IDENTIFICATION OF MOLECULAR MARKERS OF PREGNANCY SUCCESS FOR ASSISTED REPRODUCTION

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Abstract

Current infertility treatments are confounded by an inability to identify oocytes and embryos with the highest developmental potential to generate and sustain a pregnancy resulting in a live birth, reducing the efficiency of treatment cycles and resulting in low pregnancy success rates. Embryos have varying capacity to form a successful pregnancy and embryo developmental potential is particularly reliant on nuclear and cytoplasmic qualities of the oocyte from which it is derived. A biochemical marker of oocyte and therefore embryo developmental potential would improve pregnancy success rates following assisted reproductive technologies by optimising oocyte and embryo selection techniques. The communication between an oocyte and its surrounding cumulus cells is essential for growth, maturation and metabolic activity, and strengthens the rationale to utilise cumulus cells to assess oocyte quality and predict treatment outcomes and health parameters for women undergoing assisted reproduction.

The potential for cumulus cell gene expression to predict clinical embryo grade and pregnancy success was investigated in cumulus masses from single human oocytes which were fertilised and cultured individually. To make direct correlations between cumulus cell gene expression and treatment outcomes patients underwent single embryo culture and transfer. Gene expression was analysed in cumulus cells from independent oocytes that yielded a successful term pregnancy compared to those for which treatment failed and pregnancy was not established. Patient matched cumulus cell pairs were utilised to investigate a potential correlation between cumulus gene expression and clinical embryo grade. Cumulus cell gene expression was assessed using both a microarray platform for non-biased genome wide gene expression analyses and real-time RT-PCR assays focused on genes with known important functions related to oocyte maturation.

Real time RT-PCR analyses identified cumulus expressed genes which significantly correlated with pregnancy success following single embryo transfer. Specifically, cumulus cell *PTGS2*, *VCAN* and *GAS5* mRNA expression significantly ($p < 0.02$) correlated with establishment of a pregnancy resulting in a live birth, while *PTX3* mRNA expression showed a trend towards significance ($p = 0.066$). Additionally, cumulus cell levels of *VCAN*, *GREM1* and *PFKP* showed a significant correlation with birth weight in the patients who achieved pregnancy, indicating their role as potential predictors of health

outcomes for babies born from assisted reproduction. No significant differences were seen for other genes analysed in relation to pregnancy outcome or when gene expression was correlated with clinical embryo grade. The use of a microarray platform led to the identification of new genes, never before identified in the COC as markers of human oocyte quality and pregnancy success. The characterisation of *GAS5* and *PEPSINOGEN* transcripts in both human and murine follicular cells furthered the rationale for their potential as markers of oocyte quality and provided an understanding of the pattern of expression and hormonal regulation within human and murine ovarian cells. The expression of the *GAS5* transcript was confirmed by real time RT-PCR analysis and shown to be significantly correlated with live birth following initial identification by microarray experiments.

The findings demonstrate that expression of *VCAN*, *PTGS2*, *GAS5* and *PTX3* represent molecular markers of oocyte quality. The molecular markers identified in this study provide a unique tool to assess the relative potential of individual oocytes to achieve successful pregnancy from a pool of oocytes generated by one patient. In conjunction with embryo selection techniques of visual assessment and developmental milestones in culture the present biomarkers provide information to differentiate between embryos with similar appearance as viable or non-viable, to improve ART efficiency while decreasing multiple gestations and even extend to predict birth weight and hence general health expectancy for babies conceived following assisted reproduction.

Declaration

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Publications arising from this thesis

1. **GEBHARDT KM,** FEIL D, LANE M & RUSSELL DL. Cumulus Cell Gene Expression is a Biomarker of Pregnancy Success. *In preparation*.

Abstracts arising from this thesis

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1. **GEBHARDT KM,** FEIL D, LANE M & RUSSELL DL. Predicting Pregnancy Success Evaluation of Cumulus Cell Gene Expression Following Single Embryo Transfer. (2010). Annual meeting of The Fertility Society of Australia *Abs: 103.*

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2. **GEBHARDT KM,** FEIL D, LANE M & RUSSELL DL. Human cumulus cell gene expression as a biomarker of pregnancy outcome following single embryo transfer. (2009). 25th Annual meeting of The European Society for Human Reproduction and Embryology *Abs: O-078.*

3. RUSSELL DL, **GEBHARDT KM**, FEIL D, ROBKER RL & LANE M. Novel Cumulus Cell Biomarkers of an Oocyte's Potential to Achieve Pregnancy (2009) 42nd Annual Meeting for The Society for the Study of Reproduction *Abs 017624*.

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5. **GEBHARDT KM**. Are your eggs all they are cracked up to be? Picking the best egg for a successful pregnancy. Young Investigator Awards (Finalist), Adelaide, Australia.

6. **GEBHARDT KM**, FEIL D, LANE M, RUSSELL DL. Identification of Biomarkers for Pregnancy Outcome for IVF Patients. Australian Society for Medical Research Scientific Meeting, Adelaide, South Australia.

7. **GEBHARDT KM**, FEIL D, LANE M, RUSSELL DL. Human cumulus cell gene expression as a biomarker of pregnancy outcome following single embryo transfer. University of Adelaide, Faculty of Health Sciences Postgraduate Expo, Adelaide, Australia.

2008

8. **GEBHARDT KM**, FEIL D, LANE M, RUSSELL DL. Human cumulus cell gene expression as a marker of clinical embryo grade. Society for Reproductive Biology Annual Meeting, Melbourne, Australia (Podium Presentation).

9. **GEBHARDT KM**, FEIL D, LANE M, RUSSELL DL. Human cumulus cell gene expression as a marker of embryo quality. Australian Society for Medical Research, Scientific Meeting, Adelaide, Australia.

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Abbreviations

aMEM	Minimum Essential Medium alpha
Adamts1	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motifs
AHR	Aryl hydrocarbon receptor
AIHW	Australian Institute of Health and Welfare
ALDOA	Aldolase
ANOVA	Analysis of variance
AR	Androgen receptor
AREG	Amphiregulin
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BCL2L11	BCL2-like 11 apoptosis facilitator
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BMP	Bone morphogenetic protein
bp	Base pairs
BSA	Bovine serum albumin
CAMK4	Calcium/Calmodulin-dependent protein kinase 4
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CCND2	Cyclin D2
CDC42	Cell division cycle 42
cDNA	Complementary DNA
CEI	Cumulus expansion index
CL	Corpus luteum
cm	Centimetre
COC	Cumulus oocyte complex
CPB	Complement binding protein
COX2	Cyclooxygenase 2
CREB	cAMP regulatory element-binding protein
CS	Chondroitin sulphate
CT	Threshold cycle

CTNND1	Catenin delta-1
CX43	Connexin 43
CXCR4	Chemokine (C-X-C motif) receptor 4
CYP19A1	Cytochrome P450 aromatase
DET	Double embryo transfer
DEPC	Diethyl pyrocarbonate
DHCR7	7-dehydrocholesterol reductase
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DVL3	Dishevelled dish homolog 3
E2	Oestradiol
E. Coli	Escherichia coli
eCG	Equine chorionic gonadotropin
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid $[CH_2N(Ch_2CO_2H)_2]_2$
Egf	Epidermal growth factor
Egf-L	Egf-like peptide
EgfR	Egf receptor
ENOA	Alpha enolase
ErbB2	Erythroblastic leukemia viral oncogene homolog 2
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FDX1	Ferredoxin 1
FGF	Fibroblast growth factor
FIGα	Factor in the germline alpha
FSH	Follicle stimulating hormone
FSH-R	Follicle stimulating hormone receptor
g	Grams
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS5	Growth-arrest-specific transcript 5
GC	Granulosa cell
GDF	Growth differentiation factor
GLUT	Glucose transporter

GnRH	Gonadotrophin releasing hormone
GPX3	Glutathione peroxidise 3
GREM1	Gremlin 1
GV	Germinal vesicle
GVBD	Germinal vesicle break down
h	Hour
H_20	Water
HA	Hyaluronan
HAS2	Hyaluronan synthase 2
HC	Heavy chain
hCG	Human chorionic gonadotropin
HS	Heparin sulphate
HSD3β1	3-beta-hydroxysteroid dehydrogenase
HSPB1	Heatshock 27 kDa protein 1
IαI	Inter-α trypsin inhibitor
ICSI	Intra cytoplasmic sperm injection
INSL3	Insulin-like 3
i.p.	Intraperitoneal
IPA	Ingenuity pathways analysis
IU	International units
IUI	Intra uterine insemination
IVF	In vitro fertilisation
IVM	In vitro maturation
kDa	Kilodalton
KO	Knock out
L	Litre
LB	Luria broth
LDHA	Lactate dehydrogenase A-chain
LEFTY2	Left-right determination factor A
LGR8	Insl3 receptor
LH	Luteinising hormone
LHCGR	Luteinising hormone/choriogonadotropin receptor
LH-R	Luteinising hormone receptor
Lhcgr	Luteinising hormone/choriogonadotropin receptor
M	Mass

MAPK	Mitogen-activated protein kinase
mGC	Mural granulosa cells
$MgCl_2$	Magnesium chloride
MI	Metaphase I
MII	Metaphase II
Min	Minute
MIR202	MicroRNA 202
mL	Millilitre
mM	Millimolar
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
n	DNA/plasmid size (Base pairs)
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NFIB	Nuclear factor I/B
NPSU	National Perinatal Statistics Unit
nt	Nucleotide
ºC	Degrees Celsius
OHSS	Ovarian hyperstimulation syndrome
OI	Ovulation induction
OPU	Oocyte pick up
OSF	Oocyte secreted factor
p	Probability
PB	Polar body
PBS	Phosphate Buffered Saline
PCA	Principal components analysis
PCK1	Phosphoenolpyruvate carboxykinase 1
PCO	Polycystic ovary
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PDRG1	p53 and DNA damage regulated 1
PG	Prostaglandins
PGA	Pepsinogen
PGC	Progastricsin

PGD	Preimplantation genetic diagnosis
PGK1	Phosphoglycerate kinase 1
PFK L	Phosphofructokinase Liver
PFKM	Phosphofructokinase Muscle
PFKP	Phosphofructokinase Platelet
PKA	Protein kinase A
PKM2/PK	Pyruvate Kinase Muscle Variant 2
PR	Progesterone receptor
PRKO	Progesterone receptor knockout
Ptger2	Prostaglandin E receptor 2, subtype EP2
PTGS2	Prostaglandin-endoperoxide synthase 2
PTX3	Pentraxin 3
qPCR	Quantitative polymerase chain reaction
RGS2	Regulator of G-protein signalling 2
RHAMM	Receptor for HA-mediated motility
RhoA	Ras homolog gene family, member A
RMA	Robust multichip averaging
RNA	Ribonucleic acid
RNaseP	Ribonuclease P
RPA2	Replication protein A2 32 kDa
RPL19	Ribosomal protein L19
Rpm	Revolutions per minute
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SCD1	Stearoyl-coenzyme A desaturase 1
SCD5	Stearoyl-coenzyme A desaturase 5
S.E.M	Standard error of the mean
SERPINE2	Serine proteinase inhibitor clade E member 2
SET	Single embryo transfer
snoRNA/SNORD	Small nucleolar RNA
STAR	Steroid acute regulatory protein
STS	Steroid sulfatase
T	Testosterone
Taq	Thermus aquaticus
TBE	Tris/borate/EDTA

TCA	Tricarboxylic acid
TGFβ	Transforming growth factor β
TIAM1	T-cell lymphoma invasion and metastasis 1
TNFAIP6	Tumor necrosis factor alpha-induced protein 6
TPI	Triose phosphate isomerase
TRIM27	Tripartite motif-containing 27
TRIM28	Tripartite motif-containing 28
Tris	Tris(hydroxymethyl)aminomethane $(HOCH_2)_3CNH_2$
UV	Ultraviolet
V	Volts
VCAN	Versican
w/v	Weight/volume percentage solution
ZP	Zona pellucida

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Fertility is the natural capability of giving life, whereas fecundity is the potential for reproduction and is dependent on gamete production, fertilisation and carrying a pregnancy to term. Human fertility is dependent on many factors, such as lifestyle, behaviour, and endocrinology. The most notable sign of fertility is when a woman can achieve pregnancy. Conversely, this is also recognised as the most notable sign of infertility. Infertility refers to the biological inability of an individual to contribute to conception. There are many causes of infertility, and many causes can be bypassed by medical intervention. Assisted reproductiove technologies (ART) are a group of procedures used to assist women to become pregnant. ART children now account for an estimated 3.3% and 2.0% of children born in Australia and New Zealand respectively (Wang et al. 2010). The prevalence of these therapies is continually increasing; the rates of ART use have increased 9% since 2007 and 47.8% since 2004 (Wang et al. 2010). Approximately one in six couples in Australia will be faced with infertility, which is defined as a failure to conceive after one year of unprotected sex or an inability to carry a pregnancy to live birth, and tends to be attributed to male and female partners equally. Interestingly, while the use of these technologies is increasing as more people seek medical intervention in their quest to conceive, the clinical pregnancy rates following ART has remained stable at 22%. There is an immediate need to broaden our understanding of what initiates and promotes a successful pregnancy and how this can be implemented during infertility treatment to achieve more live births following ART.

The importance of the oocyte in establishing a healthy pregnancy cannot be emphasised enough. The factors which set one oocyte apart from another during follicular development, specifically the determinants of the developmental potential of individual oocytes, are continually being investigated. The growth and maturation of an oocyte is highly regulated and dependent on the supporting follicular cells and local and systemic signalling pathways. Disturbances to this maturation process can lead to follicle regression or removal of a degenerate follicle from a growing pool. Therefore, in a pool of growing follicles in a mono-ovulatory species such as the human, the single oocyte which is ovulated represents the oocyte with the greatest developmental potential, having manoeuvred a series of maturation stages and checkpoints. Following ovulation and successful fertilisation, the resultant embryo must also successfully navigate developmental milestones before implantation and subsequent foetal development. The success of this process is limited by the quality of the gametes from which the embryos were derived, specifically the oocyte. An understanding of how oocyte developmental potential is acquired during development will enable further improvement of reproductive medicine to increase pregnancy success rates and health outcomes. The ability for an embryo to establish a viable pregnancy is reflective of the follicular development and oocyte maturation prior to fertilisation (Figure

1.1). Therefore the assessment of oocyte health to predict pregnancy success has the potential to improve pregnancy success rates and health parameters for babies conceived by assisted reproduction.

During a single treatment cycle, hyperstimulation protocols mean each patient can generate a pool of oocytes available for fertilisation and subsequent embryo transfer. The inability to identify an oocyte (and embryo) with the greatest potential to achieve pregnancy is a key limitation of ART, and as such the development of additional technologies to fill this knowledge gap has the potential to improve the success rates of ART, meaning increased chances of achieving a pregnancy. As yet, no such markers with a strong correlation to live birth have been identified. While the oocyte itself is a key determinant of pregnancy success, it is not possible to investigate oocyte specific measures of quality as the oocyte cannot be manipulated or perturbed. The same applies for measures of embryo developmental potential. The next most obvious candidate is the cumulus cells which act as support cells for the oocyte. Cumulus cells represent the optimal tissue for assessing oocyte health, as they have a close, functional relationship to the oocyte during maturation to nurture the oocyte during its growth. Most conveniently, cumulus cells are seen as a by-product of ART, and are routinely discarded prior to fertilisation. A marker of cumulus cell health is an attractive non-invasive approach to evaluate oocyte developmental potential. This thesis will focus on how cumulus cell measures of oocyte quality can be used to predict pregnancy success. Ultimately, the ability to assess oocyte health and the likelihood of an oocyte to result in a successful pregnancy (subject to paternal input) has the capacity to significantly increase the success rates of ART and reduce the number of initiated treatment cycles (which also has a considerable burden on health care costs in Australia). For a couple faced with infertility, this is the most important outcome of a treatment cycle – getting pregnant and staying pregnant.

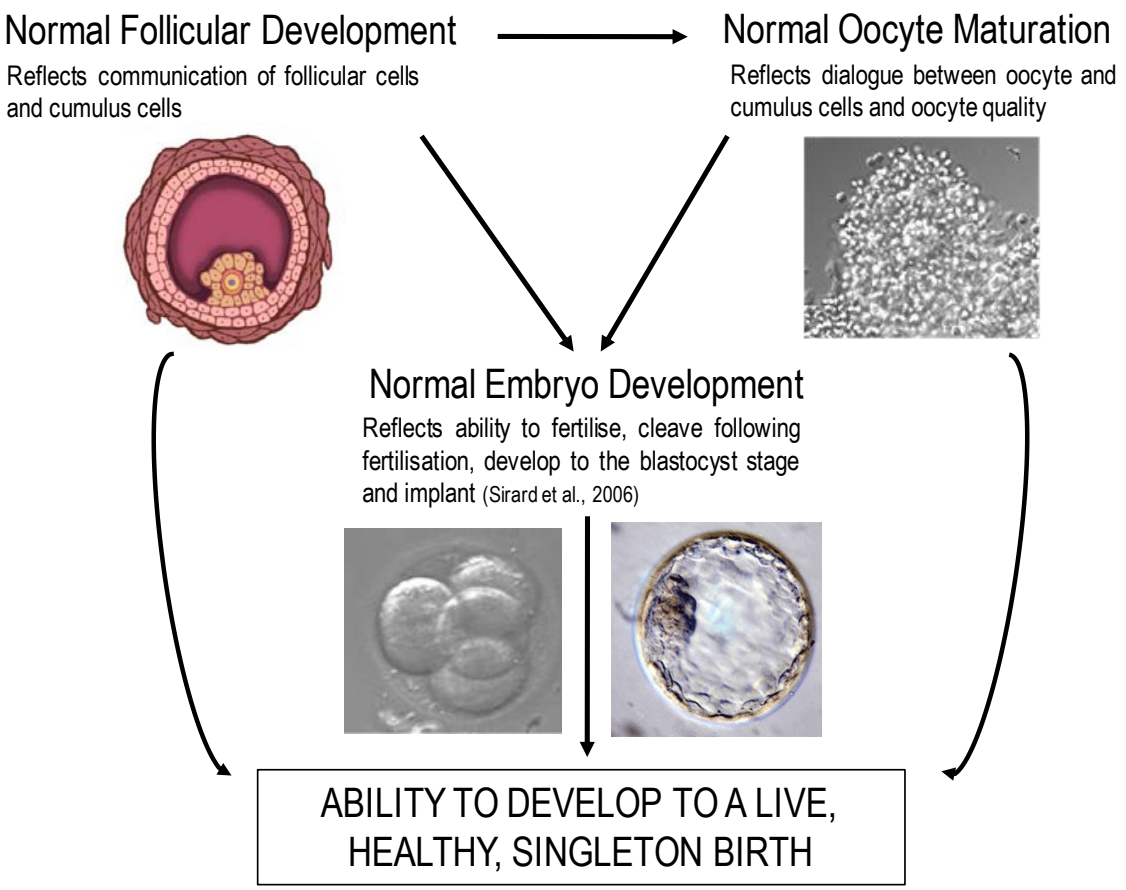

Figure 1.1 Pregnancy success is dependent on normal developmental processes occurring at several key stages of growth and development.

The ability for an embryo to establish a viable pregnancy is dependent on normal follicular development, normal oocyte maturation and normal embryo development. The ability to identify oocytes and embryos with this potential is a key limitation of assisted reproductive technology.

1.2 ASSISTED REPRODUCTIVE TECHNOLOGY

Infertility is a worldwide phenomenon, and since the first *in vitro* fertilisation (IVF) baby was born in 1978 following the pioneering work of Steptoe and Edwards (Steptoe and Edwards 1978), remarkable progress has been made in both the clinical and embryological aspects of assisted reproduction (Patrizio et al. 2007). ART is the application of laboratory or clinical technology to gametes and/or embryos for the purposes of reproduction. These technologies include IVF, intra cytoplasmic sperm injection (ICSI), preimplantation genetic diagnosis (PGD), ovulation induction (OI) and intra uterine insemination (IUI). In Australia, ART accounts for approximately 3.3% of births each year (Wang et al. 2010), a number which is continually rising. Female fertility is primarily dependent on the quality of the oocytes which make up women's ovarian reserve and how these oocytes then respond to various stimuli during development (specifically hormonal). Increased maternal age is the main compounding factor when assessing female fertility. A woman's highest reproductive capacity is between the ages of twenty to twenty four while after the age of thirty five a woman's reproductive capacity begins to decline and continues to worsen with advancing age (Menken et al. 1986; Wang et al. 2009a). Although the effect of age on fertility is well documented, the average age of women undergoing ART treatment using their own oocytes in 2008 was 35.7 years, slightly older than the average age (35.5 years) in 2007 (Wang et al. 2010). This is primarily due to a steadily rising age to first conception for mothers.

Data from the Centre for Disease Control in the United States detailing the percentage of live births per transfer in 2006 for women using their own oocytes reveals after the age of thirty two there is a decrease in the live birth rate which continues to decline until a woman in her mid-forties has only a 2.7% chance of achieving a live birth using her own oocytes (Figure 1.2). In comparison, women using donor oocytes, primarily from younger donors, can maintain a live birth rate between 50 – 55% well into their mid-forties (Toomey et al. 2008). The rates of live births are similar for women in Australia seeking fertility treatment with their own gametes, with a similar age related decline in the success of ART (Figure 1.3). As the population of women seeking infertility treatment is skewed towards older women, a population with a decreased success rate, the live birth rates following ART in Australia remain at approximately 22% (Wang et al. 2009a). The maintained birth rates for women using donor oocytes coupled with the decreased birth rates for older women using their own oocytes confirms there is something fundamentally important about the oocyte, and that the quality of the oocyte is essential to establishing a viable pregnancy. While it is clear of the role played by age, there is reason to investigate other underlying factors which contribute to fertility independent of age. In addition, the development of future technologies to treat infertility may lessen or even bypass the effects of maternal age on oocyte quality, to a point where we see a less dramatic age effect on the pregnancy success rates.

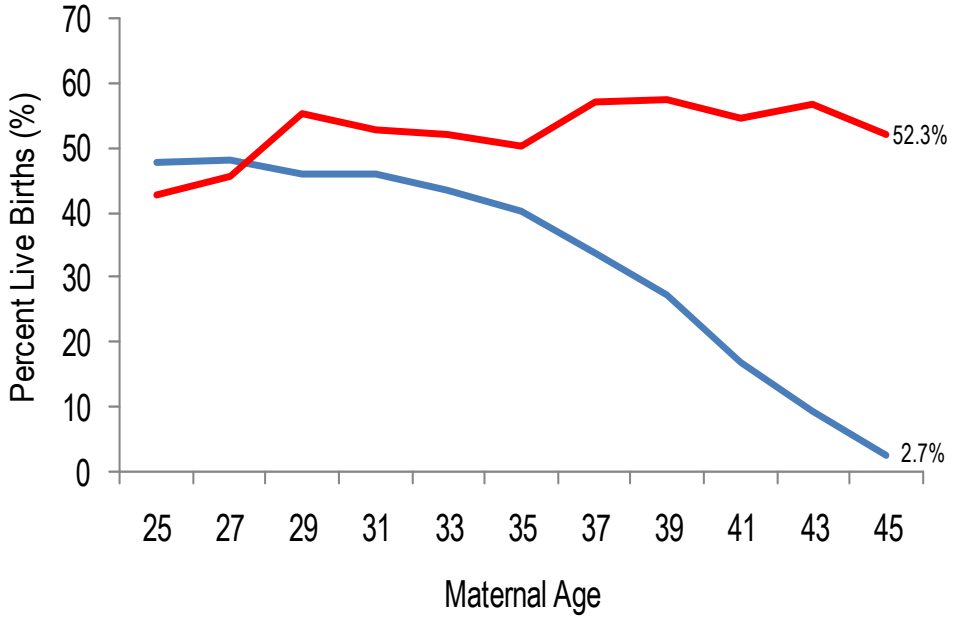

Figure 1.2 Percentages of transfers that resulted in live births for IVF/ICSI cycles using fresh embryos from own and donor oocytes, by ART maternal age, in 2006.

Percentage of transfers that resulted in live births for IVF/ICSI cycles using fresh embryos from own or donor oocytes, by women's age, 2006. The blue line represents women using their own oocytes, while the red line represents women using donor oocytes. The percentage of transfers resulting in live births for cycles using embryos from women's own oocytes declines as women get older. In contrast, donor oocytes are typically from young women, therefore the percentage of transfers resulting in live births for cycles using embryos from donor oocytes remained consistently high (red line). Data from United States of America Centre for Disease Control 2006 Assisted Reproductive Technology Success Rates National Summary and Fertility Clinic Reports.

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Figure 1.3 Age-specific live birth rates per initiated autologous fresh IVF/ICIS cycle in Australia and New Zealand in 2007.

Age-specific live delivery rates per initiated autologous fresh IVF/ICSI cycle by two-year age groups for Australia and New Zealand in 2007. The highest live delivery rates were for women aged between 23 and 30 years. The live delivery rate declined steadily for women older than 30 years. For women aged 45 years or older, only one live delivery resulted from every 100 initiated cycles. Data from Australian Institute of Health and Welfare (AIHW) National Perinatal Statistics Unit (NPSU) 2009 Assisted Reproductive Technology in Australia and New Zealand in 2007 Report (Wang et al. 2009a).

Paternal factors influenced by lifestyle, occupation or medical conditions affect ART outcomes, and must also be considered during a treatment cycle (Aitken et al. 2006). The treatment of infertility raises many challenges, from ethical and legal issues of treatment and oocyte or embryo donation, to the public funding of ART, and the allocation of funding for research into treatment options. The most prominent issues in ART are the low success rates, the rates of multiple gestations and the resultant preterm births and small birth weight babies. An inability to reliably select a high quality embryo for transfer means a continued reluctance on the part of the physician and patient to risk a single embryo transfer due to the high rate of failure, and as a result, multiple embryo transfers are common practise. The risks and ongoing poor prognoses for offspring health associated with multiple pregnancies continue unabated as clinics strive to maintain pregnancy success rates. Markers of oocyte developmental competence have the potential to alleviate many negative aspects associated with ART and improve treatment efficiency through the selection of the most developmentally competent oocytes and embryos.

1.2.1 Ovarian Hormonal Stimulation

Ovarian stimulation is performed in ART to induce the development of multiple dominant follicles and to generate a large pool of mature oocytes, and ultimately allow selection of good embryos for transfer. Ovarian stimulation is carried out with exogenous gonadotrophins, and to compensate for physiological ovarian changes induced by the stimulation protocols co-treatments including gonadotrophin releasing hormone (GnRH) analogs, oral contraceptives and luteal phase progesterone supplementation are commonly administered. The result of these approaches makes hormonal stimulation costly and complex. The superphysiological stimulation initiates multiple follicles to develop and disrupts the normal physiology of follicle development, and subsequent development of the oocyte, embryo and endometrium (Macklon et al. 2006). Ovarian stimulation causes a decrease and delay in the development of mouse one or two-cell embryos to blastocyst *in vitro*, and also delays the *in vivo* development of embryos (Ertzeid and Storeng 1992; Van der Auwera and D'Hooghe 2001). A serious consequence of ovarian stimulation is ovarian hyperstimulation syndrome (OHSS), which is a rare but life-threatening complication during controlled ovarian stimulation (Macklon et al. 2006). OHSS is characterised by ovarian enlargement, high serum sex steroids and extravascular fluid accumulation. Severe cases can result in hypotension, increased coagulability, reduced renal perfusion, severe morbidity and even mortality (Macklon et al. 2006). The aetiology of OHSS is linked with increased luteinising hormone, follicle stimulating hormone (FSH), human chorionic gonadotrophin (hCG), and oestradiol levels (Delvigne and Rozenberg 2002). The incidence of severe OHSS, as estimated by World Health Organization, is 0.2-1% of all stimulation cycles in assisted reproduction (Binder et al.

2007). Up to 80% of multiple births are considered to be due to ovarian stimulation and ART (Macklon et al. 2006). Of some concern is the lack of reliable data about the long term health risks associated with gonadotrophin administration, (Macklon et al. 2006); however ovarian stimulation remains a staple in assisted reproduction as it is necessary to generate a pool of embryos for selection for transfer. This represents an abnormal physiological process as multiple dominant follicles are matured, where normal cycles produce only a single dominant follicle, resulting in patients having a group of oocytes available for fertilisation and subsequently a number of embryos to be assessed for selection for transfer. With no appropriate quantitative tools to select an oocyte or subsequent embryo with the greatest developmental potential – and importantly a strong correlation to live birth rates - multiple embryo transfers are common practise, and often result in lower average health outcomes for babies born from ART. Markers of oocyte quality have the potential to transform clinical practises and reduce multiple embryo transfers while improving success rates.

1.2.2 Single *vs.* Multiple Embryo Transfer: Multiple Gestations

Maximising pregnancy rates by returning multiple embryos to the uterus improves ART treatment efficiency, but also dramatically increases the risk of multiple pregnancies. While Australia and New Zealand have relatively high single embryo transfer rates for younger patients, older patients are still much more likely to receive a double or triple embryo transfer (Figure 1.4). In the United States in 2006 nearly 43% of treatment cycles involved the transfer of three or more embryos (Figure 1.5). Approximately 16% of treatment cycles involved the transfer or four or more embryos, and approximately 5% of treatment cycles involved the transfer of five or more embryos. Nearly 40% of babies born following a double embryo transfer are multiple gestations, compared to only 4% following single embryo transfer (Wang et al. 2009b). The risk of a multiple gestation can be virtually eliminated when single embryo transfer is performed. Australian data comparing the pregnancy outcomes for single embryo transfer *vs.* double embryo transfer reveals replacement of one selected embryo to be as successful as double embryo transfer, without the risk of twin pregnancies (Kovacs et al. 2003; Wang et al. 2009b). Multiple pregnancies occurring after ART are associated with higher rates of preterm delivery, low birth-weight, congenital defects, infant death and disability (Wang et al. 2009b). The full health repercussions of multiple embryo transfers and resultant multiple gestations are only slowly beginning to emerge, nevertheless the excess rate of multiple pregnancies from ART has increased to epidemic levels and is entirely preventable through single embryo transfer (Gerris and Van Royen 2000; Davies 2005). For the health of both mother and baby, single embryo transfer is clearly the most beneficial approach. Single embryo transfers result in healthier babies following ART, but multiple embryo transfer remains a common practise to maintain pregnancy rates per ART treatment cycle, and

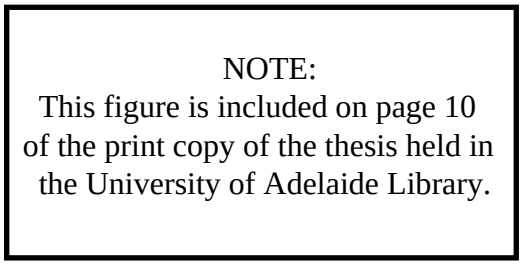

Figure 1.4 Proportion of fresh embryo transfer cycles by number of embryos transferred per cycle and women's age group, Australia and New Zealand, 2007.

Percentage of embryo transfer cycles by number of embryos transferred per cycle and women's age group in Australia and New Zealand in 2007. In general, women aged 35 years or older (grey bars) had more embryos transferred per cycle than those aged less than 35 years (red bars). Data from Australian Institute of Health and Welfare (AIHW) National Perinatal Statistics Unit (NPSU) 2009 Assisted Reproductive Technology in Australia and New Zealand in 2007 Report (Wang et al. 2009a).

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Figure 1.5 Number of embryos transferred during IVF/ICSI cycles, United States of America, 2006.

In the USA in 2006 approximately 43% of treatment cycles that used fresh non-donor oocytes or embryos and progressed to the embryo transfer stage involved the transfer of three or more embryos. Approximately 16% of treatment cycles involved the transfer of four or more embryos, and approximately 5% of treatment cycles involved the transfer of five or more embryos. *Total does not equal 100% due to rounding. Data from United States of America Centre for Disease Control 2006 Assisted Reproductive Technology Success Rates National Summary and Fertility Clinic Reports.

to reduce the number of initiated cycles per patient. Improvements in oocyte and embryo selection techniques have the capacity to reduce multiple embryo transfers and in turn the rates of multiple gestations, without compromise to success rates. The low success rates in establishing or maintaining a pregnancy after a single embryo transfer is compounded by a lack a biochemical, metabolic or other molecular marker(s), which can be used to select the most viable oocyte and resultant embryo from a cohort.

1.2.3 Single *vs.* Multiple Embryo Transfer: Legal and ethical issues

The law plays a significant role in the application of ART, as the laws of individual countries govern patient age limits, sexual orientation, marital status, as well as the number of oocytes allowed to be fertilised, embryo culture periods, number of embryos allowed to be transferred and embryo cryopreservation. Recent controversial law changes in Italy (Italian ART Law 40/2004, Republica Italianna, 2004) stated that no more than three oocytes may be fertilised at one time, and that all embryos obtained must be transferred regardless of their condition, which impacts the likelihood of a multiple gestation (Benagiano and Gianaroli 2004; Caserta et al. 2008). The choice to fertilise only three oocytes insinuates a decision with no biological relevance as there are no reliable markers of oocyte quality. Embryologists have only poor markers to gauge oocyte quality, including cumulus expansion and polar body extrusion which are poorly correlated with pregnancy success. There is controversy as to whether embryos should be cultured to the blastocyst stage for more accurate assessment of viability. The legal restrictions placed on the length of embryo culture in many countries limit an adequate assessment of embryo morphology. Pregnancy rates are higher when an embryo is cultured to the blastocyst stage, as extended culture of human embryos increases discrimination of potential embryonic viability (Rijnders and Jansen 1998; Graham et al. 2000). In comparison to countries with strict ART laws, there are only guidelines on the number of embryos which can be transferred in the United States. The consequence of no legal limit has been highlighted by the birth of octuplets in January 2009 in California (McClure 2009). The transfer of six embryos resulted in the live birth of eight babies causing worldwide media attention on both the strict and relaxed policies regarding the number of embryos transferred in a treatment cycle. In spite of the higher success rates, the reality of high order multiple gestations are the adverse health outcomes, and high neonatal mortality and morbidity rates. The overall consensus is that multiple gestations are a negative facet of infertility treatment; as such Australian clinics seek to perform more single embryo transfers where possible. Despite the knowledge that a single embryo transfer is the best approach, the rates of multiple embryo transfer are still between thirty and forty percent, especially for older patients in Australia and much higher in other jurisdictions [Figure 1.4] (Wang et al. 2009a). Improvements to oocyte and embryo culture and selection techniques

represent a major stepping stone to achieving more single embryo transfers. In countries where laws limit the number of oocytes which can be fertilised, or the number of embryos that can be transferred, robust markers of developmental potential are crucial to improving pregnancy success outcomes without compromising treatment quality.

1.2.4 Oocyte and embryo selection

Patients undergoing assisted reproduction routinely generate a pool of embryos following stimulated cycles. The transfer of multiple embryos is common due to difficulty in identifying a good quality embryo with the developmental potential to result in a live birth. The most widespread tool available for embryo selection is morphological appearance or on time development, both of which are weakly linked to pregnancy outcome (Rijnders and Jansen 1998; Graham et al. 2000; Guerif et al. 2007). Embryos considered to be of higher quality within a cohort are usually assessed to have 4 cells on day 2 of culture and 7–9 cells on day 3 of culture, with limited fragmentation and an absence of multinucleated blastomeres. Day 4 morula and blastocysts are assessed on the degree of compaction and early cavitation (Feil et al. 2008). Blastocyst assessment on day 5 utilises previously published and highly regarded criteria (Gardner and Schoolcraft 1999) based on formation of the two cell types in the blastocyst, the inner cell mass or trophectoderm. Use of morphological assessment for embryo selection for transfer has greater success in eliminating poor quality embryos that fail to keep on-time development rates or have high degrees of fragmentation, rather than selecting for developmental potential among morphologically similar embryos. The number of embryos to be transferred can be determined by the clinician prior to treatment starting, and therefore doesn't reflect the number or quality of embryos generated per cycle. It is also acknowledged that selecting embryos for transfer based on this scoring system is flawed, and that not all high grade embryos will generate a successful pregnancy and some patients will only generate poor morphology embryos which may in fact generate a successful pregnancy resulting in a live birth.

An additional constraint of the embryo grading system is that it is highly subjective as there is no standardised scoring system – with a high level of intra- and inter-clinic variations. The embryo grading system remains the most common tool available for embryo selection with any correlation to pregnancy success – even if that correlation is weak. The scoring system is predictive to some degree, and remains a quick, simple and inexpensive technique that will always remain a part of ART procedures. However, numerous studies in animal models have shown definitively that it is the health of the embryo that is important, as many embryos can appear "morphologically normal" but can be

metabolically/molecularly unviable. Therefore, embryo selection, with the goal of achieving a successful live birth, should be based on embryo health and knowledge of what makes a good embryo – that is physiologically relevant markers of embryo health. The improvements in pregnancy rates and decreases in multiple births should be natural outcomes from the understanding of what makes a "healthy" embryo. The limiting factor in embryo quality is the oocyte it was derived from (Cha and Chian 1998; Gardner and Schoolcraft 1998; Ebner et al. 2003b; McKenzie et al. 2004). This highlights the need for the development of oocyte selection techniques in addition to current embryo selection tools. Understanding oocyte physiology, and what factors contribute to oocyte health and in turn embryo quality, will help improve outcomes for ART patients.

1.3 DEVELOPMENT OF THE OVARIAN FOLLICLE

The two major functions of the ovary are the production of oocytes and the generation of bioactive molecules, primarily steroids (mainly oestrogens and progestins) and peptide growth factors, which are critical for ovarian function, regulation of the hypothalamic-pituitary-ovarian axis, and development of secondary sex characteristics (Edson et al. 2009). The ovarian follicle is the fundamental reproductive unit of the ovary. The ovarian follicle contains an oocyte and the somatic cells of the ovarian follicle, the granulosa cells that function as the ovarian "nurse" cells (Edson et al. 2009) and thecal cells that function to supply the granulosa cells with the estrogenic precursor, androstenedione. Before formation of an ovarian follicle, oocytes are present within germ cell clusters. Primordial follicle formation occurs when oocytes which survive the process of germ cell cluster breakdown are individually surrounded by a single layer of squamous pre-granulosa cells. This represents the first stage of folliculogenesis. The continued growth and development of the somatic and germ cell compartments of the ovarian follicle occur in a highly coordinated and mutually dependent manner (Gilchrist et al. 2004; Edson et al. 2009).In mammals, the population of primordial follicles serves as a resting and finite pool of oocytes available during the female reproductive life span (Edson et al. 2009). Follicle recruitment occurs through the initial activation of primordial follicles which involves the stimulation of granulosa cells to resume meiosis and assume a cuboidal shape (Fair 2010). Throughout a females' reproductive lifespan a limited number of small primordial follicles are recruited in cycles from the growing cohort, from which a subset is selected for dominance and ovulation (McGee and Hsueh 2000). There are a number of critical transcription factors during early folliculogenesis, some of which are outlined in Figure 1.6. In mice, the transcriptome of the primordial oocyte has been shown to be unique to that of oocytes obtained from subsequent stages of follicle development (Pan et al. 2005). The transition from

primordial to primary follicle showed the greatest change in the oocyte transcriptome, which was characterised by the up-regulation in the expression of members of the transforming growth factor (TGF)-β superfamily and other growth factors (Pan et al. 2005). These factors have known stimulatory effects on the primordial to primary follicle transition, which supports the hypothesis that the oocyte itself plays an important role in driving follicle activation (Fair 2010).

1.3.1 Folliculogenesis

Autocrine, paracrine, juxtacrine and endocrine factors are essential for ovarian folliculogenesis (Edson et al. 2009). Folliculogenesis occurs in response to hormonal cues which recruit follicles within the ovary to mature, and requires the expression of key genes at various stages of development, which are under the control of hormones and oocyte paracrine signals. Before birth, the cortex of the female ovary contains its peak number of follicles (about seven million), and these primordial follicles contain immature oocytes surrounded by flat, squamous granulosa cells. These oocytes are relatively quiescent, showing little biological activity (Fortune et al. 2000). The flattened layer of squamous pre-granulosa cells are activated and undergo transition to cuboidal granulosa cells, marking the beginning of the primary follicle (Hirshfield 1991; Fortune et al. 2000; Picton and Gosden 2000; Skinner 2005), activation of the oocyte genome and establishment of paracrine signalling pathways for communication between the follicle and oocyte, to support both oocyte and the follicle growth (Fortune et al. 2000). Mitotic activity is high as the follicle transitions to the secondary stage and more layers of granulosa cells are generated. Preantral folliculogenesis is characterised by oocyte growth, granulosa cell proliferation, and acquisition of the theca cell layer. Secondary follicles contain oocytes in mid-growth stages surrounded by two or more layers of granulosa cells (Edson et al. 2009). An intricate network of capillary vessels forms among the thecal layers and circulates blood to and from the follicle. The preantral follicle is marked by a fully grown oocyte surrounded by a thick zona pellucida, multiple layers of granulosa cells, a basal lamina, a theca interna, a capillary network, and a theca externa (Fortune et al. 2000). Growth of preantral follicles is dependent on autocrine and paracrine factors but appears to be gonadotropin-independent (Edson et al. 2009). During preantral folliculogenesis the complex bidirectional communication between the oocyte and the somatic compartments of the follicle become more evident. Although the oocyte relies on the surrounding somatic cells to support its growth and development, the rate of follicular development is dependent on the oocyte. Although the mechanism by which the oocyte coordinates development is not known, oocyte-secreted factors appear to have a critical role. The first oocyte-derived growth factor identified to be essential for somatic cell function *in vivo* was GDF9, which is first expressed in oocytes of primary follicles with continual expression until after ovulation (McGrath et al. 1995; Elvin et al. 1999a). Additionally, the oocyte-secreted TGFβ

superfamily member BMP15 has an mRNA expression pattern identical to Gdf9 in mouse oocytes, but is not critical to preantral folliculogenesis.

In addition to the many paracrine factors involved in the communication between the oocyte and somatic cells of a developing ovarian follicle direct communication via intercellular membrane gap junction channels are also essential for normal folliculogenesis to occur (Edson et al. 2009). Gap junctions allow the transfer of ions, metabolites, and small molecules between neighbouring cells (detailed below in section 1.4.2). The formation of a fluid-filled cavity adjacent to the oocyte, the antrum, marks the next stage of folliculogenesis to the antral or graafian stage and separates the two functionally distinct granulosa cell populations. In the mature antral follicle, specialised subpopulations of granulosa cells known as cumulus cells encapsulate the oocyte while the newly formed mural granulosa cells line the wall of the follicle and are critical for steroidogenesis and ovulation. Each oocyte is surrounded by several layers of cumulus cells (Salustri et al. 1992), which remain in a close, functional relationship with their enclosed oocyte during follicular development and ovulation, and act as mediators of oocyte paracrine signalling and developmental potential (Matzuk and Lamb 2002). The two cell types appear to be defined by opposing gradients of FSH from outside the follicle and paracrine factors from oocytes (Eppig et al. 2002; Diaz et al. 2007). During the transition from preantral to antral follicle, the hypothalamic-pituitary-gonadal (HPG) axis starts functioning as regulation of folliculogenesis becomes extraovarian (Edson et al. 2009). FSH becomes essential to maintain folliculogenesis (Kumar et al. 1997) as it prevents granulosa cell apoptosis and follicular atresia (Chun et al. 1996) and promotes granulosa cell proliferation, oestradiol production, and LH receptor expression (Richards 1994). FSH and LH are the pituitary gonadotropins that coordinate antral follicle development and ovulation. During antral follicle growth, LH acts on the LH receptor on thecal cells stimulating production of androgens, which are diffused to the granulosa cells and converted to oestrogen. FSH is required for granulosa cell oestrogen production through control of the aromatase gene. The FSH receptor, located on granulosa cells, is a G-protein coupled receptor, which activates over one hundred target genes upon binding (Grieshaber et al. 2003; Sasson et al. 2003).

While a number of follicles are recruited into the pool of growing follicles, a majority will undergo atresia and only a select few antral follicles in a developing cohort will reach the preovulatory stage. The follicles which do survive to this stage were most likely more responsive (because of higher FSH receptor expression) to decreasing serum FSH that occurs through negative feedback of oestradiol and inhibin on the pituitary (Edson et al. 2009). Preovulatory follicles express luteinising

NOTE:
This figure is included on page 17
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1.6 Folliculogenesis and classification of growing follicles in the human ovary.

Schematic of the ovary depicting the sequential events required for the origin, growth and ovulation of mature follicles. The schematic includes the key proteins which function at important stages of follicle formation, folliculogenesis, ovulation, and post-ovulatory events. *FIGα* is required for primordial follicle formation, and several proteins are needed for oocyte and granulosa cell growth and differentiation, ovulation, and the integrity of the cumulus oocyte complex. The proteins required to promote the dynamic growth and maturation of follicles and support the biological processes involved are listed. Schematic adapted from (Matzuk and Lamb 2002).

hormone/choriogonadotropin receptor (LHCGR) at high concentration in granulosa cells, enabling them to respond to the LH surge, which initiates a cascade of events leading to oocyte meiotic resumption, cumulus expansion, follicle rupture, and differentiation of the remaining granulosa and thecal cells to form the corpus luteum (CL) (Edson et al. 2009). The CL is a transient endocrine gland that produces the steroid hormone progesterone, which is required for the establishment and maintenance of early pregnancy (Wood and Strauss 2002; Havelock et al. 2004; Johnson 2007). Figure 1.6 depicts the classification of the major stages of mammalian folliculogenesis. From the primordial up to the secondary follicle stage the growth occurs in a gonadotropin-independent manner, whereas following the formation of the antrum through to ovulation, follicle growth is gonadotropin-dependent.

Expansion of the cumulus cells on a hyaluronan-rich extracellular matrix surrounding the oocyte is initiated by the LH surge and is required for normal ovulation and fertilisation. Regulation of this process is dependent on Mitogen-activated protein kinase (MAPK) signalling (Su et al. 2002; Fan et al. 2009), as well as oocyte-secreted paracrine factors (Buccione et al. 1990b). After the LH surge, a number of genes involved in the formation and stabilisation of the extracellular matrix of the cumulus oocyte complex are up-regulated (Richards 2005). A number of significant genes required for production and stability of the cumulus cell extracellular matrix and eventual ovulation are detailed below in section 1.6. The ovulatory cascade results in the release of a viable oocyte from the dominant follicle. For this to occur, LH acts on the granulosa cells to promote a cascade of gene expression changes and events, including the rapid expansion of the cumulus matrix, loss of gap junctional communication and the production of an extracellular matrix around cumulus cells and the oocyte, described further below [Reviewed in (Richards et al. 2002; Russell and Robker 2007)].

1.3.2 Cellular and molecular cascade of events during oocyte maturation

A cascade of complex events is necessary for successful ovulation to occur, initiated by a single trigger; the surge of gonadotrophins from the pituitary, specifically LH and FSH. The LH surge initiates the ovulatory cascade within the ovarian follicle, which converge on the COC (Figure 1.8). Comprehensive changes to the follicular structure and gene expression occur with overlapping control and interdependent consequences in the theca, granulosa, cumulus and oocyte compartments of the follicle (Russell and Robker 2007). The direct response to the ovulatory LH surge is predominantly in mural granulosa cells due to greater receptor levels, and it triggers the activity of multiple intracellular signalling pathways which ultimately mediates the expression of ovulatory genes. The LH receptor (LH-R) activates adenylate cyclise resulting in a large intracellular cyclic adenosine monophosphate (cAMP)

increase that activates the cAMP-dependent serine kinase protein kinase A (PKA) (Russell and Robker 2007). Downstream of PKA, the cAMP regulatory element-binding protein (CREB) is phosphorylated and recruits a transcriptional activator, CBP/p300 (Arias et al. 1994). Follicular cyclic guanosine monophosphate (cGMP) levels are increased during the process of ovulation, which has effects on granulosa cell gene expression, steroidogenesis and oocyte maturation (LaPolt et al. 2003). Induction of signalling pathways by the LH surge rapidly modify the transcriptional capacity of granulosa cells. Modification of transcriptional regulators reprogram gene expression which in turn mediates effector gene expression (Russell and Robker 2007). The cascade of periovulatory gene expression in mural granulosa cells involves the induction and recruitment of a set of transcription factors that subsequently induce effector gene products. These ovulatory genes display a signature pattern of mRNA and protein expression; a rapid, transient increase after the LH surge followed by down-regulation (Russell and Robker 2007).

The cumulus cells have a differing gene expression profile to the granulosa cells, and the cumulus cells are most directly exposed to mitogenic factors secreted by the oocyte (Erickson and Shimasaki 2000) allowing them to act as a means for the transduction of ovulatory signals. Following the LH surge cumulus cells react with a distinctive pattern of gene expression leading to production and stabilisation of a hyaluronan rich extracellular matrix that surrounds the COC. This process of mucification is crucial to ovulation (Chen et al. 1993; Hess et al. 1999; Fulop et al. 2003; Salustri et al. 2004) and subsequent fertility success (Tanghe et al. 2003; Somfai et al. 2004; Yang et al. 2005). The signalling cascade within the ovary that mediates ovulation of a mature cumulus oocyte complex is depicted in Figure 1.7. The LH *in vivo* surge initiates the ovulatory cascade within the ovarian follicle, resulting in dynamic changes to the COC resulting in ovulation. LH acts on thecal cells to induce Insl-3 and IL-1 secretion. In mural granulosa layers, Egf-Like factors: (amphiregulin, epiregulin and betacellulin) are produced and communicate the ovulatory signal to the cumulus cells. FSH action occurs through receptors expressed by mural granulosa and cumulus cells. Within the COC, GDF-9 and BMP-15 from the oocyte mediate paracrine effects on cumulus cells, initiating changes in cumulus cell gene expression. The set of genes expressed in periovulatory cumulus cells are critical for normal rates of ovulation and fertility. Disrupted expression of ovulatory genes results in perturbed oocyte maturation and ovulation failure. The expression patterns of ovulatory genes have been proposed as cumulus cell markers of oocyte quality, and the functional roles of these ovulatory genes and the significant effect to fertility following disruption of these genes are described in detail further below.

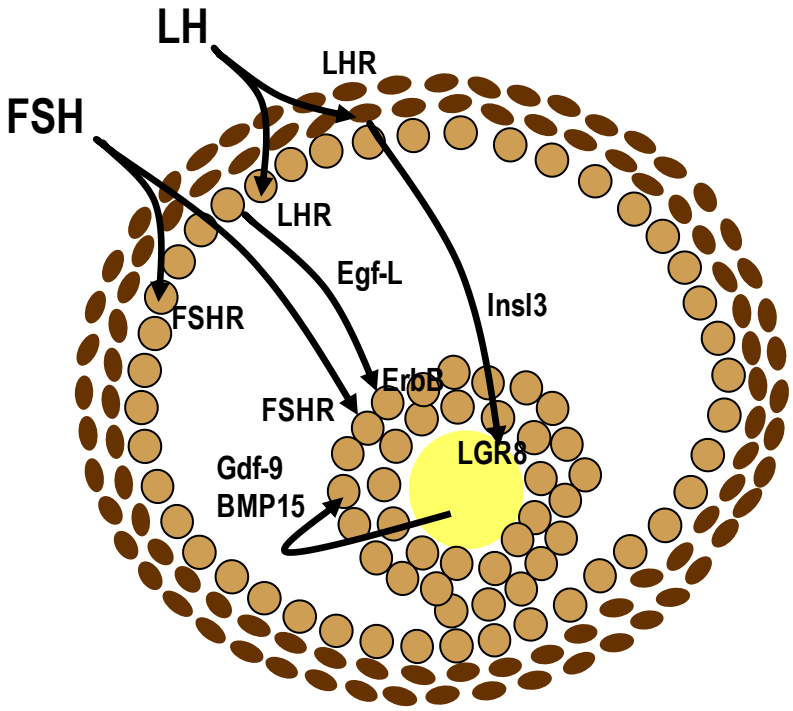

Figure 1.7 Signalling cascade within the ovary that mediates ovulation of a mature cumulus oocyte complex.

The LH *in vivo* surge initiates the ovulatory cascade within the ovarian follicle, resulting in dynamic changes to the COC resulting in ovulation. LH acts on thecal cells to induce Insl-3 and IL-1 secretion. In mural granulosa layers, Egf-Like factors: (amphiregulin, epiregulin and betacellulin) are produced and communicate the ovulatory signal to the cumulus cells. FSH action occurs through receptors expressed by mural granulosa and cumulus cells. Within the COC, GDF-9 and BMP-15 from the oocyte mediate paracrine effects on cumulus cells, initiating changes in cumulus cell gene expression. Prostaglandin E2 (PGE_2) acts in an autocrine manner via the PGE_2 receptor (EP_2). LH-R, luteinising hormone receptor; FSH-R, follicle-stimulating hormone receptor; ErbB, Egf receptor family; LGR8, Insl3 receptor; Schematic adapted from (Russell and Robker 2007).

1.3.3 Ovulation

Ovulation occurs when a mature ovarian follicle ruptures and releases an oocyte. Through a signal transduction cascade initiated by LH, proteolytic enzymes are secreted by the follicle which degrades the follicular tissue, forming a hole in the apex of the follicle called the stigma. The cumulus-oocyte complex leaves the ruptured follicle and moves out into the peritoneal cavity through the stigma, where it is caught by the fimbriae at the end of the fallopian tube (also called the oviduct). After entering the oviduct, the cumulus oocyte complex is pushed along by cilia as it travels toward the uterus. By this time, the oocyte has completed meiosis I, yielding two cells: the larger secondary oocyte that contains all of the cytoplasmic material and a smaller, inactive first polar body. Meiosis II follows at once but will be arrested in the metaphase and will so remain until fertilisation. The spindle apparatus of the second meiotic division appears at the time of ovulation. If no fertilisation occurs, the oocyte will degenerate approximately twenty-four hours after ovulation. Ovulation represents the culmination of a complex series of events known as folliculogenesis and the ovulatory cascade. Successful ovulation requires that a developmentally competent oocyte is released from the dominant ovarian follicle with appropriate timing (Russell and Robker 2007). This event is critical to fertility. Ovulation is fundamental to female fertility and the establishment of a successful pregnancy, and can also impact on the developmental potential of resultant embryos [Reviewed by (Russell and Robker 2007)].

1.4 FUNCTIONS OF THE OVARIAN FOLLICLE

1.4.1 Steroidogenesis

Steroidogenesis is vital to maintain functioning granulosa cells and correct hormone support, particularly progesterone and oestradiol, is fundamental to the development of the COC (Lucidi et al. 2003). Steroidogenesis in mammals occurs via the two cell/two gonadotrophin model in which androgens are synthesised from cholesterol in LH-stimulated theca cells, and converted to oestrogen in FSH-exposed granulosa cells (Leung and Armstrong 1980). Testosterone (T) functions in the ovary as the precursor for oestradiol (E2) biosynthesis (Walters et al. 2008). The levels of androgen, oestrogen and progesterone rise during ovulation, and oestrogen and progesterone levels increase even higher during the luteal phase to support early pregnancy. Androgen-activated androgen receptor (*AR*) stimulates the expression of target genes through a sequence of processes including ligand binding, homodimerisation, nuclear translocation, DNA binding, and complex formation with co-regulators and general transcription factors (Quigley et al. 1995). Follicle growth *in vitro* can be stimulated by androgens, including testosterone, androstenedione, and dihydrotestosterone, providing further

evidence for the role of androgens in female reproductive physiology (Murray et al. 1998; Wang et al. 2001). Hormones such as progesterone and oestrogen also play a role in follicular development and pregnancy, and progesterone is an essential mediator of ovulation (Robker et al. 2000; Chaffin and Stouffer 2002). The progesterone receptor (*PR*), a nuclear receptor transcription factor, is induced in granulosa cells of preovulatory follicles in response to the LH surge and is essential for ovulation, as *PR* knockout mice fail to ovulate (Robker et al. 2000). Cumulus cell steroidogenesis has been correlated with oocyte developmental competence, as mature oocytes are able to direct cumulus cell steroidogenesis by inhibiting E2 production *in vitro* (Lucidi et al. 2003). Steroidogenesis is vital to maintain functioning somatic cells and the development of the COC, and as such analysis of the steroidogenic activity of the follicular cells may provide a useful tool for assessing oocyte quality.

1.4.2 Oocyte Paracrine Signalling

The cumulus cells are attached to the oocyte and to each other via gap junctions, allowing cell-cell communication and mediating the transfer of small molecules (<1-2kDa) such as metabolites, ions and second messenger molecules which are critical for development and fertility (Buccione et al. 1990a; Motta et al. 1994; Simon et al. 1997; Sugiura et al. 2005; Gershon et al. 2008). Gap junctions also facilitate the transfer of amino acids, glucose metabolites and nucleotides to the growing oocyte (Eppig 1991a). Gap junctions between the oocyte and cumulus cells require trans-zonal cumulus cell projections that pass through the zona pellucida to interact with the oocyte plasma membrane (Albertini et al. 2001). The gap junctional communication between the oocyte and cumulus cells is an essential component of oocyte development and maturation, mediating oocyte paracrine signals to the cumulus cells. Locally acting paracrine factors exert important effects which influence the growth and differentiation of oocytes and cumulus cells, particularly in the activation of dormant primordial follicles and the selection of secondary follicles (Albertini et al. 2001). Paracrine signals produced by the oocyte include members of the TGF-β growth factor family growth differentiation factor-9 (GDF-9) and bone morphogenetic protein 15 (BMP-15) which act on neighbouring follicular cells to regulate a broad range of granulosa cell and cumulus cell functions, including differentiation and gene expression. The oocyte acts as a central regulator of follicular cell function, and is essential in the regulation of ovulation rate and fecundity [Reviewed by (Gilchrist et al. 2008)]. A number of genetic determinants of high fecundity in sheep have been shown to be due to polymorphisms of *Gdf-9* and *Bmp-15*, demonstrating the role of these oocyte secreted factors in follicle development and ovulation rate (McNatty et al. 2005). GDF-9 is the first oocyte-secreted paracrine factor shown to be required for ovarian somatic cell function, and regulates the expression of somatic cell genes during folliculogenesis, including cumulus cell expression of *Prostaglandin-endoperoxide synthase 2* (*Ptgs2*), *Hyaluronan Synthase 2* (*Has2*), *Pentraxin 3* (*Ptx3)*

and *Tumour necrosis factor alpha induced protein 6* (*Tnfaip6*) (Dong et al. 1996; Elvin et al. 1999a; Elvin et al. 1999b; Elvin et al. 2000; Vitt and Hsueh 2001; Yan et al. 2001; Varani et al. 2002; Pangas and Matzuk 2004; Dragovic et al. 2007). GDF-9 regulates multiple key granulosa cell enzymes involved in cumulus matrix expansion and promotes the acquisition of oocyte developmental competence (Pangas and Matzuk 2005), suggesting the expression levels of GDF-9 downstream target genes in the cumulus cells may reflect GDF-9 activity and predict oocyte quality (McKenzie et al. 2004). Mutation or knockout of the *Gdf-9* gene prevents development of follicles beyond the primary follicle stage and induces defects in oocyte meiotic competence in mice (Elvin et al. 1999b). In sheep, heterozygous mutations in the *Bmp-15* gene increase ovulation and lambing rates (McNatty et al. 2005). A decreased level of *GDF-9* signal was observed in developing polycystic ovary syndrome (PCOS) and polycystic ovary (PCO) oocytes compared with normal oocytes, suggesting that a dysregulation of oocyte *GDF-9* expression may contribute to abnormal folliculogenesis in PCOS and PCO women (Teixeira Filho et al. 2002). The *BMP-15* gene is equally critical to folliculogenesis and oocyte developmental competence, as it maintains the integrity of the COC (Dube et al. 1998; Yan et al. 2001). Knockout of the *Bmp-15* gene results in decreased ovulation and fertilisation rates in mice (Yan et al. 2001), compared to sheep in which a *Bmp-15* heterozygous mutation increases ovulation rates while homozygous mutants are infertile (McNatty et al. 2005).

Gremlin 1 (*GREM1*) is a BMP antagonist (Hsu et al. 1998) which is up-regulated in cumulus cells during ovulation (Pangas et al. 2004). A distinct change in *Grem1* mRNA compartmentalisation is seen during follicle development and ovulation, indicating a highly regulated expression pattern. Cumulus expression of *Grem1* is regulated by GDF-9 in the mouse (Elvin et al. 1999a; Pangas et al. 2004), representing a mechanism whereby cumulus gene expression may reflect oocyte function and paracrine signalling ability (Anderson et al. 2009b). It has been proposed that *Grem1* mediates the crosstalk between the GDF-9 and BMP signalling pathways necessary during follicle development, (Pangas et al. 2004). The COC partakes in bidirectional communication which sees synergistic signalling between the oocyte and surrounding cumulus cells. Therefore, measures of oocyte paracrine signalling within somatic cells, specifically the cumulus cells which are in direct physical contact with the oocyte, represent potential markers of oocyte developmental competence. The ability to assess downstream targets of oocyte paracrine signalling, such as GDF-9 and BMP-15 target genes, represent potential molecular markers of oocyte quality.

1.5 THE CUMULUS OOCYTE COMPLEX

The ovarian follicle contains several different cell types whose metabolism and function must be precisely controlled during the hours preceding ovulation (Gilula et al. 1978). Within the ovarian follicle lies the cumulus oocyte complex, the critical component of female fertility. The cumulus cells are, by definition, the cells with the closest physical association to the oocyte, and are able to interact with the oocyte via gap junctions and oocyte secreted paracrine signals, which have been detailed above. It is the close functional relationship and interaction with the oocyte which makes cumulus cells ideal for assessing the potential of their enclosed oocyte. In response to the LH surge, the COC undergoes maturation and cumulus expansion, which is required for ovulation and fertilisation (Russell and Robker 2007). In mice, if cumulus expansion fails to occur ovulation is defective and results in infertility (Lim et al. 1997; Davis et al. 1999; Varani et al. 2002; Fulop et al. 2003; Ochsner et al. 2003a; Salustri et al. 2004). The hyaluronan (HA) rich matrix is organised into a specific structure by cross-linking proteins which include Versican (Vcan), Tnfaip6 and the inter-alpha-inhibitor (IαI) which each bind HA, and are supported by Ptx3 decamers to strengthen the network of HA strands within the cumulus intercellular space (Figure 1.8) (Russell and Salustri 2006). The expression of the extracellular matrix transcripts *Has2*, *Ptgs2*, *Ptx3* and *Tnfaip6* are dependent on oocyte secreted paracrine factors such as GDF-9, and are essential for oocyte maturation, cumulus expansion and ovulation (Elvin et al. 1999a; Joyce et al. 2001; Diaz et al. 2006; Dragovic et al. 2007; Gilchrist et al. 2008). The extracellular matrix proteins are responsive to, and therefore reflective of, oocyte paracrine signalling, and represent potential cumulus cell markers of oocyte developmental competence. The functional importance of these matrix components in the acquisition of oocyte developmental competence is further supported by mouse knockout studies of key matrix proteins, which are discussed in further detail below.

