MITOCHONDRIAL DNA REPLICATION IN PRE-IMPLANTATION EMBRYONIC DEVELOPMENT

by

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A thesis submitted to
The University of Birmingham
for the degree of

DOCTOR OF PHILOSOPHY

School of Medicine
The University of Birmingham
February 2007

ABSTRACT

All eukaryotic cells possess mitochondrial DNA (mtDNA), which is maternally inherited through the oocyte, its replication being regulated by nuclear-encoded replication factors. It was hypothesised that mtDNA replication is highly regulated in oocytes, pre-implantation embryos and embryonic stem cells (ESCs) and that this may be disrupted following nuclear transfer (NT).

MtDNA copy number decreased between 2-cell and 8-cell staged porcine embryos and increased between the morula and expanded blastocyst stages, coinciding with increased expression of mtDNA replication factors. Competent porcine oocytes replicated their mtDNA prior to and during *in vitro* maturation to produce and maintain the 100000 mtDNA copies required for fertilisation. Those oocytes in which mtDNA replication was delayed had reduced developmental ability.

Expression of pluripotency-associated genes decreased as murine ESCs differentiated into embryoid bodies, although expression of mtDNA replication factors did not increase until the stage equivalent to organogenesis. Cross-species NT embryos in which the donor cell-derived mtDNA was replicated produced decreased developmental outcomes compared to those in which no mtDNA replication took place. Disruption of the strict regulation of mtDNA replication that occurs during early embryogenesis, as is likely following NT, may therefore contribute to the reduced developmental ability of embryos produced using such techniques.

ACKNOWLEDGEMENTS

I would like to say a huge thank you to my supervisor, Dr Justin St. John for encouraging me to study for a PhD in the first place and for providing me with this project. Also for his continued encouragement, enthusiasm and support throughout my PhD studies and for his advice and comments during the writing of this thesis.

I would also like to thank Professor Keith Campbell and members of his research group at the University of Nottingham for the valuable training I received in porcine *in vitro* fertilisation and nuclear transfer techniques, without which this thesis would not exist.

I also received a great deal of help, motivation and friendship from other members of our research group, particularly from my fellow PhD student, Emma Bowles, who has been an incredible support throughout my PhD studies.

Most importantly, I would like to thank my wonderful Husband, Peter for his love and support, for keeping me sane during the crazy times and for putting up with me working a ridiculous number of hours, particularly during the last year.

Finally, I would like to thank the University of Birmingham Medical School for providing me with the funding that enabled me to carry out this work.

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LIST OF ABBREVIATIONS

AMV	Avian myelovirus
ART	Assisted reproduction technology
ATP	Adenosine triphosphate
ATPase6	Adenosine triphosphate synthase subunit 6
ATPase8	Adenosine triphosphate synthase subunit 8
AZT	3'-azido-3'-deoxythymidine
BCB	Brilliant Cresyl blue
BP	Base pairs
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
$CaCl_2.2H_2O$	Calcium chloride dihydrate
CCE	CCE/R1 cell line
cDNA	Complementary deoxyribonucleic acid
CHX	Cycloheximide
CO_2	Carbon Dioxide
COXI	Cytochrome C oxidase subunit 1
COXII	Cytochrome C oxidase subunit 2
COXIII	Cytochrome C oxidase subunit 3
CSB	Conserved sequence block
CT	Cytoplasmic transfer
Ct	Cycle threshold
CytB	Cytochrome B
DAPI	4', 6-diamidino-2-phenylindole
ddC	2', 3' dideoxycytidine
ddH_2O	Double distilled water
D-loop	Displacement loop
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E	Embryonic day
EB	Embryoid body
ECNT	Embryonic cell nuclear transfer
E. coli	Escherichia coli
EDTA	Ethylenediamintetraacetic acid
EGA	Embryonic genome activation
EGF	Epidermal growth factor
ESC	Embryonic stem cell
ETC	Electron transport chain
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
G6PD	Glucose-6-phosphate dehydrogenase
GV	Germinal vesicle

GVBD	Germinal vesicle breakdown
GVII	Germinal vesicle-intact immature oocyte
GVT	Germinal vesicle transfer
hESC	Human embryonic stem cell
HSP	Heavy strand promoter
ICC	Immunocytochemistry
ICM	Inner cell mass
ICSI	Intra-cytoplasmic sperm injection
IgG	Immunoglobulin G
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen orthophosphate
LB	Luria-Bertani broth
LH	Luteinising hormone
LHON	Leber's hereditary optic neuropathy
LIF	Leukaemia inhibitory factor
LSP	Light strand promoter
MEF	Murine embryonic fibroblast
MERRF	Myoclonic epilepsy with ragged red fibres
mESC	Murine embryonic stem cell
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
MII	Metaphase of the second meiotic division
mPBS	Modified phosphate buffered saline
mRNA	Messenger ribonucleic acid
mTBM	Modified tris buffered medium
MtDNA	Mitochondrial deoxyribonucleic acid
MtRNAPol	Mitochondrial ribonucleic acid polymerase
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide, reduced
$NaHCO_3$	Sodium bicarbonate
NCSU	North Carolina State University
ND1	NADH dehydrogenase subunit 1
ND2	NADH dehydrogenase subunit 2
ND3	NADH dehydrogenase subunit 3
ND4	NADH dehydrogenase subunit 4
ND4L	NADH dehydrogenase subunit 4L
ND5	NADH dehydrogenase subunit 5
ND6	NADH dehydrogenase subunit 6
NEC	No enzyme control in a reverse transcription reaction
NRC	No ribonucleic acid control in a reverse transcription reaction
NRF1	Nuclear respiratory factor 1
NRF2	Nuclear respiratory factor 2
NRF2A	Nuclear respiratory factor 2 α subunit

NRF2B	Nuclear respiratory factor 2 β subunit
NT	Nuclear transfer
NTC	No template control in a polymerase chain reaction
O_H	Origin of heavy strand replication
O_L	Origin of light strand replication
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC	Primordial germ cell
PGD	Pre-implantation genetic diagnosis
PN	Pronucleus
PNT	Pronuclear transfer
PolG	Polymerase gamma
PolGA	Polymerase gamma catalytic subunit
PolGB	Polymerase gamma accessory subunit
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RTPCR	Reverse transcriptase polymerase chain reaction
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
TAE	Tris acetate ethylenediamintetraacetic acid
TBS	Tris buffered saline
TBS-T	Tween dissolved in tris buffered saline
TCM-19	Tissue culture medium 199
TE	Trophectoderm
TEMED	N,N,N',N'-tetramethylethylenediamine
TFAM	Mitochondrial transcription factor A
TFB1M	Mitochondrial transcription factor B1
TFB2M	Mitochondrial transcription factor B2
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	Transfer ribonucleic acid
UV	Ultraviolet
V/V	Volume/volume
W/V	Weight/volume
ZP	Zona pellucida

CHAPTER 1: GENERAL INTRODUCTION

Since the birth of Louise Brown in 1978 (Steptoe & Edwards, 1978), the use of assisted reproductive technology (ART) has become widespread for both the treatment of human infertility and for animal research. The techniques available have become increasingly invasive, ranging from artificial insemination to the highly invasive nuclear transfer (NT) technologies. Although NT is not currently used clinically, the same technology has been proposed to produce patient-specific embryonic stem cells (ESCs) for the treatment of various diseases (Smith, 1998). Worryingly, little or no mammalian animal studies were carried out before ART techniques were approved for clinical use (Leese & Whittall, 2001). However, many animals derived using NT had severe health problems and the efficiency of the technique is low (Cibelli *et al.*, 2002). It is therefore important to understand what effect techniques such as NT might have on the future health of both animal offspring derived using such techniques and of animals and human patients receiving stem cell therapy.

The majority of the research carried out so far has concentrated on the reprogramming of a somatic donor cell nucleus to behave as a newly fertilised embryo (Hiiragi & Solter, 2005; Jouneau & Renard, 2003; Morgan *et al.*, 2005). However, it is also important to understand the molecular events occurring in components of the cytoplasm, in particular the mitochondria, which contain their own genome. Mitochondria and mitochondrial DNA (mtDNA) play a vital role in the production of cellular adenosine triphosphate (ATP), which is necessary for embryogenesis (Van Blerkom *et al.*, 1995). However, little is understood about the mechanisms regulating mtDNA replication and ATP production. This thesis

investigates the occurrence and regulation of mtDNA replication during early embryogenesis and how this may be disrupted following NT.

1.1 Embryogenesis

Successful embryogenesis begins with the fusion of two haploid gametes, one male and one female, which then form a diploid cell (zygote) capable of mitosis. The haploid gametes originate in the early post-implantation embryo of the previous generation, when primordial germ cells (PGCs) are formed (Chiquoine, 1954). PGCs respond to chemotaxic signals and migrate to the genital ridge, where they lose their motility and are known as oogonia in the female and spermatogonia in the male (Chiquoine, 1954). These cells then develop into mature haploid gametes through the processes of oogenesis and spermatogenesis, respectively.

1.1.1 Oogenesis

The ovarian follicle is the structural and functional unit of a mammalian ovary (Wu *et al.*, 2001) and maturation of the gamete and follicle occur simultaneously, both requiring the other. Oogonia are contained within the primordial follicle, consisting of a single layer of somatic granulosa cells. Many cycles of replicative mitosis take place in the oogonia, which then enter meiosis shortly before birth (Leibfried & First, 1980). At this stage, the gametes become known as primary oocytes and secrete three or four different types of proteins. These cross-link to form the protective zona pellucida (ZP), which lies between the oocyte membrane and the surrounding layer of granulosa cells (Greve & Wassarman, 1985). Primary oocytes arrest at the first meiotic prophase and then remain in this quiescent state until puberty. The prophase nucleus contains partially condensed chromosomes known as the

germinal vesicle (GV), which becomes internalised by a membrane that functions to protect the DNA (Szollosi *et al.*, 1972). At puberty, increased levels of follicle stimulating hormone (FSH) result in the growth and development of the oocyte and ovarian follicle (Danforth, 1995). This includes expansion of the granulosa cell layer and development of the interstitial thecal tissue and blood capillaries. Fluid then accumulates between the granulosa cells forming a cavity known as the antrum. Formation of the antrum causes the majority of the granulosa cells to become separated from the oocyte and instead form part of the follicle wall. However, the oocyte remains surrounded by a few layers of granulosa cells, which differentiate into cumulus cells (Diaz *et al.*, 2006).

In the human, one ovarian follicle becomes dominant during each menstrual cycle and is selected, through complex mechanisms, for ovulation (Erickson & Danforth, 1995). In the pig, between 12 and 20 oocytes are ovulated during each menstrual cycle (Hunter *et al.*, 2004). The mammalian ovulatory luteinising hormone (LH) surge stimulates the competent oocyte(s) within the dominant follicle(s) to re-enter meiosis (Danforth, 1995), the first step of which is GV breakdown (GVBD), when chromosomes are released from within the nuclear envelope. The oocyte then progresses through the first meiotic division, extrudes the first polar body and begins the second stage of meiosis, arresting at metaphase II (MII). Cytoplasmic maturation also takes place, involving the early production of messenger RNA molecules (mRNAs), amino acids and lipid molecules, and subsequently, the storage of these products for use in embryonic development (Eppig, 1996). Simultaneously, cumulus cells of the dominant follicle(s) undergo proliferation and mucification, which are required for fertilisation to take place (Zhuo & Kimata, 2001). On completion of maturation of the

dominant follicle(s), the mature oocyte and its associated cumulus cells are released from the follicle ready for fertilisation.

1.1.2 Spermatogenesis and spermiogenesis

Sperm production begins in the testicular seminiferous tubules. These contain spermatogonia surrounded by Sertoli cells, which proliferate from birth until puberty. At puberty, Sertoli cells complete their mitotic phase and begin their new role as nurse cells to the developing gametes (Sylvester & Griswold, 1994). Spermatogonia exist in two major forms, the first being type A that continue to divide mitotically in order to maintain the germ cell population. Type B spermatogonia are differentiated from type A and these enter meiosis, migrate through tight junctions between the Sertoli cells to the lumen of the testis and eventually mature into functional sperm (Dolci *et al.*, 2002). Type B spermatogonia that have entered the first phase of meiosis are known as primary spermatocytes (Alberts *et al.*, 1993). On completion of the first meiotic division, diploid spermatocytes become known as secondary spermatocytes and enter the second meiotic division that results in haploid round spermatids (Alberts *et al.*, 1993). A differentiation phase, known as spermiogenesis, then begins, which, after further maturation in the epididymis, results in mature sperm that are capable of transport through the female reproductive tract and subsequently, fertilisation of an oocyte (Esponda, 1985).

