as described previously (Peura et al., 2003a) with modifications.

2.10.1 Media

Matured oocytes and cytoplasts were held or manipulated in ovine Hepes-buffered (handling medium) or ovine bicarbonate-buffered (culture medium) SOF as appropriate, containing 4 mg/ml BSA and Ovaa amino acids.

2.10.2 Donor cell preparation

Donor cells were used for nuclear transfer at either passages 2 or 3. A frozen aliquot was thawed, and the cells cultured in 35 mm culture dishes for 5 days (until confluency), before being serum starved for 2 days (see Section 2.6.6). Approximately 30 minutes before subzonal injection, a cell suspension of the donor cells was prepared by standard trypsinisation. The cells were centrifuged and resuspended in Glasgows culture medium until injection.

2.10.3 Cytoplast preparation

Enucleation was carried out as described for embryonic cell nuclear transfer (see Section 2.9.3) with care being taken to ensure removal of the polar body together with the metaphase plate.

2.10.4 Sub-zonal cell injection

Donor cells, along with a batch of 15-20 enucleated ova (cytoplasts), were placed in a drop of handling medium in the manipulation chamber. A small portion of the somatic cell suspension was place in the drop so that the cells rested on the surface of the chamber. Using a glass injection micropipette, a single donor somatic cell was placed in the space between the zona pellucida and cytoplast membrane (see Figure 2.5). 'Couplets' were then washed and kept in culture medium until the time of fusion.

2.10.5 Electrofusion

Couplets were pulsed in a fusion medium using a GenAust fusion machine as described in Section 2.9.5 for embryonic cell nuclear transfer with modifications. Zona-enclosed couplets were manoeuvred so that the cell and cytoplast were aligned perpendicular to the platinum wires before the alignment and fusion pulses were applied. For ovine and bovine donor cells, two DC fusion pulses of 1.25 kV/cm and 80 μsec followed an initial AC-alignment pulse of 400 kHz and 10 V, whereas for porcine donor cells a single fusion pulse of 1.5 kV/cm for 20 μsec was used. Fusion success was evaluated within 30 min and intact couplets that did not fuse were pulsed again. Couplets that did not fuse after the second attempt were discarded.

2.10.6 Activation treatment

Within 30 min of pulsing, fused couplets were incubated in protein free ovine Hepes-buffered SOF medium containing 10 μM of calcium ionophore for 5 min. Couplets were then washed twice in culture medium, and placed into culture medium containing 2 mM 6-DMAP, in which they were cultured in 5% CO_2 in humidified air at 38.5°C for 2 h.

Figure 2.5

Sub-zonal injection as seen through an inverted microscope. Selected somatic cells positioned on the bottom of the manipulation drop are aspirated into the injection pipette (a). A cytoplast is held with a holding pipette so that the previous hole in the zona pellucida made during enucleation is in focus at the 3 o'clock position (b). The injection pipette is manoeuvred through this hole and a single somatic cell expelled into the perivitelline space (c), ensuring that the cell and cytoplast membrane are in contact with one another.

Injection pipette

Selected somatic cells

Holding pipette
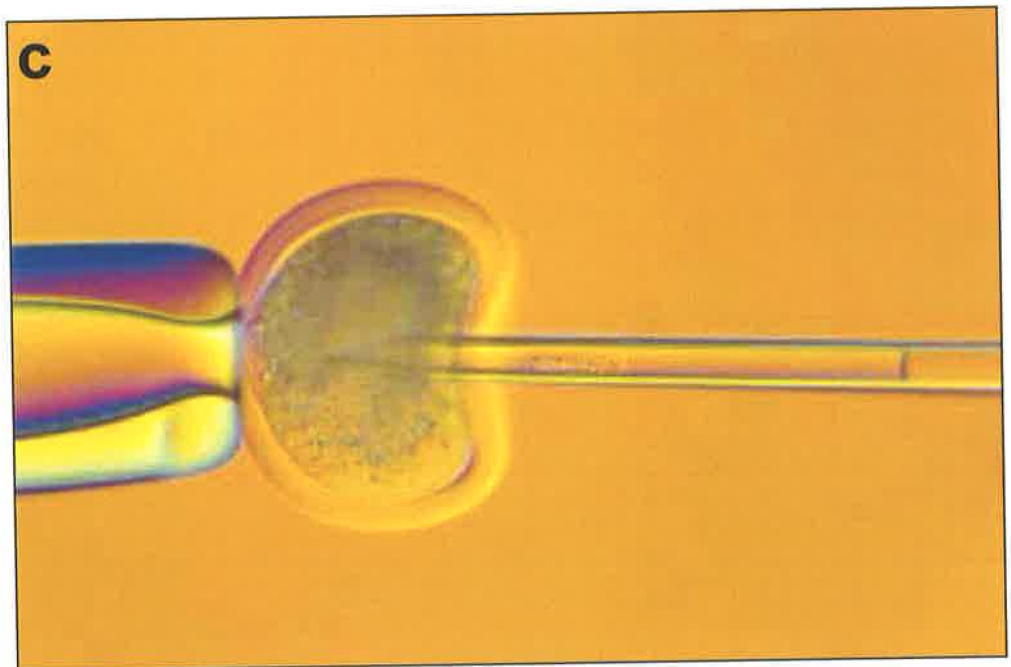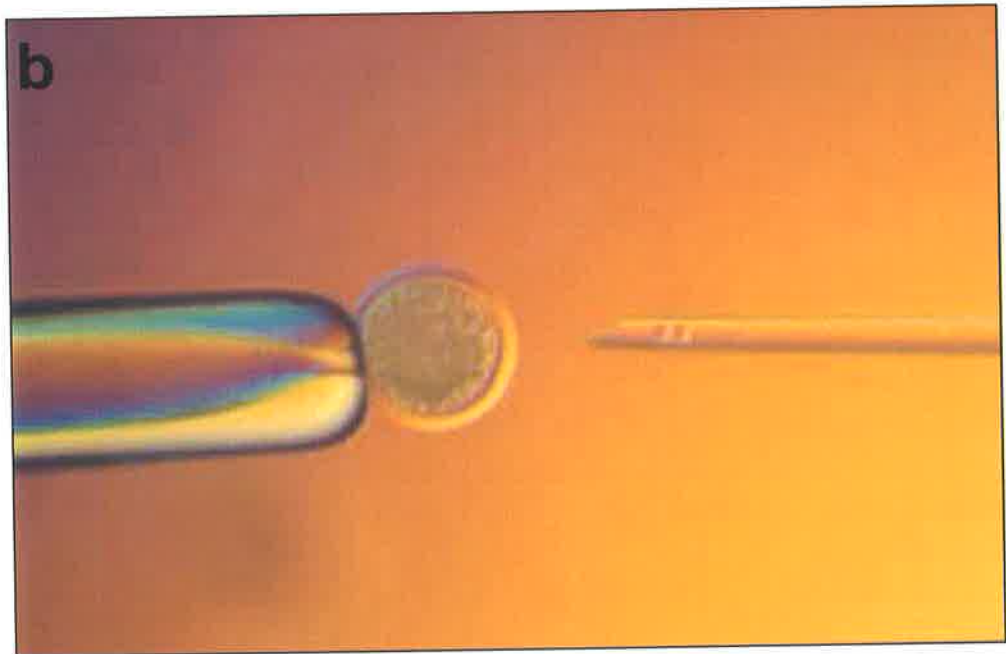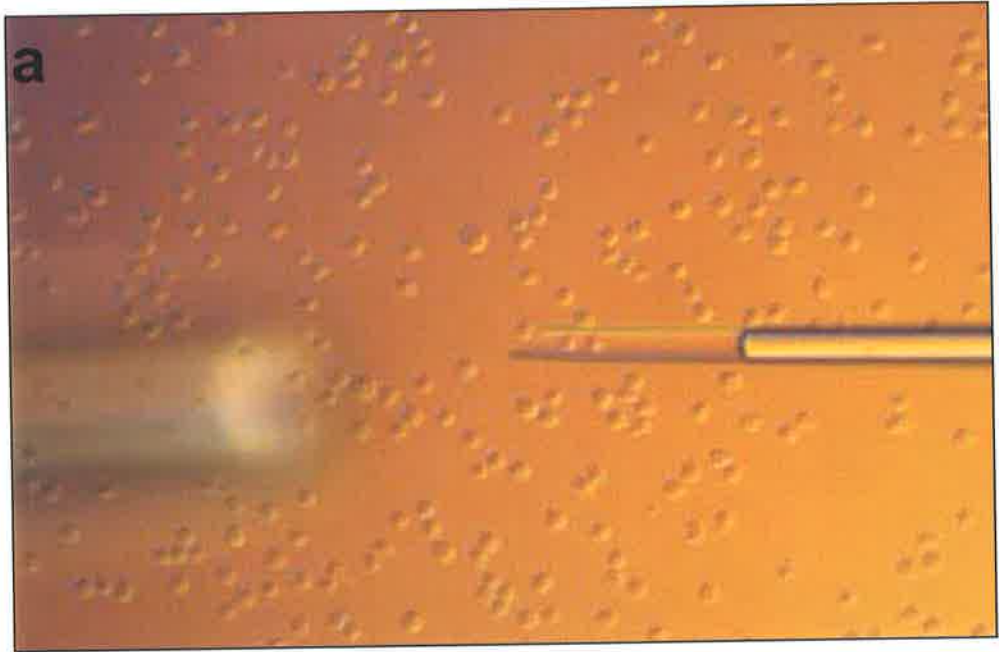
2.10.7 Embryo culture

Newly constructed embryos were washed twice in culture medium before were being placed in groups of 30-35 embryos in 0.5ml of culture medium covered with 0.38ml mineral oil in 4-well culture dishes. Incubation occurred in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C.

2.11 Transmission electron microscopy and autoradiography

2.11.1 Chemicals and reagents

0.1 M Na-phosphate buffer consisted of 3 g of sodium dihydrogen phosphate (monohydrate), and 14 g of di-sodium hydrogen phosphate (dihydrate) made up to 1 l with MilliQ water.

The primary fixative was made up of 3% (v/v) gluteraldehyde in 0.1 M Na-phosphate buffer.

Epon was made by thoroughly mixing together 98.26 g of Glycidether 100, 74.62 g of DDSA (docdecenylsuccinicanhydride), and 26.80 g of MNA (1-methyl-5-norben-2,3-dicarbxylacidanhydride). A 3 ml (1.5 %) portion of the accelerator, DMP-30 (2,4,6-tris(dimethylaminomethyl)-phenol) was added and then mixed again for 5 min. Newly made epon was placed in syringes and stored at -20°C for up to 6 months.

2.11.2 ^{3}H-uridine incubation for autoradiography

Zygotes and embryos from various developmental stages were labelled by ^{3}H-uridine (sp. act. 962 GBq/mmol; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). A final concentration of 4 Mbq/mmol (Laurincik et al., 2000) was used and labeling occurred for 20 min in gas-equilibrated ovine culture medium (see Section 2.4.1). After the incubation, specimens were repeatedly washed in ^{3}H-uridine-free culture medium and fixed as described below.

2.11.3 Processing for light microscopic autoradiography and transmission electron microscopy

2.11.3.1 Primary fixation

After labelling with the radioactive precursor, the zygotes and embryos were fixed in 3% (v/v) glutaraldehyde in 0.1 M Na-phosphate buffer (pH 7.2) for 1 h at 4°C. Subsequently, the specimens were washed three times in 0.1 M Na-phosphate buffer in which they were stored at 4°C until they were embedded in agar chips.

2.11.3.2 Agar embedding

A solution of 4% (v/v) agarose, in 0.1 M Na-phosphate buffer was prepared by weighing out 0.8 g of agarose and mixing with 20 ml of 0.1 M Na-phosphate buffer in a 50 ml flask. The mixture was heated in a microwave until completely dissolved, and kept at 45-55°C in a water bath throughout agar embedding. Using a stereomicroscope, a 2 µl drop of 0.1 M Na-phosphate buffer was placed on a glass slide into which an embryo was placed. 90 µl of liquid 4% (v/v) agarose in 0.1 M Na-phosphate buffer was carefully pipetted on to the embryo, building the drop up so that it was solidified within the agarose matrix. An agarose chip (approximately 2x2x2 mm) containing the fixed embryo was then cut from the solid drop and washed twice in 0.1 M Na-phosphate buffer, in which it was stored until post-fixation treatment.

2.11.3.3 Post-fixation treatment and embedding

Embryos embedded in agar chips were post-fixed in 1% (v/v) OsO_4 in 0.1 M Na-phosphate buffer for 1 h. After washing twice with distilled water, the samples were treated with 0.5% (v/v) uranyl acetate for 1 h and washed twice more with distilled water. Embryos were dehydrated with 10 min incubations in 70%, 96% and 99% (v/v) ethanol, followed by three 20 min incubations in 99% (v/v) ethanol (AnalR). Two 10 min incubations in propylene oxide were followed by 20 min

incubations in 2-parts propylene oxide/1-part epon, 1-part propylene oxine/1-part epon and 1 part propylene oxide/2 parts epon. Samples were kept overnight in epon until embedding.

2.11.3.4 Epon embedding

Samples were placed individually in moulds that were half-filled with freshly made epon. After the embryos were positioned appropriately, the moulds were filled with epon. Moulds were placed in an oven set to 60°C where they were kept for 48 h.

2.11.3.5 Sectioning and brightfield light microscopy

Embryos embedded in epon were cut serially into semi-thin sections (2 μm; see Figure 2.6). Every second section was stained with basic toluidine blue and evaluated by bright field light microscopy using a Leica microscope.

2.11.3.6 Re-embedding of semi-thin sections

Selected semi-thin sections were re-embedded according to Hyttel and Madsen (1987). Nail polish was painted in a circle around the selected section, and a drop of freshly made epon was placed above the section. A block of solid epon was firmly placed on the epon-covered section, and the section(s) placed in a 60°C oven for 48 h. To remove the block containing the re-embedded semi-thin section from the glass slide, the slide was immersed in liquid nitrogen causing the two to separate.

2.11.3.7 Ultra-thin sectioning and contrasting for TEM

Re-embedded semi-thin sections were ultra-thin sectioned (70 nm) using a diamond knife (Figure 2.6). A ribbon of sections was cut and carefully placed on a coated copper grid. The ultra-thin sections were contrasted with uranyl acetate and lead citrate. 2% (v/v) uranyl acetate was filtered (0.22 μm) and drops were placed on a paraffin wax film. Grids containing the ultra-thin sections

Figure 2.6

Sectioning using a) a glass knife to produce a semi-thin section (adjacent); and b) a diamond knife to produce an ultrathin section (adjacent).
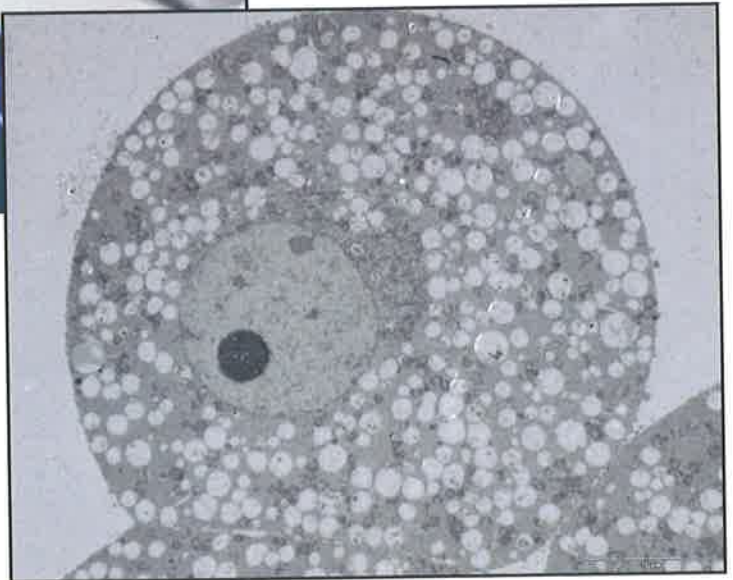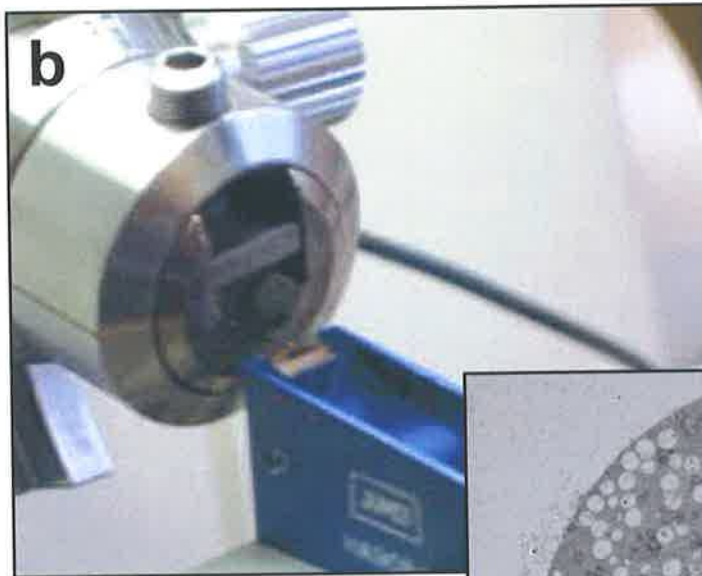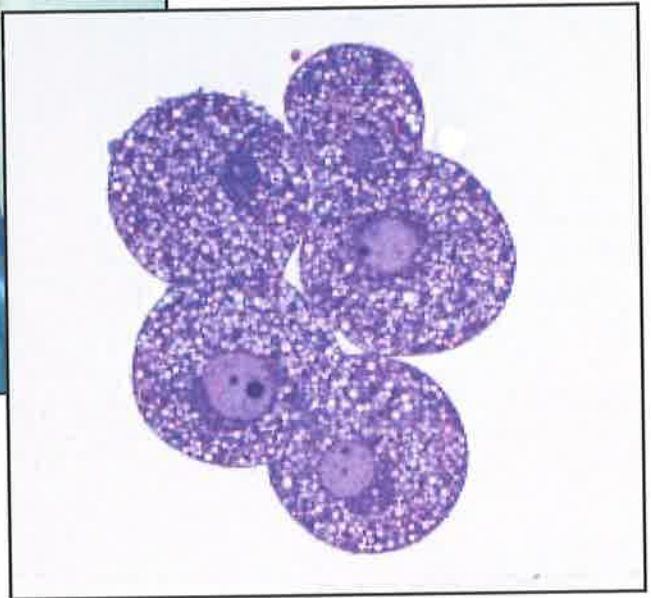
were placed inverted (sections down) on the surface of the drops where they were incubated for 40 min at 40°C. Using fine forceps, grids were washed ten times each in four separate beakers containing RO water. Following this, drops of lead nitrate were placed on a paraffin film and the same grids placed on these drops for 10 min at room temp. Sections were washed once again ensuring that all water was subsequently removed form the grids using lens paper. Grids were examined on a Philips CM100 transmission electron microscope.

2.11.4 Autoradiography

2.11.4.1 Processing of slides

Selected un-stained semi-thin sections were processed for autoradiography for detection of total RNA synthesis and nucleolus-associated RNA synthesis. This procedure was carried out in a dark room at 25°C with yellow light. Ilford K2 liquid nuclear emulsion (Ilford Imaging UK Ltd, Mobberley, Cheshire, UK) was prepared by placing in a water bath set to 40°C. It was subsequently poured into a glass cuvette into which a single glass slide could fit. One by one, each of the selected slides was dipped into the emulsion ensuring that all of the sections were coated. The emulsion was allowed to dry for 1 h in complete darkness. Slides were placed in racks, wrapped in aluminum foil and black plastic and kept at 4°C for 6 weeks.

2.11.4.2 Development of autoradiograms

The specimens were developed in Kodak D-19 at 17°C, stained with toluidine blue and evaluated by bright field and epipolarized light microscopy.

2.12 Immunocytochemistry and confocal laser scanning microscopy

2.12.1 Chemical and reagents

The following primary antibodies against key nucleolar proteins were used: mouse monoclonal antinucleolin (C23; 1:1000; Ochs et al., 1983), human antifibrillarin (1:1000; Ochs et al., 1985) and human anti-RNA polymerase I (1:500; Reimer et al., 1987).

2.12.2 Fixation

Embryos were fixed in 4% (v/v) paraformaldehyde for 1 h at 4°C. Subsequently, the specimens were washed three times in 1% (v/v) Triton X-100 in PBS.

2.12.3 Antibody labelling

Fixed embryos underwent a three-step indirect immunofluorescence procedure. Specimens were washed four times in PBS containing 1% (v/v) Triton-X, and preincubated for 2 h with 5% (v/v) rabbit serum (Dako, Glostrup, Denmark) in PBS at room temperature. Thereafter, they were incubated in primary antibodies diluted in PBS containing 5% (v/v) rabbit serum overnight at 4°C. Excess primary antibodies were removed by extensive washing of the embryos in PBS prior to a 4 h (at 4°C) and 1 h (at room temperature) incubation in rabbit antihuman-biotin (Dako; for antifibrillarin and anti-RNA polymerase I) or rabbit antimouse-biotin (Dako; for antinucleolin), diluted in PBS containing 5% rabbit serum. The secondary antibodies were visualised by streptavidin-fluorescein isothiocynate (Dako) in PBS. Finally, embryos were mounted on glass slides using Dako fluorescent mounting medium (Dako) and examined on a Leica confocal laser-scanning microscope. Control immunostaining of unspecific labelling by the secondary antibody was performed by omitting the primary antibodies.

Chapter 3

Optimising conditions for intergeneric somatic cell nuclear transfer

Chapter 3: Optimising conditions for intergeneric somatic cell nuclear transfer

3.1 Introduction

Somatic cell nuclear transfer has been used to produce offpring in a wide range of different species (and genera) including sheep (Wilmut et al., 1997), cattle (Cibelli et al, 1998; Kato et al., 1998; Wells et al., 1999), mice (Wakayama et al., 1998), goats (Baguisi et al., 1999), pigs (Polejaeva et al., 2000), rabbits (Chesné et al., 2002), cats (Shin et al., 2002) and horses (Galli et al., 2003). However, SCNT is a complex procedure involving many steps and embryological procedures (see Section 1.4; Peura and Vajta, 2003). It is technical by its very nature, and all steps and conditions must be established and optimised before experiments can be performed.

As discussed in Chapter 1, the common concept of SCNT across all species is the transfer of a somatic nucleus by electrofusion or injection into an enucleated oocyte, after which, the reconstructed embryo is artificially activated. Variations exist in the order, timing and specific detail of the procedure due to the different requirements demanded by the different species. In fact, there are examples where variations in methodology have been implemented to overcome developmental barriers. These include a report that described the successful application of SCNT to produce the first rabbit offspring (Chesné et al., 2002). This outcome was attributed to the modification of NT protocols successful in other species and included taking into account the rapid kinetics of the cell cycle of rabbit embryos, and the narrow window of time for their implantation after transfer to foster recipients. Because the rabbit zygote enters S phase of the cell cycle very early after activation, the exposure to protein synthesis inhibitors such cyclohexamide was minimised. In addition, NT embryos were transplanted asynchronously to allow for their

slow development in comparison with embryos derived from in vivo or in vitro produced zygotes.

An outline of the steps involved in SCNT is summarised in Figure 3.1. In vitro maturation of ovine oocytes (Walker et al., 1996; Peura et al., 2003) and oocyte enucleation (Campbell et al., 1996; Peura et al., 2003) procedures are required to produce ovine cytoplasts. Adult and foetal somatic cell culture (Wilmut et al, 1997; Peura et al, 2003), their serum starvation (Polejaeva et al., 2000) and sub-zonal microinjection into the perivitelline space of an enucleated oocyte (Campbell et al., 1996; Wilmut et al., 1997; Peura et al., 2003) are necessary to obtain couplets. Somatic cell electrofusion (Wilmut et al., 1997; Peura et al., 2003) followed by chemical activation of cybrids (Peura et al., 2003) need to be performed to fuse the couplets and artificially activate the resulting cybrids respectively. Finally, ovine in vitro culture (Walker et al., 1996) would be used to culture the reconstructed embryos. In the present study, the above-mentioned techniques were performed to produce reconstructed intergeneric SCNT embryos alongside ovine SCNT controls with the aim of determining whether the ovine cytoplasm could initiate embryonic development when combined with a nucleus from another genus, by assessing embryo development visually, by nuclear staining, as well as assessing the karyotype of the resulting embryos.

Fusion before activation (FBA) protocol involves leaving the donor nucleus in the ooplasm for a set period of time after fusion and before the cybrid is activated. The somatic DNA is therefore exposed directly to factors in the ooplasm, and it has been hypothesised that this improves the remodelling of the genomic DNA (Wakayama et al., 1998). It has been shown to improve outcomes in mouse (Wakayama et al., 1998) and cattle (Wells et al., 1999; Dominko et al., 1999) SCNT, in terms of embryo development and offspring production. FBA has also been implemented in ovine nuclear transfer (Campbell et al., 1996). However no significant

differences between FBA and simultaneous fusion-activation in terms of offspring were observed. In the present study, the ovum was exposed to a range of electrofusion pulses to determine the parameters that would facilitate couplet fusion without causing activation.

Fusion of ovine-ovine and bovine-ovine SCNT couplets has been shown to be successful with three DC pulses of 1.25 kV/cm, each of 80 µsec duration (Hamilton et al., 2000). However, attempts to fuse porcine granulosa cells to ovine cytoplasts using these particular electrofusion parameters have yielded high levels of porcine granulosa cell lysis (>80%, Hamilton et al., unpublished results); the vast majority of the donor cells lysed immediately. Therefore, the goal of the present study was to determine an appropriate set of electrofusion parameters that would be sufficient to fuse porcine granulosa cells to ovine cytoplasts without lysing either entity. The specific aims were to:

1) Establish electrofusion parameters that would enable the application of a FBA protocol that would fuse couplets but not cause the ovine ooplasm to activate.

2) Determine suitable electrofusion parameters that would enable fusion of porcine-ovine intergeneric couplets with minimal lysis of either the cell or cytoplast.

3) Determine whether the ovine ooplasm could initiate embryo development after nuclear transfer with a nucleus from another species.

3.2 Materials and Methods

Three separate experiments were conducted.

3.2.1 Activation of the ovine ovum (Experiment 1)

3.2.1.1 Experimental design

This experiment evaluated the ability of electrofusion pulses to activate ovine ova. In vitro matured oocytes were kept in calcium and magnesium free culture medium for between 1 and 5 hours before pulsing in a mannitol medium using a GenAust fusion machine. One to five hours after pulsing, oocytes were fixed in drops of glycerol-Hoechst 33342 and evaluated under UV-illumination. Oocytes with intact MII spindles were considered non-activated, whereas oocytes possessing anaphase II, telophase II or pronuclear chromosomes and/or a clearly extruded second polar body were considered to be activated. More than 500 oocytes were used to test various combinations of fusion pulse strengths (400, 500, 750, 1000, 1250 or 2000 V/cm), number of pulses (1, 2 or 3), pulse durations (10, 15, 20, 25, 40 or 80 μsec), fusion chambers (0.2mm stainless steel wires with 0.5mm separation or 0.1mm platinum wires with 0.2mm separation) and methods of introducing ova in fusion medium (directly or stepwise diluted).

3.2.1.2 Oocyte preparation

Ovine COCs were collected and matured in vitro as described in Section 2.4. At 20-22 hpm, cells were removed from the oocytes by hyularonidase treatment after which they were placed in calcium/magnesium free culture medium for 1-2 h.

3.2.1.3 Electrical stimulation of oocytes

Ovine oocytes were pulsed in a platinum (200 μm separation) or stainless steel (500 μm separation) fusion chamber as for couplet electrofusion described in Section 2.9.5 with the following modifications. Oocytes were first equilibrated in fusion medium for approximately 10 sec, before being transferred to the electrofusion chamber. Activation pulses were produced using a GenAust fusion machine connected to the fusion chamber. The initial AC pulse of 8V and 500

kHz for 5-10 sec, was followed by the activation pulse that was to be tested. Oocytes were then washed twice in culture medium and incubated at 38.5°C in an atmosphere of 5% CO_2 in air until they were fixed in glycerol-Hoechst 33342.

3.2.1.4 Fixing of oocytes to determine activation

At the desired timepoint, a group of embryos was taken from the incubator. Droplets of approximately 5 µl of glycerol-Hoechst staining fixative (Appendix II) were placed at one end of a glass microscope slide and, working under a stereomicroscope and using a mouth controlled glass pipette, groups of 3-5 oocytes were placed into each droplet. A glass coverslip with a portion of Vaseline-wax in each of the corners was placed above the droplets and carefully pressed, ensuring that the oocytes were not squashed. Without losing sight of the droplet, a felt-tip pen was used to circle each droplet. When all the oocytes had been fixed, the glass slides were placed on a tray, covered in aluminium foil and kept overnight at 4°C. The oocytes were examined the following day under UV fluorescence microscopy.

3.2.2 Optimisation of porcine granulosa cell to ovine oocyte fusion (Experiment 2)

3.2.2.1 Experimental design

To establish a suitable set of fusion parameters that would fuse porcine granulosa cells with ovine cytoplasts, cell fusion and lysis rates were recorded for a range of fusion parameters. Two different entities were used to test a range of electrofusion parameters, namely (1) cell 'doublets', created by adhering two porcine granulosa cells to each other and (2) 'couplets', made by attaching a granulosa cell to a zona-free oocyte. Between two platinum (0.1 mm diameter) wires, groups of 10 doublets or couplets (one group at a time) were exposed to voltages of 0.8, 1.2, 1.6 and 2.0 kV/cm for 10, 20, 40 and 80 µs in duration. The couplets were created zona-free for practical reasons so that a large number could be pulsed in a short period of time across a large

number of different pulse strengths and durations. Such large numbers of zona-intact couplets are not easily produced with a single operator. Electrofusion parameters that achieved fusion rates of 80% or higher, as well as lysis rates of 20% or lower were considered suitable. Selected suitable parameters were then retested on groups of 10 doublets to ensure repeatability. The electrofusion parameters that yielded fusion rates of 80% or higher, paralleled with lysis rates of 20% or lower were considered suitable to be used in porcine to ovine intergeneric SCNT.

3.2.2.2 Somatic cell culture

Cultures of porcine granulosa cells were established as described in Section 2.6.3. When almost confluent, the cells were frozen, and stored in liquid nitrogen. One week before the experiment, a vial of cells was thawed and cultured for 5 days until confluent. They were then serum starved for 2 days, after which they were ready for use in the pulsing experiment.

3.2.3 Initiation of intergeneric bovine-ovine embryo development (Experiment 3)

3.2.3.1 Experimental design

To determine whether the ovine cytoplasm could initiate embryonic development, intra- and intergeneric SCNT was performed using ovine or bovine foetal fibroblasts and ovine cytoplasts (summarised in Figure 3.1). Ovine oocytes were enucleated by micromanipulation 18 h after maturation, and fused to bovine foetal fibroblast cells that had been serum starved for 9 to 10 days. Successfully fused couplets were activated using calcium ionophore and 6-DMAP. In two replicates, the resulting SCNT embryos were karyotyped (metaphase arrested with 20 μM nocozadole at the 4- to 8-cell stage, fixed with ethanol+acetic acid and stained with Giemsa). The bovine and ovine fibroblast cell lines were also karyotyped. In another five replicates, the fused couplets were cultured in ovine culture medium for seven days, after which time the

Figure 3.1

Schematic diagram summarising the SCNT procedure and design for Experiment 3.

Hours Post Maturation

Step	Time
1. Ovine oocyte clean-up.	18 h
2. Enucleation.	18.5 h
3. Bovine or ovine somatic cell preparation.	20–20.5 h
4. Sub-zonal cell injection.	21 h
5. Electrofusion.	23 h
6. Activation with calcium ionophore & 6-DMAP.	24 h
7. In vitro culture at 38.5 C in reduced oxygen.	27 h
8. Cleavage assessment.	75 h
9. Nuclei number or karyotyping.	195 h
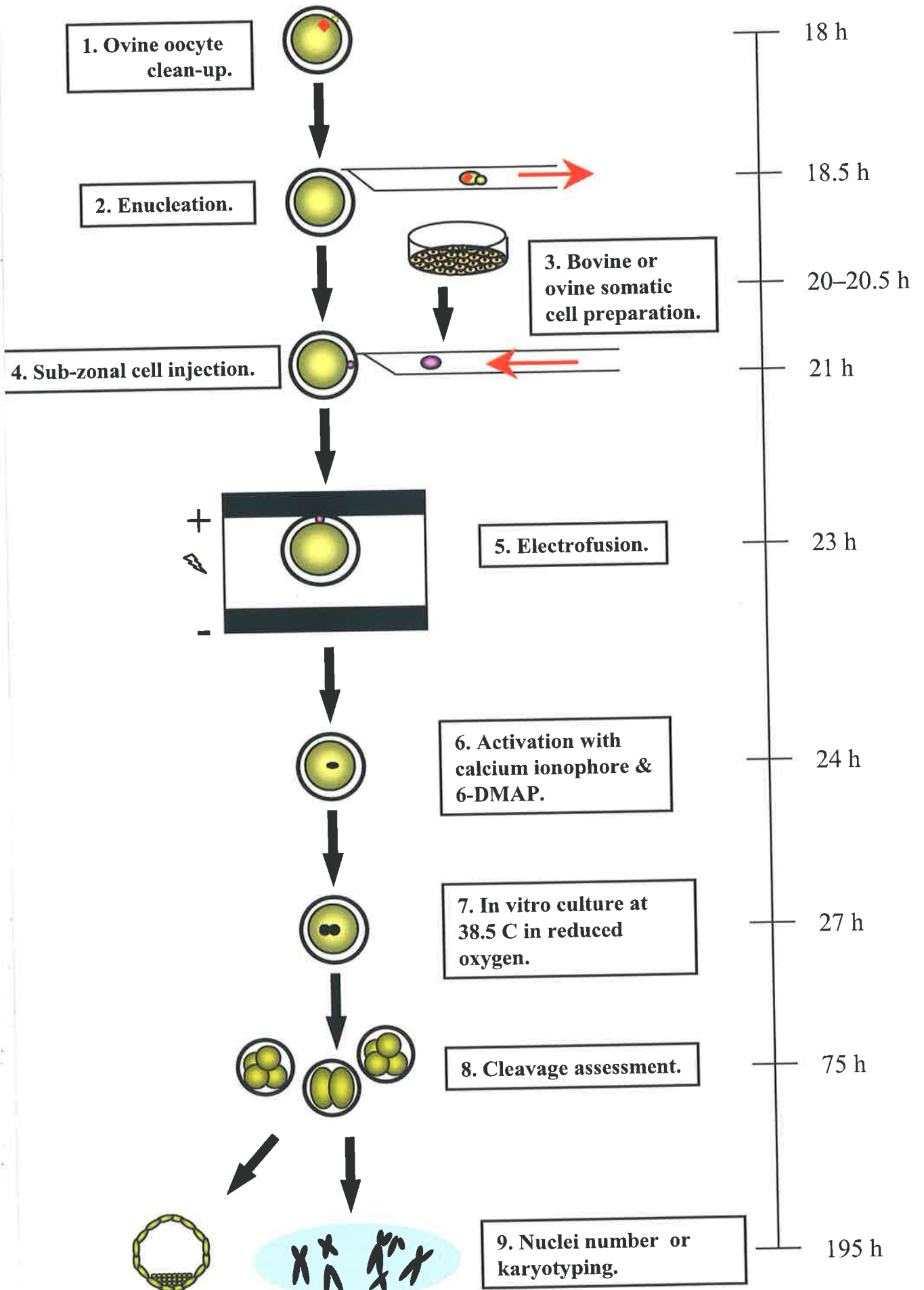
embryos were scored visually before being fixed in glycerol-Hoechst 33342 to determine the number of nuclei in each embryo.

3.2.3.2 Karyotyping of somatic cells

Foetal fibroblast cell cultures that were previously established from day 90 sheep (female) and cattle (male) foetal tissue, were thawed and cultured in a T25 culture flask for two days or until approximately 30% confluent. The cell monolayer was inspected on the day of karyotyping to ensure that individual fibroblasts appeared healthy with long pseudopods, clear cytoplasm and numerous cells in mitosis. The foetal fibroblasts were incubated for 1 h in culture medium supplemented with 100 µg/ml of colchicine to arrest cells at the metaphase stage of mitosis. The cells were harvested by first removing the culture medium and rinsing the monolayer three times with Hanks buffered salt solution (HBSS). The cells were then incubated with 0.5 ml of trypsin solution for 2 min or until cells had detached from the culture flask surface. By adding the original culture medium (which had been retained), the trypsin was inactivated. The cell suspension was then placed into 10 ml conical tubes and centrifuged at 700 g for 10 min. A 45% (w/v) hypertonic solution of potassium chloride was prepared by first diluting a 10x stock solution 1:10 in MilliQ water, before diluting this further by mixing 45 ml with 55 ml of MilliQ water. The supernatant was removed from the centrifuged tubes, and the pellets of cells resuspended in 8 ml of the hypertonic solution that had been pre-warmed to 37°C. The tubes were incubated in a 37°C water bath for 15 min, after which time, 2 ml of the fixative was added. This mixture was centrifuged at 600 g for 10 min after which the supernatant was removed, and the pellet resuspended in 5 ml of the fixative. The cells were again centrifuged at 700 g for 10 min, and then the supernatant removed leaving 0.5 ml in which the cells were resuspended. A test was carried out to ensure fixation was sufficient by placing a drop of this suspension on a pre-treated glass slide, and allowed to evaporate. The evaporated drop was observed under a light

microscope and, if there was a sufficient number of chromosome spreads, two drops of the cell suspension were placed on each glass slide until the suspension was completely used. The drops were allowed to evaporate and the glass slides were subsequently stored in a desiccated slide box at -20°C. If there were not sufficient chromosome spreads on the test slide, then the suspension was resuspended in fixative and centrifuged once again. Chromosome spreads were stained with Leishmans stain which was prepared by diluting 1 in 5 in 'buffered water'. The stain was poured over the slides and incubated for 15 min. The stain was washed off with water (ensuring that any precipitate was completely removed), and the slides stood vertically to dry. The slides were mounted using DPX medium and a glass coverslip and left overnight to dry. Slides were then examined using brightfield microscopy and each chromosome spread photographed and the number of individual chromosomes counted.

3.2.3.3 SCNT procedure

The 2-step nuclear transfer procedure used is described in detail in Section 2.10 and also outlined in Figure 3.1. Briefly, in vitro matured sheep oocytes were enucleated by aspiration 18 h after the commencement of maturation followed by fusion with bovine or ovine foetal fibroblast cells. Successfully fused couplets were activated in 10 µM calcium-ionophore before a 2 h incubation in culture medium containing 2 mM 6-DMAP. Cattle and sheep foetal fibroblasts between passages 3 and 6 were used as donor cells following serum starvation for 9 to 10 days.

3.2.3.4 Embryo karyotyping

Details on fixatives and stains can be found in Appendix II. Selected embryos were added to ovine culture medium supplemented with 20 µM of nocozadole. To determine whether a sufficient number of nuclei had arrested at the metaphase stage of mitosis, the embryos were removed after 12 hours, incubated in ovine handling medium supplemented with 10 µg/ml

Hoechst 33342 for 5 min, and viewed briefly under UV. If insufficient nuclei had arrested at metaphase, they were returned to the medium containing nocozadole. The zona pellucida was first removed by placing the embryos in medium containing 0.5% Pronase for 5 min. Embryos were placed in the hypertonic solution one at a time for 10 min at 37°C. Each embryo was then moved to a well containing Fixative I and then transferred to a pre-treated glass slide. Before the embryo was able to dry, another drop of Fixative I was added using a 1 ml syringe. To ensure that the fixed embryo did not dry out, a drop of Fixative I was dispensed when complete evaporation was approaching. This was repeated three times. Drops of Fixative II were then dispensed from a 1 ml syringe and replenished twice when evaporation of the fixative approached. When the embryo was sufficiently swollen and spread, the fixative was allowed to evaporate completely, the position of the embryo was marked with a diamond pen and the slide allowed to air-dry overnight. Giemsa-blue stain was prepared and added to the relevant area on the slide for 5 min. Slides were then rinsed with tap water and left to dry.