During ART, a pool of COCs are collected from each patient, and fertilised following a short incubation period. During this time there is some evaluation carried out based on oocyte and cumulus cell morphology. However, there are few measures that can be applied to differentiate between COCs of similar morphology. The short incubation period prior to fertilisation is seen as a window of opportunity for “catch up” growth, and only those COCs that appear to have arrested or show high degrees of fragmentation can be singled out as “poor quality”. For the remaining COCs there is no clear marker to determine the highest quality oocyte within the pool. The production and stability of the cumulus matrix is crucial to the function of the matrix immediately following ovulation and fertilisation, or in the case of oocytes collected for ART, the period of time immediately following collection and fertilisation. While the importance of the cumulus matrix has been extensively detailed in animal studies, it is only now emerging that the cumulus matrix surrounding human oocytes is of equal critical importance. While

oocytes collected following ovarian stimulation for ART procedures have been subjected to extremely high doses of hormone, the composition of the cumulus matrix surrounding human oocytes may be the key to differentiating between “high” and “low” quality oocytes and predicting pregnancy success for ART patients.

1.5.1 Components of the Cumulus Matrix

The composition and stability of the cumulus matrix is fundamental to the process of fertilisation. Cumulus matrix production and expansion is essential for female fertility, specifically for ovulation and fertilisation [Reviewed by (Russell and Robker 2007)]. This is most notable by facilitating the pick-up of the COC by the oviductal fimbria into the reproductive tract and mediating sperm-egg interactions at the site of fertilisation in vivo (Lam et al. 2000). Cumulus expansion appears to be essential for cytoplasmic maturation (Testart et al. 1983; Chen et al. 1993). Cumulus expansion may also have a role in protecting the oocyte from proteolytic, mechanical and other stresses during extrusion from the follicle (Tatemoto et al. 2000; Varani et al. 2002). Cumulus expansion has previously been applied as a visible marker of oocyte health (Vanderhyden et al. 1990), and is routinely assessed in a clinical setting during ART. In bovine COCs, a very low cumulus expansion score (1.0-2.0 on a 1.0 to 4.0 scale) is associated with poor developmental competence of in vitro cultured embryos to the blastocyst stage (Rose-Hellekant et al. 1998). The gene expression required for formation of the extracellular matrix is modulated by the oocyte, specifically through the secretion of oocyte secreted factors including GDF-9 and BMP-15 which have been discussed previously. Appropriate formation and expansion of the COC requires not only the synthesis of HA by cumulus cells but also the incorporation of proteins derived both from serum and secreted from mural granulosa and cumulus cells of the preovulatory follicle. The highly organised matrix structure, through the bonds formed between the various matrix proteins, modulates the functions of the matrix, and has a critical role during oocyte maturation and ovulation. Perturbations to the structure and integrity of the matrix, some of which are discussed below, result in sub-fertility or complete ablation of fertility. The matrix proteins each play a key role in the composition and stability of the cumulus matrix, which in turn influences the capacity for that oocyte to be fertilised. Hence, it can be reasoned that the integrity and the structure of the cumulus matrix is the limiting factor on the developmental potential of an oocyte prior to fertilisation. The requirement of key matrix proteins for COC development and fertility has been thoroughly established, details of which have been summarised below.

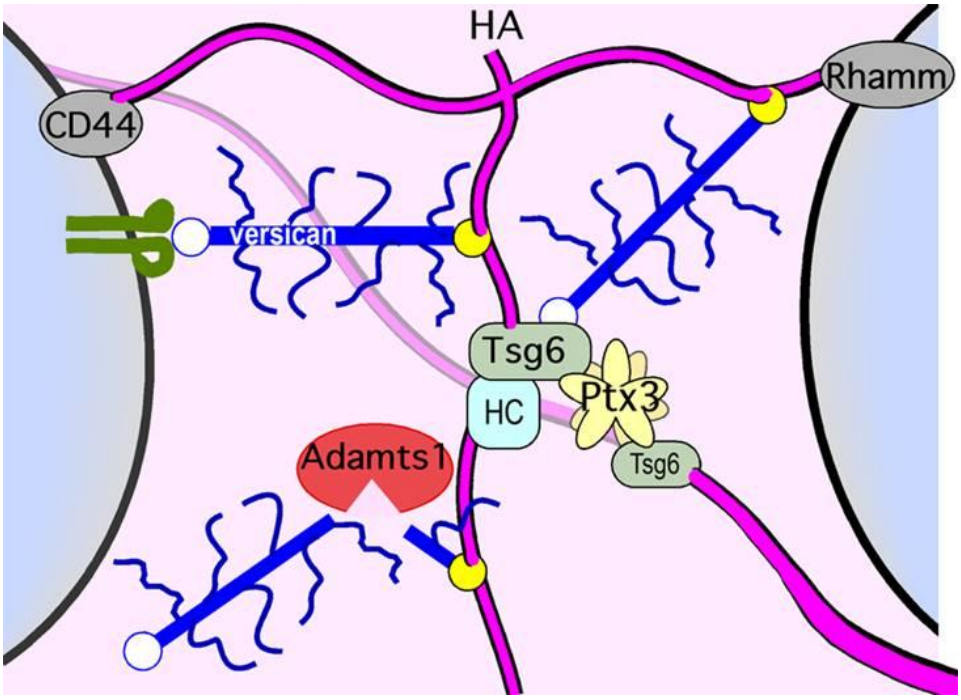

Figure 1.8 Schematic representation of the molecular structure of the expanded intercellular matrix between cumulus cells.

The intercellular matrix between cumulus cells consists primarily of hyaluronan (HA), which is stabilised and organised into a specific structure by cross-linking proteins to create a strengthened network. HA binding proteins include *Vcan*, *Tnfaip6* and *IαI*, which each bind HA, while *Ptx3* interacts with multiple *Tnfaip6* molecules. The interaction of these protein complexes results in the assembly of a network of HA strands cross-linked with matrix proteins to increase stability. Schematic from (Russell and Robker 2007).

1.5.1.1 Hyaluronan

Hyaluronan (HA) is a large glycosaminoglycan made up of D-glucuronic acid and D-N-acetyl glucosamine (Saito et al. 2000), and the main structural component of the cumulus extracellular matrix. Hyaluronan chains are stabilised to form a matrix through interactions with additional matrix proteins. The synthesis of HA within the cumulus oocyte matrix is catalysed by expression of the *Hyaluronan synthase 2* (*Has2*) enzyme (Salustri et al. 1989; Fulop et al. 1997b). Expression of *Has*2 mRNA in cumulus cells is induced during cumulus expansion in response to the ovulatory LH surge *in vivo* (Salustri et al. 1989; Fulop et al. 1997b), and recombinant GDF-9 is also capable of inducing *Has*2 expression by granulosa cells (Elvin et al. 1999a) and cumulus cells (Dragovic et al. 2007) *in vitro*. Regulation of cumulus expansion via the synthesis of HA is essential for fertility (Salustri et al. 1990b), as disruption of cumulus expansion by the addition of excess HA *in vivo* negatively affects early embryo development (Hess et al. 1999), while a lower concentration of HA in the follicular fluid of patients undergoing ART was correlated to an increased likelihood of fertilisation (Saito et al. 2000). Reduced HA in follicular fluid may suggest greater retention within the cumulus matrix, while higher concentrations of HA in follicular fluid may imply that the follicle becomes over-mature or the cumulus matrix is less able to retain HA due to a loss of or lower expression of matrix stabilising proteins, elucidating possible reasons why oocytes from these follicles have reduced developmental competence (Saito et al. 2000). Hyaluronan is able to regulate several cellular properties and functions by binding to cell surface receptors, leading to altered cell shape and behaviour which can result in altered adhesion, migration, proliferation, differentiation, cell death and anchorage (Knudson and Knudson 1993). The interaction of HA with cell surface receptors has been shown to promote anchorage independent growth and cell survival. Culture of human cumulus cells in vitro with HA decreases the rate of apoptosis (Kaneko et al. 2000; Saito et al. 2000).

1.5.1.2 Tumour-necrosis-factor-alpha-inducible-protein-6 (Tnfaip6)

Tumour-necrosis-factor-alpha-inducible-protein-6 (*Tnfaip6,* previously termed *Tsg-6*) is synthesised by cumulus and granulosa cells following the LH surge *in vivo* and its synthesis is dependent on secretion of factors such as GDF-9 from the oocyte (Mukhopadhyay et al. 2001; Varani et al. 2002). *Tnfaip6* catalyses the formation of covalent crosslinks between HA and the heavy chain of serum-derived inter-α-trypisin inhibitor ($I\alpha I$) in the mouse COC following the ovulatory stimulus and contributes to HA cross-linking and cumulus matrix stability (Carrette et al. 2001; Wisniewski et al. 2005). *Tnfaip6* mRNA expression peaks 3 h after the LH surge or hormone stimulation of cumulus expansion in COCs in the mouse (Fulop et al. 1997a). *Tnfaip6* mRNA expression is reduced in COCs isolated from fallopian tubes

soon after ovulation, however a small amount is still synthesised, possibly to maintain the stability of the matrix, further extending the role of *Tnfaip6* in COC maturation and ovulation (Ochsner et al. 2003a). *Tnfaip6* is essential for successful cumulus expansion as COCs fail to expand in *Tnfaip6* null mice, and *Tnfaip6*$^{-/-}$ female mice have a markedly lower number of oocytes in their oviducts after superovulation (Fulop et al. 2003). The impaired cumulus matrix formation is due to the lack of covalent complexes between HA and the heavy chains of the *IαI* family catalysed by *Tnfaip6* (Fulop et al. 2003). Blocking the activity of *Tnfaip6* disrupts normal COC expansion, most likely by preventing its association with IαI and other matrix molecules such as HA (Ochsner et al. 2003a).

1.5.1.3 Inter-α Trypsin Inhibitor (IαI)

Inter-alpha trypsin inhibitor (*Iαl*) is a product of the liver which diffuses from the blood into the ovarian follicle during the periovulatory period due to a gonadotrophin-dependent increase in vascular permeability (Powers et al. 1995; Nagyova et al. 2004). The *Iαl* complex contains two proteins known as heavy chains (HCs), joined through their attachment to a chondroitin sulphate (CS) chain to the proteoglycan bikunin (Salier et al. 1996). During cumulus expansion *in vivo* IαI and incorporates into the HA-rich cumulus matrix through the transfer of the heavy chains from IαI to HA (Zhao et al. 1995; Chen et al. 1996; Nagyova et al. 2004). IαI is a critical component that stabilises the COC matrix. COC expansion *in vitro* in the absence of a source of IαI results in HA becoming labile and lost into the culture media, resulting in the dispersion of cumulus cells and loss of normal COC morphology despite normal HA synthesis (Eppig 1979; Salustri et al. 1989; Chen et al. 1992; Castillo and Templeton 1993; Chen et al. 1994; Chen et al. 1996). The role of IαI in matrix stability and anchoring of HA within the cumulus matrix is significant to female fertility.

1.5.1.4 Pentraxin 3 (Ptx3)

Pentraxin 3 (*Ptx3*) is essential during oocyte maturation for the expanding cumulus matrix to retain HA molecules in the cumulus intercellular spaces, and is found in both human and mouse COCs (Salustri et al. 2004). Pentraxins are a superfamily of conserved proteins involved in innate immunity and characterised by a cyclic multimeric structure (Emsley et al. 1994). *Ptx3* is a cytokine-inducible gene and its role extends to matrix deposition, tissue remodelling and repair (Garlanda et al. 2005). *Ptx3* is produced by cumulus cells and co-localises with HA in the expanding COC matrix prior to ovulation (Varani et al. 2002; Salustri et al. 2004). *Ptx3* binds to *Tnfaip6* and may also bind cumulus cells to oviductal fimbria to help shuttle the complex into the oviduct and to the site of fertilisation (Varani et al.

2002; Salustri et al. 2004). Recent work has suggested that *Ptx3* interacts with IαI-HCs, and that such interaction is essential for organizing HA in the viscoelastic matrix of COCs, highlighting a direct functional link between the two molecules (Scarchilli et al. 2007). Homozygous *Ptx3*$^{-/-}$ male mice show normal fertility while *Ptx3*$^{-/-}$ female mice have severe defects in fertility (Varani et al. 2002). *Ptx3*$^{-/-}$ mice are unable to form a stable matrix, as cumuli are disorganised and oocytes are randomly located and often denuded, but the cells are able to synthesise a relatively normal amount of HA when compared to wild type mice. Further studies have suggested that *Ptx3*$^{-/-}$ mice are completely infertile, indicating a role for *Ptx3* in the formation and organisation of the HA rich matrix of the COC (Salustri et al. 2004), and a role in female fertility.

1.5.1.5 Versican (Vcan)

Versican (*Vcan*) is a HA-binding proteoglycan with numerous chondroitin sulphate (CS) attachments. In addition to a HA binding N-terminal link-module, *Versican* possesses a C-terminal lectin-like domain that may bind cell surface or matrix molecules. Dual epidermal growth factor (EGF)-like modules that can stimulate cell proliferation as well as a complement regulatory like protein domain complete the C terminus (Russell et al. 2003b). Among its reported activities, *Vcan* may confer structural integrity to tissues and anti-adhesive pro-migratory effects in vascular cells (Ang et al. 1999). *Vcan* mRNA expression is up-regulated in mural granulosa cells of the rodent ovary 4-12 h post-hCG (Russell et al. 2003b). *Vcan* protein was detected in granulosa cells of primary and growing follicles but was most intensely detected within the expanded COC matrix of ovulatory follicles 4-12 h post hCG. The presence of *Vcan* as well as HA in the expanded COC matrix suggests that the protein may play a role in the organisation or stabilisation of the COC matrix (Russell et al. 2003b). The protease *Adamts1* (a disintegrin and metalloprotease with thrombospondin motifs-1) cleaves *Vcan* in the CS attachment domain (Sandy et al. 2001), and *Adamts1*-mediated *Vcan* cleavage has been shown to occur in ovulating mouse COCs (Russell et al. 2003a). It has been shown that *in vitro* matured mouse COCs have 15-fold lower *Vcan* mRNA expression compared to *in vivo* matured COCs (Dunning et al. 2007). *In vitro* matured COCs also lacked intact and cleaved *Versican* protein compared to *in vivo* matured COCs. This is concurrent with the general consensus that *in vitro* matured COCs have lower developmental potential than *in vivo* matured COCs (Jones et al. 2008). The lack of *Vcan* mRNA and protein from *in vitro* matured mouse COCs coupled with the decreased developmental potential of these COCs strengthens the role of this matrix protein in the acquisition of oocyte developmental potential and as a cumulus cell marker of oocyte quality for assisted reproduction.

1.5.1.6 Prostaglandin synthase 2/Cyclooxygenase 2 (Ptgs2/Cox2)

Prostaglandin-endoperoxide synthase 2 (*Ptgs2*), also termed *Cyclooxygenase 2* (*Cox2*), is the rate-limiting enzyme in the production of prostaglandins (PG) from arachidonic acid (Sirois and Richards 1992; Wong and Richards 1992; Matsumoto et al. 2002). The induction of ovarian PG levels, cumulus expansion and ovulation are dependent on expression of *Ptgs2*, which is rapidly induced following the LH surge in mural granulosa and cumulus cells (Sirois and Richards 1992; Davis et al. 1999). Within ovarian follicles, oocyte paracrine factors promote expression of *Ptgs2* mRNA, and co-culture of COCs with mature denuded oocytes results in significant up-regulation of *Ptgs2* mRNA (Joyce et al. 2001). Acquisition of *Ptgs2* mRNA promoting activity may be developmentally regulated by oocytes and represents a mechanism to reduce the chances of immature or developmentally incompetent oocytes from being ovulated. Knockout of *Ptgs2* in mice leads to infertility, as *Ptgs2* null mice display normal follicular development but have reproductive defects including limited and unorganised cumulus expansion, little or no oocytes in the oviducts after ovulation, defective fertilisation and failure to impregnate (Lim et al. 1997; Davis et al. 1999). Similarly mice null for the PGE2 receptor subtype EP2 have fertility defects due to impaired ovulation associated with defective cumulus expansion (Richards 2005). This suggests that *Ptgs2* deficiency in the ovary is the primary cause for ovulation failure. A mutation in the *Ptgs2* or EP2 genes results in reduced *Tnfaip6* expression downstream of PGE2, impairing cumulus expansion and fertility (Ochsner et al. 2003b), supporting the essential role for both *Ptgs2* and *Tnfaip6* in cumulus matrix stability and organisation, and their potential as molecular markers of oocyte quality.

1.6 CUMULUS OOCYTE COMPLEX METABOLIC ACTIVITY

Cumulus cells play a pivotal role in the utilisation of glucose by the COC. While mature human oocytes express three facilitative glucose transporters (GLUT-1, 3 and 8) their capacity to utilise glucose is poor, therefore glucose uptake by the COC is carried out by the cumulus cells which express four facilitative glucose transporters (GLUT-1, 3, 4 and 8) (Dan-Goor et al. 1997; Augustin et al. 2001; Williams et al. 2001; Roberts et al. 2004; Nishimoto et al. 2006; Zheng et al. 2007; Pisani et al. 2008). Oocytes require a constant supply of nutrients during maturation to acquire developmental competence both *in vivo* and *in vitro*. In the absence of cumulus cells, immature mammalian oocytes demonstrate very low levels of glucose uptake, glycolytic activity and glucose oxidation (Zuelke and Brackett 1992; Rieger and Loskutoff 1994; Saito et al. 1994). Within the COC, the majority of the glucose taken up in response to gonadotrophins is metabolised to lactate which can be utilised by oocytes for energy (Downs and Utecht 1999). Rapid HA synthesis by cumulus cells required for cumulus expansion (Eppig 1979; Salustri et al. 1989) is sustained by an increase in glucose uptake and glucose flux through the hexosamine pathway to synthesise HA substrate monomers (Sutton-McDowall et al. 2004; Sutton-McDowall et al. 2005; Harris et al. 2007). The increased glucose consumption by cumulus cells for cumulus expansion highlights the importance of an appropriate supply of glucose to the COC. Additionally, glucose regulates the rate of meiotic resumption and progression and the acquisition of oocyte developmental competence (Colton et al. 2002; Sutton-McDowall et al. 2005; Sutton-McDowall et al. 2006; Hashimoto et al. 2007). Measures of cumulus and oocyte metabolic activity are therefore likely to be useful predictive markers of oocyte quality (Krisher and Bavister 1999; Spindler et al. 2000; Preis et al. 2005; Sugiura et al. 2005).

1.6.1 Glucose metabolism

Glucose enters cells via facilitated glucose transporters (GLUTs) and is rapidly phosphorylated to glucose-6-phosphate by the enzyme hexokinase (Bouche et al. 2004). The phosphorylated glucose supplies substrates for the five primary glucose utilisation pathways, which include glycogen synthesis for the storage of glucose, glycolysis for L-lactate production, the tricarboxylic acid (TCA) cycle for the production of ATP, the pentose phosphate pathway which generates ribose moieties, and the hexosamine biosynthesis pathway. Oocytes require pyruvate and lactate as an energy source for growth and resumption of meiosis (Biggers et al. 1967; Eppig 1976). The cumulus cells transfer amino acids and glucose metabolites to the oocyte via gap junction channels (Rose-Hellekant et al. 1998). Metabolite balance, supplied by the combination of maternal nutrition levels and cumulus cell metabolism, has a clear influence on oocyte development and acquisition of developmental

competence, and further illustrates that measures of metabolic activity are potential markers of oocyte quality. It has previously been shown that cumulus cell expression of key glycolytic enzymes are responsive to oocyte secreted paracrine factors *in vitro* (Sugiura et al. 2005), and suggests further investigation of these key glycolytic enzymes may identify markers of oocyte metabolic activity and developmental potential.

1.6.2 Glycolysis

Energy is essential for oocyte development and function. Oocytes obtain amino acids and products of glycolysis from cumulus cells (Biggers et al. 1967; Donahue and Stern 1968; Colonna and Mangia 1983; Leese and Barton 1984; Haghighat and Van Winkle 1990). Enzymes in the glycolytic pathway are highly expressed in cumulus cells compared with mural granulosa cells and their expression in cumulus cells is promoted by oocyte-secreted paracrine factors (Eppig et al. 2005; Sugiura et al. 2005). Oocytes regulate the metabolic cooperativity between oocytes and cumulus cells for energy metabolism through the actions of the oocyte secreted paracrine factors which is crucial for oocyte development (Sugiura et al. 2005). Little is published regarding the regulation of glycolysis in human cumulus and granulosa cells. One study details the expression profiles of selected glycolytic enzymes (triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase 1, and two isoforms of alpha enolase [ENOAi and ENOAii]) between human cumulus and granulosa cells (Gillott et al. 2008). However the human cumulus cells were analysed following 24 h of *in vitro* culture, resulting in limited expression of the glycolytic enzymes analysed. Regardless, the authors support the influence of oocyte secreted factors in maintaining the transcriptional levels of glycolytic enzymes in human follicular cells (Gillott et al. 2008).

Glycolysis, or more specifically the Embden-Meyerhof pathway, is the intracellular oxidation of glucose to pyruvate and lactate, with the rate of glucose oxidation dependent on the flux of glucose through the pathway, reflective of circulating levels of glucose and insulin (Bouche et al. 2004). Glycolysis is a sequence of ten reactions involving ten intermediate compounds in which the intermediates provide entry points to glycolysis (Figure 1.9), and regulation occurs by inhibiting or activating the enzymes within this pathway (Berg et al. 2007). In the first regulated step, hexokinase converts glucose to glucose-6-phosphate. Human 6-phosphofructokinase (PFK;' ATP: D-fructose-6-phosphate, 1-phosphotransferase) encodes muscle (M), liver (L), and platelet (P) tissue specific isoenzymes, (Davidson et al. 1983; Berg et al. 2007). Aldolase A catalyses the aldo reaction: fructose 1,6-bisphosphate (F-1,6-BP) is broken down into glyceraldehyde 3-phosphate and dihydroxyacetone

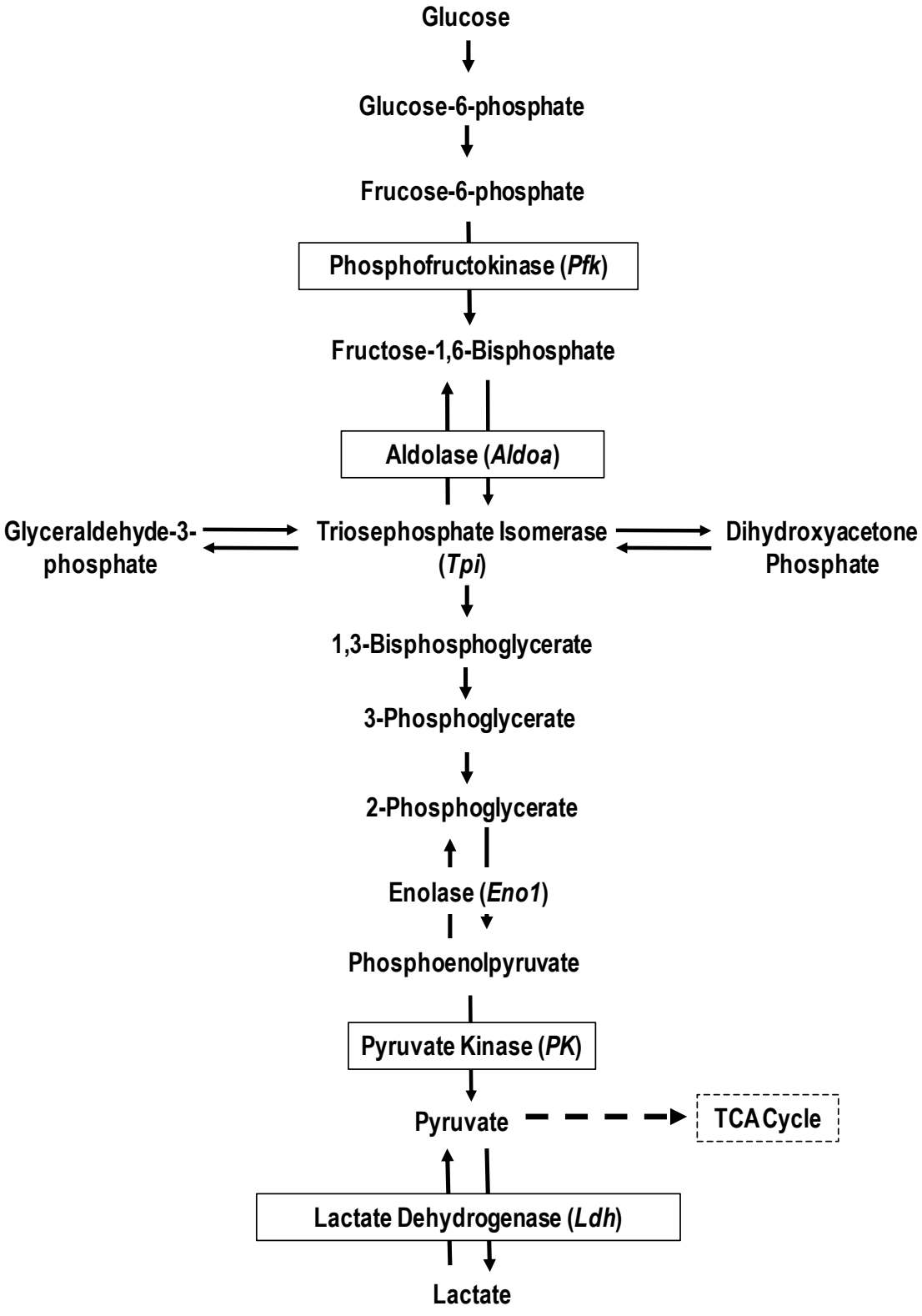

Figure 1.9 The metabolic pathway of glycolysis

Glycolysis is the metabolic pathway which oxidises glucose to pyruvate and occurs in virtually all living cells. A continuous supply of glucose is necessary as a source of energy. Glycolysis provides energy and generates precursors for the synthesis of primary metabolites such as amino acids and fatty acids.

phosphate (DHAP) (Gamblin et al. 1991). Enolase, also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for the catalysis of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate and can also catalyse the reverse reaction (Pancholi 2001). Triose-phosphate isomerase (TPI or TIM) catalyses the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Albery and Knowles 1976). Pyruvate kinase catalyses the transfer of a phosphate group from phosphoenolpyruvate to ADP for the production of pyruvate and ATP (Liapounova et al. 2006). Pyruvate kinase activity is regulated by phosphoenolpyruvate and fructose 1,6-bisphosphate, or ATP can act as a negative allosteric inhibitor (Liapounova et al. 2006). Lactate dehydrogenase catalyses the conversion of pyruvate to lactate through the oxidation of NADH and replenishing of NAD+, which is essential for the glycolytic conversion of glucose to pyruvate (Kim and Dang 2005). There are several points of regulation in glycolysis which may provide markers of oocyte quality. Oocyte secreted factors have been shown to promote the mRNA expression of rate limiting glycolytic enzymes within murine cumulus cells (Sugiura et al. 2005).

1.6.3 Altered glucose metabolism and oocyte maturation

Systemic perturbations to glucose metabolism have profound effects on female fertility, and more specifically ovarian function, oocyte maturation and follicle dynamics (Colton et al. 2003). The COC is sensitive to circulating metabolites and the dynamic process of follicle growth and maturation are highly responsive to changes in circulating glucose and insulin. Diabetic mice show delays in germinal vesicle breakdown (GVBD) and poor on-time embryo development. Hyperglycaemia slows oocyte growth through altered meiotic regulation (Colton et al. 2002), and increased apoptosis in mural granulosa and cumulus cells (Chang et al. 2005). Obesity plays a significant role in female fertility and has been associated with several reproductive disturbances including irregular menstrual cycles, reduced spontaneous and induced fertility, increased risk of miscarriage and changes in circulating sex hormones (Kirschner et al. 1982; Pasquali and Casimirri 1993; Pasquali et al. 2003). Obesity may interfere with many neuroendocrine and ovarian functions, reducing both ovulation and fertility rates in otherwise healthy women (Pasquali et al. 2003), and adversely affect embryo quality in young women (Metwally et al. 2007). Establishment of animal models which mimic diet induced obesity in humans have revealed the effects of obesity on reproductive function. Female mice fed a high fat diet until the onset of insulin resistance display defective ovarian function, including poor oocyte quality, reduced blastocyst survival rates, and abnormal embryonic cellular differentiation (Minge et al. 2008). In both human and animal studies, peri-conceptional weight loss and changes in maternal nutrition confer improvements in fertility outcomes (Metwally et al. 2008; Minge et al. 2008). Alterations in glucose metabolism have profound consequences for female fertility as the physiological balance of metabolites

contributes to ovarian function and oocyte quality. Glucose sensitive pathways and the activity of enzymes which regulate glucose uptake may facilitate in some part the acquisition of oocyte developmental competence. In turn, the activity and integrity of the glucose utilisation pathways may provide a tool for assessing oocyte developmental competence.

1.7 MARKERS OF OOCYTE DEVELOPMENTAL COMPETENCE

In the early days of assisted reproduction, the oocyte was simply seen as the precursor to the developing embryo, the first step in creating a pregnancy for an otherwise infertile couple. Only now has it started to emerge that the oocyte is in fact incredibly important in fertility outcome. It is now becoming evident that the quality of the oocyte is the key limiting factor in female fertility (Gilchrist et al. 2008). The development of the oocyte within the ovarian follicle requires input from multiple signalling pathways to fully mature and reach the point of ovulation, while the quality of the ovulated oocyte will impact its fertilisation potential and the development of a subsequent embryo. The potential of an oocyte to create a successful pregnancy is termed developmental competence, and the factors which create optimal oocyte developmental competence and how this has the potential to be measured are described below.

1.7.1 Acquisition of oocyte developmental competence

The acquisition of oocyte developmental competence is a complex process whereby an oocyte and resultant embryo acquires the capacity to resume and complete meiosis, cleave following fertilisation, develop to the blastocyst stage, and produce a normal and viable embryo capable of implantation which results in a live birth (Sirard et al. 2006). Oocyte developmental competence depends on both nuclear and cytoplasmic maturation acquired gradually throughout folliculogenesis (Lucidi et al. 2003; Thomas and Vanderhyden 2006). Oocyte meiotic competence is an oocytes' ability to resume meiosis, undergo germinal vesicle break down (GVBD) and complete meiosis (Eppig 1991b). Meiotic maturation involves a cascade of nuclear events that are induced by the LH surge and underlies a spatio-termporal synchrony of cell cycle molecules (Sirard et al. 2006). The meiotic competence of an oocyte is associated with the accumulation of important cell cycle regulatory factors and the reorganisation of the chromatin and microtubules within the oocyte (Albertini et al. 2001). When oocytes are removed from the follicle they have the ability to undergo spontaneous resumption of meiosis without the addition of stimulating agents (Edwards 1965). Meiotic resumption can be visualised under a microscope by the extrusion of the first polar body or through the ability to stain the metaphase with specific dyes (Sirard et

al. 2006). It is hypothesised that this spontaneous resumption of meiosis occurs because the oocyte is removed from the follicular environment which contains an inhibitor of meiosis. Cytoplasmic maturation is not as clearly defined as meiotic maturation, but is evident when an oocyte stops RNA and protein synthesis during the preparation phase and a redistribution of organelles such as mitochondria and the cortical granules occurs.

The capacity for an oocyte to cleave following fertilisation is acquired sequentially during development, and the capacity to undergo maturation does not assure the competence to cleave to the two-cell stage following fertilisation (Eppig and Schroeder 1989). Likewise, the ability to cleave to the two-cell stage does not guarantee an ability to develop beyond this stage. The ability for an oocyte to develop to the blastocyst stage is the most common marker of oocyte competence employed in a laboratory and clinical setting. For an embryo to have the greatest likelihood to induce a successful pregnancy there are defined time points at which this development to the blastocyst must take place under proper culture conditions. The ability to sustain the first week of embryonic development is influenced by the follicular origins the oocyte was obtained from indicating that developmental potential is intrinsic in within certain oocytes (Sirard et al. 2006). Appropriate activation of the embryonic genome is critical for further development, which is part of the early developmental program ingrained in the oocyte through the accumulation of proteins and RNA. At this stage of development, blastocysts can be evaluated based on their morphology, which classifies quality through criteria such as number of cells, trophectoderm to inner-cell mass ratio, blastocoele expansion, and overall appearance. However, the ultimate measure of embryo quality is the ability to induce a pregnancy, and to a lesser degree, survive cryopreservation. As there is a tendency for a proportion of embryos to reach a block in development prior to reaching the blastocyst stage, these embryos may also provide insight into what constitutes an embryo with low developmental competence, and how this differs from an embryo with high developmental potential.

Not all blastocysts are created equal, and therefore not all blastocysts are able to induce a pregnancy. Failure to induce a pregnancy can in part be attributed to the receptivity of the uterus. However, the low rates of gestation seen from *in vitro* matured oocytes compared to those obtained following *in vivo* maturation suggest there is a significant oocyte contribution to pregnancy success. The basis for the oocyte contribution is supported by recent bovine studies which indicate that induction of follicular differentiation by manipulation of the ovarian stimulation protocol can result in germinal-vesicle stage (immature) oocytes where most are capable of developing to the blastocyst stage (Blondin et al. 2002). The embryonic development rates obtained from manipulation of ovarian stimulation protocols was

equal to or possible surpasses the blastocyst rates obtained from *in vivo* matured oocytes (although it should be noted the two were not compared in the same experiment) (Blondin et al. 2002; Sirard et al. 2006). The follicular environment not only has an effect on oocyte quality and female fertility but on offspring health, as seen in Large Calf Syndrome seen in bovine studies (Sirard et al. 2006). Fetal development and offspring health can be influenced by the uterine environment (Lucy 2003) but less is known about the ovarian influence and how the processes of folliculogenesis effect oocyte developmental competence. Importantly, it is during oocyte maturation within the follicle that the oocyte acquires the machinery to sustain embryo development and as such, oocyte developmental competence (Sirard 2001). A pre-ovulatory surge of LH induces meiotic maturation *in vivo*, but as oocytes do not have LH receptors they rely on the surrounding somatic cells to mediate this hormonal stimulus (Buccione et al. 1990b). As cumulus cells are required to mediate the hormonal signals to induce maturation and are known to respond to oocyte signals and convey oocyte metabolic activity, the somatic cells represent a unique and valuable tissue for the assessment of oocyte quality.

1.7.2 Markers of oocyte developmental competence

Follicular oocyte growth and the acquisition of oocyte developmental competence are obtained during development under the control of key molecular and biochemical pathways at stage-specific time points (Fair 2010). As such, the quality of each oocyte which is ovulated is determined by the follicular environment in which these pathways were active and to a lesser degree, the expression of appropriate genes and how this adapted to developmental challenges (Fair 2010). The current understandings of the pathways and genes which contribute to oocyte developmental competence have led to recent gene profiling experiments to find markers of oocyte quality. The identification of such markers has the potential to improve ART success rates and broaden the field of knowledge regarding oocyte maturation and the importance of the follicular microenvironment. Improvements in pregnancy rates following ART have historically been achieved through the improvement of culture conditions and laboratory procedures; however the ability to determine the most viable gametes or embryos from a cohort has remained dependent on morphological assessment and embryo developmental checkpoints during the culture period. The inability to confidently and repeatedly identify an oocyte and embryo with high developmental potential results in low pregnancy rates and the routine practise of multiple embryo transfers, increased rates of multiple gestations and subsequent pregnancy complications. Techniques to investigate the developmental potential of an oocyte are restricted – oocytes which are used for experimentation cannot be fertilised and subsequently transferred. Markers of oocyte quality which would be applied in a clinical setting must be non-invasive, currently limiting oocyte assessment to visual morphological parameters. Therefore, the cumulus and granulosa cells present themselves as the

next best option, and can be harvested with ease during assisted reproduction treatment. Emerging research into the non-invasive measurement of oocyte quality has focussed on cumulus cell apoptosis (Yuan et al. 2005), the degree of cumulus expansion (Ebner et al. 2003b; Lucidi et al. 2003), measures of metabolic activity in cumulus cells (Sugiura et al. 2005) and more recently embryo culture medium metabolomic profiling (Gardner et al. 2001; Brison et al. 2004; Seli et al. 2009), and the composition of follicular fluid (Saito et al. 2000; Babayan et al. 2008).

An area of particular focus is the measurement of cumulus cell genes as markers of oocyte quality – as the cumulus cell respond directly to oocyte paracrine signals and are the support cells for the oocyte during development. Cumulus cell gene expression has previously been assessed as a marker of oocyte and embryo quality (McKenzie et al. 2004; Hasegawa et al. 2005; Zhang et al. 2005; Cillo et al. 2007; Feuerstein et al. 2007; Hasegawa et al. 2007; Assidi et al. 2008; Assou et al. 2008; Hamel et al. 2008; Anderson et al. 2009a; Anderson et al. 2009b; Hamel et al. 2009). The landmark study of McKenzie *et al.* correlated cumulus cell gene expression with embryo morphology and reported significant differences in *PTGS2*, *HAS2* and *GREM1* mRNA expression between high and low grade embryos (McKenzie et al. 2004). However, this groundbreaking study had only eight patients and did not extrapolate the data to include pregnancy success. The patients in this study had group embryo culture and multiple embryo transfers; hence the expression data for individual COCs was not able to be correlated with the developmental potential of the specific oocyte from which the cumulus cells were derived. The pooling of cumulus cell samples or the routine practise of group embryo culture is a shortfall of many studies in this area. Shortly after this publication, an independent research group reported microarray analyses on human cumulus cells and correlated the expression profiles with fertilisation and embryo morphology (Zhang et al. 2005). Both microarray and qPCR analysis found *PTX3* mRNA expression was indicative of oocyte quality. However, the previous study found no difference in *PTX3* expression and these contradictory reports may be due to methods based differences. This study was again not designed to correlate gene expression directly with pregnancy success as patients underwent multiple embryo transfer and the analyses were undertaken on pooled cumulus cell samples.

Following these landmark publications, a number of studies investigating cumulus cell gene expression as a marker of oocyte quality have now emerged. Cillo *et al.* reported *GREM1* and *HAS2* mRNA levels to be significantly higher in cumulus cells from oocytes that produced high quality embryos on day 3 of culture when compared to unfertilised oocytes or those which developed to poor quality embryos, but no

difference was seen in *PTX3* mRNA levels (Cillo et al. 2007). Feuerstein *et al.* investigated genes induced by the LH peak prior to ovulation, *Steroidogenic acute regulatory protein* (*STAR*), *Cyclooxygenase 2* (*COX2*/*PTGS2)*, and *Amphiregulin* (*AREG*) as well as genes involved in oocyte lipid metabolism, *Stearoyl-coenzyme A desaturase 1 and 5* (*SCD1* and *SCD5*) and *Connexin 43* (*CX43* or *GJA1*) which is important for gap junction function (Feuerstein et al. 2007). Oocyte nuclear maturation was associated with increased expression of *STAR*, *COX2*, *AREG*, *SCD1* and *SCD5* in cumulus cells. Mean expression levels of target genes in cumulus cells were not significantly different between those from oocytes yielding top quality embryos and those from oocytes yielding weak or low grade embryos in terms of early embryo development. A reduction in *progesterone receptor* (*PR*) mRNA expression in cumulus cells at the time of oocyte collection has been associated with the development of a good quality embryo (Hasegawa et al. 2005), while in a separate study *CX43* mRNA expression was lower in cumulus cells from oocytes which resulted in good morphology embryos (blastomeres >7 cells with fragmentation < 10% on day 3) when compared to those with poor morphology (Hasegawa et al. 2007). A recent microarray analysis demonstrated mRNA expression of genes involved in apoptosis and glucogenesis were significantly correlated with embryo potential and successful pregnancy (Assou et al. 2008). Cumulus cell expression of *BCL2-like 11 apoptosis facilitator* (*BCL2L11*) and *Phosphoenolpyruvate carboxykinase 1* (*PCK1*) was significantly higher in patients with a successful pregnancy, while *Nuclear factor I/B* (*NFIB*) had significantly lower expression in cumulus cells in pregnant patients. Validation of the array data by quantitative RT-PCR was unable to achieve the significance seen with the array results; however the pattern of expression was reproduced between the pregnancy outcome groups.

The combined results of these studies, as summarised in Table 1.1, show there is value in utilising cumulus cells to assess oocyte quality. Many of the genes which have been significantly associated with oocyte and embryo development have important functions related to oocyte maturation, cumulus expansion and metabolic activity of the COC. However, there have been flaws in the experimental design aspects of these studies which have left the door open for future work. Limitations of past studies include low patient numbers, pooling of cumulus cell samples from multiple follicles or even pools of patients, group culture of oocytes and subsequent embryos, development assessed only to blastocyst stage, multiple embryo transfers, and the end point being a positive hCG blood tests or eight week ultrasounds scans for pregnancy success. While each study should be commended for exploring this exciting new avenue of research, there are a number of issues which still need to be addressed to arrive at an outcome which is both biologically and clinically relevant and ultimately has the potential to differentiate between two similar looking oocytes to select one with the high developmental

competence. The ability to select a high quality, healthy oocyte through a biochemical marker in combination with existing embryo selection methods which can be easily implemented during infertility treatment has the makings to improve pregnancy success rates for patients undergoing ART far beyond what has previously been achieved through improvements in the culture media and handling techniques.

Cumulus samples are commonly collected post hyaluronidase treatment (for ICSI patients) or post-fertilisation which may cause cumulus cell and mRNA degradation. While previous studies have identified potential markers of oocyte developmental competence, no single study has looked at cumulus cell gene expression in a cohort of patients with single oocyte and embryo culture as well as single embryo transfer. The literature presents a strong case for the use of cumulus cells to assess oocyte health and predict pregnancy success, but also highlights the need for this assessment to be carried out under modified experimental conditions where individual oocytes are tracked from collection, through fertilisation, embryo cleavage and on time development checkpoints, and most importantly in a patient cohort where only single embryo transfer is performed so gene expression can be directly correlated with outcomes. Positive markers of oocyte developmental competence identified under these experimental conditions have the potential to increase the number of single embryo transfers, and maintain or even increase the pregnancy success rates for ART.

Fertility is the natural capability of giving life. Human fertility is dependent on many factors, such as lifestyle, behaviour, and endocrinology. There are many causes of infertility, and many of the causes can be bypassed by medical intervention. While the use of these technologies is increasing as more people seek medical intervention in their quest to conceive, the clinical pregnancy rates following ART has remained stable at 22%. There is an immediate need to broaden our understanding of what initiates and promotes a successful pregnancy and how this can be implemented during infertility treatment to achieve more live births following ART. The work presented in this thesis aims to address a current gap in the literature surrounding assisted reproductive technologies – through the investigation of a biochemical marker or markers of oocyte quality which can routinely predict the likelihood of successful pregnancy.

Table 1.1 Studies analysing gene expression in follicular cells to identify markers of oocyte quality, embryo development, and pregnancy success.

Authors	Markers Identified	Patient Numbers	Outcomes Measured	Method	SET
(McKenzie et al. 2004)	*PTGS2, HAS2, GREM1*	8	Day 3 embryo morphology	qRT-PCR	
(Zhang et al. 2005)	*PTX3*	39	Day 3 embryo development, Clinical Pregnancy	Microarray & qRT-PCR	**No**
(Hasegawa et al. 2005)	*PR*	44	Day 3 embryo morphology	qRT-PCR	
(Cillo et al. 2007)	*HAS2, GREM1*	45	Day 3 embryo morphology	qPCR	
(Hasegawa et al. 2007)	*CX43*	29	Day 3 embryo morphology	qRT-PCR	
(Feuerstein et al. 2007)	*STAR, PTGS2, CX43, AREG, SCD1, SCD5*	47	Oocyte nucleus maturity, embryo development in culture to blastocyst	qRT-PCR	
(van Montfoort et al. 2008)	*CCND2, CXCR4, GPX3, CTNND1, DHCR7, DVL3, HSPB1, TRIM28*	6	Early cleavage	Microarray & qRT-PCR	
(Hamel et al. 2008)	*HSD3β1, FDX1, CYP19A1, CDC42, SERPINE2*	34	Pregnancy (foetal heartbeat)	Microarray & qPCR	**No***
(Assou et al. 2008)	*BCL2L11, PCK1, NFIB*	30	Pregnancy (foetal heartbeat)	Microarray & qRT-PCR	
(Anderson et al. 2009a)	*HAS2, BDNF, PTGS2, GREM1*	67	Oocyte maturation *in vitro*	qRT-PCR	
(Anderson et al. 2009b)	*PTGS2, BDNF, GREM1*	75	Cumulus expansion, oocyte maturity, fertilisation, embryo formation, embryo quality, embryo selection for transfer	qRT-PCR	**6/75**
(Hamel et al. 2009)	*PGK1, RGS2, CDC42*	9	Pregnancy (foetal heartbeat)	qRT-PCR	**3/9**

*For Hamel *et al.*, 2008 the number of patients who underwent single embryo transfers was not specified, however the average numbers of embryos per transfer was 1.4. PTGS2, Prostaglandin-endoperoxide synthase 2; HAS2, Hyaluronan synthase 2; GREM1, Gremlin 1; PTX3, Pentraxin 3; PR, Progesterone receptor; CX43, Connexin 43; STAR, Steroid acute regulatory protein; AREG, Amphiregulin; SCD1, Stearoyl-coenzyme A desaturase 1; SCD5, Stearoyl-coenzyme A desaturase 5; CCND2, Cyclin D2; CXCR4, Chemokine (C-X-C motif) receptor 4; GPX3, Glutathione peroxidise 3; CTNND1, Catenin delta-1; DHCR7, 7-dehydrocholesterol reductase; DVL3, Dishevelled dish homolog 3; HSPB1, Heatshock 27 kDa protein 1; TRIM28, Tripartite motif-containing 28; HSD3β1, 3-beta-hydroxysteroid dehydrogenase; FDX1, Ferredoxin 1; CYP19A1, Cytochrome P450 aromatase; CDC42, Cell division cycle 42; SERPINE2, Serine proteinase inhibitor clade E member 2; BCL2L11, BCL2-like 11 apoptosis facilitator; PCK1, Phosphoenolpyruvate carboxykinase 1; NFIB, Nuclear factor I/B; BDNF, Brain-derived neurotrophic factor; PGK1, Phosphoglycerate kinase 1; RGS2, Regulator of G-protein signalling 2; CDC42, Cell division cycle 42.

1.8 SUMMARY, HYPOTHESIS AND AIMS

The most desirable approach in assisted reproduction is the transfer of a single embryo with the greatest developmental potential to establish a live birth. However, assisted reproduction practises result in a pool of embryos available for transfer through the collection of multiple oocytes. Embryologists must select an embryo with the greatest chance of establishing a successful pregnancy. This approach is limited due to a lack of robust markers of embryo quality. Embryo quality is determined by the quality of the gametes it was derived from, particularly the oocyte. Oocyte maturation and ovulation are highly controlled, tightly regulated processes, and the acquisition of oocyte developmental competence influences the quality of a subsequent embryo, and ultimately the ability for that embryo to form a successful, viable, term pregnancy. The follicular microenvironment is critical to oocyte maturation, and of particular interest are the cumulus cells which have a close, functional relationship with the oocyte. It is the presence of these cumulus cells which support the oocyte in the acquisition of developmental competence. As the cumulus cells are critical to oocyte maturation and respond directly to signals from the oocyte, it is suggested that they may also reflect the quality of their enclosed oocyte. Further studies are required to assess cumulus cells as a direct marker of oocyte developmental competence in a system which utilises individual oocyte and embryo culture and single embryo transfer.

My general hypothesis is that cumulus cell gene expression is indicative of the health of their enclosed oocyte and can be utilised in a non-invasive test of oocyte developmental potential. The expression levels of key genes in human cumulus cells can therefore be measured to determine oocyte health and predict pregnancy success in women undergoing assisted reproduction.

1.9 AIMS AND HYPOTHESES

SPECIFIC HYPOTHESES AND AIMS

Hypothesis 1: Cumulus cell gene expression is correlated with clinical embryo grade.

To address this hypothesis the following aims were completed:

- Determine whether matrix, signalling and metabolic genes show different expression levels in human cumulus cells from oocytes which developed to good morphology embryos (high grade) compared to cumulus cells from oocyte which developed to poor morphology embryos (low grade).

Hypothesis 2: Cumulus cell gene expression is a predictor of live birth following assisted reproductive treatment with elective single embryo transfer.

To address this hypothesis the following aims were completed:

- Determine whether matrix, signalling and metabolic genes show different expression levels in human cumulus cells from oocytes which formed embryos resulting in a live birth following single embryo transfer compared to those for which IVF failed.

Hypothesis 3: There are characteristic gene expression patterns in cumulus cells from oocytes which formed an embryo associated with a successful pregnancy compared to embryos in which IVF failed.

To address this hypothesis the following aims were completed:

- Determine the differential expression of genes in pools of human cumulus cells from oocytes which formed an embryo associated with a successful pregnancy compared to embryos in which IVF failed using a microarray approach.
- Validate the gene expression of microarray identified genes in human cumulus cells from oocytes which formed an embryo associated with a successful pregnancy compared to embryos in which IVF failed.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

Equine chorionic gonadotropin (eCG/Gestyl) was purchased from Professional Compounding Centre of Australia, (Sydney, NSW). Human chorionic gonadotropin (hCG/Pregnyl) was purchased from Organon, Australia (Sydney, NSW). Plastic culture dishes were purchased from SARSTEDT Australia Pty. Ltd. (Adelaide, SA). Culture media was purchased from GIBCO, Invitrogen Australia Pty. Ltd. (VIC, Australia). All other reagents were purchased from Sigma-Aldrich Pty. Ltd. (Castle Hill, NSW, Australia) unless stated otherwise.

2.2 METHODS

2.2.1 Patient Selection

Ethical approval for the use of human samples was obtained from the Women's and Children's Hospital Human Research Ethics Committee, Adelaide, Australia and the Repromed Scientific Advisory Committee, Adelaide, Australia. Written consent for the use of cumulus and mural granulosa cells for research was obtained from patients undergoing routine IVF/ICSI with at Repromed, Dulwich, South Australia. Cumulus cells were collected from thirty eight patients undergoing elective single embryo transfer. Patients with clinical indications of polycystic ovary syndrome were not included in this study. All patients had treatment with their own gametes and were not undergoing pre-implantation genetic diagnosis. Patient demographic data is described in further detail in future experimental chapters.

Table 2.1 Patient demographic characteristics for the thirty eight patients recruited to investigate cumulus cell markers of oocyte quality and pregnancy success.

Number of Patients	38
Maternal Age Mean	33.4 ± 0.56
Maternal Age Median	33.8 ± 0.56
Maternal Age Range	24.6 - 4.03
BMI Mean	25.7 ±0.66
BMI Median	25 ± 0.66
BMI Range	19.3 – 36.4

(Age data is presented as years ± SEM. All other data is presented as mean ± SEM)

2.2.2 Patient Stimulation

All patients undergoing single embryo transfer were stimulated using a long down-regulation protocol involving the administration of a Gonadotropin Releasing Hormone analog (Nafarelin (Synarel),

Organon, Australia) with down regulation confirmed by blood oestrogen levels below 0.2 nM/L as was standard within the clinic at the time and has been previously described in (Feil et al. 2008). Patients were then administered recombinant Follicle Stimulating Hormone (Gonal-F, Serono, Sydney, Australia or Puregon, Organon, Sydney, Australia) for 9-12 days, monitored by ultrasound and serum hormone levels, until the lead follicle was a size of 18 mm. Patients were then given 5,000 IU human Chorionic Gonadotropin (Pregnyl, Organon, Sydney, Australia) and the oocyte collection scheduled for 36 h later. Cumulus oocyte complexes (COC's) from follicles greater than 14mm were collected using transvaginal ultrasound and a 17 gauge needle.

2.2.3 Laboratory Procedures and Cumulus Sampling

Laboratory procedures and cumulus sampling procedures were performed as previously published by our laboratory and as per the clinical procedures in place at Repromed (Feil et al. 2008). All media and oil products were purchased from Vitrolife, Kungsbacka, Sweden, unless otherwise indicated. All cultures were performed at 37°C, 6% CO_2, 5% O_2, 89% N_2, in a humidified atmosphere, with manipulations being performed at 37°C, 6% CO_2. COC's were isolated from follicular fluid and washed in glucose supplemented (2.5 mM glucose) GFERT plus medium. COC's were stored in 1mL of the same medium for 3 h post-oocyte collection for both IVF and ICSI inseminations. Following the 3 h period, COC's were trimmed of their outer layers of cumulus cells with a 30 gauge needle on a syringe and collected individually. All cumulus cell samples were collected prior to insemination and stored frozen in a minimal volume of culture media at -80°C until required for analysis. Each cumulus mass and its respective oocyte were numbered to track which cumulus mass came from which oocyte. Oocytes undergoing ICSI were then exposed to 75 IU Hyaluronidase (Hyalase®, Aventis Pharma Pty Ltd, Lane Cove, Australia) in glucose supplemented GFERT and then ICSI performed in G1.3 plus medium and cultured singly in 10 µL drops of the same medium under oil for 16-18h. Oocytes undergoing IVF had the inner layers of cumulus left intact and were co-incubated with 1,000 motile sperm in 10 µL drops of glucose supplemented GFERT plus under oil for 17-19 h. All oocytes underwent individual culture in order to track resultant embryos with respect to fertilization, timed cleavage, embryo cell number and quality, as well as pregnancy following SET. Fertilization was assessed 16-19 h post-insemination and all embryos with two pronuclei continued culture individually in 10 µL drops of G1.3 plus medium under oil for the next 48 h. All embryos were then washed thoroughly through G2.3 Plus medium and cultured for another 48 h of culture at which point embryos were transferred to fresh G2.3 medium for the final 24 h of culture.

2.2.4 Embryo Quality Assessments

Embryo quality assessments were based on multiple observations. Embryo transfers were performed on days 2/3 (cleavage stage embryos n=30) or days 4/5 for extended culture (n=8). The day of embryo transfer was a clinical decision determined prior to the commencement of the patient's treatment by their clinician, and was independent of the response to stimulation or the number of embryos resulting from treatment. Embryo selection for transfer was based on morphology assessment, and in all cases the single highest morphological quality embryo, as determined by the appropriate scoring system (cleavage or extended culture stage), was transferred. The cleavage stage morphology scoring system used was based on the number of cells, the degree of fragmentation and the presence of multinucleated cells. Good quality embryos were assessed to have 4-cells on day 2 or 7-9 cells on day 3 with limited (< 10%) fragmentation and an absence of multinucleated blastomeres. Extended culture selections performed for day 4 embryo replacements were performed according to the scoring system described by Feil et al., 2008, with the degree of compaction and early cavitation being assessed (Feil et al. 2008). Blastocyst development on day 5 was assessed as per the grading system described by Gardner and Schoolcraft (1999), describing degree of expansion and quality of the inner cell mass and trophectoderm (Gardner and Schoolcraft 1999). Independent of the day of transfer embryos were scored using a scale of 1 to 4, where grade 1 indicates best quality and grade 4 indicates poor quality. Embryos selected for transfer were incubated in EmbryoGlue (Vitrolife) for 0.5-4 h before transfer and transferred into the uterus in a volume of approximately 10 µL, under ultrasound guidance. All oocytes were fertilised in individual culture, with resultant embryos cultured singly and all patients undergoing single embryo transfer only.

2.2.5 Pregnancy Outcomes

Clinical pregnancy was determined by a positive serum hCG test, the presence of a foetal heart beat 6 weeks following embryo transfer and was retrospectively analysed. Further to clinical pregnancy, patients who achieved pregnancy were tracked throughout gestation to the end point of live birth. Pregnancy outcomes were obtained from the obstetrician in charge of the patient care. Obstetricians were asked to provide delivery dates, birth weights, sex and whether there were any maternal or neonatal interventions or complications.

2.2.6 Animals

All mice (F1 C57Bl/6 X CBA) were maintained on a 12 h: 12 h day/night cycle with rodent chow and water provided ad libitum. All murine experiments were approved by the University of Adelaide's ethics committee and were conducted in accordance with the Australian Code of Practise for the Care and Use of Animals for Scientific Purposes.

2.2.7 Agarose gel electrophoresis

Restriction digests or PCR products were separated by a standard method of agarose gel electrophoresis in a gel containing 1% (w/v) agarose (Promega Corporation, Annandale, NSW, Australia)/0.5X TBE (44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA pH 8.0) and 2 µg/mL Ethidium Bromide. Electrophoresis was performed in 0.5X TBE buffer at 100 V. To each sample, 5X loading buffer (25% (v/v) glycerol, 50 mM EDTA, 0.25% (w/v) bromophenol blue) was added to a final 1X concentration. To determine size and concentration of resolved DNA fragments the following DNA markers were utilised, SPP1/EcoRI and pUC19/HpaII (Geneworks, Adelaide Australia) and 100 bp ladder (Promega Corporation, Annandale, NSW, Australia). Gels were visualised using a UV transilluminator and gel documentation system (Kodak DC120).

2.2.8 RNA Isolation and Real Time RT-PCR

Cumulus cell RNA was extracted using a commercial RNA isolation kit (Ambion RNAqueous Micro, Austin, TX, USA) as per the manufacturer's instructions. Total RNA was eluted in 20 µL of elution buffer and stored at –80°C. Total RNA was then treated with 1 U of DNase as per manufacturer's instructions (Ambion Inc., Austin, TX, USA). First-strand complementary DNA (cDNA) was synthesized from total RNA using random hexamer primers (Geneworks, Hindmarsh, SA, Australia) and Superscript III reverse transcriptase (Invitrogen Australia Pty. Ltd). Specific gene primers for real time RT–PCR were designed against published mRNA sequences (NCBI Pubmed database) using Primer Express software (PE Applied Biosystems, Foster City, CA) and synthesised by Sigma Genosys (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). Primer sequences that detect human V1 *VCAN* splice variant were described previously (Corps et al. 2004). Real time RT–PCR [first described by (Mullis et al. 1986)] was performed in triplicate for each sample on the Corbett Rotor Gene 6000 as per the manufacturer's instructions (Corbett Life Science, Qiagen, Doncaster, Victoria, Australia). In each reaction, 2 µL of cDNA (equivalent to 1000ng of total cDNA), 0.2 µL of forward and reverse primers and 10 µL of SYBR Green (Applied Biosystems, Scoresby, VIC, Australia) master mix were added, with H_2O added to make

a final volume of 20 µL. All primers were used at an optimised concentration of 50 µM with the exception of *18S* primers, which were used at 2 µM. PCR cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 amplification cycles of 95°C for 15 s and 60°C for 1 min. Controls included omission of the cDNA template or RT enzyme in otherwise complete reaction mixtures; each showed no evidence of product amplification or primer dimers. Following real time RT–PCR, analysis of the dissociation curves confirmed that a single product was amplified in all reactions. The most stable real time RT-PCR loading control was determined using the GeNorm housekeeper analysis software (Vandesompele et al. 2002). The housekeeper *GAPDH* was chosen as the loading control with the most stable expression between samples as determined by GeNorm. Primer sequences are as per Table 2.1.

Table 2.2 Human Real Time Primer Sequences

Gene	Primer Name	Amplicon size (bp)	Sequence (5'-3')	Accession No.
VERSICAN (V1)	hV1 F	122	TGAGAACCCTGTATCGTTTTGAGA	X15998
VERSICAN (V1)	hV1 R		CTGAATCTATTGGATGACCAATTACAC	
PTGS2	hCOX2 F	93	TGGAACATGGAATTACCCAGTTTG	NM_000963
PTGS2	hCOX2 R		AATGTTCCACCCGCAGTACAGA	
PTX3	hPTX3 F	80	ACATTAGCCTTCTCTGGGAGACTCA	NM_002852
PTX3	hPTX3 R		GCAATGAAGAGATAAGAGAGACCGG	
HAS2	hHAS2 F	120	GCAAGTCCTACTTCCGAGAATGG	NM_005238
HAS2	hHAS2 R		CCTTTCTTTCTCATTGCCACAGTAA	
TNFAIP6	hTSG6 F	77	GGATTTCATGTCTGTGCTGCTGGAT	NM_007115
TNFAIP6	hTSG6 R		GTGAAGCCAGGGCCCAACTG	
GREM1	hGREM1 F	113	CTCTGCCACAAGAATGCAATTTC	NM_013372
GREM1	hGREM1 R		CATAAGTGCAGATTTGGCTCAAGT	
AHR	hAHR F	84	TGCACCGATGGGAAATGATACTATCC	NM_001621
AHR	hAHR R		GAACTCATTTGCTGGAGGTCACCC	
STS	hSTS F	95	CACCTAACCCCCCCATATCTGTTCA	NM_000351
STS	hSTS R		GATGTCTGGTCAACTGGCCTGCCT	
LDHA	hLDHA F	80	AACACCAAAGATTGTCTCTGGCAAAGAC	NM_005566
LDHA	hLDHA R		TATCACGGCTGGGGCACGT	
PKM2	hPKM2 F	72	TGCCGCCTGGACATTGATTCA	NM_182471
PKM2	hPKM2 R		TCATCTGTACCATTGGCCCAGCT	
PFKP	hPFKP F	77	AAGTACTTGGAAGAGATCGCCACACAGA	NM_002627
PFKP	hPFKP R		CTGATCATCGGTGGATTCGAGGC	
PFKL	hPFKL F	102	CAGATGACCAAGGAAGTGCAGAAAGC	NM_001002021
PFKL	hPFKL R		AGCTTCGAGAACAACTGGAACATTTACAAG	
PFKM	hPFKM F	78	GGAGAGCGTTTCGATGATGCTTCAG	NM_000289
PFKM	hPFKM R		TGCAAGGACTTTCGGGAACGAGA	

ALDOLASE	hALDOA F	84	CAAATCCAAGGGCGGTGTTGTG	NM_000034
ALDOLASE	hALDOA R		GACAAATGGCGAGACTACCACCCA	
AR	hAR F	87	GCTGAAGGGAAACAGAAGTACCT	NM_000044
AR	hAR R		ACGACAAGATGGACAATTTTTCCT	
GAPDH	hGAPDH F	70	TTCCACCCATGGCAAATTCC	NM_002046
GAPDH	hGAPDH R		AAGCTTGTCATCAATGGAAATCC	
18S	H18S F	91	AGAAACGGCTACCACATCCAA	M10098
18S	H18S R		AGGTAGTGACGAAAAATAACAATACAGG	
MRPL19	hL19 F/R	124	n/a	NM_000479
MRPL19			n/a	

2.2.9 Standard Curves

Real time RT-PCR standard curves were produced for all genes of interest and internal controls using plasmid DNA (as described in detail in Chapter 3). PCR products were generated and inserted into TOPO 2.1 or TOPO 4.0 vectors (Invitrogen, Carlsbad, CA, USA), then transformed into chemically competent *E. coli* host strain TOP10 cells (Invitrogen) as per the manufacturer's instructions. The presence of gene inserts was confirmed by PCR and restriction digestion of recombinant plasmids. The confirmed positive recombinant plasmids were purified using the *Wizard Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, USA). The mass of plasmids containing cloned target sequences were calculated based on the size of the TOPO vector and PCR insert based on the derivation of DNA mass formula available from Applied Biosystems (PE Applied Biosystems). For each gene, a series of standard dilutions were generated ranging from 30 copies to $30x10^6$ copies of the plasmid clone per reaction then refined to an appropriate standard curve of five logarithmic concentrations matching the concentration range found in human cumulus cells. Each gene standard curve was linear and showed similar efficiency. For full standard curve generation details see Chapter 3.