During spermiogenesis, the Golgi apparatus develops into the acrosome, containing hyaluronidase and acrosin, enzymes involved in penetration of the oocyte (Huang & Yanagimachi, 1985). Nuclear condensation also occurs, with histones being replaced by protamines as the proteins required for DNA packaging (Grimes, 1986). Centrioles migrate to

the opposite pole to the acrosome and form the flagellum, the top part of which contains the cell cytoplasm, with mitochondria arranged helically (Alberts *et al.*, 1993). The mitochondria supply the ATP required for movement of the flagellum, which propels the sperm towards the oocyte (Alberts *et al.*, 1993). Mature sperm almost capable of fertilisation are then stored in the epididymis until required. Final fertilisation capability is obtained in the female reproductive tract. This process is known as capacitation and involves complex changes in sperm membrane glycoprotein structure (reviewed in Brewis *et al.*, 2005).

1.1.3 Fertilisation

On reaching the oocyte in the ampulla region of the oviduct, sperm travel through the cumulus cells and bind to the ZP. The acrosome reaction then takes place, which involves rearrangement of the sperm head membrane components and results in the release of acrosomal enzymes (Breitbart & Spungin, 1997). These enzymes break down the mucus surrounding the oocyte allowing sperm access to the oocyte membrane. Sperm and oocyte membranes then fuse and, through one of the many proposed mechanisms (reviewed in Wassarman, 1999), Ca^{2+} oscillations are initiated. Early fertilisation events include the release of cortical granules, which cause structural changes in the ZP and oocyte membrane that prevent additional sperm from gaining entry (Braden *et al.*, 1954). Completion of the second stage of oocyte meiosis also occurs, resulting in the release of the second polar body, after which the late fertilisation events can take place. These include the formation and fusion of the parental haploid pronuclei to produce a diploid zygote, followed by DNA synthesis and the first mitotic cell division of the embryo (Mori *et al.*, 1988)

1.1.4 Early embryogenesis

Embryonic blastomeres go through several rounds of mitosis, with blastomeres progressively decreasing in size due to the absence of cell growth between the cell divisions. This process is known as cleavage and takes place whilst the embryo travels through the oviduct towards the uterus. During the cleavage divisions, the embryonic genome becomes transcriptionally active in a process known as embryonic genome activation (EGA). This occurs at the 2-cell stage in mice (Bolton *et al.*, 1984), the 4-cell stage in pigs (Jarrell *et al.*, 1991), the 4 to 8-cell stage in humans (Braude *et al.*, 1988) and around the 8 to 16-cell stage in sheep (Calarco & McLaren, 1976) and cattle (Camous *et al.*, 1986). The embryo therefore reduces its dependence on maternally derived cytoplasmic components present in the oocyte at fertilisation, such as mRNA molecules (Gosden, 2002), ribosomes (Kedes *et al.*, 1969), mitochondria (Van Blerkom, 2004), and Golgi apparatus (Wang *et al.*, 2005). Blastomeres become progressively smaller during the cleavage stages and, at the 8 to 16-cell stage, they begin to flatten and increase cell-cell contacts, a process known as compaction (Pratt *et al.*, 1982). Compacted morulae then begin to accumulate fluid between blastomeres producing a cavity known as the blastocoel. At this stage, the embryo is known as a blastocyst and consists of two cell types, those of the outer layer known as the trophoblasts, that then form the placenta, and those of the inner embryo, known as the inner cell mass (ICM), that give rise to the foetal tissues (Gardner & Johnson, 1972).

On reaching the uterus approximately 7 days after fertilisation, the ZP is lost in a process known as hatching and the blastocyst is free to attempt implantation into the uterine wall. If the blastocyst successfully implants, it undergoes a process known as gastrulation, during which significant cell migration and morphological changes occur, resulting in the formation

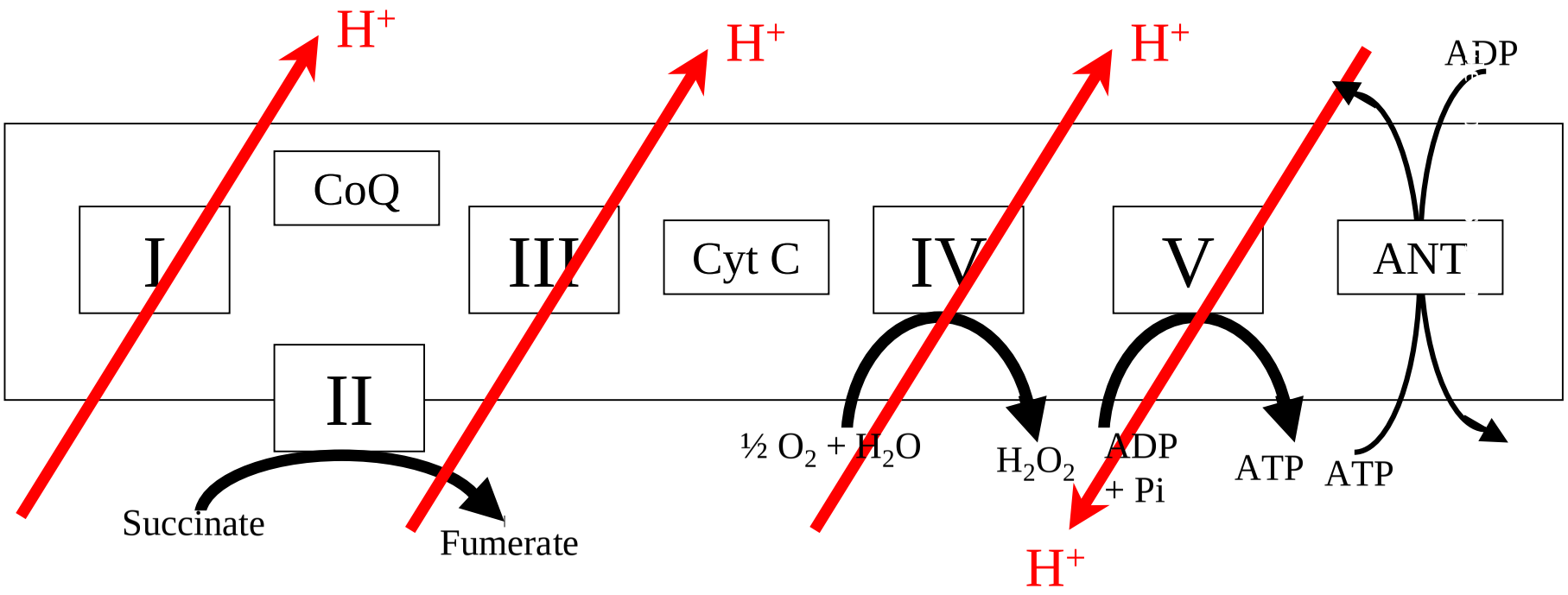
and correct localisation of cells of the endoderm, ectoderm and mesoderm layers (reviewed in Tam & Beddington, 1992). Cells within each of these layers then undergo further differentiation into the endoderm to form tissues and organs of the digestive tract (Grapin-Botton & Melton, 2000), the ectoderm, which develops into the skin and central nervous system (Tam & Zhou, 1996) and to the mesoderm that gives rise to the cardiovascular, respiratory and other internal systems (Tam & Beddington, 1987).

1.2 Mitochondria

Mitochondria are found in the cytoplasm of all eukaryotic cells and are essential to cellular function due to their ability to generate ATP, the cell's major source of energy. They comprise a double membrane, with the outer membrane separating the mitochondrial matrix from the cytoplasm of the cell whilst the inner membrane forms the cristae. Mitochondrial cristae are the site of the electron transfer chain (ETC), the final stage in cellular respiration where oxidative phosphorylation (OXPHOS) takes place (Sherratt, 1991). This aerobic process allows further metabolism of the products of anaerobic glycolysis and the citric acid cycle to produce carbon dioxide and water, with the subsequent release of 32 molecules of ATP. This is a far more economical return than provided by anaerobic respiration which generates only 2 molecules of ATP (Pfeiffer *et al.*, 2001). The ETC consists of 5 enzyme complexes, each containing multiple polypeptide subunits (see Figure 1.1). Electrons flow through the first four complexes of the ETC releasing protons from the mitochondrial matrix into the inter-membrane space. This creates the mitochondrial membrane potential. Re-entry of the protons into the mitochondrial matrix through the fifth complex results in ATP generation by ATP synthase.

Figure 1.1 (next page): The Electron Transfer Chain

The electron transfer chain (ETC) is the final stage in cellular respiration where oxidative phosphorylation (OXPHOS) takes place. It consists of 5 enzyme complexes which, with the exception of complex II, all contain polypeptides encoded by both the nuclear and mitochondrial genomes. Electrons flow through the first four complexes, releasing protons (H+) from the mitochondria and thereby setting up the mitochondrial membrane potential. Pumping these protons back into the mitochondria through the fifth complex results in ATP generation. CoQ: Coenzyme Q; Cyt C: Cytochrome C; ANT: Adenosine Nucleoside Transporter; ADP: Adenosine diphosphate; ATP: Adenosine Triphosphate.



Complex	I	II	III	IV	V
nDNA subunits	>18	4	8	10	10
mtDNA subunits	7	0	1	3	2

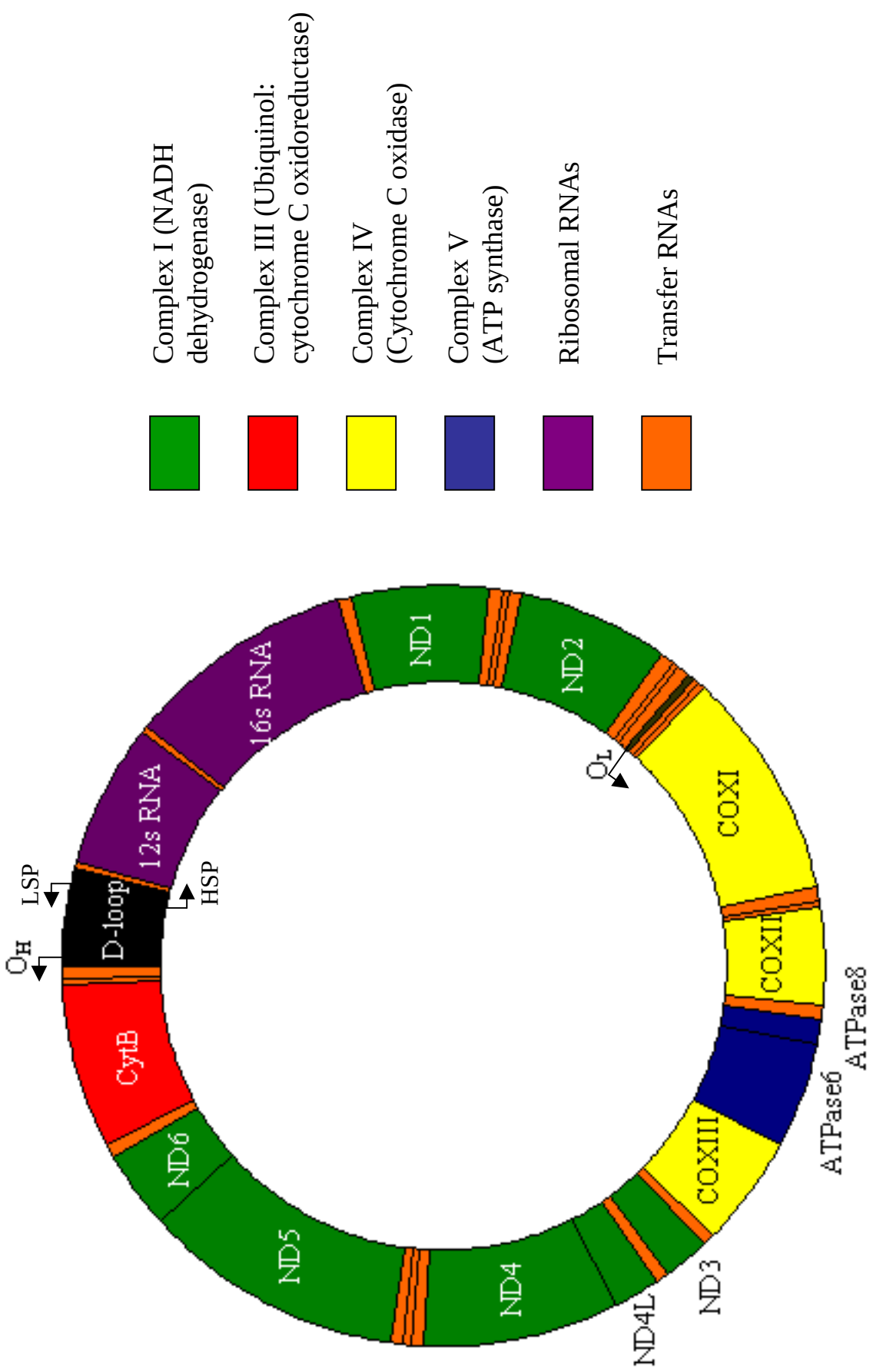
1.2.1 Mitochondrial DNA

Most of the components of the ETC are encoded by the nuclear genome and imported into the mitochondria. However, the ETC is unique in that some of its components are encoded by a genome found within the mitochondria themselves, mtDNA. All ETC components, and therefore both nuclear and mitochondrial genomes, are required for efficient ETC function and maximum capacity for production of cellular ATP (see Figure 1.1). The mitochondrial genome is a double stranded circular DNA molecule of approximately 16.5kb (see Figure 1.2), with the exact size varying between species (16569bp in humans (Anderson *et al.*, 1981), 16679bp in the pig (Ursing & Arnason, 1998) and 16295bp in the mouse (Bibb *et al.*, 1981)). Using a slightly different genetic code to that of nuclear DNA, mtDNA encodes 13 polypeptides involved in the ETC, 22 transfer (t) RNAs and 2 ribosomal (r) RNAs (Anderson *et al.*, 1981). The 13 peptides include seven subunits of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6; Complex I), one component of Complex III (cytochrome B), three subunits of cytochrome C oxidase (COXI, COXII and COXIII; Complex IV) and two subunits of the ATP synthase (ATPase 6 and ATPase 8; Complex V). All other components of the ETC are encoded by the nuclear genome (see Figure 1.1). The mitochondrial genome is highly compact, with no introns contained between the coding regions (Anderson *et al.*, 1981) and some of the coding regions even overlap (For instance, ATPase 6 and ATPase 8, ND4 and ND4L; see Anderson *et al.*, 1981). Others lack termination codons, with these sequences being provided by post-transcriptional polyadenylation, whereby the first adenine base of the poly-A tail forms the third base of a termination codon (Ojala *et al.*, 1981). The only non-coding region of the genome is the displacement loop (D-loop), containing two conserved sequence blocks (CSB), the transcription promoter regions (Light strand promoter, LSP; Heavy strand promoter, HSP) and the origin of heavy strand replication (O_H).