3.3 Results

3.3.1 Ovine oocyte pulsing experiment (Experiment 1)

The optimal pulse settings for fusion (3 DC fusion pulses of 1.25 kV/cm and 80 μs) activated 92% (46 of 50) of oocytes. With other pulse settings, the proportion of activated oocytes was consistently above 40% except in the case of direct introduction to platinum chamber (0.4 kV/cm, 80 μs and 1 or 2 pulses) where it was 37% and 17%, respectively (Figure 3.2). However, these pulse settings did not trigger the fusion of cytoplast-donor cell couplets. The lowest activation stimulus 3.6% (2 of 55) was achieved with stepwise introduction to the stainless steel chamber (0.5 kV/cm 15 μs and 2 pulses), but again these settings were not sufficient to cause cell fusion at acceptable rates based on cell to cytoplast data presented in Figure 3.3.

Figure 3.2

The percentage of oocytes that activated when exposed to a particular electric pulse setting. Across the X-axis are the four different pulse strength parameters (kV/cm) and within each parameter is the number of pulses (1, 2 or 3). On the Z-axis is the pulse duration in μs.
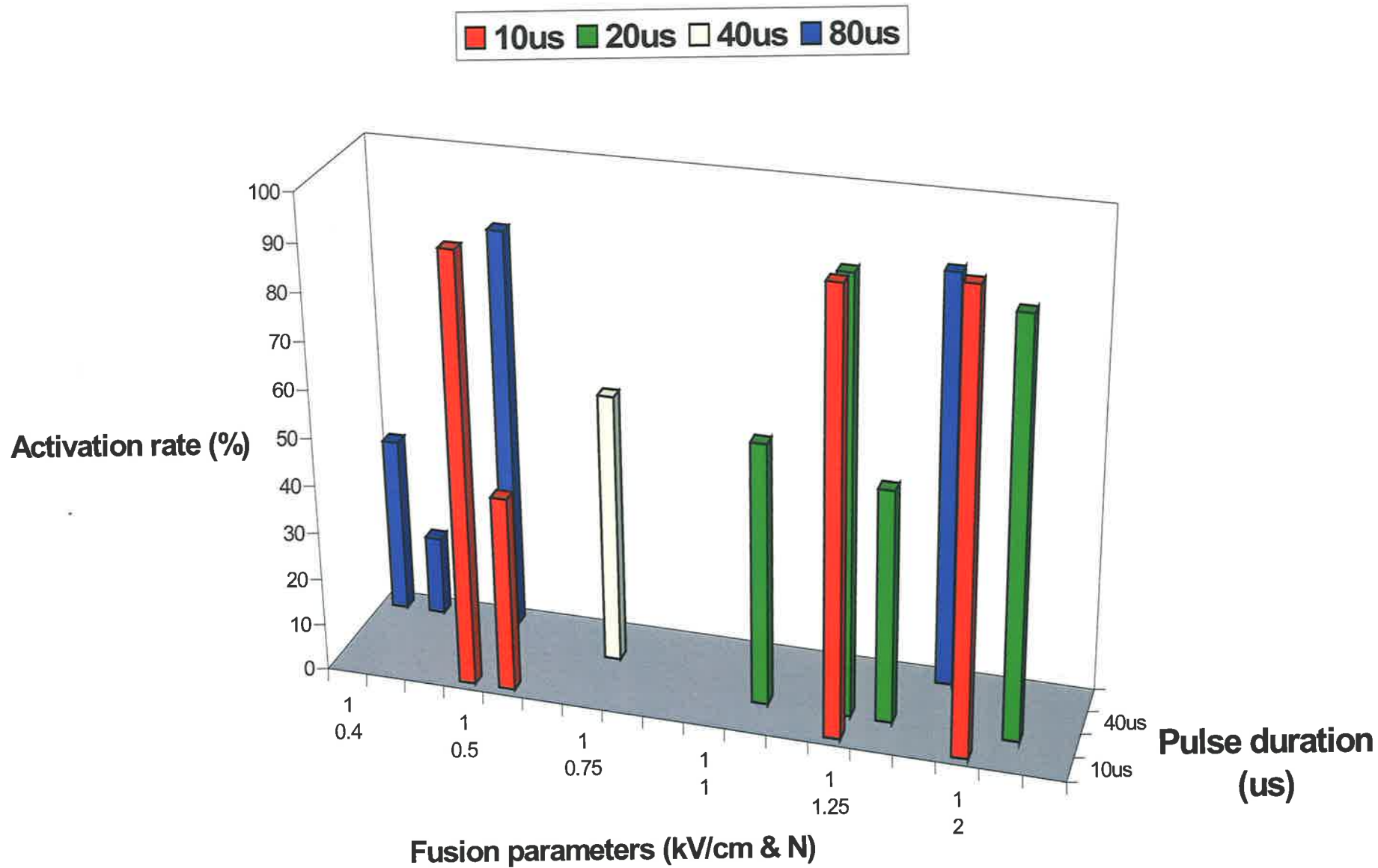
Figure 3.3

Percentage of fusion (a) and lysis (b) of porcine cell to cell couplets upon or after a range of electrofusion pulse strengths and durations.
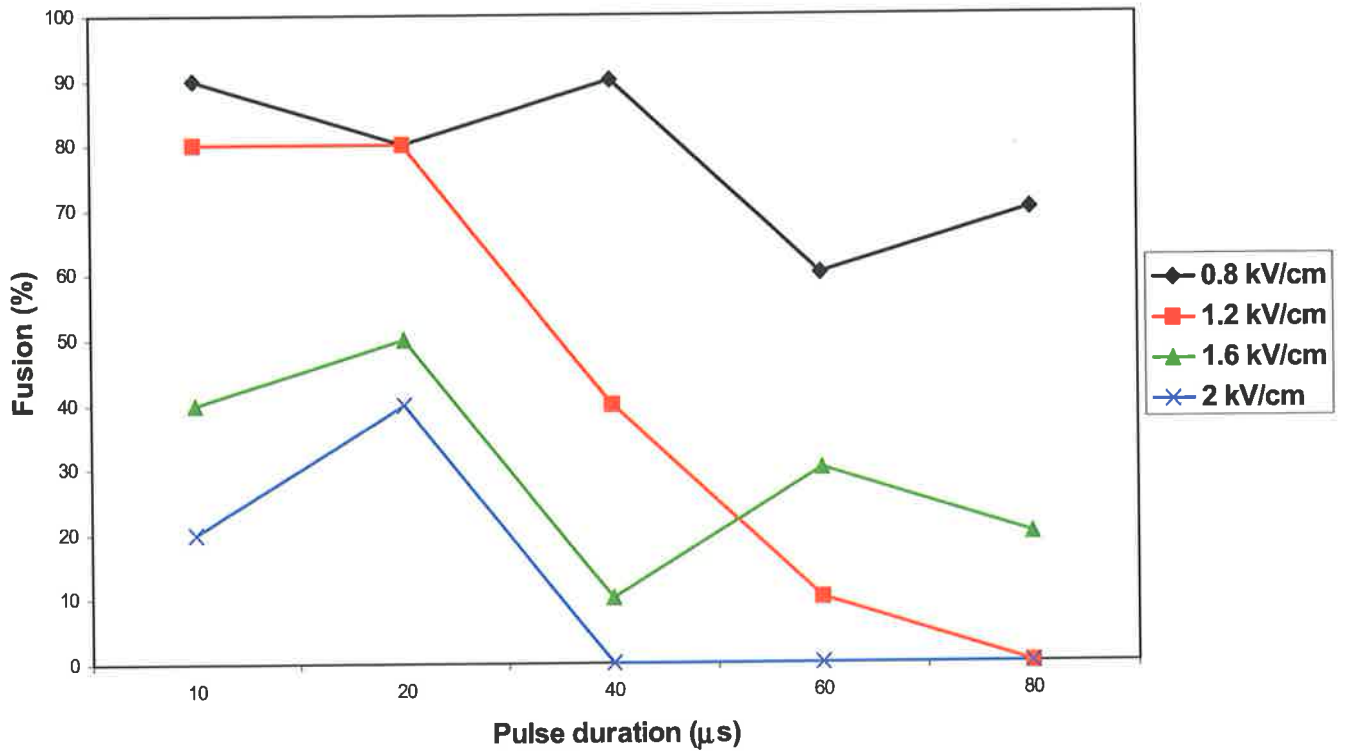
A. Fusion

B. Lysis
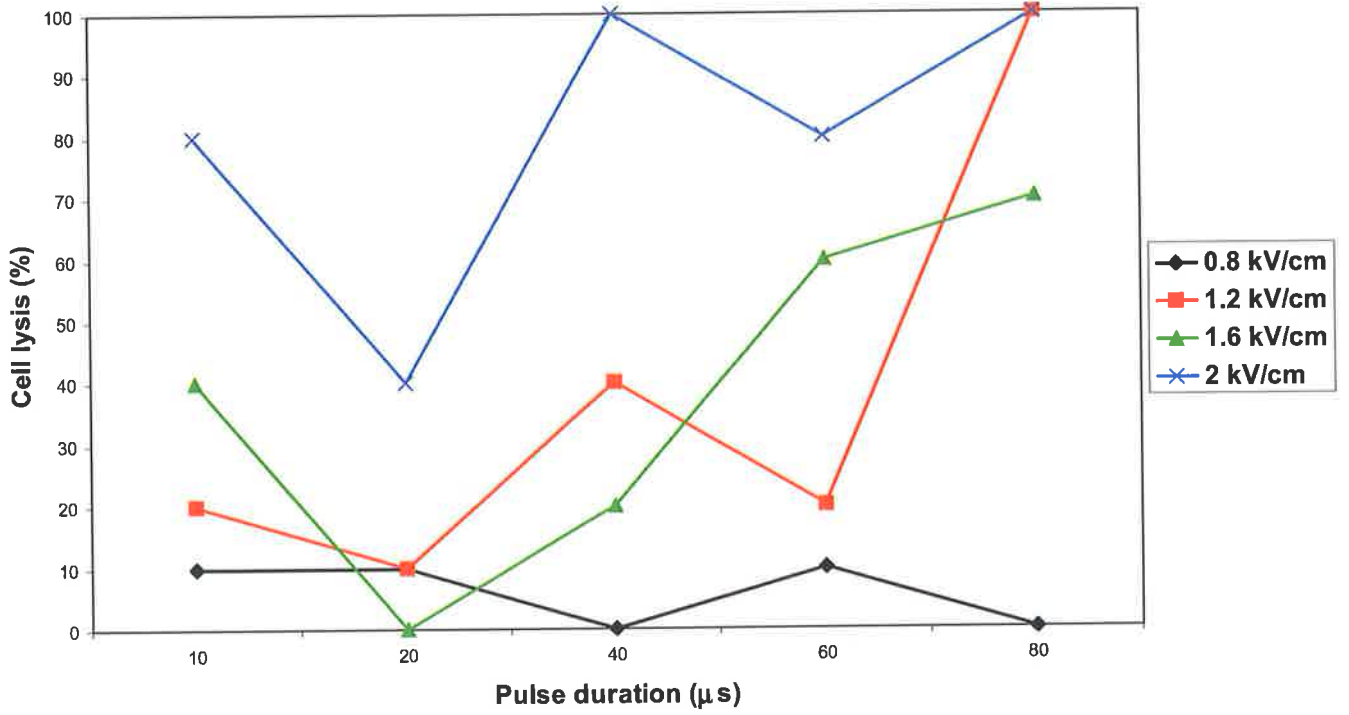
3.3.2 Optimisation of porcine granulosa cell to ovine cytoplast fusion (Experiment 2)

3.3.2.1 Cell to cell pulses

Electrofusion parameters that obtained a cell to cell fusion rate of 80% or greater were 0.8 kV/cm of 10, 20 and 40 μs duration, and 1.2 kV/cm with 10 and 20 μs duration (Figure 3.3a). Electrofusion parameters that caused cell lysis of 20% or less included 0.8 kV/cm of 10-80 μs, 1.2 kV/cm of 10, 20 and 60 μs duration, and 1.6 kV/cm of 20 and 40 μs duration (Figure 3.3b).

3.3.2.2 Zona-free oocyte to cell fusion

Electrofusion parameters that obtained a cell to oocyte fusion rate of 70% or greater, and a cell lysis rate of 20% or below were considered possible candidate parameters (Figure 3.4). An electrofusion pulse of 0.8 kV/cm for 20, 40, 60 and 80 μs duration and a pulse of 1.2 kV/cm for 10 μs duration were considered suitable parameters. Acceptable fusion and lysis rates were obtained using electrofusion pulses of 1.6 kV/cm for 10 μsec and 2.0 kV/cm for 10 and 20 μsec. However these particular fusion parameters did not produce acceptable fusion and lysis rates of cell to cell couplets (see section 3.3.2.1), and hence were not retested in the additional cell to oocyte pulsing experiment. In this final cell to oocyte pulsing experiment, parameters of 0.8 kV/cm for 40 and 80 μs duration and 1.2 kV/cm for 10 μs duration had a fusion rate 70% or greater and a lysis rate of 20% or below (Table 3.1).

Figure 3.4

Percentage of fused (a) and lysed (b) of porcine cell-ovine oocyte couplets upon, or after a range of electrofusion pulse strengths and durations.
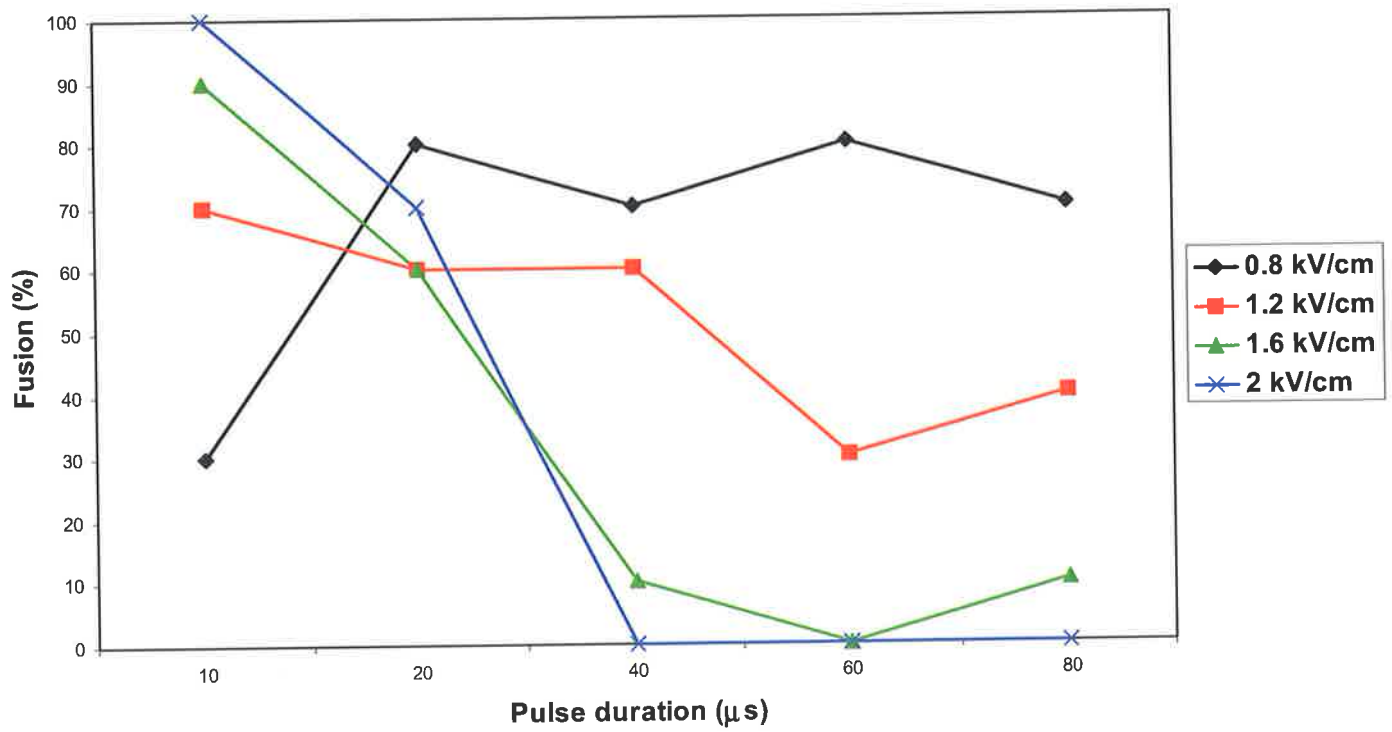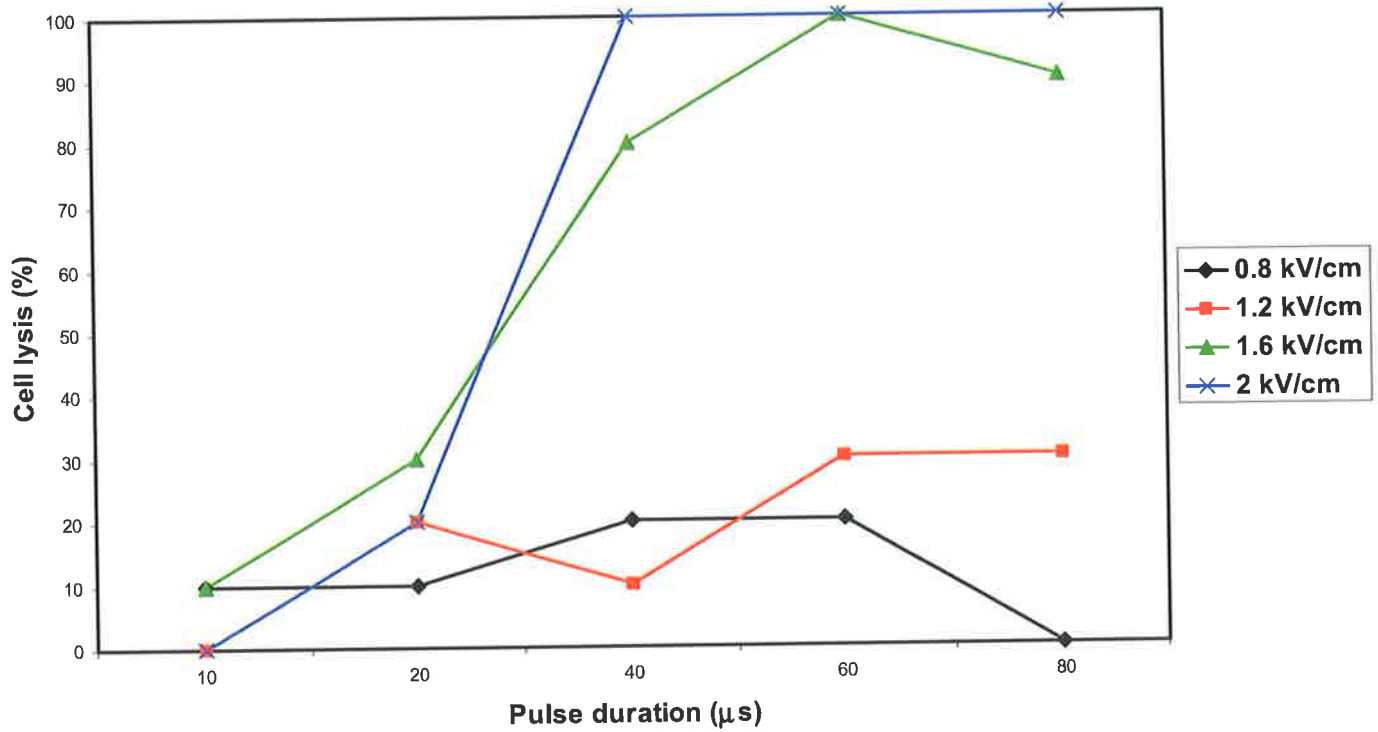
A. Fusion

B. Lysis

Table 3.1: Fusion and lysis of porcine granulosa cell to ovine oocyte upon or after being exposed to a range of electrofusion parameters.

Electrofusion parameter	Fusion rate (%)	Lysis rate (%)
0.8 kV/cm, 10 μs	30	30
0.8 kV/cm, 20 μs	40	20
0.8 kV/cm, 40 μs	90	10
0.8 kV/cm, 80 μs	80	20
1.2 kV/cm, 10 μs	90	10

Electrofusion parameters with both fusion and lysis rate in preferred range

3.3.3 Initiation of intergeneric embryo development (Experiment 3)

3.3.3.1 Karyotyping

Karyotyping of bovine and ovine foetal fibroblasts revealed the correct number of chromosomes (60XY and 54XX respectively; Figure 3.5), although one of the 30 spreads had an abnormal chromosome fragment. Twelve of 31 bovine-ovine interspecies SCNT embryos gave readable metaphase spreads, and all were derived from bovine fibroblasts (this was indicated by the presence of clearly identifiable bovine chromosomes, although exact chromosome numbers were not able to be determined; Figure 3.5).

3.3.3.2 Preliminary intergeneric SCNT developmental data

At day 7 of embryo development, a larger proportion of bovine-ovine intergeneric SCNT embryos arrested before the 16-cell stage than ovine-ovine intraspecies clones (Likelihood ratio χ^2 statistic P=0.0319; Table 3.2). The difference in cleavage rate between bovine-ovine and

Figure 3.5

Somatic cell chromosome spreads of ovine (a) and bovine (b) foetal fibroblasts, as well as chromosome spreads obtained from an ovine-ovine SCNT embryo (c) and a bovine-ovine SCNT embryo (d) constructed from the respective foetal cell line.
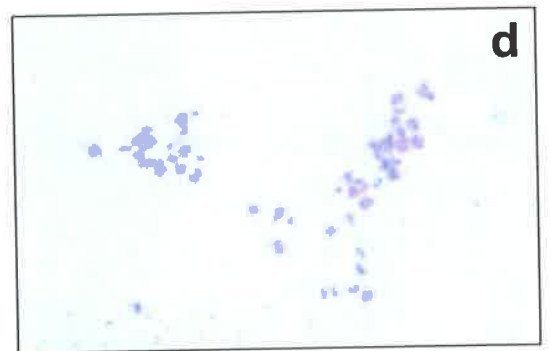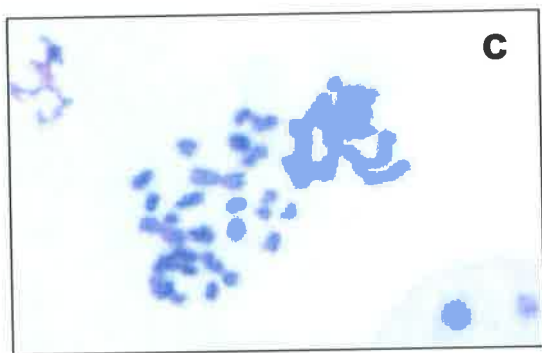
ovine-ovine populations (51% v 43%; Table 3.2) was not significantly different (χ^2 P=0.4977).

There were also a number of cleaved clones with only one nucleus (Table 3.2).

Table 3.2: Final development of cloned embryos on Day 7 derived from the transfer of bovine and ovine donor cells into ovine cytoplasts.

Donor	N	Cleaved (%)	No. of nuclei*				
			1	2-4	5-8	9-16	>16
Bovine	37	19 (51)	5 (26)*	5 (26)	5 (26)	4 (21)	0 (0)
Ovine	28	12 (43)	1 (8)	4 (33)	1 (8)	2 (17)	4 (33)

*Percentage based on the number of cleaved couplets.

3.4 Discussion

3.4.1 Ovine oocyte activation

The fusion parameters that provide an optimal fusion vary significantly between species. An AC pulse of 3 V for 5 sec followed by three DC pulses of 1.25 kV/cm for 80 μsec were used in the experiments to produce Dolly the sheep (Wilmut et al., 1997). Studies conducted in our laboratory have successfully used only two pulses of the same strength and duration (Peura et al., 2003a). The results in the present study demonstrate that these optimal cell fusion settings (platinum chamber, 1.25 kV/cm, 80 μsec, 2 pulses) used in the nuclear transfer experiments regularly caused >60% activation rate. Furthermore, the only electrofusion parameters that caused an acceptably low level of oocyte activation were those that would not cause fusion at least not at an acceptable rate. In subsequent experiments conducted by another investigator in our lab, oocytes were exposed to fusion pulses whilst in calcium and magnesium free fusion medium. These experiments failed to find electric pulse parameters that would cause couplet fusion without simultaneous oocyte activation (Peura T., 2000, personal communication). This is despite the successful implementation of FBA reported by Campbell et al. (1996). The specific detail of how FBA was achieved is not clear from the report and therefore, no conclusion can be drawn upon the reasons for this discrepancy. Consequently, no attempts were made in subsequent experiments using ovine oocytes, to delay activation of fused couplets however, the two-step calcium-ionophore and 6-DMAP activation protocol was still performed immediately after fusion (see Section 2.10.6).

3.4.2 Optimising porcine granulosa cell fusion

The results outlined in Figures 3.3 and 3.4 and Table 3.1 demonstrate that one pulse of 0.8 kV/cm for 40 or 80 μs, or one pulse of 1.2 kV/cm for 10 μs was sufficient to obtain acceptable fusion

rates of porcine granulosa cells to zona-free ovine oocytes and, in parallel, not cause donor cell lysis above 20%. Therefore, these fusion parameters were used in subsequent experiments to fuse porcine granulosa cells to ovine cytoplasts. Fusion and lysis rates were closely monitored in future experiments when fusing zona-intact couplets to ensure that these electrofusion parameters remained optimal.

3.4.3 Initiation of intergeneric development

The results in Table 3.2 show that bovine nuclei obtained from foetal fibroblast cells have the ability to initiate early pre-implantation embryo development with the support of ovine ooplasm. It should be pointed out, however, that this experiment was conducted when SCNT skills of the author were being developed and this is likely explains the poor cleavage rates (Table 3.2). Cleavage rates for sheep SCNT embryos are normally above 70% (Peura et al., 2003a).

This series of experiments provide a sound basis for the production of nuclear transfer embryos in the subsequent experiments characterising the developmental capacity of intergeneric SCNT development supported by the ovine ooplasm.

Chapter 4

Developmental kinetics of pre-implantation embryos

produced by somatic cell nuclear transfer

Chapter 4. Developmental kinetics of pre-implantation embryos produced by somatic cell nuclear transfer

4.1 Introduction

Intergeneric SCNT preimplantation embryo development has been shown to be supported by bovine (Dominko et al., 1999) and rabbit (Chen et al., 2002) ooplasm. Preliminary experiments reported in this thesis (Chapter 3) have demonstrated that the ovine cytoplasm is capable of initiating intergeneric embryonic development. Bovine-ovine embryos created using bovine fetal fibroplasts and ovine cytoplasts, exhibited bovine specific chromosomes after karyotyping, and were able to support development to at least the 16-cell stage (Chapter 3).

It has been proposed that an interspecies SCNT-model would be a useful tool to investigate nuclear reprogramming, and more specifically, the interaction between the introduced diploid nucleus and the recipient cytoplasm (Dominko et al., 1999). Thereafter, it was reported that enucleated oocytes (cytoplasts) of bovine origin possessed the ability to support embryo development to the blastocyst stage after SCNT using cells from a number of different species including those from sheep, pig and monkey (Dominko et al., 1999; Kitiyanant et al., 2001; Meirelles et al., 2001). The reconstructed embryos underwent nuclear swelling, successive cell divisions and formation of a blastocoele cavity at a time corresponding with that expected in the somatic nuclei donor species. It is perhaps more accurate to refer to SCNT embryos created in these studies as 'intergeneric' as the donor cell – cytoplast combinations differed not only in species, but in genus as well (Lee et al., 2003). More recently it has been shown

that bovine cytoplasts can support blastocyst development after intergeneric SCNT using mountain bongo (*Tragelaphus eurycerus isaaci*) somatic cells (Lee et al., 2003).

Another example of intergeneric SCNT was the construction of embryos using panda (*Ailuropoda melanoleuca*) somatic nuclei and rabbit cytoplasts. It was reported that this combination resulted in development to the blastocyst stage (Chen et al., 2002) and, furthermore, it was demonstrated that these panda-rabbit embryos could implant in the uterus of a third species, the domestic cat. Although early fetuses developed, unsurprisingly no ongoing development to term was reported.

Development of interspecies SCNT embryos, where the combination of somatic cell and cytoplast is derived from animals within the same genus but different species, has also been reported. A single pregnancy ending in an abortion was obtained when somatic nuclei from the argali wild sheep (*Ovis ammon*) were transferred to cytoplasts from domestic sheep (*Ovis aries*; White et al., 1999). In the same study, intergeneric embryos were also produced using *Bos taurus* recipient cytoplasts, but only 1.6 % of these constructed embryos developed to the blastocyst stage. Pregnancy and foetal development was likewise obtained when somatic nuclei from a gaur bull (*Bos gaurus*) were transferred into bovine cytoplasts, in spite of the donor nucleus species having a different chromosome number to that of the recipient cytoplasm species (Lanza et al., 2000; Vogel et al., 2001). In a similar study, offspring of a species of wild sheep (*Ovis orientalis musimon*) was produced using domestic sheep (*Ovis aries*) cytoplasts (Loi et al., 2001). In all these cases, although the species were not the same, they were closely related and within the same genus (i.e. intrageneric).

Despite these successes in intergeneric SCNT, there have been no reports of embryonic development following intergeneric SCNT using domestic sheep, (*Ovis aries*) cytoplasts. Consequently, the current study was conducted to test the hypothesis that, as has been demonstrated with bovine cytoplasts, the ovine ooplasm has the capacity to direct in vitro embryo development following SCNT using somatic nuclei from a different genus, in this

case those of bovine and porcine origin. The successful first pig offspring was attributed to a combination of factors including the use of granulosa cells (Polejaeva et al., 2000). Therefore, to maximise interspecies NT development, it was important to use the cell type most likely to form an NT blastocyst. Comparisons were made with ovine IVP and ovine-ovine SCNT embryos. Parameters investigated included cleavage rates, mean cell numbers over time, blastocyst rates, blastocyst quality and nuclei number.

4.2 Materials and Methods

4.2.1 Experimental design

4.2.1.1 Intergeneric somatic cell nuclear transfer

The developmental capacities of four different experimental embryo groups were compared over a seven-day period. Group 1 was the control group consisting of ovine IVF embryos (OvIVF), representing normal embryos developed under *in vitro* conditions. Group 2 consisted of ovine to ovine intrageneric SCNT embryos (ovine-ovine) representing an SCNT control group to contrast the experimental intergeneric SCNT embryos. Group 3 consisted of bovine to ovine intergeneric SCNT embryos (bovine-ovine), while Group 4 consisted of porcine to ovine intergeneric SCNT embryos (porcine-ovine). Embryos in all four groups were terminally sampled at one of seven time points: 24, 36, 48, 72, 96, 120 and 168 h post-activation/fertilisation (hpa). At each time point, embryos were fixed, stained with Hoechst 33342 in glycerol and the number of nuclei determined by viewing under UV excitation. Determining exact nuclei number was carried out to obtain a true indication of the developmental status of each to eliminate inaccuracies caused by embryo fragmentation (i.e. production of anuclear fragments). Between 3-5 replicates were completed for each time point for each group.

4.2.1.2 Intergeneric embryonic cell nuclear transfer

The developmental potential of embryos reconstructed by embryonic cell nuclear transfer (ECNT) was compared between intrageneric and intergeneric combinations. Four groups of embryos were studied in this experiment. Groups 1 and 2 consisted of ovine-ovine ECNT embryos cultured either individually in droplets (Group 1) or in WOWs (Group 2). Group 3 and 4 consisted of bovine-ovine intergeneric ECNT embryos cultured in droplets (Group 3) or in WOWs (Group 4); thus forming a 2x2 factorial design. Embryo cleavage was recorded 48 hpa, and development to morula and blastocyst stages was recorded at 168 hpa. At this time, all cleaved embryos were fixed and stained with Hoechst 33342-glycerol for nuclei number determinations.

4.2.2 Ovine *in vitro* embryo production

Ovine *in vitro* maturation, fertilisation and culture were performed as described in Section 2.4. Briefly, ovine COCs were recovered by aspiration from abattoir-derived ovaries. COCs with a compact, nonatretic cumulus investment, and a homogenous ooplasm were matured in maturation medium covered with mineral oil. The dishes were incubated at 38.5°C in an atmosphere of 5% CO_2 in humidified air for 24 h.

Outer cumulus cells were removed from matured ova whilst leaving the corona radiata layers intact, and placed in fertilisation medium covered in mineral oil. Capacitated frozen-thawed sperm were then combined with the ova, and the gametes were co-cultured at 38.5°C in an atmosphere of 5% CO_2 in humidified air for 24 h.

Cumulus cells were subsequently removed and the presumptive zygotes were placed in wells of a 4-well culture (30-35 per well) dish containing culture medium overlaid with mineral oil, and were incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in humidified air at 38.5 °C. After 48 h embryos, were assessed for cleavage and uncleaved cells removed from culture.

4.2.3 Somatic cell nuclear transfer

All aspects of SCNT are described in significant detail in Section 2.10 and outlined in Figure 4.1. Briefly, primary ovine, bovine and porcine cell lines were established from mural granulosa cells collected by aspirating ovarian antral follicles from ovaries obtained from the slaughterhouse. Cells were cultured for two passages in Glasgows medium and frozen at passage 2. Cells for SCNT were thawed, cultured for five days until confluent, and then serum starved for two days in the presence of 0.5% HIFCS. On the day of SCNT, a suspension of the donor cells was prepared by standard trypsinisation 30 min beforehand.

To produce ovine cytoplasts, COCs were in vitro matured for 18-20 h, freed from all cumulus cells and selected according to the presence of a polar body. One-step enucleation and sub-zonal cell injection was performed by removing a small portion of the oocyte cytoplasm containing the metaphase spindle and the polar body, followed by the immediate (within 30 sec) placement a single donor somatic cell into the perivitelline space.

Exposure to electric pulses between two platinum wires then fused couplets. Fusion success was evaluated within 30 min and intact couplets that did not fuse were pulsed again. Couplets that did not fuse after the second attempt were discarded. Within 30 min of pulsing, fused couplets were given an additional chemical activation stimulus using calcium ionophore and 6-DMAP treatments. Newly constructed embryos were then washed twice in culture medium, and assigned to sample groups of 7-10 embryos. Embryos were placed in 0.5 ml of culture medium covered with 0.38 ml mineral oil in 4-well culture dishes and incubated in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in humidified air at 38.5 °C.

4.2.4 Assessment of embryo nuclei number

At the desired timepoint, a group of embryos was taken from the incubator. A visual assessment of each embryo was made and recorded. Droplets of Hoechst-glycerol staining fixative (Appendix II) were placed at one end of a glass microscope slide and, working under

Figure 4.1

Schematic diagram summarising the cloning procedure and design for the SCNT experiment.

Hours Post Maturation

1. Ova clean-up. — 18 h

2. Bovine, ovine and porcine somatic cell preparation.

3. One-step enucleation and sub-zonal cell injection. — 19 h

— 21 h

4. Electrofusion. — 22-23 h

5. Activation with calcium ionophore & 6-DMAP. — 24 h

6. In vitro culture at 38.5 C in reduced oxygen. — 27 h

7. Cleavage assessment, and sampling of embryos at 24, 36, 48, 72, 96, 120 and 168 hpa for cell number assesment. — 75 h

8. Blastocyst assessment — 168 h

a stereomicroscope and using a glass pipette, single embryos were placed into each droplet. A glass coverslip with a small portion of Vaseline-wax on the corners was placed above the droplets and carefully pressed to gently squash the embryos. Without losing sight of the embryos, a felt-tip pen was used to circle each embryo, marking the location of each embryo. When all the embryos had been fixed, the glass slides were placed on a tray, covered in foil and kept at 4°C overnight. The following day, the number of nuclei in each fixed embryo was counted using UV fluorescence microscopy.

4.2.5 Bovine *in vitro* embryo production

Bovine *in vitro* maturation, fertilisation and culture were performed as described in Section 2.5. Briefly, bovine ovaries were collected and aspirated by hand with a 10 ml syringe and 18-gauge needle. COCs with a compact, nonatretic cumulus investment, and a homogenous ooplasm were selected and matured *in vitro*. They were placed in a maturation medium in groups of 30-35, in wells of a 4-well culture dish containing 0.6 ml of maturation medium covered with 0.38 ml of mineral oil and incubated at 38.5°C in 5% CO_2 in humidified air for 24 h.

Matured ova were separated from one another, washed and placed in 0.5 ml fertilisation medium in wells of a 4-well culture dish. A straw of frozen bull semen was thawed and centrifuged for 20 min at 780 *g* through a Percoll gradient to separate motile sperm. Diluted motile sperm were added to each well containing COCs at a concentration equivalent to 1 x 10^6 sperm/ml and cultured at 38.5°C in 5% CO_2 in humidified air for 24 h.

Presumptive zygotes were manually denuded using a glass pipette and washed three times in culture medium, and placed in groups of 30-35 in wells of a 4-well culture dish containing 0.5 ml of culture medium covered with 0.38 ml of mineral oil. Embryos were incubated in an atmosphere of humidified 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C. After 72 h, embryos were assessed for cleavage and 1-cell stages discarded.

4.2.6 Vitrification of embryos

Ovine and bovine embryos were vitrified at day 4 of development. Open-pulled straw (OPS) vitrification of donor ovine and bovine embryos was performed according to Section 2.7. Briefly, morulae were selected and washed twice in bench medium, followed by a 3 min incubation in bench medium supplemented with 7.5% dimethylsulfoxide (DMSO) and 7.5% ethylene glycol. Finally, the embryos were incubated for 20-25 sec in the bench medium containing 0.6 mol sucrose l^{-1}, 16.5% DMSO and 16.5% ethylene glycol, during which time they were loaded into thin pulled straws by capillary action in a 2 µl volume, followed by immersion into liquid nitrogen.

4.2.7 Embryonic cell nuclear transfer

As described in Section 2.9, on the day before ECNT, vitrified donor embryos were taken from liquid nitrogen, warmed in air for 2-3 sec before submerging the narrow end containing the embryos in bench medium with 0.3 mol sucrose l^{-1}, into which the embryos were expelled immediately and kept for one min. Embryos were then incubated for 5 min in bench medium containing 0.15 mol sucrose l^{-1}, followed by two 5 min incubations in bench medium containing no sucrose. Embryos were placed in culture medium at 38.5°C in humidified 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C for 24h.

ECNT was performed as described in Section 2.9. Briefly, COCs were obtained and in vitro matured for 18 h. Mature ova were enucleated, and cytoplasts were then activated with calcium ionophore and 6-DMAP. The zona pellucida was removed from the activated cytoplasts and donor embryos using 0.5% pronase in protein free SOF-Hepes, followed by a recovery period of 30 min. Handling of zona-free embryos was performed in SOF-Hepes containing 20 mg/ml BSA. Day 4 morulae (16-32 cell) donor embryos were disaggregated by incubation in calcium and magnesium-free handling medium containing 7.5 µg/ml cytochalasin B for 10 min, followed by further disaggregation into individual blastomeres

using a fine fire pulled pipette. For zona-free electrofusion, blastomeres and cytoplasts were equilibrated in fusion medium for 30 sec and transferred into the electrofusion chamber. The initial alignment of cells was achieved with an AC pulse of 8 V and 500 kHz for 5-10 sec, followed by a fusion pulse consisting of a single DC pulse of 100 μsec and 500 V/cm. The use of the AC alignment electrically forced the blastomere and cytoplast together against the platinum wire of the fusion chamber. Couplets were checked 20-40 min later for successful fusion before transferring to culture medium supplemented with 20 mg/ml BSA. Embryos were either cultured in groups of 30 using the WOW system (Vajta et al., 2000) or individually in 20 μl droplets to prevent aggregation of individual embryos.

4.2.8 Statistics

Differences between treatment groups in the intergeneric SCNT experiment were tested using the procedure CATMOD in SAS for parameters of categorical data, while analysis of variance (procedure GLM in SAS) was employed for nuclei number. If variance between treatments were unequal, data was log transformed prior to analysis. Data for the intergeneric ECNT experiment was analysed as a 2x2 factorial; factors were method of culture (droplets, WOW) and intergeneric ECNT (bovine-ovine, ovine-ovine). The main effects and their interaction were tested using the CATMOD procedure.