CHAPTER 3

GENERATION OF QUANTITATIVE PLASMID DNA STANDARD CURVES

3.1 INTRODUCTION

In order to measure the expression of a large number of genes in very small cumulus biopsy samples comparative methods such as the $2^{-\Delta\Delta CT}$ are impractical because they require a replicate set of PCR reactions for the internal standard run with each specific gene. Furthermore, we wanted to establish a standardised approach to the gene measurement that might be used to define a set of "reference" levels of gene expression useful for future prospective studies or cross-centre comparison of results. Thus it was essential to develop a highly sensitive, quantitative and reproducible real time PCR assay based on a set of standards to estimate gene copy numbers in each sample. The standard curves produced were robust and easily reproducible, and represent the most precise method of quantifying PCR data allowing absolute quantitation of our genes of interest.

Real time PCR analysis often relies on a calibrator or control sample, to which treatments or time points are compared. A calibrator sample acts as a single standard within a PCR run. This calibrator then remains the same for all replicates and experiments to allow comparisons between groups, and subsequent results are interpreted as "relative arbitrary units" relative to that calibrator. Analysis of real time PCR using a calibrator sample requires the use of the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). This method of analysis is regarded as relative quantification, and is a highly recognised method for PCR analysis. However, this method of arbitrary expression is undesirable when developing a reproducible standardised test. The use of the $2^{-\Delta\Delta CT}$ method for real time PCR analysis relies on the existence of a suitable calibrator. The sample set used for our experiments did not contain an appropriate calibrator. Each sample instead represented a treatment outcome, and there was no one sample which could have been labelled as a calibrator above the remaining samples without introducing possible bias or reducing the robust nature of the assay.

Cell lines are a good contender for real time PCR calibrators. A relevant cell line will often express a multitude of genes at high levels. However, for a cell line to be relevant in the present context it must express all the genes of interest for a particular experiment at a comparable expression level – i.e. within a range that is detectable roughly equivalent to the test samples. In our experimental work up the expression of each gene of interest was analysed in a human embryonic kidney cell line (293H), which is known to express a large number of genes. Through expression analysis we were able to characterise the expression of our genes of interest in this cell line, but determined it would not make an appropriate calibrator for all genes of interest. Other cell lines available to us included the breast cancer

cells lines MDA-MB231, MCF7, MCF10A and Hs578t, but each were found lacking in several of our genes of interest. As the overall aim of these experiments was not to characterise gene expression in cell lines, a standard curve approach was undertaken to determine absolute expression. The techniques to isolate and amplify genes or DNA segments and insert them into another cell with precision, creating a transgenic bacteriumuse were first described by Lobban et al (Jackson et al. 1972; Cohen et al. 1973; Lobban and Kaiser 1973).

The PCR primer design employed the *Primer Express™* (Applied Biosystems) software with very strictly constrained design parameters to ensure a rationalised and consistent approach to the design of primers for each gene. Primers were also tested empirically for their efficiency and specificity and primers that amplified only the gene of interest and with efficiency above 85% were accepted and next used to clone a plasmid standard. Generation of a plasmid DNA clone for each gene of interest allows for the estimation of gene copy numbers. This method allows each gene of interest to have a unique standard curve which fits the expression profile for that gene. Standard curves were also assessed using paired cumulus and granulosa cDNA from three independent patients to measure the effectiveness of these assays. These methods development experiments are detailed below

3.2 MATERIALS AND METHODS

3.2.1 PCR Reagents

PCR reagents were obtained from Promega Corporation (Annandale, NSW, Australia) and Applied Biosystems (Scoresby, VIC, Australia). Specific gene primers for real time RT–PCR (Table 2.1) were designed against published mRNA sequences (NCBI Pubmed database) using Primer Express software (PE Applied Biosystems, Foster City, CA) and synthesised by Sigma Genosys (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW, Australia). PCR cycling conditions were 95°C for 10 mins, followed by 35 amplification cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min, followed by 72°C for 7 mins. PCR products were separated by agarose gel electrophoresis in a gel containing 1% (w/v) agarose (Promega Corporation, Annandale, NSW, Australia)/0.5X TBE (44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA pH 8.0) and 2 µg/mL Ethidium Bromide. Electrophoresis was performed in 0.5X TBE buffer at 100 V. Gels were visualised using a UV transilluminator and gel documentation system (Kodak DC120). Genes with bands which displayed primer dimers were cut from the gel and purified with the Qiagen QIAquick gel purification kit (Qiagen, Doncaster, VIC, Australia).

3.2.2 Lysogeny Broth and Agar Plates

LB Agar and LB Broth (Luria-Bertani) were made by dissolving 10 g Tryptone, 5 g Yeast Extract, 5 g NaCl with 1 mL of 1M NaOH and made up to 1 L with additional MilliQ deionised water. Broth was autoclaved on liquid cycle and stored in a cool, dry place. LB Agar was made by the addition of 15 g/L LB Agar to broth and heated to dissolve. Mixture was cooled to ~55ºC before adding ampicillin (100 µg/mL) and poured into 10 cm plates. Agar was allowed to harden, then inverted and stored at +4°C in the dark. Plates were pre-warmed to room temperature for one hour before use.

3.2.3 Amplification and sub-cloning of gene sequences of interest

3.2.3.1 Producing a PCR product for cloning

For each gene of interest a *Taq* polymerase-amplified PCR product was generated using conventional PCR amplification methods (Table 3.1).

Table 3.1 Reactions for generation of PCR products for cloning.

PCR Reagent	Volume (µL)
5x Buffer	5
$MgCl_2$ (25 mM)	5
10mM dNTPs	0.5
Forward Primer	0.5
Reverse Primer	0.5
GoTaq Polymerase	0.125
cDNA	2 (equal to 100ng template)
PCR grade H_20	11.38
TOTAL	25

3.2.3.2 Sub-cloning of PCR products into TOPO vector

The TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) is a one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector. The cloning reaction was set up using the reagents below (Table 3.2), in the order presented.

Table 3.2 Constituents of the TOPO cloning reaction.

Reagent	Volume (µL)
Fresh PCR Product	0.5 to 4
Salt Solution	1
H_20	Add to total volume of 5µL
TOPO® Vector	1
TOTAL	6

(The amount of fresh PCR product used for each reaction was dependent on the intensity of the band visualised on the gel. An excess of PCR product or not enough reduces the efficiency of the TOPO® reaction). The TOPO® reaction was mixed gently and incubated for 5 mins at room temperature (22-23ºC) then placed on ice.

3.2.3.3 Transformation of TOPO® construct into competent E. Coli cells

For each gene of interest, 2 µL of the TOPO® Cloning reaction, as produced above, was transformed into a single vial of One Shot® Chemically Competent *E. Coli*. The reaction was incubated on ice for 5 minutes, then heat-shocked for 30 secs at 42°C and immediately transferred to ice. 250 µL of room temperature S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and the tubes were allowed to shake horizontally

(220 rpm) at 37°C for 1 h. 50-150 µL from each transformation was spread on at least two pre-warmed plates and incubated overnight at 37°C.

3.2.3.4 PCR analysis of transformed colonies containing gene insert

Following overnight incubation up to 20 colonies were picked for each gene of interest, and analysed using conventional PCR with the TOPO® *M13* forward (5´-GTAAAACGACGGCCAG-3´) and reverse (5´-CAGGAAACAGCTATGAC-3´) primers. For each colony a PCR master mix was made as described above in section 3.2.3.1 ***"producing a PCR product"*** with the following modifications: 13.38 µL of PCR grade water was added to each reaction, and the cDNA template omitted. Individual transformed cell colonies were picked from LB-Amp plates with a sterile yellow pipette tip and mixed briefly with the master mix then placed in 2 mL of inoculated Luria broth (LB, 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) containing 100 µg/mL ampicillin (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia). Colonies were numbered for later identification. The TOPO® *M13* forward and reverse primers were added at a concentration of 40 µM. The conventional PCR thermal profile remains as in section 3.2.3.1 ***"producing a PCR product"***.

PCR products were separated by agarose gel electrophoresis as described in section 2.2.7. PCR products representing the *M13* TOPO® vector without an insert migrated in the gel at 201 base pairs for the TOPO® 2.1 vector or 243 base pairs for the TOPO® 4.0 vector. A DNA band representing the TOPO® vector plus the insert for the gene of interest were identifiable by a band equal to the size of the inserted gene of interest plus the TOPO® vector flanking sequence as specified above by the *M13* TOPO® primers (i.e. Gene insert base pairs + *M13* TOPO® base pairs = expected band height).

3.2.3.5 Purification of plasmid DNA

LB broth cultures of clones positively identified by PCR (as described above) were subjected to plasmid purification using the Promega Wizard® *Plus* SV Minipreps DNA Purification System (Promega Corporation, Annandale, NSW, Australia) according to the manufacturer's instructions as follows: 1.5-3 mL of bacterial culture was pelleted by centrifugation for 5 mins at 10,000 x *g*. The supernatant was discarded and 250 µL of Cell Resuspension Solution was added and the cell pellet was completely resuspended. 250 µL of Cell Lysis Solution was added and thoroughly mixed, followed by 10 µL of Alkaline Protease Solution which was mixed by inverting the tube, then incubated for 5 mins at room

temperature. Alkaline protease inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA. 350 µL of Neutralisation Solution was added and mixed by inverting. The bacterial lysate was centrifuged at maximum speed for 10 mins at room temperature. The cleared lysate was transferred to a spin column. Care was taken to avoid disturbing or transferring any of the white precipitate with the supernatant. The supernatant was centrifuged at maximum speed for 1 min at room temperature, the flow through discarded and the spin column reused. 750 µL of Column Wash Solution, previously diluted with 95% ethanol, was added to the spin column, and the mixture was centrifuged at maximum speed for 1 min at room temperature and the flowthrough discarded. The wash procedure was repeated using 250 µL of Column Wash Solution, and the column centrifuged at maximum speed for 2 mins at room temperature. The spin column was transferred to a new, sterile 1.5 mL microcentrifuge tube. The purified plasmid DNA was eluted by adding 45 µL of Nuclease-Free Water to the spin column and centrifuged at maximum speed for 1 min at room temperature. Purified plasmid DNA was stored at -20 ºC.

3.2.3.6 PCR analysis of purified PCR product with specific gene primers

Purified plasmid DNA was again analysed using conventional PCR using the gene specific primers for each respective gene of interest (Table 2.1). For each purified plasmid sample a PCR master mix is made as described above in 3.2.3.1 ***"producing a PCR product"*** with a master mix reaction made for each set of gene specific primers. Purified plasmid DNA mass was quantified and purity (OD ratio 260/280) was assessed by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The gene specific primers were added at the optimal concentrations as determined in the real time PCR optimisation experiments. The conventional PCR thermal profile remains as in 3.2.3.1 ***"producing a PCR product"***. PCR products were separated by agarose gel electrophoresis as described in section 2.2.7. DNA bands for each plasmid of interest were assessed based on the expected height of each amplicon (Table 2.1).

3.2.3.7 Restriction Enzyme Digestion

Restriction analysis was performed to confirm the presence and correct orientation of each insert. Restriction enzymes were purchased from Promega Corporation (Annandale, NSW, Australia). Typically, restriction digests were performed in a 10 µL reaction containing 200ng-1µg of DNA, 2-5U of the appropriate restriction enzyme (*EcoR1, HindII, NotI, BamHI, EcoRV, Kpn1*), 1 µL of the manufacturer's recommended 10X Buffer (*Buffer H*) and sterile H_2O to 10 µL. Digests were incubated in

a dry block for 1 h at 37ºC. Restriction digests were separated by agarose gel electrophoresis in a gel containing 1% (w/v) agarose (Promega Corporation, Annandale, NSW, Australia)/0.5X TBE (44.5 mM Tris, 44.5 mM Boric acid, 1 mM EDTA pH 8.0) and 2 µg/mL Ethidium Bromide. Electrophoresis was performed as described in section 2.2.7.

3.2.4 Calculation of plasmid standard curves

The approach to generate the plasmid DNA standard curves was based on the Applied Biosystems "Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR" (document can be found at: www6.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf)

Standard curves of purified plasmid DNA were calculated based on the mass of the DNA:

Derivation of DNA mass formula is as follows:

$$m = (n)\left(\frac{1\ \text{mole}}{6.023e23\ \text{molecules (bp)}}\right)\left(\frac{660g}{\text{mole}}\right) = (n)\left(\frac{1.096e21\ g}{bp}\right)$$

n = DNA size (bp)

m = mass

Avogadro's number = 6.023e23 molecules / 1 mole

Average molecular weight of a double-stranded DNA molecule = 660 g/mole

To calculate the mass of a single plasmid molecule the size value of the entire plasmid (plasmid + insert) is added to the following equation:

$$m = (n)\left(\frac{1\ \text{mole}}{6.023e23\ \text{molecules (bp)}}\right)\left(\frac{1.096e21\ g}{bp}\right)$$

n = plasmid size (base pairs)

m = mass

e-21 = $x10^{-21}$

The mass of the plasmid containing the copy numbers of interest was then calculated, to determine the plasmid mass needed for 30 to 30,000,000 copies for each gene of interest:

Copy # of interest x mass of single plasmid = mass of plasmid DNA needed

The above equation determines the plasmid mass needed to achieve the copy number of interest. To do this we divided the mass needed (calculated above) by the volume to be pipetted into each reaction (in this case 2 µL per PCR reaction). Cloned sequences are highly concentrated in purified plasmid DNA stocks. A series of serial dilutions was performed to achieve a working stock of plasmid DNA for quantitative real time PCR applications. Serial dilutions of the plasmid DNA were performed to obtain plasmid DNA at workable concentrations. Once the plasmid was at a workable concentration, the following formula was use to calculate the volume needed to prepare the 30,000,000 copy standard dilution:

$C_1V_1 = C_2V_2$

This equation was used to determine the dilutions required to prepare the remaining standards, 3,000,000 to 30 copies. The calculation used to determine dilutions required to make the plasmid standards are based on some errors but are intended to provide a well standardised assay. The calculation of the mass of a single plasmid molecule was based on an assumption that across the plasmid all nucleotides are represented equally and therefore the mass of each nucleotide used for calculating the mass of each plasmid containing an insert represented an average of the mass of each nucleotide. An example calculation for the dilutions to make the standards detailed below.

Table 3.3 Mass of *VCAN* gene insert and plasmid and subsequent mass of plasmid DNA needed to generate copy number of interest.

		Copy Number	Mass DNA needed (g)
***VERSICAN* (bp)**	122	30000000	1.34085e-10
Plasmid (bp)	3956	3000000	1.34085e-11
Total bp	4078	300000	1.34085e-12
		30000	1.34085e-13
Mass (g)	4.46949e-18	3000	1.34085e-14
		300	1.34085e-15
		30	1.34085e-16

Table 3.4 Conversion of purified VERSICAN plasmid DNA concentrations to working units required for dilutions of plasmid standards.

NanoDrop ng/µL	µg/µL	mg/µL	g/µL
389.69	0.38969	0.00038969	3.8969e-07

Table 3.5 Dilutions of purified *VCAN* plasmid DNA required to generate standard curves at copy numbers of interest.

Copies	Initial Conc. (g/µL)	Volume (µL)	Diluent Volume (µL)	Final Volume (µL)	Final Conc. (g/µL)	Total mass in 2µL
	3.8969e-07	10	990	1000	3.8969e-09	
30000000	3.8969e-09	1.72	98.28	100	6.70423e-11	1.34085e-10
3000000	3.8969e-11	10	90	100	6.70423e-12	1.34085e-11
300000	3.8969e-12	10	90	100	6.70423e-13	1.34085e-12
30000	3.8969e-13	10	90	100	6.70423e-14	1.34085e-13
3000	3.8969e-14	10	90	100	6.70423e-15	1.34085e-14
300	3.8969e-15	10	90	100	6.70423e-16	1.34085e-15
30	3.8969e-16	10	90	100	6.70423e-17	1.34085e-16

3.2.5 Validation of plasmid standard curves by real time RT-PCR

Standard curve assays were validated by real time RT-PCR analysis. In each reaction, 2 µL of plasmid DNA dilutions containing the appropriate mass equivalent to 30 – $3x10^7$ plasmid copies (equivalent to mass of plasmid DNA needed to achieve the copy number of interest), 0.2 µL of forward and reverse primers, 10 µL of SYBR Green (Applied Biosystems, Scoresby, VIC, Australia) master mix were added and H_2O to a final volume of 20 µL. All primers were used at a concentration of 50 µM with the exception of human *18S* primers, which were used at a concentration of 2 µM, and human *MRPL19* Quantitect® primer assay primers (Qiagen, Doncaster, VIC, Australia), which were used as per manufacturer's instructions. PCR cycling conditions were 50ºC for 2 mins, 95ºC for 10 mins, followed by 40 amplification cycles of 95ºC for 15 secs and 60ºC for 1 min. Controls were represented by the omission of the plasmid DNA. Following real time RT-PCR, analysis of the dissociation curves confirmed that a single product was amplified in all reactions. Real time RT-PCR analysis to refine the standard curves for each gene of interest to five appropriate points was performed using cDNA from pooled human cumulus cells, cDNA from three matched cumulus cell and granulosa cell pairs, and cDNA from 293H cells.

3.3 RESULTS

The results in this chapter validate the experimental approach aimed to develop a highly sensitive, quantitative and reproducible real time PCR assay based on a set of standards to estimate gene copy numbers in each sample. The standard curves produced were robust and easily reproducible, and represented a precise method of quantifying PCR data allowing absolute quantitation of our genes of interest. The results in this chapter represent the plasmid standard curves that were generated for each gene of interest and tested for efficiency using real time RT-PCR. Each plasmid standard curve was then refined to a five-point standard curve which was most appropriate for each gene based on the copy number expression of the experimental control samples. Further validation of the quantifiable nature of each set of plasmid standards was performed on various cDNA samples available within the laboratory. These expression analyses included real time RT-PCR on three sets of patient matched pairs of human cumulus and mural granulosa cell pairs (Figures 3.1 to 3.18).

Each patient pair is represented by different coloured bars in the graphs detailed below (Patient A = yellow, Patient B = light blue, Patient C = green), with each bar representing the mean expression within that sample following real time RT-PCR analysis in triplicate (therefore each coloured bar is representative of a single measurement rather than a pooling of multiple samples or patients). An additional pooled cumulus cell sample is also represented through this series of graphs (dark blue bars) which was generated through the pooling of multiple cumulus masses from a number of patients (patients were not necessarily part of other experiments presented in this thesis). This pooled cumulus sample was generated to run as an internal control across all further real time RT-PCR runs as a quality control measure for the standard curves. Throughout this series, the patients' samples remain the same for each gene under investigation.

This analysis allowed for the extrapolation of real time RT-PCR to generate a gene copy number for each sample. For each gene of interest, this validation protocol was able to demonstrate the sensitivity of the standard curves produced, illustrating that a small change in the sample CT results in a marked change in gene copy number. Further analysis of the relative gene copy number is presented for each gene of interest relative to the copy number of the housekeeper *GAPDH* (Figure 3.19 to 3.21). For Figures 3.19 to 3.21 the independent measures for each of the cumulus and granulosa cell pairs were combined to visually assess the variability between patients across the genes of interest. The goal of these experiments was to validate the standard curves generated for further experiments and get an

indication of variability between and within patients which would help determine appropriate patient numbers for later analyses. For this reason no thorough statistical analysis was performed on the combined real time RT-PCR data, and a simple paired t-test confirmed there was no significant difference in the expression of any of the genes measured when comparing cumulus and granulosa cell gene expression (data not shown).

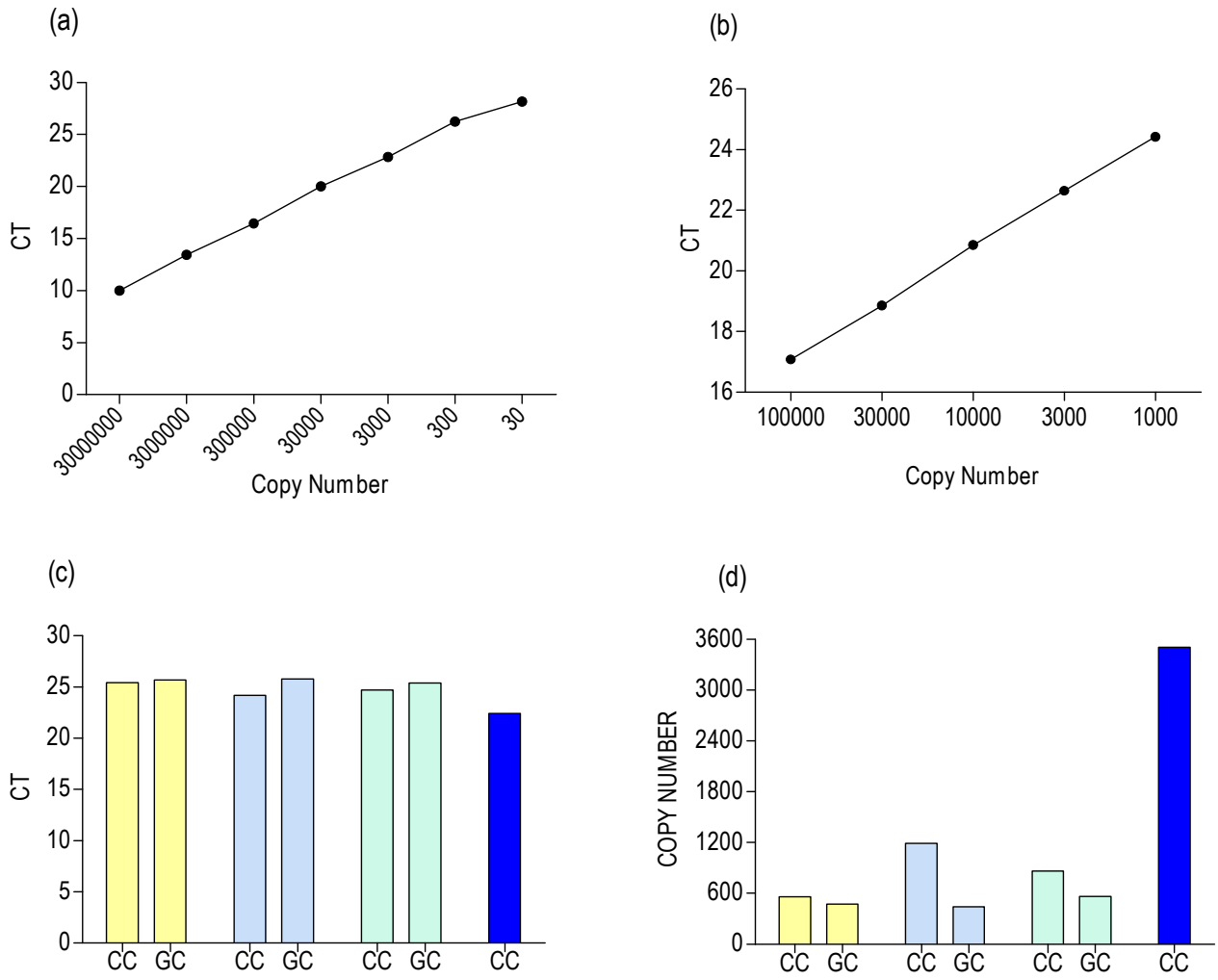

Figure 3.1 Real time RT-PCR validation of the human *VCAN* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *VCAN*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *VCAN* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following in vivo stimulation.

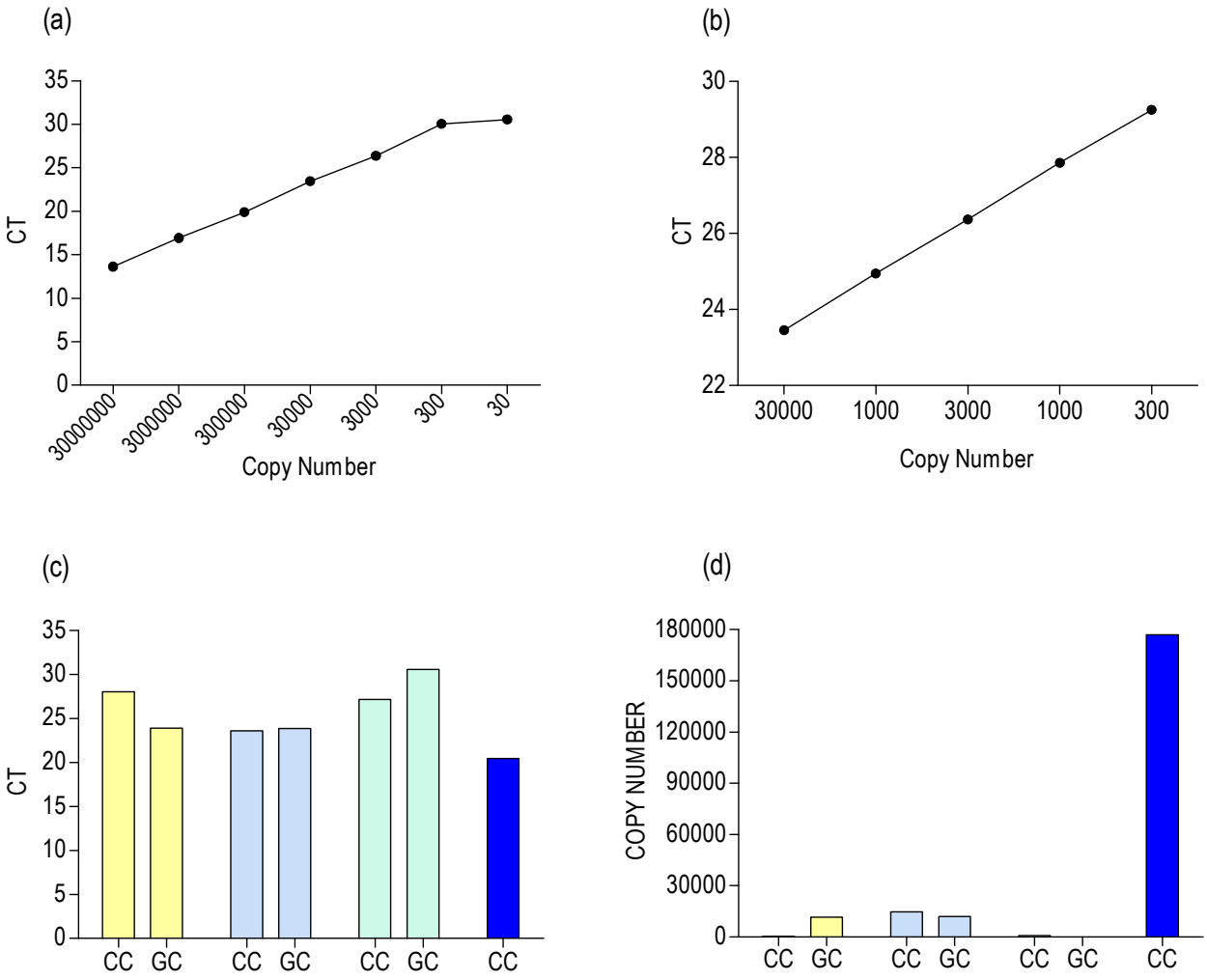

Figure 3.2 Real time RT-PCR validation of the human *PTX3* plasmid DNA standard curves. (a) Real time RT-PCR of sub-cloned *PTX3*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *PTX3* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

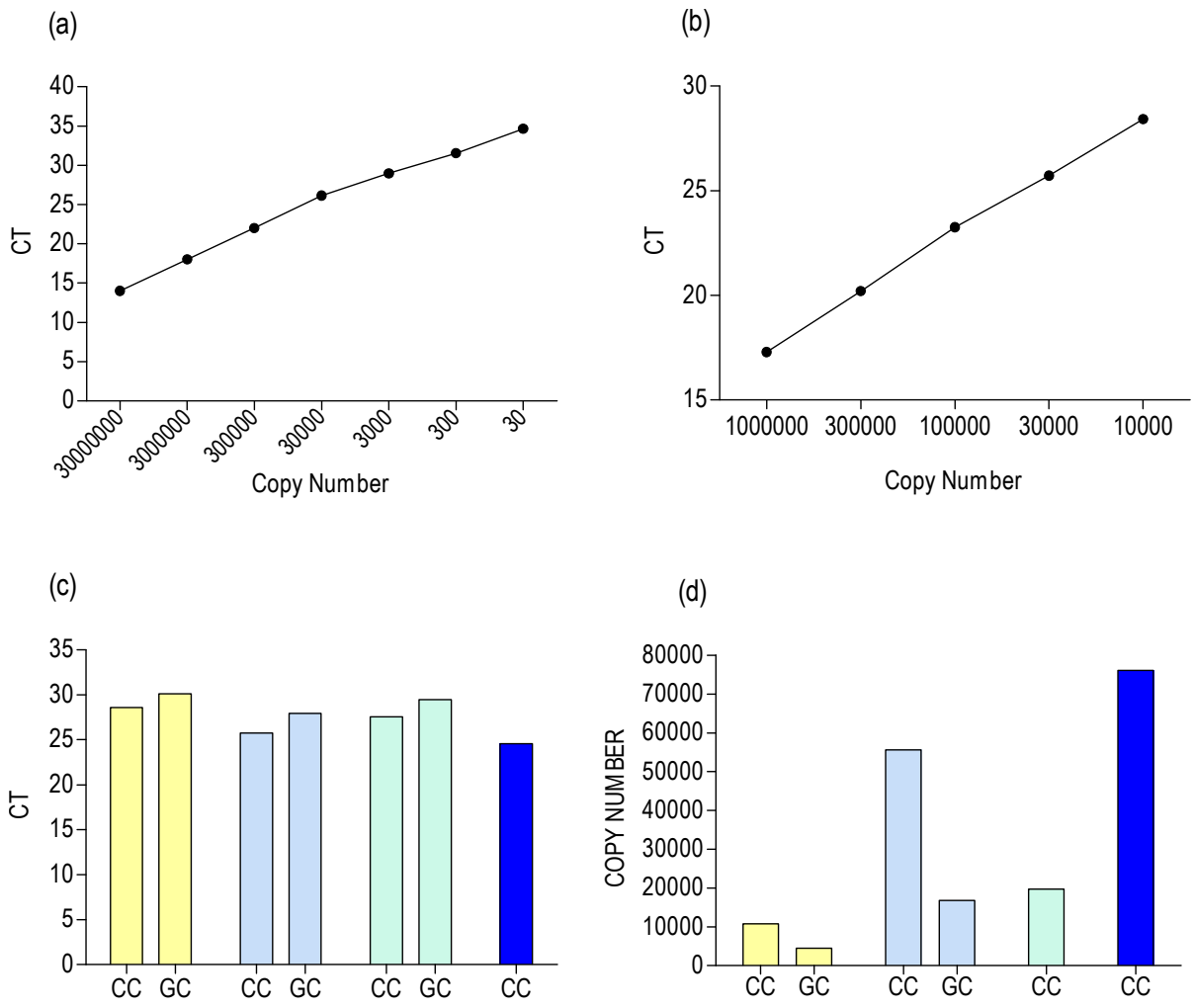

Figure 3.3 Real time RT-PCR validation of the human *HAS2* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *HAS2*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *HAS2* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation. On figure (d) the third patient pair (green) did not generate a readable measure for *HAS2* gene expression in the granulosa cell sample which could be reliably extrapolated using the *HAS2*-TOPO plasmid standard curve, therefore this data point is not presented for comparison.

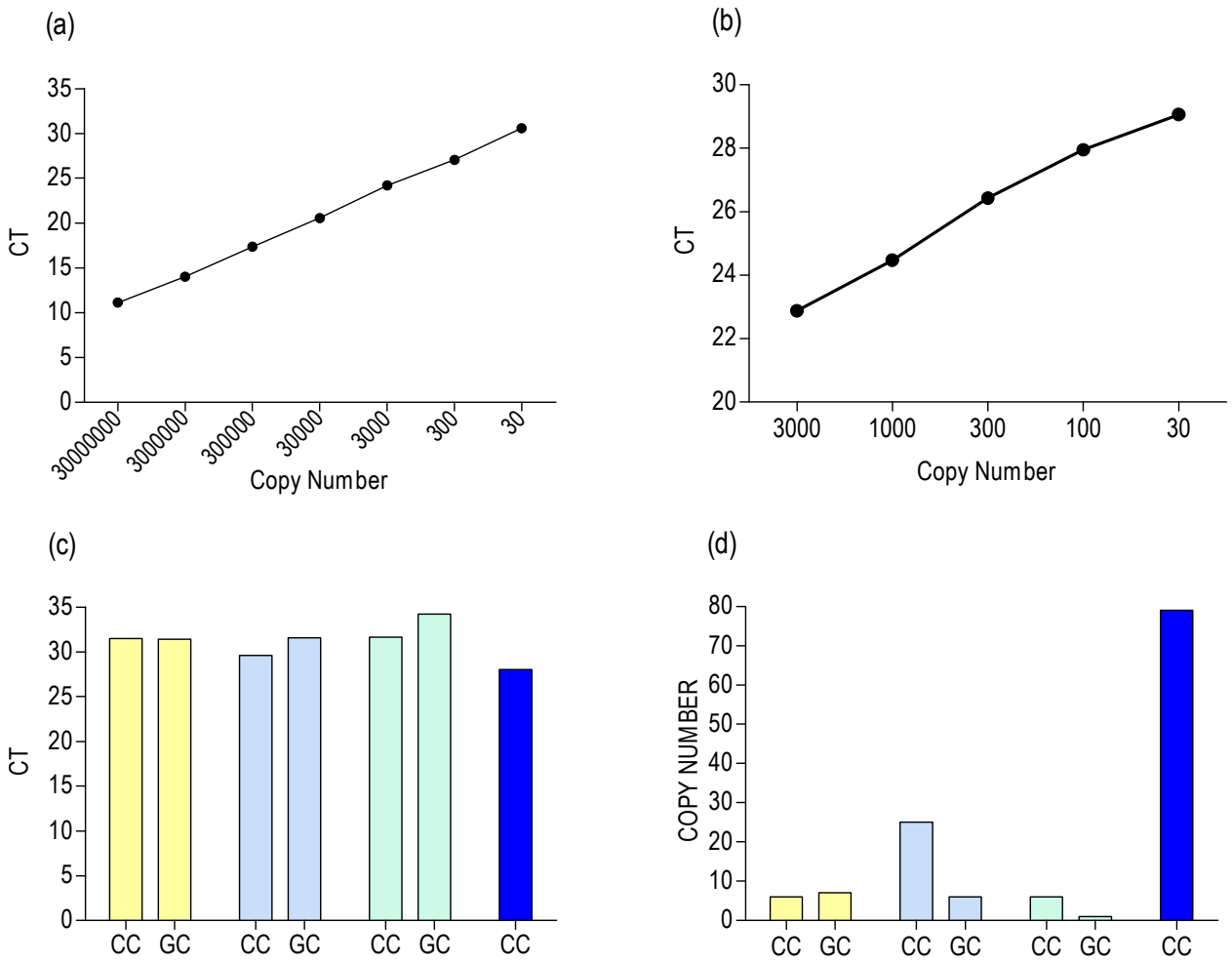

Figure 3.4 Real time RT-PCR validation of the human *TNFAIP6* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *TNFAIP6*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *TNFAIP6* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

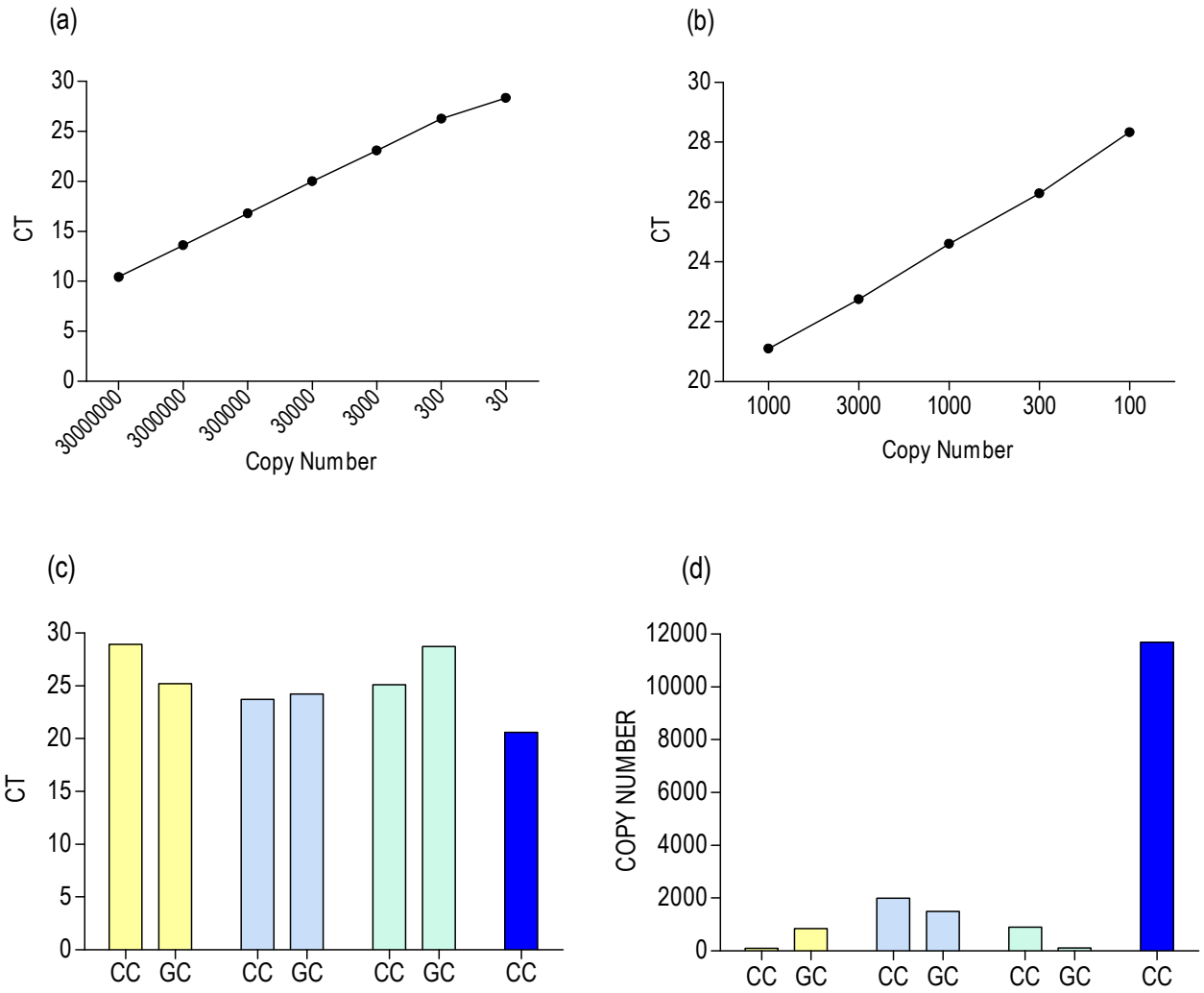

Figure 3.5 Real time RT-PCR validation of the human *PTGS2* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *PTGS2*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *PTGS2* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

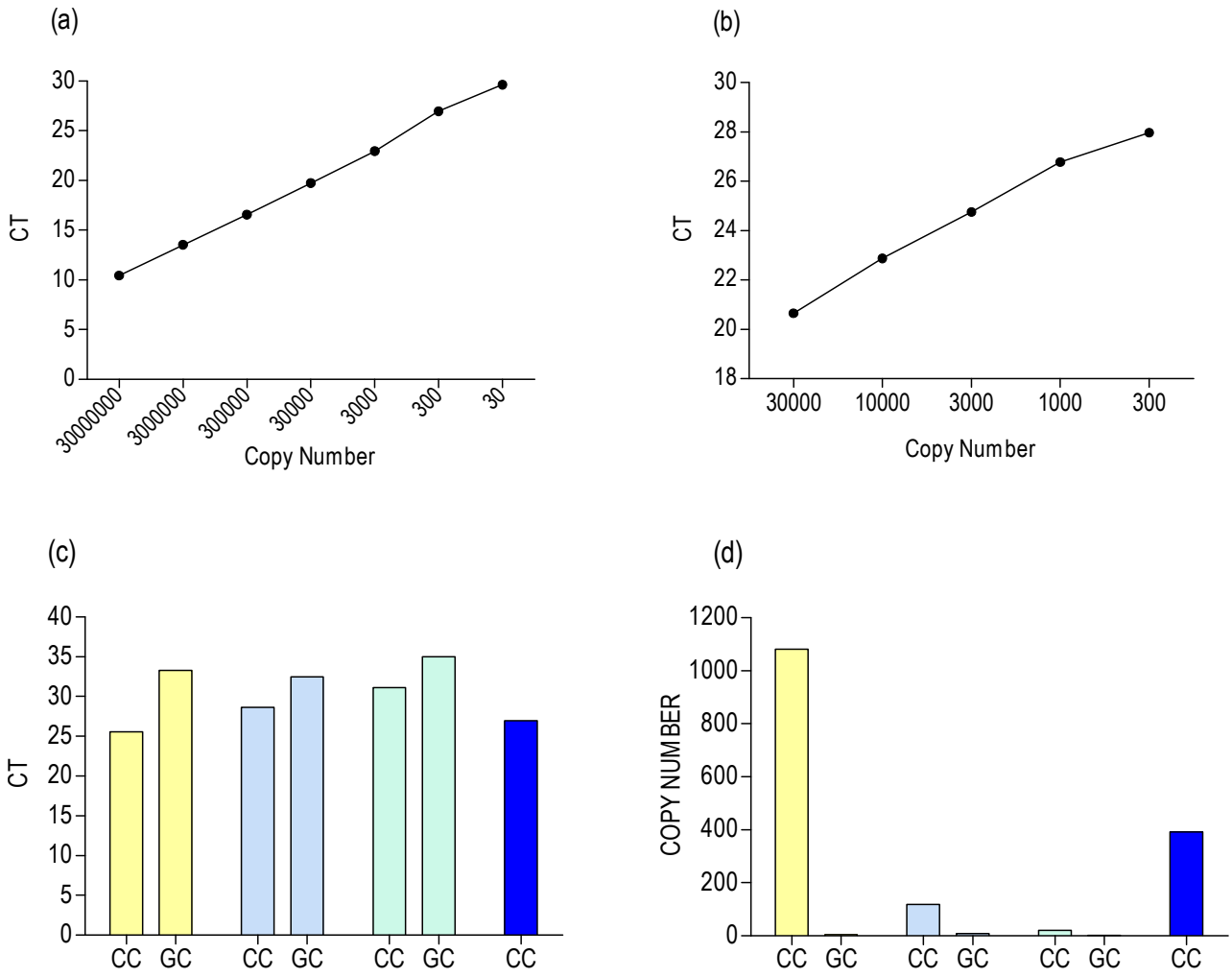

Figure 3.6 Real time RT-PCR validation of the human *GREM1* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *GREM1*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *GREM1* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

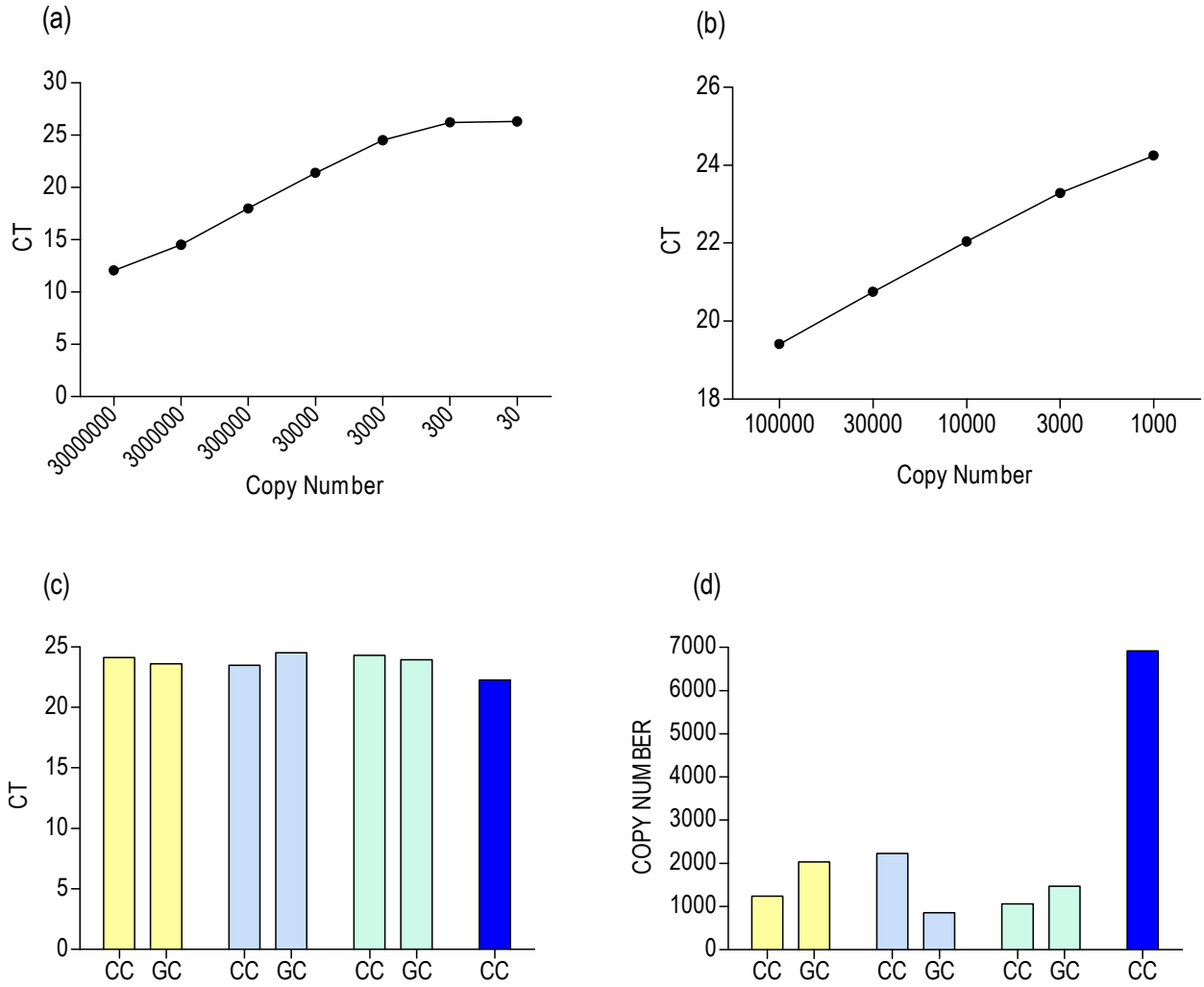

Figure 3.7 Real time RT-PCR validation of the human *AHR* plasmid DNA standard curves

(a) Real time RT-PCR of sub-cloned *AHR*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *AHR* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

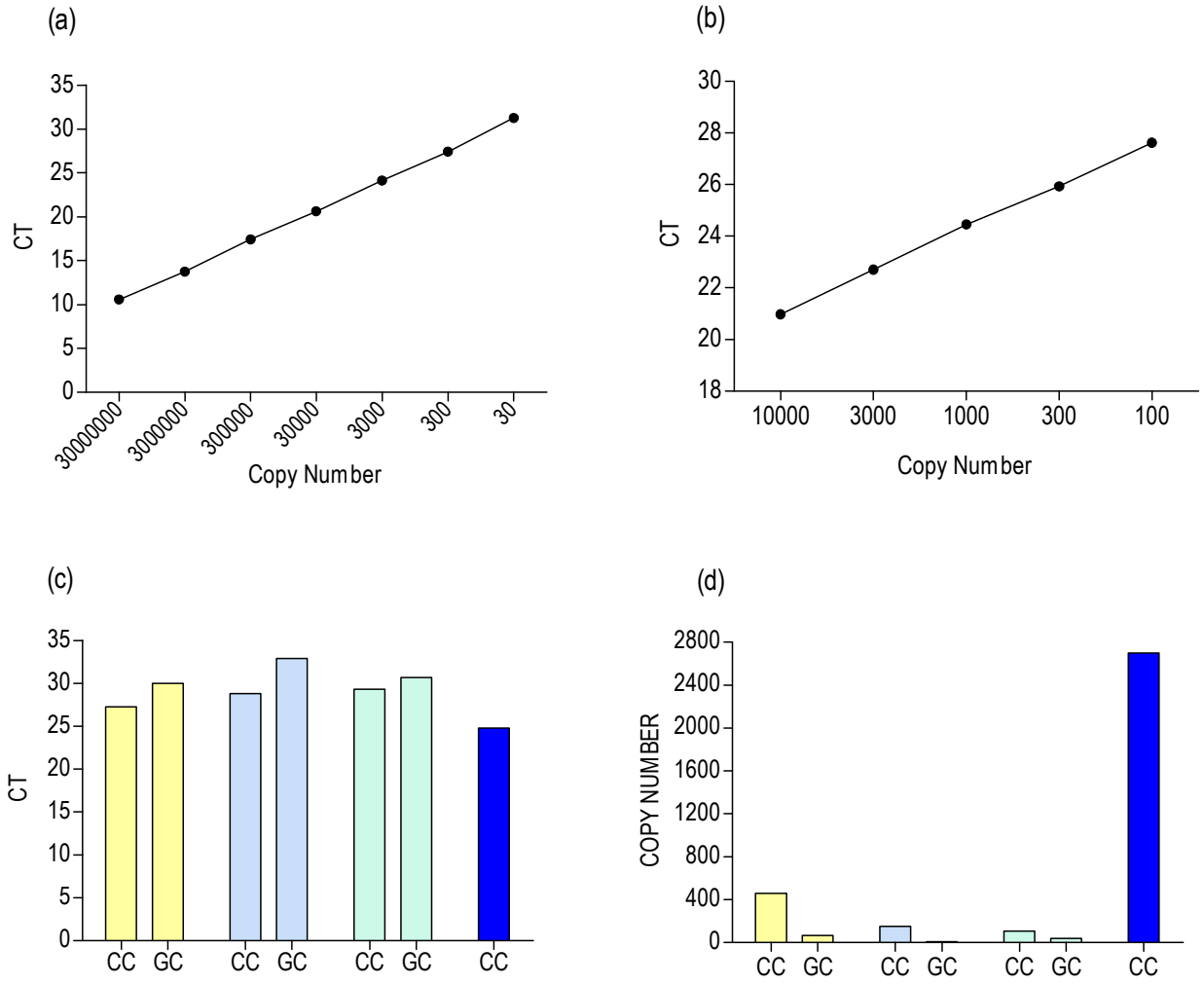

Figure 3.8 Real time RT-PCR validation of the human *AR* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *AR*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *AR* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

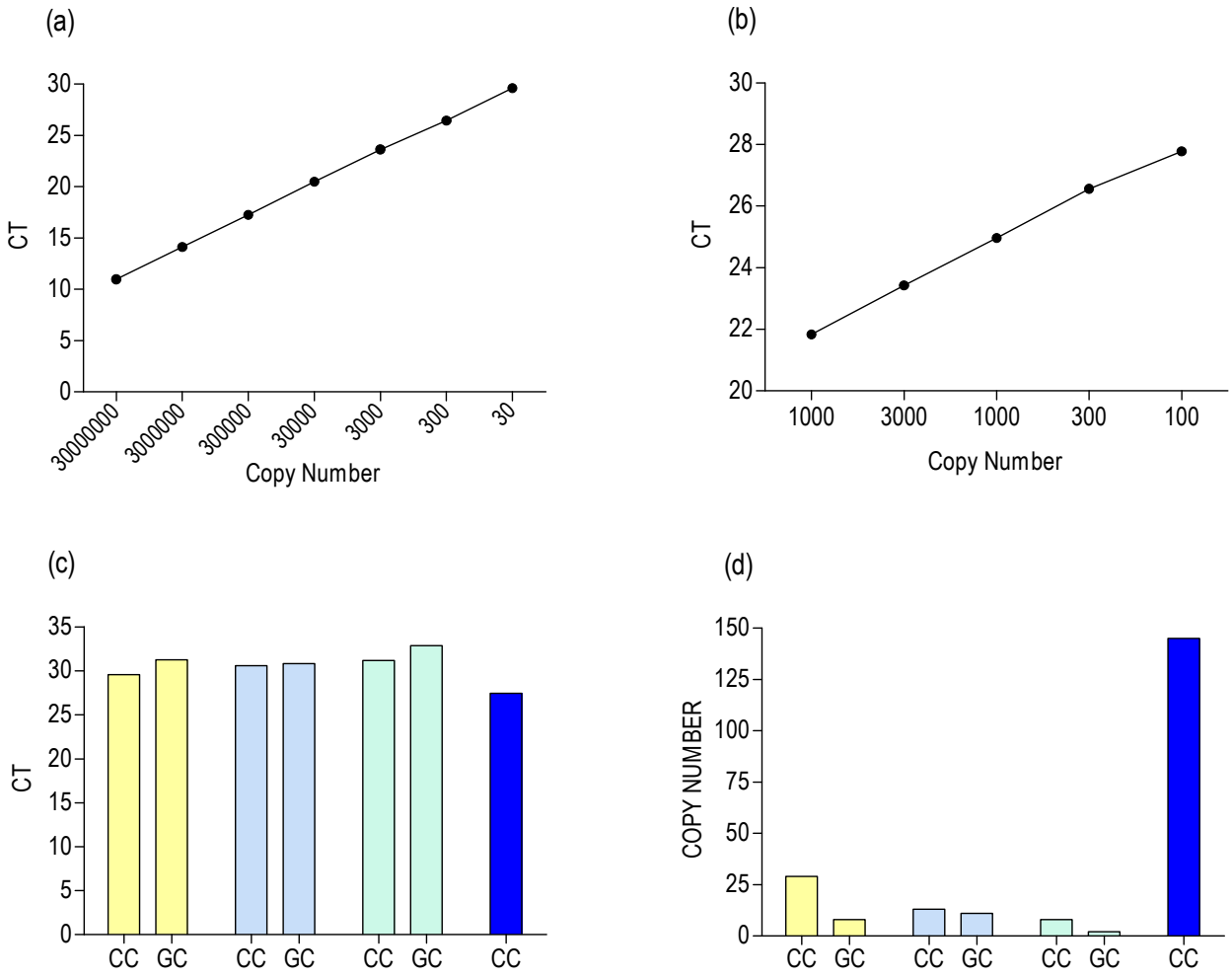

Figure 3.9 Real time RT-PCR validation of the human *STS* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *STS* **-**TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *STS* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

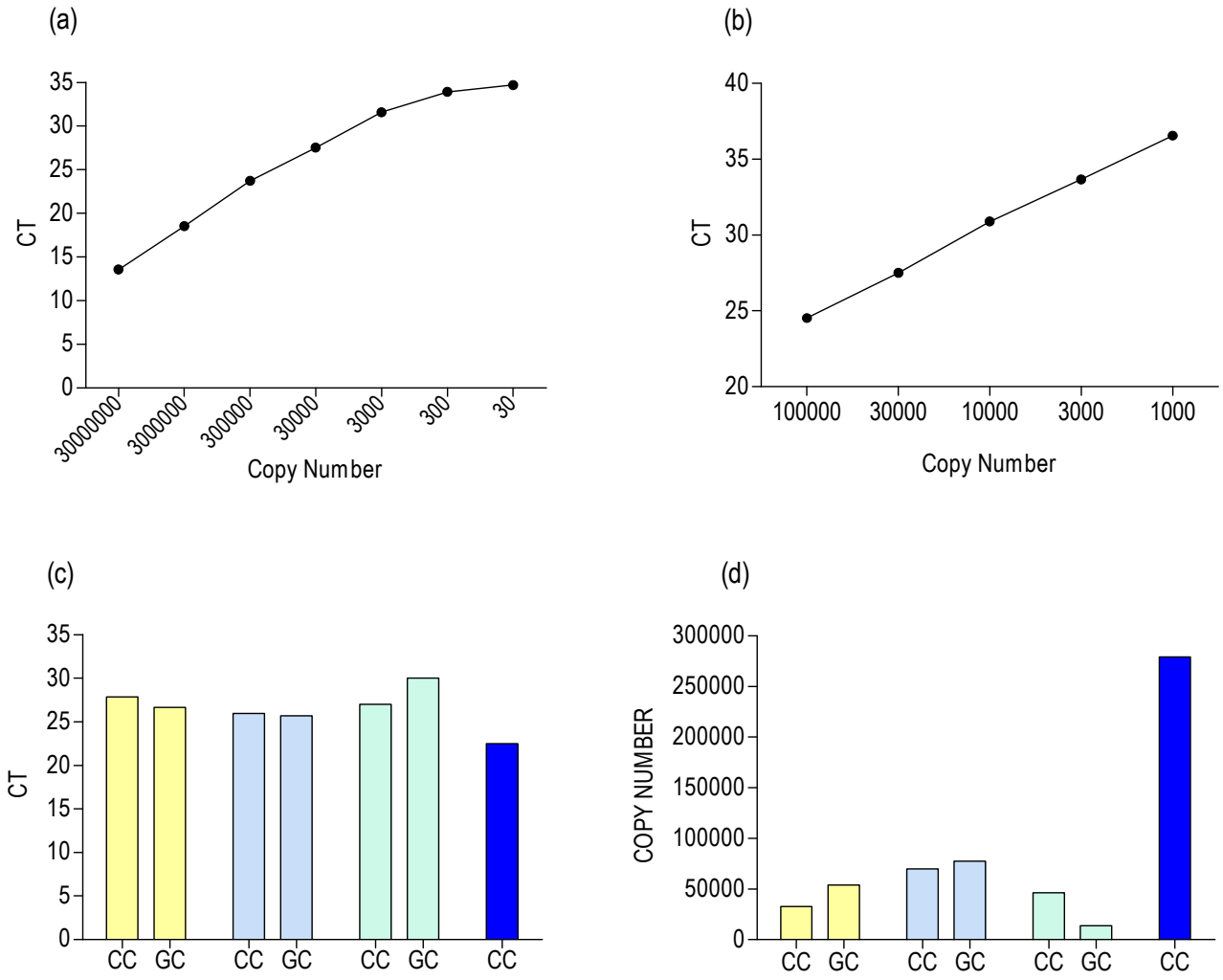

Figure 3.10 Real time RT-PCR validation of the human *LDHA* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *LDHA*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *LDHA* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

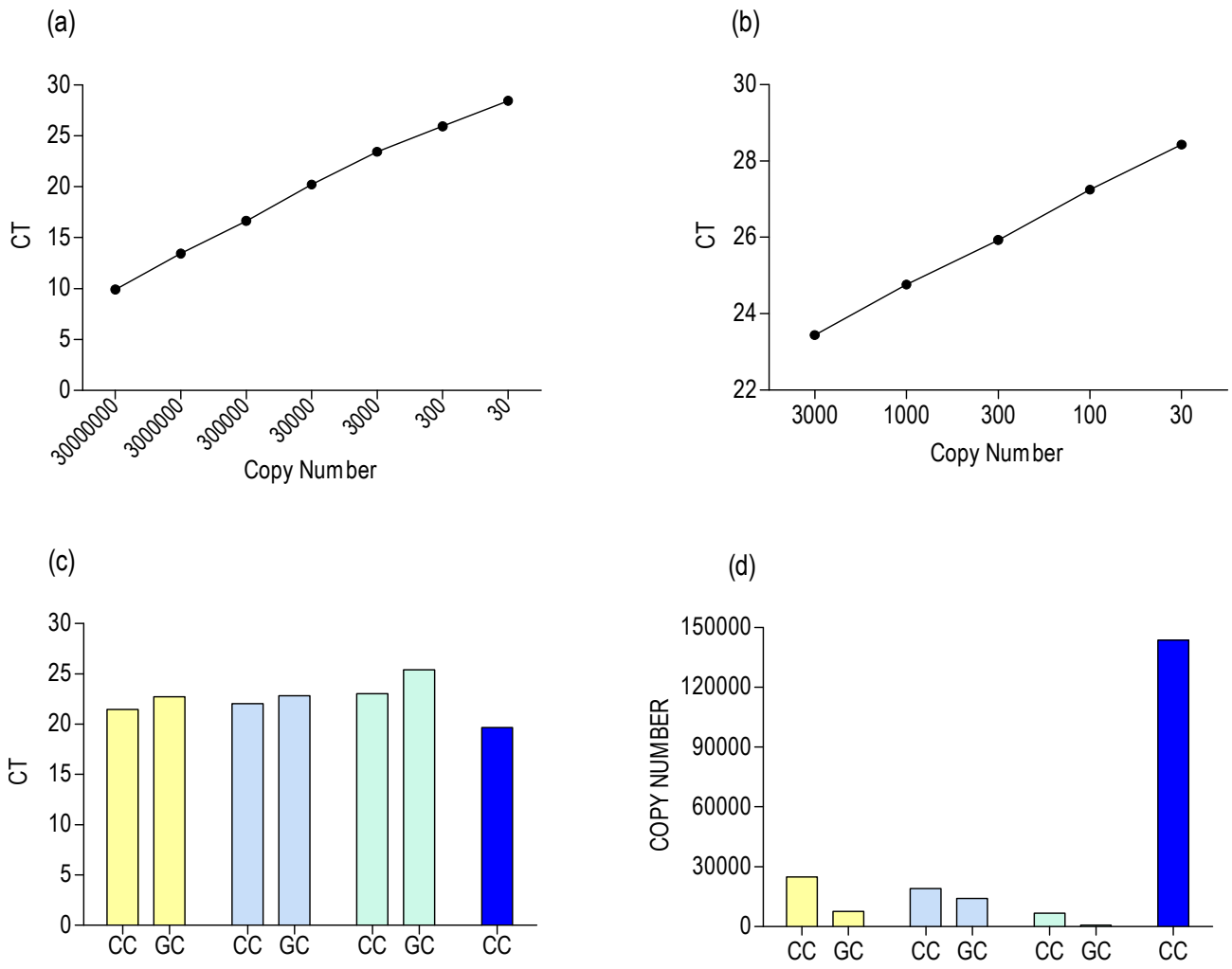

Figure 3.11 Real time RT-PCR validation of the human *ALDOA* plasmid DNA standard curves

(a) Real time RT-PCR of sub-cloned *ALDOA*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *ALDOA* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