Figure 1.2 (next page): Mammalian mtDNA

MtDNA is a double stranded circular molecule of approximately 16.5kb, consisting of a heavy (H) and a light (L) strand. The non-coding D-loop region contains the origin of heavy strand replication (O_H) and the transcription promoters for both strands (LSP, HSP), whilst the origin of light strand replication (O_L) is found approximately two thirds of the way around the genome. The remainder of the genome encodes 13 protein subunits of the electron transport chain, 12 on the heavy strand and 1 on the light strand, and also 22 tRNAs and 2 rRNAs that are necessary for mtDNA transcription and protein synthesis.

ND1, ND2, ND3, ND4, ND4L, ND5 and ND6: subunits of NADH dehydrogenase; COXI, COXII and COXIII: subunits of cytochrome C oxidase; CytB: cytochrome B; ATPase 6 and ATPase 8: subunits of ATP synthase.


Complex I (NADH dehydrogenase)
Complex III (Ubiquinol: cytochrome C oxidoreductase)
Complex IV (Cytochrome C oxidase)
Complex V (ATP synthase)
Ribosomal RNAs
Transfer RNAs
ND1
ND2
COXI
COXII
ATPase8
ATPase6
COXIII
ND3
ND4L
ND4
ND5
ND6
CytB
D-loop
12s RNA
16s RNA
LSP
HSP
OH
OL

1.2.2 MtDNA transcription and replication

Transcription of mtDNA occurs following interaction between nuclear-encoded regulatory proteins and regions within the D-loop of mtDNA (see Figure 1.3). Requirements for transcription include the mitochondrial RNA polymerase (mtRNAPol; Tiranti *et al.*, 1997), mitochondrial transcription factor A (TFAM; Fisher & Clayton, 1985; 1988), and one of transcription factor B1 (TFB1M) or B2 (TFB2M; Falkenberg *et al.*, 2002); see Table 1.1). TFAM binds to a region 10 to 40bp upstream of the promoter region within the D-loop, which separates the two strands of DNA, allowing access for mtRNAPol and TFB1M or TFB2M for initiation of transcription. Once initiated, transcription generates a polycistronic precursor RNA transcript, allowing co-ordinated transcription of all genes on the same strand. Excision of the polycistronic precursor by endonucleases produces precursor rRNAs and tRNAs which are then processed further to allow translation of the precursor mRNAs (Ojala *et al.*, 1981).

Replication of mtDNA is also dependent on nuclear-encoded regulatory proteins (see Table 1.1), in particular, the mtDNA-specific Polymerase Gamma (PolG; (Hubscher *et al.*, 1979). PolG consists of two subunits, the catalytic subunit (PolGA), responsible for elongation of the daughter DNA strands, and the accessory subunit (PolGB), responsible for primer recognition and proof reading activity (Gray & Wong, 1992). The replication process begins with light strand transcription from the LSP region to the second CSB within the D-loop region, producing an RNA sequence complementary to the light strand DNA (Xu & Clayton, 1995). This is then cleaved from the remainder of the light strand transcript by the mitochondrial RNA processing endoribonuclease (RNase MRP; Shadel & Clayton, 1997), allowing it to form a triple stranded RNA-DNA hybrid. PolGB recruits the RNA component of this hybrid as a primer for mtDNA replication by PolGA (Lee & Clayton, 1996).

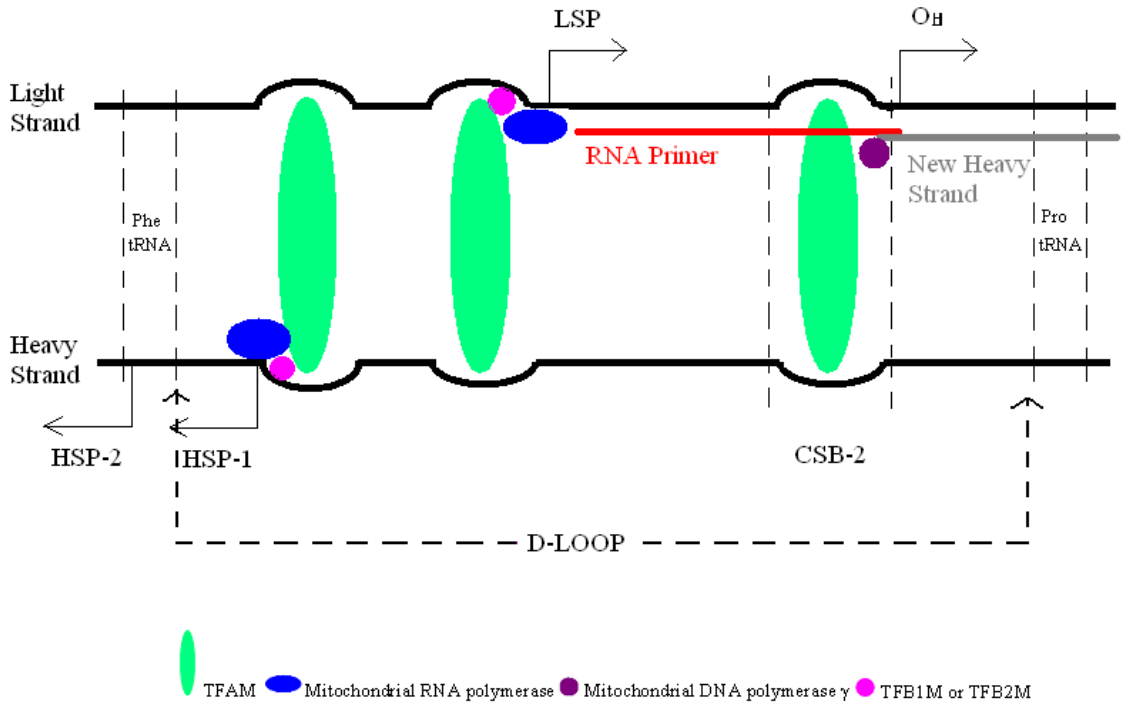


Figure 1.3: MtDNA transcription and replication origins in the D-loop

TFAM binds and separates the two DNA strands. The accessory transcription factor, either TFB1M or TFB2M allows mtRNAPol to bind specifically to the DNA and therefore produce the transcripts of the light strand mtDNA and the RNA primer for mtDNA replication by PolG (Lee & Clayton, 1996).

TFAM: Mitochondrial transcription factor A; TFB1M/TFB2M: Mitochondrial transcription factor B1 or B2; mtRNAPol: Mitochondrial RNA polymerase; PolG: Mitochondrial DNA Polymerase γ

HSP: Heavy strand promoter; LSP: Light strand promoter; CSB: Conserved sequence block; O_H: Origin of heavy strand replication; D-loop: Displacement loop; the non coding region that is the binding site for nuclear encoded transcription and replication factors, Phe tRNA: Phenylalanine transfer RNA; Pro tRNA: Proline transfer RNA.

Protein	Binding Site	Role	Reference
TFAM	Enhancer region	Causes unwinding of mtDNA, exposing the binding site for mtRNA polymerase	Fisher & Clayton, 1985; 1988
TFB1M or TFB2M	RNA Polymerase	Form heterodimer with RNA Polymerase allowing specific transcription initiation	Falkenberg *et al*., 2002
mtRNAPol	Transcription initiation region	Produces RNA copy of mtDNA sequence	Tiranti *et al*., 1997
PolG	RNA-DNA hybrid	Produces replicate copy of mtDNA	Hubscher *et al*., 1979
mtSSB	Single stranded mtDNA	Prevents re-annealing of single stranded mtDNA allowing PolG polymerisation	Farr *et al.*, 1999
mTERF	RNA Polymerase	Termination of transcription	Daga *et al.*, 1993
RNase MRP	MtDNA primary light strand transcript	Processing of primary light strand transcripts to produce RNA primer for replication	Shadel & Clayton, 1997

Table 1.1: Major nuclear-encoded regulatory factors required for mtDNA transcription and replication.

TFAM: Mitochondrial transcription factor A; TFB1M/TFB2M: Mitochondrial transcription factor B1 or B2; mtRNAPol: Mitochondrial RNA polymerase; PolG: Mitochondrial DNA Polymerase γ; mtSSB: mitochondrial single stranded binding protein; mTERF: Mitochondrial transcription termination factor; RNase MRP: Mitochondrial RNA processing endoribonuclease.

Two mechanisms of mtDNA replication have been proposed, the asynchronous-displacement mechanism (Clayton, 1982) and the coupled leading and lagging strand mechanism (Holt *et al.*, 2000). The asynchronous-displacement mechanism describes the binding of PolG to the transcribed RNA primer, thereby allowing heavy strand replication to begin from O_H in the D-loop (see Figure 1.2). After replication of approximately two thirds of the genome, the formation of the new heavy strand exposes the origin of light strand replication (O_L) on the parent heavy strand. Light strand replication is then able to commence in the opposite direction (Clayton, 1982). The more recently proposed coupled leading and lagging strand mechanism describes the replication of both mtDNA strands at the same time from the same replication origin in the D-loop. It has been hypothesised that the choice of replication mechanism may be influenced by variations in TFAM or PolGB binding or the number of dNTPs present within mitochondria in different cell types and that the asynchronous-displacement mechanism was responsible for the maintenance of mtDNA, whilst the coupled leading and lagging strand mechanism occurs primarily when mtDNA amplification is required (Holt *et al.*, 2000). More recent analysis of mtDNA replication intermediates has indicated that the majority of or even all mtDNA replication occurs via the coupled leading and lagging strand mechanism (Yang *et al.*, 2002). It has since been argued that some of the reported replication intermediates are actually mtDNA molecules undergoing transcription, indicating that these data actually support rather than discount the asynchronous-displacement mechanism of mtDNA replication (Bogenhagen & Clayton, 2003). However, further evidence for the coupled leading and lagging strand mechanism has arisen from recent reports of bi-directional replication of mtDNA in both mammals (Bowmaker *et al.*, 2003) and birds (Reyes *et al.*, 2005).

1.2.3 Regulation of mtDNA copy number

Replication of mtDNA is not linked to the cell cycle, but is dependent on cellular requirement for mtDNA synthesis (Bogenhagen & Clayton, 1976) and could be initiated to replenish damaged or degraded molecules or to increase the number of mtDNA copies within a particular cell (Moyes *et al.*, 1998). Despite its small size in comparison to the nuclear genome, the host cell depends on the mitochondrial genome for aerobic respiration as much as the mitochondria rely on proteins encoded by the host cell's nuclear DNA. In fact, as much as one third of the total DNA content of an oocyte consists of mtDNA (Piko & Matsumoto, 1976). Somatic cells usually contain between 10^3 and 10^4 copies of mtDNA, with 2 to 10 copies per organelle (Satoh & Kuroiwa, 1991), correlating to each particular cell's requirement for OXPHOS. For example, it has been reported that human subcutaneous fat cells contain more mtDNA copies than peripheral blood mononuclear cells (Gahan *et al.*, 2001) and that liver tissue contains more mtDNA than various muscle tissues (Wiesner *et al.*, 1992). Replication of mtDNA and ETC activity can be influenced by the expression of nuclear-encoded transcription and replication factors, including TFAM, PolG and the nuclear respiratory factors (NRF).