4.3 Results

4.3.1 Intergeneric SCNT

There were no significant differences in the efficiency of couplet production (after enucleation and injection) or in the total percentage of ovine-ovine, bovine-ovine and porcine-ovine SCNT embryos fused and activated (NT units) as a proportion of the number of mature ova used for cloning (Table 4.1). In addition, the fusion success in SCNT embryo production did not differ despite the granulosa cells being derived from different species (Figure 4.2). Fusion rates varied between replicates (Figure 4.3). Fusion success appeared to vary

Table 4.1

Summary of enucleation and cell injection of three different intergeneric SCNT embryos produced for the analysis of developmental capacity.

Embryo	N	Reps	Percentage enucleated & injected[1] (n)	Fusion Percentage[2]	% NT Units in culture[3] (n)
ovine-ovine	439	11	81.5 (358)	73.3	57.4 (252)
bovine-ovine	358	7	87.4 (313)	67.4	57.3 (205)
porcine-ovine	418	6	82.3 (344)	73.9	57.7 (241)

[1]Refers to the percentage of mature oocytes that were successfully enucleated and injected with a somatic cell ready for fusion.

[2]The percentage of couplets pulsed that were successfully fused.

[3]NT units constructed as a percentage of mature ooocytes used at the beginning of experiments

Figure 4.2

Percentage of fused couplets, non-fused couplets, donor cell lysis and cytoplast lysis for ovine-ovine, bovine-ovine and porcine-ovine SCNT embryos.
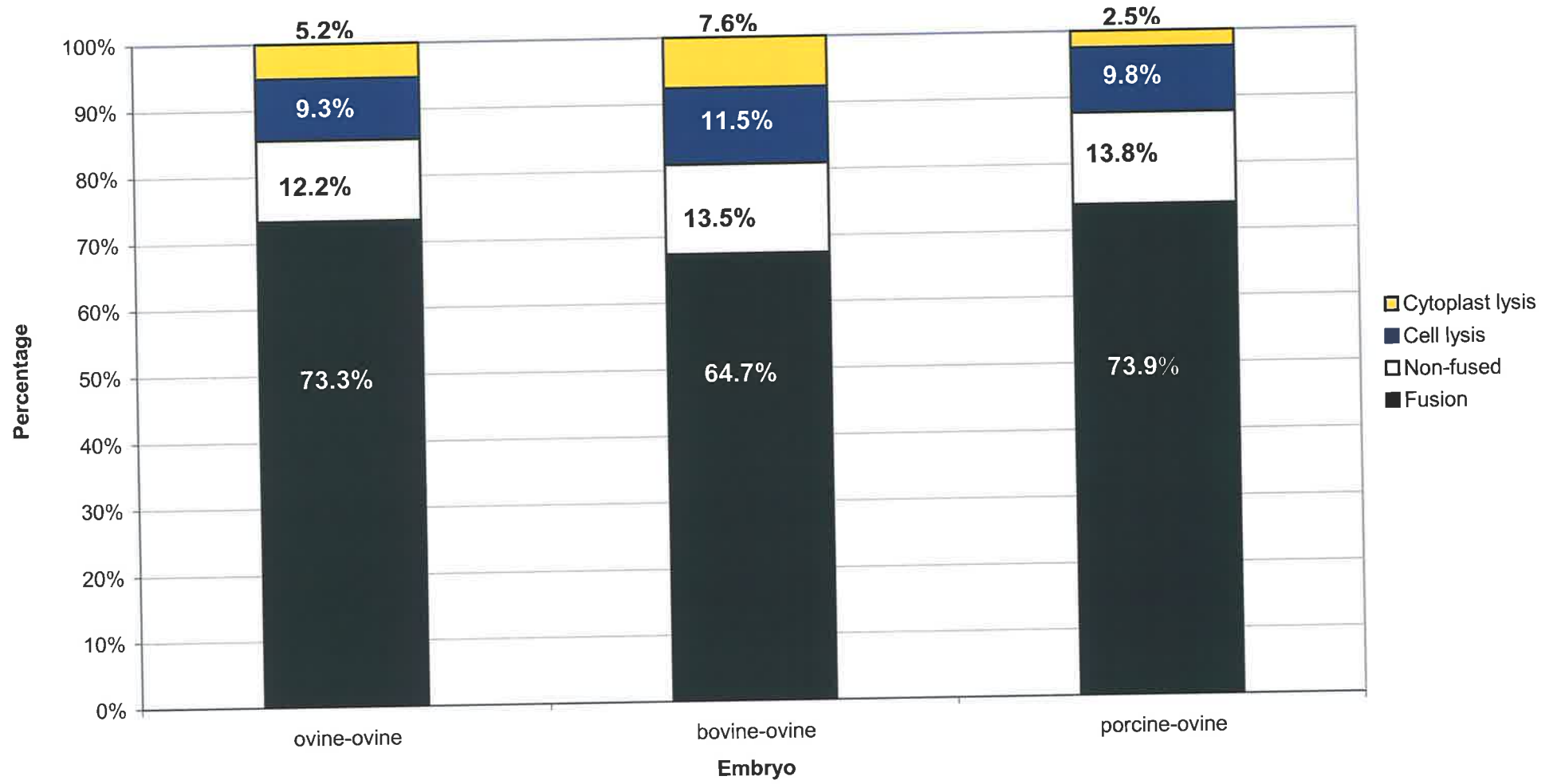
Figure 4.3

Variation, across SCNT experiments, in fusion percentages for a) bovine-ovine and b) porcine-ovine SCNT embryos. Ovine-ovine fusion percentages were determined for each of the experiments and are shown as a comparison.

A
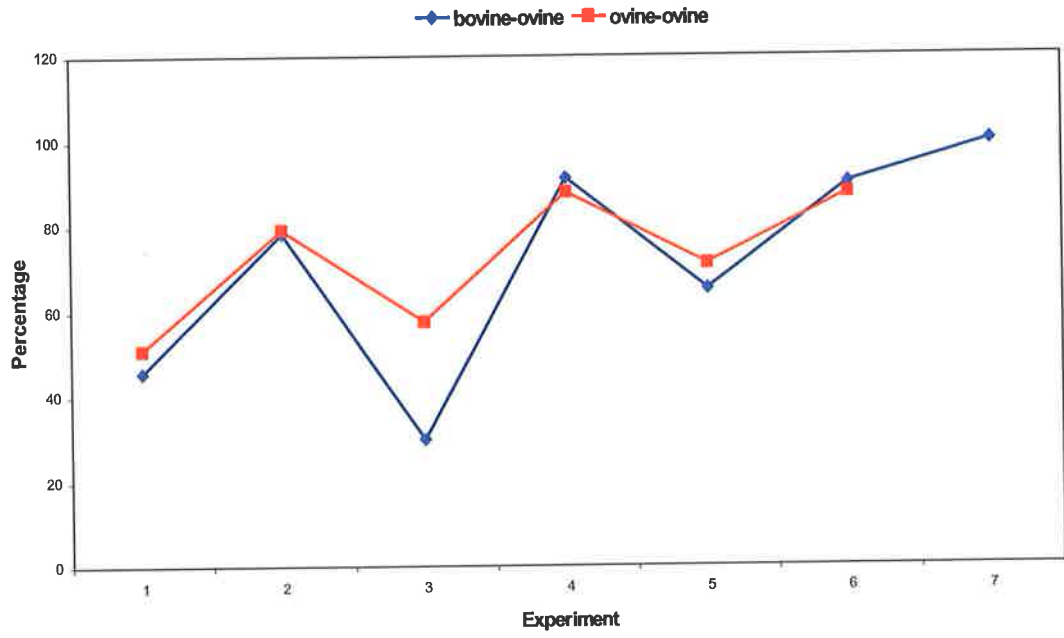
B
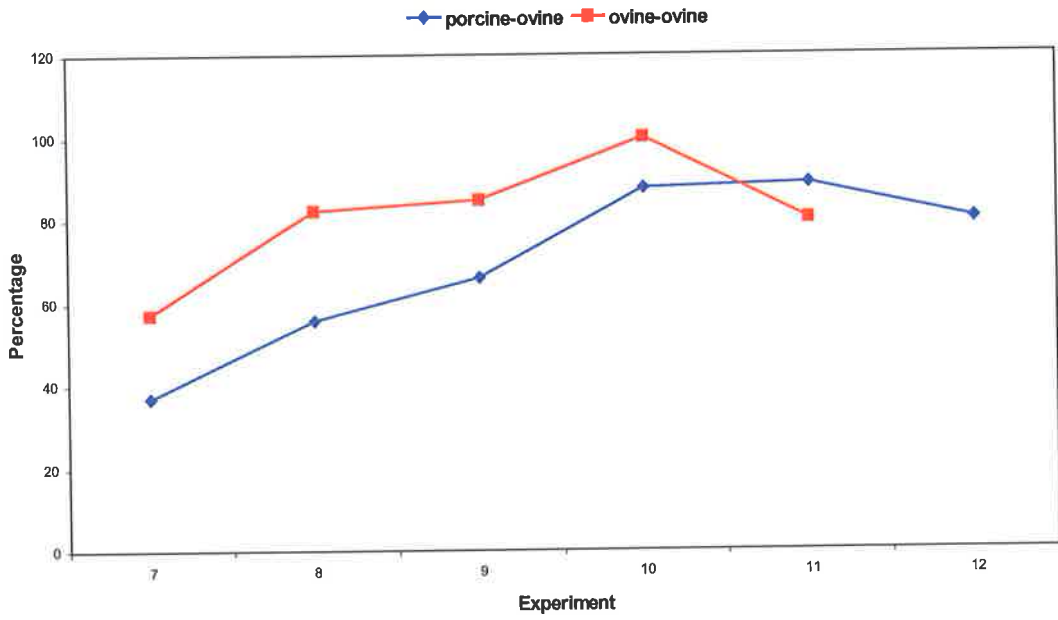
according to unspecified experimental factors, and not according to the donor cell species. Bovine and ovine cells demonstrated similar fusion rates (Figure 4.3a) but, porcine donor cells consistently showed a reduced ability to fuse compared with ovine cells (Figure 4.3b). No notable difference was observed between ovine and bovine donor cell lysis during fusion for all experiments (Figure 4.4a). However, the lysis rate was consistently higher with porcine donor cells than those of ovine cells during the fusion process (Figure 4.4b).

Some examples of glycerol-Hoechst fixed and squashed embryos, used to determine cell number are shown in Figure 4.5. The percentages of cleaved embryos containing >1 nucleus were 77.5% (145/187), 77.9% (106/136) and 76.8% (136/177) for the ovine-ovine, bovine-ovine and porcine-ovine, respectively (Figure 4.6). These values were not significantly different from each other, and were significantly ($P<0.05$) less than the OvIVF cleavage rate of 91.2% (239/262). Neither bovine-ovine nor porcine-ovine SCNT embryos formed blastocysts or compact morulae (Figure 4.7). Of those embryos allowed to develop to 168 h, 78.9% (30/38) of cleaved OvIVF embryos formed blastocysts, which was a significantly ($P<0.05$) higher rate than the 58.3% (28/48) observed in the ovine-ovine SCNT group (Figure 4.7). However, the mean cell number for ovine-ovine SCNT blastocysts (96.87 ± 8.00) was not significantly different from that of the IVF blastocysts (96.89 ± 8.81; Figure 4.8).

The average number of nuclei for all four groups over time is illustrated in Figure 4.9. All SCNT embryos were comparable with the OvIVF group at all time points up to 72 hpa, with the exception of the porcine-ovine SCNT embryos, which had significantly ($P<0.05$) fewer nuclei at 48 hpa. For the 96 and 120 h time points, intra- and intergeneric SCNT embryos had significantly ($P<0.05$) reduced nuclei numbers compared with the OvIVF embryos. However, the intra- and intergeneric SCNT embryo groups were not significantly different from each other. At 168 h post activation, the intergeneric bovine-ovine and porcine-ovine SCNT embryos had significantly ($P<0.05$) fewer nuclei than both their ovine-ovine and OvIVF counterparts.

Figure 4.4

The incidence of donor granulosa cell lysis that occurred during electrofusion of a) bovine-ovine, and b) porcine-ovine couplets. Lysis of ovine donor cells are shown as a comparison for each experiment.

A
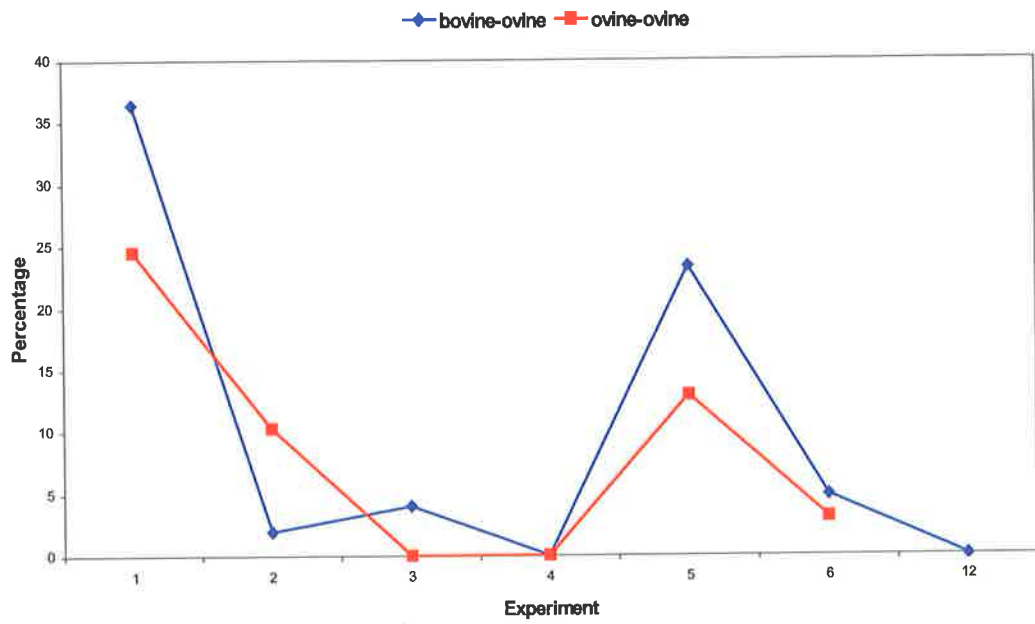
B
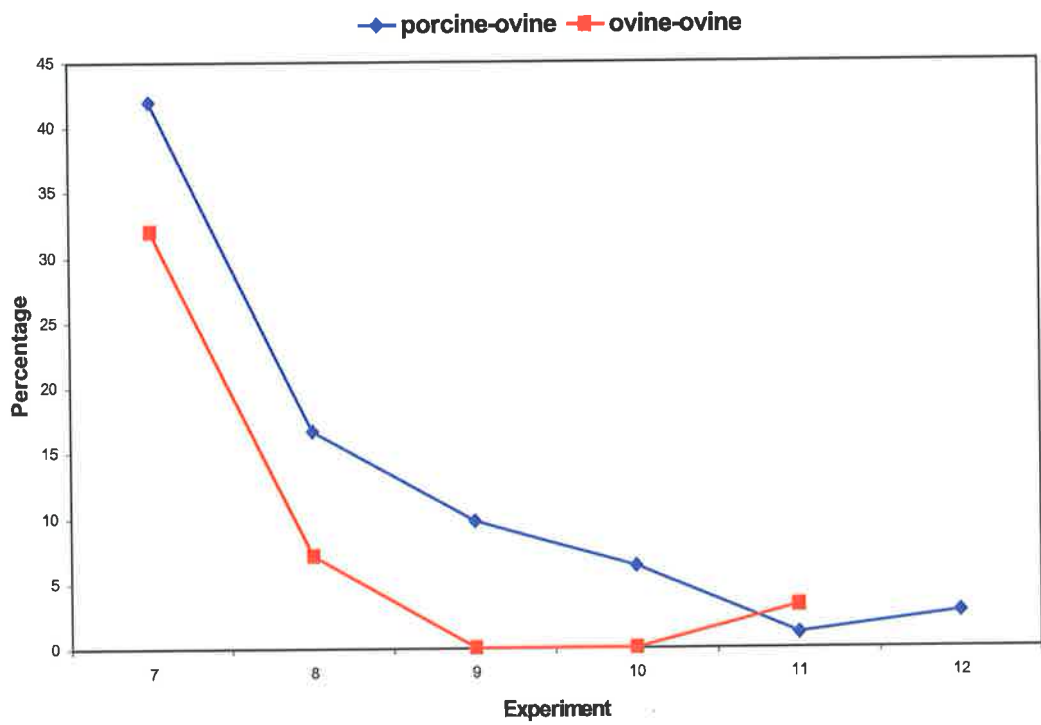
Figure 4.5

Glycerol-Hoechst fixed ovine-ovine SCNT embryos under UV fluorescence at the 4-cell (a),

8-cell (b), 26-cell morula (c) and blastocyst (d) stage of development.
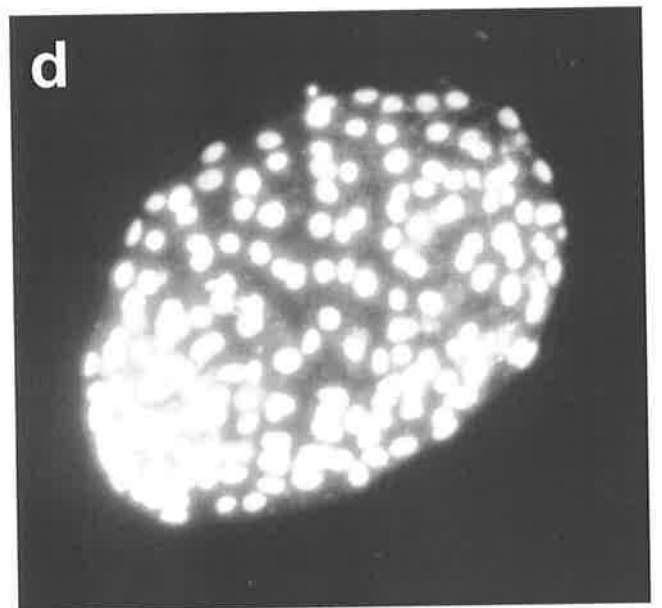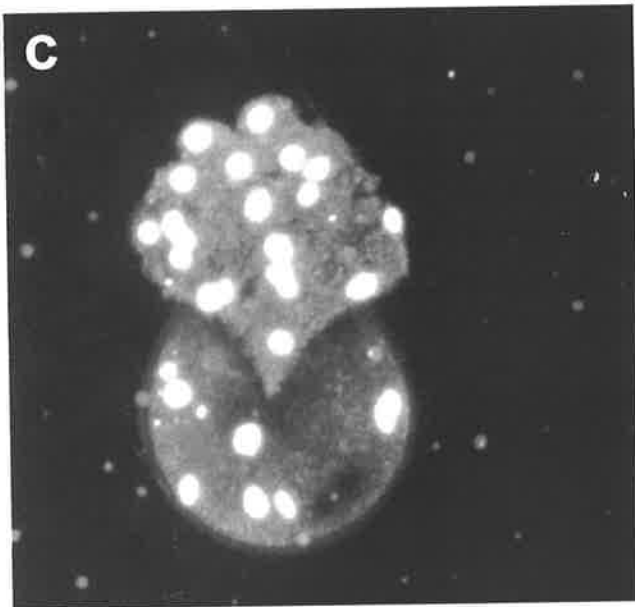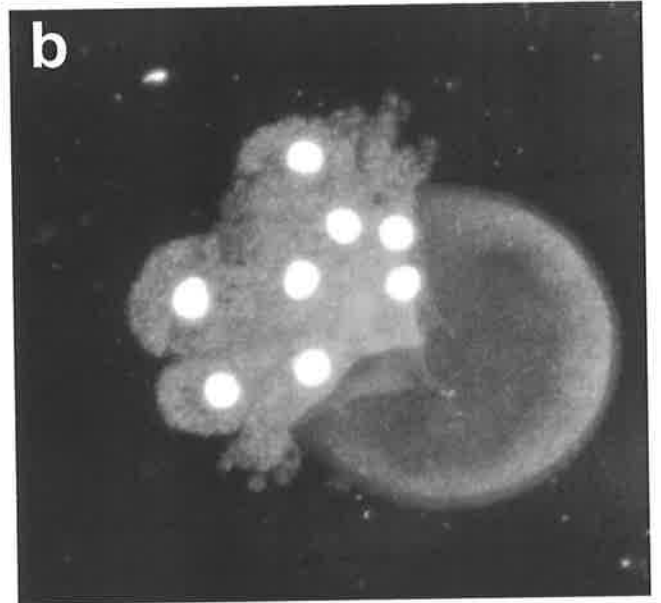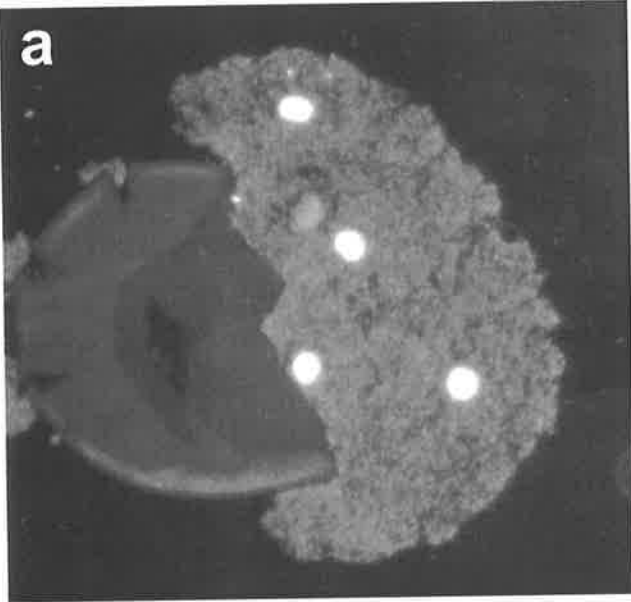
Figure 4.6

Cleavage of ovine-IVF, ovine-ovine (OvOv), bovine-bovine (BoINT), and porcine-ovine (PrINT) SCNT embryos.

*Denotes significantly different (P<0.05).
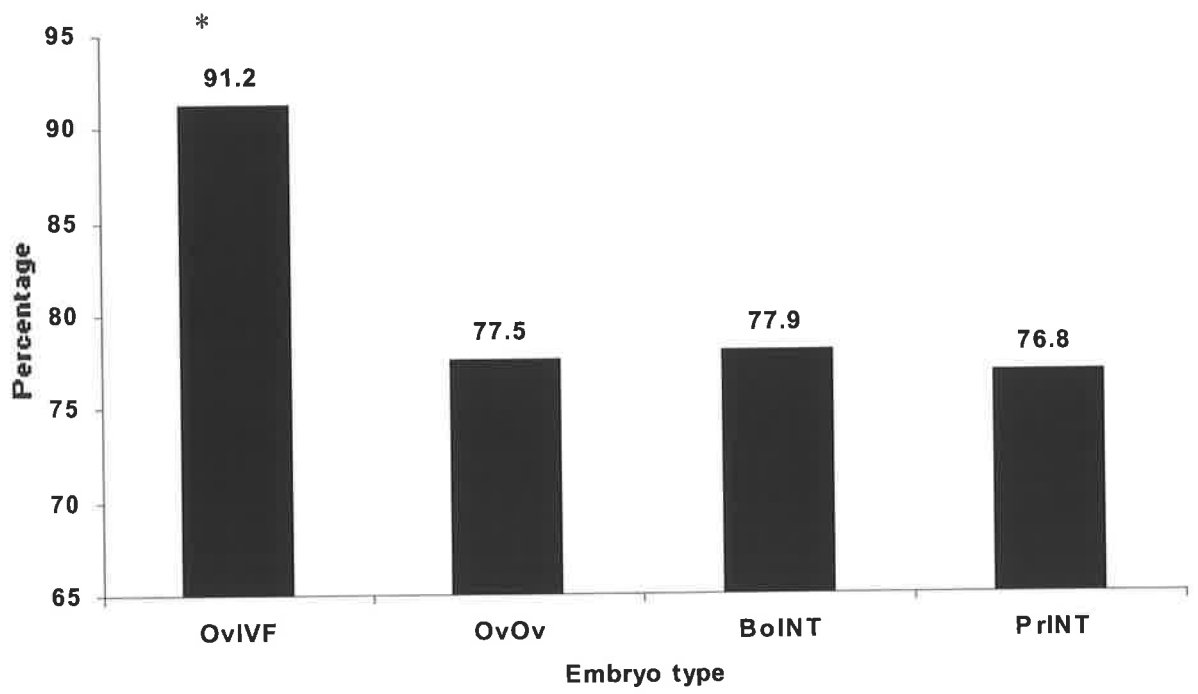
Figure 4.7

Percentage of day-7 ovine IVF (OvIVF), ovine-ovine (OvNT), bovine-ovine (BoINT) and porcine-ovine (PrINT) embryos that develop to the compact morula-hatched blastocyst stage (CM-HB), and early blastocyst to hatched blastocyst range of advanced preimplantation embryo development (EB-HB).
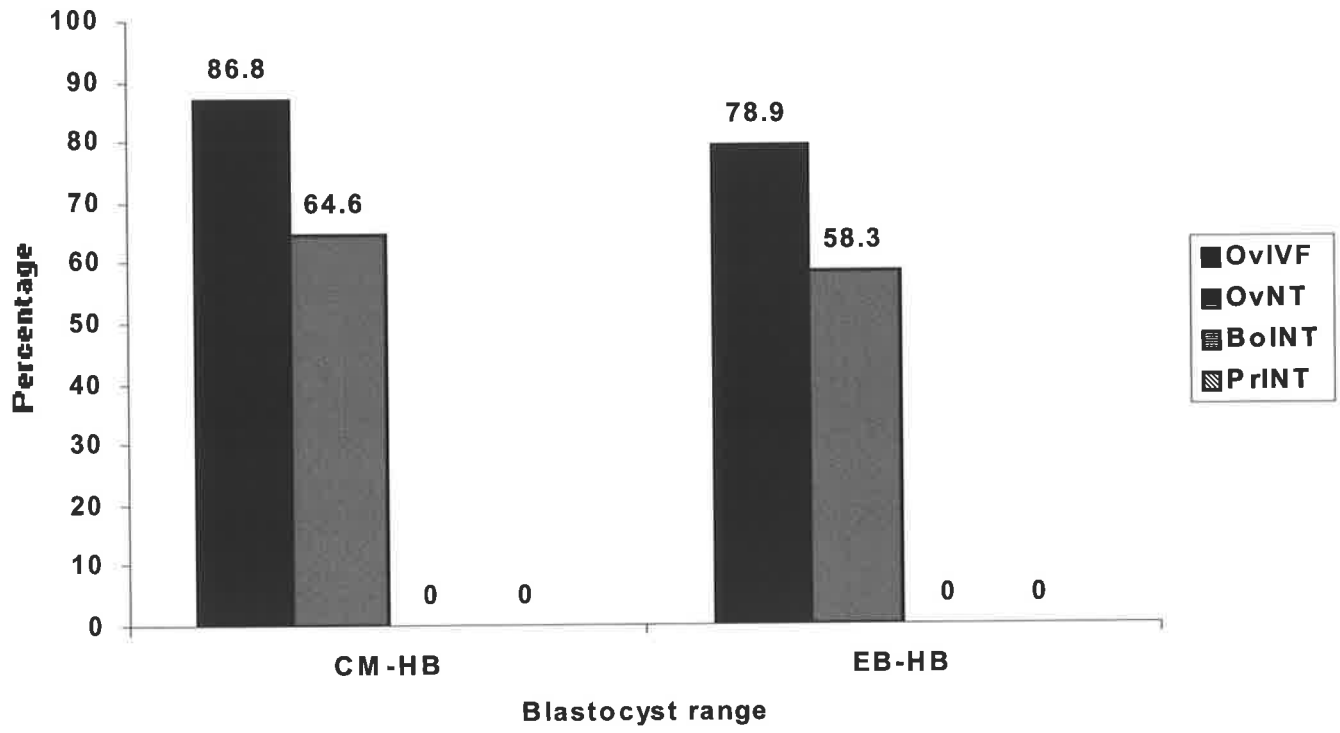
Figure 4.8

Mean nuclei number ($\pm$SEM) for ovine IVF (OvIVF) and ovine-ovine SCNT (OvNT) blastocysts.
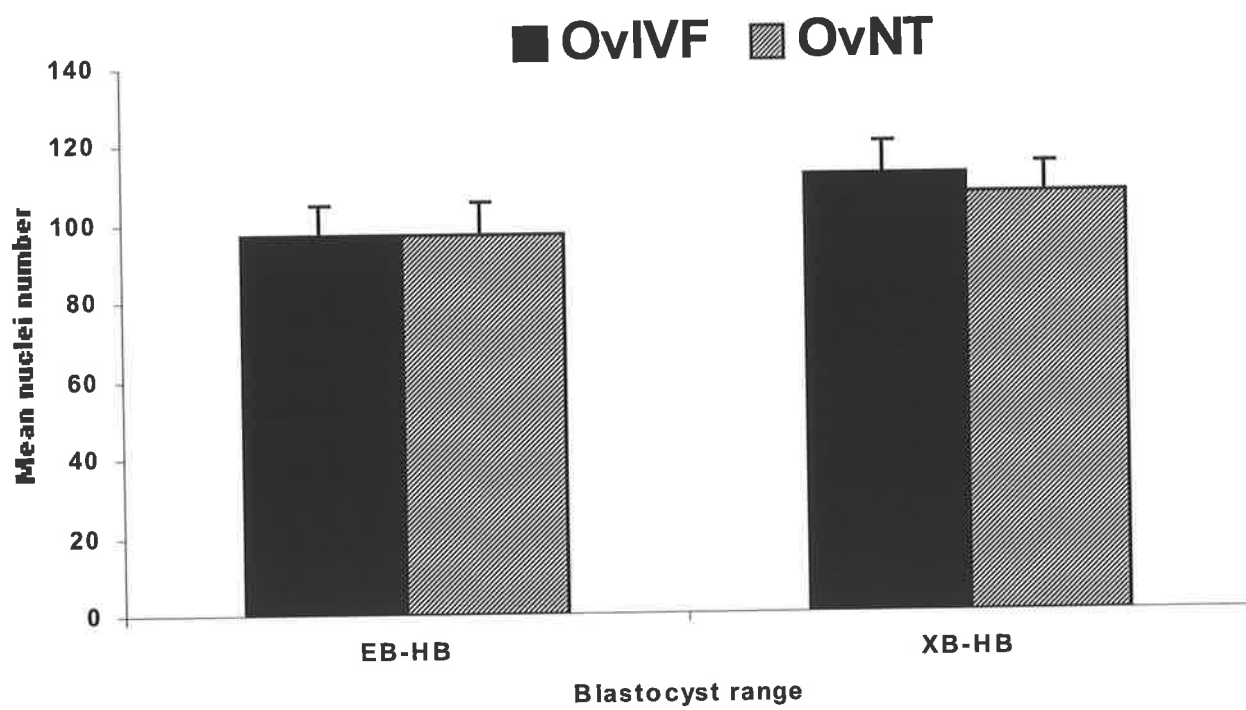
Figure 4.9

Number of nuclei per embryo (least-squares means±SEM) presented numerically and plotted at various time points over 168 hpa for ovine IVF, ovine-ovine, bovine-ovine and porcine-ovine somatic nuclear transfer embryos. Numbers are of embryos with 2 or more nuclei for all time points except at 24 hours, where 1 cells are included due to the fact that the first cleavage division may not have occurred by this time. [abc]Values for subclass means with at least one common superscript do not differ significantly (P> 0.05). Values are plotted on a logarithmic scale.
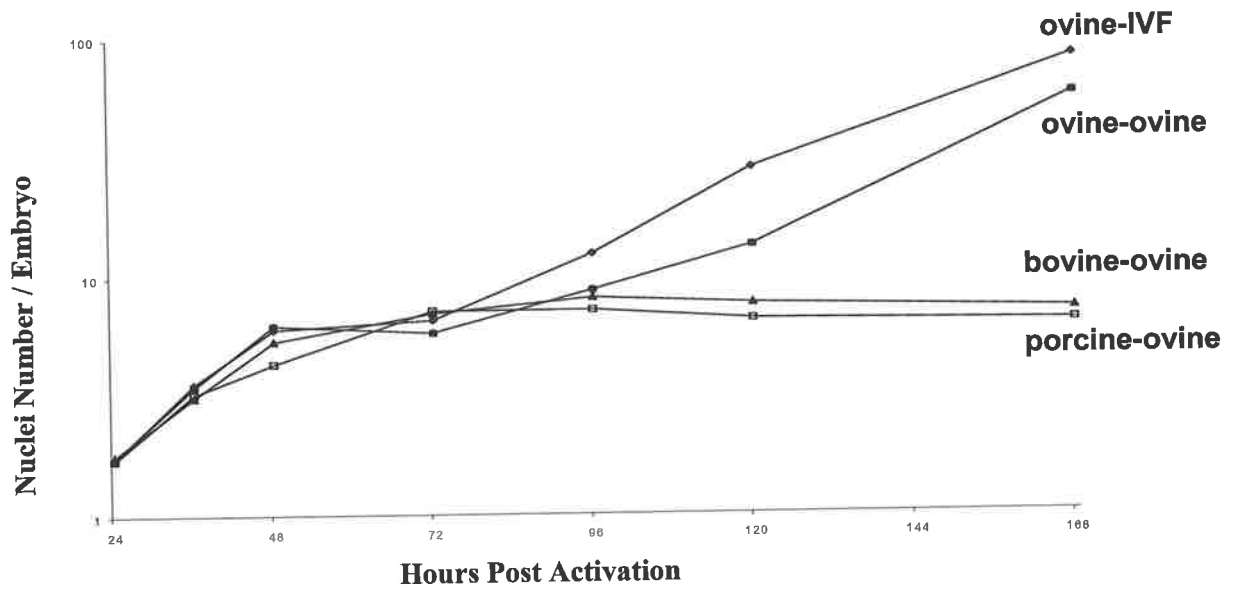
	Hours post activation						
Group	24	36	48	72	96	120	168
ovine-IVF	1.73 ± 0.13	3.57 ± 0.24	6.03 ± 0.34^a	6.56 ± 0.37	12.38 ± 1.09^a	28.10 ± 3.71^a	81.21 ± 8.21^a
ovine-ovine	1.75 ± 0.16	3.47 ± 0.30	6.25 ± 0.47^a	5.81 ± 0.56	8.70 ± 1.32^b	12.28 ± 3.88^b	$56.48 \pm 7.76\text{-}^b$
bovine-ovine	1.79 ± 0.16	3.14 ± 0.25	5.38 ± 0.46^{ab}	7.00 ± 1.06	8.09 ± 0.70^b	7.59 ± 0.98^b	7.10 ± 0.83^c
porcine-ovine	1.72 ± 0.11	3.24 ± 0.19	4.32 ± 0.44^b	7.17 ± 0.81	7.18 ± 1.00^b	6.52 ± 0.89^b	6.32 ± 0.84^c

The initial development of the different groups of embryos at 48, 72 and 96 hpa is presented in Figure 4.10. At 48 hpa, the OvIVF and ovine-ovine SCNT group showed a normal distribution with the greatest percentage of embryos having 5-8 nuclei. However, both the bovine-ovine and porcine-ovine groups had a skewed distribution with the majority of embryos having 2-4 nuclei. At 72 hpa, small proportions of bovine-ovine and porcine-ovine SCNT embryos had 17-32 nuclei, but were otherwise not dissimilar to the ovine-ovine group.

The OvIVF group had a higher percentage at the 5-8 nuclei stage than all NT groups. At 96 hpa, 28.2% of OvIVF embryos had 17-32 nuclei while only 12.5% of ovine-ovine, 0% of bovine-ovine and 6.7% of porcine-ovine SCNT embryos had reached this stage of development. OvIVF embryos were spread relatively evenly between the 5-8 nuclei, 9-16 nuclei and 17-32 nuclei stages.

4.3.2 Intergeneric ECNT

The development of intergeneric ECNT embryos in both drop and WOW culture systems are presented in Table 4.2. Figures do not include multiple blastomere embryos containing zero nuclei or their DNA remnants (hereafter referred to as anuclear embryos). For both culture systems, the percentage of embryos that were anuclear at 168 hpa, differed significantly (P<0.05) between the ovine-ovine and bovine-ovine ECNT embryos (Table 4.2). No significant difference was apparent in the number of couplets that underwent the first cleavage division, however, there was a significant difference in cleavage between the culture systems (P<0.05), irrespective of whether the embryo was intergeneric or not. In the droplets, the number of embryos that reached the 8-cell stage or greater did not differ significantly between the intergeneric embryos and the ovine-ovine ECNT controls. This was not the case with the WOW culture system, where bovine-bovine embryos reached this developmental stage at a reduced proportion compared with the ovine-ovine control. No bovine-ovine embryos reached

Figure 4.10

Development of ovine IVP (OvIVF), ovine-ovine (OvNT), bovine-ovine (BoINT) and porcine-ovine (PrINT) embryos at 48 hpa, 72 hpa and 96 hpa.
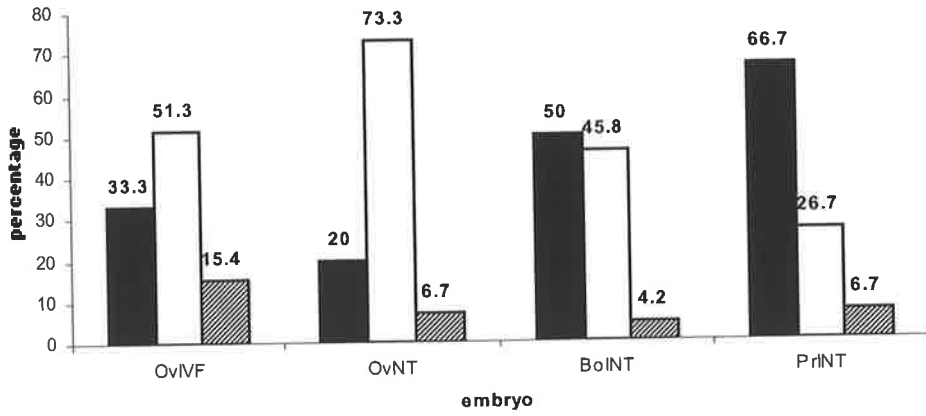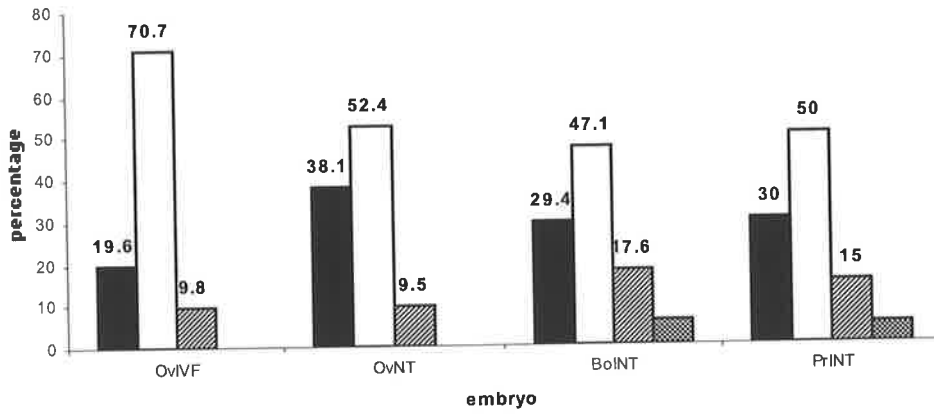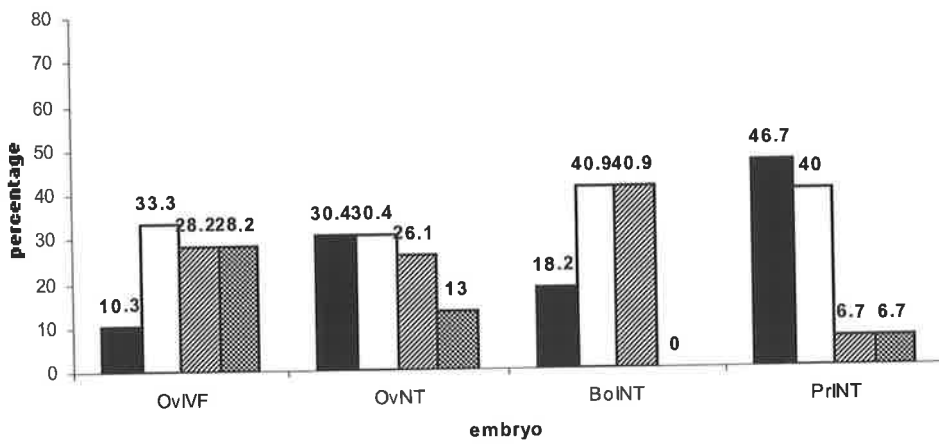
Table 4.2

In vitro development of zona-free ovine-ovine and bovine-ovine ECNT embryos in two different culture systems (droplet versus WOW).