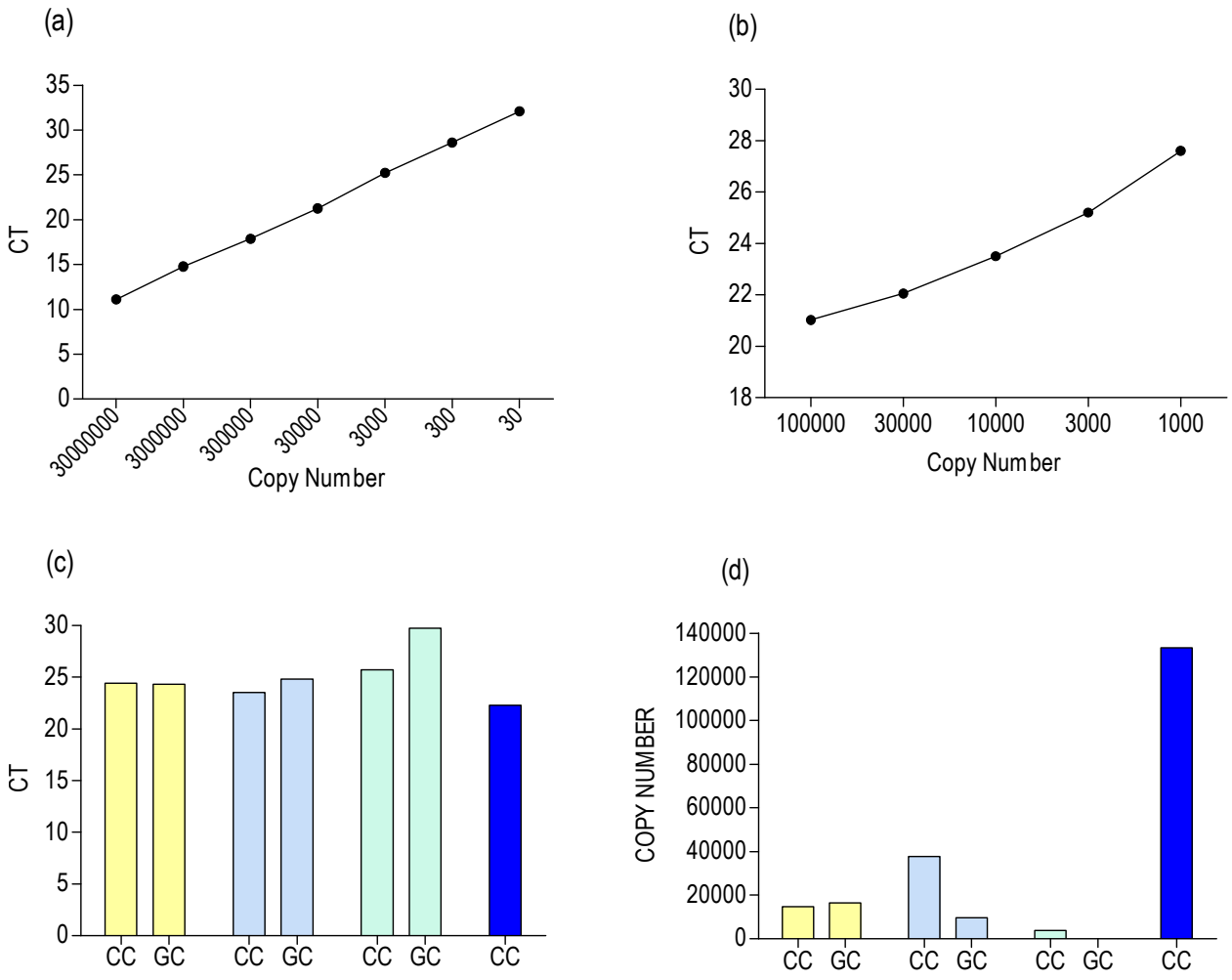

Figure 3.12 Real time RT-PCR validation of the human *PFKP* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *PFKP*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *PFKP* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

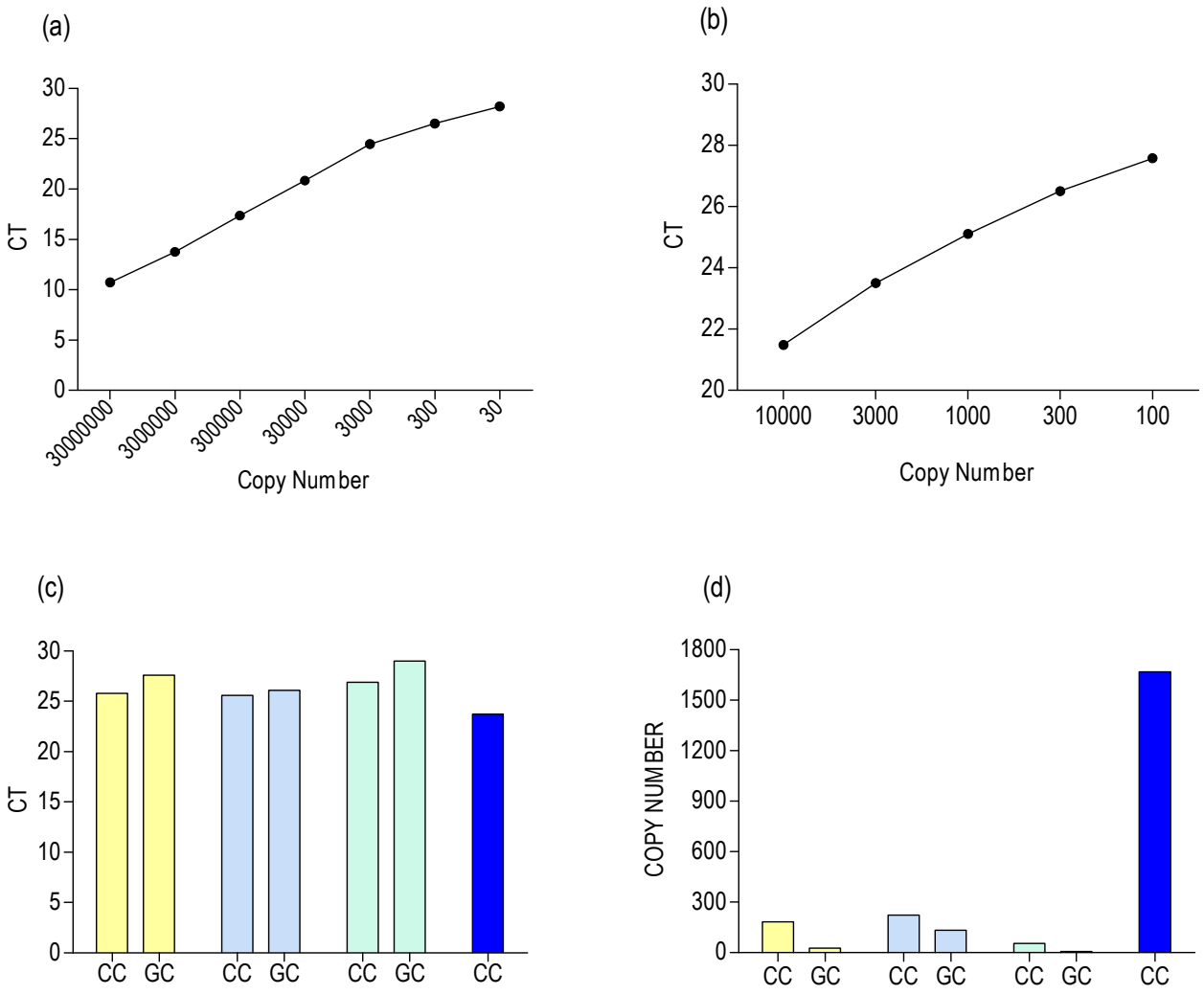

Figure 3.13 Real time RT-PCR validation of the human *PFKL* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *PFKL*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *PFKL* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

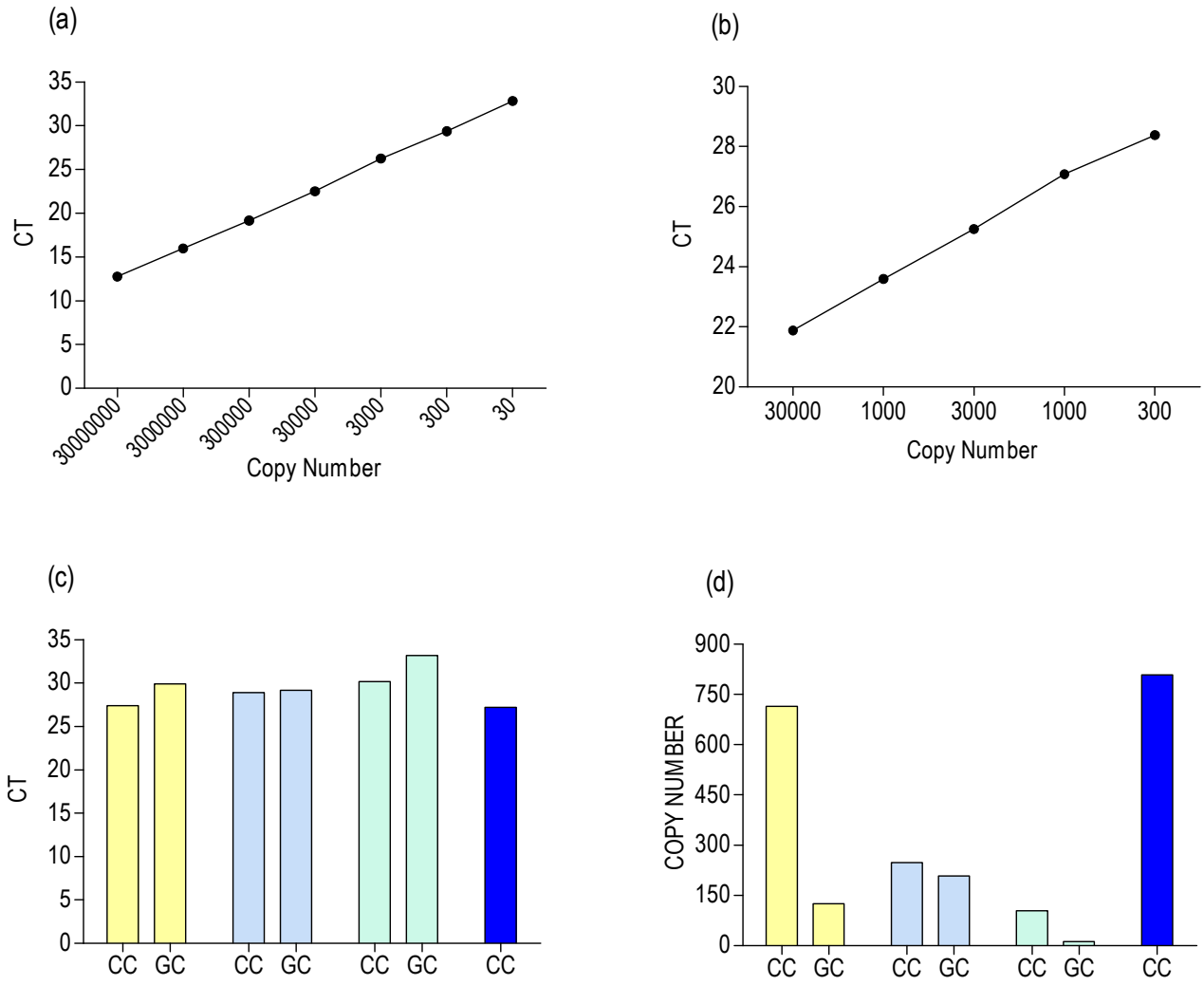

Figure 3.14 Real time RT-PCR validation of the human *PFKM* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *PFKM*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *PFKM* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

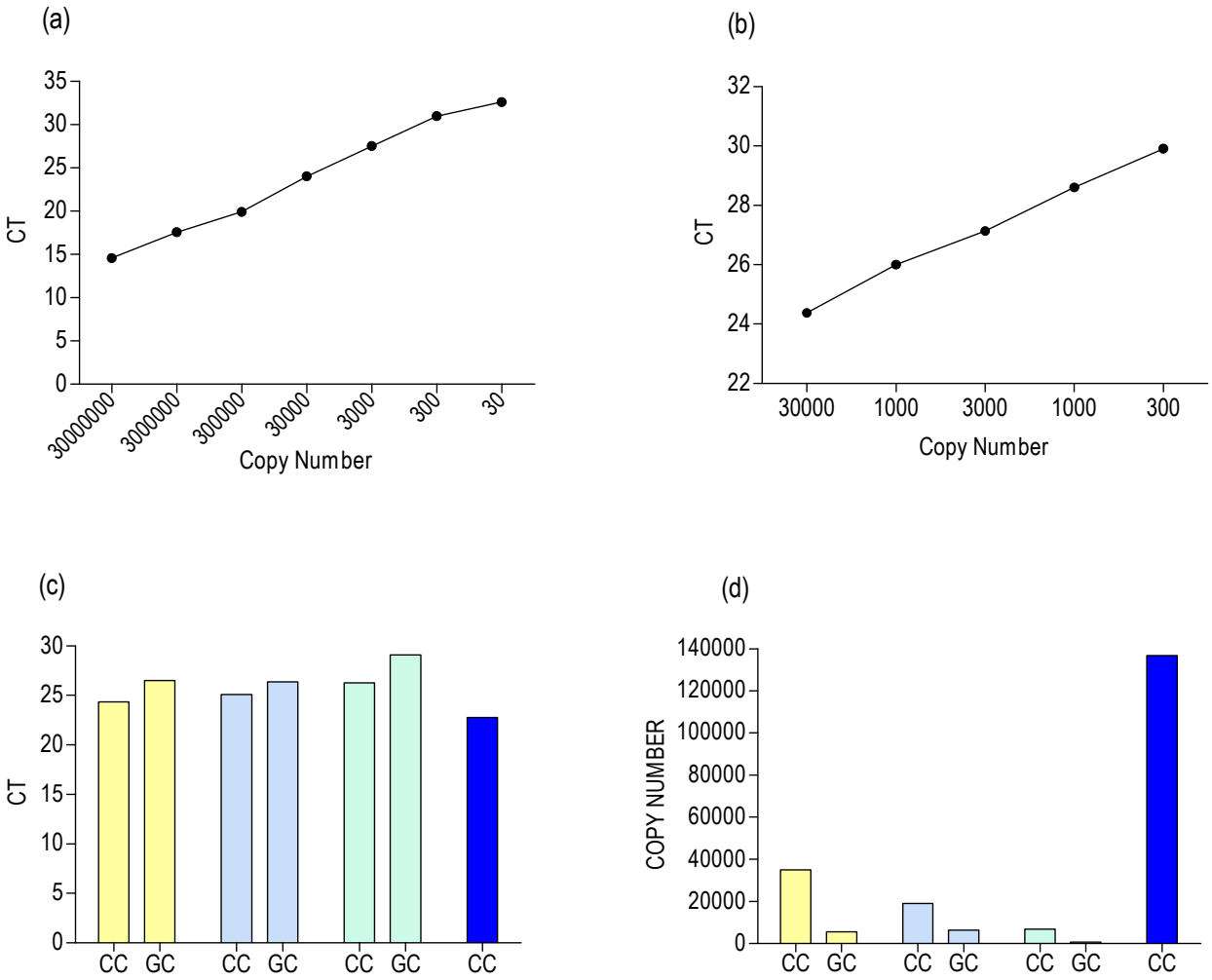

Figure 3.15 Real time RT-PCR validation of the human *PKM2* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *PKM2*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *PKM2* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

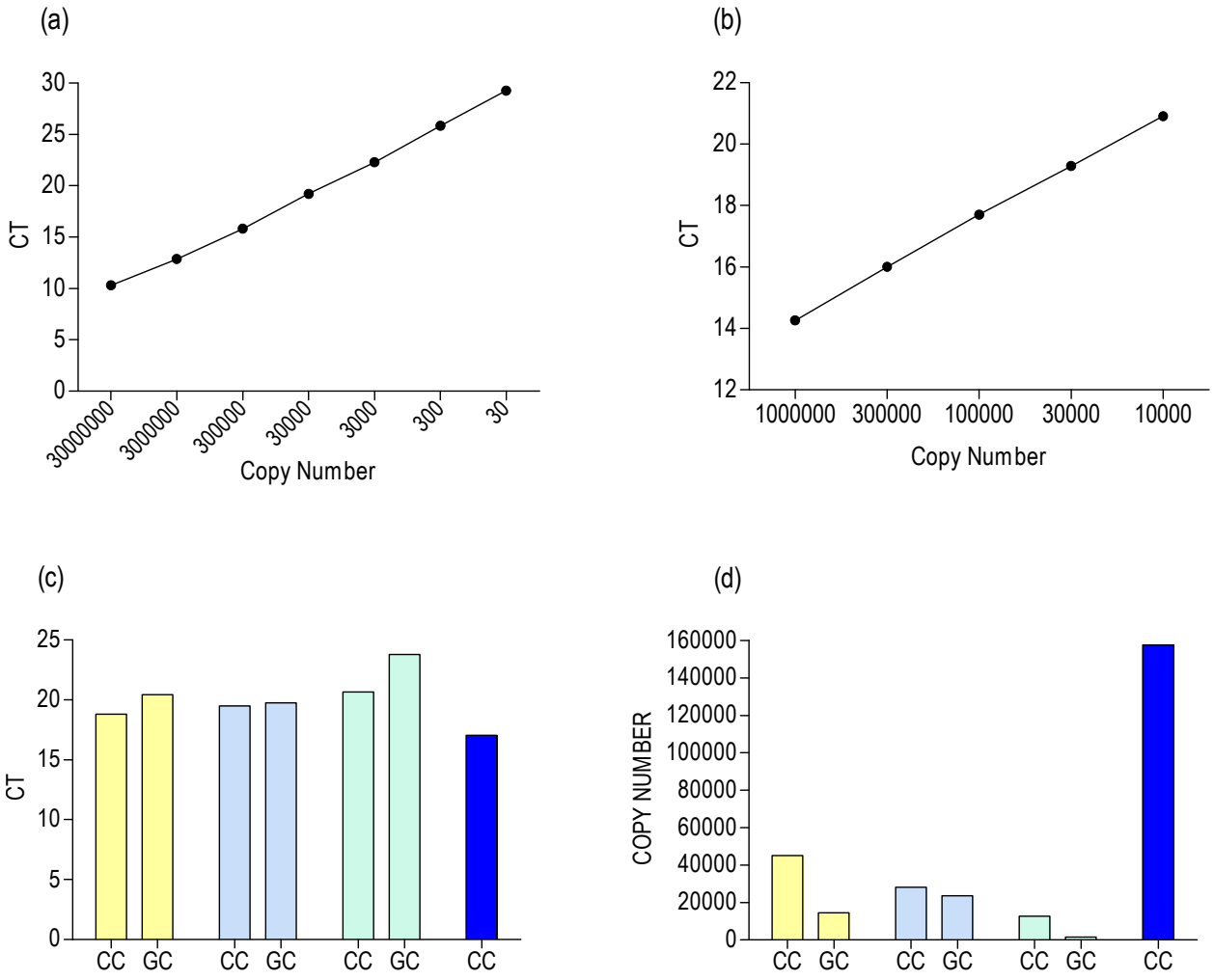

Figure 3.16 Real time RT-PCR validation of the human *GAPDH* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *GAPDH*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *GAPDH* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

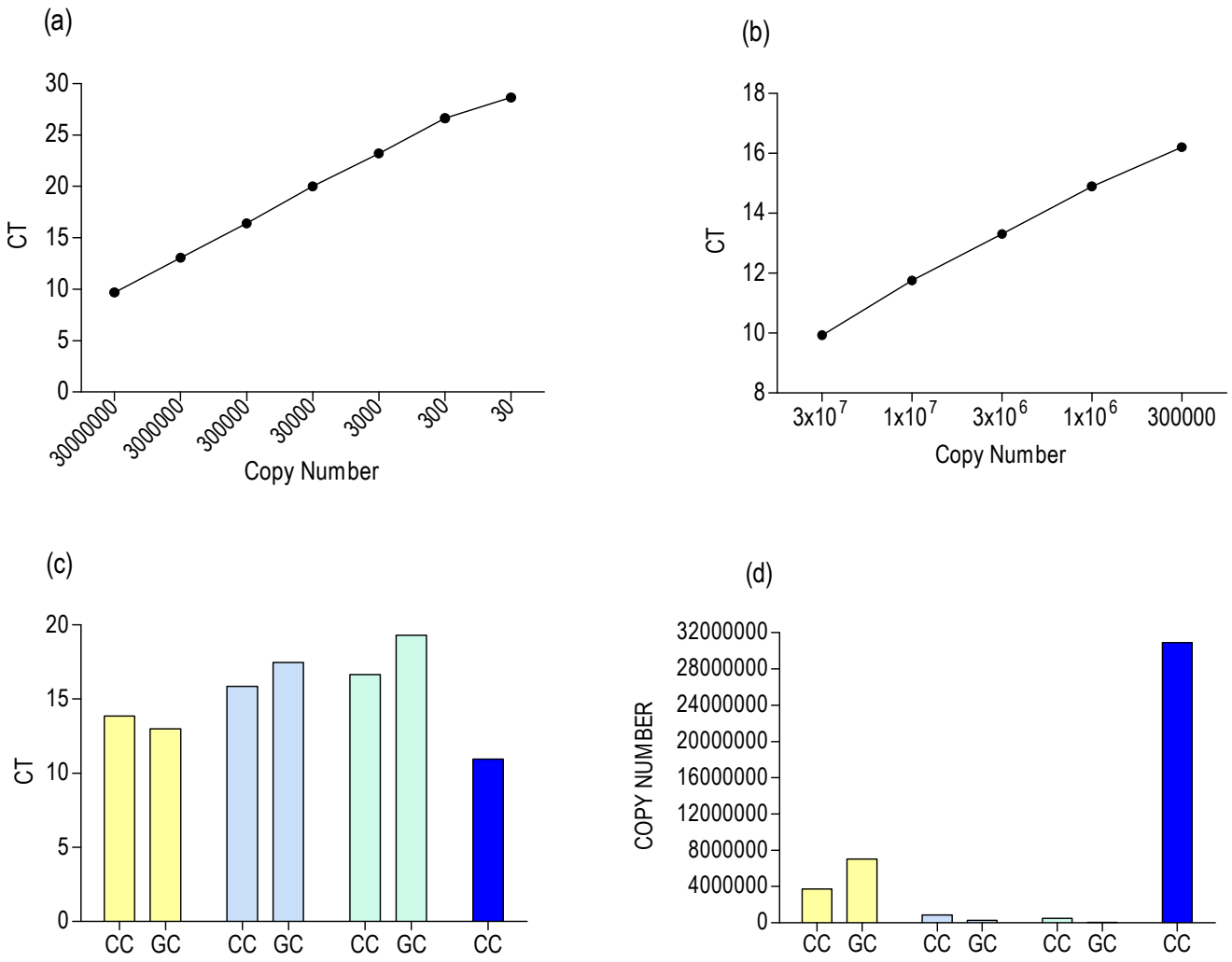

Figure 3.17 Real time RT-PCR validation of the human *18S* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *18S*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *18S* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

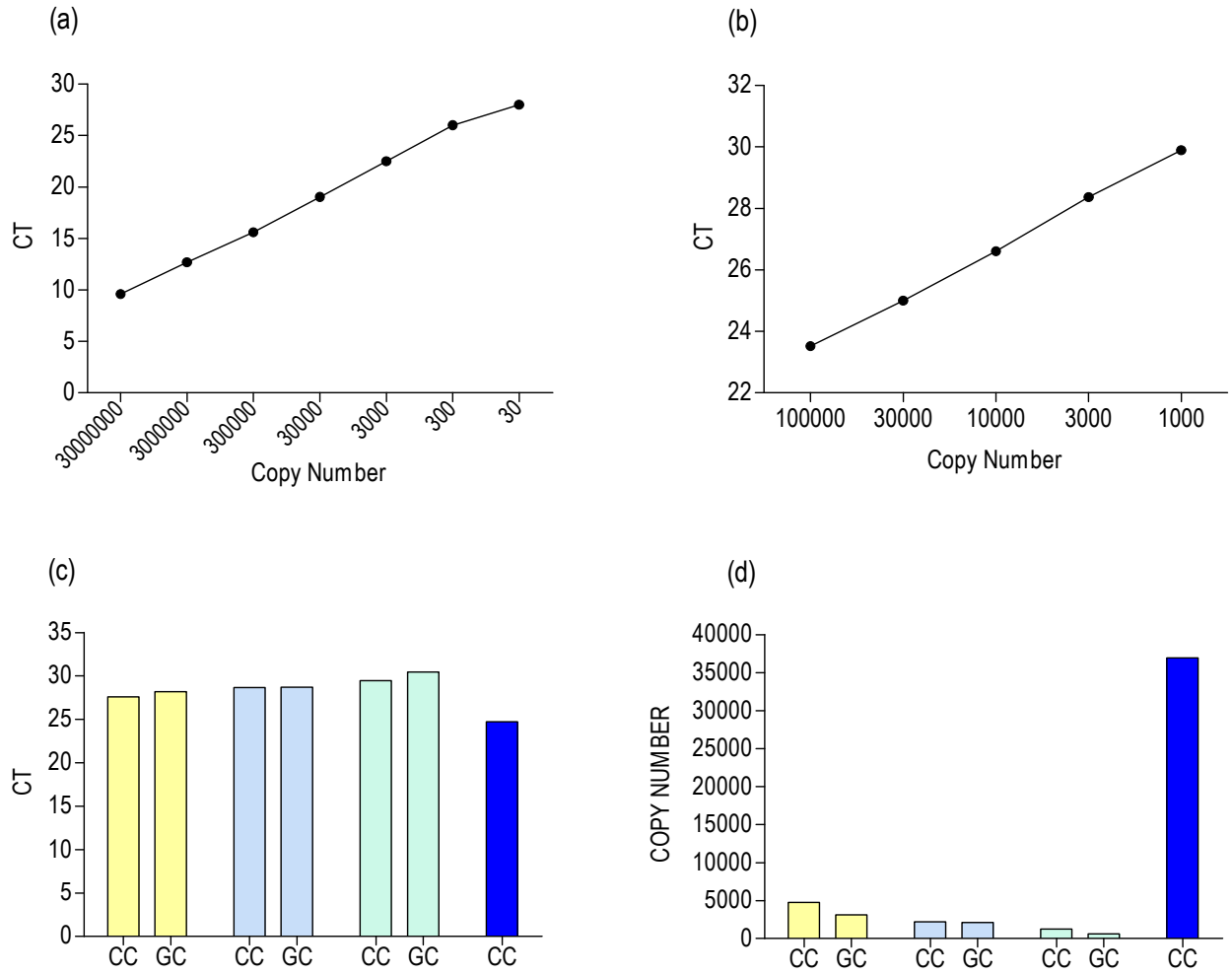

Figure 3.18 Real time RT-PCR validation of the human *L19* plasmid DNA standard curves.

(a) Real time RT-PCR of sub-cloned *L19*-TOPO plasmid following serial dilutions of plasmid DNA through 7 orders of magnitude. (b) The final standard curve dilutions implemented for the analysis of *L19* mRNA expression. (c, d) Analysis of cycle threshold (CT; c) and gene copy number inferred from the standard curve (d) in matched pairs of human cumulus and granulosa cells following *in vivo* stimulation.

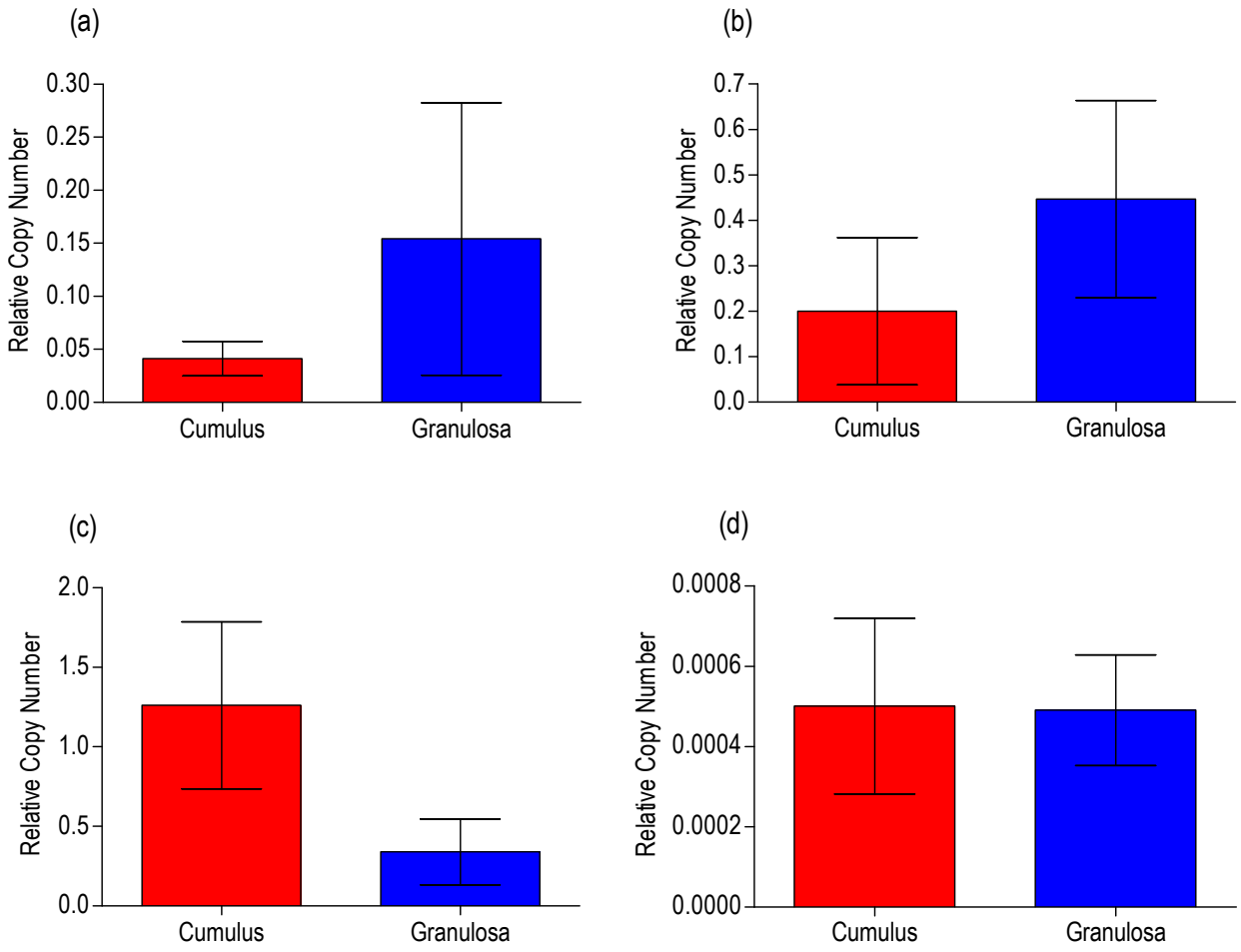

Figure 3.19 Summary of relative gene copy numbers of extracellular matrix genes in patient matched pairs of human cumulus and mural granulosa cells.

Summary of quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *VCAN*, (b) *PTX3*, (c) *HAS2* and (d) *TNFAIP6* in human cumulus and mural granulosa cell pairs. Data is representative of three patient matched pairs of cumulus and mural granulosa cells. Data is normalised to *GAPDH*.

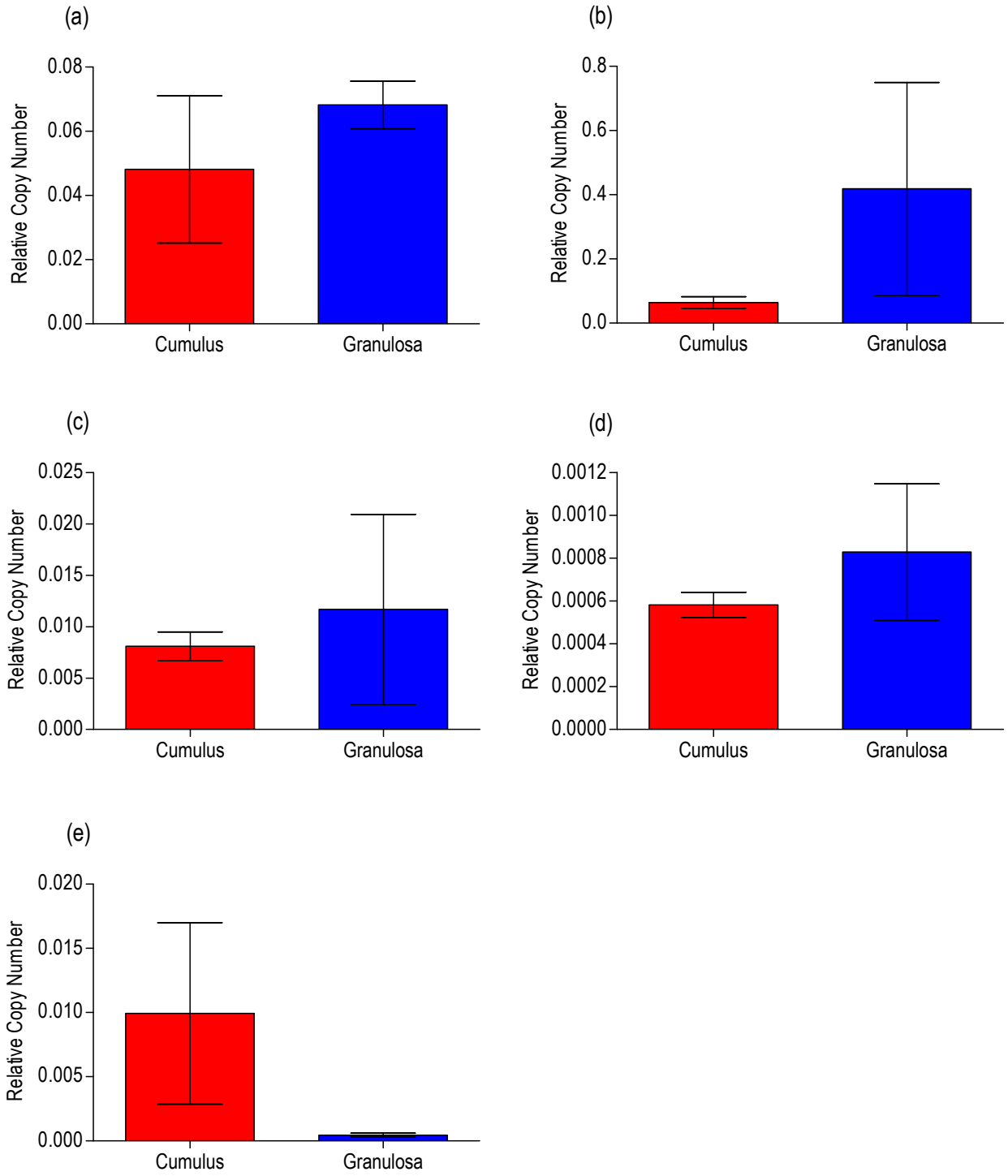

Figure 3.20 Summary of relative gene copy numbers of signalling genes in patient matched pairs of human cumulus and mural granulosa cells.

Summary of quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *PTGS2*, (b) *AHR*, (c) *AR*, (d) *STS* and (e) *GREM1* in human cumulus and mural granulosa cell pairs. Data is representative of three patient matched pairs of cumulus and mural granulosa cells. Data is normalised to *GAPDH*.

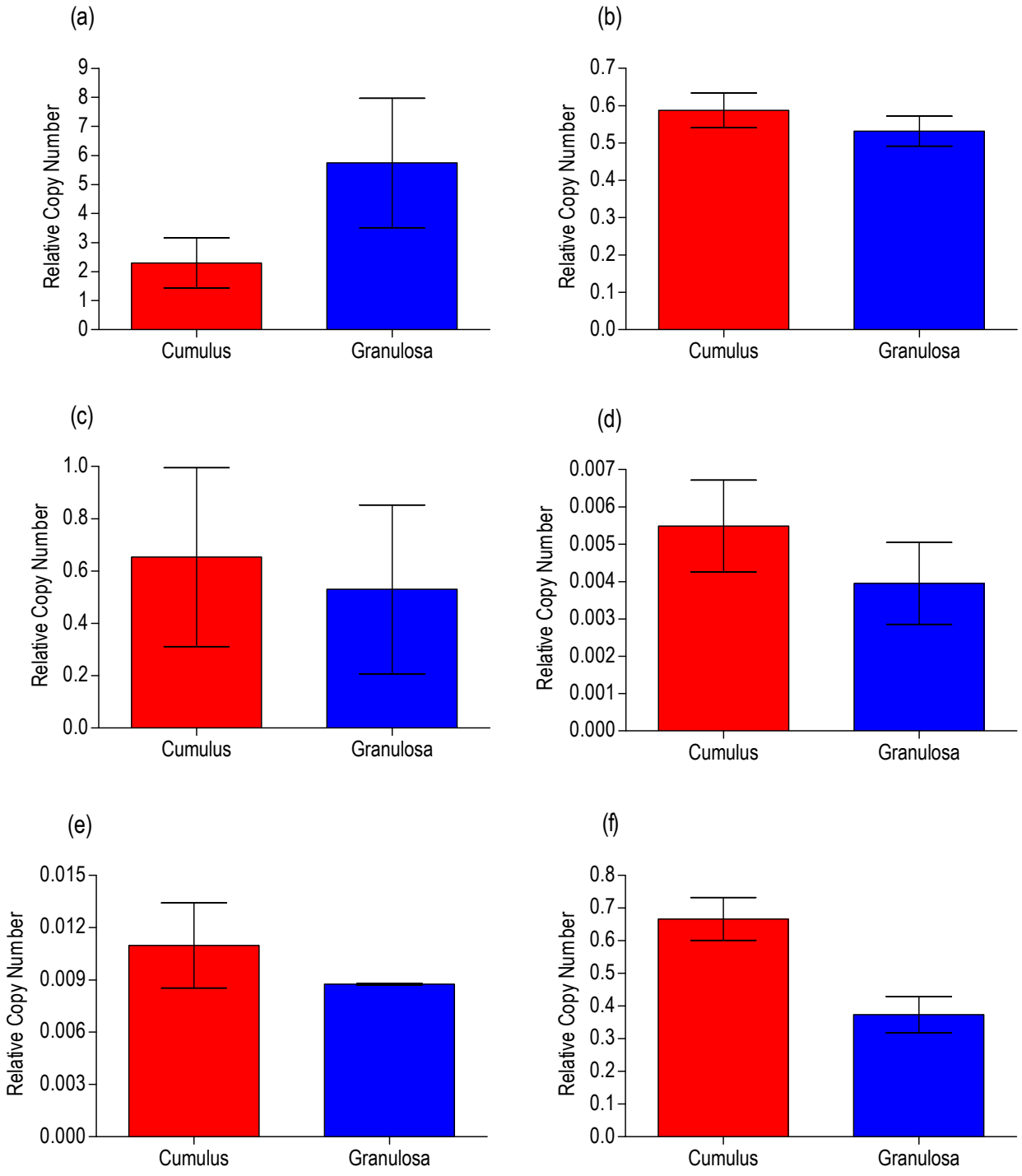

Figure 3.21 Summary of relative gene copy numbers of glycolytic genes in patient matched pairs of human cumulus and mural granulosa cells.

Summary of quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *LDHA*, (b) *ALDOA*, (c) *PFKP*, (d) *PFKL*, (e) *PFKM* and (f) *PKM2* in human cumulus and mural granulosa cell pairs. Data is representative of three patient matched pairs of cumulus and mural granulosa cells. Data is normalised to *GAPDH*.

3.4 DISCUSSION

The overall aim of our study is to identify novel molecular markers of oocyte developmental competence which are associated with pregnancy success in patients undergoing assisted reproduction. The identification of these markers will provide a valuable tool in oocyte selection during assisted reproduction. Despite numerous studies investigating cumulus cell gene expression as a marker of oocyte and embryo health, there are currently no markers of oocyte quality which are correlated with live birth rates. The results in this chapter validate the experimental approach undertaken to develop a highly sensitive, quantitative and reproducible real time RT-PCR assay which generated a set of standards to allow an estimation of the gene copy number in each sample. Thorough validation was performed on each assay to produce a robust set of PCR standards for each gene of interest. Each standard curve was initially created using a sub-cloned gene of interest-TOPO plasmid. For each gene of interest a specifically tailored standard curve was produced and calibrated to human cumulus cell samples, which will be the primary cell type examined throughout this thesis. Each standard curve was demonstrated to be highly sensitive to a small change in CT, which was then reflected in the change in gene copy number. The highly sensitive nature of the plasmid DNA standard curves also allowed for the measurement of a large number of genes in very small cumulus biopsy samples. This was advantageous as it allowed us to assess a significant number of genes to identify cumulus cell biomarkers of oocyte developmental competence. Furthermore, the development of this assay represents a standardised approach to the gene measurement that might be used to define a set of "reference" levels of gene expression useful for future prospective studies or cross-centre comparison of results. The results from this chapter were developed for the quantitative assessment of cumulus cell markers which reflect oocyte developmental competence.

CHAPTER 4

CUMULUS CELL GENE EXPRESSION AS A MARKER OF CLINICAL EMBRYO GRADE

4.1 INTRODUCTION

Pregnancy rates following assisted reproductive technologies (ART) have not increased significantly over recent years, with live birth rates remaining steady at 22% in Australia in 2007 (Wang et al. 2009a). This is primarily due to the limitations in embryo selection, as the current tools used are restricted to the visual morphological appearance of the embryo. This morphological assessment of an embryo is based on a set of criteria that can vary between clinics, but usually incorporates an embryo's capacity to complete "on time" development. On time development refers to the optimal timing at which a cultured embryo undergoes the first and second cleavage, achieves blastocyst development or the time when blastocyst hatching occurs during extended culture. Parameters such as even cell sizes and cellular granularity or fragmentation are often also considered. The visual morphological assessment of an embryo's appearance is considered highly subjective, and the judgement of embryo grade varies greatly between embryologists, and therefore between clinics. The use of multiple published embryo grading systems by various fertility clinics results in wide variation in the efficacy of ART, and as a result there is currently no standardised quantitative method for the non-invasive assessment of embryo quality or a means to predict embryo development in culture.

Single embryo transfer (SET) is the most desirable approach in ART in the interests of both the mother and babies health. There are significant differences in the health outcomes for babies born following single *vs.* double embryo transfer (DET). Double embryo transfer results in more preterm babies, more low birth weight babies and a higher peri-natal death rate (Wang et al. 2008). Despite this, the outcomes for babies born from ART in Australia have been improving each year, which corresponds to increasing numbers of single embryo transfers. However this is not the case for all countries and multiple embryo transfers remain common, especially for older patients, due to the inability to reliably select high quality embryos which will result in a successful pregnancy. At the end of the culture period an embryologist may be faced with two or three or more high grade embryos which look morphologically similar and have no obvious characteristics which set them apart from one another. The embryologist is then required to select one or two of the morphologically assessed high grade embryos for transfer. In many cases, because a single high quality embryo with the greatest developmental potential cannot be identified, multiple embryos are transferred to increase the likelihood of pregnancy success. While multiple embryo transfer may increase the chances of pregnancy success, it also significantly increases the chances of multiple gestations and the associated adverse health outcomes.

4.1.1 Relationship of on time morphological assessment and blastocyst quality

While the assessment of embryo morphology and the embryo scoring system is a fundamental part of assisted reproduction, it has only a weak correlation with pregnancy success. Blastocyst stage transfers are associated with high implantation rates, but the stresses of *in vitro* culture means not all embryos will reach the blastocyst stage, resulting in a decreased number of embryos available for transfer (Rijnders and Jansen 1998; Papanikolaou et al. 2006). Because of this, many patients will undergo day 2 or 3 embryo transfer, but at this stage in development the embryo is still in part dependent on the maternal genome and uterine receptivity may be suboptimal (Rijnders and Jansen 1998). The ability to predict embryo development in culture is limited. Studies have shown that the predictive value of embryo morphology on days 2 or 3 is limited in regards to subsequent blastocyst formation on day 5 as only half of the embryos assessed as high morphological grade on day 3 reached the blastocyst stage, and 49% of embryos preselected on day 3 were rejected on day 5, illustrating limitations to day 3 assessments for extended culture, and likely pregnancy success (Dokras et al. 1993; Rijnders and Jansen 1998). In a similar study, only 48% of embryos that would have been suitable for transfer or cryopreservation on day 3 were selected at the blastocyst stage on day 5/6 (Graham et al. 2000). Importantly, the predictive value of embryo assessment on day 3 or day 5/6 was not correlated with actual pregnancy rates in either study. Implantation and pregnancy rates have been shown to be significantly influenced by embryo cell number, and to a lesser extent the degree of fragmentation (Ziebe et al. 1997). Transfer of embryos at the 4-cell stage resulted in higher pregnancy rates than the transfer of a 2-cell embryo or an embryo which had cleaved beyond the 4-cell stage. Interestingly, transfer of embryos with minor amounts of fragmentation did not impact pregnancy rates casting doubt on this morphological criterion (Ziebe et al. 1997).

Pregnancy and implantation rates are also improved by the transfer of early cleaving embryos (25-27 h post insemination/microinjection) compared to late cleaving embryos (Lundin et al. 2001; Sakkas et al. 2001; Salumets et al. 2003; Windt et al. 2004). The use of early cleavage as a marker of developmental potential is influenced when ICSI is performed due to more rapid cleavage embryos and is limited to cycles were laboratory personnel are available to assess the timing of cleavage. Regardless of the stage of transfer, a single observation appears to be insufficient to predict the developmental capacity of an embryo; hence, multiple measures of development are required as there is still a limit on implantation and pregnancy rates following ART (Scott 2003; Borini et al. 2005). The fundamental molecular mechanisms that underpin embryo developmental potential are well known to be influenced by the quality of the gametes, especially the oocyte (Ebner et al. 2000; Ebner et al. 2003a; Ebner et al. 2003b;

Paz et al. 2004) and culture conditions. While culture conditions have been progressively advanced there remain no accepted methods to verify oocyte developmental competence.

4.1.2 Relationship between cumulus gene expression and embryo morphology

The hypothesis that embryo quality is limited by the gametes it was derived from has been investigated by a number of groups (Krisher 2004; Wang and Sun 2007; Gilchrist et al. 2008). The potential of cumulus cell gene expression to predict embryo morphology has led to the identification of genes with important functional relationships to oocyte developmental potential (Summarised in Table 1.1, Chapter 1). The landmark study by McKenzie *et al*. (McKenzie et al. 2004) correlated cumulus cell gene expression with subsequent embryo development to the blastocyst stage in a cohort of 8 patients. High embryo morphology parameters were correlated with cumulus cell expression of *PTGS2*, *HAS2* and *GREM1*. Additionally, *PTGS2* and *GREM1* were found to be predictors of fertilisation. The associations seen for cumulus cell *HAS2* and *GREM1* were later supported in a larger study (Cillo et al. 2007) comparing cumulus cells isolated from oocytes which developed to high quality embryos compared to those which did not fertilise or developed to poor quality embryos. Zhang *et al*. found *PTX3* mRNA expression was higher in cumulus cells from oocytes that fertilised *vs.* cumulus cells from oocytes which failed to fertilise (Zhang et al. 2005). The relative abundance of *PTX3* mRNA was significantly higher in cumulus cells from oocytes that resulted in 8-cell embryos on day 3 which established a successful pregnancy compared to cumulus cells from oocytes that resulted in 8-cell embryos that did not establish pregnancy, or oocytes which failed to fertilise (Zhang et al. 2005). A unifying feature of *HAS2*, *PTGS2*, *GREM1* and *PTX3* is that they are genes whose expression is regulated in cumulus cells by oocyte secreted factors (Dong et al. 1996; Elvin et al. 1999a; Elvin et al. 1999b; Elvin et al. 2000; Vitt and Hsueh 2001; Yan et al. 2001; Varani et al. 2002; Pangas and Matzuk 2004; Dragovic et al. 2007). Further studies have reported low cumulus cell *CX43* mRNA expression to be associated with good embryo morphology (Hasegawa et al. 2007), while cumulus cell expression of *STAR*, *PTGS2*, *CX43*, *AREG*, *SCD1* and *SCD5* was significantly different between oocytes which yielded top quality embryos and oocytes yielding weak or low grade embryos (Feuerstein et al. 2007).

The results from the studies investigating cumulus cell gene expression as a marker of embryo morphology occasionally contradict or fail to support one another. Small variations in methodology may be responsible for much of the variation, including the day of embryo morphology assessment and the PCR methods utilised. Feuerstein *et al*. found *PTGS2* mRNA expression to be significantly lower in cumulus cells from oocyte which developed to top quality embryos but was significantly higher in

cumulus cells from oocytes which fertilised successfully, while McKenzie *et al*. had initially found *PTGS2* was significantly higher in cumulus cells from oocytes which developed to high grade embryos (McKenzie et al. 2004; Feuerstein et al. 2007). A key difference between the independent studies was the day embryo morphology was assessed: Feuerstein *et al*. assessed embryo morphology on day 2, while McKenzie *et al*. correlated gene expression with embryo morphology on day 3. Differences in PCR methodologies may also explain the differences. Real time RT-PCR was initially used to assess the gene expression patterns between embryos of high and low morphological grade (McKenzie et al. 2004; Hasegawa et al. 2005; Cillo et al. 2007; Feuerstein et al. 2007; Hasegawa et al. 2007) while microarray analysis was employed as a whole genome approach in other studies (Zhang et al. 2005; van Montfoort et al. 2008). Of note, only one of the previous studies looked at cumulus cell gene expression in patient matched pairs for both microarray and real time RT-PCR analyses (van Montfoort et al. 2008), while other studies assessed individual cumulus cell gene expression but did not perform single embryo transfers in all patients (refer to Table 1.1, Chapter 1 for summary).

Genes which have been correlated with embryo morphology and fertilisation rates are mostly known to be gonadotrophin dependent or downstream targets of the GDF-9 signalling pathway (Buccione et al. 1990b; Elvin et al. 1999a; Calder et al. 2001; Feuerstein et al. 2007). The cumulus cell expression of GDF-9 target genes may therefore be a reflection of oocyte GDF-9 activity and the oocyte paracrine signal strength and in turn the developmental competence of that oocyte (McKenzie et al. 2004). The LH surge initiates the induction of genes such as *STAR* which is critical for steroidogenesis, while expression of *PTGS2*, which is an important mediator of cumulus expansion, is dependent on oocyte secreted factors (Davis et al. 1999; Hasegawa et al. 2000). The relationship between embryo development in culture and oocyte paracrine signalling pathways supports the hypothesis that the bi-directional communication between the oocyte and cumulus cells is essential for developmental competence. For this reason, our analyses were similarly focussed on genes which contribute to the acquisition of oocyte developmental competence and which are key mediators of cumulus expansion. Oocyte signalling also mediates cumulus cell metabolic activity and cumulus cell metabolism is a key contributor to oocyte quality. Therefore measurement of cumulus cell metabolic activity may reflect oocyte developmental potential (Sugiura et al. 2005).

While a number of studies have investigated the potential correlation between cumulus gene expression and oocyte developmental potential, the system used to define high *vs.* low quality embryos is widely variable between these studies, and is reflective of the variation seen between clinics. We wanted first

to use our highly quantitative assays of selected genes to investigate the link between cumulus cell gene expression and embryo morphological grade on day 2 on a set of twelve patient matched pairs of cumulus cells comparing only grade 1 (high grade) and grade 4 (low grade) embryos. The analysis of gene expression in paired cumulus samples from the same patients is important to facilitate a comparison of the degree of variance in gene expression between cumulus samples *vs.* that between patients.

4.2 MATERIALS AND METHODS

4.2.1 Patient Matched Cumulus Pairs

High and low grade embryos were matched within each of the twelve patients. Each embryo was scored by one embryologist. The same embryologist was responsible for the scoring of cumulus expansion, individual culture of oocytes and embryos, and the collection of the cumulus cells. The experimental model was based on previously published studies, as detailed in Table 1.1, with some modifications specific to our lab and interests.

4.2.2 Embryo Quality Assessments

The embryos involved in this analysis were not transferred; however, most high grade embryos were stored frozen for potential future use. Embryo quality assessments were based on multiple observations as described in Chapter 2. The cleavage stage morphology scoring system used for this analysis was based on the number of cells, the degree of fragmentation and the presence of multinucleated cells. Good quality embryos were assessed to have 4-cells on day 2 with limited fragmentation (< 10%) and an absence of multinucleated blastomeres. Embryos were scored using a scale of 1-4, where grade 1 indicates best quality and grade 4 indicates poor quality. This scoring system was applied to the embryos assessed in this study (see Figure 4.1).

4.2.3 qPCR Assays

qPCR assays were described as in Chapter 3. GeNorm analysis software identified *GAPDH* as the most stable internal control gene across the sample population and was utilised for the relative expression analysis in these experiments.

4.2.4 Statistical Analysis

Data are presented as box and whisker plots where the horizontal line represents the median copy number and the box encompasses 50% of data points (first quartile to third quartile). Data points within the 10th and 90th percentiles are represented by the upper adjacent and lower adjacent “whiskers” (vertical lines). Values lower than the 10th percentile and greater than the 90th percentile are indicated by closed dots. Data is normalised to the internal control *GAPDH*. Statistical analysis was performed using a Wilcoxon Signed Rank Test, a non-parametric test that does not require the normality assumption (GraphPad Prism version 5.01 for Windows, San Diego, CA, United States,

www.graphpad.com). Differences were considered significant at $p < 0.05$. A Spearman's rank order correlation assessed any possible relationships between maternal age or body mass index (BMI) and the difference in the relative copy number in cumulus cells from high grade *vs.* low grade embryos. The difference in relative copy number was calculated by subtracting the relative gene copy number (relative to the housekeeper *GAPDH*) from the cumulus cells associated with the grade 4 embryos (low quality) from the relative gene copy number from the cumulus cells associated with the grade 1 embryos (high quality) for each of the eleven patients.

NOTE:
These images are included on page 92 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.1 Embryo morphology scoring system (embryo grade).

Visual representation of embryo morphological grade on day 2 with images depicting embryos awarded scores of (a) Grade 1, (b) Grade 2, (c) Grade 3 and (d) Grade 4. The grading system weights "on-time" development very heavily. Only embryos that are "on-time" are eligible for a score of grade 1 or 2. Fragmentation and cell quality then impact an "on-time" embryo to be either a grade 1 or 2, and a not "on-time" embryo to be a grade 3 or 4. Embryo images obtained from Deanne Feil.

4.3 RESULTS

4.3.1 Patient matched cumulus pairs.

Pairs of cumulus cell samples were collected from twelve patients so that each patient was represented by cumulus cells from an oocyte which developed to a high grade embryo and cumulus cells from an oocyte which developed to a low grade embryo. Following quantitative real time RT-PCR, only eleven patients were included in the analyses as one patient sample pair yielded negligible amplification. Patient demographic data is presented in Table 4.2. Data presented as mean ± SEM for thirty eight patients undergoing elective single embryo transfer.

Table 4.1 Patient demographic characteristics for the analysis of cumulus cell gene expression as a marker of clinical embryo grade.

Number of Patients	**11**
Maternal Age	34.7±0.5
% ICSI	91%
Number of previous IVF/ICSI cycles	0.9±0.4
Number of oocytes collected this cycle	12±1.0
Number of oocytes inseminated	10.2±0.7
Number of oocytes fertilised	7.3±0.6
Infertility Diagnosis	
Male Factor	8
Ovulatory disorders	1
Unexplained	2

4.3.2 Cumulus cell gene expression is not associated with clinical embryo grade.

The gene expression patterns in cumulus cell masses from individual oocytes were analysed based on the clinical grade of resultant embryos following individual culture. No embryos utilised for this analysis were transferred in the fresh cycle at the end of the culture period. Cumulus cell gene expression of genes which are functionally related to cumulus expansion, oocyte maturation, oocyte-cumulus signalling and cumulus oocyte complex metabolism were investigated. A total of twenty two cumulus masses trimmed from individual COCs after collection were subjected to gene expression analyses. Cumulus cell gene expression showed no relationship with clinical embryo grade. There was no significant difference in the median relative gene copy number for any gene analysed in cumulus cells from oocytes yielding high grade embryos and low grade embryos in this cohort (Figures 4.2 to 4.10).

4.3.3 Maternal age or body mass index (BMI) does not influence cumulus cell gene expression in relation to clinical embryo grade.

A spearman's rank order correlation was performed to explore the effects of maternal age and Body Mass Index (BMI) on gene expression and embryo grade however no significant correlations were found (Figure 4.2 to 4.10). For each patient, the difference in the relative gene copy number between the high and low grade embryos was correlated to maternal age and BMI (i.e. the relative gene copy number from the cumulus cells associated with the high grade embryo minus the relative gene copy number from the cumulus cells associated with the low grade embryo). Maternal age trended towards a significant correlation with both *PTGS2* (p = 0.075) and *TNFAIP6* (p = 0.068), however the study population was too small to fully appreciate an effect. The results of the Spearman's rank order correlation are summarised for each gene to compare maternal age (Table 4.2) and BMI (Table 4.3).

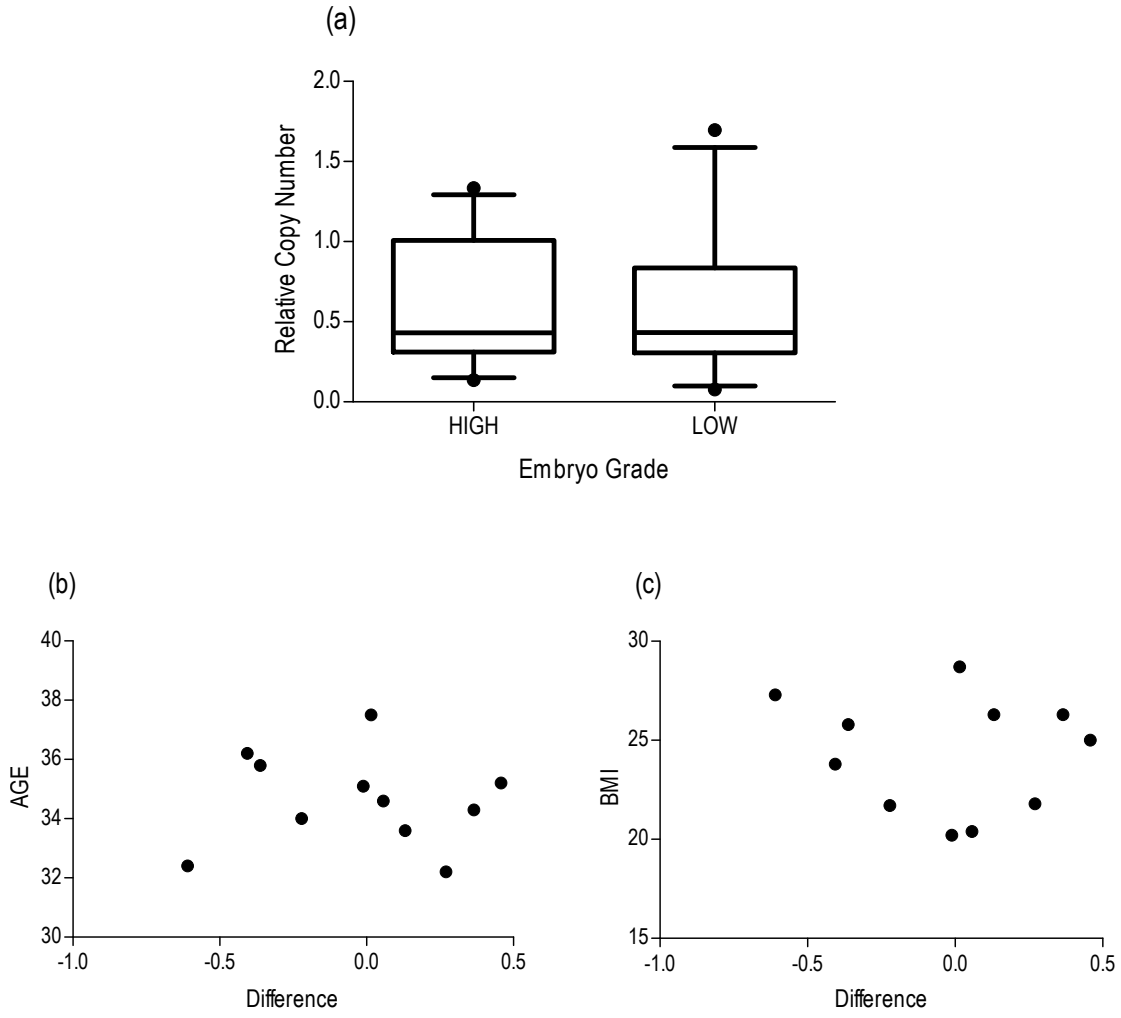

Figure 4.2 Cumulus cell *VCAN* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *VCAN* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *VCAN* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *VCAN* copy number is normalised to *GAPDH* copy number. No significant differences observed.

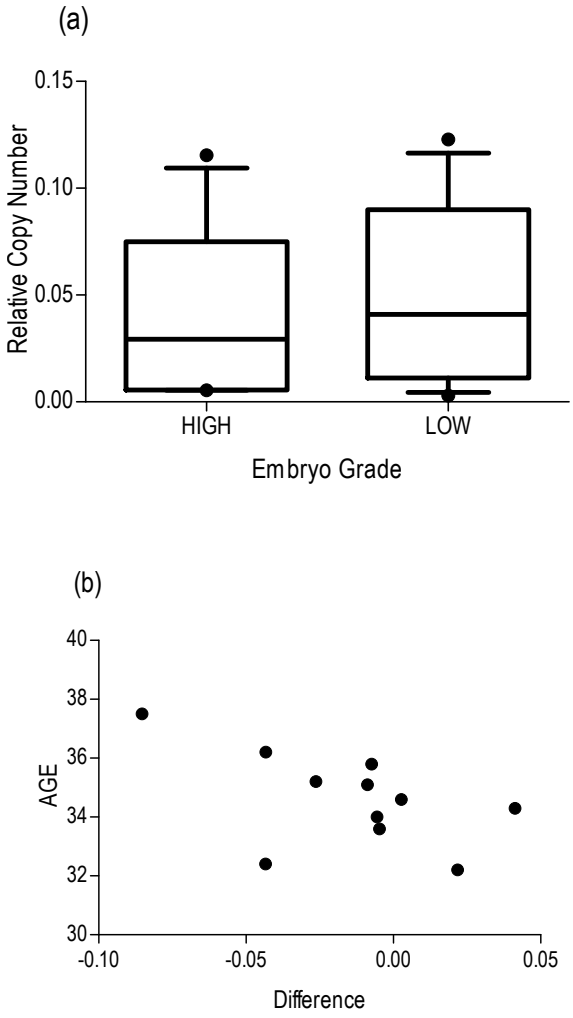

Figure 4.3 Cumulus cell *PTGS2* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *PTGS2* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *PTGS2* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *PTGS2* copy number is normalised to *GAPDH* copy number. No significant differences observed.