1.2.3.1 TFAM

TFAM plays an important role in both transcription and replication of the mitochondrial genome. In this respect, increased levels of TFAM within mitochondria can induce increased mtDNA transcription (Gensler *et al.*, 2001) and replication (Ekstrand *et al.*, 2004). It is therefore not surprising that low levels of TFAM have been associated with a variety of mitochondrial depletion syndromes such as infantile mitochondrial myopathy (Poulton *et al.*, 1994), familial mtDNA-associated liver disease (Spelbrink *et al.*, 1998), fatal childhood myopathy (Larsson *et al.*, 1994), skeletal muscle and mitochondrial encephalomyopathy

disorders (Siciliano *et al.*, 2000) and ocular myopathy, exercise intolerance and muscle wasting (Tessa *et al.*, 2000). Furthermore, there is a decrease in TFAM expression localised to the mitochondria during spermatogenesis in both the human (Larsson *et al.*, 1997) and the mouse (Larsson *et al.*, 1996). This has been proposed as a functional mechanism for specifically reducing mtDNA copy number in maturing sperm and therefore paternal mtDNA transmission to future generations. It arises at the late spermatocyte/early spermatid stage when an isoform of TFAM is expressed that lacks the mitochondrial targeting sequence.

Interestingly, the reduction in mtDNA copy number resulting from reduced TFAM levels is not mirrored by increased mtDNA copy number following overexpression of TFAM in cultured HEK cells (Maniura-Weber *et al.*, 2004). This is consistent with a role for TFAM in the initiation rather than maintenance of mtDNA replication, perhaps through its role in transcription, which is necessary to generate the RNA primer for mtDNA replication. This also indicates that low levels of TFAM may be sufficient for mtDNA replication to take place. Indeed, it has been reported that low levels of TFAM expression may even be beneficial when mtDNA copy number is low (Seidel-Rogol & Shadel, 2002). This is due to its proposed additional role in the packaging of mtDNA (Alam *et al.*, 2003). Low levels of TFAM could therefore increase the accessibility of mtDNA to mtDNA replication factors (Seidel-Rogol & Shadel, 2002). However, overproduction of TFAM does not decrease but actually increases mtDNA replication in knock-in mice (Ekstrand *et al.*, 2004). The mechanism of mtDNA copy number regulation by TFAM is therefore unclear, although its importance as an essential regulator of mtDNA copy number has been clearly demonstrated through the production of knock-out mice (Larsson *et al.*, 1998). Heterozygous knock-out mice demonstrated reduced mtDNA copy numbers and cardiac respiratory chain deficiency, whilst homozygous knock-out

mice did not develop beyond embryonic day (E) 10.5 due to severe mtDNA depletion and absence of ETC function.

1.2.3.2 PolG

Like TFAM, PolG expression may not be directly linked to mtDNA copy number. For example, overexpression of PolGA in a human cell line with a partial chromosome duplication did not result in increased mtDNA copy number (Schultz *et al.*, 1998). Furthermore, heterozygous PolGA knock-out mice contain similar mtDNA copy number levels to wild type mice, despite a 50% reduction in PolGA transcripts (Hance *et al.*, 2005). However, homozygous knock-out mice do not develop beyond E 8.5 (Hance *et al.*, 2005). Furthermore, knock-in mice expressing PolGA proteins that lack the proof-reading function contain mutated mtDNA molecules and age prematurely (Trifunovic *et al.*, 2004). These data suggest that the fully functional PolG protein is required for mtDNA copy number to be regulated successfully. This would include the accessory subunit, PolGB, mutated forms of which result in reduced DNA binding affinity and reduced processivity (Fan *et al.*, 2006). Mutation to the PolGA gene and the subsequent failure of PolGA to successfully regulate mtDNA copy number can result in mtDNA depletion syndromes such as chronic progressive external ophthalmoplegia (CPEO; Graziewicz *et al.*, 2004; Van Goethem *et al.*, 2001) and Alpers syndrome (Naviaux *et al.*, 1999). Similarly, loss of function of PolGB can also result in such syndromes. For example, CPEO can result from a mutation in the PolGB gene itself (Longley *et al.*, 2006) or from a mutation in the PolGA gene that prevents interaction with PolGB (Chan *et al.*, 2005). This mtDNA depletion effect is also mimicked by nucleoside antiretroviral analogues such as 3'-azido-3'-deoxythymidine (AZT) and 2'3'-dideoxycytidine (ddC), which can inhibit the action of PolG (Dalakas *et al.*, 2001). For example, men treated

with these drugs for 12 months had increased numbers of deleted mtDNA molecules in their sperm samples (White *et al.*, 2001).

1.2.3.3 Nuclear respiratory factors

As ETC subunits are encoded by the nuclear genome as well as mtDNA, coordinated expression of genes encoded by both genomes is required for the assembly of complete and functional ETCs. NRF1 and NRF2 have been implicated in this role. For example, NRF1 and NRF2 binding sites have been found in the promoter regions of the TFAM (Virbasius & Scarpulla, 1994), TFB1M and TFB2M (Gleyzer *et al.*, 2005) genes that are responsible for mitochondrial gene expression. NRF2 also directly initiates expression of all nuclear-encoded COX genes in rat neurons (Ongwijitwat & Wong-Riley, 2005) and NRF1 activation can directly induce cytochrome C activity (Herzig *et al.*, 2000). The requirement of NRF1 for OXPHOS function is further highlighted by the death of homozygous NRF1 knock-out mice between E 3.5 and 6.5 (Huo & Scarpulla, 2001), which is considerably earlier than TFAM and PolG homozygous knock-out mice. Strict regulation of OXPHOS function is therefore vital for embryo survival, likely due to the very different metabolic requirements of fully differentiated gametes and pluripotent early embryonic blastomeres.

1.3 Mitochondria and embryogenesis

1.3.1 MtDNA replication

Replication of mtDNA, at least in the mouse, does not occur during early pre-implantation embryo development (Piko & Taylor, 1987). However, there is one report of a small window of replication at the 2-cell stage (McConnell & Petrie, 2004). The mtDNA molecules present in the mature oocyte at fertilisation are therefore largely relied upon to provide sufficient ETC components to allow OXPHOS activity during the pre-implantation phase of embryonic development. In order to meet this demand, mtDNA is amplified during oocyte maturation within the ovary, with a 45-fold increase being reported in cattle from the PGC stage to pre-ovulating oocytes (Smith & Alcivar, 1993), resulting in a mean of 260000 copies being present in mature bovine oocytes (Michaels *et al.*, 1982). Similar amplifications have been reported in other species. For example, human premigratory PGCs possess approximately 10 mitochondria per cell (Jansen & de Boer, 1998). Mature murine oocytes contain mean mtDNA copy number values between 114000 ± 7000 (Piko & Taylor, 1987) and 159000 (range: 52920-338600; Steuerwald *et al.*, 2000). In the human, copy number ranges from 10000 to 700000 (Almeida-Santos *et al.*, 2006), with other reported mean values of 314000 (range: 90680-662300; Steuerwald *et al.*, 2000) and 193000 (range: 20000-598000; Reynier *et al.*, 2001).

Oocytes with higher copy numbers of mtDNA are known to be associated with improved fertilisation rates. For example, human oocytes failing to fertilise due to poor sperm quality, and therefore assumed to be of good quality, had significantly higher mtDNA copy number than poor quality oocytes which were fertilised by good quality sperm (mean 255000 versus 152000 copies; Reynier *et al.*, 2001). Furthermore, low quality oocytes from patients with a

variety of ovarian disorders contained an average of 100000 mtDNA molecules compared to an average of 256000 copies in oocytes from healthy individuals (May-Panloup *et al.*, 2005a). Similar results have also been reported recently in porcine (El Shourbagy, 2004) and human (Almeida-Santos *et al.*, 2006) oocytes. However, significantly different copy numbers were also observed between arrested 2-cell human embryos and those that developed further (Almeida-Santos *et al.*, 2006). A putative threshold number of mtDNA molecules has been proposed for the mature unfertilised oocyte to allow embryo development to take place after fertilisation. In the mouse, this has been predicted at approximately 100000 copies per oocyte (Piko & Taylor, 1987), with a similar value being appropriate for the pig (El Shourbagy, 2004).

The number of mtDNA copies per organelle is indicative of the number of mitochondria in an oocyte. For example, in the mouse, each mitochondrion has just one copy (Piko & Taylor, 1987). Consequently, the minimum number of mtDNA copies would indicate the minimum number of mitochondria required for fertilisation to take place. These mitochondria would support embryonic development to cavitation, after which glycolysis becomes increasingly important in many species (Human: (Leese *et al.*, 1993); Pig: (Sturmey & Leese, 2003); Cow: (Thompson *et al.*, 1996); Rat: (Brison & Leese, 1991); Mouse: (Houghton *et al.*, 1996)). Furthermore, this minimum number may be essential as it has been hypothesised that a threshold level of ATP is required to energise cell division (Brenner *et al.*, 2000) and may also synchronise mitochondrial dynamics (Van Blerkom *et al.*, 2000). The distribution of mitochondrial networks throughout the cell varies with different stages of the cell cycle (Margineantu *et al.*, 2002). Mature and immature oocytes also have distinct patterns of mitochondrial distribution (Nishi *et al.*, 2003; Stojkovic *et al.*, 2001). Consequently, those

oocytes with mtDNA copy number lower than the required threshold may be unable to form the networks required for developmental competence. In the pig, competent BCB+ oocytes contain more copies of mtDNA and are more likely to fertilise than incompetent BCB- oocytes (El Shourbagy, 2004). However, improved fertilisation outcome was achieved following injection of BCB- oocytes with supplementary mitochondria (El Shourbagy, 2004). Developmental competence therefore appears to be related to the numbers of mitochondria present (El Shourbagy, 2004).

1.3.2 Mitochondrial activity

In sea urchin oocytes, mitochondria act as calcium sinks (Eisen & Reynolds, 1985). The Ca^{2+} wave pacemaker that is necessary for completion of meiosis at fertilisation is maintained by mitochondrial uptake of Ca^{2+} (Dumollard *et al.*, 2003). Failure to maintain the Ca^{2+} wave pacemaker results in apoptotic cell death of the oocyte (Liu *et al.*, 2001). Therefore, oocytes with low levels of ATP production due to low levels of mitochondria and mtDNA may be unable to maintain the Ca^{2+} wave pacemaker and complete meiosis, and instead, undergo apoptosis. It has been hypothesised that oocytes without sufficient wild type mtDNA and therefore the capacity to generate ATP would not normally be ovulated (St. John, 2002). This is reflected in the low numbers of mtDNA observed in degenerate oocytes obtained through superovulation protocols (mean = 44,629 ± 40,729; Almeida-Santos *et al.*, 2006). Consequently, the use of such superovulation protocols during *in vitro* fertilisation (IVF) allows these low quality oocytes to undergo the maturation process and the effects of insufficient mtDNA molecules to be observed (St. John, 2002).

Mitochondrial morphology changes after oocyte maturation to the inactive state (Bavister & Squirrell, 2000). This may be due to the lack of mtDNA replication and the import of nuclear-encoded regulatory factors from the cytoplasm. However, OXPHOS is still functional during early embryo development and mitochondria are dynamic organelles, moving to provide ATP where it is most needed (Stojkovic *et al.*, 2001). Staining of mitochondria has shown that during embryo development, they cluster around the nucleus of blastomeres during the cleavage stages, providing the energy required for cell division processes such as spindle formation (Stojkovic *et al.*, 2001; Sun *et al.*, 2001; Wilding *et al.*, 2001). When cell division is not taking place, they tend to spread throughout the cytoplasm of the cell (Stojkovic *et al.*, 2001; Sun *et al.*, 2001; Wilding *et al.*, 2001). Clustering around the nuclear material during cell division would increase the likelihood of equal mitochondrial segregation following cytokinesis. Equal segregation of mitochondria following cell division would subsequently reduce the possibility of the resulting tissues of the offspring lacking the ability to produce sufficient ATP through OXPHOS. However, disproportionate numbers of mitochondria and/or mtDNA molecules amongst blastomeres has been reported in the pig (El Shourbagy, 2004) and human (Lin *et al.*, 2004; Van Blerkom *et al.*, 2000). This may result in some blastomeres with reduced ATP-generating capacity. If this occurs early on in development, blastomeres may fragment, resulting in embryo arrest or less competent blastocysts with fewer cell numbers.

In the human, TEM studies indicate that mitochondria become less dense at the 8-cell stage, indicating initiation of mtDNA transcription (Sathananthan & Trounson, 2000). This is perhaps in preparation the onset of mtDNA replication (Piko & Taylor, 1987) and the increased requirement for OXPHOS (Trimarchi *et al.*, 2000) at the blastocyst stage. Increased

use of glycolysis during early embryo development may allow cells that are no longer able to generate sufficient ATP through OXPHOS to survive for long enough to enable production and import of nuclear-encoded regulatory factors necessary for the onset of mtDNA replication post-implantation (El Shourbagy, 2004). Indeed, studies in pigs using OXPHOS inhibitors have indicated that inhibition of OXPHOS function at compaction can improve embryo viability (Machaty *et al.*, 2001). This hypothesis is supported by reports of glycolysis rather than OXPHOS metabolism occurring in other cell types with a functioning nuclear genome but low mtDNA copy number, including somatic cells being depleted of their mtDNA (King & Attardi, 1989). However, ATP production through glycolysis is far less efficient than through OXPHOS. It is therefore vital that sufficient mtDNA molecules are present at fertilisation to maintain embryo survival until mtDNA replication resumes.