Method of embryo culture	Embryo	N (Reps)	Anuclear (%)	N1	Cleavage (%)^2	$\geq$8 Nuclei^3	Blastocyst (%)
Droplets	ovine-ovine	125 (6)	46 (36.8)^a	79	65 (82.3)^a	24 (36.9)^{ab}	14 (21.5)^a
Droplets	bovine-ovine	91 (4)	9 (9.9)^b	82	65 (79.3)^a	20 (30.8)^a	0 (0)^b
WOWs	ovine-ovine	33 (2)	12 (36.4)^a	21	12 (57.1)^b	8 (66.7)^b	8 (66.7)^c
WOWs	bovine-ovine	43 (2)	5 (11.6)^b	38	21 (55.3)^b	4 (19.0)^a	1 (4.8)^{ab}

[abc]Means or properties followed by different letter within columns differ significantly ($P<0.05$).

[1]Refers to the total number adjusted to exclude all anuclear embryos. Cleavage is therefore a percentage of this number, and proportions in columns thereafter are percentages of cleaved.

[2]Cleavage visually determined at Day 3 of development.

[3]Percentages of embryos determined at day 7 after nuclei staining after Hoechst staining.

the blastocyst stage when cultured in droplets compared with 21.5% (14/65) of ovine-ovine embryos. A single bovine-ovine embryo reached the blastocyst stage (4.8%, 1/21) when cultured in the WOW system, but this success rate was reduced compared with the 66.7% (8/12) observed for the ovine-ovine embryos. The mean blastocyst nuclei number did not differ significantly between the WOWs and droplets for ovine-ovine ECNT embryos.

4.4 Discussion

Results of this study demonstrate that intergeneric nuclear transfer embryos produced by transferring bovine or porcine somatic nuclei into ovine cytoplasts were incapable of developing to the blastocyst stage. Furthermore, following nuclear transfer of pluripotent bovine embryonic nuclei into ovine cytoplasts, only a very small proportion (4%, n=1) were capable of developing to the blastocyst stage. These results contrast those reported using bovine cytoplasts, which have been shown to be capable of supporting advanced stages of embryonic development following nuclear transfer of somatic cells from different species (Dominko et al., 1999).

The ability of most couplets to develop to at least the 8-16 cell stage is not unexpected given that, in ruminant embryos, the first three cell cycles, are known to be regulated primarily by maternally inherited gene products stored in the oocyte (Telford et al., 1990). Development subsequent to activation of the embryonic genome relies on the successful transfer of control to a fully functional embryonic genome. The combination of the ovine cytoplasm with bovine or porcine somatic cells in this study clearly did not produce embryos that were capable of such transfer, even when the donor cell was of a pluripotent nature. This suggests that the ovine ooplasm may not be capable of reprogramming granulosa cell nuclei from another genus, or alternatively, that the two entities are incompatible to such an extent that normal cellular processes cannot occur.

It is unlikely that technical differences contributed to discrepancies in the intergeneric developmental ability of embryos constructed with ovine ooplasm compared with other studies using bovine ooplasm. One technical difference was the timing of fusing and activation, which occurred simultaneously in this study. It has been reported that allowing the donor nucleus to be exposed to the recipient cytoplasm for a period of 4 to 6 h prior to activation (referred to as fusion before activation; FBA) can improve the developmental success in cattle (Wells et al., 1999). FBA was used in the intergeneric NT experiments performed by Dominko et al. (1999). It was not implemented in this study due to the inability to electrically fuse couplets without simultaneously activating the ovine cytoplast (see Chapter 3). Minor differences in NT and culture procedures exist between the ovine and bovine, such as a longer incubation of bovine NT embryos in the protein kinase inhibitor 6-DMAP (between 4-6 h) to inhibit the activity of maturation promoting factor (MPF). In ovine NT embryos, it has been demonstrated that a 2 h incubation in 6-DMAP is equally effective and yields blastocysts in the same proportions as a longer incubations (Peura et al., 1999). It is not clear whether the intergeneric NT embryos require a longer incubation period in 6-DMAP due to the different species of the donor nucleus, but it is unlikely that such a failure in developmental competence could be attributed to this fact.

It could be argued that the retardation in the development of intergeneric nuclear transfer embryos is a result of the culture medium being best suitable to sheep embryos, and not to bovine or porcine embryos. After the major activation of the embryonic genome occurs, the oocyte cytoplasm is no longer the only source of proteins and mRNA. Therefore, the requirements of intergeneric embryos might differ according to the donor nucleus as embryonic genome takes control of development. Dominko and colleagues (1999) cultured interspecies embryos to the blastocyst stage in CR1aa medium, which is designed for bovine embryos. The use of species-specific sequential media was considered as a line of investigation in the present study to improve the *in vitro* development capacity of the

interspecies embryos. For example, culturing bovine-ovine intergeneric SCNT embryos in SOF medium designed for the sheep embryo for the first 48 hours whilst the cytoplasm is responsible for supporting development, and then moving them into SOF medium designed for cow embryos (donor nucleus species) for the remaining 5 days when the donor nucleus would be expected to be controlling the developing embryo. SOF is a "simple" culture medium based on the concentration of salts and energy sources in the oviductal fluid of sheep (Restall and Wales, 1966). It is a very common medium used successfully with modifications in both bovine and ovine embryo culture. In this experiment, the SOF culture medium was supplemented with amino acids at sheep oviductal fluid concentrations (Walker et al., 1996). In previous experiments, this SOF culture medium supplemented with amino acids at oviductal fluid concentrations was shown to support bovine *in vitro* embryo production to a high level of blastocyst development (40-50%; J.M. Kelly, personal communication). Therefore, the use of sequential species-specific media is not likely to have improved the developmental capacity of bovine-ovine NT interspecies embryos and thus was not implemented. Had there been successful blastocyst development in these embryos, such a species-specific sequential media would have been the subject of an investigation to improve the porcine-ovine NT embryos.

In the intergeneric ECNT experiment, the anuclear embryos were most likely to have resulted from the fusion of anuclear blastomeres. Results presented in Table 4.2 show that the number of anuclear embryos in bovine-ovine ECNT embryos was significantly less than the ovine-ovine embryos. This suggests that perhaps the ovine embryos from which donor blastomeres were obtained had a much higher proportion of anuclear blastomeres than their bovine counterparts. As with the SCNT intergeneric embryos, no significant difference in cleavage rate was observed between bovine-ovine and ovine-ovine reconstructs. However, cleavage rate appeared to be severely hampered when embryos were cultured individually in microdrops compared with the WOWs.

For *in vivo* bovine and porcine embryos, it has been demonstrated that the major activation of the embryonic genome occurs at the late 8-cell stage (Camous et al., 1986) and late 4-cell stage (Tomanek et al., 1989) respectively. The major activation of the sheep embryonic genome is known to occur between the 8- and 16-cell stage (Crosby et al., 1989), a stage that may not coincide with either of the two species that have been used in the construction of intergeneric embryos, especially that of the porcine. The fact that the timing of this crucial event differs between species may be a reason for the failure of these embryos to develop past the 16-cell stage of development. However, interspecies embryos constructed with ovine and porcine nuclei into bovine oocytes yielded embryos that developed beyond the late 8-cell stage where activation of the bovine embryonic genome occurs (Dominko et al., 1999). The timing and further characterisation of embryonic genome activation in these intergeneric embryos is investigated in Chapter 5 by examining nucleolus ultrastructure using transmission electron microscopy, in combination with autoradiographic detection of heterogenous rRNA synthesis (Hyttel et al., 2000a). Such studies enable a determination of the ovine ooplasm's ability to support critical cellular events prior to genome activation; a cellular event such as nucleologenesis. In addition, by examining the porcine-ovine intergeneric embryos, it will enable the elucidation of what influence the cytoplasm has on such a process.

In conclusion, NT embryos created by fusing porcine and bovine somatic cells to ovine cytoplasts were unable to develop beyond the 16-cell stage, at least in the circumstances employed in this study. They do, however, develop to this particular stage at comparable rates to those obtained with ovine NT and IVF control embryos.

It would appear that the molecular and biochemical events that underlie this poor rate of development also exist in intergeneric nuclear transfer embryos produced by transferring pluripotent bovine embryonic nuclei into ovine cytoplasts. Studies presented in the following chapter are thus aimed at examining the role of the cytoplasm in the development of embryos produced by SCNT.

Chapter 5

The role of ovine ooplasm in initial nucleolar

assembly in intergeneric SCNT embryos

Chapter 5. The role of ovine ooplasm in initial nucleolar assembly in intergeneric SCNT embryos

5.1 Introduction

The reprogramming capacity of the ooplasm, at least to some degree, appears to be conserved between mammalian species. Hence, intergeneric SCNT has been reported to sustain blastocyst development using bovine ooplasm and nuclei from a range of different animals including those from sheep and the pig (Dominko et al., 1999). In contrast, it has been demonstrated in Chapter 4 that intergeneric SCNT embryos, created by transferring a porcine or bovine somatic nucleus into ovine ooplasm, do not develop beyond the 16-cell stage, raising the question of the differences in oocyte reprogramming capability between mammalian species.

In mammals, the ooplasm supplies the necessary mRNA and polypeptide requirements for initial embryonic development until major activation of the genome occurs, the timing of which varies between species (Shultz, 1993). Following one to three cleavage divisions, expression of specific genes in the embryonic genome influence development as the maternally derived transcripts and proteins are gradually degraded (Telford et al., 1990). This transfer of control is known as the major activation of the embryonic genome, an event that encompasses the activation of the ribosomal RNA (rRNA) genes, and which is distinct from the minor transcriptional activity that has been detected in a number of different species (Thompson, 1996; Plante et al., 1994; Hyttel et al., 1996; Viuff et al., 1996). In sheep (Crosby et al., 1988) and cattle (Camous et al., 1986) embryos, the major genome activation occurs in the 8-16-cell embryo, while in pigs it occurs in the 4-cell embryo (Tománek et al., 1989).

The transcription of rRNA genes and the subsequent rRNA transcript processing results in the development of a fibrogranular nucleolus, the nuclear organelle where ribosomal subunit synthesis occurs. Consequently, genome activation can be analysed by transmission electron microscopy (TEM) using the nucleolus ultrastructure as a marker. A functional nucleolus contains three main ultrastructural components: fibrillar centres (FCs) that carry the enzymatic machinery for transcription, a dense fibrillar component (DFC) representing the nascent rRNA transcripts under initial processing, and a granular component (GC) made up of pre-ribosomal subunits (Hyttel et al., 2000b).

For in vivo produced ovine embryos, the first fibrillo-granular nucleoli appear in the 16-cell embryo (Calarco and McLaren, 1976). In stark contrast, equivalent porcine embryonic nucleoli appear in the 4-cell embryo (Tománek et al., 1989; Hyttel et al., 2000). In addition to temporal differences, the ultrastructure of nucleolar development in the embryo differs significantly between the pig on the one hand and ruminants on the other (Hyttel et al., 2000). In the early embryo, before the formation of the fibrillo-granular nucleolus, nucleoli are absent but so-called nucleolus precursor bodies (NPBs), appearing as spheres of densely packed fibrillar material, define the site of subsequent nucleolar formation. Initially, the NPBs are significantly larger in porcine embryos (3-4 µm) than in ruminant embryos (1-1.5 µm). Moreover, in ruminants, vacuoles often appear in the NPBs even in the 1-cell embryo, and when final nucleolar formation occurs in the 8-cell bovine (Laurincik et al., 2000) and the 16-cell ovine embryos (Calarco and McLaren, 1976), more extensive vacuolisation occurs. In parallel, FCs and a DFC appear in the periphery of the NPBs, and subsequently the GC is formed. In porcine embryos on the other hand, the NPBs remain unvacuolated and when nucleolar formation occurs, FCs, a DFC and a GC form on the surface of the NPBs (Hyttel et al., 2000a).

The aim of studies in this chapter was to investigate the role of the ooplasm versus the nucleus in the events leading up to the major genome activation and initial nucleolar formation in SCNT embryos derived from somatic ovine, bovine and porcine cells and ovine ooplasm.

5.2 Methods

5.2.1 Experimental design

Firstly, an ultrastructural point of reference for the two different models of nucleolar formation, namely vacuolated (ruminants) versus non-vacuolated (porcine), was established. This involved the processing of in vivo developed 2- and 4-cell porcine and in vitro produced (IVP) 8-cell ovine embryos for TEM.

To establish the rRNA synthesis status of intrageneric SCNT ovine embryos, five ovine-ovine NT embryos were analysed for the presence and ultrastructure of NPBs or nucleoli, at each of the following time points: 8 (1-cell), 24 (2-cells), 36 (4-cells), 48 (early 8-cells), 72 (late 8-cells), and 96 (8-16-cells) hours following activation (hpa). Further, in order to detect total RNA synthesis and nucleolus-associated RNA synthesis, ^{3}H-uridine incubation followed by light microscopical autoradiography was performed. Intergeneric porcine-ovine SCNT embryos were similarly processed and fixed in the same numbers and at the same time points and cell stages as for the ovine-ovine SCNT embryos. In order to examine the degree of ooplasm-nucleus compatibility in ruminant intergeneric SCNT embryos, six bovine-ovine SCNT embryos were processed and fixed at each of the early and late 8-cell stages.

5.2.2 Collection of porcine *in vivo* produced embryos

Porcine embryos were collected as previously described (Hyttel et al., 2000a). Briefly, gilts were fed Regumate (Altrenogest, Iffa Merieux, France), injected with 1500 IU eCG (Pregnennolon; Dessau, Germany) and 72 h later with 500 IU hCG (Ovogest; Intervet,

Tönisvorst, Germany). Insemination occurred at 24 and 36 h after the hCG injection and animals were slaughtered at 48-74 h after this injection. Embryos were flushed from the oviducts/uteri and 2-cell embryos were either fixed immediately (n=2) or cultured and fixed at 10 h after the next cell division (n=2).

5.2.3 Production of ovine *in vitro* produced embryos

Ovine IVP embryos were obtained as described in Section 2.4. Briefly, cumulus-oocyte complexes (COCs) were aspirated from abattoir-sourced ovaries and cultured in groups of 30 in Nunc 4-well dishes (Nunc, Roskilde, Denmark) in Tissue Culture Medium 199 (TCM199) containing 20% heat inactivated sheep serum (HISS), 5 μg/ml LH (Lutropin-V, Vetrepharm, London, Canada), 5 μg/ml FSH (Folltropin-V, Vetrepharm) and 1 μg/ml oestradiol. Maturation occurred at 38.5°C in a humidified atmosphere of 5% CO_2 in air for 24 h. COCs were then fertilised with frozen-thawed sperm in synthetic oviduct fluid (SOF) containing 2% HISS. Twenty-four hours later, presumptive zygotes were denuded of all cumulus cells and cultured in groups of 30 in Nunc 4-well dishes in culture medium (SOF-bicarbonate containing 4 mg/ml of BSA and amino acids at oviductal fluid concentrations; Walker et al., 1996) for 48 h or 72 h at which time the embryos were fixed at the 8-cell stage of development (n=5).

5.2.4 Somatic cell nuclear transfer

Passage one ovine, bovine and porcine granulosa cells were thawed, cultured to confluency in Glasgow MEM + 10% fetal calf serum (FCS; CSL, Parkville, VIC, Australia), and serum starved for 2 days. Zona-free SCNT was performed as described previously (Peura, 2003) with modifications and is summarised in Figure 5.1. COCs were aspirated from abattoir-sourced ovaries and in vitro matured as described above. After maturing for 18-23 h, ova were first cleaned of all cumulus cells by pipetting and then incubated for 5 min in 7.5 μg/ml cytochalasin B and 10μg/ml Hoechst 33342 in handling medium (SOF-Hepes with amino

Figure 5.1

Schematic diagram summarising the zona-free SCNT technique.

Hours Post Maturation

1. Ova clean-up. — 18 h

2. Enucleation. — 18.5 h

4. Ovine, bovine or porcine somatic cell preparation. — 20–20.5 h

3. Zona removal with Pronase. — 21 h

5. Cell attachment with PHA. — 23 h

6. Electrofusion. — 24 h

7. Activation with calcium ionophore & 6-DMAP. — 27 h

8. In vitro culture at 38.5 C in reduced oxygen in agar wells. — 75 h

9. Uridine pulsing and fixation at 1-cell, 2-cell, 4-cell, 8-cell (early), 8-cell (late) and 16-cell. — 195 h

acids and 4 g/l BSA). Ova were subsequently enucleated in handling medium containing only cytochalasin B using Narishige micromanipulators (Narishige International, Tokyo, Japan) attached to Olympus IX70 inverted microscope (Olympus Optical Co. Ltd, Tokyo, Japan). The exact location of the metaphase plate within an ovum was established with < 1sec exposure to UV illumination, after which it was removed by aspiration using a bevelled glass pipette (approximately 20 µm in diameter). No attention was paid to the removal of the first polar body, but the success of removing the metaphase plate was confirmed by viewing the contents of the pipette under UV. The zona pellucida was removed enzymatically with 0.5% pronase followed by repeated washing in handling medium. All subsequent ova handling and culture procedures were performed in a medium supplemented with 20 g/l BSA instead of 4 g/l to prevent the attachment of zona-free oocytes to the dish.

Fusion was performed according to the method of Vajta et al. (2001) with modifications described more recently (Peura, 2003). Briefly, each cytoplast was placed for 1-2 sec in a drop of protein-free SOF-Hepes containing 200 µg/ml of phytohemagglutinin (PHA; ICN Pharmaceuticals Seven Hills, NSW, Australia) and then moved to a handling medium drop where it was rolled onto an individual granulosa cell, leading to adhesion of the two membranes. When a group of 10-15 couplets had been prepared, they were then transferred to a fusion chamber consisting of two parallel 0.1 mm diameter platinum wires separated by 0.2 mm and covered with a mannitol medium (0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$). Each couplet was individually pulsed with a 400 kHz alignment pulse and then either two 80 µsec 1.25 kV/cm, or one 20 µsec 1.5 kV/cm DC electric pulses for ovine and porcine cells respectively. Within 30 min of fusion, reconstructed embryos were activated with calcium ionophore, and incubated in 6-dimethylaminopurine (6-DMAP) for 2 h. The reconstructed zona-free embryos were cultured in modified culture medium containing 20 g/l

BSA in agar wells as previously described (Peura, 2003) until they were fixed at the desired stages of development.

5.2.5 ^{3}H-Uridine incubation

Embryos at desired developmental stages were labelled with ^{3}H-uridine (specific activity 962 GBq/mmol; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) at a final concentration of 4 Mbq/mmol (Tománek et al., 1989). Embryos were incubated for 20 min in gas-equilibrated SOF-bicarbonate culture medium containing ^{3}H-uridine, before repeated washing in ^{3}H-uridine-free culture medium.

5.2.6 Processing for transmission electron microscopy (TEM) and autoradiography

The techniques employed in TEM analysis are summarised in Figures 5.2, 5.3, and 5.4. After labelling with the radioactive precursor where appropriate, embryos were fixed in 3% gluteraldehyde in 0.1 M phosphate buffer. Subsequently, specimens were washed in buffer, post-fixed in 1% OsO_4 in phosphate buffer, dehydrated with ethanol, and embedded in freshly prepared epon. Individual embryos were serially sectioned into semi-thin (2μm) sections and every second section was stained with basic toluidine blue for brightfield light microscopy. Selected semi-thin sections were re-embedded (Hyttel and Madsen, 1987) and ultra-thin (70nm) sections cut, contrasted and examined on a Phillips CM100 transmission electron microscope. Neighbouring unstained semi-thin sections were processed for autoradiography for detection of total RNA synthesis and nucleolus-associated RNA synthesis. These sections were coated with Ilford K5 liquid nuclear emulsion (Ilford; Basildon, Essex, UK) and exposed for six weeks at 4°C. Finally the sections were developed in Kodak D19 at 17°C, stained with toluidine blue, and evaluated by brightfield and epipolarized light microscopy.

Figure 5.2

Schematic diagram summarising embryo fixation and epon embedding processing for TEM analysis.

1. Embryos at desired developmental stage removed from culture and given ^{3}H-uridine pulse. — 0 h

2. Primary fixation in 3% gluteraldehyde at 4°C. — 0.5 h

3. Agar embedding — 1.5 h

4. Secondary fixation in osmium & dehydration in ethanol, propanyl oxide & Epon — 2.5 h

5. Epon embedding — 26.5 h
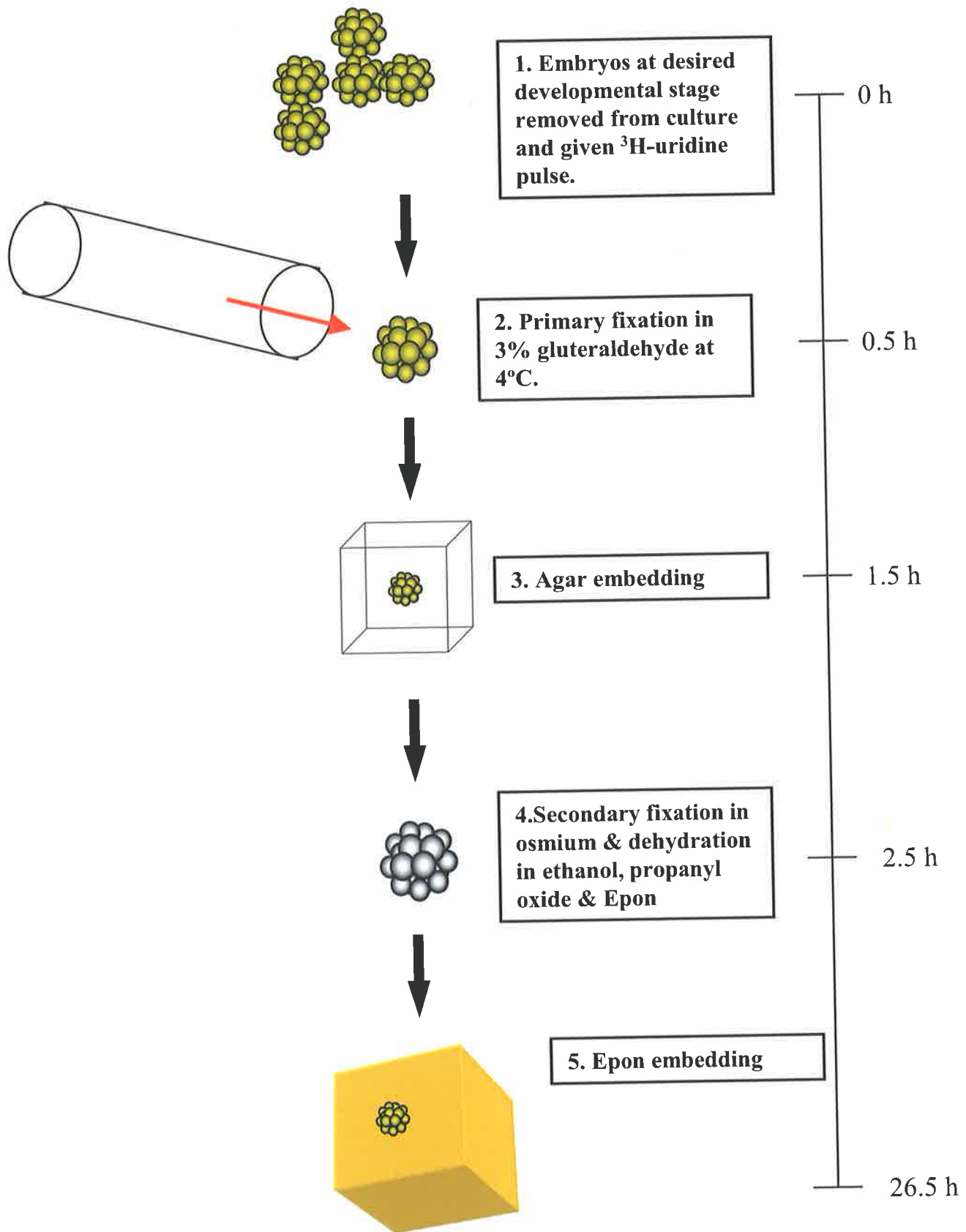
Figure 5.3

Schematic diagram summarising the semi-thin serial sectioning and section re-embedding procedures employed in TEM analysis.

1. Embryo embedded in Epon.

2. Embryo in epon serial semithin sections cut with a glass knife.
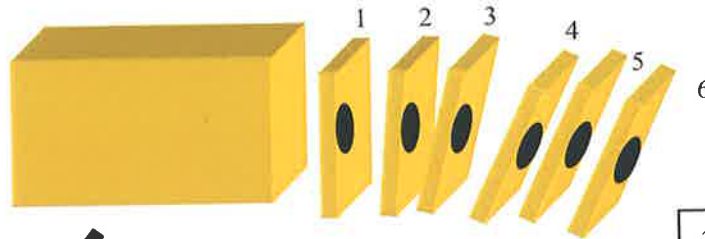
3. Alternate sections placed on separate glass slides as shown
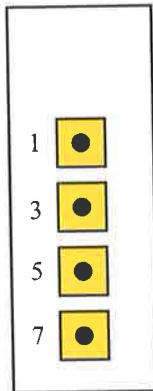
5. Even numbered sections processed for autoradieography.
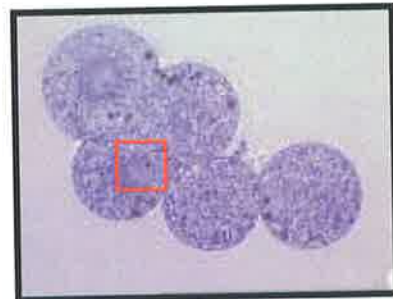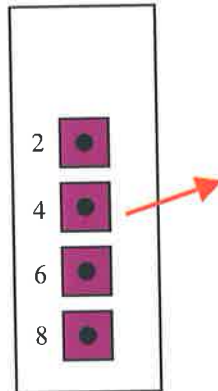
4. Even numbered sections stained with Toulidine blue observed under brightfield microscopy.
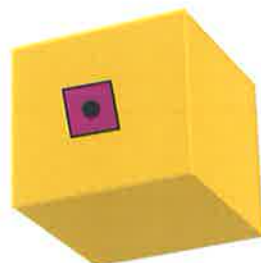
6. Selected sections with nucleoli re-embedded in epon and processed for TEM.

Figure 5.4

Schematic diagram summarising the ultra-thin sectioning technique used in TEM analysis.
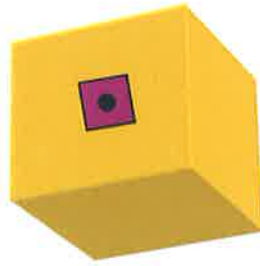
1. Semithin section re-embedded in epon.

2. Ultrathin sections cut with diamond knife and placed on copper grids.

3. Ultrathin sections stained with uranyl acetate and lead nitrate.
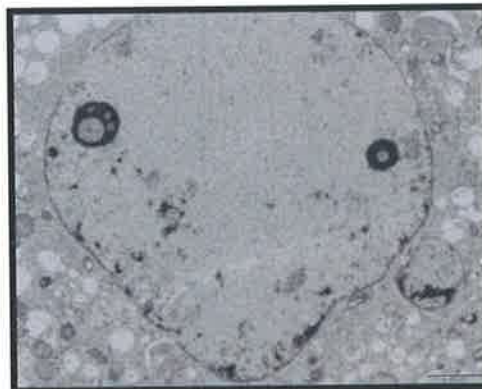
4. Sections viewed under Phillips CM100 transmission electron microscope.

5.3 Results

5.3.1 Intergeneric embryo production

Figure 5.5 shows zona-free intergeneric porcine-ovine SCNT embryos before further processing for TEM and autoradiographical analysis.

5.3.2 Ovine control embryos

All nuclei from 8-cell ovine IVP embryos presented NPBs appearing as electron-dense spheres consisting of tightly packed fibrillar material (Figure 5.6). The NPBs presented numerous vacuoles (Figure 5.6) that were compact. No autoradiographical analysis was carried out on these embryos.

5.3.3 Porcine control embryos

As with the ovine NPBs, nuclei from 2- and 4-cell in vivo produced porcine embryos presented NPBs appearing as electron-dense spheres consisting of tightly packed fibrillar material (Figure 5.7). The NPBs did not present numerous vacuoles unlike the ovine NPBs (Figure 5.6). No autoradiographical analysis was carried out on these embryos.

5.3.4 Somatic donor cells

Ovine granulosa cells serum starved for two days that were used as nuclear donors in the SCNT experiments presented nucleoli with FCs, a DFC and a GC signalling ribosome subunit-synthesis (Figure 5.8). Autoradiography was not performed on these samples.

5.3.5 Ovine-ovine SCNT embryos

5.3.5.1 One, two and four-cell stages

In 1-, 2- and 4-cell ovine-ovine SCNT embryos, 80, 40, and 47% of the nuclei presented NPBs or nucleoli respectively (Table 5.1). NPBs for 1-, 2-, and 4-cell embryos appeared as

Figure 5.5

Plate showing 1-cell (a), 2-cell (b), 4-cell (c) and 8-cell (d) zona-free embryos before processing for TEM.
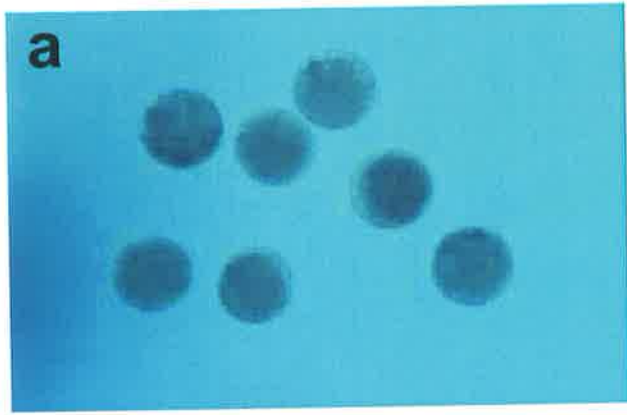
Figure 5.6

Transmission electron micrographs of a nucleus (N) from an 8-cell ovine IVP embryo (a) and a nucleolus precursor body (b). Note the numerous vacuoles (V) in the latter.

a

N

5 μm

b

V

V

V

1 μm
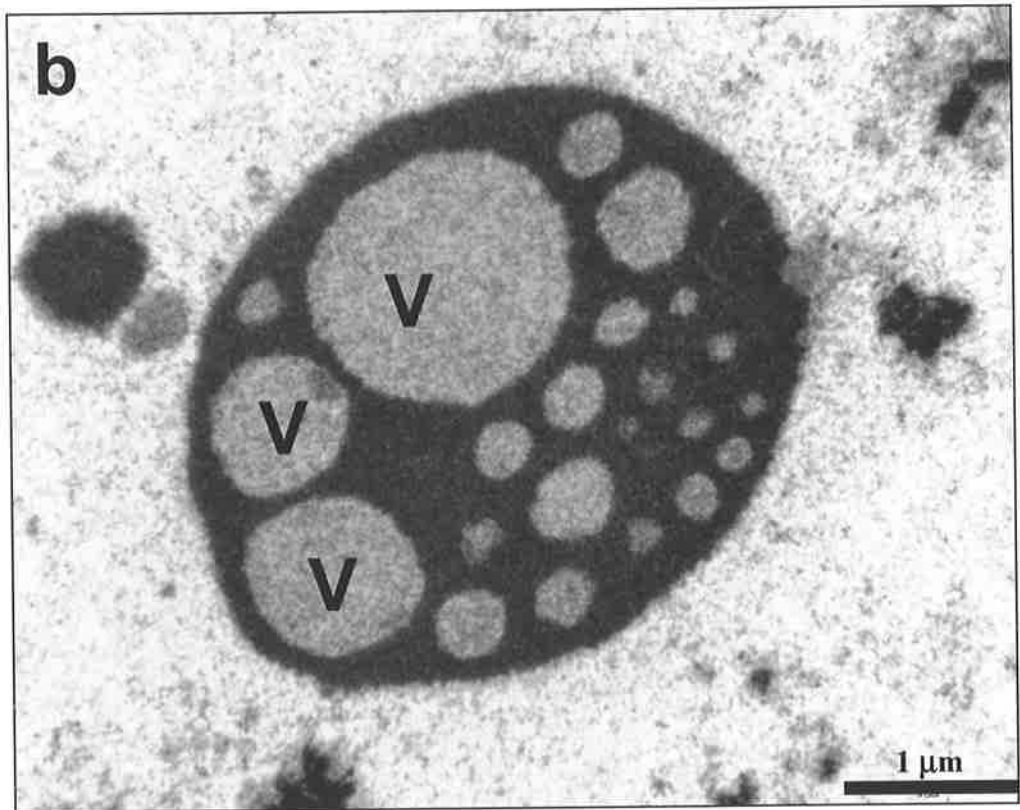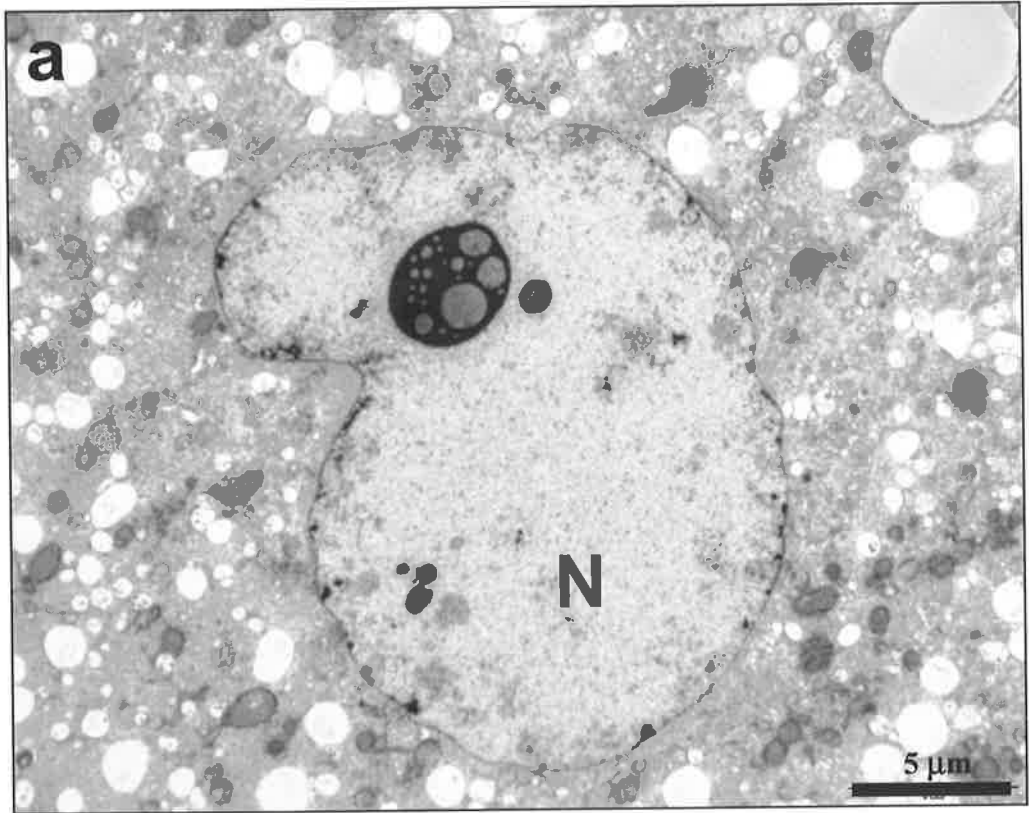
Figure 5.7

Transmission electron micrographs of a nucleus (N) from a porcine in vivo produced 4-cell

embryo a) and nucleolus precursor body (b). Note the lack of any vacuoles in the NPB.

a

N

2 μm

b

NPB

500 nm
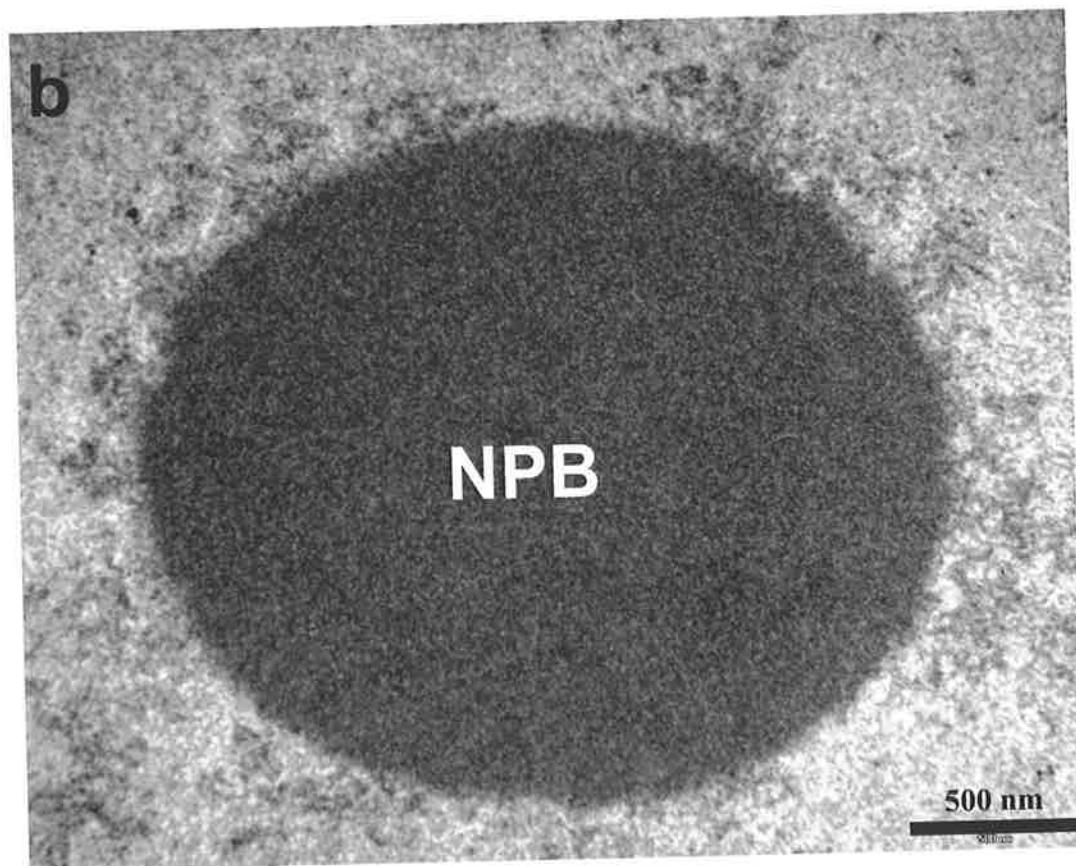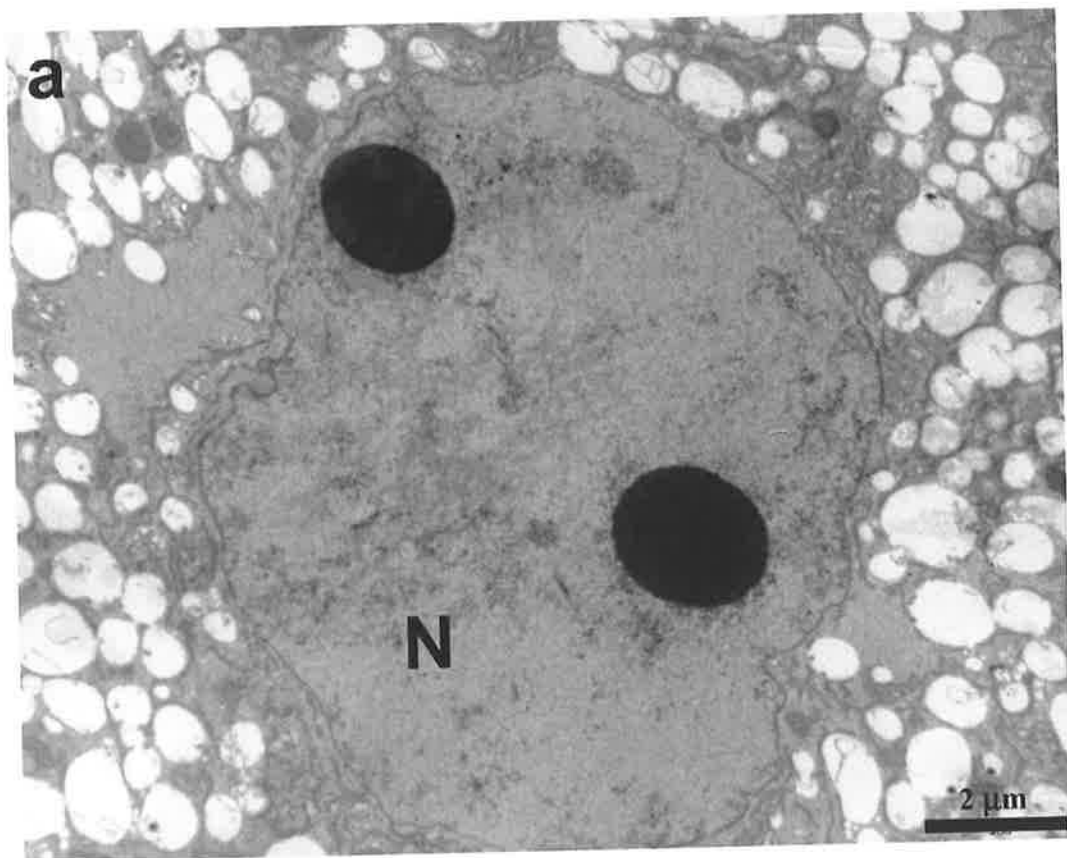
Figure 5.8