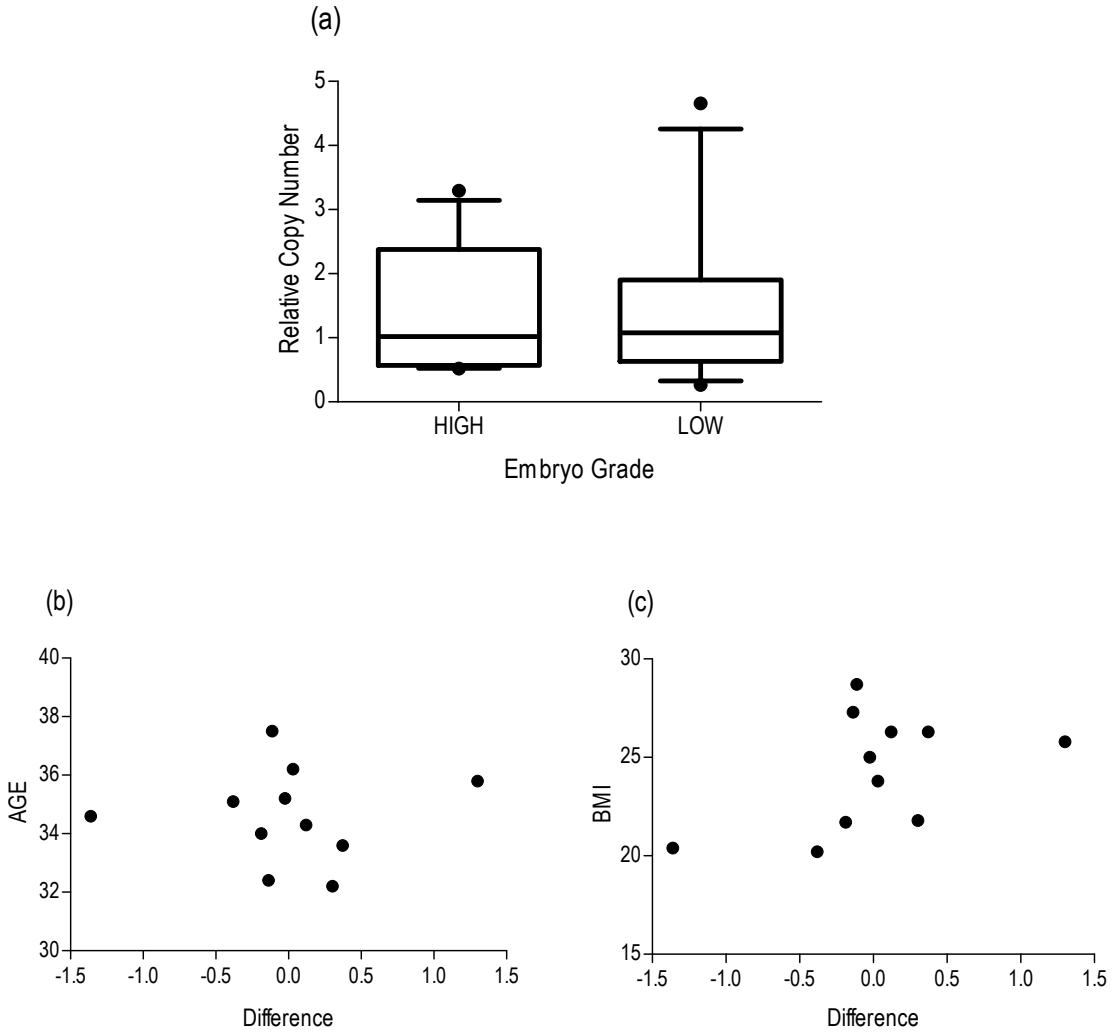

Figure 4.4 Cumulus cell *PTX3* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *PTX3* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *PTX3* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *PTX3* copy number is normalised to *GAPDH* copy number. No significant differences observed.

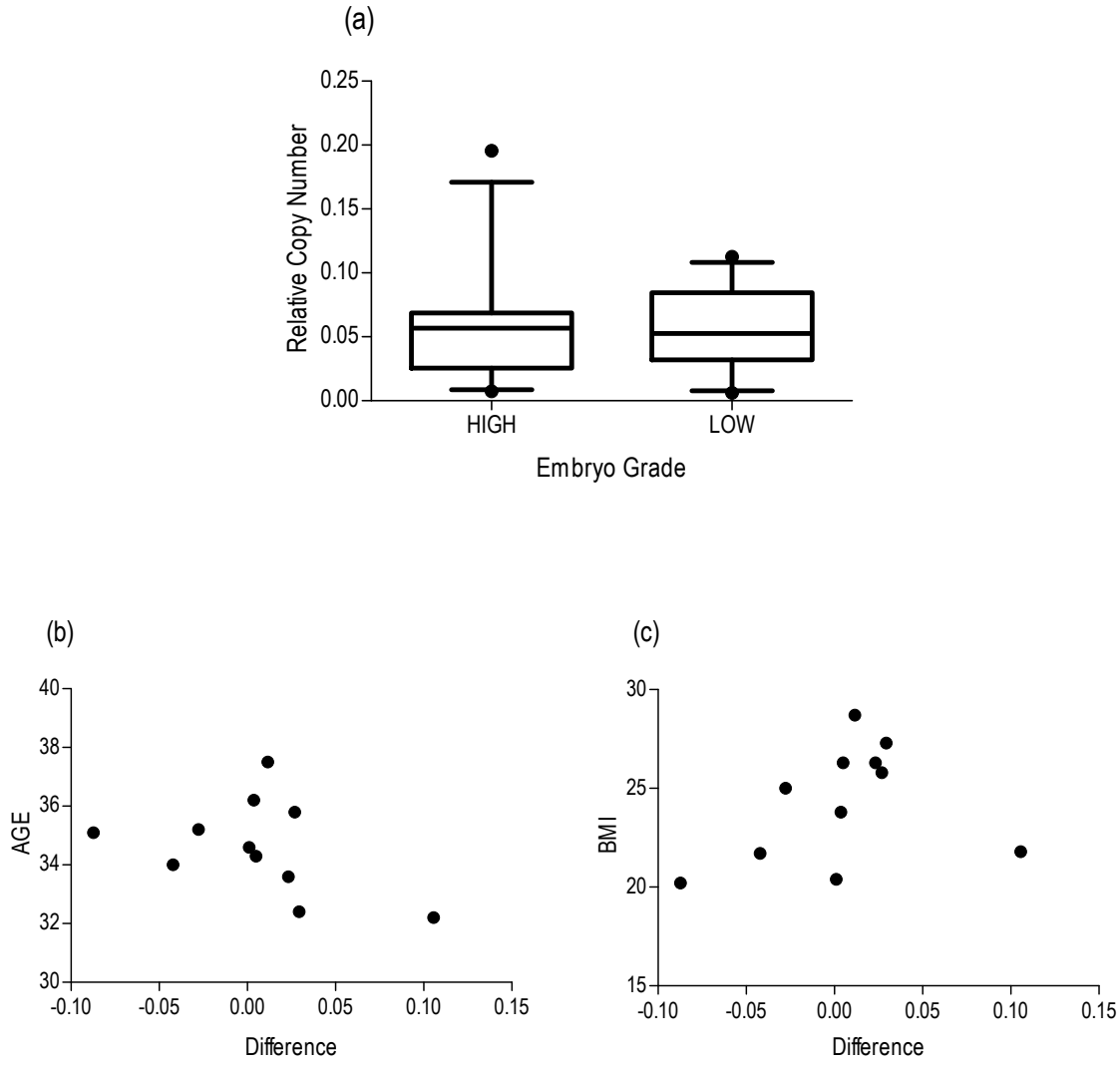

Figure 4.5 Cumulus cell *GREM1* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *GREM1* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *GREM1* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *GREM1* copy number is normalised to *GAPDH* copy number. No significant differences observed.

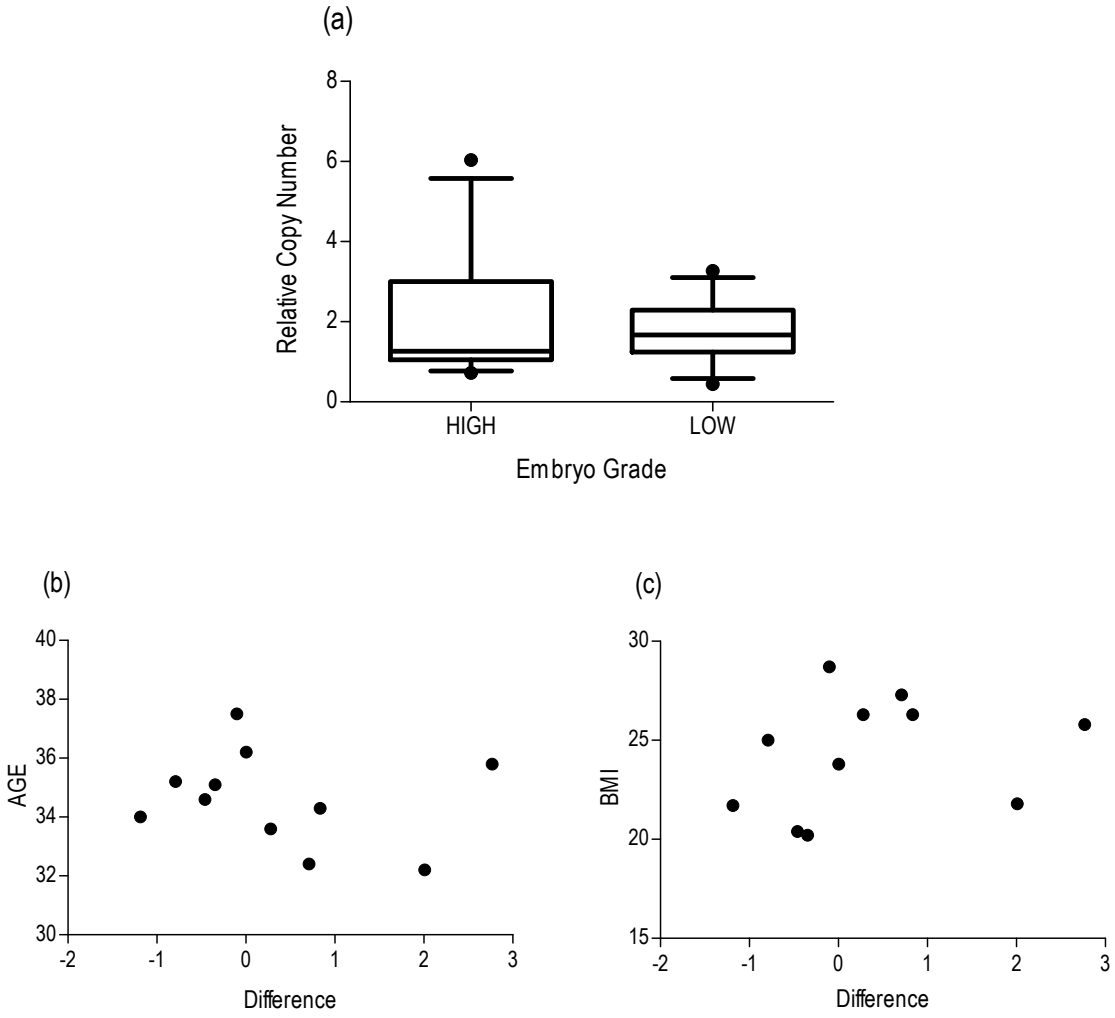

Figure 4.6 Cumulus cell *HAS2* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *HAS2* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *HAS2* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *HAS2* copy number is normalised to *GAPDH* copy number. No significant differences observed.

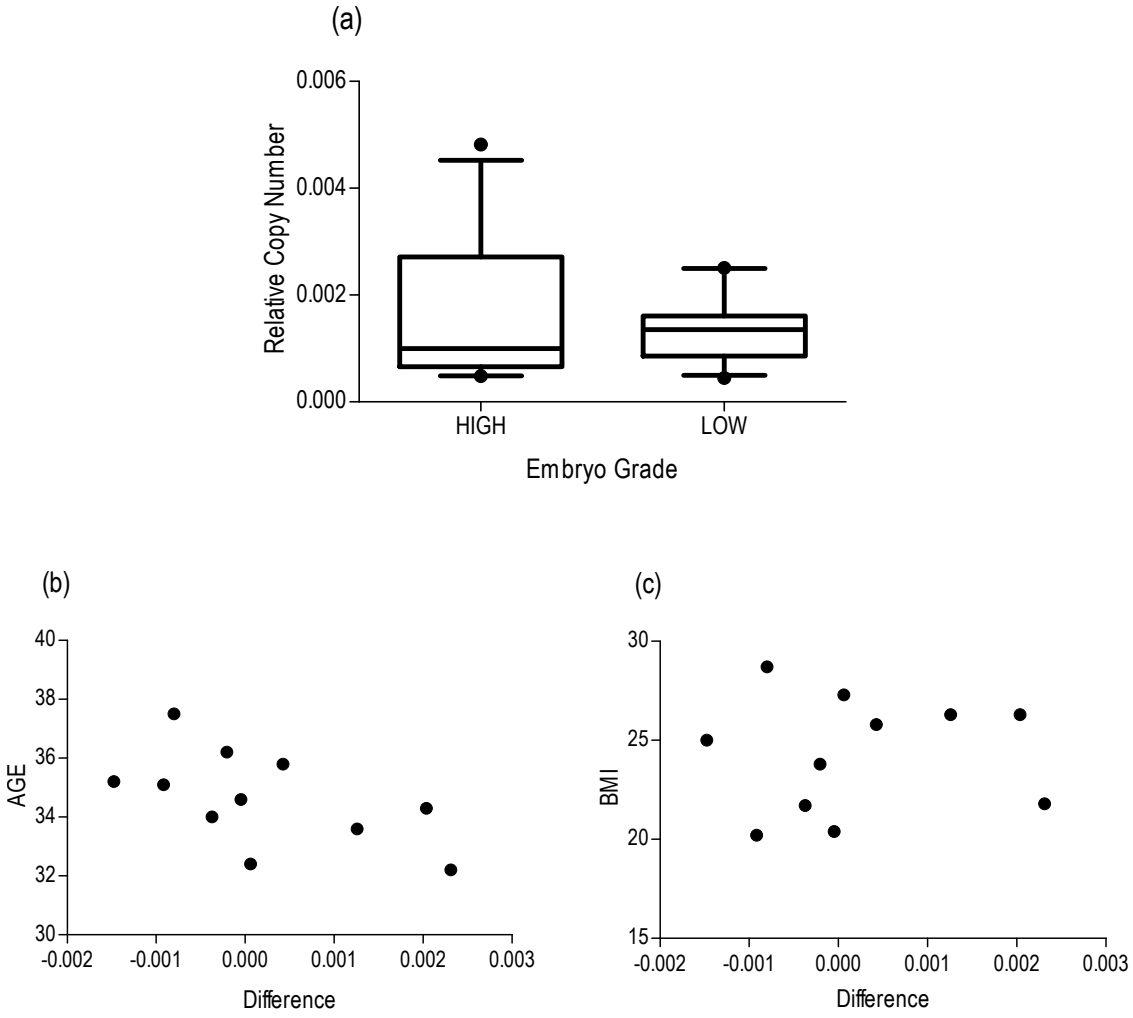

Figure 4.7 Cumulus cell *TNFAIP6* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *TNFAIP6* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *TNFAIP6* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *TNFAIP6* copy number is normalised to *GAPDH* copy number. No significant differences observed.

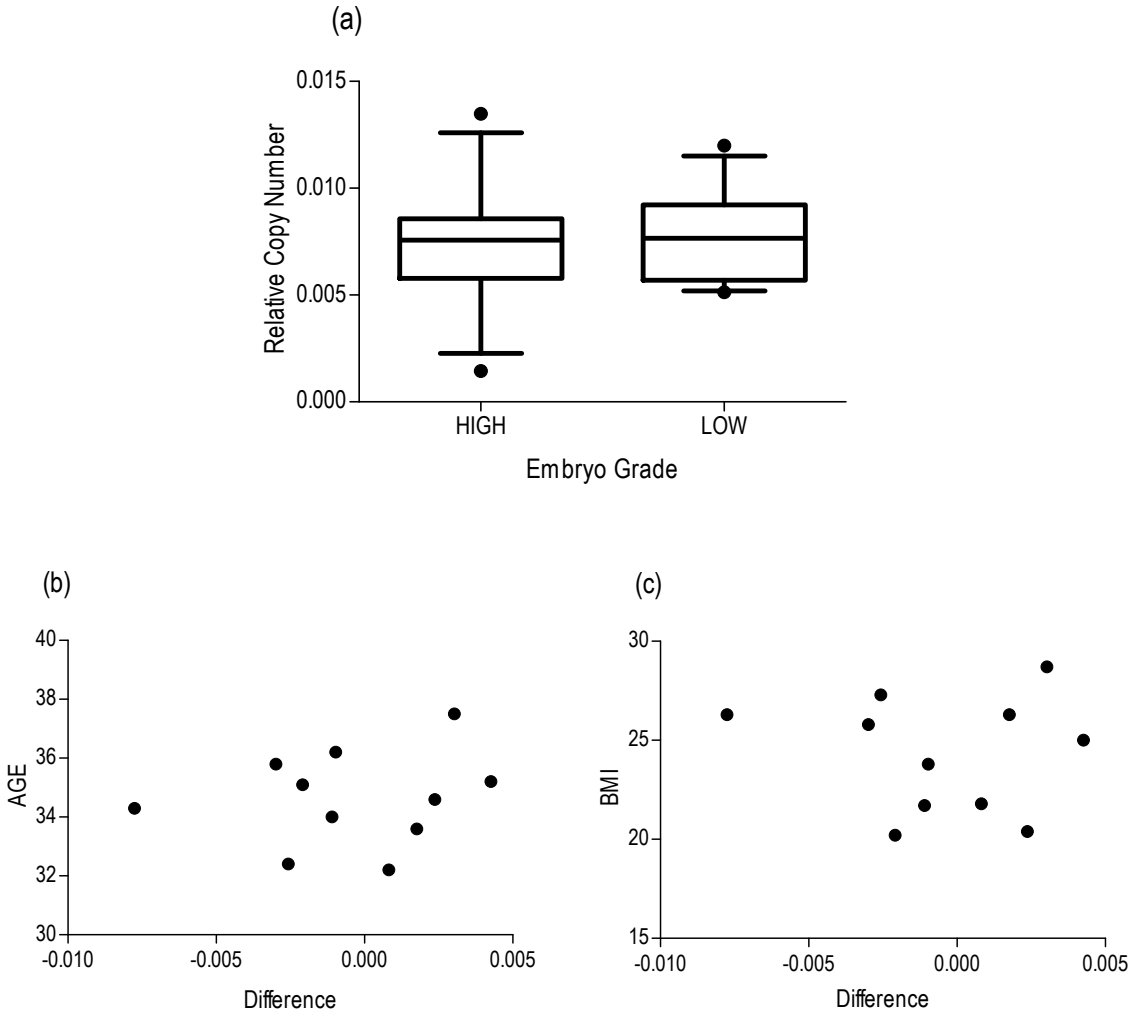

Figure 4.8 Cumulus cell *AR* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *AR* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *AR* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *AR* copy number is normalised to *GAPDH* copy number. No significant differences observed.

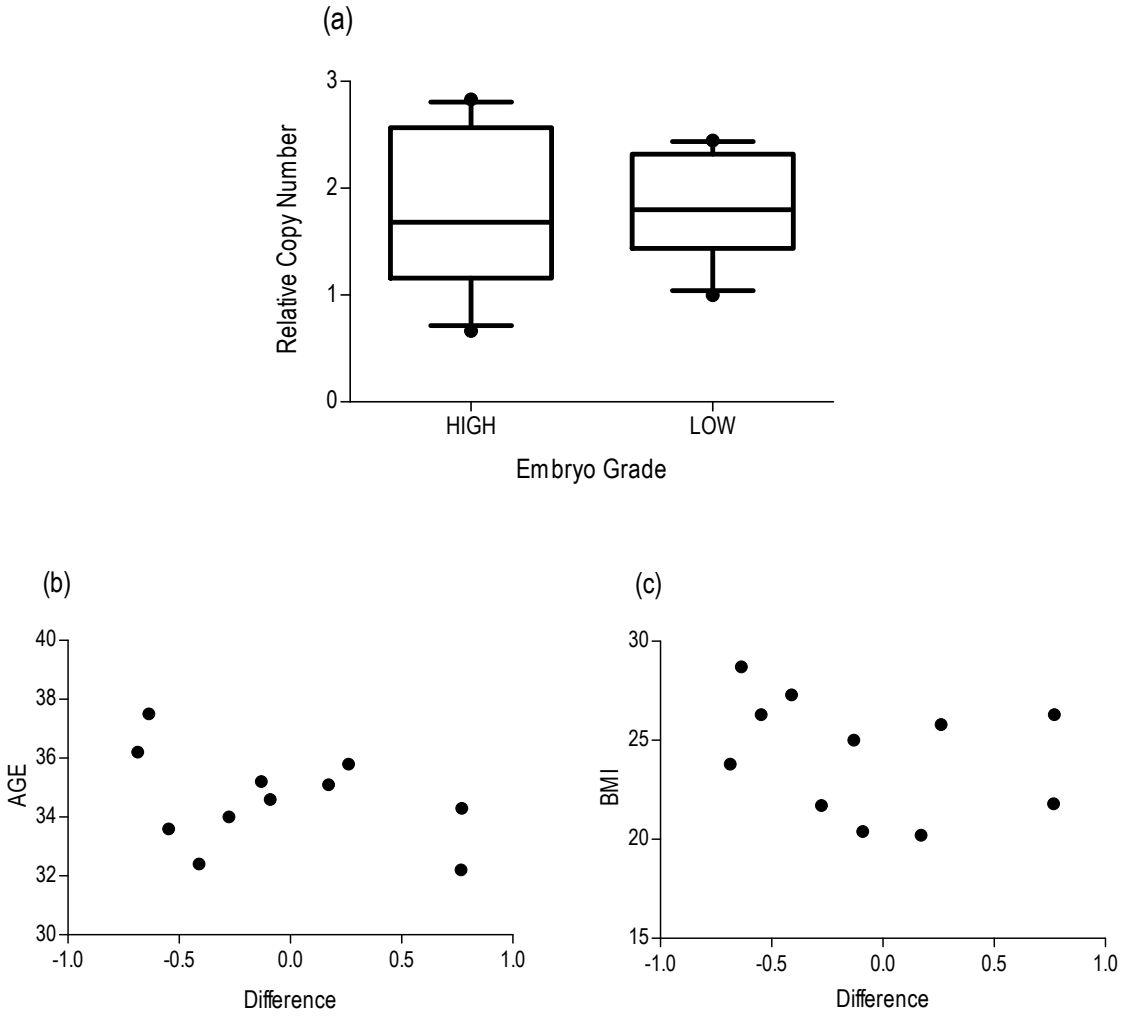

Figure 4.9 Cumulus cell *LDHA* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *LDHA* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *LDHA* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *LDHA* copy number is normalised to *GAPDH* copy number. No significant differences observed.

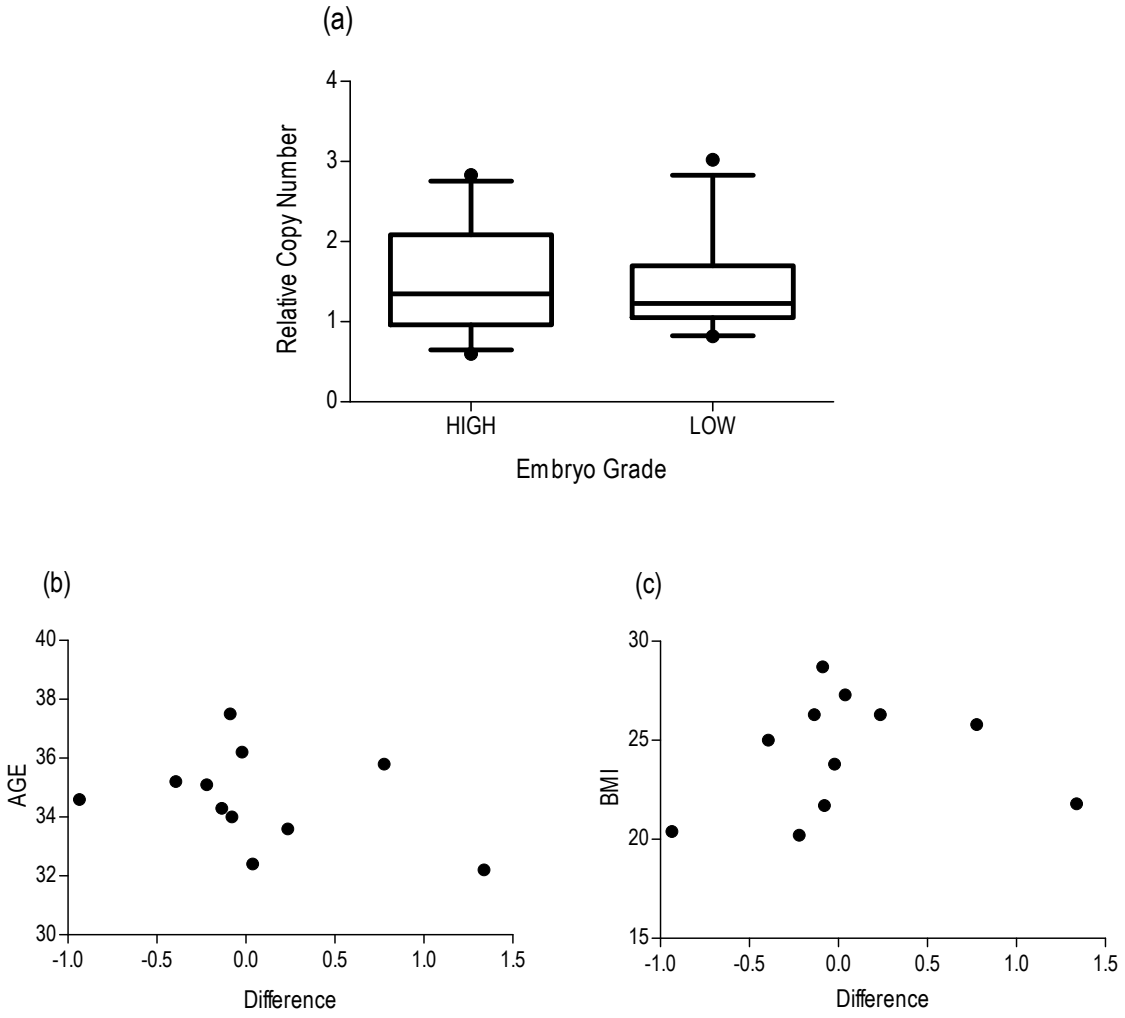

Figure 4.10 Cumulus cell *PFKP* is not indicative of clinical embryo grade, and is not correlated with maternal age or body mass index.

(a) Cumulus cell *PFKP* mRNA expression (gene copy number) is not associated with clinical embryo grade following quantitative real time RT-PCR analysis. The difference in *PFKP* mRNA expression (gene copy number) in cumulus cells between high *vs.* low grade embryos was not correlated with maternal (b) age or (c) BMI following a Spearman's rank order correlation. *PFKP* copy number is normalised to *GAPDH* copy number. No significant differences observed.

Table 4.2 Spearman r-value and p-value summary to assess the association between the difference in relative gene copy number when comparing high grade vs. low grade embryos and age (age in years at OPU date).

Gene of Interest	Spearman r^2	*p*-value
VCAN	0.0010	0.92
PTGS2	0.3099	0.075[#]
PTX3	0.1629	0.22
GREM1	0.1610	0.22
HAS2	0.0434	0.54
TNFAIP6	0.3225	0.068[#]
AR	0.0357	0.58
LDHA	0.1399	0.26
PFKP	0.1128	0.31

([#] denotes a trend towards a significant correlation where $p < 0.08$ following a Spearman rank order correlation).

Table 4.3 Spearman r-value and p-value summary to assess the association between the difference in relative gene copy number when comparing high grade vs. low grade embryos and body mass index (BMI).

Gene of Interest	Spearman r^2	*p*-value
VCAN	0.0116	0.75
PTGS2	0.2078	0.16
PTX3	0.0364	0.57
GREM1	0.0868	0.38
HAS2	0.0648	0.45
TNFAIP6	0.0035	0.86
AR	0.0053	0.83
LDHA	0.0895	0.37
PFKP	0.0166	0.71

4.3.4 Inter- and intra-patient variability in relative gene copy number in cumulus cells associated with high grade *vs.* low grade embryos.

Individual patient plots of the quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of each gene of interest were used to assess the inter- and intra-patient variability (Figure 4.11 to 4.13). Each of the eleven patients is represented by a different colour, and is represented by a cumulus cell relative gene copy number associated with a high grade embryo and a cumulus cell relative gene copy number associated with a high low embryo. The individual patient plots reveal the degree of variation between patients is on a similar scale but usually higher than the degree of variation between high and low grade embryos within a patient. The degree of inter- and intra-patient variation is gene dependent; that is the degree of variation is not similar for each patient across all the genes analysed. For most genes analysed, the greatest degree of variation is strikingly between cumulus cells from oocytes which developed to high grade embryos, possibly indicating that a high morphological grade embryo can be associated with a wide range of molecular conditions, while a poor morphological embryo is less molecularly active.

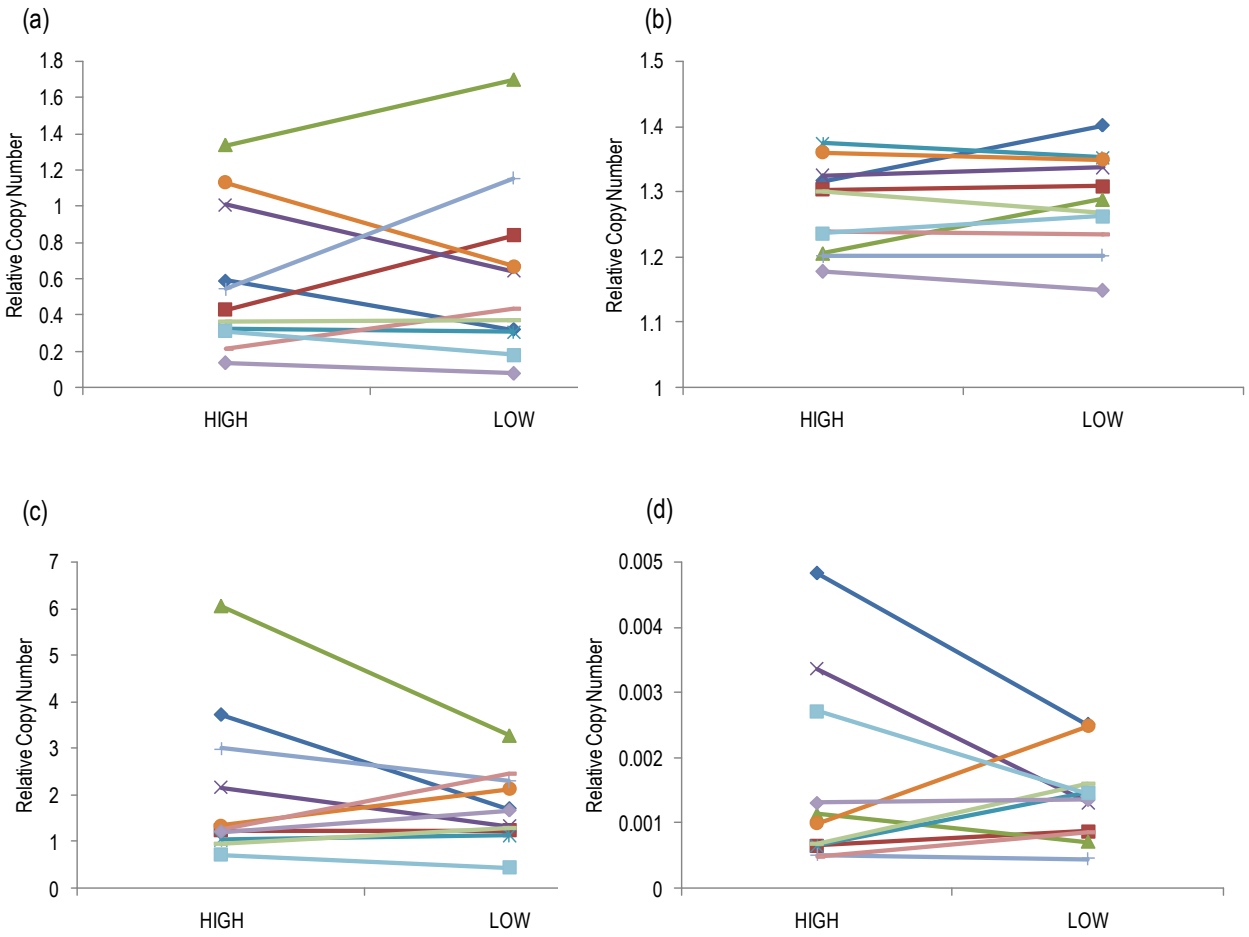

Figure 4.11 Inter- and intra-patient variation in the expression of extracellular matrix related genes in cumulus cells associated with high grade vs. low grade embryos.

Individual patient plots of the quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *VCAN*, (b) *PTX3*, (c) *HAS2* and (d) *TNFAIP6* in matched pairs of human cumulus cells from oocytes which formed a high grade *vs.* low grade embryo. Copy number of each gene is normalised to *GAPDH* copy number. Each of the eleven patients is represented by a different colour.

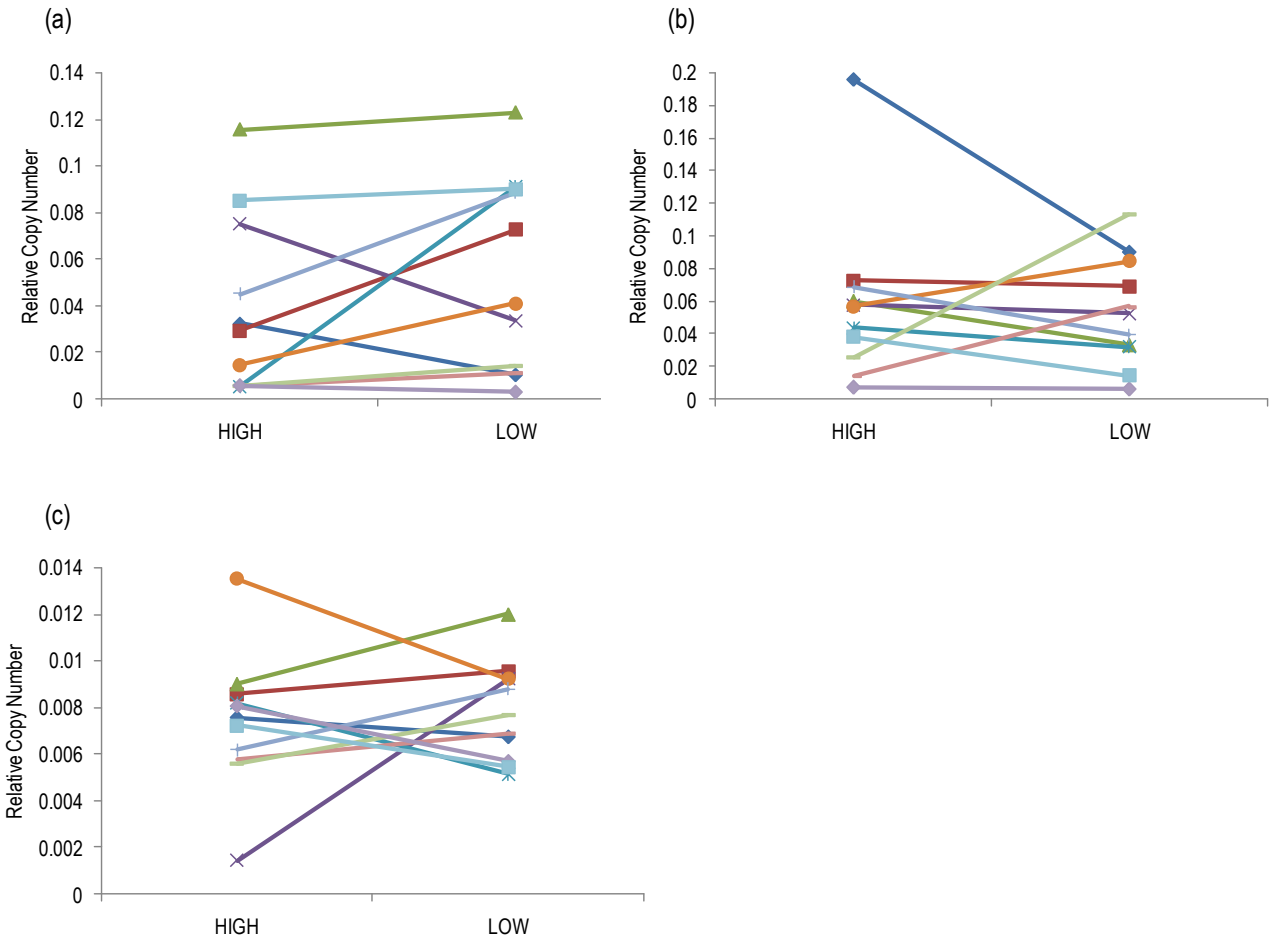

Figure 4.12 Inter- and intra-patient variation in the expression of signalling related genes in cumulus cells associated with high grade vs. low grade embryos.

Individual patient plots of the quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *PTGS2*, (b) *GREM1*, and (c) *AR* in matched pairs of human cumulus cells from oocytes which formed a high grade *vs.* low grade embryo. Copy number of each gene is normalised to *GAPDH* copy number. Each of the eleven patients is represented by a different colour.

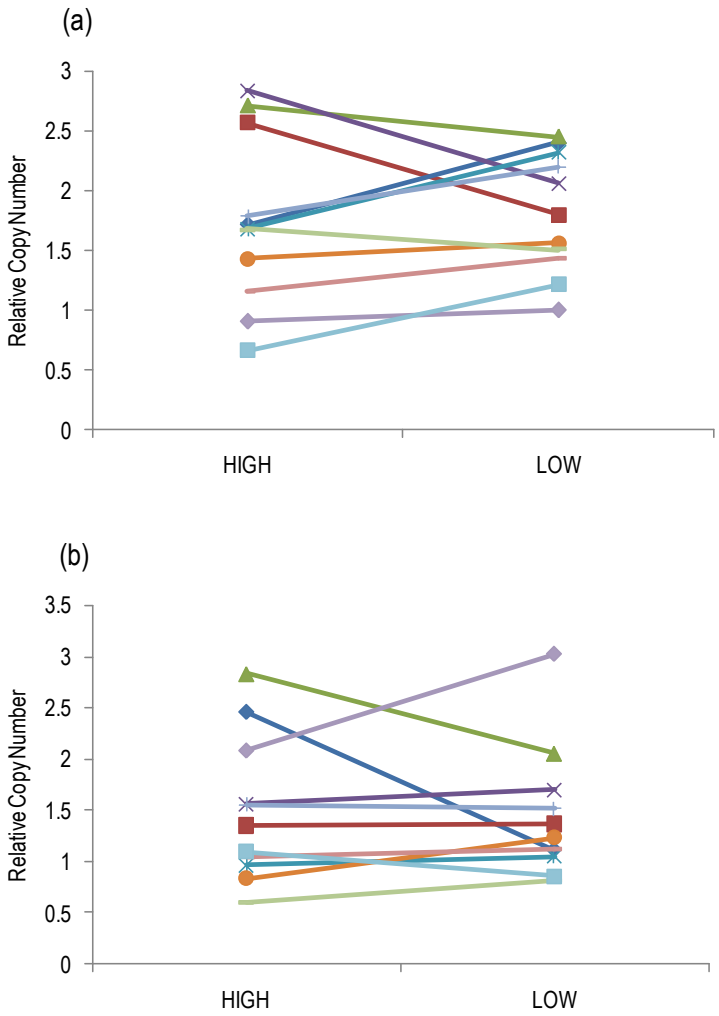

Figure 4.13 Inter- and intra-patient variation in the expression of metabolic (glycolysis) related genes in cumulus cells associated with high grade vs. low grade embryos.

Individual patient plots of the quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *LDHA*, and (b) *PFKP* in matched pairs of human cumulus cells from oocytes which formed a high grade *vs.* low grade embryo. Copy number of each gene is normalised to *GAPDH* copy number. Each of the eleven patients is represented by a different colour.

4.4 DISCUSSION

Embryo morphological appearance is routinely used as a measure of embryo potential in a clinical setting. However, the correlation between embryo morphology and pregnancy success is weak, as poor grade embryos can still form a viable pregnancy while high grade embryos frequently fail to implant (Rijnders and Jansen 1998; Graham et al. 2000; Guerif et al. 2007). Bearing this in mind, we investigated a potential correlation between the expression patterns of selected genes and clinical embryo grade. Our assessment of cumulus cell gene expression in eleven patient matched pairs of high *vs.* low grade embryos showed widely variable expression and no significant correlations between embryo morphology on day 2 of culture and cumulus cell gene expression. Interestingly, when comparing the ranges of expression between and within patients the most consistent observation was similar gene expression in the low grade embryos while the high grade embryos showed a widely variable range of expression. This may be interpreted as an indication that morphologically high grade embryos may arise from a range of different follicle/cumulus origins and may in fact have wide ranging developmental potential. Consideration of the variability within and between patients impacts the efficacy of any non-invasive test of oocyte quality which may be applied to a large number of patients across multiple treatment centres. Even within our experimental approach, where the embryos were assessed by a single embryologist, there was significant variation in the relative gene copy numbers both within and between patients. It seems that the current embryo grading system based on morphological appearance and "on time" development provides a better tool for the elimination of poor quality embryos, rather than selecting "top quality" embryos for transfer from a cohort of morphologically similar embryos.

The experimental approach matched the specific outcome for each oocyte with the gene expression of its derivative cumulus cells, but found results contradictory to those of a landmark study, which found a positive correlation between cumulus cell gene expression and clinical embryo grade in groups of embryos cultured together (McKenzie et al. 2004). In replicating aspects of this study with slight differences in the experimental design, cumulus cell expression of *HAS2*, *PTGS2* and *GREM1* was assessed, however no significant difference in the expression patterns were found when comparing embryo grades. Previously the cumulus cell expression of these transcripts was found to be significantly higher in high grade embryos compared to low grade embryos. Our study correlated cumulus cell gene expression with day 2 embryo morphological grade (48 hours post fertilisation) whereas the above study assessed day 3 embryo grade culture (72 hours post fertilisation). Zhang *et al.* found *PTX3* mRNA expression correlated with embryo development on day 3 of culture and the establishment of pregnancy following embryo transfer, but did so in pooled cumulus cell samples limiting the capacity to make direct

correlations with patient outcomes (Zhang et al. 2005). The significant relationships for *HAS2* and *GREM1* mRNA expression was supported in an independent study using individual cumulus masses from oocytes which resulted in high grade embryos; however the relationship between *PTX3* mRNA expression and clinical embryo grade was not supported (Cillo et al. 2007).

The various studies provide conflicting results as to whether embryo grade is reflected in cumulus cell gene expression and which particular genes are robust markers. Several possible explanations exist: differences in real time RT-PCR methods and embryo scoring systems, the range of patient demographics, sample sizes and pooling of the embryos in culture or the cumulus samples or both. Most experimental variations emphasise the need for more standardised methodologies, such as the assessment of embryo morphology as discussed above. For this reason we established highly sensitive and quantitative PCR assays based on standards that can be easily replicated. A further variable of note is the use of different housekeeping genes for real time RT-PCR analysis. The housekeeper *18S* rRNA was used for two studies (McKenzie et al. 2004; Zhang et al. 2005) but the source of the primers differed (a previously referenced set of primers *vs.* a ready-made commercial primer set) while Cillo *et al.* used the housekeeper *28S* rRNA (Cillo et al. 2007). Appropriate housekeeper selection affects the analysis of real time PCR data, and as such we utilised multiple housekeepers and used a non-biased algorithm to select the most appropriate control that varied least within the whole sample cohort to normalise for errors in assay input, efficiencies of reverse transcription and PCR reactions which influence this measurement.

The limited relationship between cumulus cell gene expression and embryo morphology may not only be an artefact of differing clinical procedures and experimental protocols. Metabolomic profiling of spent embryo culture media developed to non-invasively assess embryo developmental potential in culture fails to find differences in an embryo's metabolomic profile and morphology (Gardner et al. 2001; Brison et al. 2004; Seli et al. 2009). Viability scores or indices obtained from metabolomic profiling of spent embryo culture media do not correlate with clinical embryo grade. This may in part substantiate our finding that the expression of the metabolic genes *LDHA* and *PFKP*, which encode enzymes of the glycolytic pathway, were not different between the two embryo grades. The embryo metabolomic profile has, in contrast, been significantly correlated with pregnancy success, which is the most desirable end point in assisted reproduction (Brison et al. 2004; Seli et al. 2009). It is important to consider that any expensive technical method that only correlates with morphology is of little value as embryo morphology can already be evaluated cheaply and quickly by visual assessment. As embryo morphology is only

weekly correlated with pregnancy success, in actual fact the ultimate marker of oocyte quality is that which is correlated with pregnancy success.

The current analysis found no relationship between maternal age and the difference in relative gene copy number between embryo grades. However, the age range for the eleven patients was quite small (mean 34.7±0.5) and possibly not powerful enough to properly assess the effect of age. Similarly there was no relationship between maternal body mass index (BMI) and the difference in relative gene copy number between embryo grades. However, the range of BMI was also limited in this cohort, although it has previously been reported that BMI does not affect markers of embryo quality in women over thirty five (Metwally et al. 2007). However, in women less than thirty five years of age obesity results in significant adverse effects on embryo grade, embryo utilisation rate, and the number of embryos discarded and cryopreserved. Live birth rates are significantly decreased among overweight and obese women compared to normal weight women, which could be due to an impaired response to ovarian stimulation, altered oocyte quality, or implantation abnormalities (Lenoble et al. 2008). The current results do not extend the existing knowledge on the relationship between maternal age or BMI and embryo morphology.

The genes analysed in this study are critical for oocyte maturation, oocyte-cumulus cell signalling and cumulus extracellular matrix production and function. It was the functional relevance of these genes which led to their selection for analysis. While no association between cumulus cell gene expression and clinical embryo grade was identified, our data indicate that the cumulus cell gene expression patterns were widely variable both within and between patients regardless of the clinical embryo grade. The overall aim of this study is to identify markers of oocyte developmental competence, which is partly defined by the ability of an oocyte to fertilise, cleave and develop to blastocyst, but far more importantly determined by the ability of an embryo to implant and reach a healthy, term delivery. Differences in gene expression profiles between high and low grade embryos are likely to be highly sensitive to the morphological criteria used and possibly only characterised by genes which regulate embryo development and “on time” cleavage. These events do not need to be predicted as they are directly observed in scoring the embryo morphological grade. Our research endeavours to reveal genes that predict the most profound definitions of oocyte quality and developmental competence – the ability to result in a successful pregnancy and ultimately a live, term birth. This resonates with the many studies that show only a poor correlation between embryo grade and pregnancy success, highlighting why new biomarkers are important for assisted reproductive technology to improve overall outcomes.

CHAPTER 5

CUMULUS CELL GENE EXPRESSION IS A MARKER OF PREGNANCY SUCCESS

5.1 INTRODUCTION

The most desirable approach for patients undergoing assisted reproductive therapies, including *in vitro* fertilisation (IVF) and intra cytoplasmic sperm injection (ICSI), is the transfer of a single, high quality embryo with the greatest developmental potential. In terms a patient can understand, it is safer to transfer a single embryo with the greatest chance of achieving a singleton live birth with a healthy, term baby. Oocyte quality is the key limiting factor in embryo developmental potential (Ebner et al. 2000; Ebner et al. 2003a; Ebner et al. 2003b; Paz et al. 2004), yet there is still no clear understanding of what determines human oocyte developmental competence and how this can be assessed non invasively. There are currently no markers of oocyte developmental competence with a strong correlation to live birth which can be used in a clinical setting. The ideal marker should also incorporate parameters which better indicate the health outcomes for the mother and baby, which are equally as important as improving pregnancy success rates.

Australian data comparing the pregnancy outcomes for single embryo transfer *vs.* double embryo transfer reveals replacement of one selected embryo to be as successful as double embryo transfer in terms of pregnancy rates, without the risk of twin pregnancies (Kovacs et al. 2003). The multiple birth rate for babies born in Australia between 2002 and 2006 following single embryo transfer was approximately ten times lower than for women who had double embryo transfer (Wang et al. 2009b). The mean birth weight for all live born babies conceived by single embryo transfer was significantly higher than for those conceived by double embryo transfer, and babies conceived by single embryo transfer were less likely to be born preterm (Wang et al. 2009b), consistent with previous studies illustrating that babies born following single embryo transfer have better peri-natal outcomes than those conceived by double embryo transfer (De Sutter et al. 2006; Heijnen et al. 2007; Poikkeus et al. 2007). Multiple embryo transfer remains a common practise to overcome the challenge of selecting a single embryo with the greatest potential to maintain pregnancy rates, and to reduce the number of initiated cycles per patient. Improvement to oocyte and embryo selection, through newly identified methods, has the capacity to reduce multiple embryo transfers and in turn the rates of multiple gestations. As pregnancy rates are not significantly improved following double (or triple) embryo transfer but the rates of multiple gestations are greatly increased, the identification of novel markers of oocyte quality will address both maintenance of pregnancy success rates and reduction in multiple gestation rates.

As discussed extensively in the previous chapter, the only tool available for embryo selection is embryo morphological appearance and on time development, which have a poor correlation with pregnancy success (Rijnders and Jansen 1998; Tesarik and Greco 1999; Gardner and Sakkas 2003; Borini et al. 2005; Guerif et al. 2007). The success rates of ART have improved over time with developments in clinical procedures – such as culture media and ovarian stimulation protocols – to a point where approximately 41% of women under thirty eight years of age can achieve a clinical pregnancy following one cycle of ART (data correct for Repromed, Dulwich, South Australia [www.repromed.com.au]). However, there are still a large number of patients who do not fall pregnant. This highlights the need for identification of non-invasive markers of oocyte and embryo health, particularly markers to predict pregnancy success. Markers of oocyte and embryo quality will augment embryo selection and in turn reduce multiple embryo transfers. Selection of the highest "quality" oocyte should by definition mean selection of an embryo with the greatest likelihood of achieving a pregnancy which results in a healthy, term delivery.

The follicular environment is highly regulated to precisely control oocyte maturation. At the time of ovulation, in normal fertile women a single follicle will ovulate a single, mature cumulus oocyte complex (COC). Of the many oocytes within the ovary, this single oocyte somehow expresses its superiority to achieve selection to ovulate. What makes this oocyte developmentally superior and what signals it receives which favours it over others involves the cell signalling between the oocyte and the follicle. It is known that during the course of antral follicle development, the oocyte gradually and sequentially acquires meiotic and developmental competence (Eppig 1992), and the capacity to fully support embryo development (Gandolfi and Gandolfi 2001; Sirard et al. 2006). We know that the oocyte participates in the processes governing its growth and maturation through a feedback relationship with the cumulus cells surrounding it (Buccione et al. 1990a; Eppig et al. 2002; Senbon et al. 2003; Gilchrist et al. 2004; Makabe et al. 2006; Sirard et al. 2006). During oocyte growth *in vivo* the COC is maintained in a highly regulated follicular microenvironment in which hormonal stimuli initiate the stages of development, growth and maturation. Sirard and colleagues argue that oocyte and embryo developmental competence is represented in five key steps; the ability for an oocyte to resume meiosis, cleave following fertilisation, develop to the blastocyst stage, induce a pregnancy, and finally the ability to develop that pregnancy to term in good health (Sirard et al. 2006). These steps are necessary for meiotic maturation (Hunter and Moor 1987), activation of the zygotic genome (Barnes and First 1991) and blastocyst formation (De Sousa et al. 1998). Each of these steps are equally important when considering the development of techniques for the assessment of oocyte and embryo quality to be applied in a clinical setting.

Many critical cumulus-expressed genes are under the control of oocyte-secreted growth factors as well as endocrine hormones, and as such it is proposed that their expression is representative of the oocytes' maturational stage and also of the maternal environment (Russell and Robker 2007). Experiments investigating oocyte control of metabolic cooperativity between oocytes and their surrounding cumulus cells in murine COCs *in vitro* show isolated cumulus cells exhibited decreased expression levels of genes encoding subunits of glycolytic enzymes, glycolysis and activity of the tricarboxylic acid cycle (Sugiura et al. 2005). Gene expression patterns could be restored by culturing the isolated cumulus cells with fully grown oocytes, which secrete paracrine factors. Thus the oocyte controls the metabolic cooperativity between itself and the cumulus cells needed for energy production by granulosa cells and required for oocyte and follicular development (Sugiura et al. 2005). This study demonstrates that the presence of an oocyte can affect the cellular signalling of cumulus cells *in vitro*, even if the COC is not intact. This supports the utilisation of cumulus cell gene expression to evaluate the level of signalling emanating from the associated oocyte, which can be used as a non-invasive measure of oocyte quality and developmental competence.

Previous studies investigating cumulus cell parameters as markers of oocyte health have evaluated apoptosis (Yuan et al. 2005), the degree of cumulus expansion (Ebner et al. 2003b; Lucidi et al. 2003), and cumulus cell metabolic activity (Brison et al. 2004; Sugiura et al. 2005) as well as the dynamics of the follicular environment (Saito et al. 2000; Babayan et al. 2008). It is important to consider that in a clinical setting, any tests of oocyte quality would have to be non-invasive, as damage cannot be caused to any oocyte that may develop to an embryo which is then transferred. Additionally, any procedure for assessing oocyte quality would need to be expeditious for patients who undergo embryo transfer on day 2. Quantitative real time RT-PCR is a fast experimental method which can provide a large, multifactorial and precise data output. The development of a PCR assay which measures a unique signature of cumulus cell genes as markers of oocyte developmental competence, specifically pregnancy success and live birth, would be a practical tool for gamete selection in addition to existing embryo selection tools.

Cumulus cells are a readily available by-product of standard IVF and ICSI procedures that can be utilised for non-invasive tests of oocyte competence. A multitude of studies have assessed cumulus cell gene expression and its' relationship to oocyte quality, embryo morphological grade and three have

assessed pregnancy success (refer to Table 1.1, Chapter 1). While the most imperative end point of ART remains a successful term pregnancy resulting in a live birth of a healthy, normal birth weight baby, no previous studies have assessed this in a model in which all patients have undergone single oocyte and embryo culture, and most importantly single embryo transfer. The use of group oocyte and embryo culture, and double or multiple embryo transfers in these studies voids any information that was collected on the cumulus cells as there can be no direct association with the oocytes that result in embryos which implant. In a singleton gestation there is no way of knowing which embryo implanted following multiple embryo transfers, and therefore no way of making a direct correlation between the cumulus cell parameters assessed and pregnancy success. The present study is the first to assess cumulus cell gene expression from oocytes which were cultured individually, as were all resultant embryos, in a cohort of patients who underwent elective single embryo transfer. Therefore all gene expression analyses can be directly correlated with the oocyte and resultant embryo from which the cumulus cells were trimmed, and in turn establish a relationship between cumulus expressed genes and pregnancy success. In this study we sampled individual cumulus masses, utilising highly quantitative standardised real-time PCR assays to directly associate gene expression in the cumulus cells with the outcome for that particular oocyte. We investigated cumulus cell gene expression in cumulus cells from a cohort of thirty eight patients' individual oocytes that yielded a successful term pregnancy (n = 12) compared to those for which pregnancy was not established (n = 26).

5.2 MATERIALS AND METHODS

5.2.1 Embryo Quality Assessments

Embryo quality assessments were based on multiple observations. Embryo transfers were performed on days 2/3 (cleavage stage embryos n=30) or days 4/5 for extended culture (n=8). The day of embryo transfer was a clinical decision determined prior to the commencement of the patient's treatment by their clinician, and was independent of the response to stimulation or the number of embryos resulting from treatment. Embryo selection for transfer was based on morphology assessment, which is heavily weighted on day 2/3 morphology. In all cases the single highest morphological quality embryo, as determined by the appropriate scoring system (cleavage or extended culture stage), was transferred (73.7% grade 1/2 embryo transfer *vs.* 26.3% grade 3/4 embryo transfer). The cleavage stage morphology scoring system used was based on the number of cells, the degree of fragmentation and the presence of multinucleated cells. Good quality embryos were assessed to have 4-cells on day 2 or 7-9 cells on day 3 with limited fragmentation and absence of multinucleated blastomeres. Extended culture selections performed for day 4 embryo replacements were performed according to the scoring system described by Feil *et al.*, 2008, with the degree of compaction and early cavitation being assessed (Feil et al. 2008). Blastocyst development on day 5 was assessed as per the grading system described by Gardner and Schoolcraft (1999), describing degree of expansion and quality of the inner cell mass and trophectoderm (Gardner and Schoolcraft 1999). All embryos were transferred immediately after culture (no frozen/cryopreserved embryos were transferred). Embryos were scored using a scale of 1 to 4, where grade 1 indicates best quality and grade 4 indicates poor quality. Embryos selected for transfer were incubated in EmbryoGlue (Vitrolife) for 0.5-4 h before transfer and transferred into the uterus in a volume of approximately 10 µL, under ultrasound guidance. All oocytes were fertilised in individual culture, with resultant embryos cultured singly and all patients undergoing single embryo transfer only.

5.2.2 Pregnancy Outcomes

Clinical pregnancy was determined by a positive serum hCG test, the presence of a foetal heart beat 6 weeks following embryo transfer and was retrospectively analysed. Live birth outcomes were obtained from the obstetrician in charge of the patient care. Obstetricians were asked to provide delivery dates, birth weights, sex and whether there were any maternal or neonatal interventions or complications. Data analysis was limited to ten of the twelve patients who achieved a successful pregnancy. For two of the pregnant patients birth weight was not provided to Repromed by the obstetricians.

5.2.3 qPCR Assays

qPCR assays were utilised as described in Chapter 3. GeNorm analysis software identified *GAPDH* as the most stable internal control gene across the sample population and was utilised for the relative expression analysis in these experiments.

5.2.4 Statistical Analysis

Relative copy number and relative CT data are normalised to *GAPDH* copy number and CT respectively. Data are presented as box and whisker plots where the horizontal line represents the median copy number and the box encompasses 50% of data points (first quartile to third quartile). Data points within the 10th and 90th percentiles are represented by the upper adjacent and lower adjacent "whiskers" (vertical lines). Values lower than the 10th percentile and greater than the 90th percentile are indicated by closed dots. Associations with pregnancy outcomes were analysed using a Wilcoxon-Mann-Whitney test (GraphPad Prism version 5.01 for Windows, San Diego, CA, United States, www.graphpad.com). Differences were considered significant at a p-value < 0.05. A Spearman's rank order correlation was performed to examine a possible relationship between age, body mass index (BMI) or birth weight (grams) and the relative gene copy number for each of the genes of interest. Body mass index values were available for thirty seven of the thirty eight patients. Birth weights were available for ten out of the twelve patients with a live birth outcome. Further analyses were performed to assess whether gene expression of a selected gene in addition to a second gene increased the predictive power of the current model. A logistic regression model was used to examine whether the interaction between one gene measure and a second gene significantly predicts pregnancy outcome better than a model with a single gene measure only. To test whether adding the main effect and the interaction between the variable and a second gene changes the model fit significantly, the change in the -2 log likelihood of the models was obtained, and that value was compared to a chi-square distribution. A significant chi-square value would indicate that the model with one gene alone is not as good.

RESULTS

5.2.5 Live birth rate following single embryo transfer.

The live birth rate for patients following one single embryo transfer cycle for this study was 31.6% (Table 5.1). Maternal age was not significantly different between pregnant and non-pregnant patients (Mann-Whitney t-test, data not shown). There was no difference in the day 2 embryo grades of the transferred embryo, the number of oocytes collected, inseminated and fertilised, or the infertility diagnoses between pregnant and non-pregnant patients. Data presented as mean ± SEM for thirty eight patients undergoing elective single embryo transfer.

5.2.6 Cumulus cell gene expression is a molecular marker of pregnancy success.

The gene expression patterns in cumulus cell masses from individual oocytes were analysed with reference to the pregnancy outcomes of patients following single embryo transfer. Cumulus cell expression of two genes showed a significant association with pregnancy success; these were *VCAN* (Figure 5.1; a) and *PTGS2* (Figure 5.2; a). The median relative gene copy numbers of these genes were significantly ($p < 0.02$) higher (3-fold and 2-fold respectively) in cumulus cells from oocytes which established a successful pregnancy compared to cumulus cell gene expression from oocytes which failed to establish a pregnancy. Cumulus cell *PTX3* median relative gene copy number showed a trend ($p = 0.066$) towards significance with a higher relative gene copy number in cumulus cells from oocytes which resulted in a successful pregnancy and live birth (Figure 5.1; b). In cumulus cells from oocytes which resulted in a live birth compared to cumulus cells not associated with pregnancy the median relative gene copy number of *VCAN* was 1.9-fold higher (0.4553 *vs.* 0.239 respectively), *PTGS2* relative gene copy number was 1.82-fold higher (0.326 *vs.* 0.1788 respectively) and *PTX3* relative gene copy number was 1.74-fold higher (3.115 *vs.* 1.455 respectively).

Additional genes analysed include the matrix genes *HAS2* and *TNFAIP6* which showed no significant difference in the median relative copy numbers between pregnant and non-pregnant patients (Figure 5.1; c, d). There was no significant difference in the expression of the signalling genes *GREM1*, *AHR*, *AR* and *STS* between the pregnant and non-pregnant patient cohorts (Figure 5.2; b-e), nor cell median copy numbers of *ALDOA*, *PFKP*, *LDHA* and *PKM2* genes which encode subunits/isoforms of rate limiting enzymes of the glycolytic metabolic pathway which were not significantly different between patients with a successful pregnancy and those for which treatment failed and pregnancy was not achieved (Figure 5.3; a-d).

Table 5.1 Patient demographic characteristics for the analysis of cumulus cell gene expression as a marker of pregnancy success as assessed by a live birth following single embryo transfer.