1.3.3 Maternal mtDNA inheritance

Unlike nuclear DNA, mtDNA does not follow the Mendelian pattern of inheritance, where one allele is inherited from each parent nucleus. Instead, mtDNA molecules are maternally inherited through the oocyte cytoplasm (Giles *et al.*, 1980). Due to its size, a mature oocyte can contain far more mitochondria in its cytoplasm than the fertilising sperm cell. Coupled with replication of the mitochondrial genome during oocyte maturation in the ovary, this results in an oocyte to sperm mtDNA ratio of at least 1000:1 (Ankel-Simons & Cummins, 1996), with sperm mtDNA contributing approximately only 100 (Hecht & Liem, 1984) or even just 1 (May-Panloup *et al.*, 2003) mtDNA molecule(s) at fertilisation.

MtDNA is usually homoplasmically transmitted, i.e. only one mtDNA genotype being present (Monnat *et al.*, 1985). Occasionally, two or more mtDNA genotypes can be present, often due

to either a pathological or non-pathological rearrangement, resulting in a state of heteroplasmy, as in the oocytes of women harbouring an mtDNA disease (Blok *et al.*, 1997). MtDNA has a much higher mutation rate than nuclear DNA (Wallace *et al.*, 1987). This may be partially explained by the production of mutagenic free radicals within the mitochondria and by limited mtDNA repair mechanisms (Yakes & Van Houten, 1997). Furthermore, mtDNA is not stabilised by histones as is the case for nuclear DNA, although TFAM is now thought to be involved in mtDNA packaging (Alam *et al.*, 2003). Despite the high mutation rate, mtDNA has been highly conserved throughout evolution and when it arises, mtDNA heteroplasmy tends to revert to the homoplasmic, although not necessarily wild type, state, sometimes within one generation (Koehler *et al.*, 1991). This is thought to be due to the segregation process that would result from the proposed absence of mtDNA replication during pre-implantation embryogenesis (Piko & Taylor, 1987).

MtDNA replication does not occur in synchrony with nuclear DNA replication and therefore some mtDNA genomes are not replicated during one cell cycle whilst others are copied many times (Sazer & Sherwood, 1990). It has been reported that there is preferential replication of those mtDNA molecules that are closer to the nucleus (Davis & Clayton, 1996), perhaps due to faster transport of nuclear-encoded regulatory factors. The introduction of exogenous mtDNA into zygotes either close to the nucleus or at the periphery influences the amount of exogenous mtDNA observed at the blastocyst stage, with higher proportions arising from karyoplast injections than from injections at the periphery of the zygote (Meirelles & Smith, 1998). Consequently, the position of mitochondria in the early embryo may play a role in determining which mtDNA genomes are selected for amplification.

Contradictory data have recently shown that mtDNA replication is not just restricted to mitochondria in close proximity to the nucleus (Magnusson *et al.*, 2003). Here, it was demonstrated that all components of the replication machinery were available in sufficient quantities throughout the cell, allowing replication to take place throughout the entire cytoplasm. This included PolG and TFAM. These authors also suggested that mitochondria were more dense around the nuclei than at the periphery of the cell, providing an explanation for the preferential labelling of replicating mtDNA near to cell nuclei that was reported previously (Davis & Clayton, 1996). There is currently no evidence for selection against pathogenic mtDNA mutations in oogenesis or early embryonic development (reviewed by (Shoubridge, 2000). It is therefore likely that the selection of mtDNA template molecules for replication is random and is not limited to a particular mtDNA sequence or to a particular part of the cell's cytoplasm.

When mtDNA replication resumes following implantation (Piko & Taylor, 1987), each cell type of the developing embryo contains a few copies of mtDNA, as indicated by the few mitochondria present in undifferentiated ESCs (Sathananthan *et al.*, 2002; St. John *et al.*, 2005b). Consequently, the combination of segregation followed by amplification of selected templates produces an mtDNA purification process, known as the bottleneck (Hauswirth & Laipis, 1982), which is important for the maintenance of mtDNA homoplasmy. If blastomeres inherit mutated mtDNA or are depleted of mitochondrial genomes, the resulting adult tissues would be deficient in OXPHOS capacity, which could result in the onset of mitochondrial-type disease (Wallace, 1992).

1.3.3.1 Maternally inherited mtDNA disease

There are many diseases caused by mutations in mtDNA and symptoms of these diseases generally appear in tissues highly dependent on OXPHOS (Wallace, 1992). These include Leber's Hereditary Optic Neuropathy (LHON), which can result from mutations in the gene for the NADH-Q oxidoreductase component of Complexes I and III (Brown *et al.*, 1992). Myoclonic Epilepsy with Ragged Red Fibres (MERRF) is also transmitted by maternal inheritance and results in myoclonus, epilepsy and ataxia. This can result from mutations in mitochondrially-encoded tRNA genes (Hanna *et al.*, 1995; Mancuso *et al.*, 2004; Nakamura *et al.*, 1995; Nishigaki *et al.*, 2003) and protein genes (Naini *et al.*, 2005). As well as point mutations, some mitochondrial diseases are caused by large-scale deletions to the genome. The most well known in this group is Kearns-Sayre Syndrome, which includes symptoms of progressive external ophthalmoplegia, pigment retinopathy and disorders of cardiac conduction (De Block *et al.*, 2004; Klopstock *et al.*, 1995; Lertrit *et al.*, 1999). The clinical phenotypes resulting from mtDNA mutations are dependent on the proportion of mutated mtDNAs (Wallace, 1992). In the case of LHON, >60% mutant mtDNA load is required before the disease phenotype presents (Chinnery *et al.*, 2001). In some mitochondrial diseases, including a case of MERRF, over 85% mutant mtDNAs need to be present before symptoms are observed (Boulet *et al.*, 1992).

1.3.4 Paternal mtDNA inheritance

There is increasing evidence demonstrating that maternal only mtDNA transmission is not merely due to dilution of the paternal mitochondria beyond the level of detection. It has been suggested that paternal mtDNA is actively eliminated by ubiquitination in the oocyte cytoplasm and subsequent proteolysis during embryonic development (Nishimura *et al.*, 2006;

Sutovsky *et al.*, 1999). The elimination hypothesis is supported by reports demonstrating reduced paternal mtDNA content as embryonic development progresses (Cummins *et al.*, 1997). Paternal mtDNA was detected only as far as the pronucleus stage following intra-specific murine crosses, compared to detection in neonates following inter-specific murine crosses (Kaneda *et al.*, 1995). This suggests that the elimination mechanism is species specific. However, the elimination mechanism is not only species specific but also tissue specific, as donor sperm mtDNA was eliminated whilst exogenous liver mtDNA injected into the oocyte persisted at least until the neonatal period (Shitara *et al.*, 2000).

The destruction of paternal mtDNA (Sutovsky, 2003), and the presence of the genetic bottleneck should usually result in homoplasmic individuals. Recently, the accepted theory of uniparental mtDNA inheritance has been questioned. This is especially so in the case of a male patient presenting with a mitochondrial myopathy who was found to have inherited his muscle mtDNA from his father. However, the 2bp deletion giving rise to his myopathy was *de novo* (Schwartz & Vissing, 2002). This outcome is further supported by evidence of sperm mtDNA persistence in human polyploid IVF-generated blastocysts (St. John *et al.*, 2000). Paternal leakage of mtDNA has also been reported following inter-specific crosses in fruit flies (Kondo *et al.*, 1990), mice (Gyllensten *et al.*, 1991), honeybees (Meusel & Moritz, 1993), birds (Kvist *et al.*, 2003), sheep (Zhao *et al.*, 2004) and rhesus monkeys (St. John & Schatten, 2004), and following intra-specific crosses in mussels (Zouros *et al.*, 1992) and fruit flies (Kondo *et al.*, 1992). The ubiquitin-related paternal mtDNA destruction process has therefore proven ineffective in these cases. It is as yet unknown how paternal mtDNA might escape the destruction process, and how paternal mtDNA might be preferentially replicated in a particular tissue to such an extent that it resulted in a mitochondrial disease (Schwartz & Vissing, 2002).

In this latter instance, a considerable selective advantage must have taken place to out-compete the maternal source of mtDNA in the affected tissue.

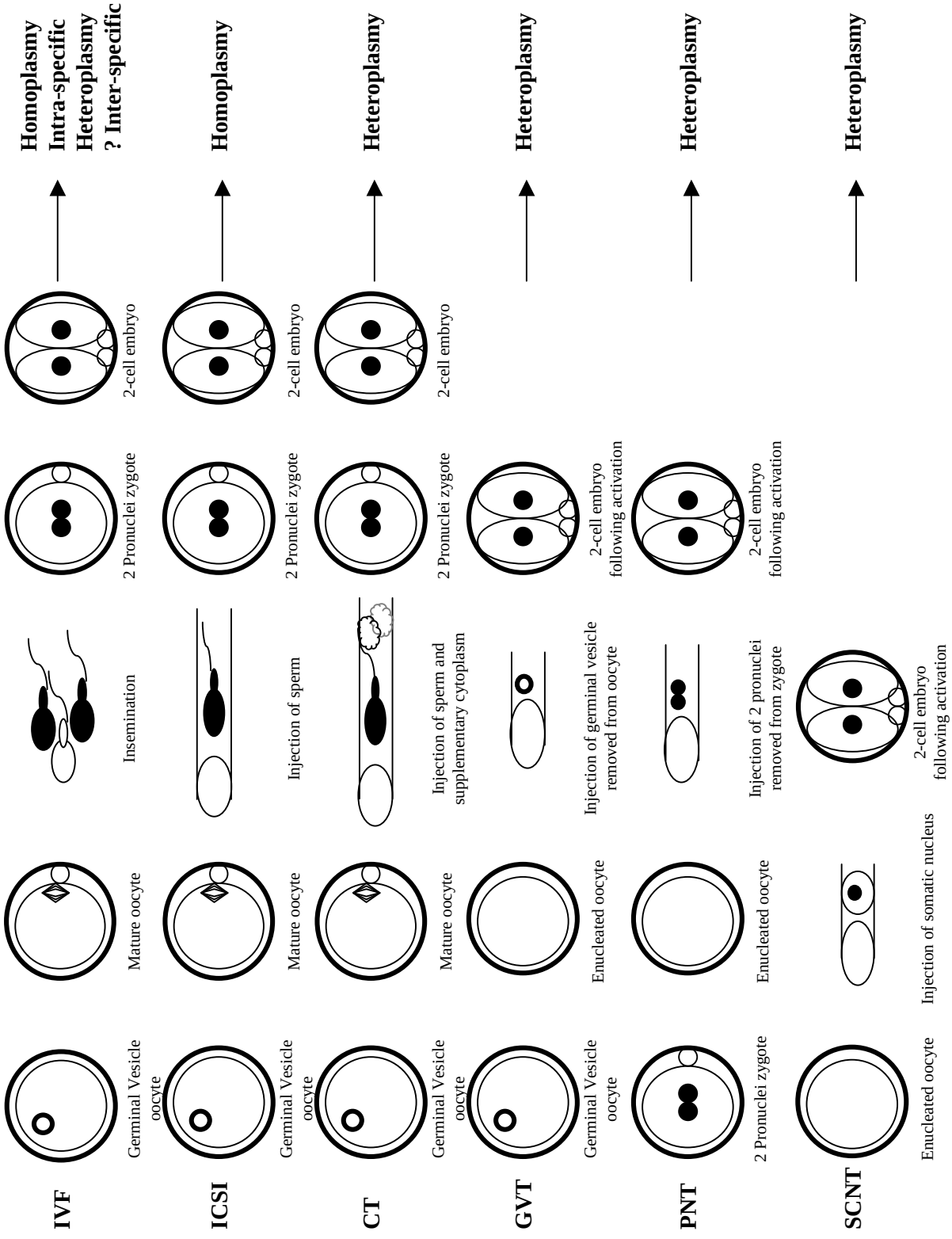
A possible mechanism for bypassing the paternal mtDNA destruction process is the fusion of parental mitochondria followed by recombination of the two genomes. Recombination of mammalian mtDNA was originally thought not to arise, although it is known to occur in yeast. However, more recent evidence suggests it may also occur in mammals. For example, Ono and colleagues created two respiration-deficient cell lines from patients with mitochondrial diseases caused by different mutations (Ono *et al.*, 2001). Fusion of the two cell lines resulted in morphology and respiratory enzyme activity returning to normal within 10 to 14 days. This outcome was proposed to have arisen from mtDNA recombination and genome compensation. Mammalian mtDNA recombination has since been confirmed in human cells using PCR based techniques (D'Aurelio *et al.*, 2004), and recent sequence analysis of invertebrate and vertebrate species, including primates (Tsaousis *et al.*, 2005). It was suggested that recombination may have been previously missed due to the mtDNA bottleneck process prohibiting the transmission of new mtDNA haplotypes to future generations (Tsaousis *et al.*, 2005). Recombination of sperm and oocyte mtDNA is now thought to explain the presence of the 2bp deletion in the male myopathy patient (Kraytsberg *et al.*, 2004).