Transmission electron micrographs of the nucleus (N; a) and the nucleolus (b) of a two day serum-starved ovine granulosa cell. Note the fibrillar centres (FC), the dense fibrillar component (DFC) and the granular component (GC) of the nucleolus.

a

N

2 μm

b

DFC

GC

FC

500 nm
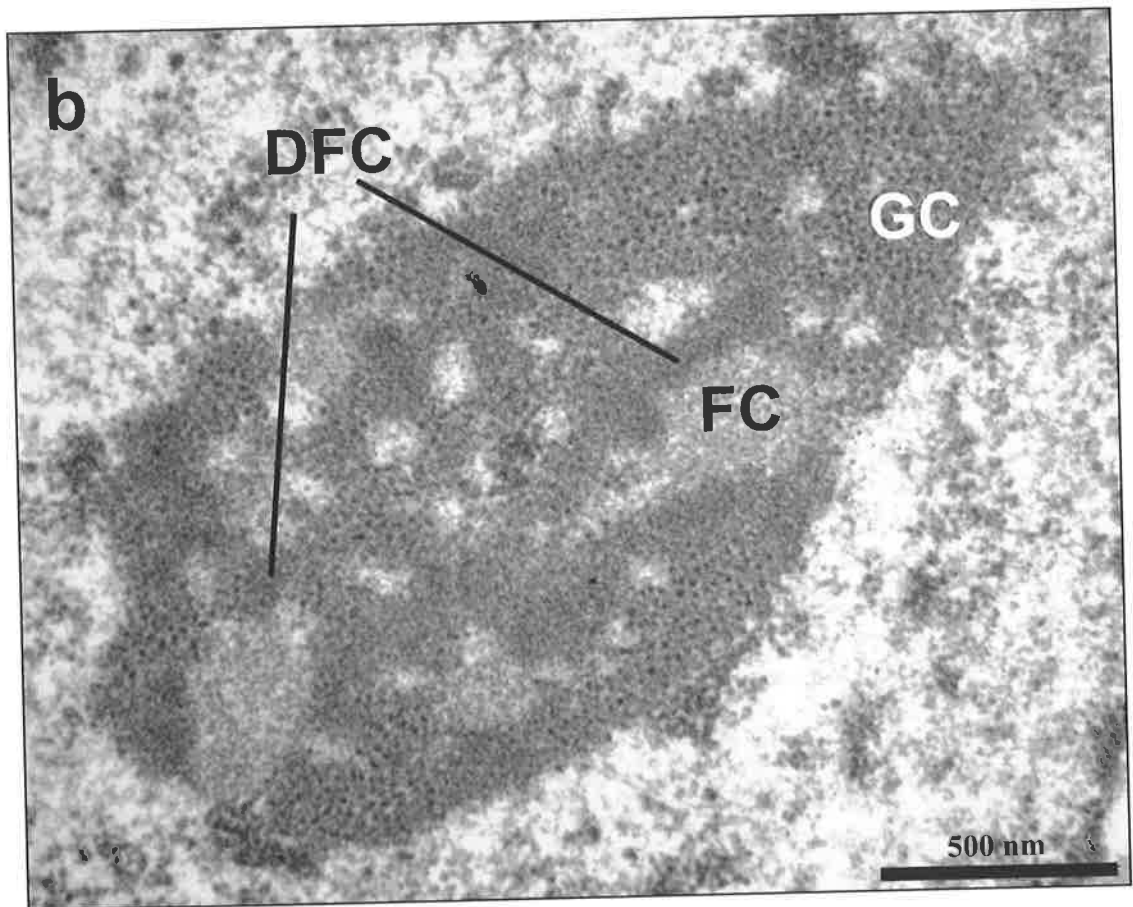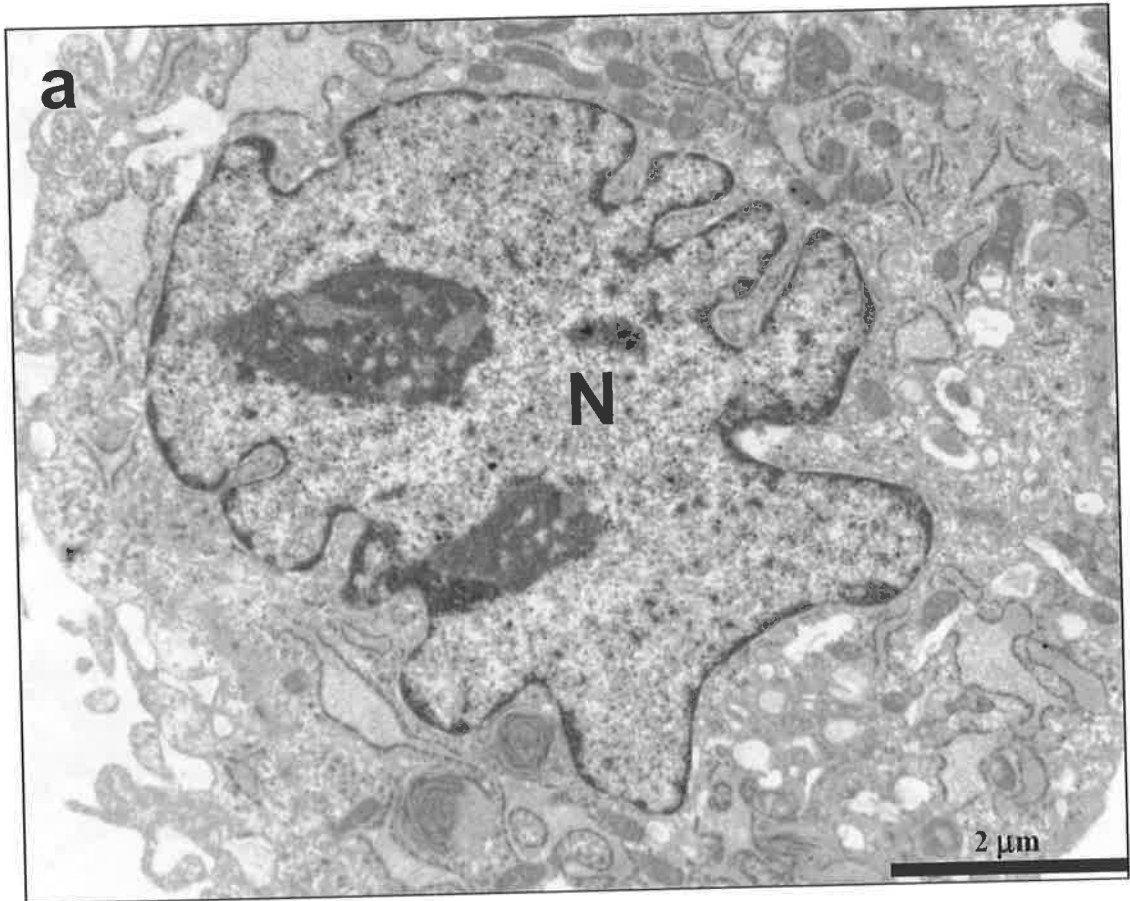
electron-dense spheres of approximately 1.2 μm in diameter consisting of tightly packed fibrillar material and are presented in Figures 5.9, 5.10, and 5.11 respectively.

5.3.5.2 Early and late eight-cell stages

In early 8-cell and late 8-cell embryos, 68 and 85% of the nuclei presented NPBs respectively (Table 5.1). At this stage of development, the NPBs displayed numerous vacuoles characteristic of a typical ruminant NPB during initial nucleolar formation (Figure 5.12). All embryos up to and including the late 8-cell stage lacked autoradiographic labelling of their nuclei.

5.3.5.3 Sixteen-cell stage

In 16-cell embryos, 72% of nuclei displayed NPBs or nucleoli. Two 16-cell embryos displayed fibrillo-granular nucleoli with FCs, a DFC and a GC (Figure 5.13). Correspondingly, these embryos showed autoradiographical labelling over nuclei and, in particular, nucleoli (Figure 5.14). Thus, at least in some of the 16-cell ovine-ovine SCNT embryos, ribosome subunit-synthesising nucleoli developed.

5.3.6 Porcine-ovine SCNT embryos

5.3.6.1 One, two and four-cell stages

For 1-, 2- and 4-cell porcine-ovine SCNT embryos, NPBs were identified in 26, 50, and 13% of the nuclei analysed respectively (Table 5.2). The ultrastructure and size of the NPB resembled those observed in ovine-ovine SCNT embryos (Figure 5.15). In addition, unusual and non-functional nucleolar-type structures were observed. A 1-cell embryo presented an electron dense structure made up of tightly packed fibrillar material (as with an NPB), but was instead hollow and appeared shell-like (Figure 5.16a). In addition, an unidentified nucleolar-type structure was observed in a 2-cell embryo and it is likely that this was a remnant of a former functional nucleolus from the donor cell (Figure 5.16b).

Table 5.1: Number of ovine-ovine (OvOv) embryos and nuclei containing nucleoli or nucleolar precursor bodies (NPBs).

Embryo stage	N	No. nuclei	No. nuclei with nucleoli/NPBs (%)	No. embryos with visible nucleoli/NPBs (%)
1 cell	5	5x1	4 (80)	4 (80)
2 cell	5	1x3, 3x2, 1x1	4 (40)	4 (80)
4 cell	4	3x4, 1x3	7 (47)	4 (100)
8 cell (early)	5	2x7, 2x6, 1x5	21 (68)	5 (100)
8 cell (late)	5	1x10, 2x8, 1x7, 1x6	33 (85)	5 (100)
16 cell	5	1x14, 1x13, 1x12, 2x11	44 (72)	4 (80)

Figure 5.9

Transmission electron micrographs of an ovine-ovine (Ov-Ov) one-cell SCNT embryo. (a) Nucleus (N) of a one-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) NPB of the same one-cell embryo.

a

N

5 µm

b

NPB

500 nm

Figure 5.10

Transmission electron micrographs of an ovine-ovine (Ov-Ov) 2-cell SCNT embryo. (a) Nucleus of 2-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) NPB of the same 2-cell embryo displaying two vacuoles (V).
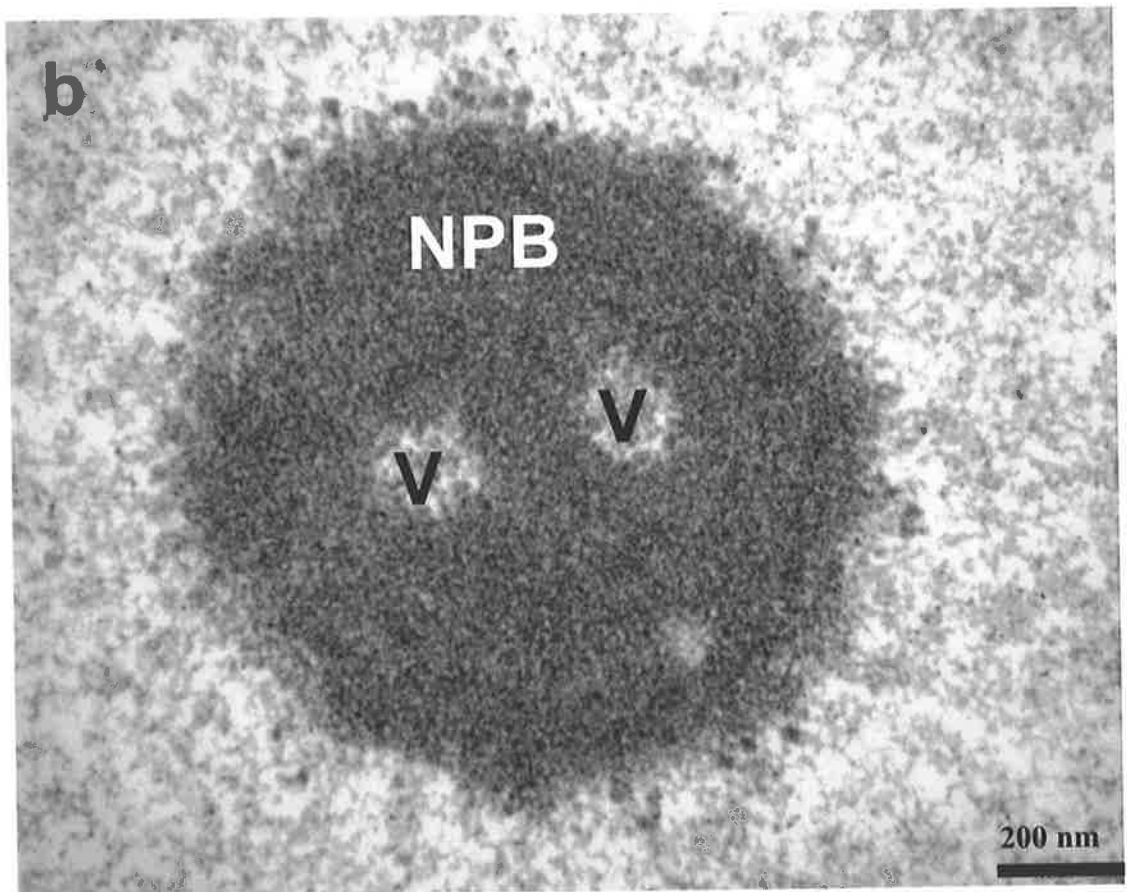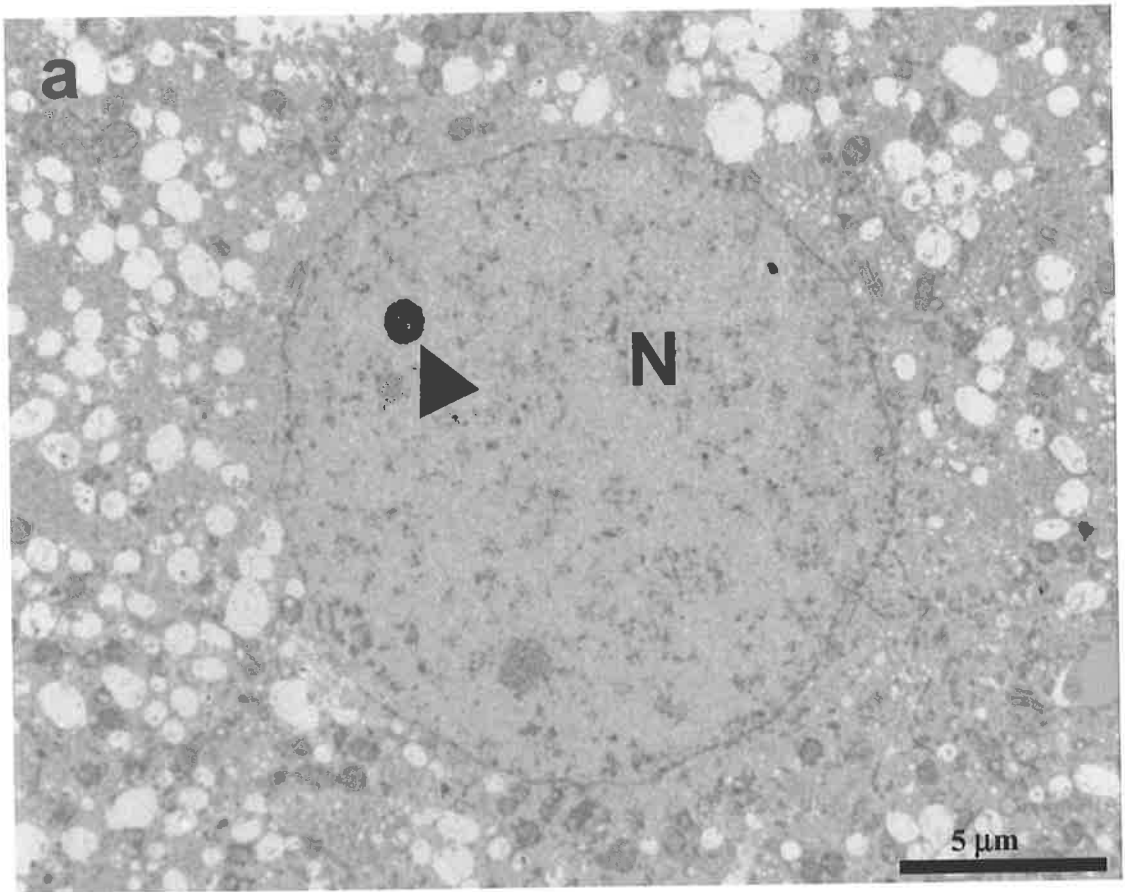
Figure 5.11

Transmission electron micrographs of an ovine-ovine (Ov-Ov) 4-cell SCNT embryo. (a) Nucleus (N) of 4-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) NPB of the same 4-cell embryo displaying vacuoles (V).

a

N

5 µm

b

V

V

200 nm
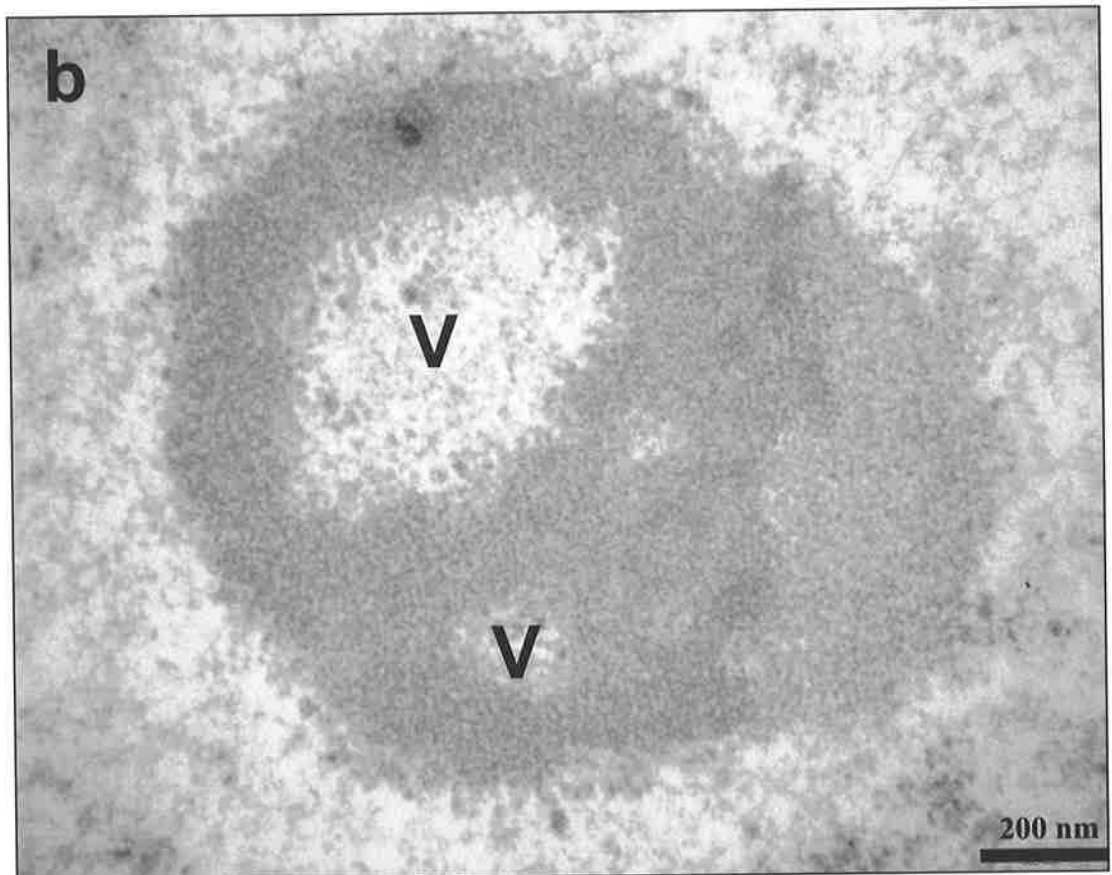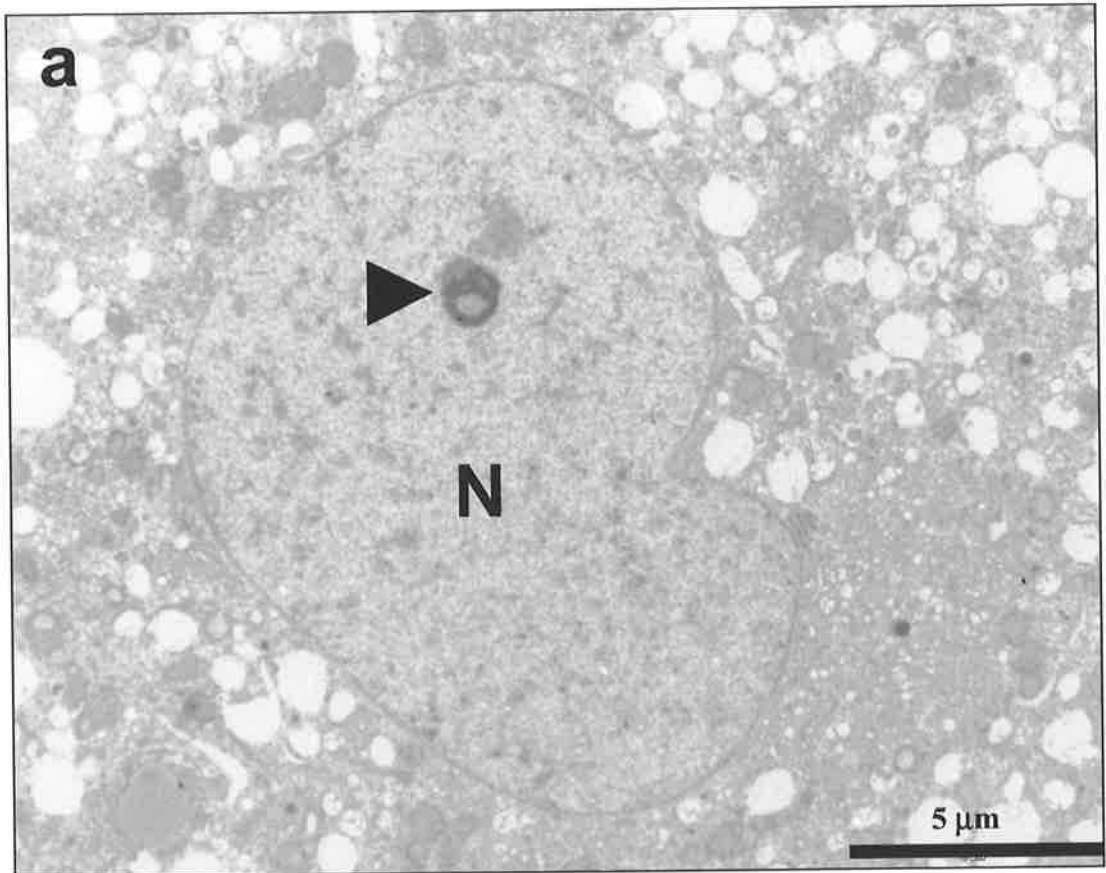
Figure 5.12

Transmission electron micrographs of an ovine-ovine (Ov-Ov) 8-cell SCNT embryo. (a) Nucleus (N) of 8-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) NPB of the same 8-cell embryo displaying several vacuoles (V).

a

N

5 μm

b

V

V

V

500 nm
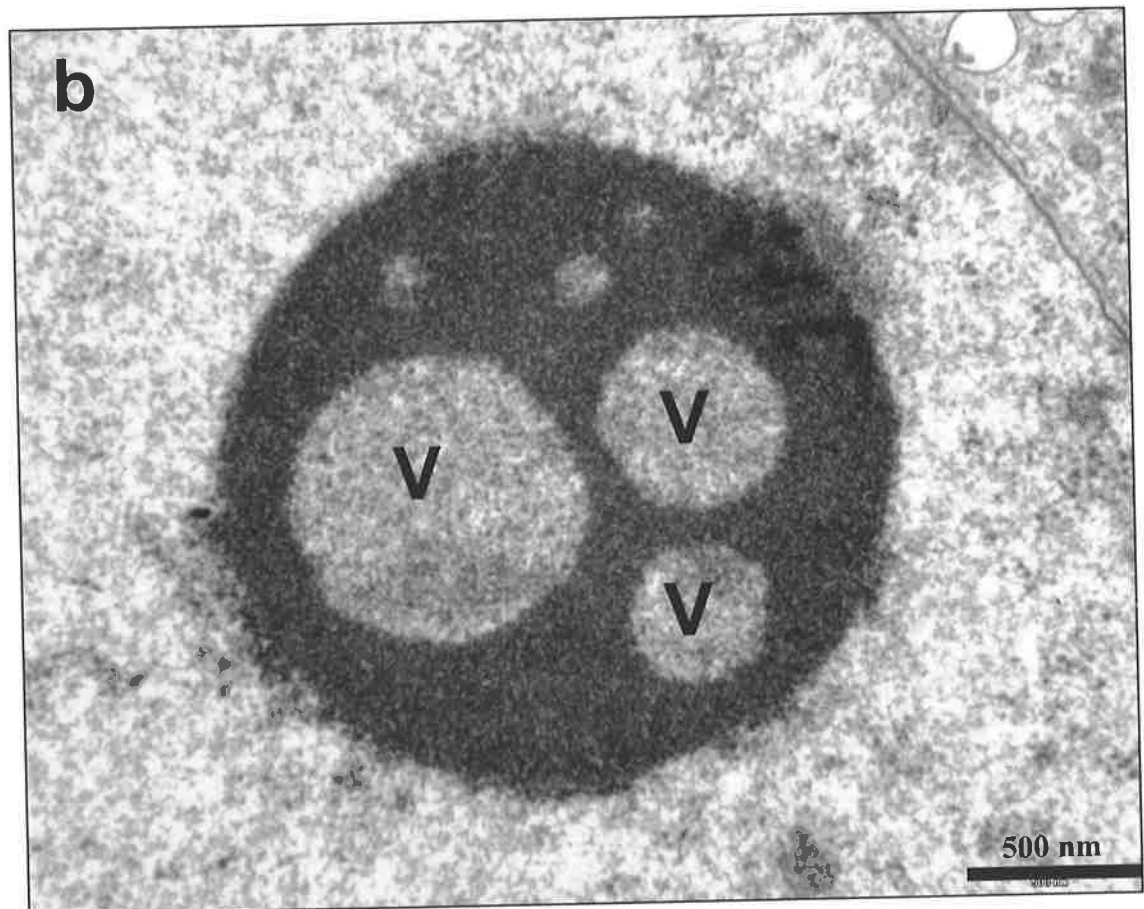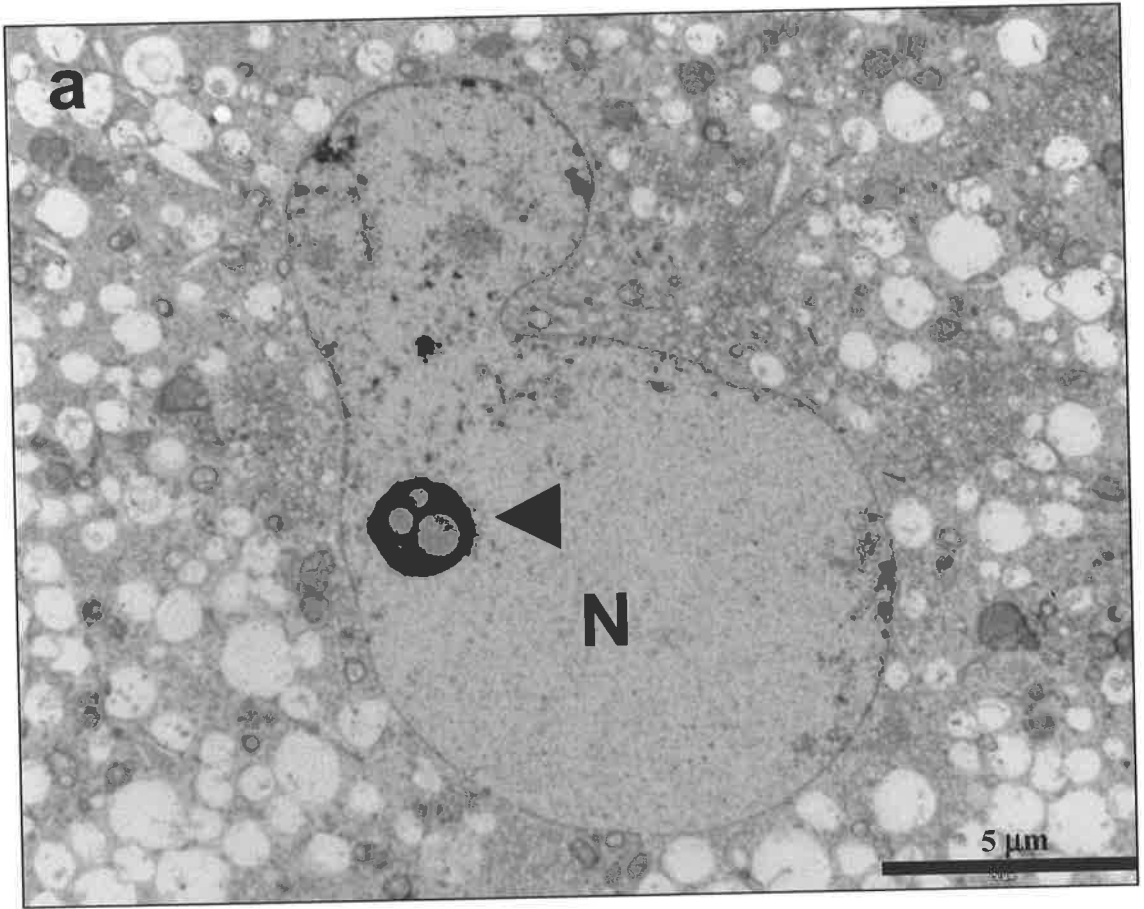
Figure 5.13

Transmission electron micrographs of an ovine-ovine (Ov-Ov) 16-cell SCNT embryo. (a) Nucleus (N) of 16-cell embryo with nucleolus (arrowhead). (b) Nucleolus of the same 16-cell embryo presenting fibrillar centres (FC), a dense fibrillar component (DFC) and granular component (GC).

a

N

2 μm

b

GC

FC

DFC

200 nm
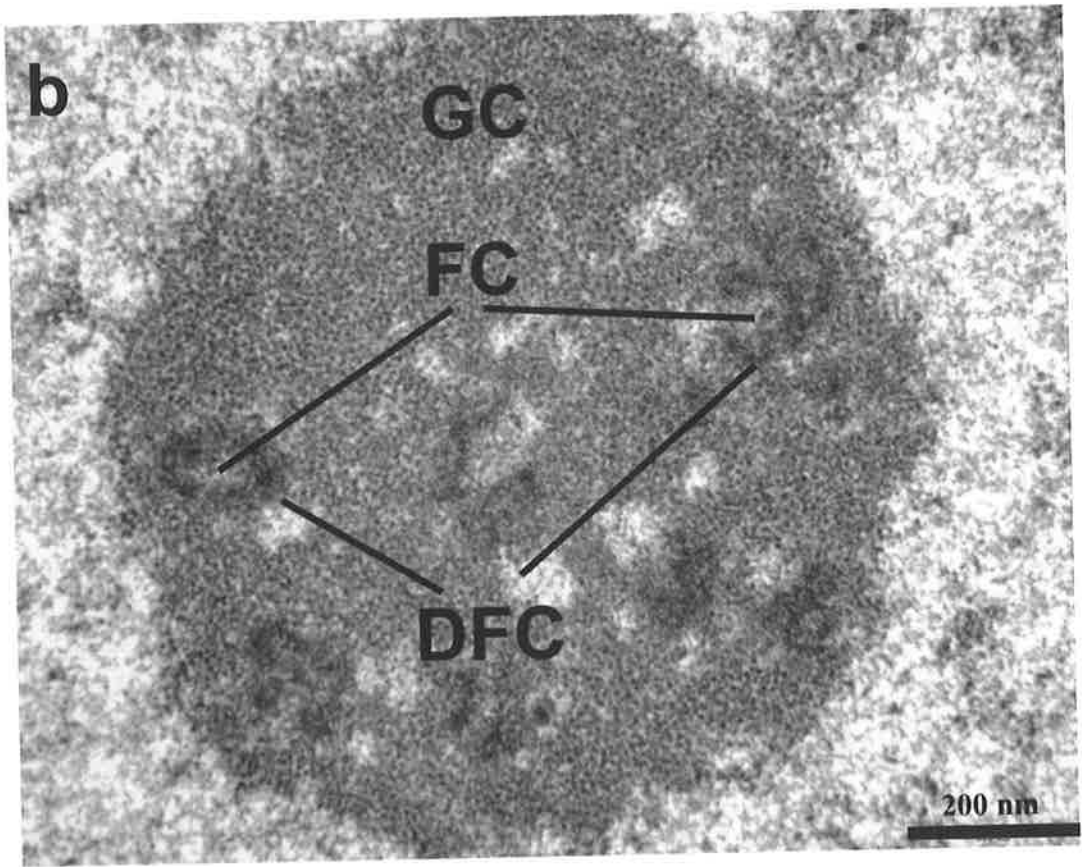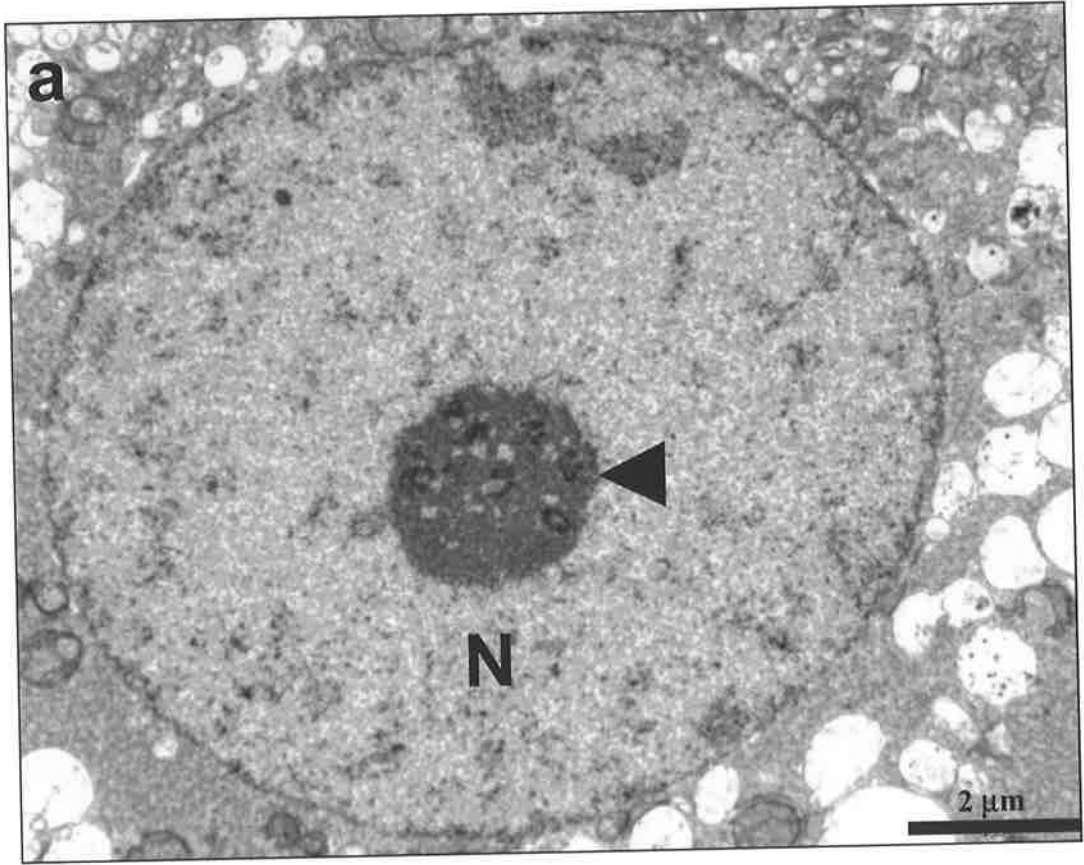
Figure 5.14

Autoradiograms of an ovine-ovine (Ov-Ov) 16-cell embryo (same embryo as for Figure 5.13) with (a) and without (b) brightfield exposure, displaying two nuclei with autoradiographic labelling over the nucleoplasm and, in particular, the nucleoli (arrowheads).
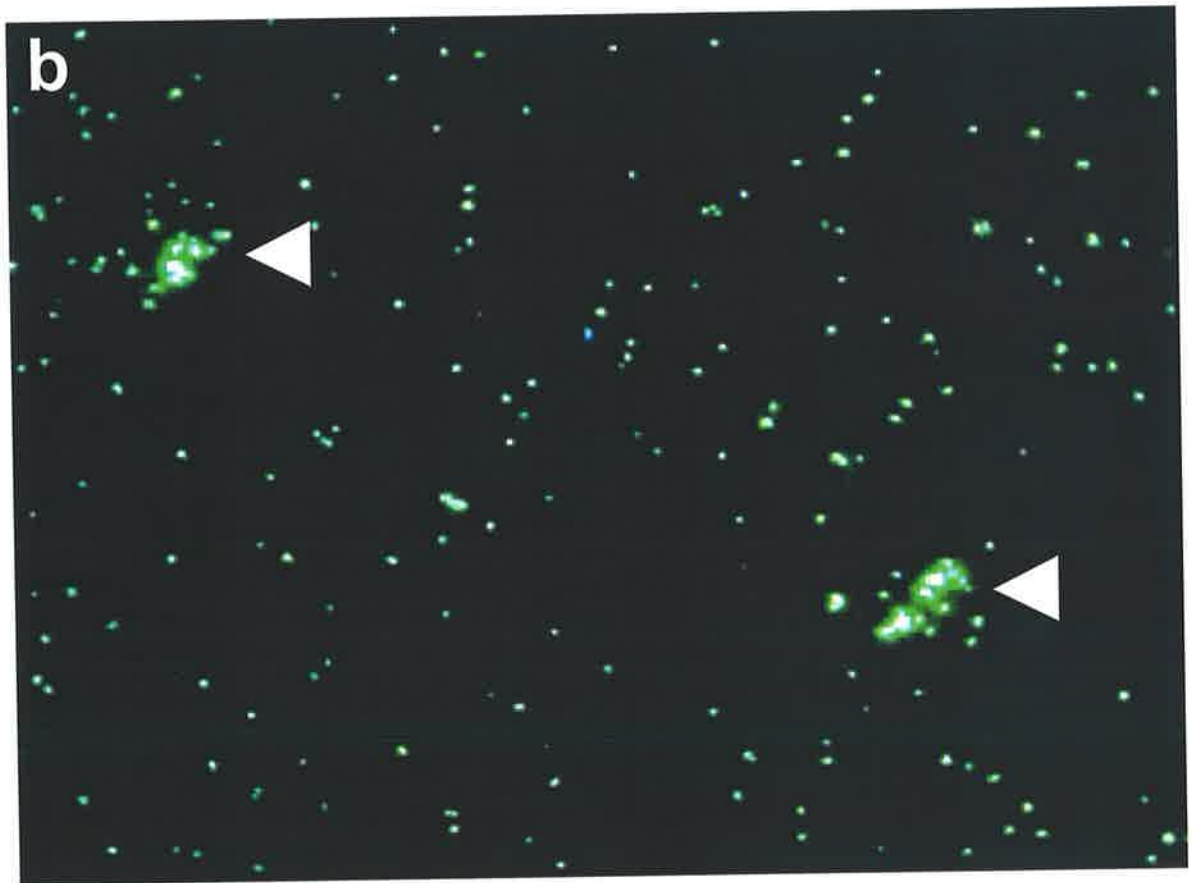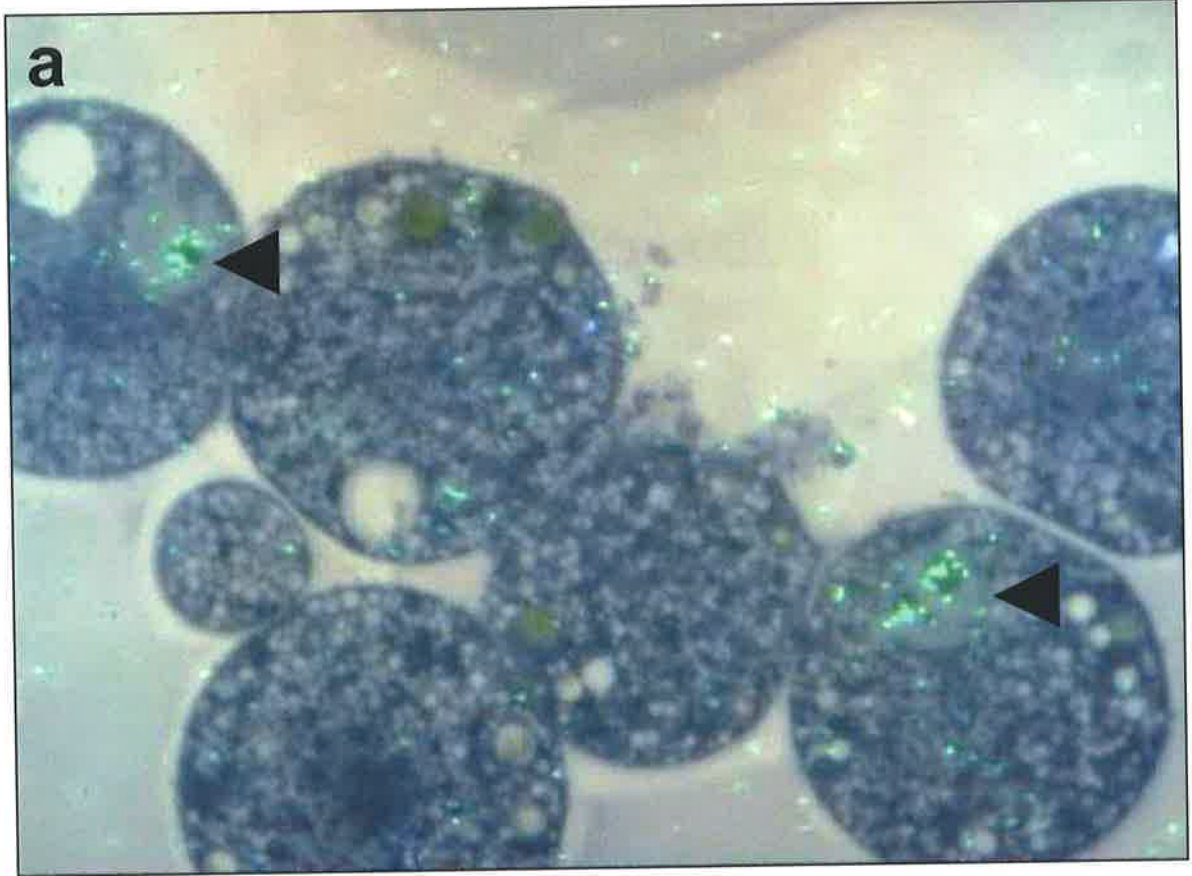
Table 5.2: Number of porcine-ovine (PrOv) embryos and nuclei containing nucleoli or nucleolar precursor bodies (NPBs).