	LIVE BIRTH	NO PREGNANCY
Number of Patients	12	26
Maternal Age Mean	32.9 ± 1.13	33.6 ± 0.64
Maternal Age Median	33.7 ± 1.13	33.9 ± 0.64
Maternal Age Range	24.6 – 37.7	24.6 – 40.3
BMI	26.4 ± 0.93	25.6 ± 0.9
% ICSI	91	73
Number of previous IVF/ICSI cycles	0.7 ± 0.3	0.6 ± 0.2
Number of oocytes collected	10.1 ± 1.3	11.2 ± 1
Number of oocytes inseminated	8.5 ± 1.3	9.4 ± 0.9
Number of oocytes fertilised	5.8 ± 1	6.0 ± 0.7
Day 2 grade of embryo transferred	1.9 ± 0.6	2.0 ± 0.15
Infertility Diagnosis		
Male Factor	8	16
Endometriosis	-	1
Tubal	-	2
Ovulatory	1	1
Unexplained	3	4
Combined	-	2

(Age data is presented as years ± SEM. All other data is presented as mean ± SEM)

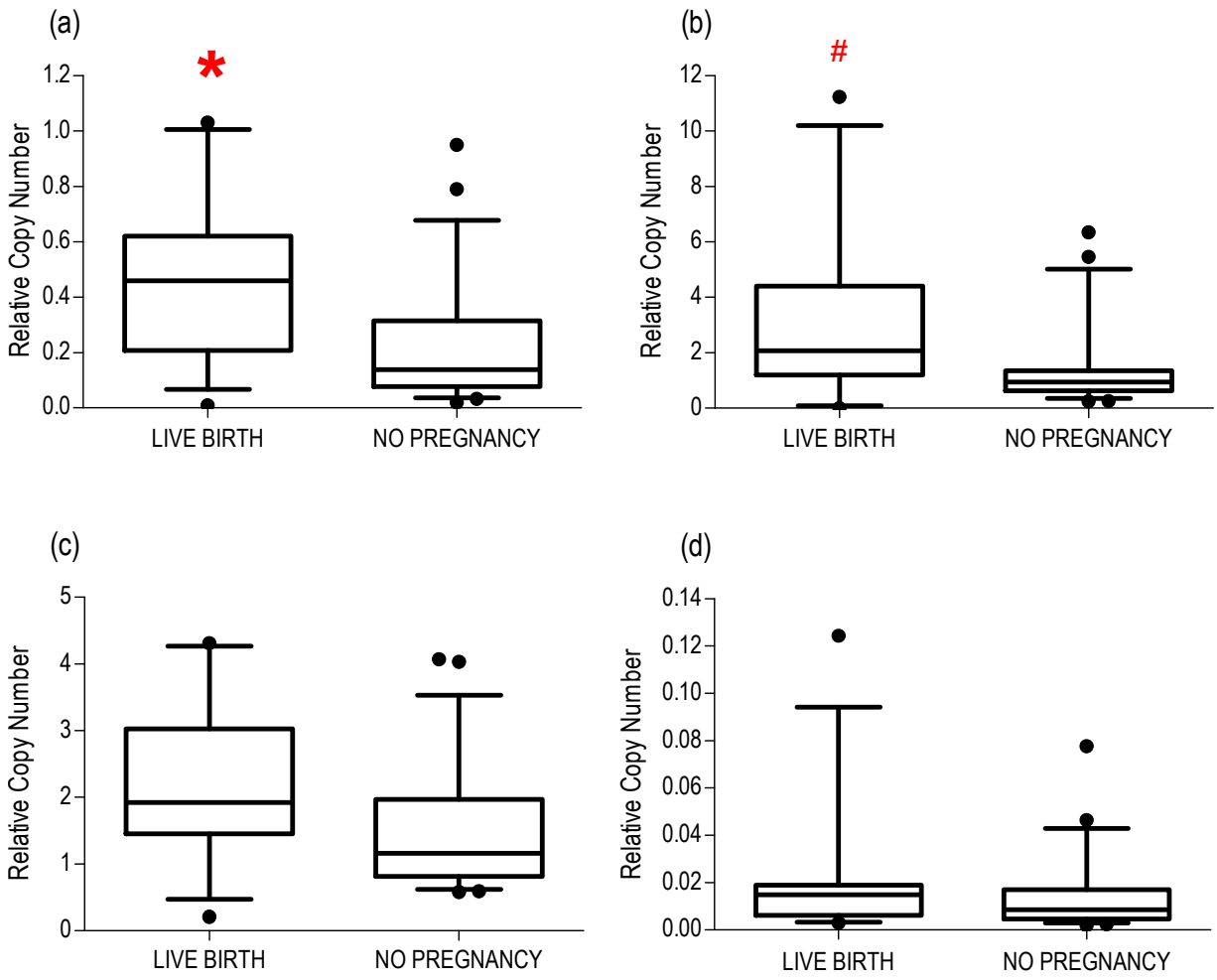

Figure 5.1 Extracellular matrix gene expression in cumulus cells associated with pregnancy success following single embryo transfer.

Quantitative real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *VCAN,* (b) *PTX3*, (c) *HAS2* and (d) *TNFAIP6* in human cumulus cells from oocytes which went on to establish pregnancy and live. Copy number of each gene is normalised to *GAPDH* copy number. Asterix (*) indicates significant difference (p = 0.02), # denotes a trend (p = 0.06).

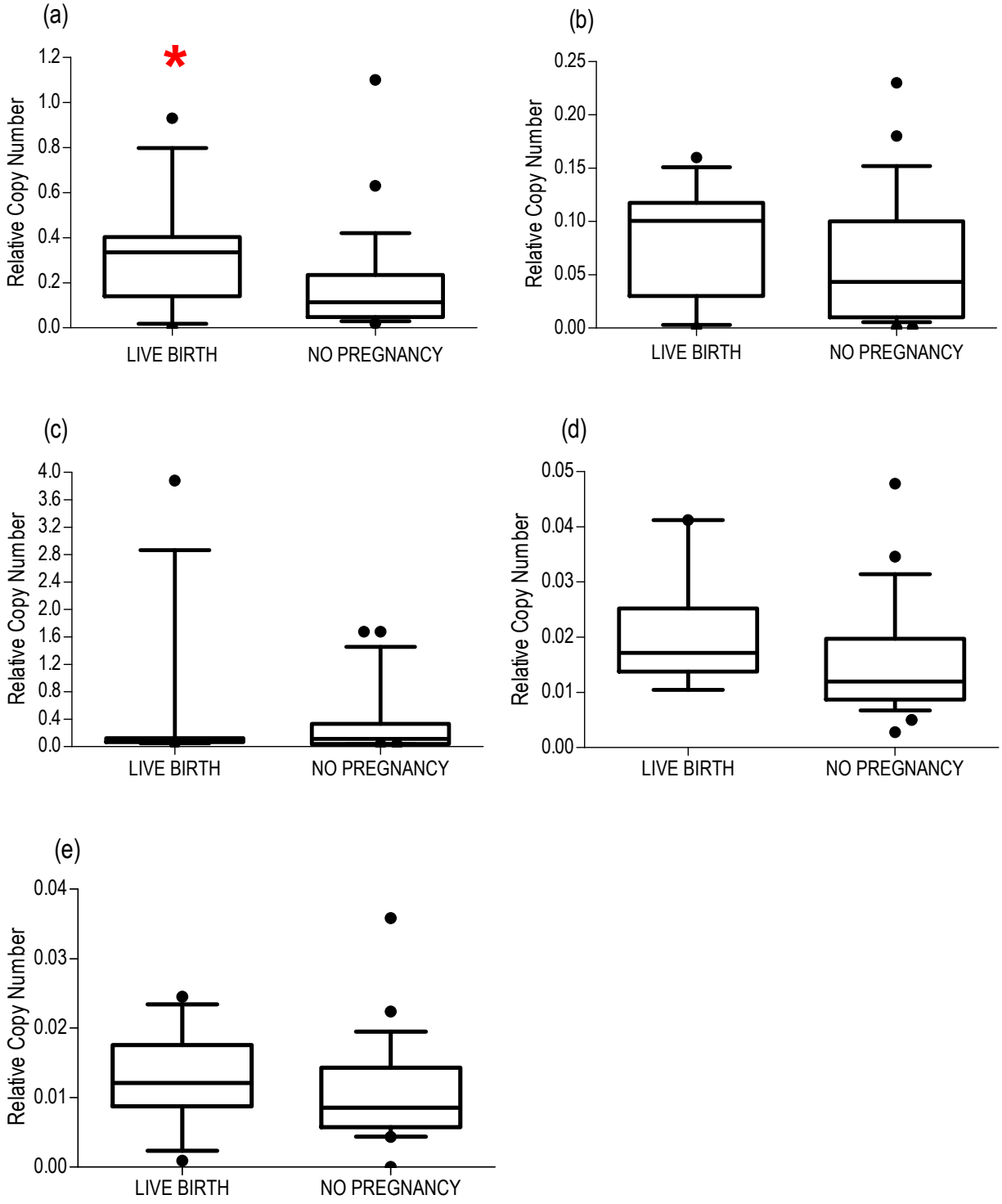

Figure 5.2 Analysis of signalling related genes in cumulus cells associated with pregnancy success following single embryo transfer.

Quantitative Real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *PTGS2*, (b) *GREM1*, (c) *AHR*, (d) *AR* and (e) *STS* in human cumulus cells from oocytes which went on to establish pregnancy and live. Copy number of each gene is normalised to *GAPDH* copy number. Asterix (*) indicates significant difference (p = 0.02).

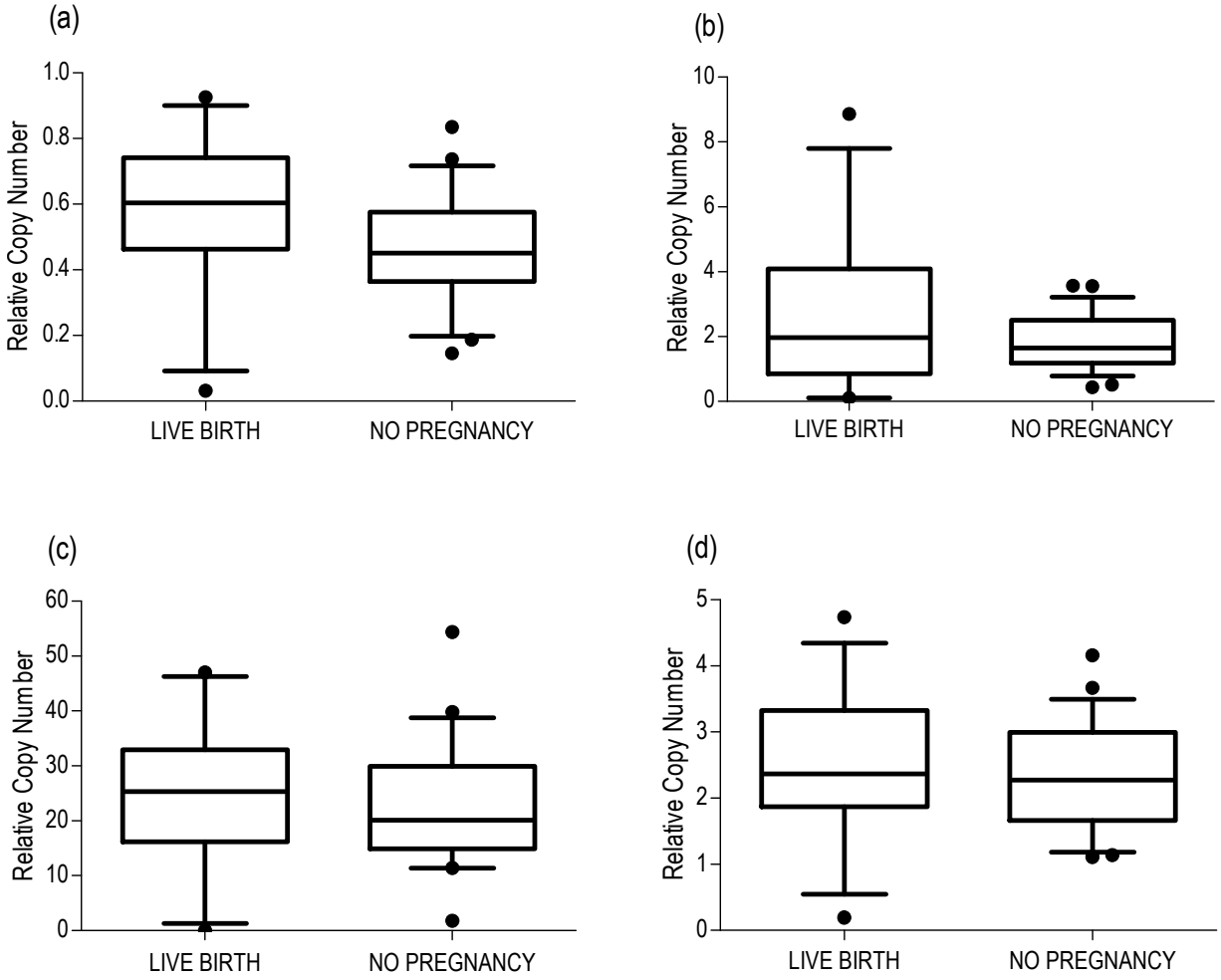

Figure 5.3 Analysis of metabolic related genes in cumulus cells associated with pregnancy success following single embryo transfer.

Quantitative Real time RT-PCR analysis of mRNA expression (gene copy number) of (a) *ALDOA,* (b) *PFKP*, (c) *LDHA* and (d) *PKM2* in human cumulus cells from oocytes which went on to establish pregnancy and live. Copy number of each gene is normalised to *GAPDH* copy number. No significant differences observed.

5.2.7 Cumulus cell gene copy number is not correlated with maternal age or body mass index (BMI).

Spearman's rank order correlations identified no associations between most genes and maternal age or BMI. Interestingly, a trending positive correlation (p = 0.051) was seen between *ALDOA* gene copy number and maternal age (Figure 5.4; a). Increased maternal age was associated with higher cumulus cell *ALDOA* relative gene copy numbers. The genes analysed showed no correlation between cumulus cell gene copy number and maternal age or BMI (Tables 5.2 and 5.3 respectively).

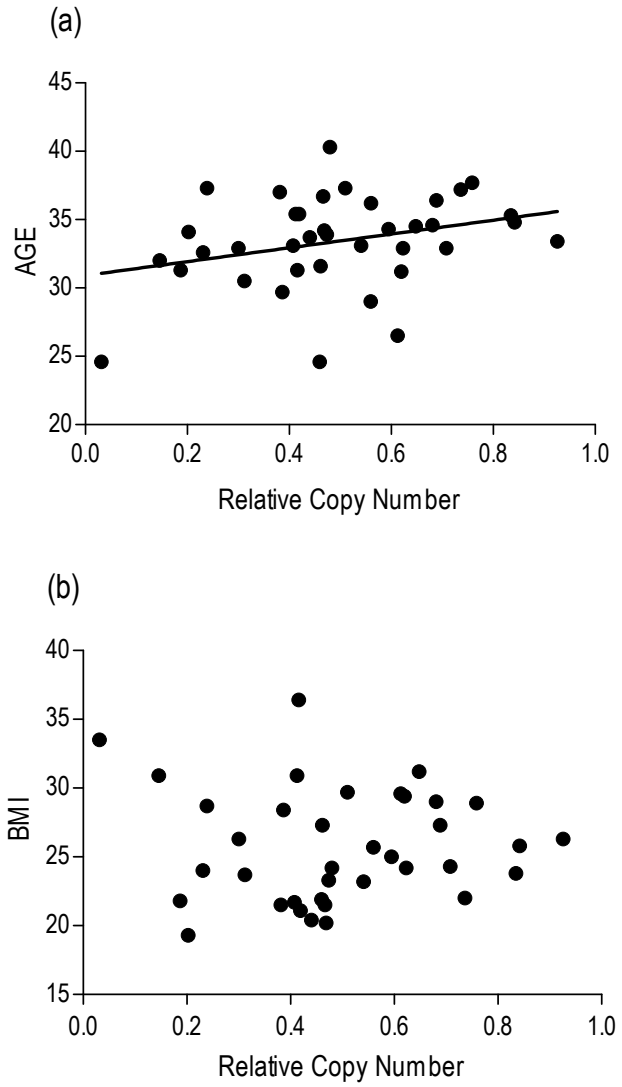

Figure 5.4 Cumulus cell *ALDOA* gene copy number is weakly correlated with maternal age but not body mass index.

A Spearman's rank order correlation was performed to compare *ALDOA* relative gene copy number and maternal (a) age and (b) BMI in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation. Copy number of each gene is normalised to *GAPDH* copy number. A trending correlation was seen between *ALDOA* gene copy number and maternal age (p = 0.05).

Table 5.2 Association between relative gene copy number and maternal age following single embryo transfer.

	Spearman *r*-value	*p*-value
VCAN	0.0851	0.61
PTX3	0.0884	0.60
HAS2	-0.0004	1.0
PTGS2	0.0141	0.93
TNFAIP6	-0.0595	0.72
STS	0.1361	0.43
GREM1	0.0778	0.64
AHR	0.0407	0.81
AR	-0.0969	0.58
ALDOA	0.3194	0.051#
LDHA	0.2133	0.20
PFKP	0.0031	0.98
PKM2	0.1994	0.23

Table 5.3 Association between relative gene copy number and maternal body mass index following single embryo transfer.

	Spearman *r*-value	*p*-value
VCAN	0.0945	0.58
PTX3	0.0037	0.98
HAS2	0.1653	0.33
PTGS2	0.0706	0.68
TNFAIP6	0.2644	0.11
STS	0.0439	0.80
GREM1	0.0450	0.79
AHR	0.2039	0.23
AR	0.2598	0.14
ALDOA	0.1181	0.49
LDHA	-0.2332	0.16
PFKP	-0.0997	0.56
PKM2	-0.1777	0.29

5.2.8 Cumulus cell relative gene copy number and delta CT are positively correlated with birth weight.

Our unique experimental approach, in which cumulus cell RNA was collected from individual oocytes that were tracked from collection through to single embryo transfer and live birth, allowed us the opportunity to assess the association between cumulus gene expression and health outcomes of the babies, specifically birth weight. A Spearman's rank order correlation was performed to assess a link between the relative cumulus cell copy number or delta CT (normalised to *GAPDH*) for each gene and birth weight (Tables 5.4 and 5.5 respectively). The delta CT was determined by the CT of the gene of interest minus by the *GAPDH* CT. Birth weight correlated with the cumulus cell expression three genes. Remarkably, cumulus cell *VCAN* delta CT was significantly ($p < 0.005$) correlated with birth weight, while *VCAN* relative mRNA gene copy number trended towards a significant ($p = 0.08$) positive correlation with birth weight (Figure 5.5). The cumulus cell relative mRNA gene copy number of *GREM1* also showed a trend ($p = 0.06$) towards a significant positive correlation with birth weight (Figure 5.6; a).The cumulus cell relative mRNA gene copy number of the glycolysis-related gene *PFKP* was significantly ($p < 0.05$) correlated with birth weight, while the delta CT showed a trend ($p = 0.06$) towards a significant correlation (Figure 5.7). Additional genes analysed showed no correlation between cumulus cell gene copy number or CT and birth weight (Tables 5.4 and 5.5 respectively). Delta CT was analysed to further ensure that any correlation observed was not influenced by additional processing of the data for the relative copy number. The correlation of CT values after simple normalisation for input variations supports the surprising result that the level of cumulus cell gene expression was indeed associated with birth weight.

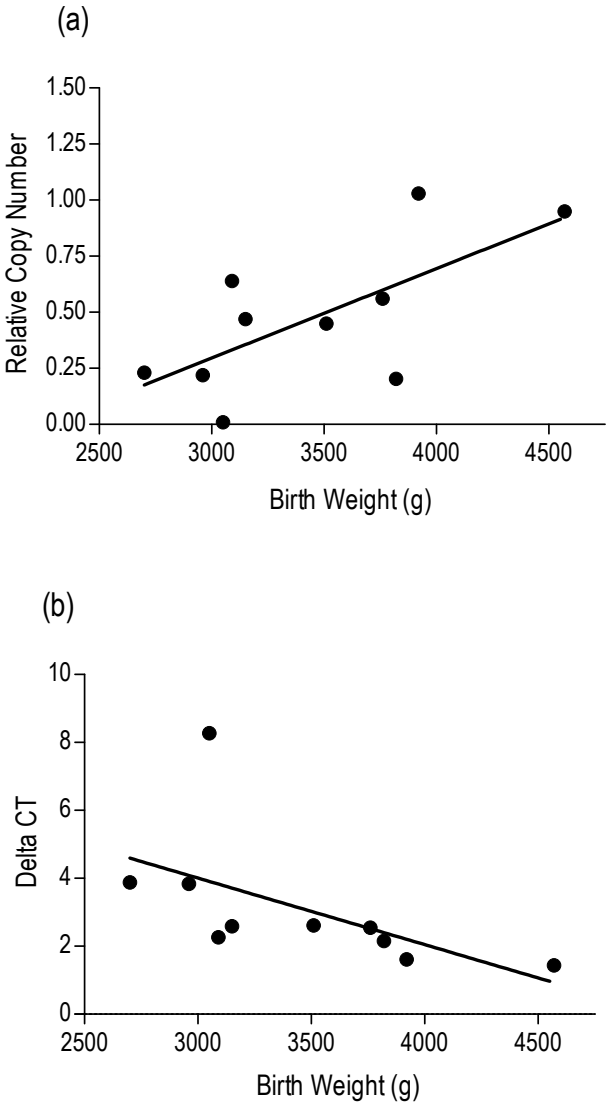

Figure 5.5 Cumulus cell *VCAN* mRNA abundance correlates with birth weight following single embryo transfer.

A Spearman's rank order correlation of birth weight (grams) and *VCAN* (a) relative gene copy number and (b) delta CT in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation. Relative copy number and delta CT data are normalised to *GAPDH* copy number and CT respectively. Cumulus cell relative gene copy number showed a trend towards significance ($p = 0.08$) while cumulus cell delta CT was significantly correlated ($p < 0.005$) with birth weight.

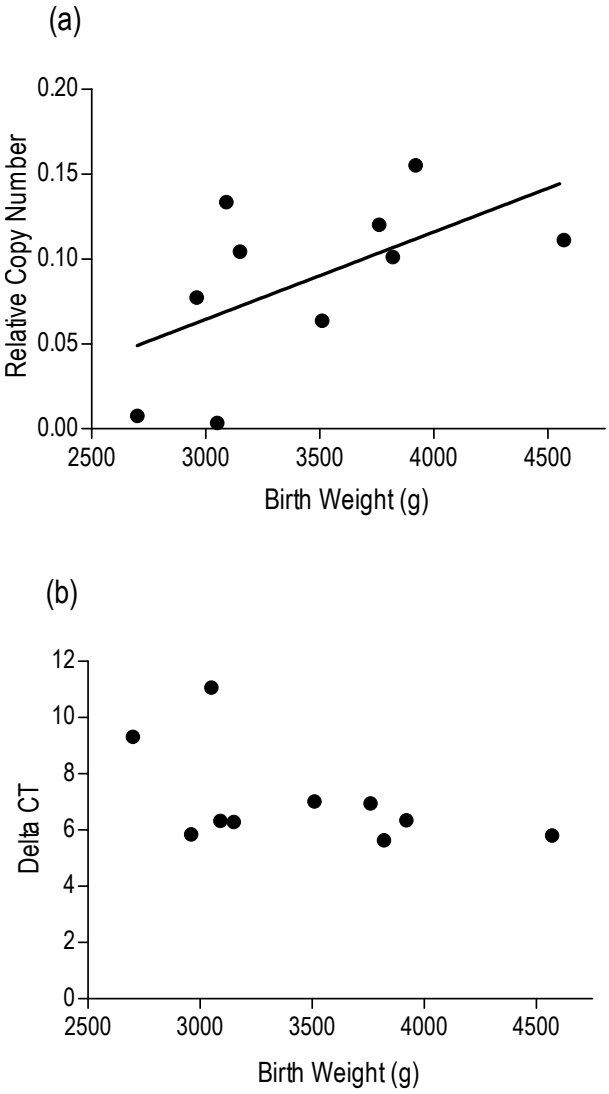

Figure 5.6 Cumulus cell *GREM1* relative gene copy number showed a trend towards significance in association with birth weight following single embryo transfer.

A Spearman's rank order correlation of birth weight (grams) and *GREM1* (a) relative gene copy number and (b) delta CT in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation. Relative copy number and delta CT data are normalised to *GAPDH* copy number and CT respectively. Cumulus cell relative gene copy number showed a trend towards significance (p = 0.06) while cumulus cell delta CT was not associated with birth weight.

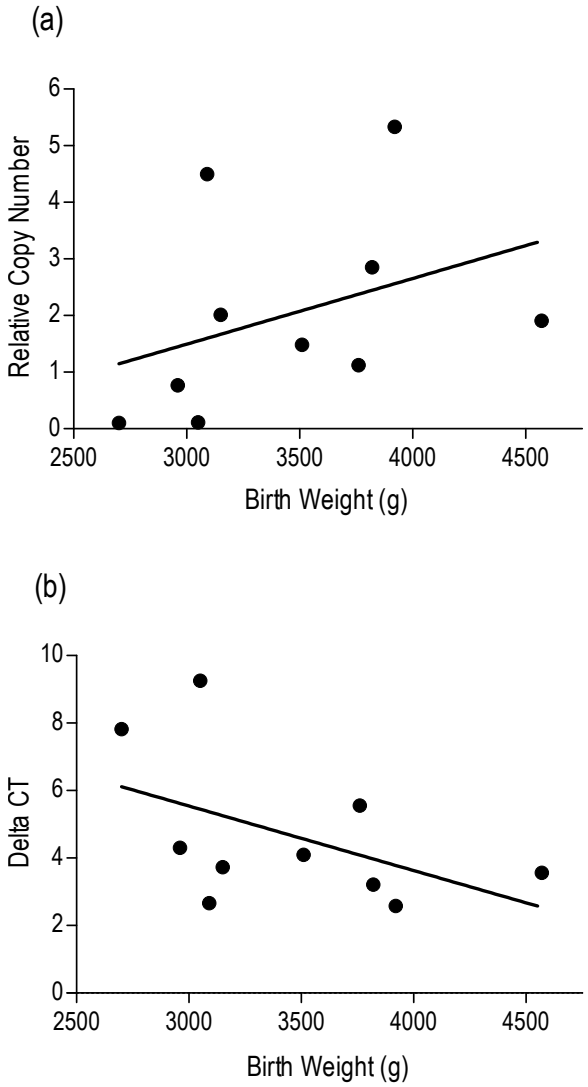

Figure 5.7 Cumulus cell *PFKP* mRNA abundance correlates with birth weight following single embryo transfer.

A Spearman's rank order correlation of birth weight (grams) and *PFKP* (a) relative mRNA gene copy number and (b) delta CT in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation. Relative copy number and delta CT data are normalised to *GAPDH* copy number and CT respectively. Cumulus cell relative mRNA gene copy number was significantly correlated ($p < 0.05$) with birth weight while cumulus cell delta CT trended towards significance ($p = 0.06$) in association with birth weight.

Table 5.4 Association between relative mRNA gene copy number and birth weight in patients with a live birth outcome following single embryo transfer.

	Spearman *r*-value	*p*-value
VCAN	0.5758	0.082#
PTX3	-0.3333	0.39
HAS2	0.3161	0.37
PTGS2	-0.0060	0.99
TNFAIP6	0.4667	0.18
STS	0.2848	0.43
GREM1	0.6121	0.060#
AHR	0.3095	0.46
AR	0.2848	0.43
ALDOA	0.4182	0.23
PFKP	0.6485	0.043*
LDHA	-0.0909	0.81
PKM2	0.5515	0.1

Table 5.5 Association between ΔCT and birth weight in patients with a live birth outcome following single embryo transfer.

	Spearman *r*-value	*p*-value
VCAN	-0.8788	0.001*
PTX3	0.2333	0.55
HAS2	-0.5237	0.12
PTGS2	-0.4545	0.19
TNFAIP6	-0.1030	0.79
STS	-0.6201	0.06#
GREM1	-0.4667	0.18
AHR	0.5106	0.13
AR	-0.1261	0.78
ALDOA	-0.3455	0.33
PFKP	-0.6242	0.06#
LDHA	-0.4061	0.25
PKM2	-0.4620	0.18

5.2.9 No evidence for a correlation between gene expression and gestation length following single embryo transfer.

A Spearman's rank order correlation was performed to determine a possible correlation between the length of gestation and relative mRNA gene copy number or relative CT in human cumulus cells. Data analysis was limited to eight of the twelve patients who achieved a successful pregnancy resulting in a live birth due to data collection limitations specified previously. Only the genes which showed a previous significant or trending correlation with birth weight were analysed. Cumulus cell *VCAN* (Figure 5.8), *GREM1* (Figure 5.9) or *PFKP* (Figure 5.10) did not show an association with gestation length.

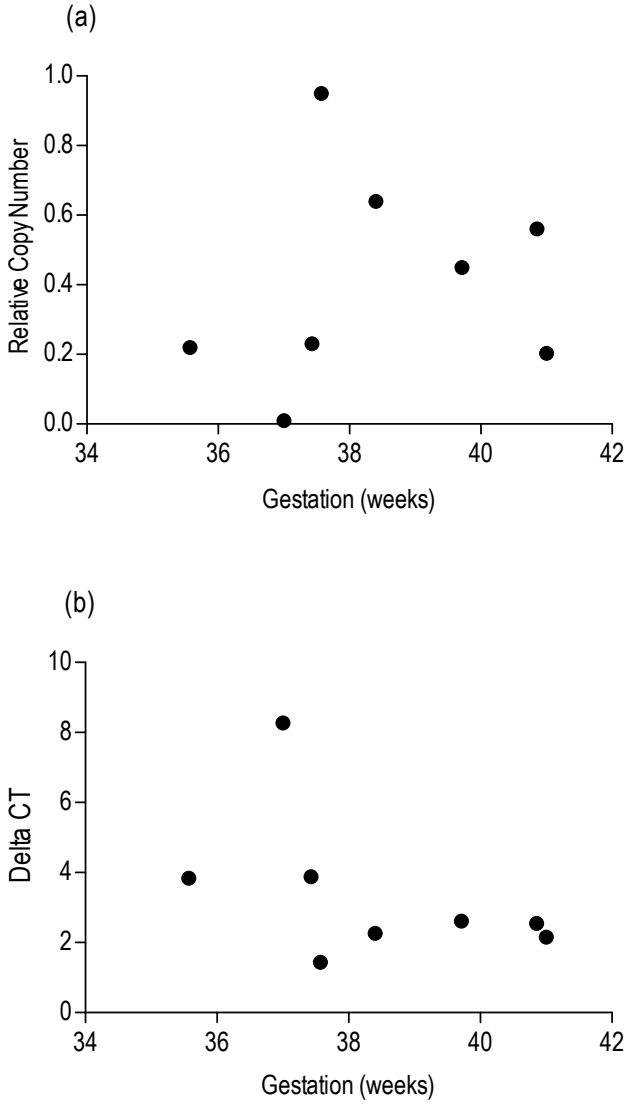

Figure 5.8 Cumulus cell *VCAN* mRNA abundance does not correlate gestation length following single embryo transfer.

A Spearman's rank order correlation of gestation (weeks) and *VCAN* (a) relative mRNA gene copy number and (b) delta CT in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation. Relative copy number and delta CT data are normalised to *GAPDH* copy number and CT respectively. No significant associations seen.

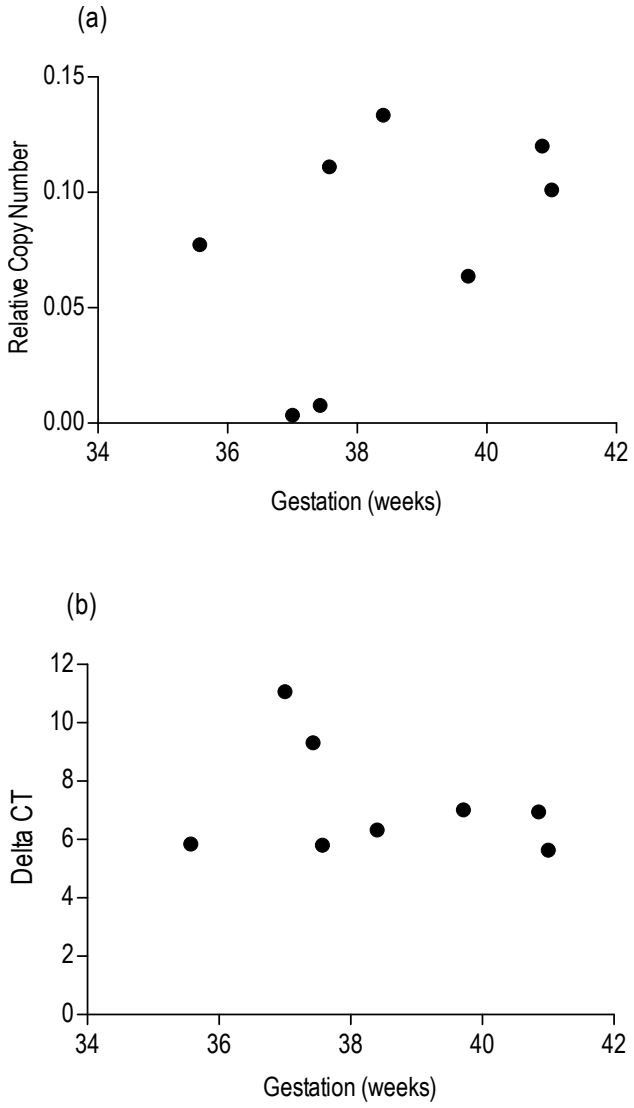

Figure 5.9 Cumulus cell *GREM1* mRNA abundance does not correlate gestation length following single embryo transfer.

A Spearman's rank order correlation of gestation (weeks) and *GREM1* (a) relative mRNA gene copy number and (b) delta CT in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation. Relative copy number and delta CT data are normalised to *GAPDH* copy number and CT respectively. No significant association seen.

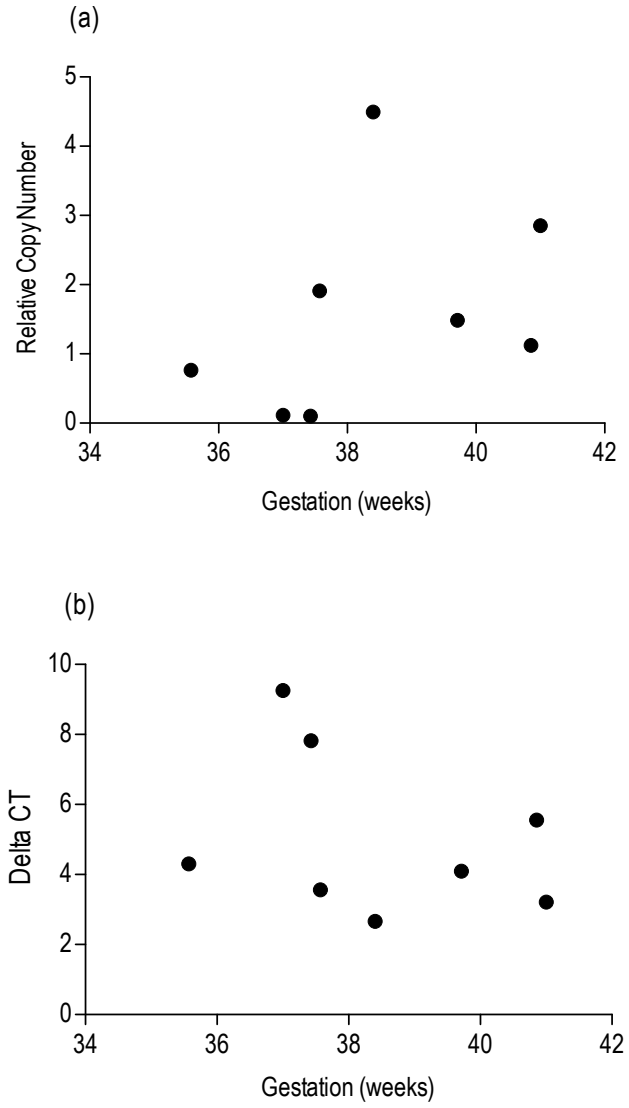

Figure 5.10 Cumulus cell *PFKP* mRNA abundance does not correlate gestation length following single embryo transfer.

A Spearman's rank order correlation of gestation (weeks) and *PFKP* (a) relative mRNA gene copy number and (b) delta CT in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation. Relative copy number and delta CT data are normalised to *GAPDH* copy number and CT respectively. No significant association seen.

5.2.10 Pregnancy outcome is not better predicted using a combination of genes.

A logistic regression model was employed to examine whether an interaction between two genes significantly predicts pregnancy outcome better than a model with a single gene measure only. Models were created to look at possible interactions between the genes already identified to correlate with pregnancy success and a second gene. That is, *VCAN*, *PTGS2* and *PTX3* were analysed using this model to determine if the predictive power of the current test could be significantly increased through the addition of a second gene. The models of *VCAN* including *PTX3* and *AR* (Table 5.6) showed a significant ($p < 0.05$) change in the log likelihood. However, the parameter estimates for both of these models (as well as the model with *STS*, though it was not significant) show that the model with the interaction is not valid (data not shown). Investigation into a possible interaction between *PTGS2* and a second variable indicated the model with *STS* as a potential better fit (Table 5.7) however this model was not significant ($p = 0.055$). Lastly, the model using *PTX3* suggests there may be some value in adding *VCAN* and *AR* to increase the prediction value (Table 5.8) however there is not enough data to form a reliable, robust conclusion.

5.6 Predicting pregnancy success from *Gapdh* normalised *Versican* and a second variant

Additional Variable	-2 log likelihood	Difference in LL from *VCAN*-only model	*p*-value for difference
PTX3	31.2655	11.4785	0.0032*
HAS2	42.3632	0.3808	0.8266
PTGS2	40.3289	2.4151	0.2989
TNFAIP6	41.7574	0.9866	0.6106
STS	38.9134	3.8306	0.1473
GREM1	41.0301	1.7139	0.4244
AHR	41.3282	1.4158	0.4927
AR	33.3009	9.4431	0.0089*
ALDOA	41.7611	0.9829	0.6117
PFKP	42.0304	0.7136	0.6999
LDHA	41.9561	0.7879	0.6744
PKM2	42.5589	0.1851	0.9116

(* denotes a significant interaction where $p < 0.05$, # denotes a trend towards a significant interaction)

5.7 Predicting pregnancy success from *Gapdh* normalised *Prostaglandin Synthase 2* and a second variant

Additional variable	-2 log likelihood	Difference in LL from *PTGS2*-only model	*p*-value for difference
VCAN	40.3289	4.0271	0.1335
PTX3	43.6025	0.7535	0.6861
HAS2	44.3193	0.0367	0.9818
TNFAIP6	42.7171	1.6389	0.4407
STS	33.9343	10.4217	0.0055*
GREM1	43.9210	0.4350	0.8045
AHR	44.1463	0.2097	0.9005
ALDOA	42.7915	1.5645	0.4574
PFKP	43.6430	0.7130	0.7001
LDHA	43.5338	0.8222	0.6629
PKM2	42.3694	1.9866	0.3703

(* denotes a significant interaction where $p < 0.05$)

5.8 Predicting pregnancy success from *Gapdh* normalised *Pentraxin 3* and a second variant

Additional variable	-2 log likelihood	Difference in LL from *PTX3*-only model	*p*-value for difference
VCAN	31.2655	14.2165	0.0008*
HAS2	44.7298	0.7522	0.6865
PTGS2	43.6025	1.8795	0.3907
TNFAIP6	45.1580	0.3240	0.8505
STS	43.7401	1.7419	0.4186
GREM1	43.6382	1.8438	0.3978
AHR	43.0124	2.4696	0.2909
AR	36.4732	9.0088	0.0111*
ALDOA	43.4766	2.0054	0.3669
PFKP	43.8184	1.6636	0.4353
LDHA	44.9295	0.5525	0.7586
PKM2	45.1786	0.3034	0.8592

(* denotes a significant interaction where $p < 0.05$)

5.3 DISCUSSION

The ability to select a single embryo with the greatest developmental potential is the most desirable approach for patients undergoing ART. There are currently no biomarkers of oocyte developmental competence demonstrated to reliably and repeatedly predict either successful implantation or pregnancy outcome. The acquisition of high oocyte developmental competence is critical to embryo development and the establishment of a successful pregnancy, for both naturally and artificially conceived pregnancies (Gilchrist et al. 2008). The identification of novel markers of oocyte developmental competence will augment the selection of gametes and embryos in assisted reproductive technology. Cumulus cells are routinely discarded during infertility treatment, and the close, functional relationship cumulus cells have with the oocyte during folliculogenesis makes them an ideal candidate for interpreting oocyte health. The developmental potential of oocytes to produce a high morphological grade embryo has been shown to be reflected in the gene expression profile of its surrounding cumulus cells (McKenzie et al. 2004; Zhang et al. 2005; Feuerstein et al. 2007; Hasegawa et al. 2007; van Montfoort et al. 2008). However, a morphologically normal embryo does not guarantee a successful pregnancy outcome (Rijnders and Jansen 1998; Graham et al. 2000; Guerif et al. 2007). Those studies to date have assessed pregnancy success using groups of oocytes and could not relate the expression to individual outcomes.

In this study we have performed analysis of cumulus cell gene expression from oocytes tracked individually from oocyte pick up and cumulus cell sampling through to single embryo transfer and any subsequent live births. By being able to track the fate of oocytes from collection through to birth and associate these parameters with gene expression in matched samples we have identified genes that may predict better ART success rates and, excitingly, healthier outcomes. These results show that cumulus cells from single oocytes can be harvested and rapidly subjected to multiple gene analysis using very routine technologies. Thus, this test has all the necessary qualities of a very useful, easy to apply clinical test.

Three genes identified as useful biomarkers expressed in cumulus cells were *PTGS2*, *VCAN* and *PTX3*. By selectively focusing on genes with important roles in cumulus cell matrix and metabolism we have been able to identify genes which indicate a successful pregnancy outcome. The observed correlation between GDF-9 target gene expression of *PTGS2* and *PTX3* and oocyte developmental competence supports the conclusion that oocyte factor enhanced COC gene expression is important for oocyte

developmental competence. The data shows that expression of genes that modulate cumulus matrix function contributes to oocyte developmental competence and pregnancy success. Metabolic enzyme genes did not change between the groups. This surprising outcome suggests that this enigmatic matrix plays a profound role in oocyte developmental competence.

Acquisition of oocyte developmental potential is thought to be dependent on the function of the cumulus cells which are in turn controlled by signals emanating from the oocyte, as well as endocrine signals (Buccione et al. 1990a; Salustri et al. 1990a; Salustri et al. 1990b; Vanderhyden et al. 1990). The expression level of genes responding to oocyte derived signals can be expected to indicate the strength of that signal. A part of our underlying hypothesis is that a strong signal from the oocyte is an expression of its quality that communicates readiness to ovulate to the follicular somatic cells, especially cumulus cells, helping to induce expression of genes required for ovulation. Hence the expression patterns of these genes represent the veracity of the oocyte and potential biomarkers through which oocyte potential may be interpreted. Supplementation with recombinant murine *Gdf-9* during *in vitro* oocyte maturation increases the developmental competence of bovine (Hussein et al. 2006) and mouse COCs (Yeo et al. 2008) suggesting that a robust oocyte signal also promotes gene expression in cumulus cells which in turn improves oocyte quality. We therefore focussed our analysis on cumulus genes involved in a range of cumulus cell functions that are known to be responsive to oocyte secreted Tgfβ family members; GDF-9 and BMP-15 (Dong et al. 1996; Aaltonen et al. 1999; Elvin et al. 1999a; Elvin et al. 1999b).

Cumulus expansion is essential for ovulation, fertilisation, and hence, fertility (Russell and Robker 2007) and genes we have identified as biomarkers of oocyte developmental competence and pregnancy success are each important for cumulus expansion. The mechanism by which *VCAN* is regulated in cumulus cells remains unknown. Our laboratory has recently shown that *VCAN* is more highly expressed in the cumulus cells of human ovaries compared to mural granulosa cells (Dunning et al. 2007). Furthermore, *in vitro* matured mouse COCs lack versican, potentially contributing to the perturbed development of embryos from *in vitro* maturation (Dunning et al. 2007). Together with our present data, this suggests the *VCAN* encoded versican protein is an important component of the cumulus matrix and its presence directly influences oocyte developmental competence. Interestingly, our result is further supported as *VCAN* expression was found to be altered in a microarray study comparing early *vs.* late cleaving embryos (van Montfoort et al. 2008). The expression of *PTX3* showed a strong trend towards an association with pregnancy outcome. The *PTX3* gene product interacts with

TNFAIP6 in the cumulus matrix, and this specifically binds hyaluronan throughout the cumulus matrix (Varani et al. 2002; Salustri et al. 2004; Russell and Salustri 2006). The relationship between cumulus cell *PTX3* mRNA expression and oocyte competence remains ambiguous. The oocyte secreted factor GDF-9 has been shown to induce *PTX3* mRNA expression in cultured cumulus cells from oocytectomised COCs and during *in vitro* culture (Elvin et al. 1999a; Elvin et al. 1999b; Elvin et al. 2000; Varani et al. 2002; Salustri et al. 2004; Dragovic et al. 2007). Previous studies have postulated a significant relationship between *PTX3* expression and oocyte fertilisation ability (Zhang et al. 2005), while other studies found no difference in *PTX3* expression when comparing fertilisation rates or embryo morphology (McKenzie et al. 2004; Cillo et al. 2007). Our results add further weight to a relationship between *PTX3* expression in cumulus cells and the acquisition of oocyte developmental competence.

The *PTGS2* gene encodes the COX2 enzyme which synthesises prostaglandins. *PTGS2* expression in bovine and primate COCs may participate in the timing of maturation (Duffy 1987; Sirois and Richards 1992) and determine oocyte quality (Calder et al. 2001). In mice, *Gdf-9* is able to facilitate cumulus expansion via the induction of *PTGS2* and the resultant prostaglandin E_2 (PGE2) production (Elvin et al. 2000). *Ptgs2* deficient mice have defects in cumulus matrix stability, ovulation, fertilisation, decidualisation and implantation (Lim et al. 1997), and *Ptgs2* is important for survival of mouse cumulus cells (Takahashi et al. 2006). Therefore our observation of a correlation between *PTGS2* expression and pregnancy outcome supports our understanding of the role of *PTGS2* in cumulus expansion and promoting oocyte maturation, and further indicates a key role of the COC matrix composition and function in the acquisition of oocyte developmental competence. Higher levels of cumulus cell *PTGS2* expression has previously been associated with embryos assessed to be of high morphological grade compared to low grade embryos (McKenzie et al. 2004), and low cumulus cell *PTGS2* expression has been associated with embryos which developed to the blastocyst stage in culture compared to embryos which stopped developing at the embryo stage on day 6 (Feuerstein et al. 2007).

A near significant association was seen between maternal age (years at date of oocyte pick up) and cumulus cell *ALDOA* relative mRNA gene copy number, but not with other genes within the glycolytic pathway, or any other genes analysed. Expression of the aldolase 1A isoform has been reported to be up-regulated in the testes of a mouse model with accelerated senescence compared to control mice (Chiba et al. 2007). This expression was indicative of active cell division and proliferation of the germ cells to produce round spermatids. However, this association has not previously been correlated with maternal age or oocyte developmental competence. Maternal age plays a significant role in fertility (and

therefore infertility), as from the age of about 38 years onwards, the rate of follicular depletion accelerates leading to a decreased number of selectable follicles while age related changes in the ovary also account for loss of reproductive function (Gougeon 2005). Oocytes, all of which are present at birth, decline in number and quality with age. The endocrine function of the ovary declines with age, and the ovary becomes unable to sustain its normal neuroendocrine function (Fitzgerald et al. 1998). Evidence from treatment with young oocytes donated to older women indicates that the major responsibility for the decline in fertility with age can be attributed to aging oocytes (Speroff 1994; Sauer 1998). Oocyte age appears to specifically impair embryo implantation potential, which begins to decline gradually in women at 30 years of age and is reduced by half by 40 years of age (Hull et al. 1996). The effect of age on oocyte quality is best demonstrated by Figure 1.2 previously described in Chapter 1.

A significant association between birth weight (grams) and cumulus cell *PFKP* ($p < 0.05$) relative gene copy number, along with a strong trend towards significance for cumulus cell *GREM1* ($p = 0.06$) and *VCAN* ($p = 0.08$) relative mRNA gene copy number was seen in patients for whom live birth weight was recorded. However, as only ten birth weights were available this exciting result must be investigated in a larger cohort. As *VCAN* relative mRNA gene copy number was also significantly correlated with successful pregnancy, the expression levels of this gene may not only indicate oocytes with better implantation quality but these higher quality embryos may have greater developmental capacity right through gestation. The association of *PFKP* and *GREM1* with birth weight may also provide a useful clinical tool to be further explored in a larger study. While these genes may be predictive of birth weight, we see that some babies born in this cohort were of a low birth weight, which was associated with low expression levels, and that a better quality gamete with higher expression within the patient's cohort may have resulted in a live birth of a healthy birth weight baby. Changes in environmental conditions both *in vivo* and *in vitro*, mediated by a range of epigenetic, cellular and metabolic mechanisms in the preimplantation embryo, may alter cell division, gene expression, morphology and potential (Watkins et al. 2008). *In vitro* culture of embryos has been associated with changed in fetal growth (Bowman and McLaren 1970; Caro and Trounson 1984; Khosla et al. 2001; Thompson et al. 2007), gene expression and regulation (Khosla et al. 2001; Reik et al. 2003; Lucifero et al. 2004), postnatal behaviour (Ecker et al. 2004; Fernandez-Gonzalez et al. 2004), and raised systolic blood pressure (Watkins et al. 2007).Short term effects of embryo culture alter the global pattern of gene expression within blastocysts, indicating a sensitivity of several gene pathways (Rinaudo and Schultz 2004).

The consequences of assisted reproductive technology on the epigenetics of embryos and the incidence of imprinting disorders is an important consideration during the development and refinement of this technology (Horsthemke and Ludwig 2005). While the risk of an imprinting disorder following ART is very low and there is no reason for alarm, the increase seen compared to naturally conceived children is fitting with an adverse effect of preimplantation manipulation and culture on proper establishment of epigenetic marks (Watkins et al. 2008). The effect of *in vitro* manipulation and culture of early preimplantation stage embryos on epigenetic changes is indicative of the sensitivity of the embryo during this stage of development. The current results show a correlation at an earlier stage of development, between cumulus cell gene expression and birth weight, which is suggestive that even the oocyte is susceptible to reprogramming during the short culture period prior to and during fertilisation. We see that even in the patients who achieved a successful pregnancy there is a large scope of birth weights following birth. While obvious obstetric interventions and factors specific to each pregnancy cannot be ignored, there is a possible link between oocyte-cumulus cell communication and the developmental program of an embryo in relation to birth weight. While the current cohort has insufficient numbers to make definite conclusions it is a possible mechanism which should be the focus of detailed future research.

While the current test investigated the predictive value of individual genes, it was worth consideration that a combination of genes would have the potential to increase the predictive power of such a test. The logistic regression model used to assess interactions between genes with the potential to increase the predictive power of the test showed promising outcomes however the small sample size available meant there was not adequate data to draw a reliable conclusion. *AR* was consistently reported to have the capacity to increase the predictability of this test when combined with either *VCAN* or *PTX3*. This finding merits further investigation in a larger patient cohort when possible, as it may dramatically improve the effectiveness of this test. The current results have limited predictive value due to a small patient cohort and validation using a larger independent sample set is an important future aim of this research.

These results support our hypothesis that certain cumulus cell genes are candidate markers for high oocyte developmental potential and hence predict pregnancy outcome. The results also support the hypothesis that the bi-directional communication between the oocyte and cumulus cells mediates oocyte developmental competence. The assessment method employed here can be rapidly applied in a clinical laboratory setting, and combined with embryo morphological assessment could give a better

overall prediction of the success of ART cycles for each embryo. This study is the first to assess the correlation between cumulus cell gene expression from individual oocytes with pregnancy outcome in patients who underwent single embryo transfer only. The remarkable observation that some cumulus cell genes correlated with birth weight suggests that cumulus cell gene expression may be an even more powerful indicator of oocyte quality than we first predicted. We conclude that cumulus cell gene expression has a high potential to improve success rates and health outcomes if included as a diagnostic tool in ART clinics.

CHAPTER 6

MICROARRAY IDENTIFICATION OF MOLECULAR MARKERS OF PREGNANCY SUCCESS

6.1 INTRODUCTION

Microarray technology plays a vital role in both basic and diagnostic research, where increased understanding of basic biological processes can be gained through genome wide non-biased detection of genes differentially regulated under changing conditions. Through this approach new and unpredicted biomarkers of health or disease may be discovered. Microarray can aid in the understanding of complex or poorly described biological pathways and their regulation, as well as the discovery of pathways not previously identified in various tissues. More common is the use of microarray to compare a treatment or condition to understand changes in the system which can affect outcomes. A number of studies have now used microarray to assess cumulus and mural granulosa cell gene expression associated with oocyte fertilisation ability (Zhang et al. 2005), embryo morphology and embryo viability (van Montfoort et al. 2008) or pregnancy success (Assou et al. 2008; Hamel et al. 2008; Hamel et al. 2009) as well as comparisons of cumulus cell masses obtained from bovine oocytes matured either *in vivo* or *in vitro* (Tesfaye et al. 2009). *In vitro* maturation of bovine oocytes led to an up-regulation of stress response genes while *in vivo* matured oocytes had a greater abundance of cumulus expansion and oocyte maturation genes. Such analyses may lead to a greater understanding of the pathways affected by *in vitro* culture and subsequent improvements to developmental competence of *in vitro* matured oocytes through enhanced culture conditions.

Microarray studies have identified a large set of cumulus cell genes as potential biomarkers of oocyte health. In the first study utilising a microarray approach, human cumulus cells were pooled depending on oocyte fertilisation ability and embryo developmental outcome at the 8-cell stage (Zhang et al. 2005). Functional gene groups with higher expression in cumulus cells from oocytes that developed to 8-cell embryos on day 3 compared to cumulus cells from oocytes which failed to fertilise included cell cycle and cell proliferation, apoptosis and oncogenesis, energy metabolism, and proteases. Functional gene groups with higher expression in cumulus cells from oocytes that fertilised compared to those which failed to fertilise included proteases, transmembrane transporters and signal transduction, and metabolism and catabolism (Zhang et al. 2005). Pentraxin 3 (*PTX3*) expression was investigated further using qPCR following identification by microarray analysis and was found to be significantly higher in cumulus cells from oocytes associated with embryo development to the 8-cell stage compared to oocytes which failed to fertilise. Subsequent microarray studies have identified functional gene groups associated with cell cycle, angiogenesis, apoptosis (Assou et al. 2008; van Montfoort et al. 2008), epidermal growth factor, fibroblast growth factor, chemokine and cytokine signalling (van Montfoort et al. 2008), and steroidogenesis (Hamel et al. 2008). Validation of these independent microarray results by qPCR has substantiated the potential of these markers of oocyte quality. Hamel *et al.* identified

steroidogenic genes in granulosa cells to be significantly associated with pregnancy success (Hamel et al. 2008). The genes 3-beta-hydroxysteroid dehydrogenase (*3βHSD*), Ferredoxin 1 (*FDX1*), Serine proteinase inhibitor clade E member 2 (*SERPINE2*), Cytochrome P450 aromatase (*CYP19A1*), and cell division cycle 42 (*CDC42*) were significantly higher in mural granulosa cells from follicles which contained an oocyte associated with a positive pregnancy outcome (Hamel et al. 2008). However, the qPCR validation, which was carried out in the same pools of granulosa cells used for the microarray studies, did not assess granulosa cells from individual follicles and not all patients underwent single embryo transfer.

Cumulus cells from oocytes which developed to late cleaving embryos were reported to have higher expression of genes which most likely indicated hypoxic conditions and delayed oocyte maturation compared to cumulus cells from oocytes that developed to early cleaving embryos following microarray analysis (van Montfoort et al. 2008). In a set of independent samples, qPCR confirmed eight genes had differential expression between the cumulus cells from oocytes which formed early cleaving embryos compared to late cleaving embryos. The genes identified are regulators of cell cycle and stress response. The eight genes, Cyclin D2 (*CCND2*), Catenin delta-1 (*CTNND1*), CXC chemokine receptor 4 (*CXCR4*), 7-dehyrocholesterol reductase (*DHCR7*), Dishevelled dsh homolog 3 (*DVL3*), Glutathione peroxidise 3 (*GPX3*), Heatshock 27 kDa protein 1 (*HSPB1*) and Tripartite motif-containing 28 (*TRIM28*), showed variable differential expression in cumulus cells from oocytes which developed to early cleaving embryos compared to late cleaving embryos. The third microarray study assessing cumulus cells as markers of oocyte health identified BCL2-like 11 apoptosis facilitator (*BCL2L11*) and phosphoenolpyruvate carboxykinase 1 (*PCK1*) as up-regulated genes in cumulus cells from patients which achieved a clinical pregnancy, while nuclear factor I/B (*NFIB*) was down-regulated in cumulus cells from oocytes associated with a clinical pregnancy (Assou et al. 2008). These results were confirmed by qPCR validation to be statistically significant between the pregnancy outcome groups. However, patients did not undergo single embryo transfer although the cumulus masses were analysed individually, therefore the individual gene expression patterns cannot be fully correlated with the embryo which created a successful pregnancy. Nevertheless, the identification of these markers add value to the utilisation of cumulus cell gene expression to assess oocyte developmental competence and help to build a total gene signature associated with competence. Further validation in individual cumulus masses from patients who underwent single embryo transfer will reveal the full potential of these markers.

The results presented in this chapter were collected through the utilisation of a microarray platform to examine the genome wide expression pattern present in cumulus cells which may identify molecular markers of oocyte developmental competence leading to pregnancy success. Cumulus cells collected from patients who achieved a live birth outcome were compared to cumulus cells from patients where IVF failed and pregnancy was not established. Through this comparison, the experimental aims were to identify additional novel markers of oocyte developmental competence to augment the establishment of a "gene signature" which can be applied to cumulus cells to assess the health of their enclosed oocyte and potentially predict pregnancy outcomes. Our initial analysis using highly quantitative real time RT-PCR centred on genes within pathways known to be controlled by the oocyte and important for oocyte maturation and the acquisition of developmental competence. Following thorough investigation of these selected gene markers, our utilisation of a microarray platform has identified additional molecular markers of oocyte developmental competence associated with pregnancy success. Assessment of oocyte and embryo health should take into account the physiology of oocytes and embryos and not be solely based on pregnancy rates; therefore the clinical relevance of these markers extends beyond the improvement of pregnancy success rates. By utilising microarray technology this study may shed light on new pathways which are important for normal oocyte physiology.

6.2 MATERIALS AND METHODS

Microarray experiments were carried out by the Adelaide Microarray Centre (AMC, IMVS Main Building, SA, Australia). A useful review of general microarray standard operating procedures is outlined in (Forster et al. 2003). Detailed below is the more specific methods utilised for the experiments presented in this thesis as performed by AMC.

6.2.1 RNA cleanup and concentration

In order to generate RNA samples with sufficient quantity to complete microarray hybridisation without the potentially confounding step of pre-amplification, cumulus cell RNA from at least three patients was pooled into six pools of RNA representing three replicate samples from individual oocytes that either resulted in pregnancy success (n=3) or no pregnancy (n=3). Patients were allocated to pools based on the amount of RNA sample available. Previous gene expression data was not considered when allocating patients to various pools. Total RNA was further purified and concentrated using the Qiagen RNeasy Micro RNA Isolation kit as per the manufacturers' instructions (Qiagen, Doncaster, VIC, Australia). RNA quantity was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA integrity was further confirmed with an Agilent 2100 Bioanlyzer (Agilent Technologies, Forrest Hill, VIC, Australia). RNA was not amplified due to possible confounding effects such as transcript loss due to the amplification process which have been found to be not randomly distributed, and have higher GC-content and hairpins in the sequence mainly found in the lower intensity range which includes many transcription factors from specific signalling pathways (van Haaften et al. 2006).

6.2.2 cDNA synthesis and dye coupling

Total RNA was dissolved in 20 µL of Diethyl pyrocarbonate (DEPC) H_2O. 2 µL of anchored polyT(V)N (2 µg/µL) was added and incubated at 70ºC for 10 mins. The sample was then placed on ice, mixed with 6 µL of 5X Superscript II buffer, 2 µL of 0.1 M DTT, 2 µL of Superscript II (200 U/µL) and 0.6 µL of aminoallyl (aa) dNTP mix (25 mM dATP, 25 mM dGTP, 25 mM dCTP, 10 mM dTTP and 15 mM aa dUTP) then incubated at 42ºC for 2.5 h. The RNA was hydrolysed by adding 10 µL of 0.25 M NaOH, 10 µL of 0.5 M EDTA (pH 8) and incubating the mix at 65ºC for 15 mins. The reaction was neutralised by adding 15 µL of 0.2 M acetic acid. The cDNA was purified using a QIAquick PCR purification kit. Briefly, the cDNA was mixed with 300 µL of buffer PB, applied to the Qiagen column and centrifuged at 6500 x *g* for 1 min. The eluent was re-passed through the column and the column was washed twice

with 600 µL of buffer PE with residual buffer removed by spinning the column at 6500 x *g* for 1 min. The sample was eluted into a clean tube with 90 µL of MilliQ Q water, and the purified cDNA was dried under reduced pressure and dissolved in 9 µL of 0.1 M $NaHCO_3$ (pH 9.0). The mixture was added to a Cy dye aliquot (Amersham, GE Healthcare, Rydalmere, NSW, Australia), mixed, then left to incubate at room temperature for 60 mins in the dark. The labelled cDNA was mixed with 41 µL of MilliQ Q water then purified using a QIAquick PCR purification kit. Briefly, the cDNA was mixed with 250 µL of buffer PB then applied to the Qiagen column and centrifuged at 6500 x g for 1 min. The eluent was re-passed through the column. The column was then washed twice with 600 µL of buffer PE with residual buffer removed by spinning the column at 6500 x *g* for 1 min. The sample was eluted into a clean tube with 90 µL of MilliQ Q water. The purified labelled cDNA samples appear purple after being dried under reduced pressure.

6.2.3 Hybridisation and washing

For microarray analysis we used the Affymetrix GeneChip 1.0 ST Array System for Human, which contains 764,885 sets of oligonucleotide probes ("probeset") which corresponds to an estimated 28,869 human genes or predicted genes. Prior to hybridisation each microarray was immersed in 50 mL hot MilliQ water (80-95ºC) and agitated gently for 5 mins. The slides were then dried by centrifugation at 750 rpm. The labelled cDNA was mixed with 0.64 µL of 25 mg/mL yeast tRNA, 4 µL of 2 mg/mL poly A and 20 µL of 1 mg/mL Cot-1 DNA. The mix was dried under reduced pressure then dissolved in formamide and 6.25 x SSC microarray hybridisation buffer. The mixture was heated to 100ºC for 3 mins and transferred directly to the centre of the coverslip. The array was lowered onto the cover slip then incubated at 42ºC overnight in a humidified chamber. Following incubation the array was immersed in Solution A (0.2 x SSC, 0.01% SDS) for 5 mins until the coverslip disengaged the surface. The array was washed in Solution A for 5 mins then Solution C (0.2 x SSC) for 3 mins. The slide was dried in a centrifuge at 750 rpm for 5 mins and scanned.

6.2.4 Analysis

Analysis was performed using Partek Genomics Suite as per the manufacturer's software instructions. For the data set background correction was performed by robust multichip averaging (RMA) followed by probe affinity adjustment (based on the probe GC content). Quantile normalisation was performed to make the arrays comparable, and a mean probeset summary was obtained by averaging the intensity values for the multiple probes representing a gene. Linear modelling statistics using a mixed model

Analysis of Variance (ANOVA) were performed and an adjusted *p*-value obtained. To uncover functional biological networks and top canonical pathways, the data set was imported into the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA).

6.2.5 Ingenuity Pathway Analysis

To investigate the biological processes correlated with cumulus cells from oocytes which achieved a pregnancy resulting in a live birth, IPA software was used to annotate approximately 350 genes from the data set which showed greater than to 1.2-fold change in expression following analysis by Partek Genomics Suite. Genes were analysed using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA). The genes with known gene symbols and their corresponding expression values were uploaded into the software, and each gene symbol was mapped to its corresponding gene object in the IPA knowledge base. Networks of genes were algorithmically generated based on their connectivity and assigned a score. The score was used to rank networks according to how relevant they are to the genes in the input dataset but not necessarily an indication of the quality or significance of the network. The score takes into account the number of focus genes in the network and the size of the network to approximate how relevant this network is to the original list of focus genes. Networks identified are graphically presented indicating the molecular relationships between genes/gene products. Canonical pathways analysis identified the pathways, from the IPA library of canonical pathways which were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on two parameters: (1) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway and (2) a p-value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone. Uncoloured genes were not identified as differentially expressed in this experiment but were integrated into the networks for computational grounds and represent potential interacting factors in each pathway that are not regulated at the mRNA level.