1.4 Invasive oocyte reconstruction protocols

The introduction of more advanced ART techniques and their current use in the clinical setting and in animal production further confounds the unimaternal transmission of mtDNA. These technologies include: i) Intracytoplasmic Sperm Injection (ICSI) which is used clinically; ii) Cytoplasmic Transfer (CT) which has been used clinically but is now, for example, banned in the UK and USA; and iii) GV Transfer (GVT), Pronuclear Transfer (PNT), and NT, which have not been used to treat humans (see Figure 1.4). These more invasive ART protocols present further opportunities for the violation of the normal pattern of mtDNA inheritance. If uniparental mtDNA inheritance is not maintained following these techniques, offspring may harbour various forms of mtDNA heteroplasmy. Indeed, the introduction of these new technologies results in heteroplasmy being redefined. In this instance, either a wild type molecule can co-exist with a rearranged molecule or two wild type molecules may persist in the reconstructed oocyte.

Figure 1.4 (next page): Assisted reproductive techniques

Predicted outcomes associated with mtDNA transmission following *in vitro* fertilisation (IVF), intracytoplasmic sperm injection (ICSI), cytoplasmic transfer (CT), germinal vesicle transfer (GVT), pronuclei transfer (PNT) and somatic cell nuclear transfer (SCNT).


IVF
Germinal Vesicle oocyte
Mature oocyte
Insemination
2 Pronuclei zygote
2-cell embryo
Homoplasmy
Intra-specific
Heteroplasmy
? Inter-specific
ICSI
Germinal Vesicle oocyte
Mature oocyte
Injection of sperm
2 Pronuclei zygote
2-cell embryo
Homoplasmy
CT
Germinal Vesicle oocyte
Mature oocyte
Injection of sperm and supplementary cytoplasm
2 Pronuclei zygote
2-cell embryo
Heteroplasmy
GVT
Germinal Vesicle oocyte
Enucleated oocyte
Injection of germinal vesicle removed from oocyte
2-cell embryo following activation
Heteroplasmy
PNT
2 Pronuclei zygote
Enucleated oocyte
Injection of 2 pronuclei removed from zygote
2-cell embryo following activation
Heteroplasmy
SCNT
Enucleated oocyte
Injection of somatic nucleus
2-cell embryo following activation
Heteroplasmy

1.4.1 Intracytoplasmic Sperm Injection

Unlike IVF, the ICSI procedure varies dramatically from the natural fertilisation process. For example, the intact, non-capacitated, non-acrosome reacted sperm is injected into the oocyte. There are also delays and alterations in post-fertilisation processes following ICSI, including male pronucleus formation (Luetjens *et al.*, 1999) and the calcium oscillations essential for the activation process (Ludwig *et al.*, 2001). This delayed response after sperm entry means that ICSI also has the potential to delay degradation of sperm mtDNA. Furthermore, couples that have male factor infertility and require ICSI are at a higher risk of using sperm containing abnormal mtDNA. This is demonstrated in those men harbouring large numbers of mtDNA deletions who are more likely to exhibit poorer semen quality (Lestienne *et al.*, 1997; Reynier *et al.*, 1997; St. John *et al.*, 2001) as are those exhibiting point mutations (Spiropoulos *et al.*, 2002). Despite the many diversions from the natural fertilisation processes following ICSI, to date, it appears that sperm mtDNA is eliminated in offspring generated using this technique (Danan *et al.*, 1999; Marchington *et al.*, 2002). Furthermore, it appears to be eliminated prior to the blastocyst stage in ICSI-derived polyploid human embryos (St. John *et al.*, 2000).

1.4.2 Cytoplasmic Transfer

CT is an extension of ICSI and involves the injection of supplementary cytoplasm, as well as a sperm, into the oocyte (see Figure 1.4). The rationale for using CT is that donor cytoplasm will contain supplementary, often younger, non-mutated mtDNA and will possibly contain other important cytoplasmic components to compensate for the defective cytoplasm in the oocyte (Cohen *et al.*, 1997). In the human, it has resulted in higher pregnancy rates and live births (Barritt *et al.*, 2001; Cohen *et al.*, 1997). CT can also result in heteroplasmy, as demonstrated by the analysis of various tissues of human CT offspring (Brenner *et al.*, 2000).

Consequently, it remains to be demonstrated whether the donor mtDNA out-competes the recipient mtDNA in the heteroplasmic offspring. It also needs to be ascertained whether the coupling of two distinct genomes accounted for the two cases of Turner's syndrome, one electively aborted and the other spontaneously aborted, and the one case of pervasive development disorder (Barritt *et al.*, 2001).

In order to avoid the possible harmful effects of heteroplasmy in the offspring, cytoplasm from granulosa cells of the mother rather than cytoplasm from donor oocytes has been used (Kong *et al.*, 2003; Kong *et al.*, 2004). These are likely to have the same mtDNA sequence as the recipient oocyte. However, one possible problem with this procedure is that mtDNA deletions in granulosa cells are more common in older women (Seifer *et al.*, 2002), i.e. those that are likely to have defective oocytes and require CT. Therefore, the treatment may be unsuccessful due to continued lack of wild type mtDNA and may even result in transmission of mitochondrial disease to the offspring that would not have occurred without CT. Another likely problem is that granulosa cells are fully differentiated, whilst newly fertilised oocytes are pluripotent. Cells at different stages of differentiation are known to be different in terms of their mitochondrial activity and content. For example, undifferentiated human (h) ESCs (Sathananthan *et al.*, 2002; St. John *et al.*, 2005b) and PGCs (Jansen & de Boer, 1998) have been observed to contain low numbers of mitochondria. However, differentiated cells contain higher numbers of mitochondria, depending on OXPHOS requirements of specific cell types (Moyes *et al.*, 1998). The mitochondria contained within differentiated cells also differ morphologically from those in undifferentiated stem cells. For example, undifferentiated hESCs contain round mitochondria with very few cristae, whilst those that have been differentiating for just one week contained more elongated mitochondria with an electron

dense matrix and higher numbers of cristae (Cho *et al.*, 2006). Mitochondria derived from granulosa cells would therefore need to be 'reprogrammed' to match those of the oocyte. Developmentally incompetent porcine oocytes have been supplemented with pure populations of mitochondria from developmentally competent oocytes from maternal relatives. Improved fertilisation outcome and the maintenance of unimaternal inheritance was observed following this technique (El Shourbagy, 2004).

1.4.3 Germinal Vesicle and Pronuclear transfer

GVT and PNT have also been proposed as techniques to overcome problems associated with aged oocytes, whereby the GV or pronucleus (PN) is transferred into an enucleated recipient oocyte (see Figure 1.4). Ideally, the enucleated recipient should be from the same stage of development as the donor nucleus (Liu *et al.*, 1999). In aged oocytes, there is likely to be a larger number and higher proportion of mutant mtDNA (Keefe *et al.*, 1995). It has been hypothesised that mitochondria are important not only for the production of ATP but also for correct chromosomal segregation during meiosis (Schon *et al.*, 2000). Furthermore, it has been suggested that aberrant mitochondrial function in oocytes from women of advanced reproductive age may have a role in oocyte aneuploidy (Eichenlaub-Ritter *et al.*, 2004), a common cause of infertility in older women (Dailey *et al.*, 1996; Munne *et al.*, 1995). The chances of success may therefore increase if the nuclear component of the aged oocyte was transferred into the enucleated ooplasm of a younger, healthier oocyte, rather than supplement the existing ooplasm. Indeed, studies using human oocytes have shown that transfer of the GV from aged oocytes into the enucleated ooplasm of young oocytes can overcome oocyte aneuploidy, with the majority of reconstructions having normal karyotypes (Takeuchi *et al.*, 2001; Zhang *et al.*, 1999). There may also be non-cytoplasmic components of the GV that

influence chromosome segregation. This has been indicated in a series of mouse experiments combining GVs and ooplasms of varying ages (Cui *et al.*, 2005). A significantly greater number (57.1 %) of chromosomal abnormalities were found in reconstructions when the GV was transferred from older females to the enucleated ooplasm of younger females. This compares to abnormalities found in reconstructions derived from the transfer of younger GVs into aged ooplasms (16.7 %).

Mitochondria are known to be involved in chromosome organisation and movement. For example, induction of mitochondrial damage in murine oocytes prevented oocyte maturation, chromosomal segregation and spindle formation (Takeuchi *et al.*, 2005). This damage was overcome by the transfer of the karyoplast from a GV derived from a damaged oocyte into the ooplasm of a healthy enucleated oocyte. Cytogenetic analysis showed that 20/21 of these reconstructions had a normal number of chromosomes. Furthermore, performing ICSI on GVT reconstructions and transfer of the resulting 2-cell embryos led to the generation of live mouse offspring. It has also been demonstrated that an intact murine ooplasm could rescue mitochondrially damaged karyoplasts, induced by photoirradiation, with 62% of these reconstructions maturing to MII (Palermo *et al.*, 2002). PNT is essentially the same procedure, except for the nuclear material being removed after fertilisation. This technique has also been used in mice with considerable success, as determined by the birth of live offspring (He *et al.*, 2003; Meirelles & Smith, 1997; 1998; Sato *et al.*, 2005). This procedure however, involves the destruction of a zygote, which may restrict its usage due to ethical and moral considerations.

GVT and PNT have been proposed as techniques for treating mtDNA disease (Cummins, 1998; Trounson, 2001). However, potential problems could arise as the transferred GV or PN

is still surrounded by mitochondria, which will also be carried over into the donor ooplasm. These mitochondria remain close to the centre of the immature reconstruction at first but then disperse throughout the cytoplasm as maturation ensues (Fulka, 2004). Due to segregation during embryogenesis, this provides an opportunity for mitochondria derived from the donor karyoplast to be present in all cell types of the offspring. Any disease-associated mitochondria transferred with the nuclear material may therefore continue to be transmitted.

It is further possible that, due to its initial close proximity to the nucleus, mtDNA associated with the donor karyoplast would be preferentially replicated in the reconstructions. Indeed, fusion of enucleated somatic cells with mtDNA-depleted somatic cells can lead to the preferential expansion of one particular type of mtDNA such as the mutant molecule (Dunbar *et al.*, 1995). Although, to date, there has been no analysis of mtDNA transmission following GVT, results are likely to be similar to those reported following PNT. In this instance, exogenous mtDNA accompanying the murine zygotic karyoplast is found in higher concentrations at blastocyst than mtDNA from transferred cytoplasts (Meirelles & Smith, 1998). This has been further highlighted where zygotic karyoplast transfer into zygotes resulted in varying amounts of mtDNA being transmitted to offspring (0 - 69%; Meirelles & Smith, 1997). Consequently, it would be difficult to predict to what extent a mutated or deleted mtDNA molecule would be selected. However, a recent report suggests that the varying levels of mtDNA deletion in murine zygotes could be reduced to levels lower than those expected for the phenotypic onset of mtDNA disease when the zygotic nucleus is transferred into an enucleated healthy oocyte (Sato *et al.*, 2005). The subsequent offspring also remained under the level for pathological onset even with the accumulative increase of the deletion with time. It was however, stated that, in the human, this form of nuclear

transplantation could not be applied to those mtDNA diseases where the pathogenic mutation had significant replicative advantage over wild type mtDNA. This is most likely as, in humans, the prenatal period is 13 times longer than in the mouse and this represents the window in which mutant mtDNA replication is most proliferative (Sato *et al.*, 2005).

1.4.4 Nuclear transfer

NT involves the injection of a donor nucleus or a whole cell into an enucleated recipient oocyte (see Figure 1.4). Most commonly, either embryonic cell (EC) NT, using a blastomere, or somatic cell (SC) NT is performed. Resulting embryos have then been used to generate blastocysts and offspring in a variety of species (Campbell *et al.*, 2005). However, NT is renowned for its low efficiency, with low proportions of embryos developing to the blastocyst stage (White *et al.*, 1999) and even fewer progressing far enough to implant, survive pregnancy and produce live offspring (Wilmut *et al.*, 1997). One of the major abnormalities associated with NT offspring is the production of abnormal placentae which are thought to be the cause of many foetal deaths (Cibelli *et al.*, 2002). Incorrect reprogramming of imprinted genes has also been cited as one of the major factors leading to early foetal death. This was highlighted by non-surviving NT calves having incorrect expression of imprinted genes, namely Igf2, Igf2r and H19 in some tissues, whereas surviving adult clones had normal expression at these loci except for Igf 2 in muscle (Yang *et al.*, 2005). Furthermore, in murine NT embryos and offspring, irregular patterns of gene expression have been observed, most likely as a result of inappropriate reprogramming (Humpherys *et al.*, 2002).