Embryo stage	N	No. nuclei	No. nuclei with nucleoli/NPBs (%)	No. embryos with visible nucleoli/NPBs (%)
1 cell	21	2x2, 19x1	6 (26)	6 (29)
2 cell	4	1x4, 3x2	5 (50)	3 (75)
4 cell	5	1x4, 3x3, 1x2	2 (13)	1 (20)
8 cell (early)	6	4x8, 1x7, 1x6	18 (40)	6 (100)
8 cell (late)	6	1x10, 4x8, 1x7	24 (49)	6 (100)
16 cell	5	1x16, 1x13, 1x12, 1x11, 1x10	16 (26)	5 (100)

Figure 5.15

Transmission electron micrographs of a porcine-ovine (PoOv) 1-cell SCNT embryo. (a) Nucleus (N) of 1-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) NPB of the same 1-cell embryo.

a

N

5 μm

b

NPB

500 nm
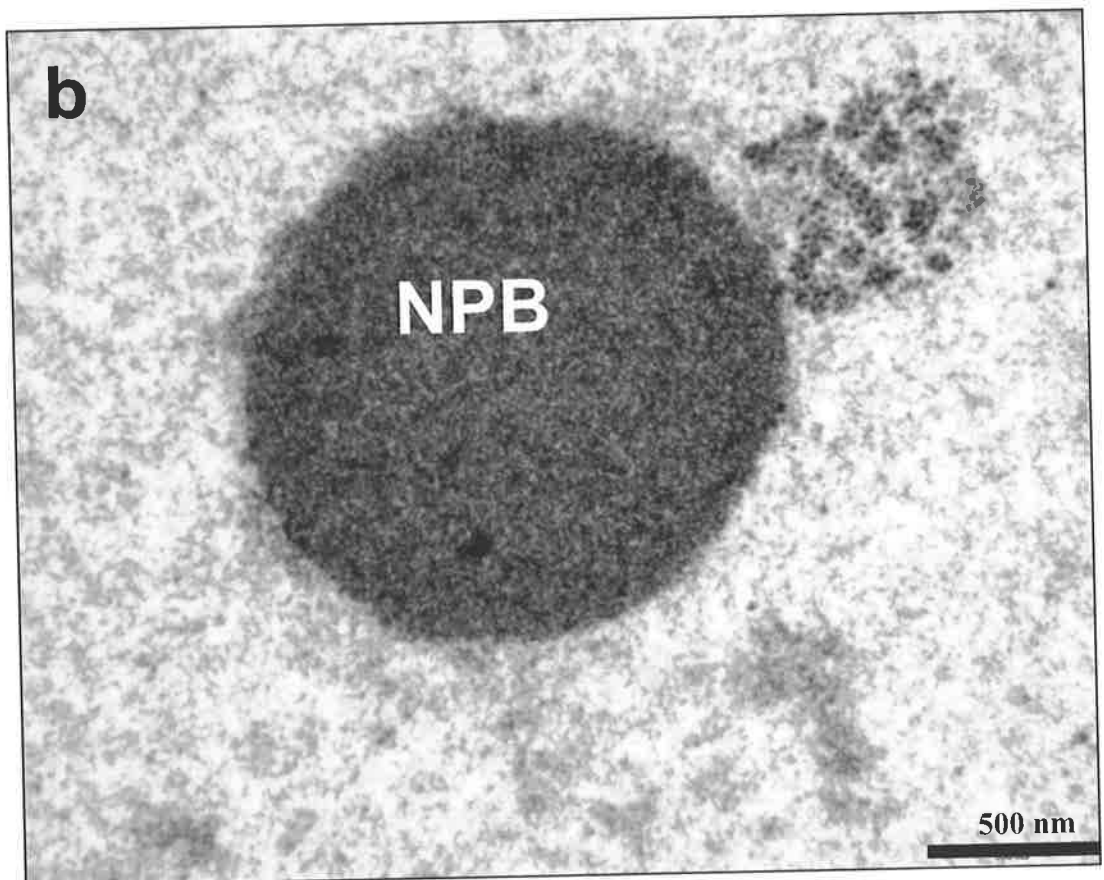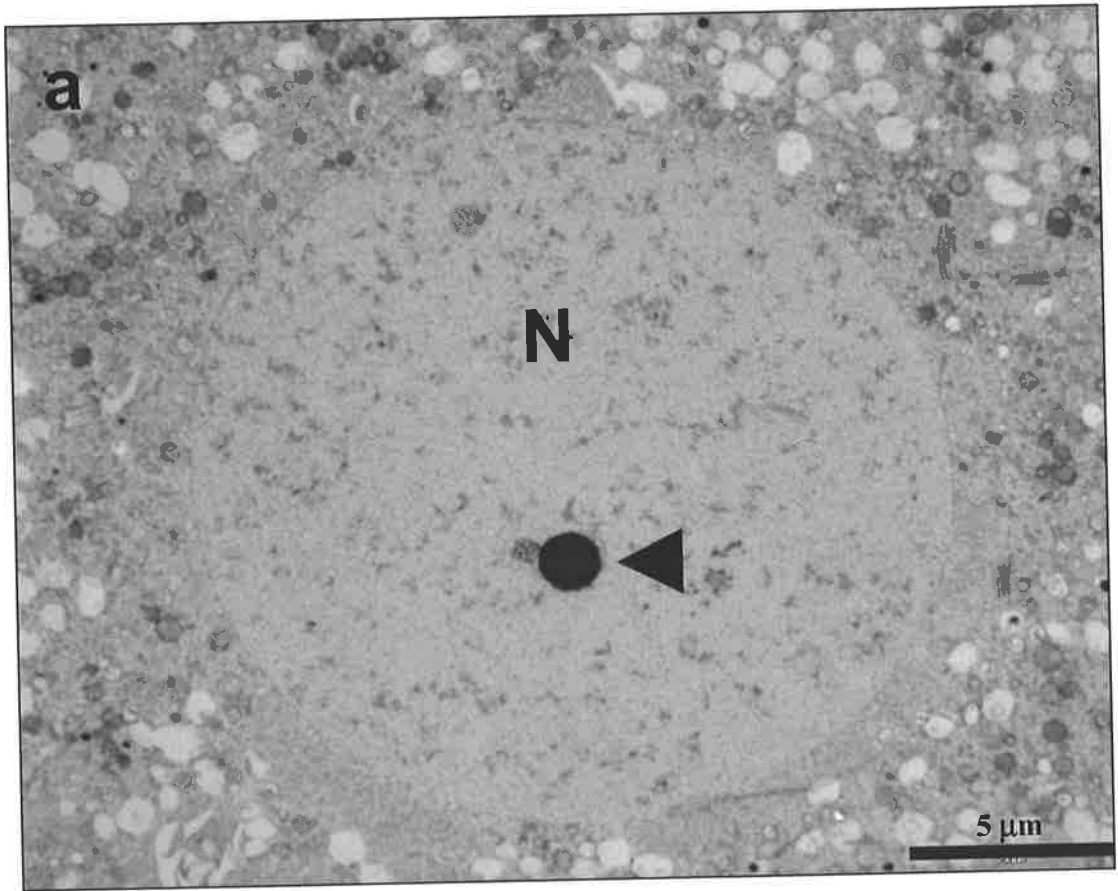
5.3.6.2 Early and late eight-cell stages

NPBs for early and late 8-cell porcine-ovine SCNT embryos were identified in 40 and 49% of the nuclei analysed respectively (Table 5.2). The ultrastructure and size of the NPBs were similar to ovine-ovine SCNT embryos (i.e. vacuolated electron-dense spheres consisting of tightly packed fibrillar material) (Figure 5.17).

5.3.6.3 Sixteen-cell stages

In 16-cell porcine-ovine SCNT embryos, 29% of the nuclei examined presented NPBs. Fibrillo-granular nucleoli did not develop in any of the 16-cell embryos and, correspondingly, all nuclei lacked autoradiographic labelling. The NPBs appeared as described for the early and late 8-cell porcine-ovine embryos. In addition, observations were made of scattered cytoplasmic organelles including mitochondria in the nuclei and unidentified fibrillar masses (Figure 5.18a & b).

5.3.7 Bovine-ovine embryos

In some cases, the ultrastructure of the NPBs found in 8-cell bovine-ovine SCNT were similar to that described in the ovine-ovine SCNT embryos (Figure 5.19). However, development of ribosome producing fibrillo-granular nucleoli was again not observed in any of the embryos. On the other hand, complex structures consisting of long strands of densely packed fibrillar material coated by granules were observed in some late 8-cell embryos indicating tentative achievement of a certain level of nucleolar development followed by disintegration (Figure 5.20).

5.4 Discussion

The data presented in this Chapter show that the ooplasm, and not the nucleus, is principally responsible for the initial stage of nucleolar formation in the preimplantation embryo. This

Figure 5.16

Transmission electron micrograph of a 1-cell (a) and a 2-cell porcine-ovine (Po-Ov) SCNT embryo. (a) Fibrous hollow nuclear entity found within the nucleus of a one-cell embryo. (b) A nuclear entity that is possibly the remnants of a former nucleolus.

a

500 nm

b

500 nm
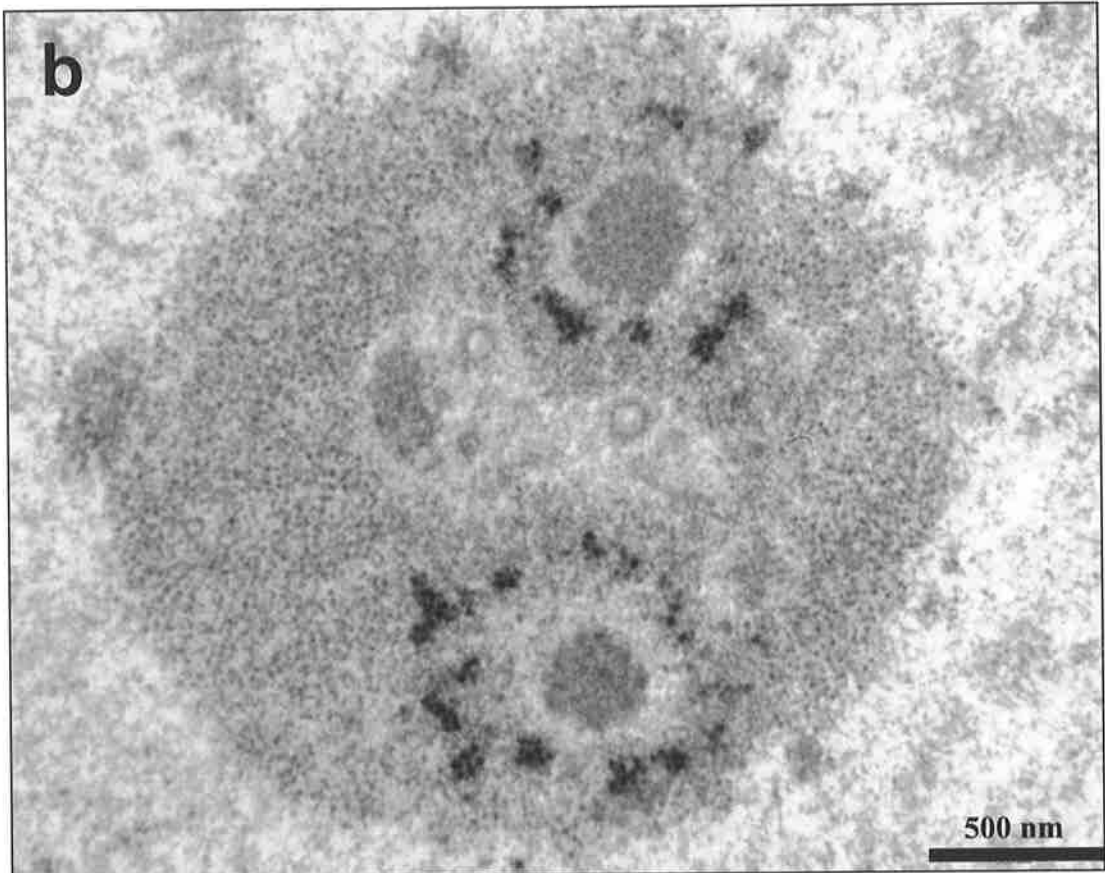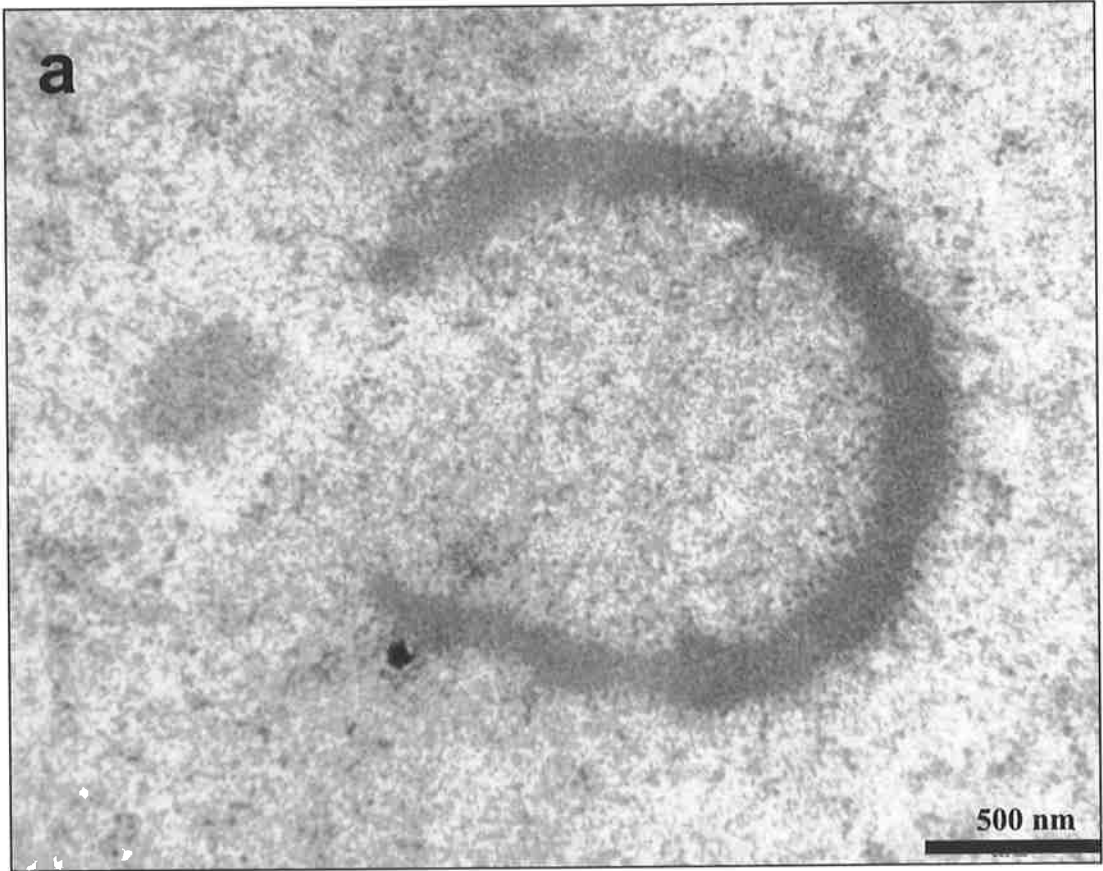
Figure 5.17

Transmission electron micrographs of a porcine-ovine (Po-Ov) 8-cell SCNT embryo. (a) Nucleus (N) of 8-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) NPB of the same eight-cell embryo displaying several vacuoles (V).

a

N

2 μm

b

NPB V

V V

500 nm
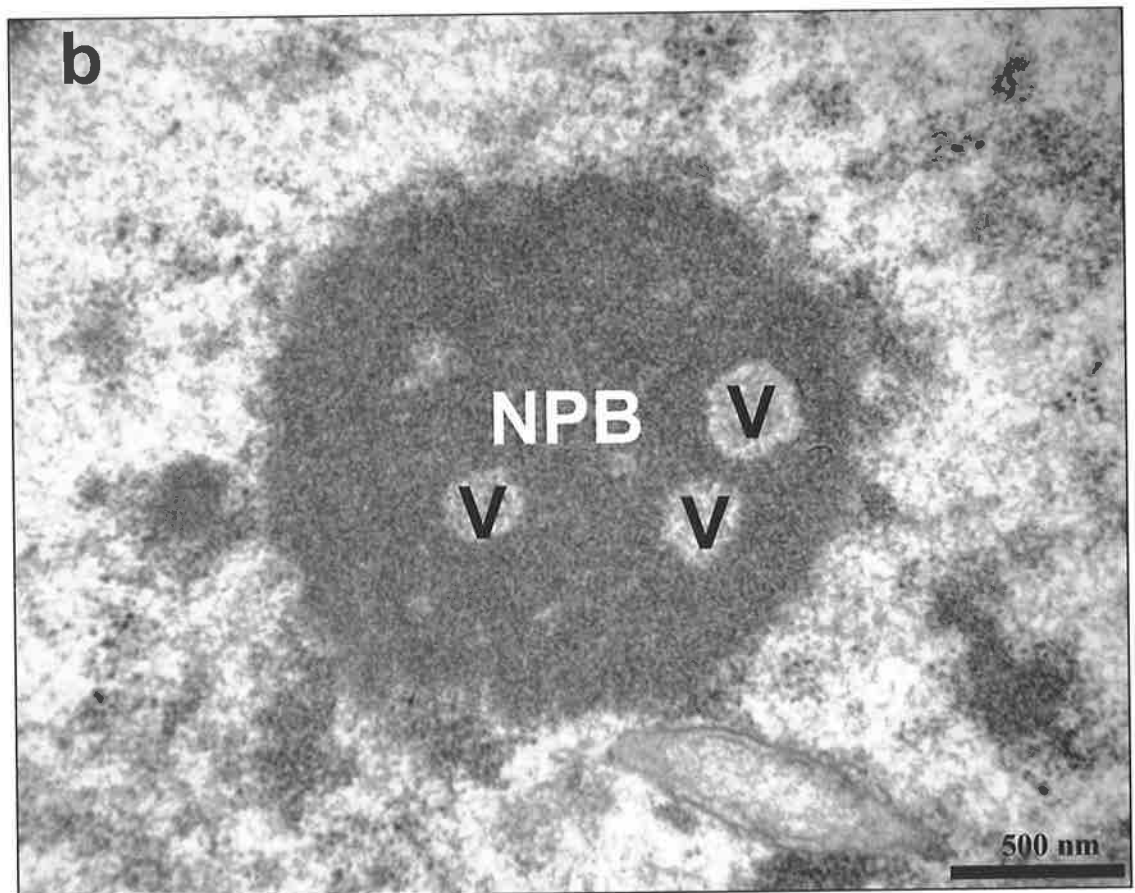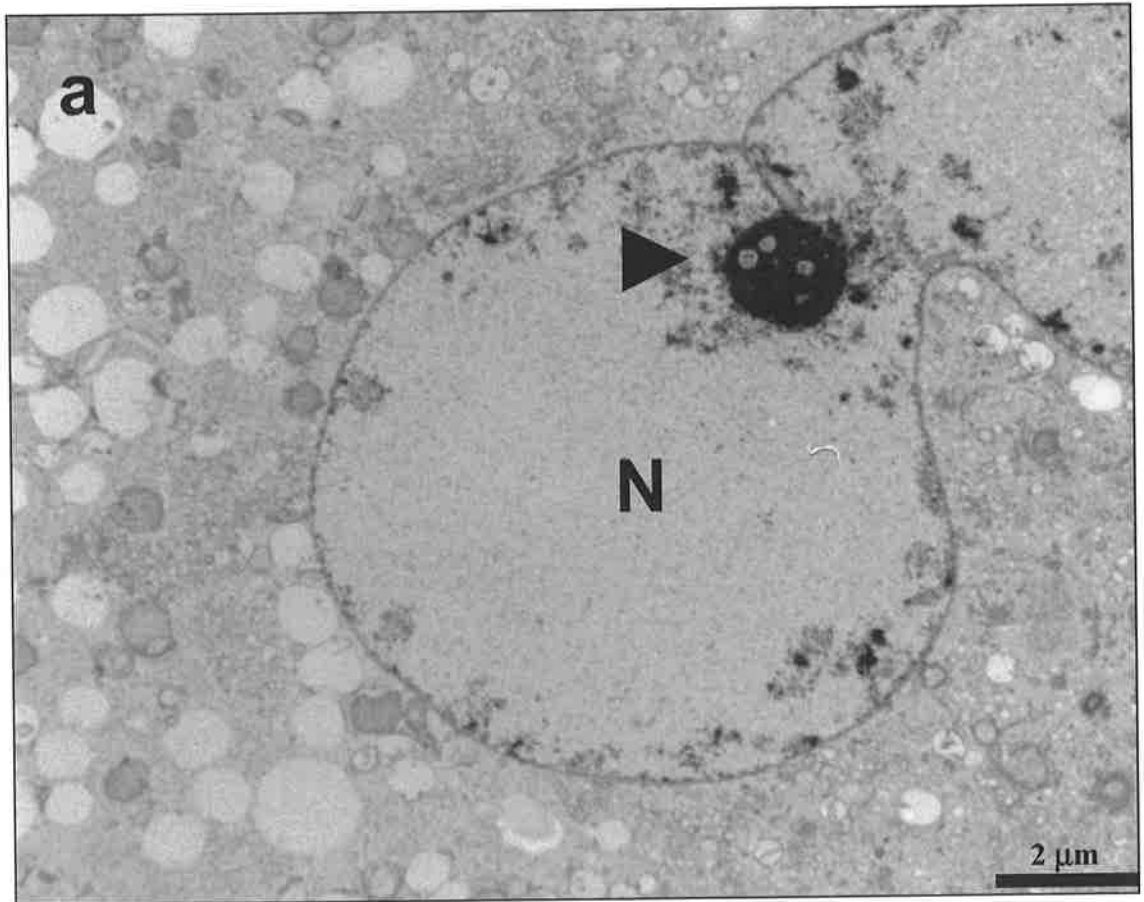
Figure 5.18

Transmission electron micrographs of porcine-ovine (PoOv) SCNT embryos. (a) Cellular organelles including mitochondria surrounded by profiles of smooth endoplasmic reticulum, found within the nucleoplasm of a 16-cell embryo. (b) Unknown fibrillar, chromatin-associated entities found within the nucleoplasm of a 16-cell embryo.

a

2 μm

b

2 μm
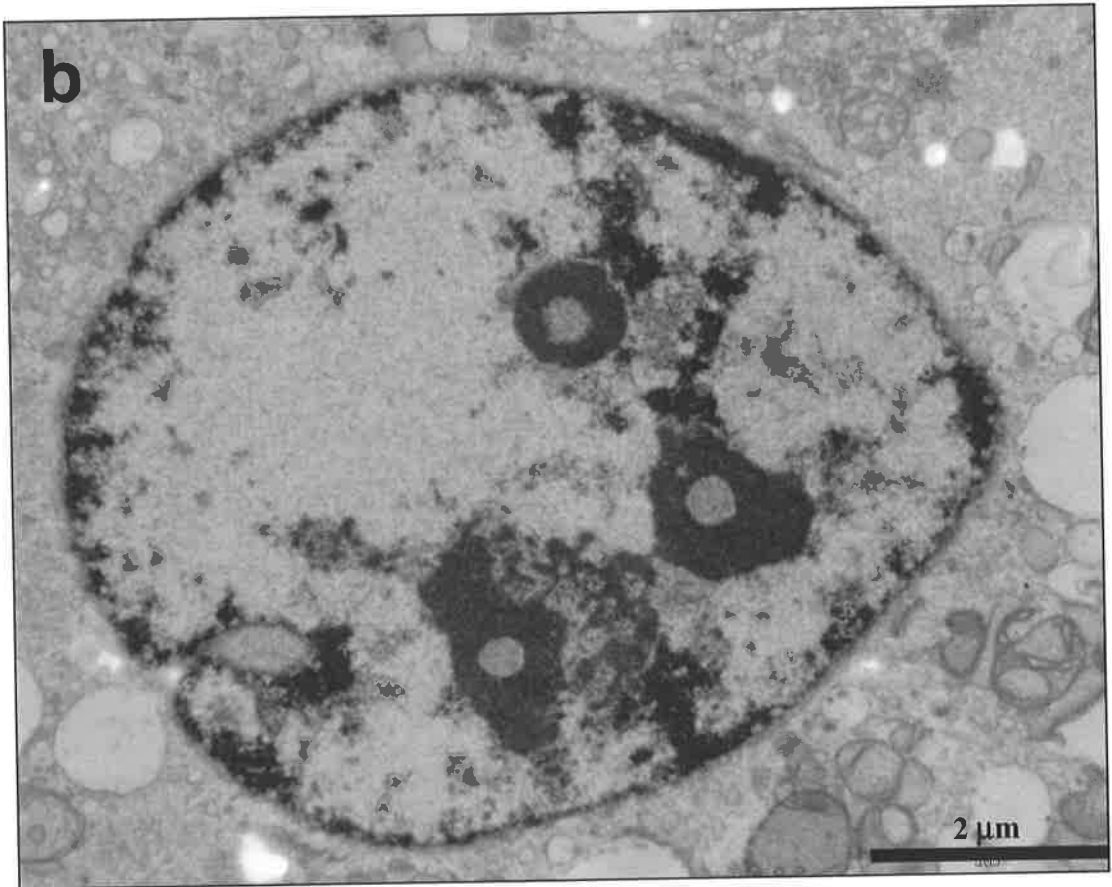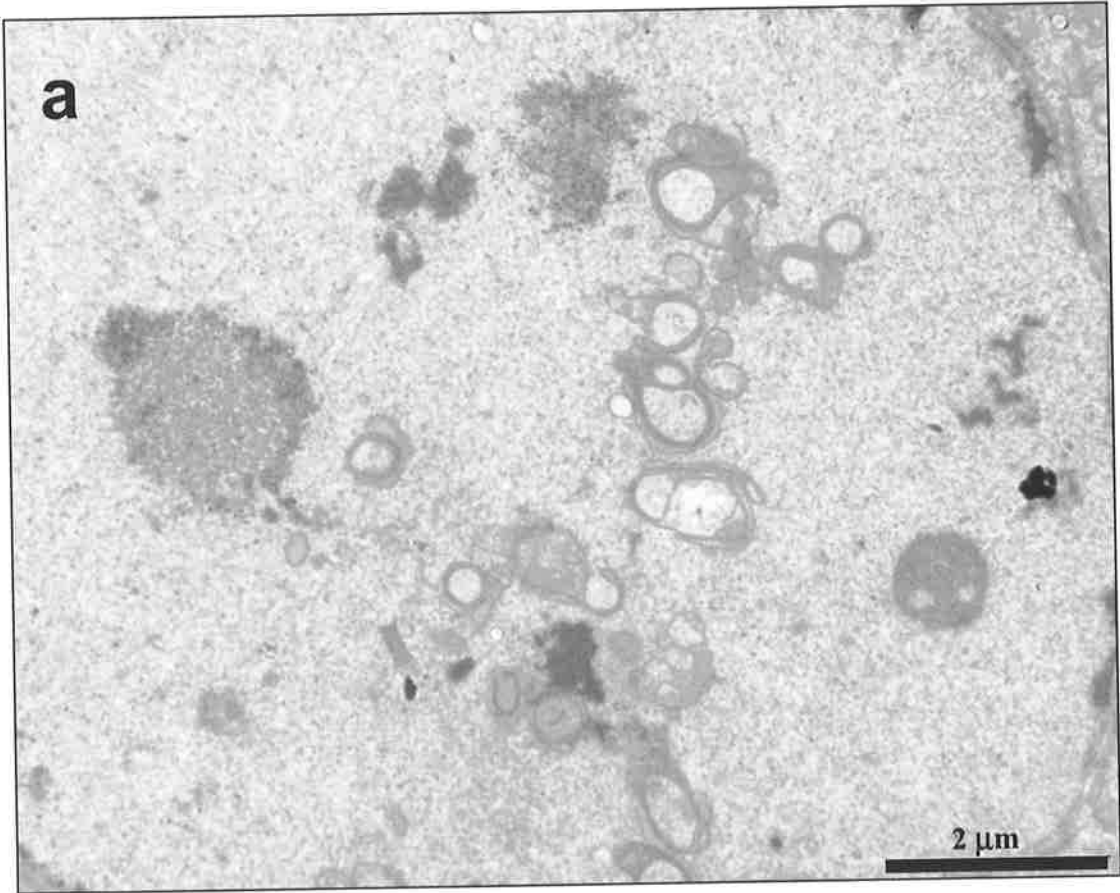
Figure 5.19

Transmission electron micrographs of a bovine-ovine (Bo-Ov) 8-cell SCNT embryo. (a) Nucleus (N) of 8-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) NPB of the same 1-cell embryo displaying several vacuoles (V).
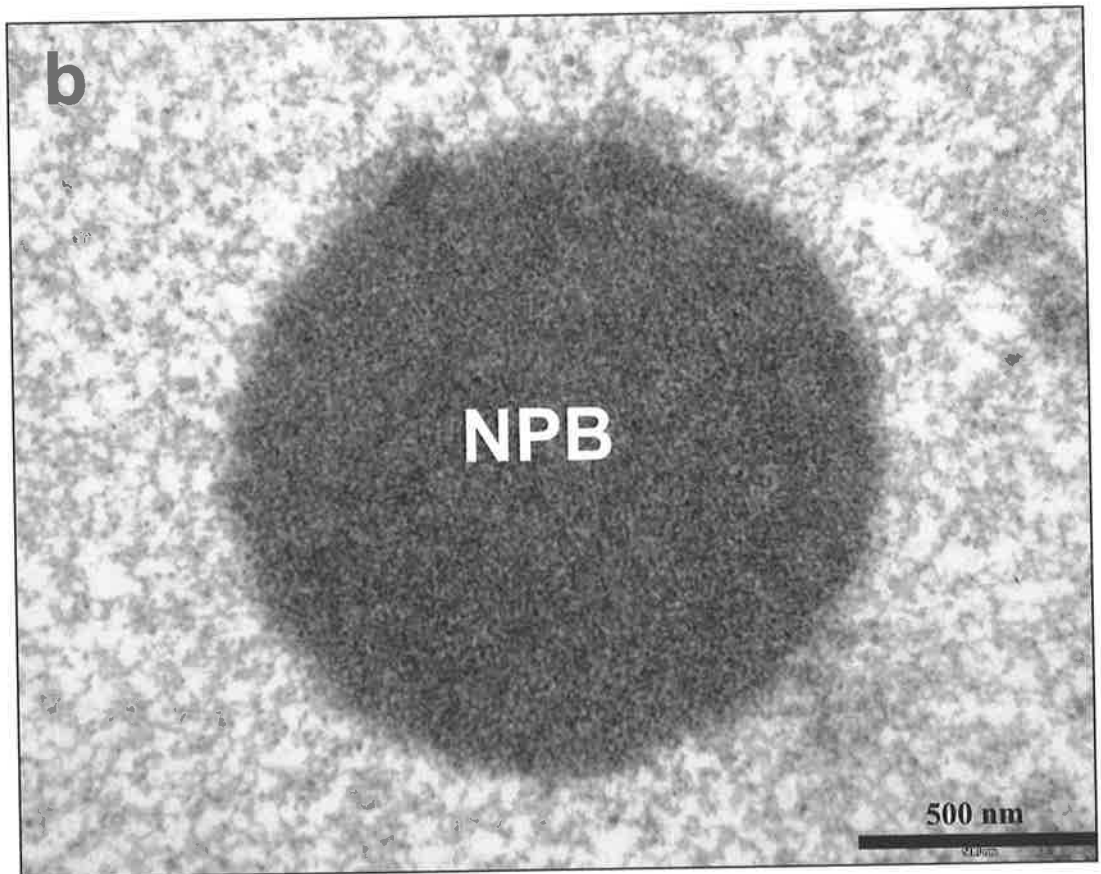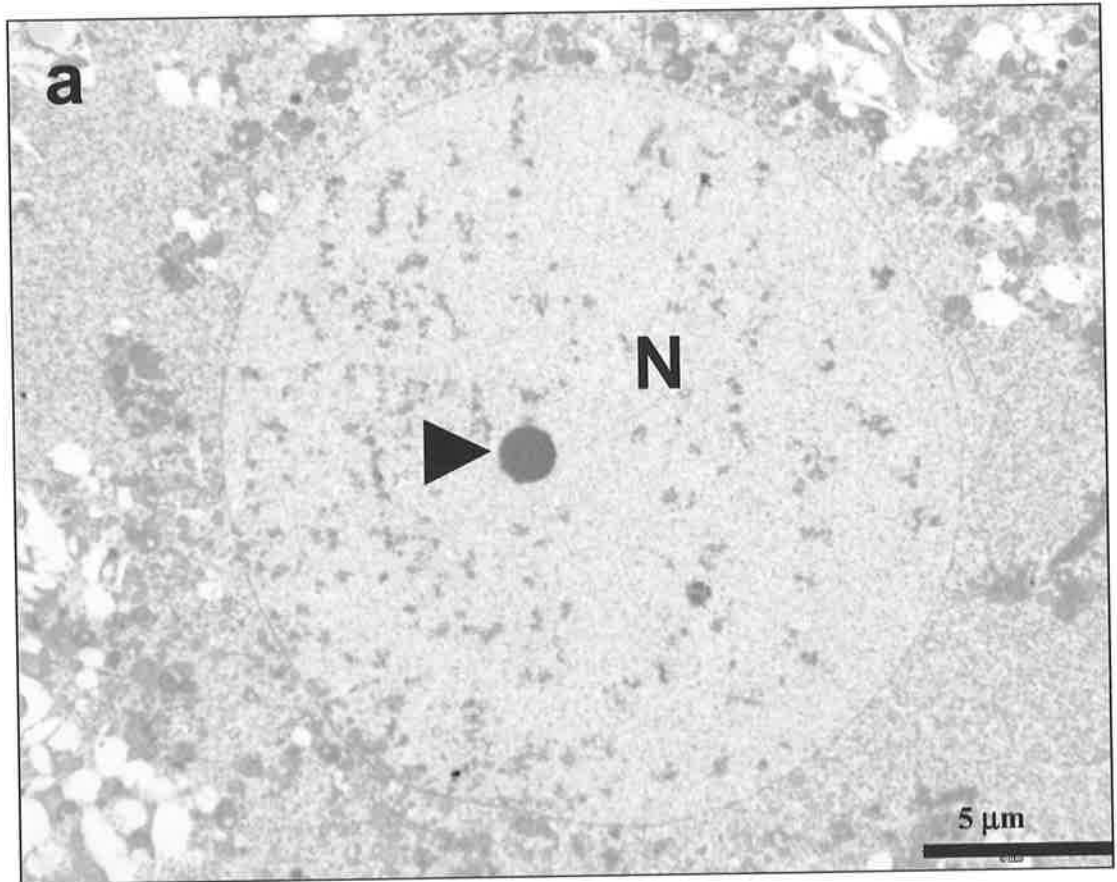
a