6.3 RESULTS

6.3.1 Analysis of microarray cDNA pools.

Principal components analysis was performed on the six microarray chips representing two treatments, pregnant (n=3) *vs.* non-pregnant (n=3) (Figure 6.1). The PCA chart reported a 47% range of variation in the principal component #1 along the X-axis, which was the amount of variation between the three pools representing cumulus cells from pregnant patients. The non-pregnant pools showed a much narrower range of variation and lined up comparably along the PC#1 axis. The variation between the three pools of cumulus cells from the pregnant patients most likely impacted the effectiveness of subsequent data analysis but is also consistent with the higher variation in individual gene expression seen in the PCR analyses in pregnant patient samples detailed in previous chapters.

6.3.2 Differential expression of genes comparing cumulus cells pregnant *vs.* non-pregnant patients.

No significant differential gene expression resulted following analysis of the microarray chips using the adjusted ANOVA statistical analysis which adjusts for multiple measures. This was most likely due to the high variation in the gene expression from the pregnant pools which showed up to 47% variation, as well as a low n value which was unavoidable for these experiments due to the limited amount of sample that can be obtained from a human COC. However, of the most highly differentially expressed genes (Table 6.1) a number were of particular interest. The data presented within Table 6.1 contains the fold change of the expression between the pregnant and non-pregnant pools of the ten most significant transcripts. The average expression is the average intensity of both channels (in log2). The logFC represents the log2 ratio of the absorbencies, and a minus in front of the logFC value indicates down-regulation in the pregnant pools (i.e. lower expression in pregnant pools). The t value is representative of a moderated student's t-test, and the B value equals log odds of differential expression. The t-test *p*-value provides a simple statistical comparison between the expression of the pregnant *vs.* non-pregnant expression values for each probe represented. The accession number is the unique identification for each gene sequence.

Two unique genes showed a strong and highly reproducible difference between pregnant *vs.* non-pregnant cumulus cells. Particularly the three isozymogens for human Pepsinogen (*PGA3*/*PGA4*/*PGA5*), which are known to be co-expressed transcripts, all showed highly similar expression levels and fold change between groups. The reproducibility of this result supports the

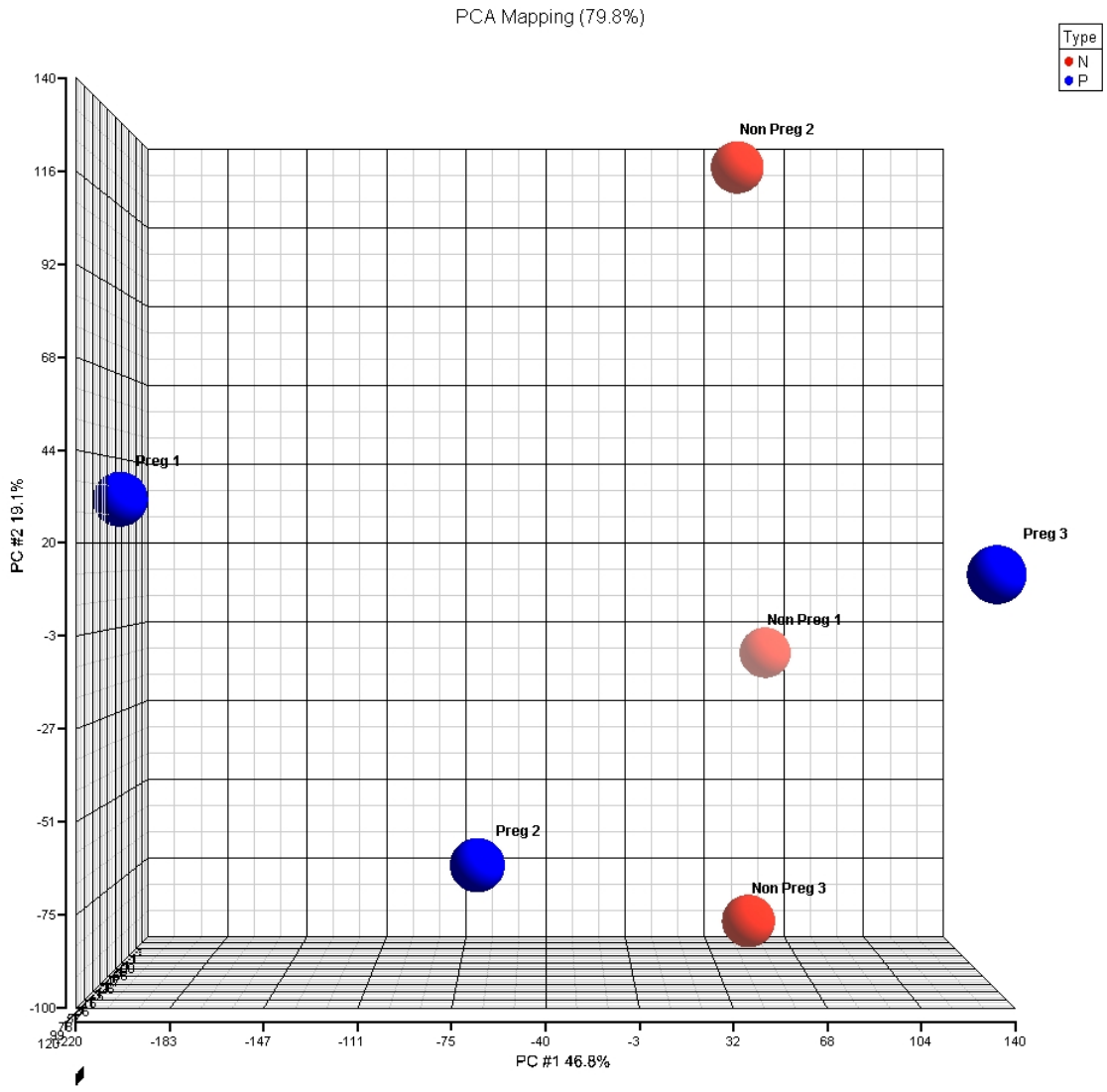

Figure 6.1 Microarray Principal Components Analysis (PCA) Chart.

PCA mapping of the samples to three dimensions to allow interactive visualisation. The microarray platform represents a global analysis of the genome; therefore samples that are close together are similar across the whole genome, whereas samples that are far apart are divergent across the whole genome. The blue circles represent the pregnant gene chips, while the red circles represent the non-pregnant gene chips. The non-pregnant gene chips show little variation, however the pregnant gene chips shows approximately 47% variability along the PC#1 axis.

Table 6.1 Top genes identified by microarray analysis to be different between human cumulus cells from pregnant *vs.* non-pregnant patients.

Gene	Gene Symbol	Fold Change	Average Expression	logFC	t	B (log odds)	t-test *p*-value	Accession No.
Pepsinogen 3	*PGA3*	2.05	5.37	1.03	7.44	-3.65	0.0007	NM_001079807
Pepsinogen 4	*PGA4*	1.99	5.31	0.99	6.94	-3.67	0.001	NM_001079808
Pepsinogen 5	*PGA5*	1.88	5.16	0.91	6.36	-3.70	0.001	NM_014224
Growth arrest-specific 5 (snoRNA, C/D box 47)	*GAS5/SNORD 47*	2.52	4.53	-1.33	-6.16	-3.71	0.005	NR_002746
MicroRNA 202	*MIR202*	2.02	4.61	-1.01	-5.66	-3.75	0.005	hsa-mir-202
p53 and DNA damage regulated 1	*PDRG1*	1.57	5.66	-0.65	-5.63	-3.75	0.00004	NM_030815
Similar to mCG4465	*LOC653665*	1.62	6.34	-0.70	-5.43	-3.76	0.001	XM_001732839
Replication protein A2, 32 kDa	*RPA2*	1.74	5.80	-0.80	-4.77	-3.83	0.008	NM_002946
Poly(A) binding protein interacting protein 1	*PAIP1*	1.93	5.81	-0.95	-4.65	-3.84	NS	NM_006451
Small nucleolar RNA, C/D box 78	*SNORD 78*	1.96	6.33	-0.97	-4.60	-3.84	0.015	NR_003944

conclusion that this is a valid expression difference. Growth Arrest-Specific transcript 5/small nucleolar RNA, C/D box 47 (*GAS5*/*SNORD47*) and Growth Arrest-Specific transcript 5/small nucleolar RNA, C/D box 78 (*GAS5*/*SNORD78*) also showed a similar pattern of expression between the groups. Small nucleolar RNAs (snoRNAs/SNORDS) result from splicing of the *GAS5* gene, which is a non-coding protein gene that hosts small nucleolar RNAs of the C/D box snoRNA family (Meier et al. 2009). Probes for the three pepsinogen isozymogens (*PGA3*/*PGA4*/*PGA5*) were individually associated with cumulus cells from patients who achieved pregnancy success. *PGA3*/*PGA4*/*PGA5* was reported to be expressed up to 2.05-fold higher in cumulus cells from patients with a successful pregnancy outcome. The three Pepsinogen isozymogens are independent coding sequences of the human Pepsinogen gene lined up together on 11q13 (Chromosome 11). While the regulation mechanism for the human Pepsinogen isozymogens is not clear, the current data suggests that *PGA3*, *PGA4* and *PGA5* are under common transcriptional control. The apoptosis related gene p53 and DNA damage regulated 1 (*PDRG1*) was 1.57-fold lower in cumulus cells from patients with a successful pregnancy outcome, while Poly(A) binding protein interacting protein 1 (*PAIP1*) expression was 1.93-fold lower in cumulus cells associated with pregnancy success. *MIR202*, a microRNA found on chromosome 10, was 2-fold lower in cumulus cells from pregnant patients.

6.3.3 Differential expression of *GAS5* encoded snoRNAs between cumulus cells from pregnant *vs.* non-pregnant patients.

The pattern of differential expression of a number of small nucleolar RNAs (snoRNAs/SNORDS) was consistently reported to be down-regulated in the cumulus cells from pregnant patients compared to non-pregnant patients. The family of snoRNAs encoded by the gene known as *GAS5* all showed greater than 1.2-fold down-regulation in the microarray analysis of cumulus cells from pregnant patients, as summarised in Table 6.2. Nine of the ten *GAS5*-encoded snoRNAs were identified by the microarray analysis, all showing higher expression in cumulus cells from patients who did not achieve pregnancy. As with the Pepsinogen transcripts, this consistently repeated observation with different probes targeting related transcripts strongly supports the conclusion that the splicing of *GAS5* to form snoRNAs is indeed differentially expressed between cumulus cell samples from pregnant *vs.* non-pregnant patients.

Table 6.2 Summary of the differential expression of *GAS5* encoded small nucleolar RNAs.

Gene	Gene Symbol	Fold Change	Average Expression	t	t-test *p*-value	Accession No.
Small nucleolar RNA C/D box 47	SNORD47	-2.32	4.53	-4.13	0.0057	NR_002764
Small nucleolar RNA C/D box 80	SNORD80	-1.88	4.02	-2.14	0.062	NR_003940
Small nucleolar RNA C/D box 44	SNORD44	-1.60	5.22	-3.06	0.04	NR_002750
Small nucleolar RNA C/D box 78	SNORD78	-1.58	6.33	-4.16	0.015	NR_003944
Small nucleolar RNA C/D box 76	SNORD76	-1.58	7.56	-3.28	0.026	NR_003942
Small nucleolar RNA C/D box 79	SNORD79	-1.47	4.02	-3.46	0.062	NR_003939
Small nucleolar RNA C/D box 75	SNORD75	-1.42	5.24	-2.61	NS	NR_003941
Small nucleolar RNA C/D box 77	SNORD77	-1.41	3.25	-2.70	0.07	NR_003943
Small nucleolar RNA C/D box 74	SNORD74	-1.21	6.73	-1.64	NS	NR_002579

6.3.4 Functional annotation of the pregnancy outcome gene list using Ingenuity Pathways Analysis (IPA).

To investigate the biological processes correlated with cumulus cells from oocytes which achieved a pregnancy resulting in a live birth, IPA software was used to annotate approximately 350 genes from the data set which showed greater than to 1.2-fold change in expression following analysis by Partek Genomics Suite. The analysis with IPA was carried out in an attempt to discover additional gene interactions with previously identified genes which may predict pregnancy success. However, as the initial microarray analysis did not yield significant results, the IPA analysis represents hypothetical gene associations and not actual data. For this reason, only a selected series of IPA generated networks and canonical pathways are presented below. These pathways were selected based on the overall functional characterisation of the pathway or particular signalling pathways which are significant to the acquisition of oocyte developmental competence. However, as the pathways generated by IPA are hypothetical, it is not possible to draw definitive conclusions about the gene interactions and the figures below illustrate potentially interesting gene interactions and pathways. A number of high-level functional categories of particular interest were identified by IPA analysis as represented by the bar graph in Figure 6.2. The high level functional categories are displayed along the x-axis, while the y-axis represents the -(log) significance. Taller bars are more significant than shorter bars, and functions are listed from most significant to least while the orange horizontal line denotes the cut off for significance ($p < 0.05$). Functions of particular interest identified to be significant and with well characterised roles in COC development, maturation and function include carbohydrate metabolism (Downs et al. 2002; Harris et al. 2007), cell death (Hussein et al. 2005; Zhang et al. 2005; Assou et al. 2008; Hamel et al. 2008; van Montfoort et al. 2008), cell morphology (Ebner et al. 2000) and gene expression (McKenzie et al. 2004; Hasegawa et al. 2005; Zhang et al. 2005; Cillo et al. 2007; Feuerstein et al. 2007; Guerif et al. 2007; Hasegawa et al. 2007; Assou et al. 2008; Hamel et al. 2008; van Montfoort et al. 2008; Anderson

et al. 2009a; Anderson et al. 2009b; Hamel et al. 2009). It is noteworthy that the IPA analysis also reported embryonic development to be significantly over-represented. Analysis of the microarray data using IPA also generated a series of networks and canonical pathways summarising various biological pathways identified in cumulus cells associated with pregnancy success. Networks of particular interest to COC function and oocyte developmental competence are described in further detail below. The top ranked functional network was associated with Cell Death Haematological System Development and Function and Tissue Development, (Figure 6.3). This network contained a significant number of genes which were both up- and down-regulated (26 of 31) in cumulus cells associated with pregnancy. It is noteworthy that this network includes hCG the luteinising hormone analog, as a node hypothetically interacting with this network of up- and down- regulated genes. hCG is a well known contributor to cumulus oocyte complex functions and oocyte maturation, and may indicate a higher level of oocyte developmental competence. This network also reports Tripartite motif-containing 27 (*TRIM27*), an apoptosis related gene, to be down-regulated in cumulus cells from pregnant patients which may indicate lower levels of apoptosis, and therefore increased developmental competence of the enclosed oocyte. Low levels of cumulus cell apoptosis may also infer robust oocyte signalling, as it has been shown oocyte-secreted factors maintain a low incidence of cumulus cell apoptosis by establishing a localised gradient of bone morphogenetic proteins (Hussein et al. 2005).

The second highest ranked functional network identified by IPA was associated with Cellular Assembly and Organisation, Nervous System Development and Function and Cellular Development (Figure 6.4). This network primarily contained genes which were down-regulated (22 of 34) in cumulus cells associated with pregnancy. Within this network, *RPA2* was down-regulated in the cumulus cells associated with pregnancy and was also amongst the most differentially expressed genes from the microarray analysis. *RPA2* is an apoptosis related gene and may represent an appropriate cellular stress response within these cells, as well as the acquisition of appropriate machinery for cell development.

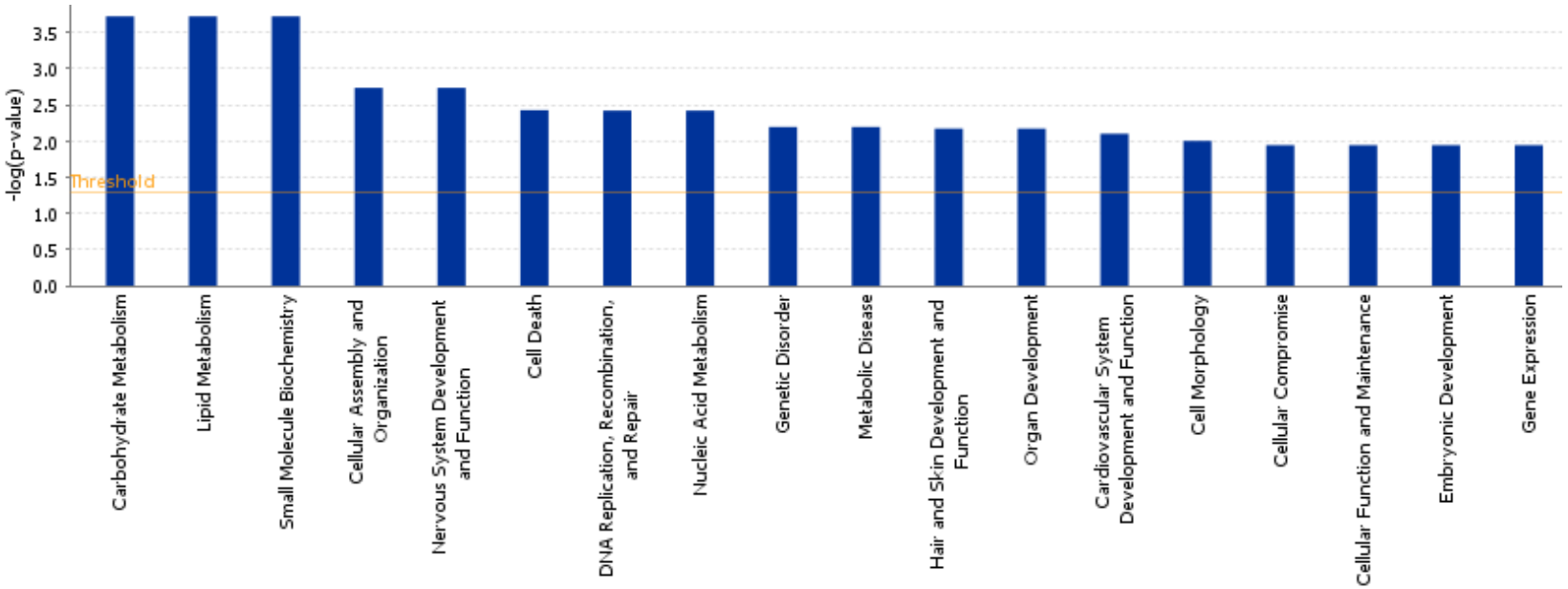

Figure 6.2 Identification of over-represented functional networks associated with a pregnancy outcome gene signature using Ingenuity Pathways Analysis.

The bar chart identifies the high-level functional categories from the IPA analysis. Functions with possible associations to COC function were identified by IPA to be over-represented in cumulus cells associated with pregnancy success.

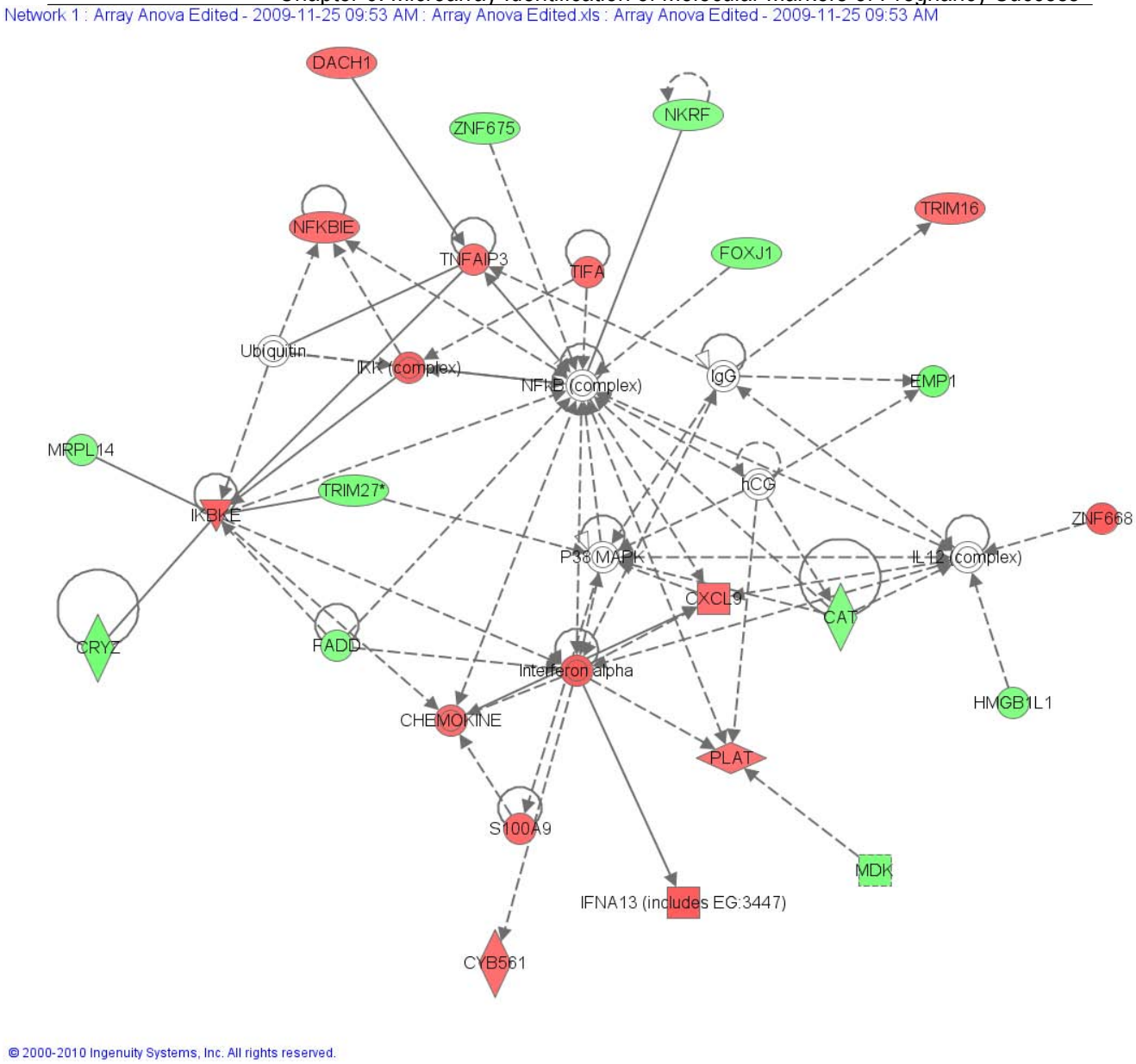

Figure 6.3 Highest significant gene ontology network associated with Cell Death, Haematological System Development and Function, Tissue Development evaluated by Ingenuity Pathways Analysis.

Top ranked functional network obtained from comparisons of differential expression in cumulus cells associated with pregnancy success *vs.* no pregnancy. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The red colour intensity of the node indicates the degree of up-regulation in the pregnancy group, while the green colour intensity of the nodes indicates the degree of down-regulation in the pregnancy group. Uncoloured nodes were not identified as differentially expressed in this experiment. A plain line indicates a direct interaction, a dashed line indicates an indirect interaction, a line without an arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, a line with an arrowhead indicates “acts on”.

A gene ontology network associated with Gene Expression, Connective Tissue Development and Function and Tissue Morphology of interest identified by Ingenuity Pathways Analysis links together a number of genes known to be important for oocyte development and maturation, as well as cumulus matrix production, to be up-regulated (21 of 36) in the cumulus cells associated with pregnancy success (Figure 6.5). Most noteworthy within this network is the up-regulation of *HAS2*, *BMP15* and Left-right determination factor A (*LEFTY2*) in the cumulus cells from patients who achieved pregnancy success. A number of genes important for TGFβ/BMP family and hormone signalling were down-regulated in cumulus samples from non-pregnant patients. This network (Figure 6.6) contained the genes *SMAD6*, Protein Kinase A (*PKA*), Calcium/Calmodulin-dependent Protein Kinase 4 (*CAMK4*) and Extracellular-signal-regulated Kinase 1/2 (*ERK1/2*). These proteins are functionally important for apoptosis, proliferation, migration, invasion, adhesion, growth and chemotaxis. SMAD6 is a signal transducer and transcriptional modulator. The importance of BMP, LH and FGF signalling for oocyte mediated cumulus cell survival as well as growth and development is well characterised (Lawson et al. 1999; Galloway et al. 2000; Fujiwara et al. 2001; Gilchrist et al. 2004; Pangas et al. 2004; Pangas and Matzuk 2004; McNatty et al. 2005; Hussein et al. 2006). Within the COC, the convergent actions of LH/hCG and oocyte secreted factors, *Egf-like* ligands and *FGF* are all likely to be reduced due to lower expression of key signal transducing genes potentially resulting in a biologically important reduction in cumulus cell function as depicted in Figure 6.6.

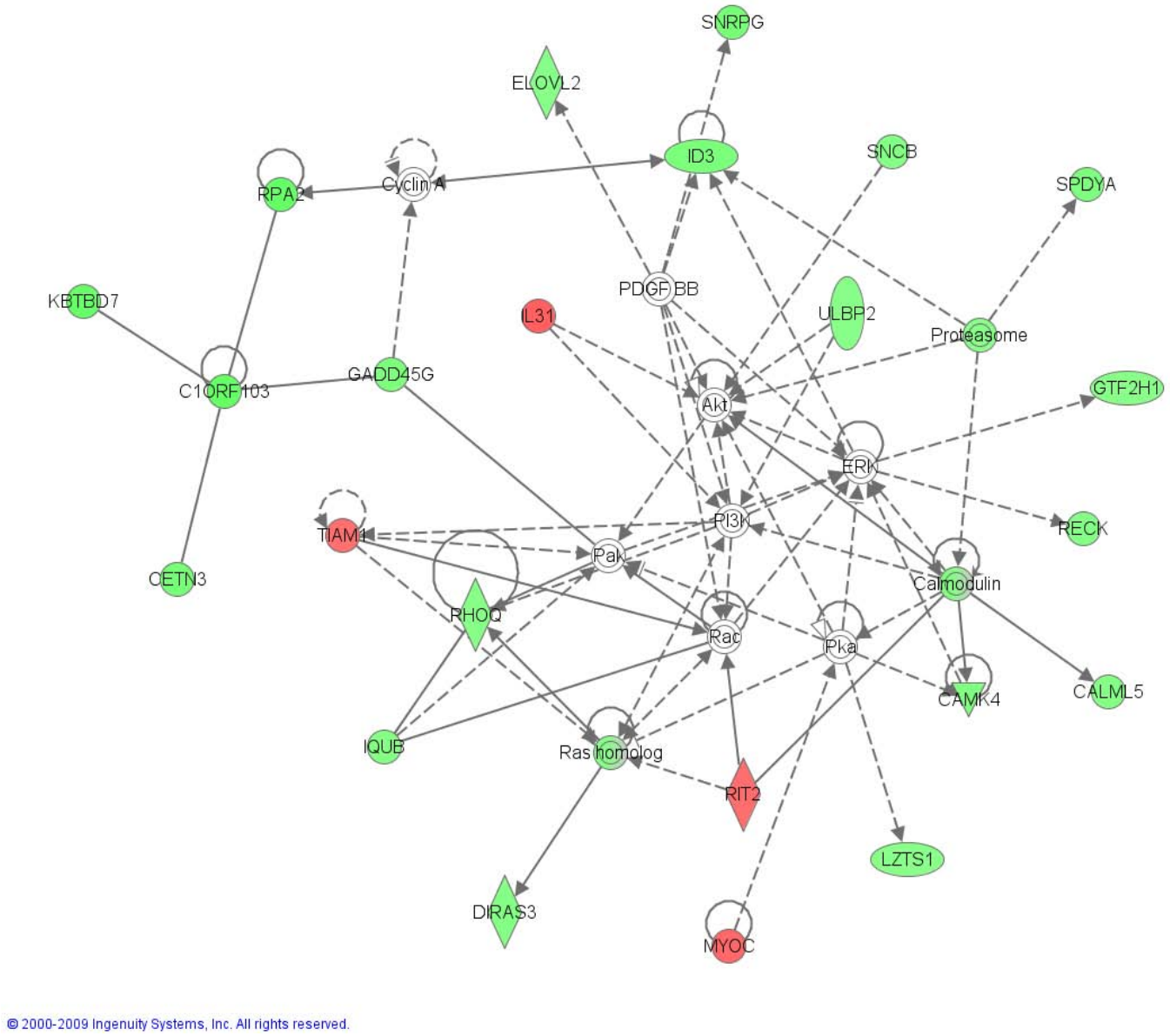

Figure 6.4 Second highest significant gene ontology network associated with Cellular assembly and organisation, Nervous System Development and Function and Cellular Development evaluated by Ingenuity Pathways Analysis.

Second ranked functional network obtained from comparisons of differential expression in cumulus cells associated with pregnancy success *vs.* no pregnancy. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line).The red colour intensity of the node indicates the degree of up-regulation in the pregnancy group, while the green colour intensity of the nodes indicates the degree of down-regulation in the pregnancy group. Uncoloured nodes were not identified as differentially expressed in this experiment. A plain line indicates a direct interaction, a dashed line indicates an indirect interaction, a line without an arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, a line with an arrowhead indicates “acts on”.

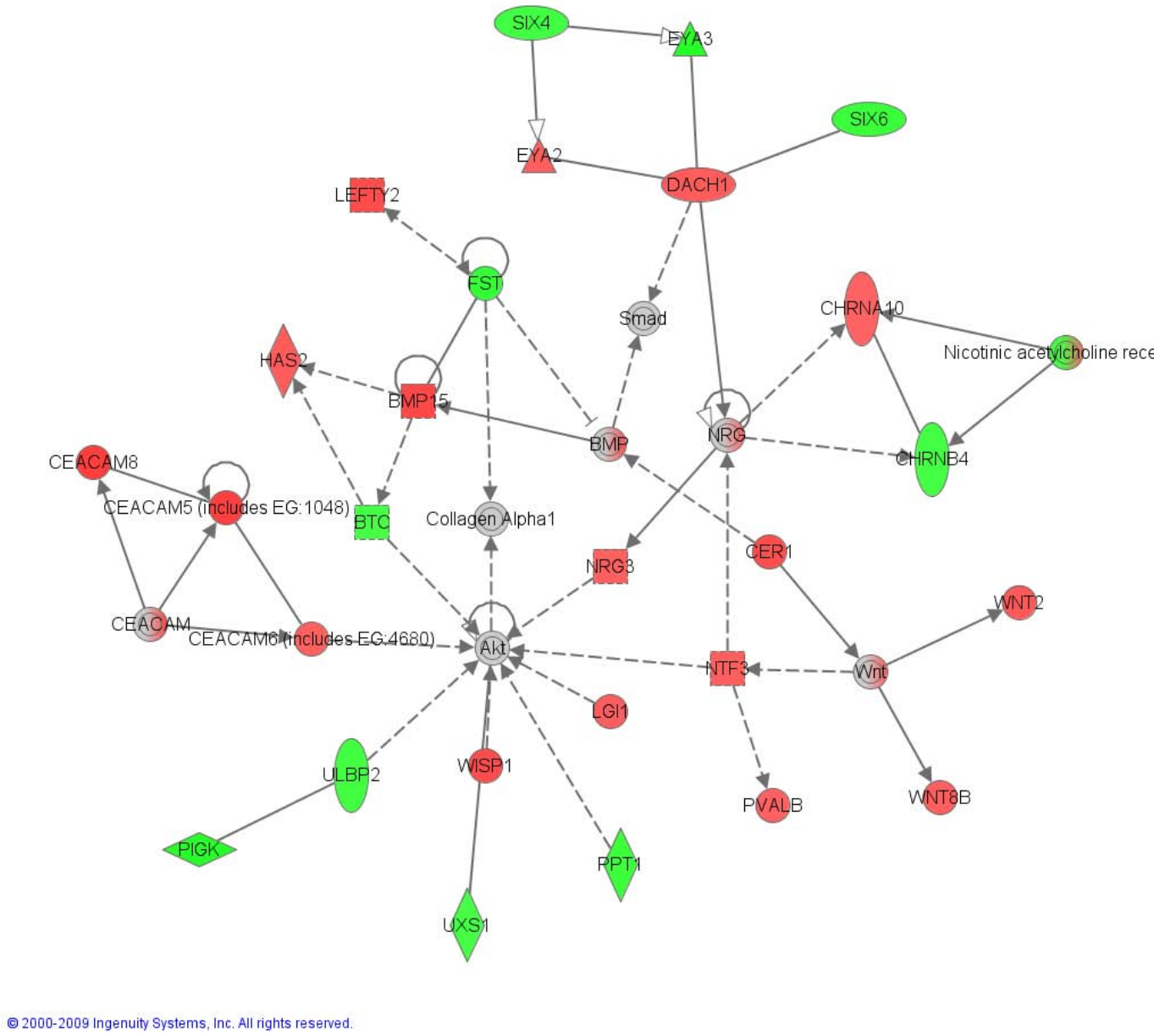

Figure 6.5 Gene ontology network associated with Gene Expression, Connective Tissue Development and Function and Tissue Morphology evaluated by Ingenuity Pathways Analysis.

Gene ontology network obtained from comparisons of differential expression in cumulus cells associated with pregnancy success *vs.* no pregnancy. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line).The red colour intensity of the node indicates the degree of up-regulation in the pregnancy group, while the green colour intensity of the nodes indicates the degree of down-regulation in the pregnancy group. Uncoloured nodes were not identified as differentially expressed in this experiment. A plain line indicates a direct interaction, a dashed line indicates an indirect interaction, a line without an arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, a line with an arrowhead indicates “acts on”.

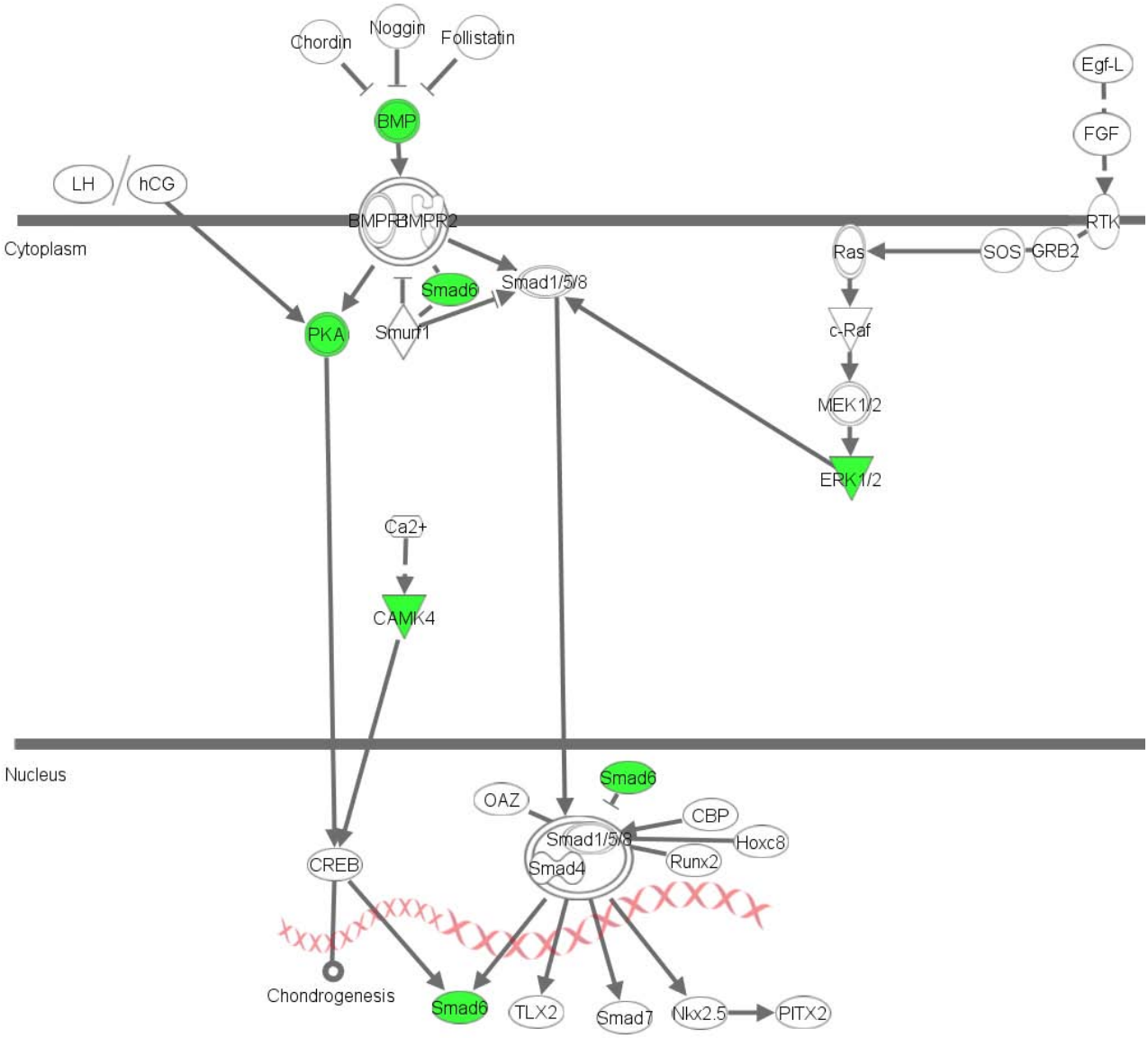

Figure 6.6 Bone Morphogenic Protein (BMP) signalling canonical network of genes associated with pregnancy success as evaluated by Ingenuity Pathways Analysis.

BMP signalling canonical functional network of gene expression in cumulus cells associated with pregnancy success *vs.* no pregnancy. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line).The green colour intensity of the nodes indicates the degree of down-regulation in the pregnancy group. Uncoloured genes were not identified as differentially expressed in this experiment. The red structure represents DNA. A plain line indicates a direct interaction, a dashed line indicates an indirect interaction, a line without an arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, a line with an arrowhead indicates "acts on".

6.4 DISCUSSION

Very few genes with any link to successful pregnancy are known, and to date most analysis has relied upon selection of genes based on animal models of experimentation. In this study we have utilised our unique human RNA samples from pregnant *vs.* non-pregnant patients in a microarray platform to identify novel cumulus cell gene markers of human oocyte health which may predict pregnancy success for women undergoing assisted reproduction. Due to limitations on samples our microarray analysis did not yield any significant results for genes which were differentially expressed between our pregnancy outcome groups. This was not unexpected as the number of replicates was small for such a study design and variation in the pregnant groups was known to be high from our PCR studies. However, our study succeeded in identifying a number of genes with highly reproducible differential expression between the two pregnancy outcomes which are of interest and may represent novel biomarkers of subsequent IVF success worthy of further study. Our analysis between cumulus cells from patients who achieved pregnancy success with a live birth outcome and patients who did not achieve pregnancy resulted in the identification of two unique genes showing a strong and highly reproducible difference between pregnant *vs.* non-pregnant cumulus cells. The human *PEPSINOGEN* isozymogens (*PGA3/PGA4/PGA5*) and the *GAS5* encoded SNORDs showed highly differential expression according to IVF outcome and pregnancy success.

Small nucleolar RNAs (snoRNAs) are short (50-100bp) nonmessenger RNAs that do not code for proteins but have several functions, including 2'-O-methylation and psuedoridinylation (conversion of uridine to psuedouridine) of rRNA (Bachellerie et al. 2002). SnoRNAs regulate protein synthesis, guidance modification and processing by targeting other RNAs such as rRNAs, tRNAs and possibly mRNAs (Bachellerie et al. 2002; Rogelj and Giese 2004). *Gas5* biological activity is thought to be mediated through its introns, which encode the snoRNAs (Smith and Steitz 1998; Mourtada-Maarabouni et al. 2009). *Gas5* is a multi-snoRNA host gene which encodes 9 (in mouse) or 10 (in human) snoRNAs (Smith and Steitz 1998). In all species investigated, snoRNAs are encoded within introns and are not independently transcribed but processed by the RNA polymerase II as portions of the pre-mRNA (Bachellerie et al. 2002). Of the 10 SNORDs encoded by *GAS5*, 9 were identified to be differentially expressed between the pregnancy outcome groups following microarray analysis. All nine of the *GAS5* encoded SNORDs identified by the microarray analysis were found to have greater than 1.2-fold lower expression in the pregnant group. This highly reproducible pattern of expression suggests the independently identified *GAS5* encoded SNORDS are candidate cumulus cell markers of pregnancy success.

The three human Pepsinogen A isozymogens (*PGA3/PGA4/PGA5*) were ranked as the top three genes with the most significant differential expression from this analysis. Each of the isozymogens was significantly higher in cumulus cells from oocytes associated with pregnancy success by independent T-test. Pepsinogen is primarily located within the gastric mucosa of the stomach but has recently been reported in human breast cancer (Sanchez et al. 1992), human ovarian carcinoma (Merino et al. 2000) and follicular cells in trout ovary (Bobe and Goetz 2001). Pepsinogen belongs to the family of aspartic proteinases, and has not been investigated during human folliculogenesis. However, other unrelated proteases have been shown to be highly expressed in the cumulus matrix during cumulus expansion and following the LH surge (Russell et al. 2003a). This identification of Pepsinogen in human cumulus cells is entirely novel and may be of considerable biological importance, particularly in light of the highly reproducible (though not significant) increased expression in pregnant patient samples. The current analysis of Pepsinogen in human cumulus cells remains limited and further characterisation is required to assess the role of these isozymogens in predicting oocyte health.

MIR202, a microRNA found on chromosome 10, was 2-fold lower in cumulus cells from pregnant patients. MicroRNAs (miRNAs) are short (20-24 nt) non-coding RNAs involved in post-transcriptional regulation of gene expression miRNAs by affect both the stability and translation of mRNAs. miRNAs are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) that can be either protein-coding or non-coding. The specific function and mRNA targets of *MIR202* are not clear. *PDRG1* and *RPA2* were also among the most differentially expressed genes between the pregnancy outcome groups, and were both identified to be higher in cumulus cells from non-pregnant patients. *RPA2* (replication protein A2, 32kDa) is important for DNA replication and repair (Anantha et al. 2007). *RPA2* is part of a heterotrimeric single-stranded DNA-binding complex, and is critical for DNA replication, recombination, and repair (Aboussekhra et al. 1995; Park et al. 1996; Golub et al. 1998; Iftode et al. 1999; Perrault et al. 2001; Ramilo et al. 2002; Binz et al. 2004) which is facilitated through its interactions with other proteins (Oakley et al. 2009). The higher expression of *RPA2* in cumulus cells from non-pregnant patients may indicate an up-regulated cellular response to stress and DNA damage, restricting the appropriate cellular signalling required for the acquisition of oocyte developmental competence A downstream target of the p53 gene, p53 and DNA-damage regulated 1 (*PDRG1*) was lower in cumulus cells from oocytes associated with pregnancy success. The *p53* gene mediates its own effects by down-regulating the expression of target genes (Luo et al. 2003). This may suggest cumulus cells from oocytes associated with pregnancy success had lower levels of apoptosis and DNA damage and may be reflective of robust signalling of oocyte secreted factors to the cumulus cells, as it has been shown that oocytes maintain a low level of cumulus cell apoptosis (Hussein et al. 2005). *BMP15* is an oocyte secreted factor, critical to folliculogenesis and oocyte

developmental competence which maintains the integrity of the COC (Yan et al. 2001), while *LEFTY2* is a growth factor which regulates TGFβ family signalling. *LEFTY2* is associated with polarisation, and has previously been linked with infertility (Tabibzadeh et al. 2000). Previous studies have reported significantly lower levels of apoptosis related genes in cumulus cells from pregnant patients (Assou et al. 2008; van Montfoort et al. 2008). The expression of these genes as markers of oocyte quality requires further validation, but shows consistency with previously published findings.

Microarray technology has become a powerful tool to analyse the gene expression of tens of thousands of genes simultaneously. However, it is important to note the limitations of microarray analysis, specifically in the current study. The small sample size and limited RNA amount was a key weakness in these experiments, as it lead to the pooling of RNA. Microarrays are primarily a screening tool. A major limitation is a decreased sensitivity of the arrays to the detection of genes with low expression levels (low-abundance genes) (Bunney et al. 2003). Another disadvantage is that microarrays do not measure posttranslational modifications (e.g., phosphorylation) (Luo and Geschwind 2001). In contrast, the strength of microarrays is that they provide the means to repeatedly measure the expression levels of a large number of genes at a time, and relatively small amounts of total RNA can be analysed. While the current analysis did not yield significant results, it is not to say that valuable information was not obtained. And through the validation of these findings is the potential to identify more novel cumulus specific biomarkers of oocyte developmental competence.

Our microarray analyses were designed to identify new markers of oocyte health worthy of further analysis by more sensitive methods. Although initial analyses did not find genes with significantly different expression, numerous genes of interest with differential expression between the pregnancy outcome groups were identified, several of which were consistent observations. A shortlist of genes associated with pregnancy success was generated. Further validation and detailed characterisation of genes which have been newly identified within the ovary will further confirm the present results, and the usefulness of the genes identified as markers of oocyte quality and pregnancy success. While limited, the current analysis has provided new and interesting avenues which will be further explored in the identification of gene markers of oocyte quality, through further validation to provide a more robust indicator of the diagnostic potential of microarray identified genes. A series of experiments to validate the current microarray findings are detailed further in Chapter 7.

CHAPTER 7

INDEPENDENT VALIDATION OF MICROARRAY EXPERIMENTS

7.1 BACKGROUND

Through microarray analysis we have identified novel transcripts present in cumulus cells which indicate the developmental potential of their enclosed oocyte. While significant changes in the genome wide expression were not found, a number of genes of interest were reported through both the analysis of the microarray data and the use of Ingenuity Pathways Analysis software. By looking at the genes with the greatest fold change in expression following microarray analysis, we have identified two particular genes of interest which warrant further investigation in relation to their potential as markers of oocyte developmental competence and pregnancy success. The genes identified had limited or no characterisation in follicular cells, however further investigation of these transcripts identified by the microarray analysis was supported by the literature available. The specific genes identified were the human pepsinogen isozymogens (*PGA3*/*PGA4*/*PGA5*) and growth arrest specific transcript 5 (*GAS5*). Further background on these transcripts is provided below. To make full use of microarray data further validation is required; therefore for this study we further assessed the potential of *PGA3*/*PGA4*/*PGA5* and *GAS5* as markers of oocyte developmental potential and pregnancy success for women undergoing assisted reproduction.

7.1.1 Growth Arrest Specific Transcript 5 (*GAS5*)

Growth arrest specific transcript 5 (*GAS5*) was first identified among a group of genes termed the growth arrest specific (*GAS*) genes, which consists of *Gas1* to *Gas6*, and the mRNAs of these genes accumulate when cells exit the cell cycle (Coccia et al. 1992). *Gas5* was initially discovered in a screen for potential tumour suppressor genes as it exhibited high levels during growth arrest (Schneider et al. 1988). *Gas5* biological activity is mediated through its introns, which encode small nucleolar RNAs (snoRNAs) (Smith and Steitz 1998; Mourtada-Maarabouni et al. 2009). *Gas5* is a multi-snoRNA RNA host gene which encodes 9 (in mouse) or 10 (in human) antisense snoRNAs as seen in Figure 7.1 for human *GAS5* (Smith and Steitz 1998). *Gas5* hosts snoRNAs of the C/D box snoRNA family (Meier et al. 2009), which are short (50-100 bp) nonmessenger RNAs that do not code for proteins but have several functions including 2'-O-methylation and psuedoridinylation (conversion of uridine to psuedouridine) of rRNA (Bachellerie et al. 2002). snoRNAs regulate protein expression, guidance modification and processing by targeting other RNAs such as tRNAs and possibly mRNAs (Bachellerie et al. 2002; Rogelj and Giese 2004). Of the ten snoRNAs encoded by GAS5, nine were detected by our microarray analysis, with two appearing amongst the top differentially expressed genes – *SNORD47* and *SNORD78*. Both of these SNORDs were down-regulated in cumulus cells from patients who achieved pregnancy compared to those for which IVF failed. Furthermore, seven other SNORDs encoded by

GAS5 probed through our microarray analysis each showed a similar pattern of expression, with lower levels in cumulus cells from patients who achieved pregnancy success. This pattern of expression is of particular interest, and indicates a role for *GAS5* and the *GAS5*-encoded SNORDs as cumulus cell markers of oocyte developmental competence.

In all species investigated, snoRNAs are encoded within introns and are not independently transcribed but processed by the RNA polymerase II as portions of the pre-mRNA (Bachellerie et al. 2002). The functional snoRNAs are then produced by exonucleolytic trimming that follows either splicing or endonuceolytic cleavage of intronic sequences (Raho et al. 2000). It is the presence of only short open reading frames (ORFs) and numerous stop codons which suggests that *Gas5* does not generate a protein product (Smith and Steitz 1998). The murine *Gas5* produces a ubiquitous, polyadenylated, alternatively spliced message which is almost undetectable in actively growing cells yet is highly expressed in cells undergoing serum starvation or density arrest (Ciccarelli et al. 1990; Coccia et al. 1992). *Gas5* is critical to the control of mammalian apoptosis and cell population growth (Mourtada-Maarabouni et al. 2009). *Gas5* is widely expressed during mouse embryo development (Coccia et al. 1992), and nutrient deprivation to murine embryonic cells results in a significant increase in *Gas5* (Fleming et al. 1998; Fontanier-Razzaq et al. 2002). The implicated relationship between *GAS5* encoded snoRNAs and oocyte competence is supported by previously identified markers of pregnancy success associated with apoptosis and cellular stress (Assou et al. 2008; van Montfoort et al. 2008). However, as *GAS5* is a non-coding RNA the exact functional role for this transcript in cumulus cells is yet to be determined. Similarly, the function of the intron-encoded SNORDs within follicular cells has not previously been reported. The repeated observation of lower SNORD expression in cumulus cells from patients who failed to achieve pregnancy is noteworthy. It is this repeated observation which has led to further investigation of *GAS5* within human and murine cumulus and mural granulosa cells to determine a functional relationship to oocyte developmental competence.

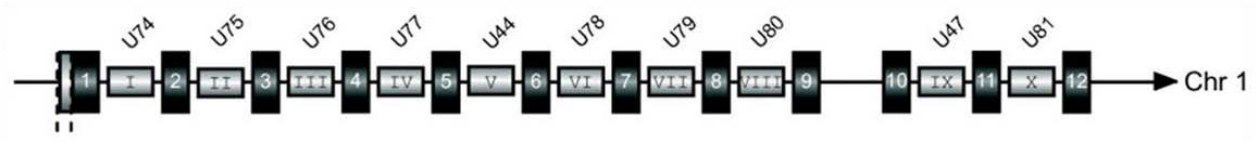

Figure 7.1 Structure of the snoRNAs encoded by the human *GAS5*.

The exons and snoRNA regions (not drawn to scale) are denoted by black and gray boxes, respectively. The human *GAS5* gene contains twelve exons and encodes ten snoRNAs. The snoRNA names are indicated above the gene structure. The snoRNAs are indicated by the same Roman numerals. Image obtained from (Shao et al. 2009).

7.1.2 Pepsinogen (*PGA*)

The second gene identified by microarray analysis with high differential expression between the pregnancy groups was Pepsinogen; a protein produced primarily by chief cells within the gastric mucosa, but which has also been found in blood, urine and other body fluids (Hirschowitz 1967; Gritti et al. 2000). The human stomach contains two immunochemically and biochemically distinct groups of pepsin precursors, pepsinogen A and C (Taggart et al. 1989). In humans, PGA is the principal pepsinogen found in the stomach, whereas, in rodents, it appears that PGC is the only form of pepsinogen present (Taggart et al. 1989). Human pepsinogens are designated as PGA-1 through PGA-5 (pepsin I group, pepsinogens) and PGC-6,7 (pepsin II group, gastricsinogens) (Samloff 1969). The PGA and PGC pepsinogen groups are considerably different in their amino acid composition and sequence (Taggart et al. 1989). The PGA gene complex includes numerous different haplotypes that contain variable numbers of PGA genes each of which encodes an electrophoretically discernible isozymogen; Pg 5 (*PGA5*), Pg 4 (*PGA4*), and Pg 3 (*PGA3*) (Nakai et al. 1986).

Pepsinogen belongs to the protease family of aspartic proteinases, and although historically associated with digestion, pepsinogens have been suggested to play a role in the development of tumour processes (Sanchez et al. 1992). While the exact role relating pepsinogens with carcinomas remains unclear, secreted pepsinogen proteinases may contribute to degradation of the extracellular matrix leading to tumour growth, invasion and metastasis (Tryggvason et al. 1987; Liotta 1988). Pepsinogens also promote cell proliferation, reduce cell adhesiveness, modify cell membranes, increase cell motility, activate hormones and growth factors, and stimulate the secretion and activation of other proteinases (Duffy 1987; Scher 1987). Pepsinogen C has been identified in extragastric tissue and carcinoma in both human and animal studies. Pepsinogen C expression has been reported in ovarian and extragastric carcinomas (Merino et al. 2000). A significant association has been reported between pepsinogen C and the expression of oestrogen receptors, with higher proteinase levels in oestrogen receptor positive breast tumours compared to oestrogen receptor negative tumours (Diez-Itza et al. 1993). Pepsinogen is involved in ovulation in fish, as a trout progastricsin which is 67% similar to human Pepsinogen C has been immunolocalised to the granulosa cells of the postovulatory trout ovary and also found in high quantities in the coelomic fluid 4–10 days postovulation (Bobe and Goetz 2001). The extragastric role of pepsinogen in the ovary, and specifically within ovarian follicular cells, has not previously been described, however the presence of this proteinase in the ovary has been briefly explored (Merino et al. 2000). An understanding of the roles of pepsin I group pepsinogens (PGA1 to PGA5) in the ovarian follicular cells, as they are less well characterised in extragastric locations, may lead to the identification of new novel biomarkers of oocyte quality.

7.2 MATERIALS AND METHODS

7.2.1 Human *vs.* Mouse Pepsinogen

The microarray analysis detailed in Chapter 7 utilised the Affymetrix GeneChip 1.0 ST Array System. The probes for human pepsinogen detected the three isozymogens which are co-expressed as a metagene which is continuous along chromosome 11. The human pepsinogen isozymogens share 99% homology, and therefore cannot be detected individually by real time RT-PCR methods consistent with those previously utilised for our experiments. For this analysis we designed an assay which detected pepsinogen expression, but not the expression of each individual isozymogen. For this analysis, the pepsinogen expression will be labelled *PEPSINOGEN 3/4/5*. The murine pepsinogen consists of two isoforms, pepsinogen 5, group I (also called as Pepsinogen F), and pepsinogen C (Progastricsin) which are approximately 50% similar when the two sequences are aligned using NCBI Blast and Geneious Software. Each of these murine pepsinogens will be investigated in this chapter. The *GAS5* encoded SNORDs were each detected by individual probes, and therefore reported as independent results. For the current analysis, an assay has been designed to detect both the human and murine *GAS5* transcript, but not the individual intron encoded snoRNAs. This analysis does not differentiate between individual snoRNAs.

7.2.2 Patient Selection

Further to previously collected cumulus cell samples, cumulus and mural granulosa cells were obtained from twenty three patients, however only twelve (*PEPSINOGEN*) to fourteen (*GAS5*) patients were included in this study due to poor RNA quality which failed to yield acceptable expression measures following real time RT-PCR analysis. Patients with clinical indications of polycystic ovary syndrome were not included in this study. All patients had treatment with their own gametes and were not undergoing pre-implantation genetic diagnosis.

7.2.3 Isolation and culture of murine cumulus oocyte complexes

The discovery of *GAS5*-related snoRNAs and *PEPSINOGEN* in human cumulus cells was entirely unexpected. To better understand the pattern of expression and regulation we turned to a mouse model of folliculogenesis which has previously been utilised and published by our laboratory (Dunning et al. 2007). For in vitro maturation (IVM), COCs were isolated from pre-pubertal 23-day-old F1 C57Bl/6 X CBA mice 44-48 h after i.p. injection of 4 IU eCG. COCs were collected from 6 – 8 female mice per treatment group, which yielded on approximately 20 - 30 COCs per ovary. COCs were washed in

complete MEM (alpha MEM media supplemented with 5% (v/v) fetal calf serum (FCS), 0.25 mM Sodium Pyruvate (GIBCO, Invitrogen Australia Pty Ltd, VIC, Australia) and penicillin G (100 U/mL), streptomycin sulphate (100mg/mL) and cultured in groups of 30 in 37ºC, 5%CO2, 95% air for either 6 or 20 h in drops of 100 mL of complete MEM media with suitable treatments as previously described (Dunning et al. 2007) and overlaid with sterile mineral oil (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia). Culture treatments involved media supplemented with either 50 mIU/mL recombinant human FSH (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) or 3 ng/mL Epidermal growth factor (Egf) (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia) or both Egf and FSH at the same doses. Expression analysis was performed following 6 h or 20 h of IVM culture. After 6 h the expression of ovulatory genes have previously been shown to be strongly up-regulated in the ovary (Fulop et al. 1997b; Robker et al. 2000; Ochsner et al. 2003a; Russell et al. 2003b; Dunning et al. 2007), while 20 h IVM culture has previously been shown to be the time for completion of oocyte maturation in vitro (Eppig 1979; Downs 1989). In vivo matured COCs were obtained from prepubertal mice treated with i.p. administration of eCG (4 IU) followed after 44 h by hCG (5 IU) and collected after 6 h or 12 h (preovulatory) or 16 h (postovulatory). Gene expression at these times was compared to in vivo matured COCs obtained by follicle puncture following 6 h, 12 h (preovulatory) or from oviducts 16 h (postovulatory) hCG treatment. Preovulatory mGC and COCs were collected by puncture of large antral follicles with a 26-gauge needle to release cells. COCs were cleaned of large adherent masses of mGCs before collection for RNA or protein isolation. Ovulated COCs (16 h after hCG) were isolated by puncture of the ampulla of the oviduct. Corresponding mGCs were also collected for RNA or protein isolation by puncture of the large follicles, cell pellets were snap frozen and stored at -80ºC for later mRNA extraction and PCR analysis as described below.

7.2.4 Real Time RT-PCR

Total RNA was isolated using Trizol (Invitrogen Australia Pty. Ltd., Mt Waverly, VIC, Australia), as per manufacturer's instructions with the inclusion of 7.5 μg Blue Glycogen (Ambion, Applied Biosystems, Scoresby, VIC, Australia) during precipitation, or by the RNAqueous Micro RNA isolation kit (Ambion, Applied Biosystems, Scoresby, VIC, Australia) as per the manufacturer's instructions. Total RNA was then treated with 1 IU of DNase as per the manufacturer's instructions (Ambion, Applied Biosystems, Scoresby, VIC, Australia). Complimentary first strand DNA (cDNA) was synthesised from total RNA using random hexamer primers (Geneworks, Hindmarsh, SA, Australia) and Superscript III reverse transcriptase (Invitrogen Australia Pty. Ltd., Mt Waverly, VIC, Australia).

Specific gene primers for real time RT-PCR were designed against published mRNA sequences (Table 7.1 and 7.2) using Primer Express software (PE Applied Biosystems, Scoresby, VIC, Australia) and synthesised by Sigma Genosys (Sigma-Aldrich Pty. Ltd, Castle Hill, NSW, Australia). Primer pairs and sequences for murine *Gas5*, *Pepsinogen C*, *Pepsinogen 5* and *RpL19*, and human *GAS5*, *PEPSINOGEN 3/4/5* and *GAPDH* are listed in Tables 7.1 and 7.2 respectively. The real time primers for murine *RpL19* were sourced from a previously published study from our laboratory (Dunning et al. 2007). Real time RT-PCR was performed in triplicate for each sample on a Corbett Rotor Gene 6000 (Corbett Life Science, Qiagen, Doncaster, Victoria, Australia). In each reaction, 2 μL of cDNA, 0.2 μL of forward and reverse primers and 10 μL of SYBR Green (Applied Biosystems, Scoresby, VIC, Australia) master mix were added, with H_2O added to make a final volume of 20 μL. All primers were used at an optimised concentration of 50 μM with the exception of murine *RpL19* and human *PEPSINOGEN 3/4/5* primers, which were used at 25 μM. PCR cycling conditions were 50°C for 2 mins, 95°C for 10 mins, followed by 40 amplification cycles of 95°C for 15 secs and 60°C for 1 min. Controls included omission of the cDNA template in otherwise complete reaction mixtures; each showed no evidence of product amplification or primer dimers. Following real time RT–PCR, analysis of the dissociation curves confirmed that a single product was amplified in all reactions.

Table 7.1 Murine Real Time Primer Sequences.

Gene	Primer Name	Amplicon size (bp)	Sequence (5'-3')	Accession No.
Gas5	mGas5 F	76	ACAGAGCGAGCGCAATGTAAG	NR_002840.2
Gas5	mGas5 R		GAATACCCAATGGCAAATGAGC	
Pepsinogen 5	mPep5 F	71	TGGGTCCTTGGGCTTGTG	NM_021453
Pepsinogen 5	mPep5 R		CTGATGAAGATTAAGTCCATGCGT	
Pepsinogen C	mPepC F	79	GCAAGTTTGGTGACTACAGTGTACTCTAT	NM_025973
Pepsinogen C	mPepC R		ATGGTGAGATCAGCATCGGG	
Rpl19	L19 F	103	CATGCCAAATGGACCAATGTC	NM_014763
Rpl19	L19 R		TGCTCAGGTTCCATGCTCATTA	

Table 7.2 Human Real Time Primer Sequences.

Gene	Primer Name	Amplicon size (bp)	Sequence (5'-3')	Accession No.
GAS5	hGas5 F	75	CTGAAGTCCTAAAGAGCAAGCCTAA	NR_002578
GAS5	hGas5 R		CAGAAAGCTGGAAGTTGAAATGG	
PEPSINOGEN	hPEP F	71	GCTCCCACCCTGGTAGATGA	NM_001079807
PEPSINOGEN	hPEP R		TACTTCGGCACTATCGGCATC	
GAPDH	hGAPDH F	69	TTCCACCCATGGCAAATTCC	NM_002046
GAPDH	hGAPDH R		AAGCTTGTCATCAATGGAAATCC	

However, for the murine real time experiments, the expression patterns of key cumulus cell genes within these samples has been previously published normalised to the internal control *RpL19* (Dunning et al. 2007). For this reason, the two different housekeepers were utilised, so that a comparison could be drawn between the previous findings of the human samples (Chapters 4 and 5) and the murine samples (Dunning et al. 2007). For some analyses the normalised gene expression was calibrated to a reference sample for use with the $2^{-(\Delta\Delta}$CT) method (K. Livak PE-ABI, Sequence Detector User Bulletin 2) (Livak and Schmittgen 2001). Results for each PCR were normalised to the calibrator, which was given the arbitrary value of 1. For murine *Gas5*, *Pepsinogen C* and *Pepsinogen 5* real time RT-PCR, the calibrator was RNA from mGCs after eCG + hCG 12 h *in vivo* treatment. For human *GAS5* and *PEPSINOGEN 3/4/5* real time RT-PCR on cumulus cells and matched pair mural granulosa cells as well as cumulus cells from oocytes that developed to good *vs*. poor embryos, the calibrator was RNA from the human cell line 293T which expressed appreciable levels of each of these genes. Following real time RT-PCR, analysis of the dissociation curves confirmed that a single product was amplified in all reactions. A real time RT-PCR standard curve was produced for the human *GAS5* gene using plasmid DNA (as

described in Chapter 3) for the analysis of *GAS5* in human cumulus cells from patients who underwent single embryo transfer.