Although the process of NT for human reproductive purposes is ethically unacceptable and is banned in many countries (UK- Human Fertilisation and Embryology Act 1990, USA- H.R.

534 Human Cloning Prohibition Act of 2003, South Korea- see Normile & Mann, 2005), its use as a means of producing histocompatible stem cells for treatment of various diseases is likely to have a significant impact. Cell replacement therapy has great potential for the treatment of certain conditions such as myocardial infarction (Rubart & Field, 2006) and Parkinson's disease (Taylor & Minger, 2005) and as a replacement for tissue/organ transplantation (Cortesini, 2005). To date, encouraging results have been obtained in animal studies whereby hESC-derived neural precursors (Wernig *et al.*, 2004) and cardiomyocytes (Laflamme *et al.*, 2005) were successfully transplanted into animal organs. However, considerable characterisation and validation of precursor cells derived from hESCs still need to be undertaken before cell replacement can be used as an effective therapy in humans. Some success at generating NT-derived ESCs has so far been achieved in mouse models (Munsie *et al.*, 2000; Wakayama *et al.*, 2001) and its use as a means of delivering the patient's own chromosomal DNA with the corrected gene for a single gene disorder has also been demonstrated in the mouse (Rideout *et al.*, 2002).

1.4.4.1 MtDNA and NT

One of the rate-limiting factors in generating autologous ESCs, i.e. stem cells possessing the patient's own chromosomal and mtDNA compositions is regulation of their mtDNA composition. In this respect, a considerable amount can be learnt from those offspring derived from NT through either SCNT or ECNT. For SCNT, this ranges from homoplasmy for the recipient oocyte mtDNA that was observed in many tissues of sheep (Evans *et al.*, 1999) and cattle (Hiendleder *et al.*, 2003) offspring to heteroplasmy including up to 59% donor cell-derived mtDNA in calf kidney tissue (Takeda *et al.*, 2003). For ECNT-derived cattle offspring, up to 57% (Hiendleder *et al.*, 1999) donor cell-derived mtDNA has been reported, although

some individuals contained only 0.4% donor cell-derived mtDNA (Steinborn *et al.*, 1998). This variability is probably due to the random pattern of mtDNA segregation during embryogenesis (reviewed in Shoubridge, 2000). Consequently, it is apparent that no real mechanism is currently understood that can account for the irregular patterns of mtDNA transmission observed. One parallel that can be drawn from both ECNT and SCNT is that the range of donor mtDNA transmitted is relatively similar at 0 to 57% (Hiendleder *et al.*, 1999) and 0 to 59% (Takeda *et al.*, 2003), respectively. However, two explanations could exist for those studies not detecting donor mtDNA in live offspring: i) no donor mtDNA is actually present; or ii) too few tissues have been analysed. Consequently, the true extent of heteroplasmy is difficult to determine. This is perhaps exemplified by the recent studies of pig (Takeda *et al.*, 2006), cow (Takeda *et al.*, 2003) and mouse (Inoue *et al.*, 2004) NT-derived offspring, where varying levels of heteroplasmy have been observed between the tissues. This would indicate that if all tissues are not analysed then these studies are not truly subjective. Furthermore, investigations should include analysis of the surrogate mother's mtDNA as leakage through the placenta into foetal blood has been reported in cattle (Hiendleder *et al.*, 2004).

The initial number of donor mtDNA molecules introduced into the recipient oocyte also does not necessarily influence whether donor mtDNA is actually transmitted. The use of blastomeres from more advanced embryonic stages in bovine NT has resulted in reduced levels of heteroplasmy being reported (Steinborn *et al.*, 1998). However, this was not a uniform pattern as two offspring derived from 52-cell morula blastomeres contained less donor mtDNA than offspring derived from 92-cell morula blastomeres. A further issue is the stage of embryonic development of the donor cell. A recent analysis of two NT-derived monkey offspring (St. John & Schatten, 2004) demonstrated mtDNA transmission from both the

recipient oocyte and the donor cell. In this instance, the early IVF blastomere (8 to 12 cell stage) still contained sperm mtDNA. Consequently, the offspring possessed three different populations of mtDNA. Furthermore, depletion of donor cell mtDNA to residual levels (<0.02% of its original content) still resulted in the persistence of some mtDNA at the blastocyst stage in sheep NT (Lloyd *et al.*, 2006).

The use of a somatic donor cell with accompanying mitochondria not only raises interesting questions with regard to the differentiated status of the nucleus but also of the mitochondria. During development, from the ICM to a fully differentiated cell, mitochondria increase in number to meet the metabolic requirements of that specialised cell. The proliferation of mitochondria further discriminates between the number of mtDNA copies per mitochondrion and cell type, with higher ATP users possessing greater copy numbers (Moyes *et al.*, 1998). Therefore, for SCNT, the mitochondria accompanying the donor cell should also be reprogrammed to the embryonic state, as is the case for the donor nucleus. Failure to do this may result in embryos containing mitochondria with insufficient plasticity to suit the multitude of cell types that they will contribute to. The persistence of donor mitochondria could also influence the metabolic pathways used during early development and explain why improved developmental rates are observed in those embryos cultured in media that enhance anaerobic respiration (Chung *et al.*, 2002). It is further evident that injection of somatic donor mitochondria into oocytes which are then parthenogenetically activated is detrimental to embryonic development though this is not observed with donor ooplasm (Takeda *et al.*, 2005).

1.4.4.2 Interaction between multiple wild type mitochondrial genomes

It has not been clearly demonstrated whether heteroplasmy resulting from two wild type mtDNA molecules can be detrimental to the offspring and result in mitochondrial disease. This is partly because many post-mortem results have not been published and the cause of many deaths following NT is not documented. Consequently, it is unclear whether those offspring that survive are the exception to the rule. However, some common causes of death in NT offspring are similar to symptoms of mitochondrial diseases, including brain, kidney and liver defects in sheep (McCreath *et al.*, 2000) and in a series of other species (Cibelli *et al.*, 2002). Sequence differences due to mtDNA from a 'foreign' source can give rise to proteins with altered amino acid sequences. This has been demonstrated in both cattle (Steinborn *et al.*, 2002) and pigs (St. John *et al.*, 2005a) and may result in inadequate interaction between the individual subunits of the ETC. This would result in reduced energy production capacity and symptoms common to mitochondrial disease and influence embryo and foetal survival. The question is whether hand-made cloning, the fusion of one or more oocyte cytoplasts with a somatic cell (Vajta *et al.*, 2001), would resolve such issues or complicate them.

Intergenomic communication is vital for efficient cellular function as much interaction occurs between nuclear-encoded proteins and those encoded by the mtDNA genome (reviewed by Scarpulla, 2002). The ETC requires nuclear-encoded proteins to be transported to the mitochondria and to assemble appropriately (Stojanovski *et al.*, 2003). Failure of TFAM, TFB1M and TFB2M to coordinate transcription would also have serious implications for a functional ETC (Falkenberg *et al.*, 2002). Furthermore, failure of TFAM and PolG to coordinate replication would have similar outcomes for replication (Hance *et al.*, 2005; Larsson *et al.*, 1998). However, NT appears to result in the continued expression of both these

factors during embryo development and could explain why even residual levels of donor mtDNA persist up to the blastocyst stage (Lloyd *et al.*, 2006). Failure of TFAM or PolG to interact with the mtDNA genome, post-implantation, through the diversity of the donor nucleus and the recipient oocyte's mtDNA would result in compromised transcription or replication and mimic mtDNA-depletion syndromes (Poulton *et al.*, 1994).

1.4.4.3 NT-derived ESCs

Patient specific ESCs derived by NT would require large numbers of good quality human oocytes which, due to medical, ethical, legal and moral issues, are a limited resource. Added to this, the methods of recruitment of donor oocytes for the derivation of such ESCs have caused considerable concern recently (Holden, 2005). Cross-species NT offers the opportunity to derive ESCs that would carry the chromosomal DNA from a patient propagated in a surrogate ooplasm of another species. Encouragingly, there is a single report of the generation of a hybrid ESC line following transfer of a human nucleus into a rabbit oocyte (Chen *et al.*, 2003). However, outcomes from cross-species NT further exemplify the importance of nucleo-mitochondrial compatibility for ESC derivation. Use of both human cord fibroblasts with bovine oocytes (Chang *et al.*, 2003) and chicken blastodermal cells with rabbit oocytes (Liu *et al.*, 2004) resulted in elimination of the donor mitochondrial genome by the morula or blastocyst stage of development. Rhesus macaque donor cells injected into rabbit oocytes resulted in heteroplasmic embryos but with only the rabbit mtDNA being replicated after the blastocyst stage (Yang *et al.*, 2003), suggesting that these too could have soon become homoplasmic for the recipient oocyte mtDNA. This suggests either preferential replication of the oocyte mtDNA or degradation of the mtDNA from the nuclear donor cell.

1.4.4.4 Cross-species NT

Analysis of cross-species NT offspring provides further indications as to potential outcomes for cross-species ESCs. In two combinations of inter-species bovine NT experiments, the donor nucleus was capable of interaction with both types of mtDNA with the production of apparently healthy heteroplasmic calves (Han *et al.*, 2004; Steinborn *et al.*, 2002). However, the combination of *Bos indicus* donor cells with *Bos taurus* oocytes resulted in homoplasmic embryos and one offspring (Meirelles *et al.*, 2001). There has also been one report of preferential replication of mtDNA from the nuclear donor cell following cross-species NT (Chen *et al.*, 2002). Here, nuclei from giant panda somatic cells were injected into rabbit oocytes and preferential replication of the panda mtDNA was observed after analysis of the foetuses. This suggests that some combinations of cross-species NT, particularly where the species are closely related or are sub-species, may result in a functional ETC and efficient mitochondrial respiration. However, more distantly related species might preferentially replicate their own mtDNA genome due to incompatibility with that in the recipient oocyte.

1.4.4.5 Cybrids and xenomitochondrial offspring

Much can also be learnt from somatic cell-cell fusion studies and xenomitochondrial offspring in relation to outcomes associated with cross-species NT. For example, one study involved combinations of cells depleted of their mtDNA and exogenous mitochondria from rats and two sub-species of mouse (McKenzie & Trounce, 2000). Transfer of *Mus spretus* mtDNA into *Mus musculus* cells resulted in heteroplasmy and no obvious OXPHOS defects, probably due to the two types of mice being closely related. However, when rat mtDNA was transferred into *Mus musculus* cells, its replication and transcription were unaffected, but OXPHOS capacity was reduced. Other studies have also demonstrated increased respiratory chain defects following

crosses of greater evolutionary distance both in mice (McKenzie *et al.*, 2003) and primates (Kenyon & Moraes, 1997). Effects of species differences, and also mitochondrial disease, can now be studied *in vivo* following the production of xenomitochondrial mice (McKenzie *et al.*, 2004; Sokolova *et al.*, 2004). Xenomitochondrial mice can be produced by: i) direct injection of foreign mitochondria into zygotes (Irwin *et al.*, 1999); ii) pronuclei fusion with enucleated oocytes (Meirelles & Smith, 1997) followed by transfer to surrogate mothers; or iii) production of ESC cybrids for injection into blastocysts (Levy *et al.*, 1999). Again, it has been demonstrated that greater evolutionary distance between species results in offspring with increased respiratory chain defects (Trounce *et al.*, 2004). It is most likely that OXPHOS defects arose from abnormal combinations of nuclear and mitochondrially encoded ETC proteins resulting in abnormal structure of ETC complexes, a scenario very similar to outcomes that arise through cross-species NT.

1.4.5 Safety of assisted reproduction technologies

There are many cases where inter-species NT embryos implant and survive pregnancy but die shortly after birth (Hammer *et al.*, 2001; Han *et al.*, 2004). These may be a result of inefficient ETC activity due to species differences. However, should these combinations result in apparently healthy live offspring, it is possible that phenotypic alterations may still be present. Such outcomes could affect those mtDNA haplotypes that influence, for example, milk quality in cattle (Brown *et al.*, 1989), growth and physical performance in mice (Nagao *et al.*, 1998) and a reduction in fertility in beef cattle (Sutarno *et al.*, 2002) and sperm motility in men (Ruiz-Pesini *et al.*, 2000). Perhaps, the biggest note of caution is exemplified by the result of introducing a common carp nucleus into an enucleated goldfish oocyte, which resulted in the offspring developing phenotypic features of the goldfish (Sun *et al.*, 2005).

Consequently, the use of cross-species NT for the derivation of hESCs needs considerable investigation although it offers great potential.