N

2 µm

b

V

V

V

200 nm
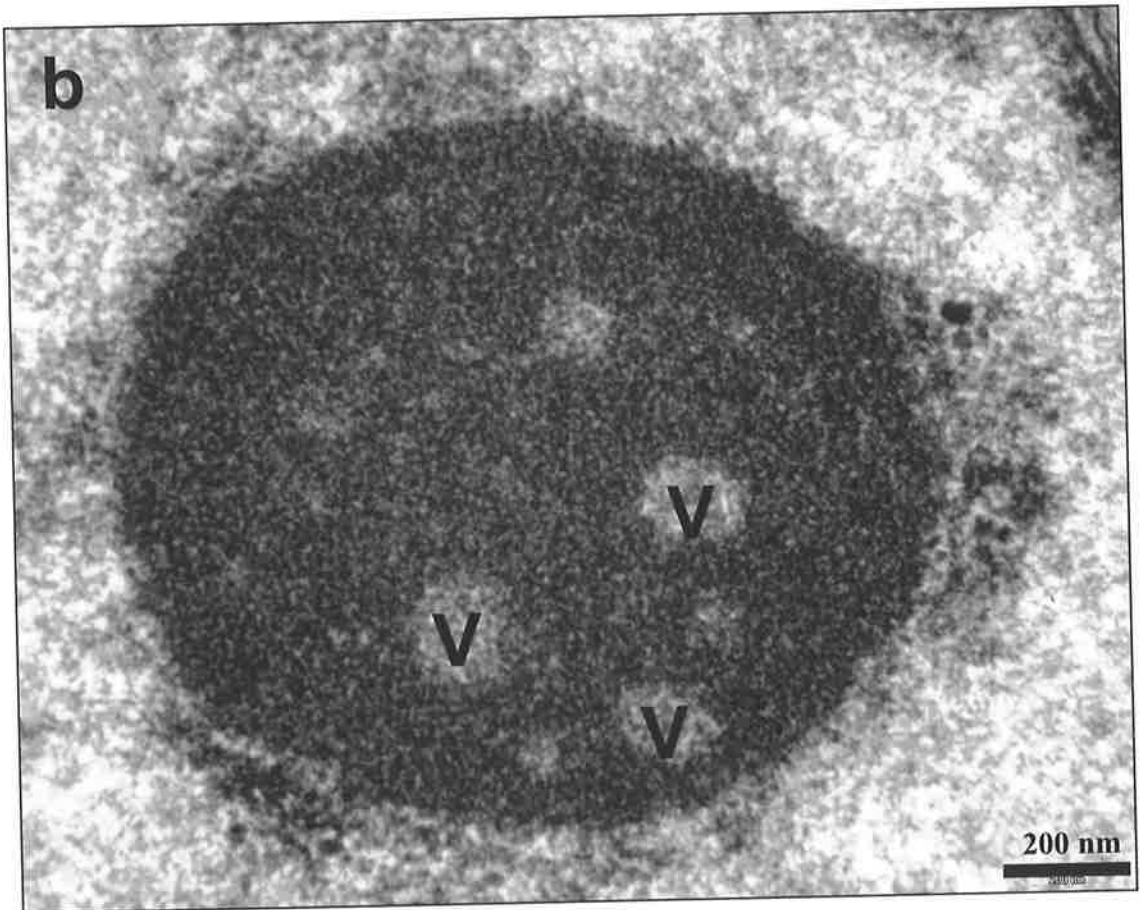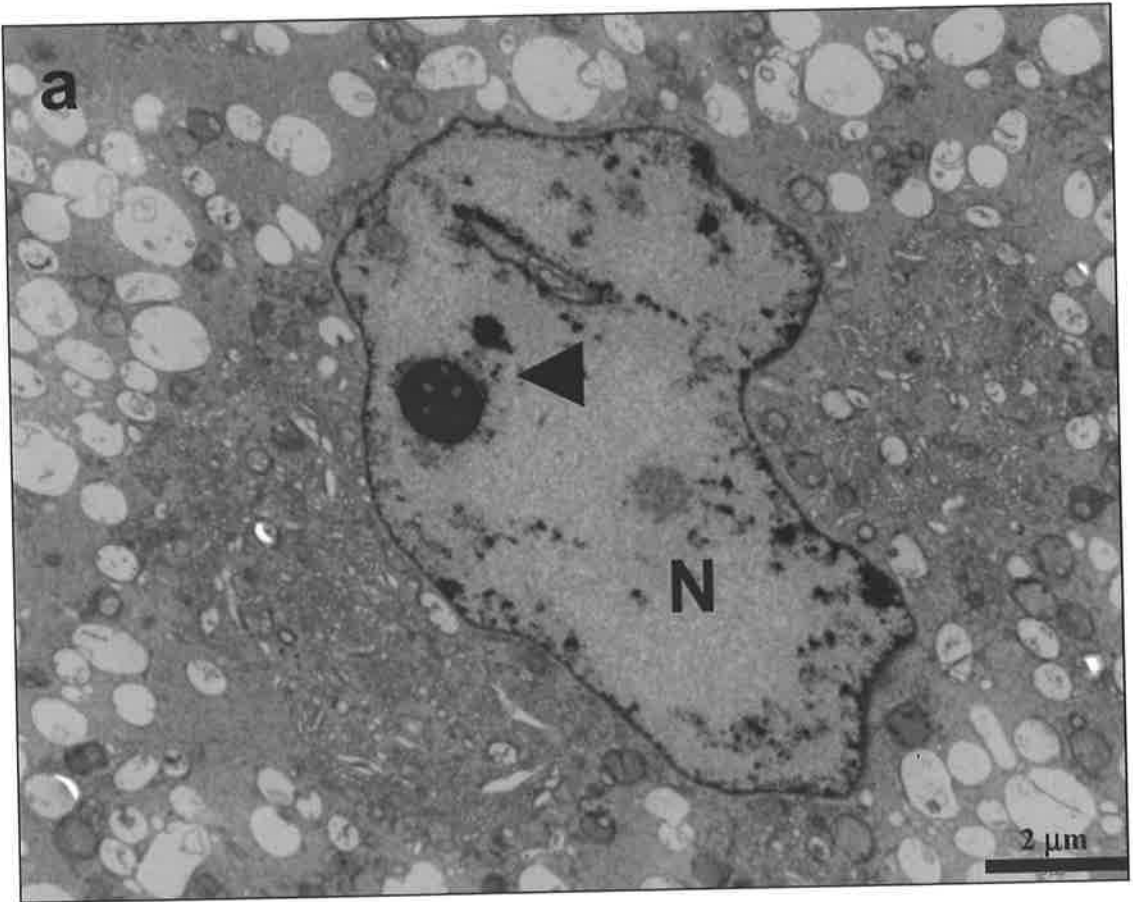
Figure 5.20

Transmission electron micrographs of a bovine-ovine (Bo-Ov) 8-cell SCNT embryo. (a) Nucleus (N) of 8-cell embryo with nucleolar precursor body (NPB; arrowhead). (b) Complex structure of long strands of densely packed fibrillar material coated by granules.

a

N ▶

5 μm

b

500 nm
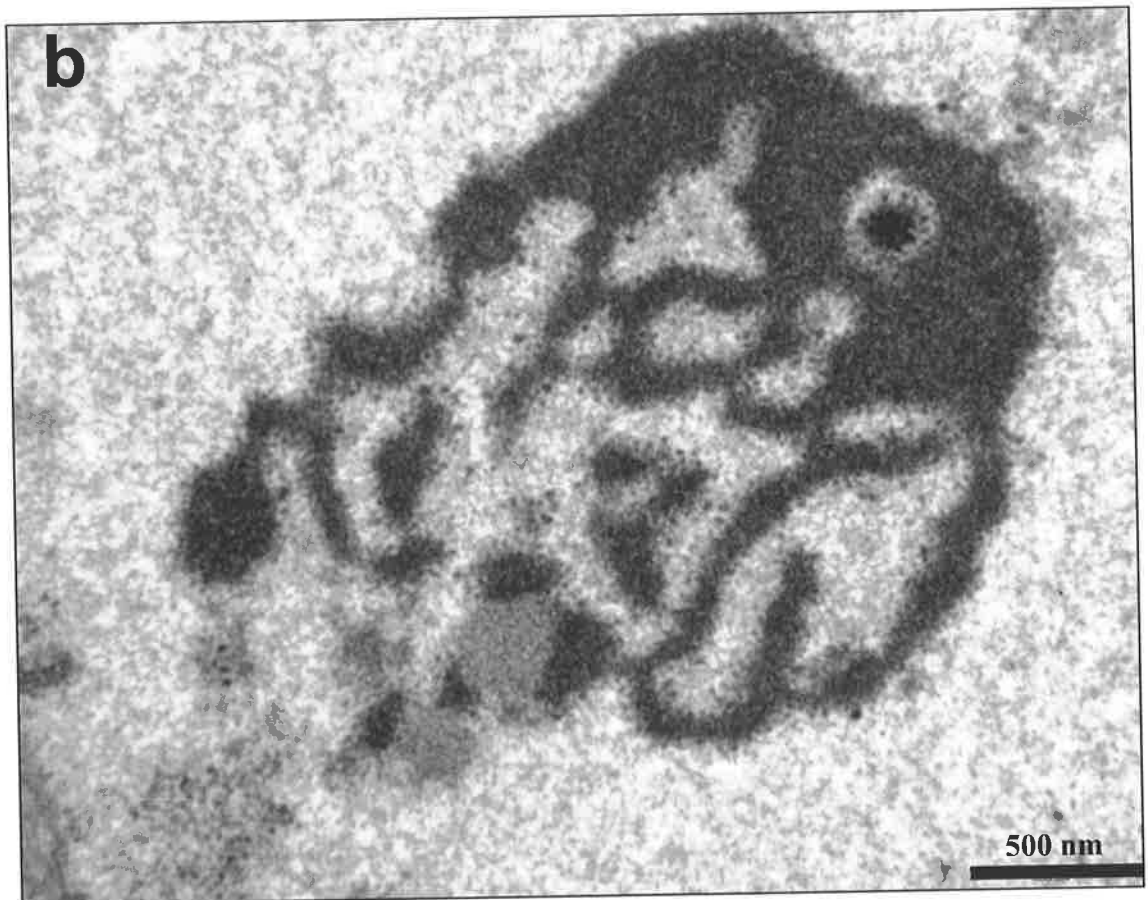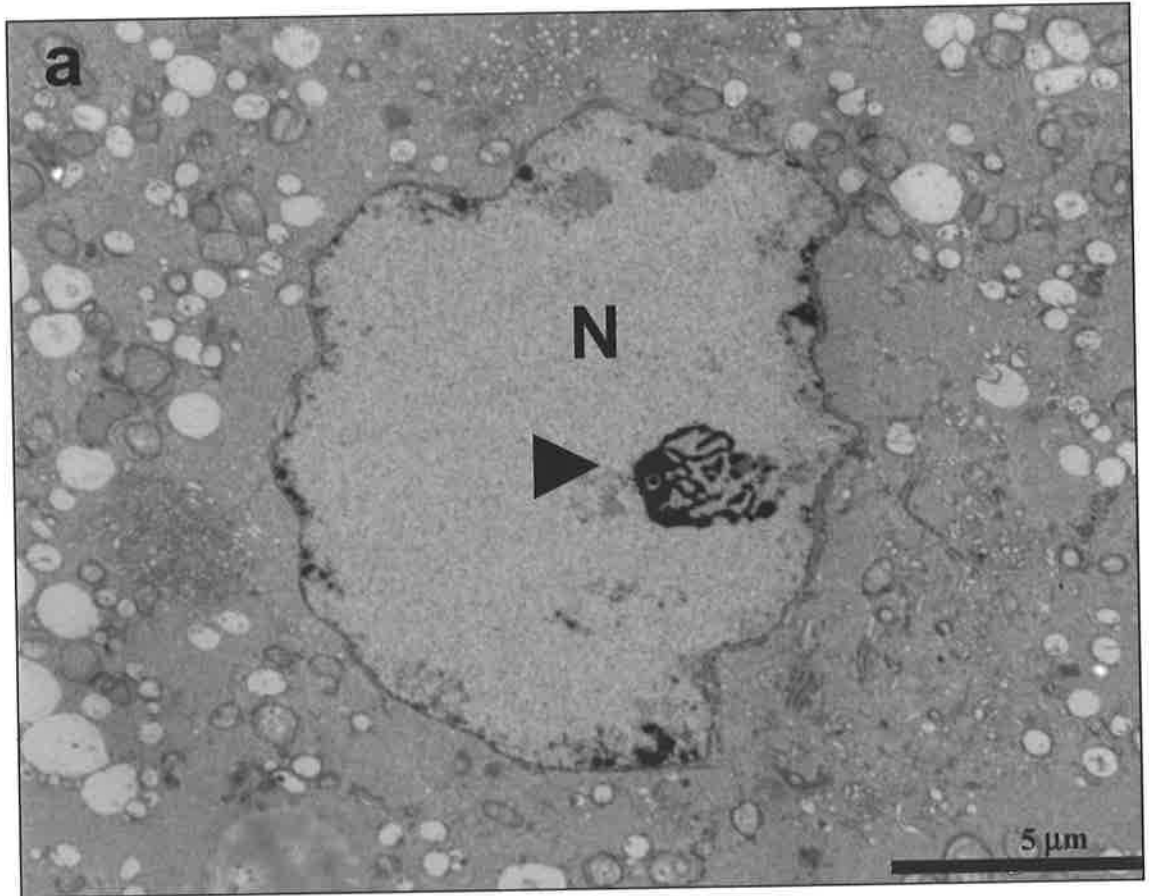
was illustrated with the development of ruminant-type nucleolus precursor bodies in the intergeneric porcine-ovine SCNT embryos. It is well known that the cytoplasm supplies the early embryo with RNA transcripts and numerous polypeptides (Shultz, 1993). Here, the influence that the ooplasm has on the structural formation of a nuclear organelle has been demonstrated, a phenomenon that is perhaps a consequence of the abundant supply of ooplasm transcripts and proteins.

The gradual differentiation of the nucleolus over the early cell cycles after fertilisation is referred to as nucleologenesis. The advent of rRNA transcription, as part of the major activation of the embryonic genome, coincides with the formation of a fibrillogranular nucleolus. Preceding this, the nucleolus is an electron-dense fibrillar sphere known as the NPB. The transformation from the fibrillar sphere into the fibrillogranular nucleolus occurs at the early 4-cell stage in porcine embryos (Hyttel et al., 2000a), and not before the 8-cell stage in ovine embryos (Calcaro and McLaren, 1976; Crosby et al., 1986). Moreover, the two species each display their characteristic pattern of nucleogenesis. Ovine nucleogenesis involves vacuolisation of the NPB with subsequent formation of FCs, a DFC and a GC in the outer area of the NPB, whereas, porcine nucleolgenesis involves the formation of these components on the surface of the NPB. The vacuolised appearance of the ovine (ruminant) NPB and the compact porcine NPB type were clearly demonstrated using TEM.

The intergeneric porcine-ovine NT embryos displayed NPBs in 8-cell embryos and indeed beyond this stage. Significantly, these spheres were vacuolated and of a size more similar to that found in the ruminant embryo. This observation indicates that the ovine ooplasm has a profound influence on the formation of the NPB in the porcine nucleus.

The three main ultrastructural components found in the nucleolus that is transcribing rRNA after the major activation of the embryonic genome are the FCs, the DFC and the GC. At no stage, in the intergeneric embryos, was any one of these three ultrastructrural components

observed. This is not altogether surprising, as a previous study, undertaken to determine the developmental potential of such embryos, found that no significant proportion of embryos developed beyond the 16-cell stage (Chapter 4). In addition, the autoradiograms for the porcine-ovine SCNT embryos that exhibited no ^{3}H-uridine incorporation associated with any nucleolus or indeed any nuclei, indicates that rRNA transcription could not be restored after nuclear transfer, therefore supporting the ultrastructural findings. The observation that fibrillo-granular nucleoli did not develop in any of the porcine-ovine embryos, and that all nuclei lacked autoradiographic labelling is in accordance with the poor developmental capacity of these embryos. It also signals that the ovine ooplasm, on the one hand, directs initial nucleolar formation but on the other hand, is incompatible with the porcine nucleus for completing this event. This ooplasm-nucleus incompatibility within the porcine-ovine SCNT embryos was highlighted by observations of scattered cytoplasmic organelles including mitochondria in the nuclei.

Bovine-ovine SCNT embryos exhibited both ruminant type NPBs as well as complex structures consisting of long strands of densely packed fibrillar material coated by granules in some 16-cell embryos. This observation may suggest tentative achievement of a certain level of nucleolar development followed by disintegration. With the exception that fibrillogranular nucleoli form in ovine embryos at the 16-cell stage as compared with the late 8-cell bovine embryo, nucleolgenesis in these two ruminant species appears to be ultrastructurally similar. However, the fact that the bovine fibrillo-granular nucleolus is unable to form in the bovine-ovine SCNT embryo suggests that, despite the similarities, molecular differences exist or demands for crucial proteins differ between the nuclear and cytoplasmic entities in this species.

Intrageneric ovine-ovine SCNT embryos did display ruminant-type NPBs as expected in the 8-cell embryos. However, unlike the porcine-ovine embryos, FCs, a DFC and a GC were

observed in at least some of the early 16-cell stage embryos suggesting that the transition to a fully functional fibrillo-granular nucleolus was sometimes successfully achieved. This was supported with the detection of ^{3}H-uridine incorporation associated with nuclei and concentrated in the nucleolus of these embryos indicating that the major activation of the embryonic genome had commenced.

By using an intergeneric SCNT model, it has been demonstrated that maternal factors within the ovine ooplasm govern the initial steps in developing the embryonic nucleoli during the major genome activation. The actual factor(s) that are responsible for this event remain to be determined. Abnormalities were also shown to exist in intergeneric embryos (Figure 5.18), and that such embryos do not develop functional fibrillo-granular nucleoli as has been reported in bovine intrageneric SCNT embryos (e.g. Laurincik et al., 2002). A proportion of the ovine-ovine NT embryos, on the other hand, were shown to undergo major genome activation and nucleolus development between the 8- to 16-cell stage, a finding that is consistent with normal embryonic development in this species (Calarco and McLaren, 1976; Crosby et al., 1988).

Chapter 6

Immunocytochemical detection of nucleolar proteins

Chapter 6. Immunocytochemical detection of nucleolar proteins

6.1 Introduction

Since the birth of the first animal created by somatic cell nuclear transfer (SCNT; Wilmut et al., 1997), the manner of genomic reprogramming of the donor nucleus by the recipient oocyte cytoplasm (ooplasm) has been an enigma. SCNT offers a unique opportunity to manipulate the nucleo-cytoplasmic interaction allowing analysis of the communication between the two compartments. The reprogramming capacity of the ooplasm, at least to a certain degree, appears to be conserved between mammalian species. Hence, intergeneric SCNT has been reported to sustain blastocyst development using bovine ooplasm and nuclei from a range of different animals, including those from porcine and ovine cells (Dominko et al., 1999; Lee et al., 2003). By contrast, studies presented in Chapter 4 indicated that intergeneric SCNT embryos, created by transferring a porcine or a bovine somatic nucleus into ovine ooplasm, do not develop beyond the 16-cell stage. A detailed analysis of nucleolus ultrastructure and the activation of the embryonic genome was made with porcine-ovine intergeneric SCNT embryos found that the ovum cytoplasm is influencing nucleolus formation.

The nucleolus contains a number of proteins that control transcription of rRNA genes, process the transcripts, assemble the transcripts with other ribosomal proteins and transport newly synthesised ribosomal subunits to the cytoplasm (Wachtler and Stahl, 1993). RNA polymerase I plays a well-defined role in mRNA transcription. Nucleolin (C23) is a protein that acts in the processing of the rRNA transcript to produce ribosomal subunits and which correspondingly has been localised to the GC of the nucleolus. Nucleolin has also been

localized to bovine NPBs and nucleoli practically throughout initial embryonic development (Laurincik et al., 2000). On the other hand, nucleolin has been localised to porcine embryonic nucleoli only from the time when fibrillo-granular nucleoli have emerged (Hyttel et al., 2000) and interestingly, not to NPBs. Fibtillarin is characterised by its special localisation to the DFC and FCs of the nucleolus.

Therefore, this study aims to extend the findings in Chapter 5 by localising expression of nucleolar proteins RNA polymerase I, nucleolin and fibrillarin to 8-16-cell porcine-ovine and ovine-ovine SCNT embryos using immunocytochemistry and confocal laser scanning microscopy.

6.2 Methods

6.2.1 Experimental design

Immunofluorescent staining for nucleolin, fibrillarin and RNA polymerase I was performed on IVP ovine embryos (ovine IVP), ovine-ovine SCNT embryos, and porcine-ovine SCNT embryos at 48 (early 8-cell), 72 (late 8-cell) and 96 (8-16-cell) hours post insemination or activation. Between 6 and 19 embryos were processed within each group at each time point. Small numbers of bovine IVP blastocyst embryos (n=4-5) were included as positive controls for fibrillarin and RNA polymerase.

6.2.2 Control ovine in vitro embryo production

Ovine IVP embryos were obtained as described in Section 2.4. When the embryos were at the desired stage of development, the zona pellucida was removed (see Section 2.9.5.1) and the embryos fixed in 4% (w/v) paraformaldehyde (see below).

6.2.3 Somatic cell nuclear transfer

The zona-free SCNT technique employed in this studied is described in Section 5.2.4

6.2.4 Immunocytochemistry and confocal laser scanning microscopy

A three-step indirect immunofluorescence protocol was performed on embryos at various stages of development for the following primary antibodies against key nucleolar proteins: mouse monoclonal antinucleolin (C23; 1:1000) (Ochs et al., 1983), human antifibrillarin (1:1000) (Ochs et al., 1985) and human anti-RNA polymerase I (1:500) (Reimer et al., 1987).

Zona-free embryos were fixed in 4% (w/v) paraformaldehyde in phosphate buffer for 3 h at 4°C. Subsequently, specimens were washed in 1% (v/v) Triton X-100 in PBS for 3 h at 4°C and then pre-incubated for 2 h at room temperature in 5% (v/v) rabbit serum (Dako, Glostrup, Denmark) in PBS (blocking buffer, BB). Embryos were then incubated with the primary antibodies diluted in BB overnight at 4°C. To remove excess primary antibody, embryos were thoroughly washed before a 4 h incubation at 4°C in rabbit antimouse-biotin (Dako; for antinucleolin) or rabbit antihuman biotin (Dako; for anti-RNA-polymerase I and antifibrillarin), diluted in BB. The secondary antibodies were visualised by streptavidin-fluorescein isothiocyanate (Dako) in PBS. Embryos were mounted on glass slides using a mounting medium (Dako) containing propidium iodide and examined on a Leica confocal laser-scanning microscope. Control immunostaining of unspecific labelling by the secondary antibody was performed by omitting the primary antibodies.

6.3 Results

6.3.1 SCNT embryo production

The total numbers of embryos obtained for immunocytochemistry analysis are found in Table 6.1.

Table 6.1: Number of embryos selected for immunocytochemistry

| | Sample time point (hpa) | | | | |
Embryo	48 h	72 h	96 h	120 h	144 h
Ovine IVP	40	32	22	34	-
Bovine IVP	-	-	-	-	14
Ovine-ovine	57	20	21	24	5
Porcine-ovine	30	31	26	-	-

6.3.2 Immunocytochemistry and confocal laser scanning microscopy

6.3.2.1 Nucleolin

The number of embryos examined by confocal microscopy for the presence of nucleolin is found in Table 6.2. For ovine IVP, ovine-ovine and porcine-ovine SCNT embryos, immunofluorescent staining for nucleolin was detected in the nucleoplasm of embryonic nuclei, at all time points investigated and with particular intensity on the surface of presumptive nucleoli or NPBs (Figure 6.1). Negative controls, in which the primary antinucleolin antibody was omitted, did not show any immunofluorescent labelling.

6.3.2.2 Fibrilarin

The number of embryos examined by confocal microscopy for the presence of fibrillarin is found in Table 6.3. For ovine IVP, ovine-ovine and porcine-ovine SCNT embryos, immunofluorescent staining for fibrillarin was not detected in the nucleoplasm at any of the stages, including blastocysts (Figure 6.2). For bovine IVP blastocysts, fibrillarin was detected in substantial quantities throughout the nucleoplasm (Figure 6.2).

6.3.2.3 RNA polymerase I

The number of embryos examined by confocal microscopy for the presence of RNA polymerase I is found in Table 6.4. For ovine IVP, ovine-ovine and porcine-ovine SCNT embryos, immunofluorescent staining for RNA polymerase I was not detected in the nucleoplasm at any of the stages analysed, including ovine IVP blastocysts (Figure 6.3). For bovine IVP blastocysts, RNA polymerase I was detected in substantial quantities throughout the nucleolplasm (Figure 6.3).

6.4 Discussion

Nucleolin is a phosphorylated protein present in large quantities in nucleoli with active ribosomal biogenesis (Lapeyere et al., 1987). It has RNA binding properties (Ghisolifi-Nieto

Table 6.2: Number of embryos analysed for Nucleolin labelling.

| | Sample time point (hpa) | | | | |
Embryo	48 h	72 h	96 h	120 h	144 h
Ovine IVP	14	13	7	11	-
Bovine IVP	-	-	-	-	-
Ovine-ovine	19	7	7	8	2
Porcine-ovine	10	10	9	-	-

Figure 6.1

Confocal laser scanning microscopy of nuclei from early 48 h (8-cell), 72 h (late 8-cell), and 96 h (8-16 cell) ovine IVP, ovine-ovine and porcine-ovine SCNT embryos, labelled with antibodies against the nucleolar protein nucleolin.

	48 h	72 h	96 h
Ov-IVP			
Ov-Ov SCNT			
Po-Ov SCNT			
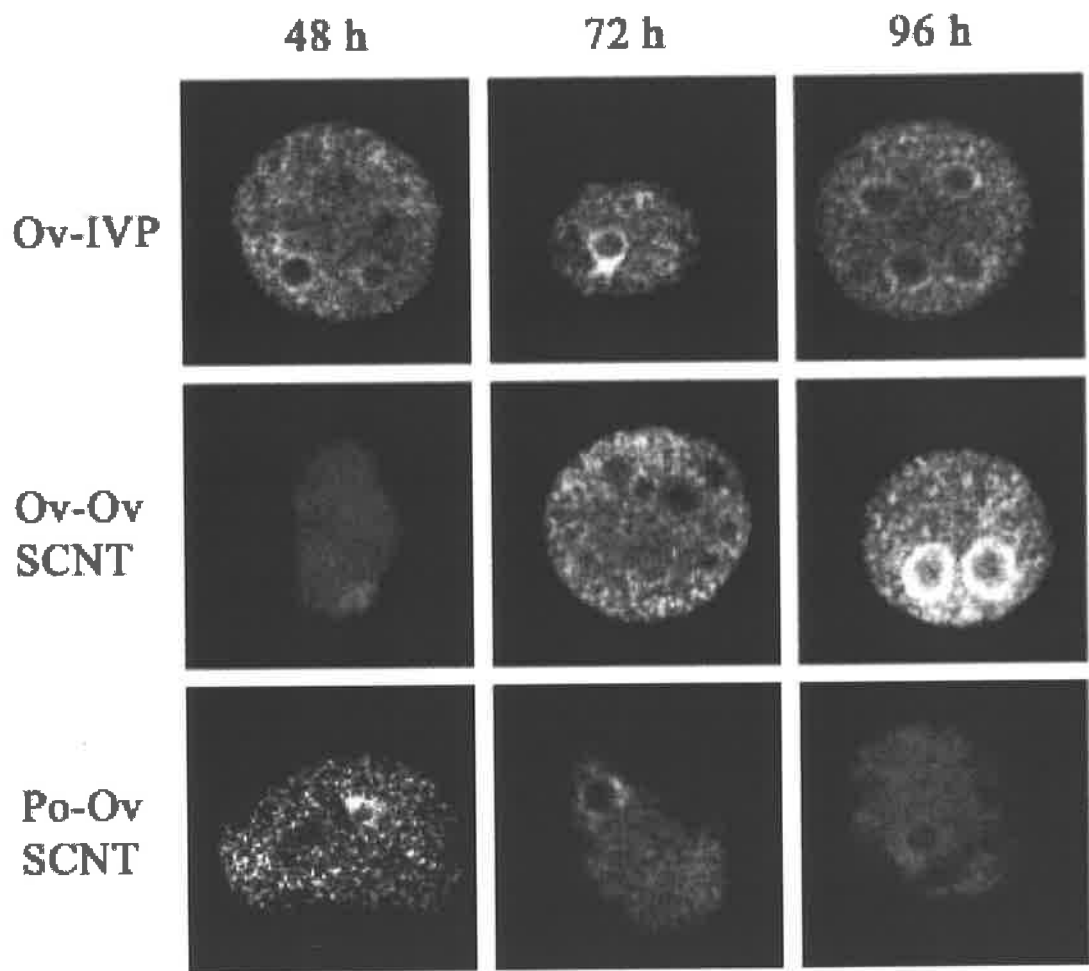
Table 6.3: Number of embryos analysed for fibrillarin labelling.

Embryo	Sample time point (hpa)				
	48 h	72 h	96 h	120 h	144 h
ovine IVP	8	10	8	11	-
bovine IVP	-	-	-	-	5
ovine-ovine	14	7	7	9	-
porcine-ovine	10	11	9	-	-

Figure 6.2

Confocal scanning microscopy of nuclei from ovine and bovine in vitro produced, and ovine-ovine and porcine-ovine SCNT embryos, labelled with antibodies against nucleolar proteins fibrillarin. (a) ovine IVP blastocyst 120 hpa. (b) Bovine IVP blastocyst 144 hpa. (c) ovine-ovine SCNT blastocyst 144 hpa. (d) porcine-ovine SCNT 16 cell 96 hpa. (e) negative control ovine IVP blastocyst.
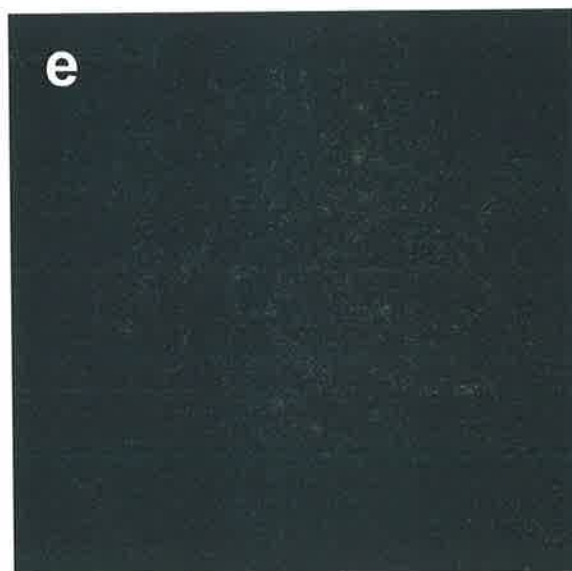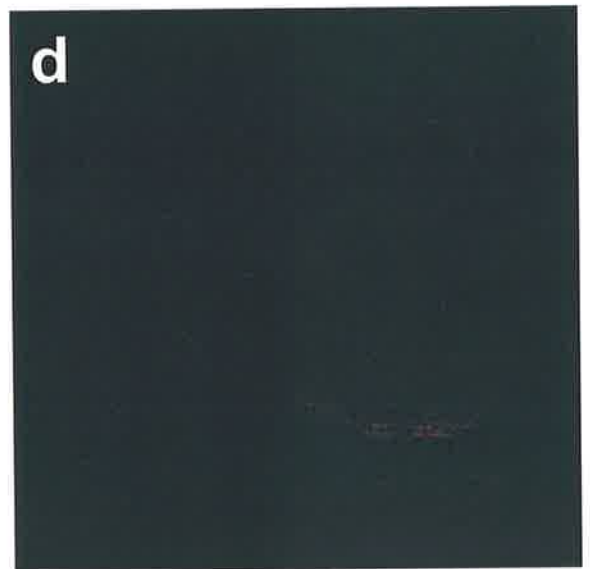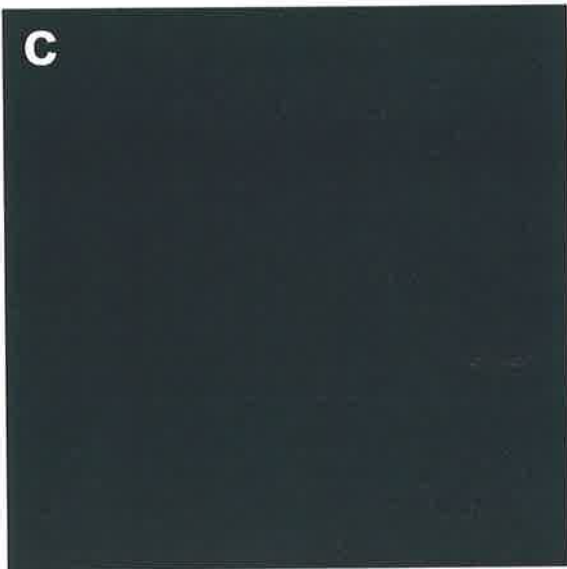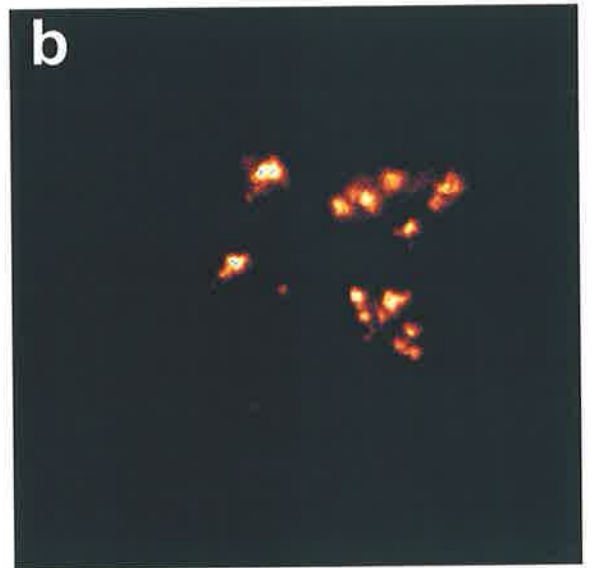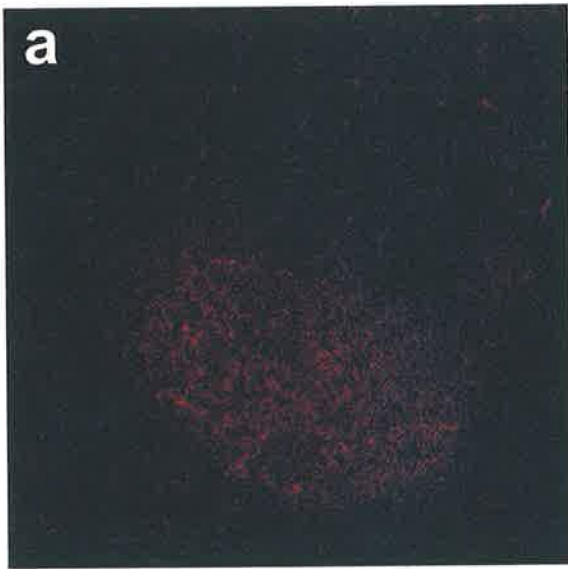
Table 6.4: Number of embryos analysed for RNA polymerase I labelling.

Embryo	Sample time point (hpa)				
	48 h	72 h	96 h	120 h	144 h
Ovine IVP	8	9	7	12	-
Bovine IVP	-	-	-	-	9
Ovine-ovine	14	6	7	7	3
Porcine-ovine	10	10	8	-	-

Figure 6.3

Confocal scanning microscopy of nuclei from ovine and bovine in vitro produced, and ovine-ovine and porcine-ovine SCNT embryos, labelled with antibodies against RNA polymerase I. (a) ovine IVP blastocyst 120 hpa. (b) bovine IVP blastocyst 144 hpa. (c) ovine-ovine SCNT blastocyst 144 hpa. (d) porcine-ovine SCNT 16-cell 96 hpa. (e) negative control ovine IVP blastocyst.
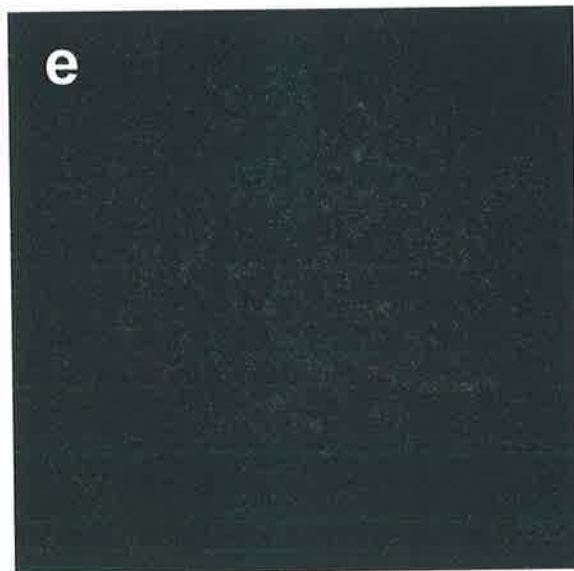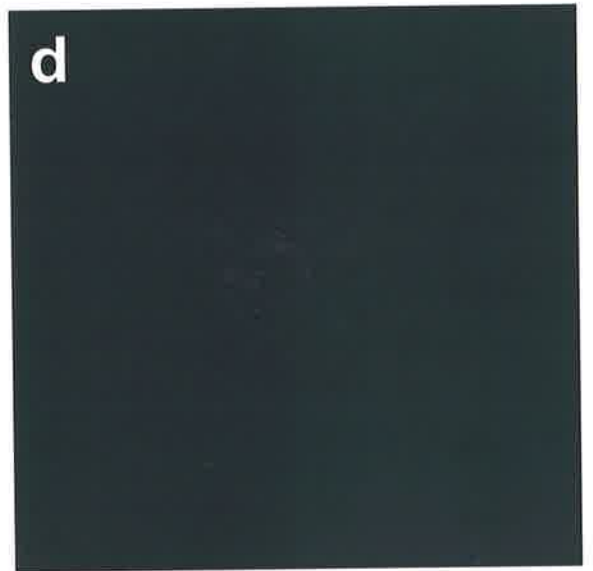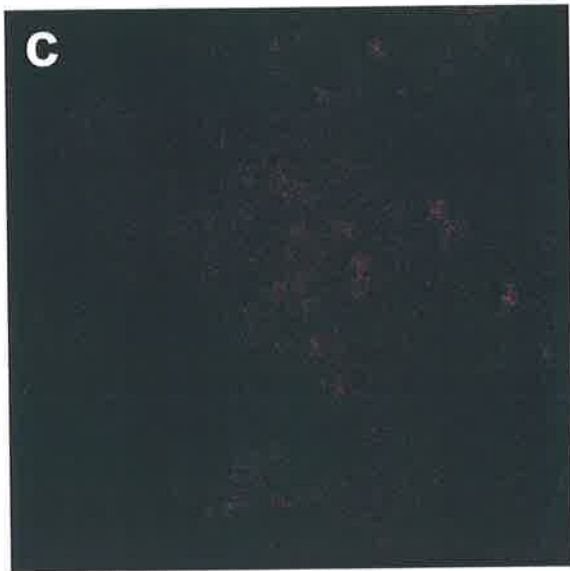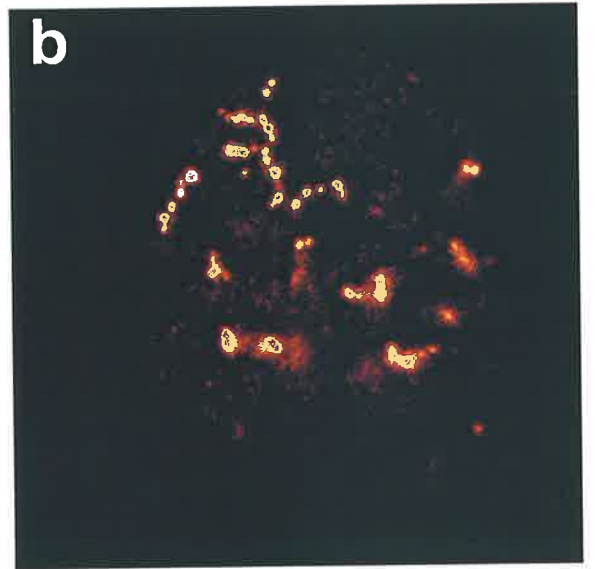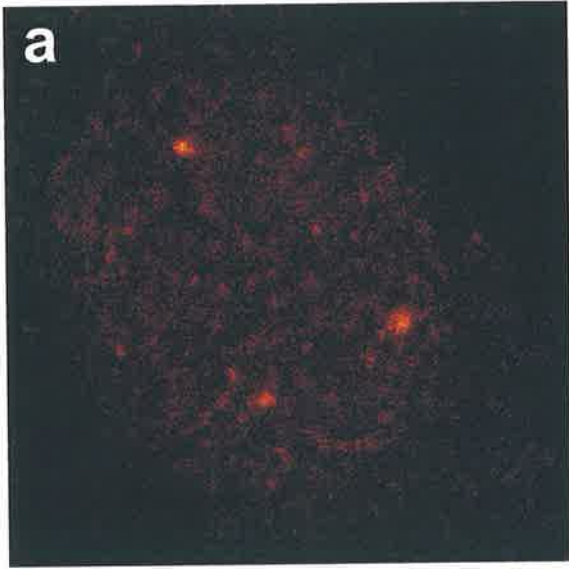
et al., 1996) and is identical to human DNA helicase IV, which unwinds RNA-RNA, DNA-DNA and DNA-RNA duplexes (Teteja et al., 1995). The protein is associated with 18S and 28S sequences. It has been suggested that nucleolin may act in promoting the functional secondary structures of 18S and 28S RNA that are necessary for the assembly of preribosomal particles (Ghisolifi et al., 1990). Nucleolin was immunocytochemically detected in early 8-cell, late 8-cell, and 16-cell of both ovine-ovine and intergeneric porcine-ovine SCNT embryos. The protein was also detected in ovine-ovine SCNT morulae and blastocysts. Labelling was dispersed throughout the nucleolplasm excluding the presumptive NPBs. However, in both ovine-ovine and porcine-ovine SCNT embryos, the labelling was particularly concentrated on the surface of these nucleolar compartments. This indicates that nucleolin is present in nuclei before the major activation of the embryonic genome and, in the case of the ovine-ovine SCNT embryos, after this event as well. In porcine embryos, dispersed nucleolplasmic labelling of nucleolin was detected in the first three cell cycles, but localisation to the surface of the nucleolus structure only occurred during the 4th cell cycle upon formation of a fibrillo-granular nucleolus (Hyttel et al., 2000a). In bovine IVP embryos, nucleolin was detected in the first, absent in the second and reappeared in the third cell cycle and onwards (Laurincik et al., 2000). No information has been published on the localisation of nucleolin in ovine embryos. The fact that nucleolin is present in such abundance in the nucleoplasm before the major activation in porcine-ovine embryos suggests that the nucleolin is most likely derived from maternal proteins or transcripts. It is also possible that some would remain from the donor nucleus, and that after genome activation, nucleolin would be sourced from the embryonic genome transcripts.