7.2.5 Sequencing

Sequencing of plasmids was performed using BigDye Terminator 3.1 kit (Applied Biosystems, Scoresby, VIC, Australia). Sequencing reactions consisted of 190 ng of plasmid DNA, 2.4 pmol of designated primer, 0.4 µL Terminator Ready Reaction Mix 3.1, 2 µL of 5X Big Dye Sequencing Buffer and H_2O to 20 µL. Cycling conditions used for sequencing were as follows, 96ºC, 1 min (1 cycle); 96ºC, 10 secs; 50ºC, 5 secs; 60ºC 4 mins (25 cycles). Sequencing products were precipitated by addition of 80 µL of 75% isopropanol (v/v), brief vortexing and precipitation at room temperature for 15 mins, followed by centrifugation at 13,000 rpm for 20 mins and removal of the supernatant. The pellets were washed with 250 µL of 75% isopropanol (v/v) and re-centrifugated at 13,000rpm for 5 mins. The supernatant was aspirated and residual isopropanol evaporated by incubating the tubes, with the lids open, in a 90ºC heat block for 1 min. Samples were analysed at the Molecular Pathology Sequencing Facility (Institute of Medical and Veterinary Science, IMVS, Adelaide Australia) by an ABI 3730 Capillary sequencer (Applied Biosystems, Scoresby, VIC, Australia). Resultant chromatograms were aligned and compared with published sequences for homology.

7.2.6 Statistical Analysis

Murine real time RT-PCR expression analysis experiments were performed in triplicate and analysed by performing a One-Way ANOVA and Tukey post-hoc test using GraphPad Prism (GraphPad Prism version 5.01 for Windows, San Diego, CA, United States, www.graphpad.com). For the human real time RT-PCR experiments, the expression of each gene was assessed between cumulus and mural granulosa cells was analysed using a Wilcoxon matched pairs test, the expression between cumulus cells associated with a good embryo *vs.* a poor embryo were analysed using a matched pairs t-test and pregnancy outcomes following single embryo transfer were analysed using a Wilcoxon-Mann-Whitney test. Differences were considered statistically significant at a p-value < 0.05. A Spearman's rank order correlation was performed to examine a possible relationship between age, body mass index (BMI) or birth weight (grams) and the relative gene copy number for each of the genes of interest. Body mass index values were available for thirty seven of the thirty eight patients. Birth weights were available for eight out of the twelve patients with a live birth outcome, due to data collection limitations and limited availability of template for PCR analyses.

7.3 RESULTS

7.3.1 Induction of *Gas5* in IVM *vs. in vivo* matured cumulus complexes and *in vivo* matured mural granulosa cells.

The *in vivo* expression of *Gas5* was first investigated in COCs and mGCs from mice which were hormonally stimulated. The level of *Gas5* in mGCs was seen to be significantly up-regulated 4.3-fold 6 h post-hCG treatment *in vivo* but declined rapidly after 12 h hCG treatment and remained low after 16 h hCG treatment (Figure 7.2). The expression of *Gas5* was higher in mGCs ($p = 0.08$) compared to COCs from the same follicles 6 h post-hCG (Figure 7.2). There was negligible induction of *Gas5* in COCs, and at 16 h post-hCG the expression of *Gas5* was barely detectable (Figure 7.2). The expression of *Gas5* was compared between *in vivo* and *in vitro* matured COCs and mGCs to determine if IVM conditions alter the formation of the COC matrix, and to compare this to the *in vivo* expression pattern of *Gas5*. No significant induction of *Gas5* was seen after IVM treatment for 6 h by FSH, egf or a combination of the two (Figure 7.3). After 20 h, which is the accepted time required for complete expansion and maturation *in vitro* (Eppig 1979; Downs 1989; De La Fuente et al. 1999), the expression of *Has2* mRNA and the degree of cumulus expansion was previously shown to follow the *in vivo* pattern in response to IVM conditions (Dunning et al. 2007). The expression of *Gas5* mRNA in IVM COCs was negligible after 20 h of culture with or without treatment (Figure 7.4) compared with immature control COCs. The expression of *Gas5* was significantly reduced in COCs after 16 h hCG treatment *in vivo* compared to eCG treated control COCs.

7.3.2 Characterisation of *GAS5* expression in human cumulus and granulosa cells

Expression of *GAS5* was investigated in human cumulus and granulosa cells collected from women undergoing assisted reproduction. In matched cumulus and mural granulosa cell samples from 14 patients, *GAS5* was abundantly present (Figure 7.5). The expression of *GAS5* mRNA was significantly higher (1.45-fold) in granulosa cells than their matched cumulus cell pairs (Figure 7.5), as was seen in mouse COCs and mGCs. The expression of *HAS2* has previously been reported in these human samples, indicating the purity of each isolated cell type (Dunning et al. 2007)

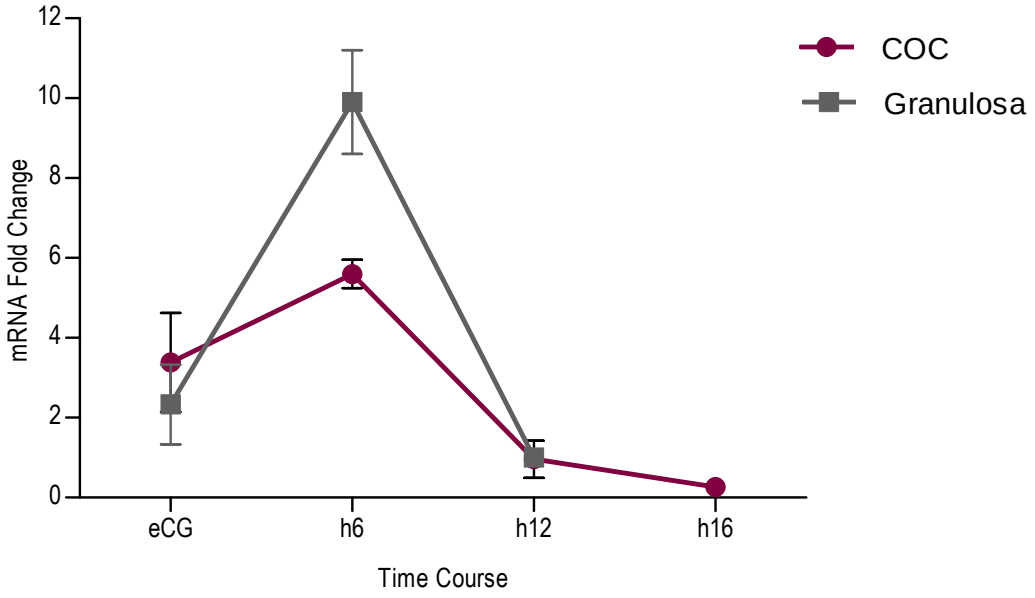

Figure 7.2 Induction of Gas5 mRNA in vivo in response to 6 h oocyte maturation stimuli in both mural granulosa cells and cumulus oocyte complexes.

Analysis of *Gas5* mRNA expression in murine mural granulosa cells (mGCs) or cumulus oocyte complexes (COCs) stimulated for 6, 12 or 16 h *in vivo* by hCG treatment. The mRNA expression of *Gas5* is normalised to the *Rpl19* internal control and presented as mean ± S.E.M. (n=3 independent experiments). *Gas5* is expressed relative to the calibrator (mGCs treated in vivo with eCG + 12 h hCG), which was set at 1. Differences in gene expression were considered significantly different at $p < 0.05$.

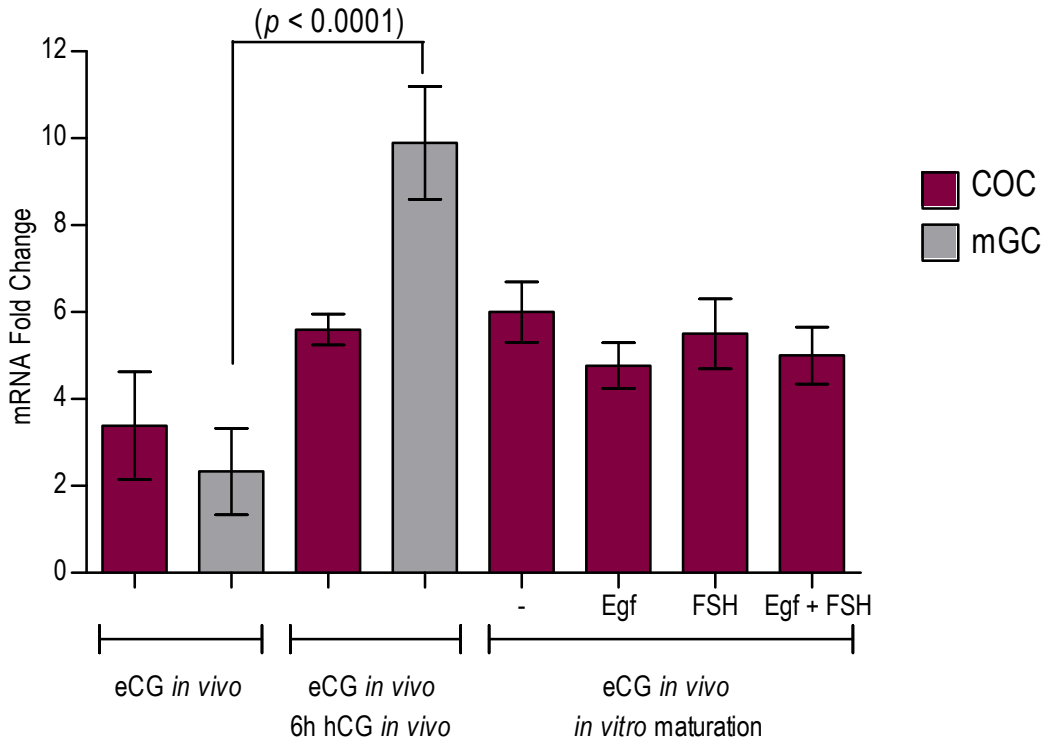

Figure 7.3 Induction of *Gas5* mRNA in vivo and in vitro in response to 6 h oocyte maturation stimuli.

Analysis of *Gas5* mRNA expression in mural granulosa cells (mGCs) or cumulus oocyte complexes (COCs) from equine chorionic gonadotrophin 44 h (eCG) stimulated mice after 6 h of maturation by in vivo hCG treatment or IVM culture with Egf, FSH or a combination of the two. The mRNA expression of *Gas5* is normalised to the *Rpl19* internal control and presented as mean ± S.E.M. (n=3 independent experiments). *Gas5* is expressed relative to the calibrator (mGCs treated in vivo with eCG + 12 h hCG), which was set at 1. Differences in gene expression were considered significantly different at $p < 0.05$.

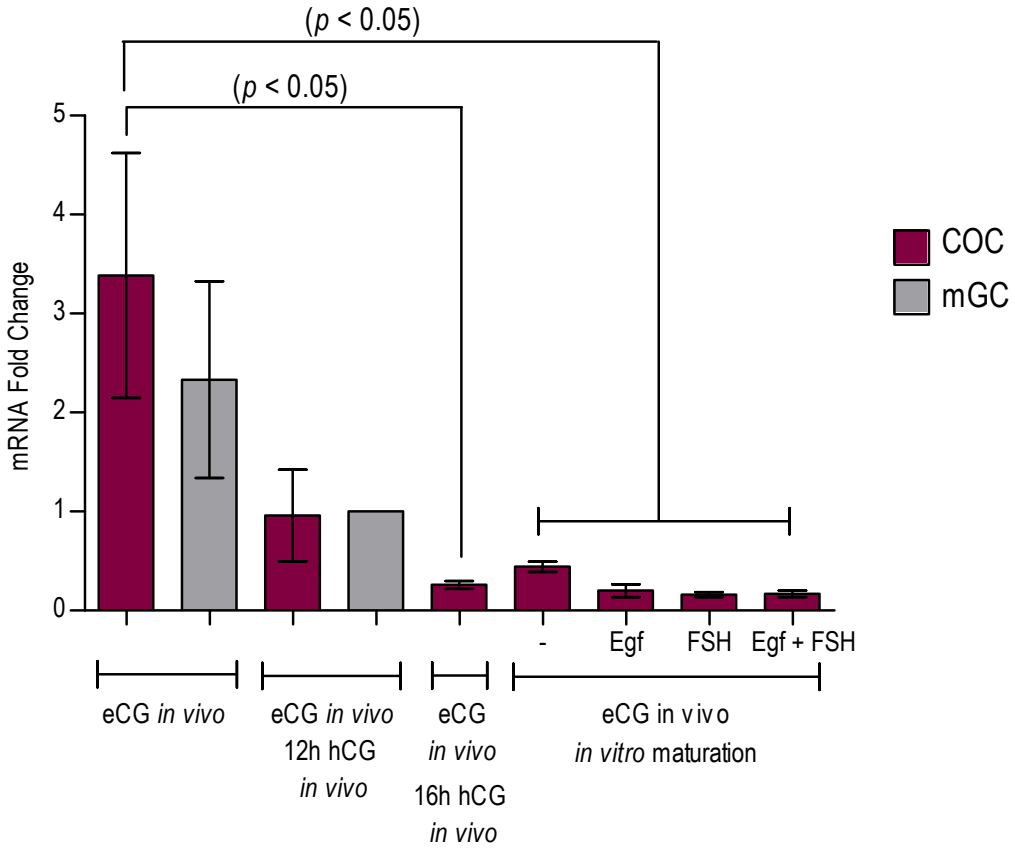

Figure 7.4 No induction of Gas5 mRNA in vivo or in vitro in response to 20 h oocyte maturation stimuli.

Analysis of *Gas5* mRNA expression in murine mural granulosa cells (mGCs) or cumulus oocyte complexes (COCs) stimulated for 12 or 16 h *in vivo* by hCG treatment or in IVM culture with Egf, FSH or a combination of the two for 20 h. The mRNA expression of *Gas5* is normalised to the *Rpl19* internal control and presented as mean ± S.E.M. (n=3 independent experiments). *Gas5* is expressed relative to the calibrator (mGCs treated in vivo with eCG + 12 h hCG), which was set at 1. Differences in gene expression were considered significantly different at $p < 0.05$.

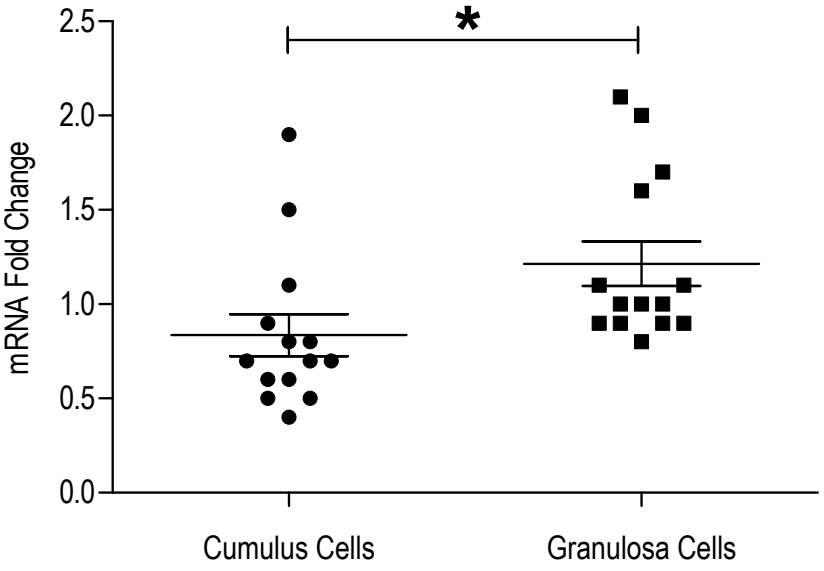

Figure 7.5 Induction of *GAS5* in mural granulosa cells and cumulus cells following in vivo stimulation in patients undergoing assisted reproduction.

Real Time RT-PCR analysis of human *GAS5* expression in paired human cumulus and mural granulosa cells following in vivo stimulation in patients (n = 14) undergoing fertility treatment. The data is expressed as a scatter plot, with the horizontal line representing the mean ± S.E.M. and normalised to *GAPDH*. GAS5 is expressed relative to the calibrator (pooled human cumulus cells from an independent patient cohort), which was set at 1. Asterix indicate significant difference ($p < 0.04$).

7.3.3 Induction of *Pepsinogen 5* and *Pepsinogen C* mRNA in *in vivo* matured cumulus oocyte complexes and mural granulosa cells.

Induction of murine *Pepsinogen 5* mRNA was negligible in COCs following *in vivo* stimulation with eCG and hCG (Figure 7.6) and was almost non-detectable at h 12 post-hCG administration. The expression in mural granulosa cells was also unchanged following hormonal stimulation *in vivo*. The mRNA expression at h 6 post-hCG treatment showed a trend towards significance between COCs and mGCs from the same follicles. Murine *Pepsinogen C* mRNA expression was not detectable in COCs at any time point; while the mRNA in mGCs was constitutively expressed at all time points (Figure 7.7). Both isoforms of murine Pepsinogen displayed higher expression in mural granulosa cells compared to COCs (Figures 7.6 and 7.7).

7.3.4 *PEPSINOGEN 3/4/5* mRNA expression in human cumulus and granulosa cells.

Expression of *PEPSINOGEN 3/4/5* mRNA was similarly investigated in human cumulus and granulosa cells collected from women undergoing assisted reproduction. In matched cumulus and mural granulosa cell samples from 14 patients, *PEPSINOGEN 3/4/5* mRNA was abundantly present (Figure 7.8). The expression of *PEPSINOGEN 3/4/5* mRNA was significantly higher in granulosa cells compared to the matched cumulus cells (Figure 7.8), with 6.7-fold more mRNA in the granulosa cell population. The expression of *HAS2* has previously been reported in these human samples, indicating the purity of each isolated cell type (Dunning et al. 2007).

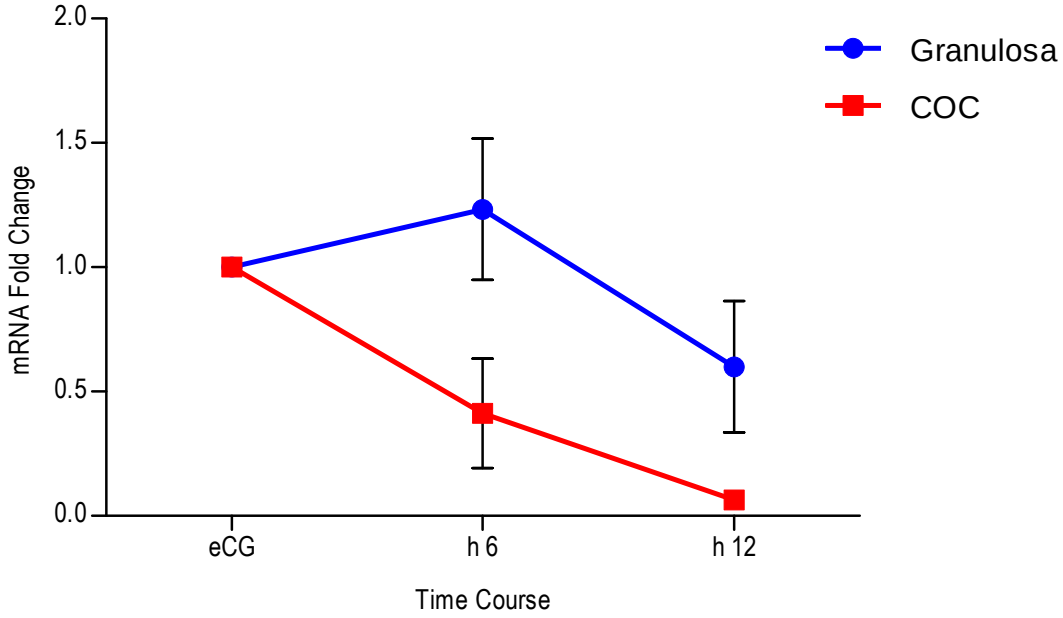

Figure 7.6 Murine *Pepsinogen 5* is not induced in vivo following oocyte maturation stimuli.

Analysis of *Pepsinogen 5* mRNA expression in murine mural granulosa cells (mGCs) or cumulus oocyte complexes (COCs) stimulated for 6 or 12 h *in vivo* by hCG treatment. The mRNA expression of *Pepsinogen 5* is normalised to the *Rpl19* internal control and presented as mean ± S.E.M. (n=3 independent experiments). *Pepsinogen 5* is expressed relative to the calibrator (mGCs treated in vivo with eCG), which was set at 1. A trend towards significance (p = 0.08) was seen between mGCs and COCs following a 6 h oocyte maturation stimuli *in vivo*.

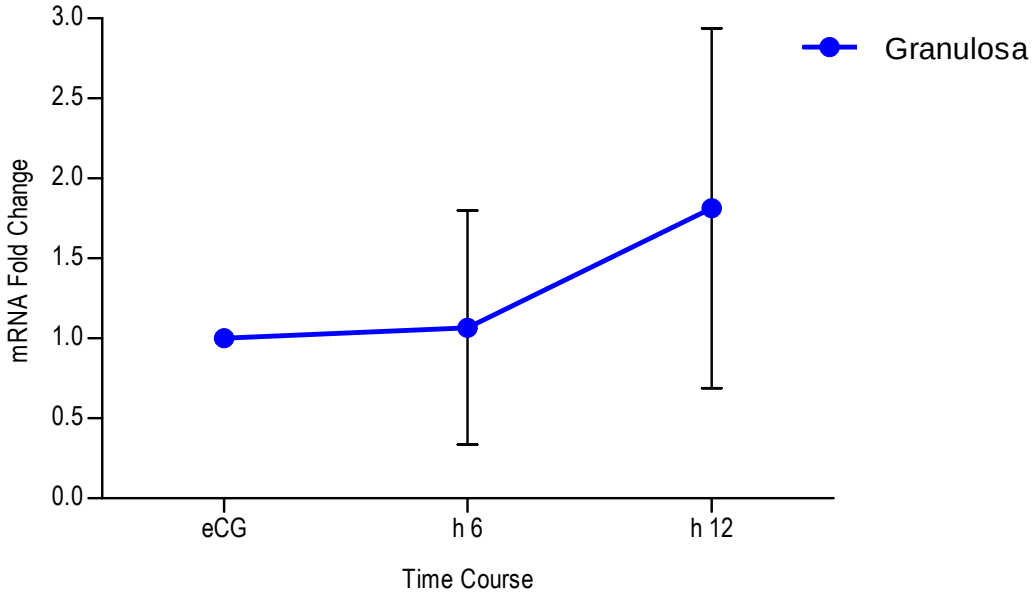

Figure 7.7 Murine *Pepsinogen C* is not induced in vivo following oocyte maturation stimuli.

Analysis of *Pepsinogen C* mRNA expression in murine mural granulosa cells (mGCs) stimulated for 6 or 12 h *in vivo* by hCG treatment. The mRNA expression of *Pepsinogen C* is normalised to the *Rpl19* internal control and presented as mean ± S.E.M. (n=3 independent experiments). *Pepsinogen C* is expressed relative to the calibrator (mGCs treated in vivo with eCG), which was set at 1. Murine *Pepsinogen C* was not detectable in COCs. No significant difference in *Pepsinogen C* mRNA expression seen.

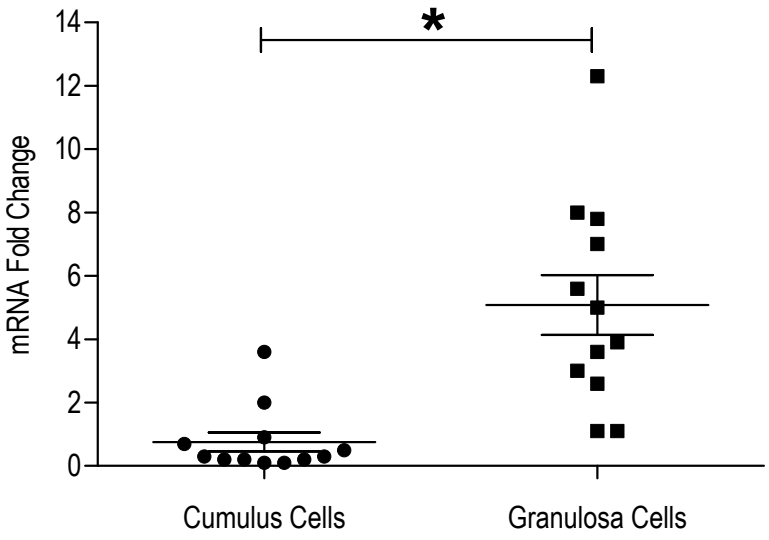

Figure 7.8 Induction of *PEPSINOGEN 3/4/5* mRNA in mural granulosa cells and cumulus cells following in vivo stimulation in patients undergoing assisted reproduction.

Real Time RT-PCR analysis of human *PEPSINOGEN 3/4/5* mRNA expression in paired human cumulus and mural granulosa cells following in vivo stimulation in patients (n = 14) undergoing fertility treatment. The data is expressed as a scatter plot, with the horizontal line representing the mean ± S.E.M. and normalised to *GAPDH*. *PEPSINOGEN 3/4/5* is expressed relative to the calibrator (pooled human cumulus cells from an independent patient cohort), which was set at 1. Asterix indicate significant difference ($p < 0.002$).

7.3.5 *GAS5* and *PEPSINOGEN 3/4/5* mRNA expression as a marker of clinical embryo grade in matched pair human cumulus cells.

Expression of *GAS5* and *PEPSINOGEN 3/4/5* mRNA was investigated in eleven patient matched pairs of human cumulus cells from oocytes that developed to high *vs.* low grade embryos from women undergoing assisted reproduction. Each patient contributed cumulus cells from an oocyte that developed to a high quality embryo and cumulus cells from an oocyte that developed to a low quality embryo as previously described in Chapter 4. Cumulus cell expression of both *GAS5* and *PEPSINOGEN 3/4/5* showed no relationship with clinical embryo grade (Figure 7.9 and 7.10 respectively).

7.3.6 Human cumulus cell *GAS5* as a biomarker of pregnancy success following single embryo transfer.

Expression of *GAS5* was investigated in human cumulus cells from oocytes that developed to high grade embryos which were selected for transfer to women undergoing assisted reproduction. Each patient underwent single embryo transfer, and each individual oocyte and its cumulus mass, along with resultant embryos, were tracked individually. Expression of *GAS5* relative gene copy number was significantly ($p < 0.01$) higher in cumulus cells from patients who achieved a live birth outcome compared to those where no pregnancy was established (Figure 7.11).

7.3.7 Relationships between human cumulus cells GAS5 mRNA expression and clinical patient data.

Expression of *GAS5* relative gene copy number in human cumulus cells from oocytes which resulted in embryos selected for transfer showed no correlation with maternal age or body mass index (Figure 7.12). In cumulus cells from patients who achieved a live birth, the cumulus cell *GAS5* relative gene copy number showed no significant correlation with maternal age or body mass index (Figure 7.13). However, birth weight for the patients who achieved a live birth showed a trend ($p = 0.08$) towards a significant correlation with cumulus cell *GAS5* relative gene copy number (Figure 7.14).

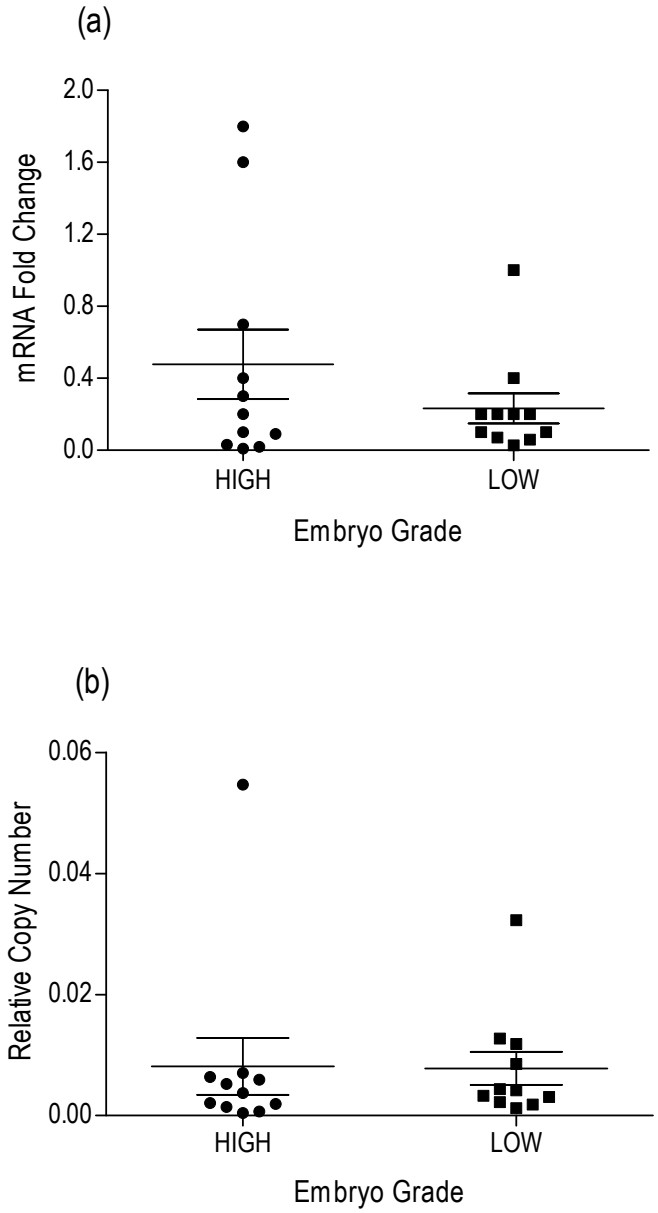

Figure 7.9 Clinical embryo grade is not correlated with cumulus cell expression of *GAS5* following in vivo stimulation of patients undergoing assisted reproduction.

Real Time RT-PCR analysis of human *GAS5* expression in paired human cumulus cells from oocytes which developed to high grade embryos *vs.* oocytes which developed to low grade embryos as determined by a clinical embryo grading system (n = 11 patients). The data is expressed as a scatter plot, with the horizontal line representing the mean ± S.E.M. and normalised to *GAPDH*. (a) GAS5 is expressed relative to the calibrator (pooled human cumulus cells from an independent patient cohort), which was set at 1, or as the (b) relative gene copy number. No significant difference seen.

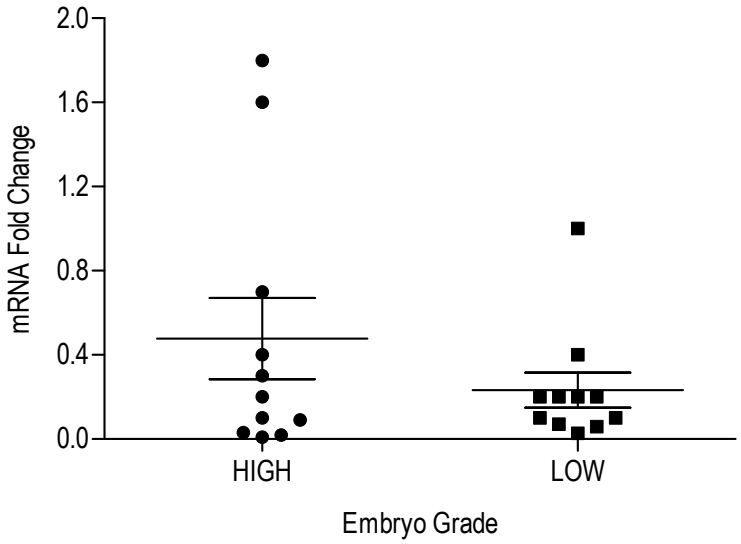

Figure 7.10 Clinical embryo grade is not correlated with cumulus cell mRNA expression of *PEPSINOGEN 3/4/5* in vivo stimulation of patients undergoing assisted reproduction.

Real Time RT-PCR analysis of human *PEPSINOGEN 3/4/5* mRNA expression in paired human cumulus cells from oocytes which developed to high grade embryos *vs.* oocytes which developed to low grade embryos as determined by a clinical embryo grading system (n = 11 patients). The data is expressed as a scatter plot, with the horizontal line representing the mean ± S.E.M. and normalised to *GAPDH*. *PEPSINOGEN 3/4/5* is expressed relative to the calibrator (pooled human cumulus cells from an independent patient cohort), which was set at 1. No significant difference seen. A plasmid standard curve was not produced for *PEPSINOGEN 3/4/5*.

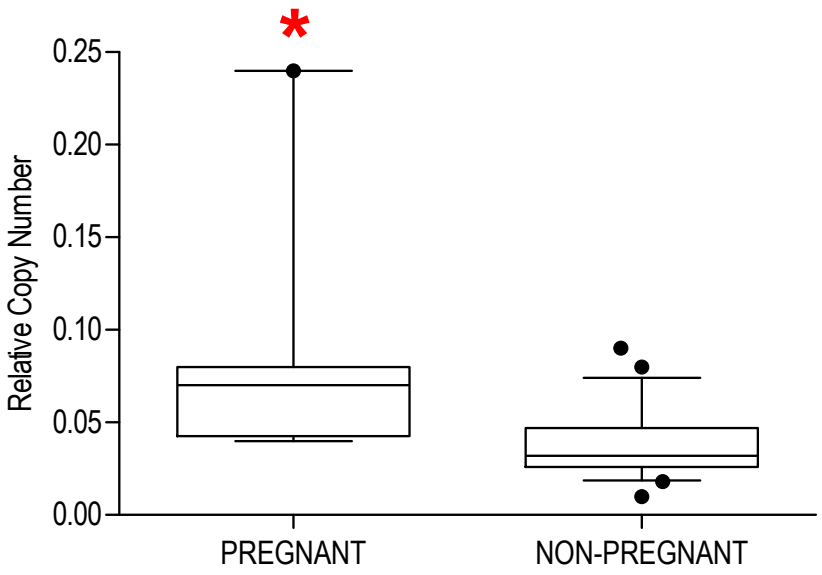

Figure 7.11 Cumulus cell relative gene copy number of *GAS5* is significantly associated with a successful pregnancy resulting in a live birth following in vivo stimulation of patients undergoing assisted reproduction.

Real Time RT-PCR analysis of human *GAS5* (relative gene copy number) expression in cumulus cells from cumulus oocyte complexes which went on to form embryos associated with a live birth outcome *vs.* embryos from which pregnancy was not established following single embryo transfer. Data is normalised to *GAPDH*. Asterix denotes a significant difference $p = 0.0024$ following a Wilcoxon Mann Whitney test.

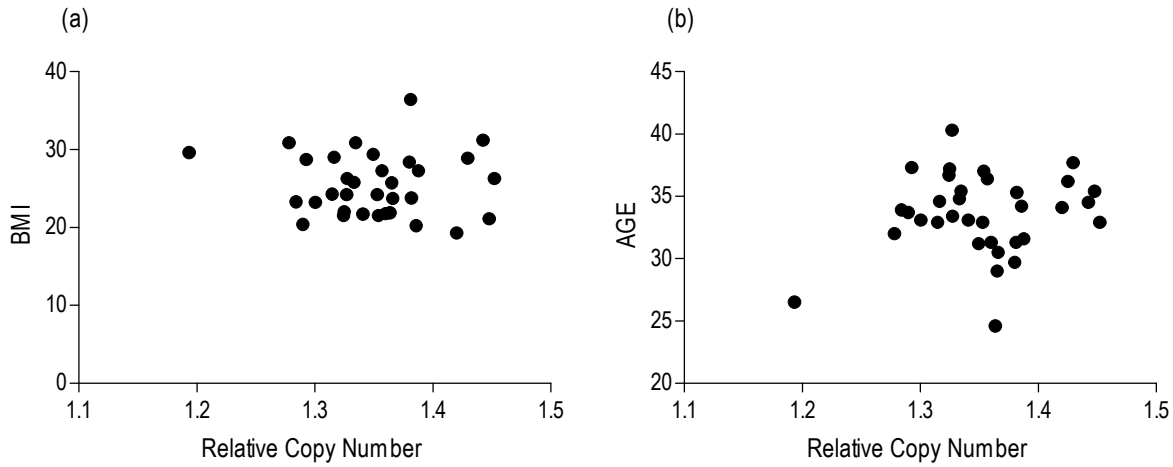

Figure 7.12 No significant association between the relative gene copy number of *GAS5* following single embryo transfer and body mass index (BMI) or maternal age.

A Spearman's rank order correlation was performed to compare maternal (a) BMI and (b) age with *GAS5* relative gene copy number in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer following *in vivo* stimulation (data available for 34 patients). Relative copy number data is normalised to *GAPDH*. No significant association seen. Spearman's r = -0.06403 (a) and -0.006726 (b).

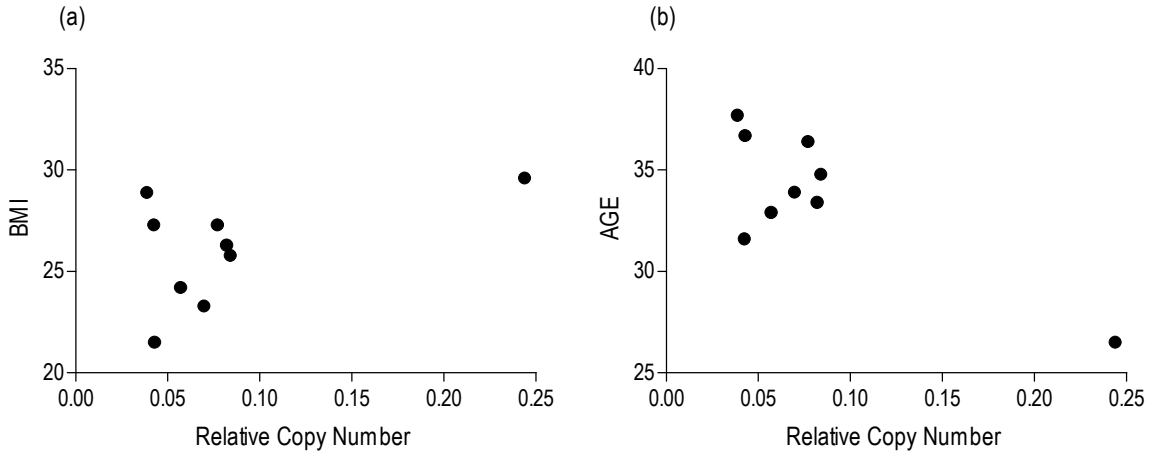

Figure 7.13 Cumulus cell *GAS5* relative gene copy number does not correlate with maternal body mass index (BMI) or age in cumulus cells from oocytes associated with a live birth.

A Spearman's rank order correlation was performed to compare maternal (a) BMI and (b) age with *GAS5* relative gene copy number in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer which resulted in a live birth (data available for 8 patients). Relative copy number data is normalised to *GAPDH*. No significant association seen. Spearman's r = 0.1339 (a) and -0.4000 (b). This analysis was carried out on information and expression data from 9 patients. Limited sample availability was the key limitation for the reduced patient numbers.

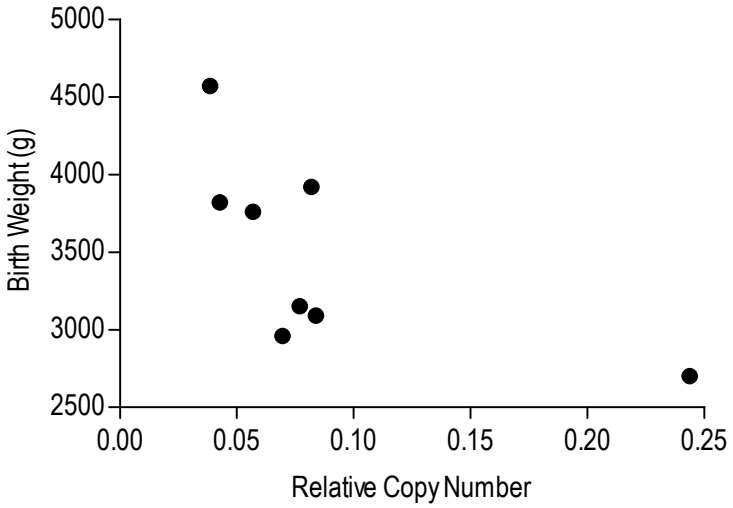

Figure 7.14 Cumulus cell *GAS5* relative gene copy number showed a trend towards significance in association with neonatal birth weight following single embryo transfer.

A Spearman's rank order correlation was performed to compare neonatal birth weight (grams) and *GAS5* relative mRNA gene copy number in human cumulus cells from cumulus oocyte complexes which resulted in embryos selected for single embryo transfer and resulting in a live birth (data available for 8 patients only). Relative copy number data is normalised to *GAPDH*. Cumulus cell relative gene copy number showed a trend towards significance (p = 0.08). Spearman r = -0.6667.

7.4 DISCUSSION

GAS5 and *PEPSINOGEN* transcripts were initially identified in human cumulus cells through microarray experiments, and their novel expression was investigated further in both murine and human cumulus and mural granulosa cells to characterise their expression in these tissues and investigate the pattern of hormonal regulation using real time RT-PCR. In the present study, we have shown for the first time that both *Gas5* and *Pepsinogen* are expressed in murine cumulus and mural granulosa cells collected following *in vivo* and *in vitro* maturation, and that both genes are present in human cumulus and mural granulosa cells recovered from women undergoing assisted reproduction. Further to this, we have validated the microarray findings that *GAS5* expression in human cumulus cells is reflective of oocyte quality and is a predictor of pregnancy success following single embryo transfer. This finding supports *GAS5* as a novel molecular marker of oocyte developmental potential.

The pattern of human *GAS5* and *PEPSINOGEN* expression appears similar to that in mouse follicles, with greater expression in granulosa cells. *Gas5* was induced by hCG in mGCs in the mouse, and a similar high abundance in human mGCs suggests it is also hCG responsive in the human. In patient matched pairs of cumulus cells from oocytes that developed to low *vs*. high grade embryos, both *GAS5* and *PEPSINOGEN* showed no difference in expression. These results are consistent with previous analyses that cumulus cell gene expression is not a candidate marker of clinical embryo grade (refer to Chapter 4). However, when cumulus cell *GAS5* transcript abundance was compared in patients achieving pregnancy success *vs.* those failing to establish a pregnancy following single embryo transfer, it was found to be significantly higher in cumulus cells from oocytes which resulted in a live birth. The relative *GAS5* gene copy number also trended towards a significant correlation with birth weight in the eight patients for which data was available. The decrease in patient numbers available to perform this correlation was primarily due to the limited amount of cumulus cell cDNA, but also due to the limited collection of clinical patient data. However, the association between birth weight and *GAS5* relative gene copy number adds to the significance of *GAS5* as a biomarker of pregnancy success – which encompasses not only live birth rates but neonatal health parameters.

The cumulus cell relative gene copy number expression is contradictory to the expression of the snoRNAs encoded by the *GAS5* gene identified by the microarray analysis in Chapter 6. The nine snoRNAs encoded by the *GAS5* introns detected in our microarray analysis consistently had lower expression in cumulus cells from oocytes that resulted in pregnancy (refer to Chapter 6). Previously

GAS5 overexpression has been shown to induce apoptosis and growth arrest (Fleming et al. 1998). The expression of *Gas5* was suppressed in mouse preimplantation embryos by granulocyte-macrophage colony-stimulating factor (GM-CSF), possibly to inhibit the cellular stress response and apoptosis pathways to facilitate embryo growth and survival (Chin et al. 2009). The data suggesting that higher *GAS5* is associated with pregnancy success is intriguing. A possible explanation for the differences seen between the microarray data and the real time RT-PCR validation experiments lies in the differences in methods. The microarray probes detected the individual snoRNAs, while our real time RT-PCR assay detected the whole *GAS5* transcript and not the individual snoRNAs. The contradictory data regarding pregnancy success and *GAS5*/snoRNA expression may be explained by the processing of small nucleolar RNAs by ribonuclease P (RNase P), a conserved endoribonuclease which removes the 5' leader sequence from precursor transfer RNAs (Coughlin et al. 2008). It has been found that box C/D snoRNAs copurify with RNase P, and that RNase P may be responsible for the splicing of snoRNAs. In RNase P deficient yeast, an accumulation of an unusual processing form of the box C/D intron-encoded snoRNAs was seen by Northern blot (Coughlin et al. 2008). Maturation of box C/D snoRNAs occurs through two pathways (in yeast), either by splicing of the pre-mRNA to free a snoRNA or a splicing independent pathway, which may involve cleavage by RNase P upstream of the snoRNA. The accumulation of snoRNAs due to the RNase P defect described in this study appears to be due to a loss of an essential cleavage that initiates the processing of the 5' side of the snoRNAs identified - as the intron sequence 3' of the snoRNA and 3' exons are absent in the accumulated RNA. The proposed snoRNA processing mechanism by RNase P is depicted the schematic below (Figure 7.15). The inverse relationship between the array probes for the snoRNAs and the real time RT-PCR analysis of *GAS5* may represent a bioassay for RNase P activity within human cumulus cells as well as a new novel marker of oocyte developmental competence. A second explanation takes into account the fact that the cumulus cells are collected 36 h post hCG administration, and undergo a 3 - 4 h incubation period to allow catch up maturation and expansion of COCs which are slightly delayed. *In vitro* culture of human COCs remains undeveloped and suboptimal and the conditions the COC is exposed to may induce the expression of the growth arrest genes.

As *GAS5* does not code for a protein, it is possible the spliced snoRNAs are the functional components of the transcript. The snoRNAs may have a role in the acquisition of oocyte developmental competence which is not yet understood. This study was limited by human cumulus sample availability and therefore was not able to investigate each of the ten snoRNAs encoded by the introns of *GAS5*. Further investigation of the expression patterns of the snoRNAs may explain the different expression pattern of *GAS5* compared to the microarray analysis. The expression of nine *GAS5* encoded snoRNAs identified

NOTE:
This figure is included on page 193 of the print copy of the thesis held in the University of Adelaide Library.

Figure 7.15 Intron-encoded snoRNA processing pathways in yeast.

Maturation of box intron-encoded C/D snoRNAs can proceed through two distinct processing pathways in yeast, as depicted above. The primary splicing-dependent pathway requires linearization of the intron lariat product by debranching enzyme (Dbr1p), allowing subsequent release of the snoRNA by endonucleases and exonucleases. The splicing-dependent pathway produces the mature mRNA and snoRNA after the intron lariat form has been opened by Dbr1p and further processing. The splicing-independent pathway produces only the mature snoRNA and may require RNase P. The dashed lines indicate the step affected by RNase P. Schematic from (Coughlin et al. 2008).

by the microarray platform may result in the identification of cumulus cell biomarkers of pregnancy success, however further investigation is needed to understand how the snoRNAs function in human and mouse follicular cells, and how the expression of the snoRNAs is affected by hormonal stimulation, *in vitro* culture and treatment with oocyte maturation stimuli. The full reciprocal relationship between *GAS5* transcript levels and the abundance of the snoRNAs has only recently become apparent (Coughlin et al. 2008). The current results are supportive of a role for *GAS5* and its intron encoded snoRNAs as predictors of pregnancy success for patients undergoing assisted reproduction.

The identification of *Pepsinogen* mRNA in human and mouse ovarian follicular cells is a novel finding, which adds increased value to the utilisation of cumulus (and even mural granulosa) cells to determine the quality of their enclosed oocytes Following identification by a microarray platform, both human and mouse Pepsinogen was detected by real time RT-PCR in the cumulus and mural granulosa cells, with the greatest levels in the granulosa cells for both species. It was not possible to validate the human Pepsinogen mRNA expression as a potential cumulus cell marker of live birth outcome, as the amount of template was limited. However, it remains a worthwhile line of investigation for future experiments. Classically, all recognised aspartic proteases have been considered to have proteolytic activity (Bobe and Goetz 2001). The presence of Pepsinogen, an aspartic protease, within the ovarian follicular cells may infer a role related to ovulation, as in the trout ovary. Proteases are required to degrade the apical wall of the follicle to allow ovulation of a mature cumulus oocyte complex encapsulated within the dominant antral follicle (Ohnishi et al. 2005). However, this remains speculation at the current time as the current findings do not allude to a definitive role for Pepsinogens during ovulation in the mouse and human ovary and further investigation is necessary.

This study has identified and characterised the expression and hormonal regulation of *Gas5* and *Pepsinogen* in mouse and human follicular cell populations for the first time. Human cumulus cell *GAS5* was then validated as a biomarker of oocyte quality with the potential to predict pregnancy success for women undergoing assisted reproduction. The functionality of *GAS5* in cumulus cells is not known, but the novel identification of *GAS5* encoded snoRNAs is captivating, and furthers our knowledge regarding the mechanisms which determine oocyte competence and pregnancy success. Although we have endeavoured to validate the results of the microarray analysis, some results were collected using the same samples as the microarray experiments. It would therefore be beneficial to repeat these findings with an independent cohort of cumulus samples. The collection of an independent sample cohort was impractical for this study due to time constraints, but would add strength to the results already presented in this chapter. In addition, analysis of the protein levels of these novel genes (where possible) would

confirm the expression patterns and hormonal control seen following real time RT-PCR analysis, and possibly shed light on the level at which regulation of these transcripts occurs. Due to sample limitations this was not possible in the current study but is an important follow up experiment to undertake to fully understand the role of these transcripts in ovarian follicular cells. The ability to non-invasively select the highest quality oocyte with the greatest potential to achieve a successful pregnancy has far reaching clinical implications. This analysis has identified the *GAS5*-encoded snoRNAs as potential markers of oocyte developmental competence.

These results further reinforce our hypothesis that novel cumulus cell genes are candidate markers for predicting pregnancy outcome. The results support the hypothesis that the bi-directional communication between the oocyte and cumulus cells mediates oocyte developmental competence and provides a unique opportunity to assess the relative potential among a cohort of oocytes through measuring genes responding to oocyte signals. This chapter further confirms the correlation between cumulus cell gene expression from individual oocytes and pregnancy outcome in a patient cohort with individual embryo culture and single embryo transfer.

CHAPTER 8

FINAL CONCLUSIONS AND FUTURE DIRECTIONS

8.1 DISCUSSION AND CONCLUSIONS

Australia has one of the highest rates of assisted reproduction, resulting in over 10,000 live births in 2007 (Wang et al. 2009a). Currently, embryos are selected for transfer based on morphological appearance and the timing of key developmental milestones, both of which have a limited association with pregnancy success. In an effort to overcome low success rates patients often have multiple embryos transferred to the uterus to improve treatment efficiency, a practise which dramatically increases the risk of multiple gestations. Multiple gestations are the most common cause of ART complications, including higher rates of preterm delivery, low birth weight babies, congenital defects, infant death and disability (Wang et al. 2009a; Wang et al. 2009b). Therefore the most desirable approach for patients undergoing assisted reproduction is the transfer of a single, embryo. But even without the complications of multiple embryo transfer ART currently achieves lower average birth weight than from natural conceptions. Both of these shortcomings can be improved through development of methods to identify gametes with the greatest developmental potential. There are currently no robust markers of embryo developmental potential with a strong correlation to pregnancy success. Oocyte quality is the key limiting factor in embryo developmental potential (Ebner et al. 2000; Ebner et al. 2003a; Ebner et al. 2003b; Paz et al. 2004), therefore markers of human oocyte developmental competence with a strong correlation to live birth have the potential to transform ART clinical practises. Without such markers, there is continued reluctance on the part of the physician and patient to risk a single embryo transfer due to the high rate of failure. Previous studies have investigated cumulus cells markers of oocyte quality; however the combined results of these studies have only alluded to potential markers of oocyte quality, with few linked to pregnancy success and none tracking individual oocytes from collection to birth. Cumulus cells represent an ideal tissue for investigating markers of oocyte developmental potential, as they are routinely discarded during ART cycles. Studies have previously found a significant relationship between cumulus cell gene expression and clinical embryo grade, however there is a lack of consistency between these studies which was the basis for undertaking these experiments. However these have not been able to make direct correlations to individual oocytes or embryos, nor have live birth outcomes been assessed in a cohort of patients with only single embryo transfers performed.

The studies described in this thesis are the first to identify novel, non-invasive cumulus cell markers of oocyte developmental potential in single cumulus masses from individually tracked oocytes. We used both informed selection of targets with known dynamic regulation and important function during oocyte maturation as well as a non-discriminatory global gene survey. The unique experimental approach utilised for these experiments broadens existing knowledge of the molecular mechanisms which

influence the acquisition of oocyte developmental competence. The markers identified were indicative of pregnancy success following single embryo transfer, and remarkably, were further found to correlate with birth weight. As all oocytes and resultant embryos were individually tracked from collection through to transfer, the cumulus cell markers identified have the potential to augment gamete selection and increase pregnancy success rates for patients seeking infertility treatments. The ability to directly and absolutely extrapolate the cumulus cell genes expression to live birth outcomes is a key strength of this study, and reinforces the significant clinical application of the markers identified. The development of highly sensitive, quantitative and reproducible real time RT-PCR assays allowed robust and easily reproducible estimation of gene copy numbers in each sample representing a precise method of quantifying the marker genes and allowing the measurement of the expression of a large number of genes in very small cumulus biopsy samples.

Analyses were performed in patient matched pairs of cumulus cells trimmed from single oocytes which were cultured and fertilised individually, as were all resultant embryos – a distinction from many of the previous studies (McKenzie et al. 2004; Hasegawa et al. 2005; Zhang et al. 2005; Cillo et al. 2007; Feuerstein et al. 2007; Hasegawa et al. 2007; van Montfoort et al. 2008; Anderson et al. 2009b). The most consistent observation was widely variable expression, seen both within and between patients. The variation in experimental outcomes emphasises the need for a standardised method for embryo grading, and a non-biased experimental method for assessing embryo developmental potential. It is known that the correlation between embryo morphology and pregnancy success is weak, as poor grade embryos may still form a viable pregnancy while high grade embryos frequently fail to implant (Rijnders and Jansen 1998; Graham et al. 2000; Guerif et al. 2007). The most definitive conclusion is that the current embryo grading system provides a better tool for the elimination of poor quality embryos than selection of “top quality” embryos for transfer. The assessment of embryo morphology and the embryo scoring system is a fundamental part of assisted reproduction; however there has been a need for identification of molecular markers which predict the most profound definitions of oocyte quality and developmental competence – the ability to result in a live, term birth.

My subsequent analysis of the association of cumulus gene expression with pregnancy outcomes identified novel molecular markers in cumulus cells which correlate with successful gestation to live birth, as well as birth weight. This experimental approach definitively linked the pregnancy outcome for each patient with the oocyte each transferred embryo was derived from. The data presented within this thesis suggests cumulus cell gene expression is a promising tool for assessing oocyte quality and

predicting pregnancy success, as cumulus cell expression of *PTGS2*, *VCAN* and *PTX3* had a significant correlation with live birth outcome, while cumulus cell levels of *VCAN*, *GREM1* and *PFKP* showed a significant correlation with birth weight. The correlation between birth weight and cumulus cell gene expression represents possible evidence of reprogramming in the embryo as the preimplantation stage of embryo development is a sensitive "window" when *in vitro* and *in vivo* manipulations, such as culture conditions or maternal diet, may have critical consequences (Watkins et al. 2008). The genes reflect potential predictors of health outcomes for babies born from assisted reproduction and support the hypothesis that cumulus cell genes are candidate tools for predicting ART outcomes. The ability to reliably select the highest quality oocyte and resultant embryo will improve both pregnancy success rates and health outcomes following assisted reproduction.

Microarray platforms are a recognised method for determining somatic cell markers of oocyte quality and numerous genes with this potential have been reported by other groups using microarray (Zhang et al. 2005; Assou et al. 2008; Hamel et al. 2008; van Montfoort et al. 2008; Hamel et al. 2009). Microarray analysis identified genes with high levels of differential expression between cumulus cells from patients who achieved a live birth outcome compared to those who did not become pregnant as prospective biomarkers of oocyte quality and subsequent pregnancy success. The repeated observations for the Pepsinogen isozymogens (*PGA3*/*PGA4*/*PGA5*) and significant or trending negative correlation of nine of the ten *GAS5*-encoded snoRNAs detected by microarray probes specified these transcripts for further investigation. Characterisation in both mouse and human follicular cells showed both transcripts had a greater abundance in mural granulosa cells; a further follow up analysis of the *GAS5* RNA transcript was found to be significantly positively associated with pregnancy success and correlated with birth weight following single embryo transfer.

Pregnancy success is dependent on numerous developmental processes which occur at key stages of follicle growth and development (Figure 8.1). Normal follicular development reflects the communication between follicular cells and systemic signalling to initiate oocyte maturation and induce changes within the ovary to support oocyte development. The acquisition of oocyte developmental competence occurs sequentially during oocyte maturation and reflects the dialogue between the oocyte and its surrounding cumulus cells. Both follicular development and oocyte maturation influence subsequent embryo development, and the ability for an embryo to cleave on time, implant and establish a viable pregnancy. Each of these key processes contributes to the ability for an oocyte and resultant embryo to develop to a live, healthy birth (Sirard et al. 2006). Oocyte quality is reflected by an embryos capacity to develop to

the blastocyst stage and establish a successful pregnancy, suggesting that markers of oocyte health and subsequent embryo development in culture may be predictive of pregnancy success. The studies described in this thesis have for the first time shown a significant relationship between cumulus cell gene expression and pregnancy success, paving the way for the development of genetic based tests of oocyte quality

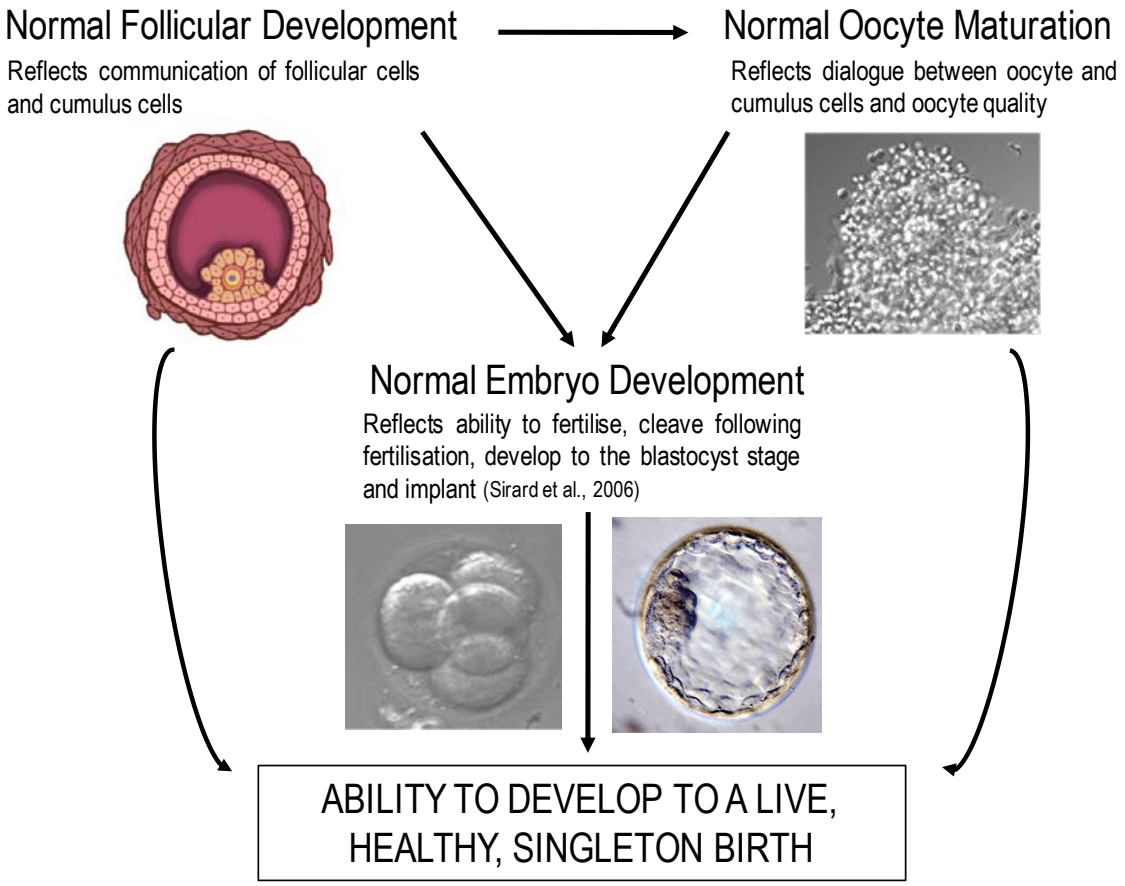

Figure 8.1 Pregnancy success is dependent on normal developmental processes occurring at several key stages of growth and development.

The ability for an embryo to establish a viable pregnancy is dependent on normal follicular development, normal oocyte maturation and normal embryo development. The ability to identify oocytes and embryos with this potential is a key limitation of assisted reproductive technology.

8.2 CLINICAL SIGNIFICANCE AND FUTURE DIRECTIONS

The ability to reliably and repeatedly select the single oocyte (and subsequent embryo) with the greatest potential to result in a live birth has considerable clinical implications for patients seeking fertility treatments, primarily through increasing pregnancy success rates while reducing multiple embryo transfers and subsequently the incidence of multiple births. The identification of an oocyte within a patient's cohort with the potential to establish a viable pregnancy will help clinics confidently implement single embryo transfer as a standard practise without compromising success rates, decrease multiple embryo transfers and virtually eliminate multiple gestations and the associated health burden.

To fully appreciate the potential of the molecular markers of oocyte quality identified in this thesis, a much larger patient population is needed in a prospective study which uses the markers identified to make a transfer selection decision for a patient who has a pool of identical embryos which have developed in a near identical manner and cannot otherwise be differentiated. A comparison of cumulus cell gene expression for all oocytes collected from each patient will better reflect the clinical situation where patients generate a pool of oocytes, and further confirm the efficacy of these markers. Gene expression comparisons of all oocytes from individual patients, which has been assessed by only one group (Anderson et al. 2009b) will aid in the development of a commercial test which will augment the selection oocytes and embryos for patients and increase the number of single embryo transfers. In the current experiments all cumulus masses were collected prior to hyaluronidase treatment, regardless of the fertilisation technique employed. The use of IVF and ICSI may result in changes to the predictability of pregnancy success, which should be investigated further in a larger study population. The current study was not able to investigate the influence of IVF vs. ICSI on the predictive potential of this test. As patients often generate a pool of embryos, those which are graded high but not transferred are often cryopreserved, and it is not yet established whether cumulus cell markers of pregnancy success are applicable following cryopreservation and thawing of selected embryos.

The most steadfast method to validate the predictive value of these cumulus cell markers is to prospectively select oocytes for transfer and assess the pregnancy success and live birth rates, as well as health parameters of the babies. The most immediate step in validating the current findings is through the implementation of this test into a prospective clinical trial for patients with a number of morphologically similar embryos and using the gene measures described throughout this thesis to select a single embryo for transfer. Real time RT-PCR is a fast and affordable technology which can easily

output the necessary data between oocyte fertilisation and embryo transfer. A large patient cohort is required to get an accurate overview of the efficacy and predictability of this test to a level that could be marketed commercially. The most important outcome from this test is the live birth of a healthy, term, singleton baby. Though the test requires further validation in both a laboratory and clinical setting, the use of the cumulus cell markers identified in this thesis to assess oocyte quality in conjunction with current morphological assessment tools has the potential to transform current clinical practise, and most importantly increase pregnancy success rates. From a patient's point of view, the likelihood a falling pregnant and carrying that pregnancy to term is the most important factor when faced with an infertility diagnosis.

CHAPTER 9

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