Despite localising RNA polymerase I and fibrillarin to nuclei of bovine blastocysts, it would appear that the antibodies for these respective proteins lacked the specificity to bind to ovine-type proteins. It is reasonable to suggest that ovine IVP embryos contain these proteins as they are critical to the process of embryonic genome activation. Therefore, the ovine RNA

polymerase and fibrillarin may differ from the bovine and porcine molecules to the extent that the epitope onto which the antibody binds does not exist on the ovine molecule.

In conclusion, nucleolin was found to exist in porcine-ovine intergeneric SCNT embryos both in the nucleoplasm and localised to the presumptive NPBs.

Chapter 7

General Discussion

Chapter 7. General Discussion

Broadening our current knowledge of the molecular and structural basis of nuclear reprogramming that occurs during SCNT will enable a further understanding of this important biological process. Understanding the principles of nuclear reprogramming may not only allow improvements of success rates in the cloning of genetically or economically valuable animals, but may also provide opportunities to understand and characterise cell differentiation and its reversibility. This knowledge has application in other fields of research, including stem cell biology and, potentially, therapeutic cloning that would create "tailor made" pluripotent stem cells for use in medicine. In addition, there is the possibility of using nuclear transfer to assist in efforts to conserve endangered species and this has prompted the investigation of the existence of a "universal recipient cytoplast" that could support SCNT embryo development when combined with cells from other species (Dominko et al., 1999).

Research described in this thesis uses an intergeneric SCNT model to determine the ability of the ovine ooplasm to support preimplantation embryo development when combined with nuclei from different genera, and in doing so, realise the potential (or lack there of) of the ovine ovum as a "universal recipient cytoplast". Furthermore, by characterising the activation of the embryonic genome in these intergeneric SCNT embryos, the influence of the ovine ovum cytoplasm on nucleolar formation was determined.

The status of NT at the commencement of these studies

When this research began, mammalian SCNT was a research field experiencing rapid growth over a short period of time. Offspring had been reported in sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998; Kato et al., 1998; Wells et al., 1999) and mice (Wakayama et al., 1998). The successful production of the first SCNT animal, Dolly the sheep, had been attributed to the use of

donor nuclei from cells that had exited the cell cycle, and were in the quiescent G_0 stage. Soon after, this was disputed with the successful production of cattle where the donor cells used were actively growing, and largely in the G_1 phase (Cibelli et al., 1998). In vitro development of SCNT embryos was also improved with fusion before activation (FBA) protocols (Wakayama et al, 1998; Wells et al., 1999). However, despite this, the success rates in terms of healthy live offspring obtained per embryo created and transferred did not exceed 3% (Wilmut et al., 1997; Wakayama et al., 1998), which is considered extremely low. This figure is a reflection of the lack of understanding of the molecular and structural reprogramming of the donor nucleus that occurs during and after nuclear transfer. This event is thought to be critical in obtaining normal healthy offspring. Factors that were suspected to be affecting the success of SCNT included the cell cycle coordination of the donor nucleus and recipient cytoplast, telomere length and mitochondrial DNA heteroplasmy (see Chapter 1). In recent times, it has become clear that the epigenetic status of the donor nucleus is a crucial factor in the success of SCNT (Alberio and Campbell, 2003).

A perceived problem associated with SCNT success was the specificity of recipient cytoplasm. For most mammals, there is an incomplete understanding of oocyte maturation, and a limited availability and high cost associated with obtaining recipient cytoplasts. If a common "universal recipient cytoplast" donor was capable of reprogramming and supporting SCNT embryo development, then SCNT technology would be possible in many more species. The bovine ovum cytoplasm was found to possess the ability to support intergeneric SCNT blastocyst development when combined with somatic nuclei from a range of other species (Dominko et al., 1999; Kitiyanant et al., 2001; Lee et al., 2003; see Chapter 1). *In vitro* maturation techniques for ovine oocytes, like those for bovine oocytes, are well understood and developed. Therefore, the ovine ovum cytoplasm is a potential "universal recipient cytoplast" candidate. It was the aim of the present study to assess the ability of the ovine ovum cytoplasm to support intergeneric SCNT embryo development.

Outcomes of studies reported in this thesis

The intergeneric SCNT model was established, and the ovine ovum cytoplasm was shown to be able to initiate embryo development when combined with donor nuclei bovine cells (Chapter 3). In a detailed developmental analysis, Chapter 4 demonstrated that, despite possessing this ability to initiate and support intergeneric SCNT embryo development through the first three cleavage divisions, the ovine ooplasm was not capable of reprogramming somatic nuclei from other genera, specifically bovine and porcine nuclei beyond the 16-cell stage. In addition, donor cell potency was found to have minimal effect on developmental potential, as demonstrated when pluripotent embryonic nuclei were fused to ovine cytoplasts in embryonic cell nuclear transfer (ECNT). In this experiment, despite observing a single (1/21) bovine-ovine blastocyst (4.8%), the developmental block remained with the large majority of embryos not developing beyond the 16-cell stage. These findings are in stark contrast with those reported using bovine (Dominko et al., 1999; Lanza et al., 2000) and rabbit (Chen et al., 2002; Yang et al., 2003) ovum cytoplasm, which support development to the blastocyst stage. Yang et al. (2003) also experienced a developmental block in intergeneric macaca-rabbit embryos coinciding with the timing of genome activation. However, this was overcome by culturing the embryos after the 8-cell stage in culture medium designed for the nuclear donor species. Such a solution was not applicable in the present study given that bovine *in vitro* produced embryos are routinely cultured in SOF medium similar to that used in ovine *in vitro* culture system.

In this thesis, confluent granulosa cells were serum starved for two days before nuclear transfer. It has been suggested that the quiescent stage of the cell cycle (G_0), induced by serum starvation, plays an important role in the success of nuclear transfer (Campbell *et al.* 1996; Wilmut *et al.* 1997). Although cloning success has been achieved with non-starved donor cells (Cibelli *et al.* 1998), subsequent studies indicate the beneficial effects of serum starvation (Zakhartchenko *et al.*

1999; Hill *et al.* 2000). On the other hand, serum starvation has also been observed to induce DNA degradation in cells (Kues *et al.* 2000; Peura 2000). Such subtle changes may not cause any obvious short-term effects at the preimplantation stage but may be linked to adverse effects at a later stage of development. Likewise, mutations accumulating during *in vitro* culture may not be repaired in non-dividing cells because of an absence of DNA replication (Bielas and Heddle 2000), possibly affecting the developmental potential of embryos derived from such cells. A study conducted by our lab (Peura et al., 2003b) reported that in the sheep at least, no differences in cloning outcomes were observed between starved and growing cells. In this study we treated the cells according to the method described by Polejaeva and colleagues (2000).

In Chapter 5, intergeneric porcine-ovine SCNT embryos exhibited nucleolar precursor bodies (NPBs) of an ovine (ruminant) ultrastructure rather than a porcine ultrastructure. This is evidence that the ovine ooplasm is directing the initial assembly of the nucleolus independent of the species of the nuclear donor. This demonstrates an example of the ooplasm influencing the structural formation of a nuclear organelle leading up to the activation of the embryonic genome. In addition, these porcine-ovine intergeneric embryos did not show any signs of activation of the embryonic genome. Therefore, the developmental block illustrated by experiments in Chapter 4 can be explained by the inability of the embryonic genome to be activated in these intergeneric embryos. However, the ovine cytoplasmic factors that influence events in the porcine nucleus such as nucleolus formation remain unidentified. What is also clear is that the nuclear and cytoplasmic entities combined in the present study appear to be incompatible with each other.

To further characterise the influence of the cytoplasm on events leading up to the major activation of the embryonic genome demonstrated by the TEM and autoradiographical work, immunocytochemical localisation of nucleolin to the presumptive NPBs in porcine-ovine intergeneric embryos was demonstrated. This pattern of expression occurs in bovine (Laurincik et

al., 2000) and here in ovine embryos, but only after the major activation of the embryonic genome in *in vivo* produced porcine embryos (Hyttel et al., 2000a). The lack of RNA polymerase I and fibrillarin expression in any ovine-ovine, porcine-ovine or indeed ovine in vitro produced embryos was interesting in that the porcine and bovine RNA polymerase I and fibrillarin molecules may differ from the ovine molecule, at least in terms of the epitopes that these antibody molecules bind to. This provides a possible explanation for the incompatability between the ovine ooplasm and the porcine and bovine somatic nuclei. RNA polymerase I, fibrillarin and perhaps a number of other proteins that are stored in the ovine ooplasm and crucial to the formation of an active ribosome subunit producing nucleolus are perhaps incompatible with the porcine and bovine nuclei.

An obvious question that presents itself in this discussion is whether the observed donor nucleus-oocyte cytoplasm interaction is restricted to the ovine oocyte only. Performing comparable ultrastructural and protein studies on other SCNT nuclear and cytoplasmic combinations would determine if oocyte cytoplasm from other species also direct initial nucleolar assembly in the SCNT embryo. Successful preimplantation embryo development of intergeneric SCNT embryos obtained using bovine oocytes (Dominko et al., 1999) as observed in context of the results outlined in this thesis presents exciting possibilities. Cytoplasmic influence on the initial nucleolar assembly would be reaffirmed by examining the nucleolar ultrastructure and detecting the presence of nucleolar proteins in an 8-cell and 16-cell porcine-bovine intergeneric SCNT embryo. The outcome of nucleolar ultrastructure from intergeneric embryos known to be capable of developing beyond the stage of the major activation of the embryonic genome, would present the nucleolus from a viable embryo.

How conserved is cytoplasmic regulation of nuclear function – key to the future

The TEM and autoradiographical examinations of the intergeneric SCNT embryos presented in this study showed that the ovum cytoplasm is influencing the formation of the nucleolus, a crucial nuclear organelle central to the successful activation of the embryonic genome (Chapter 5). The ovum cytoplasm is known to play a large role in controlling the first cleavage divisions leading up to the activation of the embryonic genome. However, these results provide a specific example of the important influence the ovum cytoplasm has in the formation of a nuclear structure. However, the specific cytoplasmic factors that are responsible for this nucleolar assembly are thus far unknown. In addition, the influence that factors in the ovum cytoplasm have on embryo development beyond the activation of the embryonic genome is not known. The fact that some combinations of intergeneric SCNT embryos develop to the blastocyst stage but are unable to develop into live offspring suggests that complications exist beyond those encountered with intrageneric SCNT embryos (e.g. ovine-ovine). It is known that mitochondrial DNA from the ovum cytoplasm is the source of mitochondrial DNA in all subsequent cells that make up that individual. Furthermore, the inability of the sheep cytoplasm to support intergeneric preimplantation embryo development, as demonstrated in Chapter 4, demonstrates that the cytoplasmic factors responsible for nuclear reprogramming are not conserved across all species. Alternatively, successful intergeneric SCNT embryo development to the blastocyst stage obtained using bovine (Dominko et al., 1999; Kitiyanant et al., 2001; Lee et al., 2003) and rabbit (Chen et al., 2002; Yang et al., 2003) ooplasm indicates that the cytoplasmic factors in these two cases provide the necessary factors to support development beyond the activation of the embryonic genome. Therefore, an alternative explanation for the developmental block experienced in the present study is that the ovine oocyte, as distinct from bovine and rabbit oocytes, lacks crucial cytoplasmic factors, or a factor that prevents the activation of the embryonic genome when combined with any nuclei from other genera.

Recent advances in intergeneric SCNT and future directions

Since this project began, bovine cytoplasts (of the genus Bos) have become the most successful 'universal recipient cytoplasm' for mammalian SCNT due to its ability to support embryo development when combined with nuclei from other species and genera. For example interspecies (intrageneric) SCNT has been used to produce a gaur bull (*Bos gaurus*) pregnancy. Intergeneric SCNT using bovine cytoplasts was implemented to obtain advanced stages of preimplantation development at acceptable frequencies following transfer of somatic cells from the genus Bubalus (Kitiyanant et al., 2001), and Tragelaphus (Lee et al., 2003).

It has been proposed that reproductive biotechnologies have a role to play in the conservation of threatened mammalian species (Corley-Smith and Brandhorst, 1999). Indeed, it has been suggested that SCNT could be implemented to increase the population of threatened species, and perhaps even restore extinct species (The cloning debate. Could the Thylacine be cloned? http://www.austmus.gov.au/thylacine/09.htm). Early unsuccessful attempts to clone endangered mammals included those using somatic cells obtained from argali wild sheep (*Ovis ammon*; White et al., 1999), and gaur (*Bos frontalis*) when using ovine (*Ovine aries*) and bovine (*Bos taurus*) oocytes as a source of recipient cytoplasm respectively. An example of a successful application of SCNT was the production of a wild endangered mouflon (*Ovis orientalis musimon*) using ovine (*Ovis aries*) oocytes as a source of recipient cytoplasts (Loi et al., 2001). These results illustrate that at the present time, success with species preservation using current SCNT techniques may only be possible if the recipient cytoplast is within the same genera, and even if that is the case, an incompatibility between the donor nucleus and recipient cytoplasm may exist in some cases making the combination lethal. Before species preservation using SCNT can be implemented, the structural and molecular events responsible for successful nuclear reprogramming that occur during nuclear transfer (as discussed in Chapter 1) must be elucidated.

Other difficulties associated with intergeneric SCNT may include sourcing appropriate recipient into which embryos would be transferred. Unless two species of animals were closely related, it would be difficult to foresee an animal maintaining a cloned pregnancy to term from an embryo reconstructed with a donor nucleus from another species. The different lengths in gestation and diverse *in utero* environments would make such a scenario improbable. Therefore, collectively, it appears unlikely that SCNT will be used widely in the near future. Indeed it should be emphasised that assisted reproductive technologies in general are only one approach to species conservation, and should be designed to supplement conventional programs directed at *in situ* conservation (Ryder and Benirschke, 1997).

Studies reported in this thesis clearly indicate that SCNT, using ovine cytoplasts, facilitates embryo development to the 8-16 cell stage but not beyond, irrespective of the species of the donor. Failure to develop past this stage is associated with various ultrastructural abnormalities in the nucleolus indicating that the ovine cytoplast lacks the molecular mechanisms to facilitate normal embryo development. Given this limitation, it appears unlikely that SCNT, using the ovine cytoplast, has a role to play in the production and/or resurrection of endangered or extinct species. However, this SCNT model provides a sound basis for further studies into the interactions between the nucleus and cytoplasm and in gene regulation and function. To this extent, intergeneric/interspecies SCNT is likely to become a valuable scientific tool in relevant research programs.

Chapter 8

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Chapter 8. Bibliography

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Appendix I: Embryo IVP stocks and media

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1. Phosphate Buffered Saline (PBS) – for ovary collection from abattoirs

OXOID PBS	5 tablets
MilliQwater	500 ml

- Make 2 lots of 500 ml in 500 ml glass flasks.
- To be made the day before ovary collection.
- Take 1 x 500 ml flask with you to abattoir and leave the other for washing before, and holding ovaries during aspiration.

2. Hormone Stocks

2.1 LH Stock

- (Vetrepharm – Lutropin)
- NIH-LH-S19 Std (each mg=0.92mg S19-LH) = 92%
- Each vial contains 25 mg of armour standard but armour standard is not relevant to weight.
- 13.8 mg of actual weight in vial therefore for 1 mg/ml stock add 13.8 ml of saline (0.9% NaCl) to one vial.
- Aliquot into 150 µl volumes and freeze
- Want a final media concentration of 5 µg/ml, therefore for every 10 ml media add 50µl of 1 mg/ml stock which is the equivalent to adding 50 µg of LH to 10 ml.
- **Note:** Each mg of NIH-LH-S19 = 0.92 mg of actual LH, strictly this should be taken into account when working out the stock concentration of 1 mg/ml.
  - ⇒ i.e. for 10.0 mg of actual weight = 9.2 mg of LH, therefore add 9.2 ml sterile saline to give 1 mg/ml LH stock. Similarly, given 13.8 mg of actual weight = 12.696 mg of LH, therefore should add only 12.696 ml saline to give 1 mg/ml LH stock.

2.2 FSH Stock

- (Vetrepharm – Folltropin), NIH-FSH-P1 Std.
- 400 mg per vial. Therefore add 40 ml of sterile saline to give a stock concentration of 10 mg/ml.
- Aliquot in 20 µl volumes and freeze.
- Want a final media concentration of 5 µg/ml. Therefore for every 10 ml media add 5µl FSH stock. For 25 ml media add 12.5 µl of stock.

2.3 Estradiol

- Stock solution is 50 mg of estradiol in 10 ml absolute ethanol, stock concentration is 5 µg/l (5 mg/ml).
- Make up in glass.
- Store at 4°C for up to 2 months.
- Want a final media concentration of 1µg/ml, therefore for every 5 ml media add 1 µl of 5 µg/µl. For a final media volume of 25 ml, add 5µl of stock.

3. Ovine Aspiration medium

3.1 Ovine aspiration stock

- Measure and combine the constituents in the following order and proportions:

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TCM199 stock (10x)	9.5 ml
Milli Q	91.0 ml
NaHCO$_3$	0.04 g
Hepes (Na salt)	0.5206 g
Heparin	2.0 ml
Osmolarity	280($\pm$5) mOsm
Filter (0.22 μm)	

- Make up in T80 culture flask.
- Store up to 2 weeks at 4°C.

3.2 Ovine Aspiration Media
- Measure and combine the constituents in the following order and proportions:

Aspiration stock (see Section 3.1)	39.2 ml
Sheep serum	0.80 ml
Pen/Strep	0.80 ml
Filter (0.22μm)	

- Make up in T25 Nunc culture flask.
- To be made the day before oocyte collection.
- Place in incubator (5% CO$_2$) to equilibrate overnight.

4. Ovine maturation medium

4.1 Ovine maturation stock
- Measure and combine the constituents in the following order and proportions:

Milli Q	55.0 ml
TCM199 stock (10x)	5.6 ml
NaHCO$_3$	0.126 g
Glutamine	0.006 g
Osmolarity	272 ($\pm$5) mOsm
Filter (0.22 μm)	

- Make up in T80 Nunc culture flask.
- Store up to 2 wks at 4°C.

4.2 Ovine maturation medium
- Measure and combine the constituents in the following order and proportions:

HISS	2.0 ml
Maturation Stock (See Section 4.1)	8.0 ml
Pen Strep	200 μl
LH Stock (1mg/mL)	50 μl
FSH Stock (10mg/mL)	5 μl
Filter (0.22μm)	
Estradiol Stock	2 μl

- Gently mix media with 1000mL pipette (as estradiol won't have mixed properly).
- To be made the day before oocyte collection and used fresh.
- Place tube in the incubator (5% CO$_2$) to equilibrate overnight.

5. Bovine maturation medium

5.1 Bovine maturation stock
- Measure and combine the constituents in the following order and proportions:

Milli Q	41.75 ml

10 x TCM199 Stock	4.8 ml
Stock B.2 (See Section 7.1.2)	5.0 ml
Stock C.1 (See Section 7.1.3)	0.25 ml
Glutamine	0.005 g
Osmolarity	272 (±5) mOsm
Filter (0.22 μm)	

- Make up in T80 Nunc culture flask.
- Store up to 2 wks at 4°C.

5.2 Bovine maturation medium

- Measure and combine the constituents in the following order and proportions:

HIFCS	2.0 ml
Bovine maturation Stock (See Section 5.1)	8.0 ml
Pen Strep	200 μl
LH Stock (See Section 1.2)	50 μl
FSH Stock (See Section 1.1)	5 μl
Filter (0.22μm)	
Estradiol Stock (See Section 1.3)	2 μl

- Gently mix media with 1000mL pipette (as estradiol won't have mixed properly).
- To be made the day before oocyte collection and used fresh.

Place tube in the incubator (5% CO_2) to equilibrate overnight.

6. Ovine fertilisation and culture media

6.1 Synthetic oviduct fluid (SOF) stock solutions

6.1.1 Stock A with Calcium and Magnesium
- Measure and combine the constituents in the following order and proportions:

NaCl	6.290 g
KCl	0.534 g
KH_2PO_4	0.162 g
$MgSO_4.7H_2O$	0.182 g
Penicillin G	0.060 g
Milli Q	99.4 ml
Na Lactate	0.6 ml
Filter (0.22 μm)	

- Make up in glass bottle.
- Store for 3 months at 4°C.

6.1.2 Modified Stock A without Calcium and Magnesium
- Measure and combine the constituents in the following order and proportions:

NaCl	6.290 g
KCl	0.534 g
KH_2PO_4	0.162 g
Penicillin G	0.060 g
Milli Q	99.4 ml
Sodium Lactate	0.6 ml
Filter (0.22 μm)	

- Make up in glass bottle.
- Lasts 3 months at 4°C.

6.1.3 Stock B

- Measure and combine the constituents in the following order and proportions:

NaHCO$_3$	0.210 g
Phenol red	2/3 grains
Milli Q	10.0 ml

- Make up in 10 ml Conical Falcon tube.
- Store for 2 weeks at 4°C.

6.1.4 Stock C

- Measure and combine the constituents in the following order and proportions:

Pyruvic acid	0.051 g
Milli Q	10 ml

- Make up in 10 ml Conical Falcon tube.
- Make up fresh Stock B and Stock C on the day of use.

6.1.5 Stock D

- Measure and combine the constituents in the following order and proportions:

CaCl$_2$.2H$_2$O	0.262 g
Milli Q	10 ml
Filter (0.22 µm)	

- Make up in 10 ml Conical Falcon tube.
- Store for 3 months in fridge.

6.1.6 Stock E

- Measure and combine the constituents in the following order and proportions:

Hepes (Acid Hepes)	0.5958 g
Phenol Red	2/3 grains
Milli Q	8 ml
Adjust to pH 7.5 with 3N NaOH after adding Milli Q	
Make up to 10 ml	
Filter (0.22 µm)	

- Make up in 10 ml Conical Falcon tube.
- Store 3 months in fridge.

6.2 Synthetic oviduct fluid (SOF) Base Media

6.2.1 Bicarb SOFM with Calcium and Magnesium

- Measure and combine the constituents in the following order and proportions:

Milli Q	155.0 ml
Stock A	20.8 ml
Stock B	20.0 ml
Stock C	1.4 ml
Stock D	1.0 ml
Osmolarity – 280 ± 5 (adjust with Milli Q if over, Stock A if under)	
Filter (0.22 µm)	

- Make up in T80 Nunc culture flask
- Store in fridge for 1 week.

6.2.2 Bicarb SOFM without Calcium and Magnesium

- Measure and combine the constituents in the following order and proportions:

Milli Q	7.75 ml
Modified Stock A	1.04 ml

Stock B 1.00 ml

Stock C 0.07 ml

Osmolarity – 280 ± 5 (adjust with Milli Q if over, Stock A if under)

Filter (0.22μm)

- Make up in T25 Nunc culture flask.
- Store in fridge for 1 week

6.2.3 Hepes-SOFM with Calcium and Magnesium

- Measure and combine the constituents in the following order and proportions:

Milli Q 7.75 ml

Stock A 1.08 ml

Stock B 0.20 ml

Stock C 0.07 ml

Stock D 0.10 ml

Stock E 0.80 ml

Osmolarity – 280 ± 5 (adjust with Milli Q if over, Stock A if under)

Filter (0.22 μm)

- Make up in T25 Nunc culture flask.
- Store in fridge for 1 week.

6.2.4 Hepes-SOFM without Calcium and Magnesium

- Measure and combine the constituents in the following order and proportions:

Milli Q 7.75 ml

Modified Stock A 1.08 ml

Stock B 0.20 ml

Stock C 0.07 ml

Stock E 0.80 ml

Osmolarity – 280 ± 5 (adjust with Milli Q if over, Stock A if under)

Filter (0.22 μm)

- Make up in T25 Nunc culture flask.
- Store in fridge for 1 week.

6.3 Ovine amino acid stock solution

- The following amino acids are weighed out individually:

Amino acid	Mass (g) per 2.5 ml stock
Arginine	0.0125
Aspartic acid	0.0019
Asparagine	0.0019
Alanine	0.0318
Cystine	0.0086
Glutamic acid	0.0053
Glutamine	0.0218
Glycine	0.0805
Histidine	0.0056
Isoleucine	0.0094
Leucine	0.0188
Lysine	0.0230
Methionine	0.0053
Ornithine	0.0019
Phenylalanine	0.0118

Proline	0.0041
Serine	0.0008
Taurine	0.0045
Threonine	0.0009
Tyrosine	0.0142
Valine	0.0226
Total stock volume	52.5 ml

- Arginine and Lysine (0.1 N HCl) and Cystine and Tyrosine (0.1 N NaOH) are made up in individual Falcon tubes so that they can be dissolved in their respective media.
- All other amino acids are combined in a Falcon tube and dissolved in SOF

6.4. Ovine fertilisation medium
- Measure and combine the constituents in the following order and proportions:

Sheep Serum	200 μl
Bicarb-SOFM (base media)	9.8 ml
Filter (0.22μm)	

- to be made the day before in vitro fertilisation.
- Place in the incubator (5% CO_2) to equilibrate overnight.

6.5 Ovine culture medium
- Measure and combine the constituents in the following order and proportions:

Bicarb-SOFM	9.265 ml
TRC amino acids	0.735 ml
BSA (fatty acid free)	0.04 g
Mix and Filter (0.22μm)	

- Place in the incubator (5% CO_2) to equilibrate overnight.
- To be made the day before in vitro culture.

7. Bovine embryo culture and handling media

7.1 Bovine synthetic oviduct fluid (SOF) stock solutions

7.1.1 Stock A
- Measure and combine the constituents in the following order and proportions:

NaCl	6.29 g
KCl	0.534 g
KH_2PO_4	0.162 g
$MgSO_4.7H_2O$	0.162 g
Milli Q	99.4 ml
Na Lactate	0.6 ml
Filter (0.22 μm)	

- Make up in glass bottle.
- Store for 3 months at 4°C.

7.1.2 Stock B
- Measure and combine the constituents in the following order and proportions:

	B.1	B.2
$NaHCO_3$	0.21 g	0.21 g
Phenol red	0.001 g	-
Milli Q	10 ml	10 ml

- Make up in 10 ml Conical Falcon tube.
- Store for 2 weeks at 4°C.

7.1.3 Stock C
- Measure and combine the constituents in the following order and proportions:

	C.1	C.2
Pyruvic acid	0.051 g	0.08 g
Milli Q	10 ml	10 ml

- Make each up in 10 ml Conical Falcon tube.
- Make up fresh on the day of use.

7.1.4 Stock D
- Measure and combine the constituents in the following order and proportions:

$CaCl_2.2H_2O$	1.31 g
Milli Q	50 ml
Filter (0.22 μm)	

- Make up in T25 Nunc culture flask.
- Store for 3 months in fridge.

7.1.5 Stock E
- Measure and combine the constituents in the following order and proportions:

Hepes (Na salt mwt. 260.3)	3.255 g
Phenol Red	0.005 g
Milli Q	50 ml
Adjust to pH 7.5 with 1N HCl after adding Milli Q	
Filter (0.22 μm)	

- Make up in T25 Nunc culture flask.
- Store 3 months in fridge.

7.2 Bovine synthetic oviduct fluid (SOF) Base Media

7.2.1 Bicarb SOFM
- Measure and combine the constituents in the following order and proportions:

Milli Q	38.75 ml
Stock A	5.2 ml
Stock B.1	5.0 ml
Stock C.2	0.50 ml
Stock D	0.50 ml
Osmolarity – 280 ± 5 (adjust with Milli Q if over, Stock A if under)	
Filter (0.22 μm)	

- Make up in T25 Nunc culture flask
- Store in fridge for 1 week.

7.2.2 Hepes-SOFM
- Measure and combine the constituents in the following order and proportions:

Milli Q	38.85 ml
Stock A	5.1 ml
Stock B.1	1.0 ml
Stock C.2	0.50 ml
Stock D	0.50 ml
Stock E	4.0 ml

Osmolarity – 280 ± 5 (adjust with Milli Q if over, Stock A if under)

Filter (0.22 µm)

- Make up in T25 Nunc culture flask.
- Store in fridge for 1 week.

7.3 Bovine fertilisation medium

- Measure and combine the constituents in the following order and proportions:

BSA	0.06 g
Bicarb-SOFM (base media)	10 ml
Pen Strep	200 µl
Filter (0.22µm)	

- To be made the day before in vitro fertilisation.
- Place in the incubator (5% CO_2) to equilibrate overnight.

7.4 Bovine sperm preparation medium

- Measure and combine the constituents in the following order and proportions:

BSA	0.05 g
Caffeine	0.005 g
Glutathione	0.003 g
Hepes-SOFM (base media)	10 ml
Filter (0.22µm)	
Heparin (sodium salt)	50 µl/10 ml(adjust to filtered volume)

- To be made the day before in vitro fertilisation.
- Place in the incubator (5% CO_2) to equilibrate overnight.

7.5 Bovine culture medium

- Measure and combine the constituents in the following order and proportions:

BSA (fatty acid free)	0.06 g
Bicarb-SOFM	10 ml
Myo Inositol	0.005 g
Essential Amino Acids (50x Stock)	300 µl
Non Essential Amino Acids (100x stock)	100 µl
Pen Strep	200 µl
Glutamine	10 µl
Mix and Filter (0.22µm)	

- Place in the incubator (5% CO_2) to equilibrate overnight.
- To be made the day before in vitro culture.

Appendix II: SCNT stock and working solutions

1. Culture media

1.1 Ovine culture medium
- Measure and combine the constituents in the following order and proportions:

Bicarb-SOFM	9.265 ml
Ovine amino acids	0.735 ml
BSA (fatty acid free)	0.06 g
Mix and Filter (0.22μm)	

- Stored for 1 week at 4°C.
- Place in the incubator (5% CO_2) to equilibrate overnight.

1.2 Zona-free Embryo Culture Media
- Measure and combine the constituents in the following order and proportions:

Bicarb-SOFM	9.265 ml
Ovine amino acids	0.735 ml
BSA (fatty acid free)	0.20 g
Mix and Filter (0.22μm)	

- Stored for 1 week at 4°C.
- Place in the incubator (5% CO_2) to equilibrate overnight.

2. Handling media

2.1 Embryo handling medium
- Measure and combine the constituents in the following order and proportions:

Hepes-SOFM	9.265 ml
Ovine amino acids	0.735 ml
BSA (fatty acid free)	0.06 g
Mix and Filter (0.22μm)	

- Stored for 1 week at 4°C.
- Place in the incubator (5% CO_2) to equilibrate overnight.

2.2 Zona-free embryo handling medium
- Measure and combine the constituents in the following order and proportions:

Hepes-SOFM	9.265 ml
Ovine amino acids	0.735 ml
BSA (fatty acid free)	0.20 g
Mix and Filter (0.22μm)	

- Stored for 1 week at 4°C.
- Place in the incubator (5% CO_2) to equilibrate overnight.

2. Fusion medium

2.1 Stock solutions:
- $MgSO_4$ (10 mM):
  0.0602 g $MgSO_4$ into 50 ml of MilliQ-water, filter (Store 3 months in fridge).

- $CaCl_2$ (5 mM):

0.0368 g CaCl$_2$ into 50 ml of MilliQ-water, filter (store 3 months in the fridge).

2.2 Working solution (0.3 M mannitol, 0.1 mM MgSO$_4$, 0.05 mM CaCl$_2$)

- For 100ml:
    1. Measure 1 ml of CaCl$_2$ stock into 100ml bottle.
    2. Measure 1 ml of MgSO$_4$ stock into same bottle.
    3. Add approx. 10-20 ml of MilliQ-water into the bottle.
    4. Weigh 5.466 g mannitol into 50 ml tube, add approx. 50 ml MilliQ-water, mix and add to the bottle.
    5. Top up to the 100 ml level with more water.
    6. Measure pH, adjust to 7.4-7.8 with 0.1 M Trizma-base stock (approx. 1 drop sufficient).
    7. Measure osmolarity, adjust to approx. 280mOsm by adding more water if necessary (re-measure pH).
    8. Filter (0.22 μm).
    9. Store for 1 month at 4°C.
    10. Before using always re-adjust the pH on the day.

3. Calcium-Ionophore

3.1 Stock solution (2 mM)
- Dissolve 995μL of DMSO into 1mg bottle of Calcium-Ionophore.
- Store at room temperature, tightly closed and sealed with Parafilm.

3.2 Working solution (10 μM)
- Add 5 μl of stock solution into 995 μl of protein free SOF-Hepes with Ca^{++} and Mg^{++} (See Appendix I).
- Use on same day.

4. 6-DMAP

4.1 Stock solution (100 mM)
- Weigh 16.32 mg of 6-DMAP and mix with 1 ml of protein free SOF-Hepes in a heat durable inert tube/container.
- Heat on hot water bath until completely dissolved.
- Aliquot into sterilised eppendorf tubes, 20 μl per tube (don't filter).
- Label and freeze, store at -20°C freezer.

4.2 Working solution (2 mM)
- Take a tube of stock solution from the freezer.
- Add 980 μl of the desired culture media into tube, mix well with vortex.
- Use on the same day.

5. Cytochalasin B
5.1 Stock solution (1 mg/ml)
- Dissolve contents of a 1 mg bottle into 1 ml of DMSO.
- Aliquot into sterilised Eppendorf tubes, 50 μl per tube.
- Label and freeze, store at -20°C freezer.
5.2 Working solution (7.5 μg/ml)
- Take one tube of stock solution from the freezer (store in the fridge and use until finished).
- Add 7.5 μl of stock solution into 1 ml of desired medium.

6. Nocodazole

6.1 Stock-solution (10 mM)
- Dissolve the contents of a 2 mg bottle into 664 µl of DMSO.
- Aliqot into sterilised Eppendorf tubes, 10 µl per tube.
- Label and freeze, store at -20°C.

6.2 Working solution (10 µM)
- Add 990 µl of equilibrated culture medium to stock solution tube, mix well.
- Add 50 µl of this secondary stock-solution into 450 µl of equilibrated culture medium.
- Use on the same day.

7. Hoechst 33342 (bisBenzimide) (Sigma B-2261)

7.1 Stock solution (1 mg/ml)
- Weigh 1 mg Hoechst 33342 and dissolve it into 1 ml of protein free SOF-Hepes.
- Aliquot into sterilised Eppendorf tubes, 10 µl per tube.
- Label and freeze, store at -20°C.

7.2 Working solution (10 µg/ml)
- Take one tube from the freezer.
- Measure 5 µl of stock solution into 495 µl of desire medium.
- Use on the same day.

8. Pronase

8.1 Stock solution (0.5%)
- Weigh 50 mg Pronase and dissolve into 1 ml of SOF-Hepes (no protein).
- Filter (0.22 µm)
- Aliquot into sterised Eppendorf tubes, 50 µl per tube.
- Label and freeze, store at -20°C.

8.2 Working solution (0.5%)
- Take one tube from freezer.
- Add 450 µl of desired medium.
- Can be stored for a few days in the fridge.

9. Hyaluronidase

9.1 Stock solution (2250-4500 U/ml)
- Weigh 3 mg of hyaluronidase (750-1500 units/mg solid) and dissolve into 1 ml of SOF-Hepes.
- Filter (0.22 µm)
- Aliquot into sterilised eppendorf tubes, 30 µl per tube.
- Label and freeze, store at -20°C.

9.2 Working solution (450-900 U/ml)
- Take one tube from the freezer.
- Add the cumulus cell-oocyte complexes in approx. 100 µl of the maturation medium into the tube.

10. Phytohemagglutinin PHA

10.1 Stock solution (2 mg/ml)
- Dissolve 2 mg vial into 1 ml of PBS (without protein).
- Aliquot into sterilised Eppendorf tubes, 20 µl per tube.
- Label and freeze, store at -20°C.

10.2 Work solution (200 µg/ml)
- Add 180 µl PBS into one tube of stock solution.
- Use on the same day.

11. Glycerol-Hoechst 33342 Stain/Fixative

11.1 Stock solution (1 mg/ml)
- Dissolve 1 mg Hoechst 33342 into 1 ml of MilliQ-water.
- Aliquot into Eppendorf-tubes, 100 µl per tube.
- Label and freeze, store at -20°C.

11.2 Working solution (10 µg/ml)
- Add 1 ml PBS into one tube of stock solution and vortex for 1 min.
- Change the contents of the tube into 15 ml tube.
- Add 9 ml of glycerol and vortex 3-4 min.
- Store in cold room, protect from light.
- Use for a month.

12. Embryo karyotyping working solutions

12.1 Hypotonic solution (1% sodium citrate)
- Disolve 0.03 g of sodium citrate in 3 ml of MilliQ water.
- Cool down in the fridge before use.
- Just before use pour into 4-well dish.

12.2 Fixative I (3:1 ethanol : acetic acid)
- Draw 3 ml of ethanol and 1 ml of acetic acid into 10 ml syringe with 18G needle attached.
- Draw 0.3 ml of ethanol and 0.1 ml of acetic acid into 1 ml syringe with 26G needle attached (needle tip cut blunt).
- Cool down in freezer before use.

12.3 Fixative II (4:3:1, ethanol : acetic acid : water)
- Draw 0.4 ml ethanol, 0.3 ml acetic acid and 0.1 ml water into 1 ml syringe with 26G needle attached (needle tip cut blunt).
- Cool down in freezer before use.

12.4 Giemsa stain (10% Geimsa in PBS)
- Measure 800 µl of PBS (pH 6.8) and 200 µl of Giemsa stain into Eppendorf tube
- Centrifuge at high speed for 3-5 min.
- Use only supernatant for staining.