Although some experiments have been carried out in animals and a small number of groups practice invasive ART protocols in humans, little is known about the likely outcomes of these techniques in relation to the long term effects on offspring and future generations. It is essential that the risks of these techniques are understood before their therapeutic use becomes widespread. The use of pre-implantation genetic diagnosis (PGD) has recently been proposed for early diagnosis of mitochondrial disease (Poulton & Marchington, 2002) and its use for chromosomal genetic analysis has been implemented in many clinics (Ao *et al.*, 1996; Ban *et al.*, 2001; Bermudez *et al.*, 2003; Verlinsky & Kuliev, 1994; 1996). However, its effectiveness needs to be demonstrated for the detection of a representative sample of mtDNA content that would be necessary to detect potential mtDNA disease. Some early work has indicated its usefulness in this respect. For example, in the mouse, mtDNA genotypes in polar bodies were compared with their respective oocytes, and in different blastomeres within the same early embryo (Dean *et al.*, 2003). The level of heteroplasmy present in polar bodies and blastomeres was representative of that in the associated oocytes and embryos, leading to the conclusion that PGD was feasible for the diagnosis of mitochondrial disease. However, cloned mouse offspring (Inoue *et al.*, 2004), various farm animal species such as pigs (Takeda *et al.*, 2006) and cattle (Hiendleder *et al.*, 2003) and patients with mitochondrial mutations (Kirches *et al.*, 2001) and diseases (Ponzetto *et al.*, 1990) often demonstrate varying levels of heteroplasmy in different tissues. Furthermore, considerable variation in oocyte-derived mtDNA copy number among intra-embryo blastomeres has been demonstrated in both the pig (El Shourbagy, 2004) and the human (Lin *et al.*, 2004). Therefore, even if the proportion of

heteroplasmic mtDNA molecules were the same in each blastomere, the actual number of mutant molecules is likely to be different.

Blastomeres containing lower numbers of mtDNA molecules will have reduced ATP-generating capacities compared to their counterparts with higher overall numbers of mtDNA. Therefore, the effects in tissues resulting from these blastomeres will be more severe than those resulting from blastomeres with a larger mtDNA complement. Furthermore, different tissues require different ATP-generating capacity (Moyes *et al.*, 1998). Low levels of wild type mtDNA in some blastomeres may therefore go unnoticed if that particular blastomere develops into a low ATP requiring tissue. However, this could result in severe mitochondrial disease should that blastomere develop into a tissue highly dependent on OXPHOS. It is therefore unlikely that PGD would be a suitable technique to determine the risk of mitochondrial disease following oocyte reconstruction, especially following GVT, PNT and NT as all have reported preferential replication of donor mtDNA. Consequently, an alternative is required.

1.5 Conclusions

NT has great potential as both a method of reproducing endangered species and animals with desired characteristics and also as a means of producing histocompatible ESCs for human therapies. However, despite the success achieved in reprogramming various nuclear donor cells, little attention has been given to the requirement for nuclear-mitochondrial compatibility and transmission of homoplasmic mtDNA and how these processes occur naturally. This is particularly important for the development of hESC therapies as the limited availability of human oocytes may lead to the use of cross-species NT with, for example, porcine oocytes. I

have therefore investigated mtDNA replication patterns during oogenesis, embryogenesis and in potential donor cells for NT, as well as in cross-species NT embryos.

1.6 Hypothesis

As for somatic cell types, oocytes and embryos have specific mtDNA replication requirements that are met through the strictly regulated expression of mtDNA replication factors. NT disrupts the regulation of mtDNA replication, which might therefore contribute to the low developmental potential of embryos produced using this method.

1.7 Aims

Experiments described in this thesis aim to determine whether:

1. mtDNA replication factors are expressed and whether mtDNA replication occurs during porcine pre-implantation embryogenesis (Chapter 3).
2. there are differences between mtDNA replication factor expression and mtDNA copy number in developmentally competent and less competent maturing porcine oocytes (Chapter 4).
3. expression of mtDNA replication factors in potential NT donor cells varies at different stages of differentiation (Chapter 5).
4. replication of mtDNA derived from the donor cell influences the developmental potential of cross-species NT embryos (Chapter 6).

CHAPTER 2: GENERAL MATERIALS AND METHODS

All reagents were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. All media were prepared, filtered and incubated at the appropriate temperature in 5% CO_2 for at least 2 hrs before use.

2.1 Cell culture

Cells were cultured in a cabinet incubator at 37°C (Jencons, Leighton Buzzard, Bedfordshire, UK) in an atmosphere of 5% CO_2.

2.1.1 Preparation and culture of feeder cell plates

Feeder cells such as murine embryonic fibroblasts (MEFs) that have been treated to prevent cell division are often used to aid attachment of ESCs. An additional advantage of feeder cells is the release of nutrients into the culture media for use by the ESCs. MEFs derived from CD-1 mice were donated by Dr Martyn Bell of the University of Birmingham for use as feeder cells. MEFs were cultured in 10ml MEF media in 75ml flasks (Sarstedt, Leicester, Leicestershire, UK) at 37ºC in 5% CO_2. MEF media consisted of high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS), supplemented with 2mM L-Glutamine and 1% non-essential amino acids (NEAA; see Appendix I). When cells had become confluent, they were washed in 2ml Ca^{2+} and Mg^{2+}-free DPBS and treated with 0.25% trypsin-EDTA solution at 37ºC for 2 to 3 mins. 8ml MEF media was then added to inactivate the trypsin and the cell suspension was transferred to a 15ml conical tube (Becton Dickinson (BD) Biosciences, NJ, USA) and centrifuged at 500 rpm for 5 mins (Harrier Centrifuge, MSE, Kent, UK). The supernatant was removed and the

pellet resuspended in 10ml MEF media. The cell suspension was then divided equally between five 75cm^3 flasks (Sarstedt) with each flask being supplemented with 10ml with fresh media.

Inactivation of MEFs to prevent cell division was carried out by treatment of a confluent 75cm^3 flask (Sarstedt) with 10μg/ml mitomycin C for 2 hrs at 37ºC in 5% CO_2. Cells were then washed 3 times in media without mitomycin C before being trypsinised as described above and were then ready to be plated as feeders. 6-well plates (Scientific Laboratory Supplies (SLS), Nottingham, UK) for ESC culture were coated with 0.1% (w/v) gelatin for 30 mins at 37ºC. Excess gelatin was then removed and the plates were incubated at 37ºC for 5 to 10 mins to allow them to dry before the suspension of inactivated MEFs, obtained from one culture plate, was added. The feeder cells were incubated for at least 12 hrs to allow them to attach to the gelatin before ESCs were added.

2.1.2 Murine ESC culture

R1 murine (m) ESCs were donated by Dr Martyn Bell of the University of Birmingham and were cultured at 37ºC in 5% CO_2 on mitomycin C-treated MEF feeder layers. The culture medium consisted of Knock-out DMEM (Invitrogen, Paisley, UK) containing 15% serum replacement (Invitrogen), supplemented with 1% penicillin/streptomycin solution, 2mM L-Glutamine, 1% NEAA (Invitrogen), 0.1mM β-Mercaptoethanol and 10ηg/ml leukaemia inhibitory factor (LIF; Chemicon, California, USA; see Appendix I). The media was changed every 24 hrs and cells were passaged at a ratio of 1:4 every 2 to 3 days by trypsinisation, as described above, and replated onto new feeder layers. Darker, differentiating colonies were

scraped from the plate during media changes with the tip of a Pasteur pipette. This left only the undifferentiated cells for further culture.

CCE/R1 (CCE) mESCs were donated by Dr Laura O'Neil of the University of Birmingham and cultured at 37ºC in 5% CO_2 on gelatin-coated plates without feeder layers. The culture medium consisted of high glucose DMEM containing 15% Hyclone FBS (Fisher Scientific, Loughborough, Leicestershire, UK), supplemented with 1% NEAA, 2mM glutamine, 1% penicillin/streptomycin solution, 0.1mM β-Mercaptoethanol and 10ηg/ml LIF (see Appendix I). Media was changed every 24 hrs and cells were passaged at a ratio of 1:4 every 2 to 3 days by trypsinisation, as described above, and replated onto new gelatin-coated plates. Darker, differentiating colonies were scraped from the plate and removed during media changes.

2.1.3 Cryopreservation of cultured cells

Cryopreservation was carried out when necessary at the time of passage. After trypsinisation, cells were centrifuged at 350 rpm for 5 mins and the supernatant was removed. The pellet was resuspended in 0.4ml of the relevant culture media and transferred to a cryovial (Nunc, Roskilde, Denmark). 0.6ml freeze media was added which consisted of Hyclone FBS containing 22.5% (v/v) DMSO (see Appendix I). Cells were stored at -20ºC for 2 hrs, -80ºC until frozen (approximately 14 hrs) and then transferred to liquid nitrogen (LN_2). When required, cells were removed from LN_2 and allowed to thaw in a water bath at 37ºC. The cell suspension was transferred to a 15ml conical tube and 5ml of the relevant media was added. The cells were centrifuged at 350 rpm for 5 mins and the supernatant was removed. The cell pellet was resupsended in the relevant media and transferred to a culture plate or flask. After 24 hrs, non-attached cells were removed and the media replaced.

2.1.4 Spontaneous ESC differentiation and embryoid body production

ESCs were cultured without feeder layers in media that did not contain LIF. Darker patches of differentiating cells known as migratory cells then began to form within the colonies. A sterile Pasteur pipette was used to detach these cells from the rest of the colony every 24 hrs. After 3 to 4 weeks, migratory cells began to form embryoid bodies (EBs). These were transferred to a new plate coated with feeder cells and cultured in media without LIF. Some of the EBs attached to the feeder cells and individual cells migrated away from the centre of the EB.

2.1.5 Directed production of embryoid bodies and ESC differentiation

EBs were formed from R1 mESCs using the hanging drop method (Keller, 1995). After trypsinisation, cells were resuspended in their culture media (see above) without LIF and a sample was pipetted onto a haemocytometer for counting. The cells in the central $1mm^3$ area of the haemocytometer were counted. If the central $1mm^3$ area contained fewer than 200 cells, the cells in the corner $1mm^3$ areas were also counted. A mean count for $1mm^3$ area was then determined and used to calculate the cell concentration:

$$\text{Cells/ml} = \text{No. cells in } 1mm^3 \text{ area} \times 10^4 \times \text{dilution factor}$$

The suspension was diluted to a concentration of 2.25×10^4/ml and fifty 20µl drops containing approximately 450 cells were prepared on a 10cm diameter petri dish. The plates were inverted and cultured at 37ºC in 5% CO_2 for 2 days to allow the cells to form EBs. After 2 days, the plates were inverted and 10ml media was added. The EBs were then maintained in suspension for a further 6 days before being plated onto gelatin-coated 6-well plates (Nunc)

and the cells were allowed to attach and migrate for 10 days. The attached EBs were harvested from Days 1-10 after plating for analysis.

2.2 Embryo production and culture

2.2.1 In vitro maturation

Ovaries were collected from a local slaughterhouse (F.A. Gill Ltd., Wolverhampton, UK) in a vacuum flask containing DPBS supplemented with 75µg/ml potassium penicillin G and 50µg/ml streptomycin sulphate at 25-30ºC. The ovaries were rinsed three times with DPBS at 39ºC and transferred to a laminar flow hood where follicular fluid, containing oocytes, was aspirated from ovarian follicles and transferred to a 15ml conical tube containing 1ml basic oocyte culture media. Basic oocyte culture media consisted of TCM-199 with Earle's salts and sodium bicarbonate, supplemented with 25mM hepes, 3mM L-glutamine, 0.1% (w/v) bovine serum albumin (BSA), 0.57mM cysteine, 50 IU/ml penicillin and 50µg/ml streptomycin (see Appendix I). The aspirated oocytes were incubated for approximately 10 mins to allow the oocytes to settle at the bottom of the tube. The majority of the 'supernatant' was then removed and the 'pellet' was diluted in an equal volume of basic oocyte culture media before being transferred to a 92 x 10 mm petri dish (Nunc). Oocytes with a homogeneous cytoplasm, surrounded by three or more layers of cumulus cells were considered good quality and were selected for *in vitro* maturation. Selected oocytes were washed 3 times in basic oocyte culture media and once in *in vitro* maturation (IVM) media. IVM media consisted of basic culture media excluding hepes, supplemented with 10ηg/ml epidermal growth factor (EGF), 0.2µg/ml LH and 50ηg/ml FSH (see Appendix I). Groups of 50 oocytes were cultured in 500µl media covered with a layer of mineral oil in 4 well dishes (Nunc) at 39ºC in 5% CO_2. Oocytes used in the experiments described in Chapter 4 were matured in IVM media for 44 to 46 hrs, with one media change after 16 to 18 hrs. Oocytes used in the experiments described in Chapter 3 and Chapter 6 were matured in IVM media containing 5µg/ml cycloheximide (CHX) for 16 to 18 hrs to arrest oocytes prior to GVBD and

were then washed 3 times in basic oocyte culture media before being transferred to IVM media without CHX. These oocytes were then incubated for a further 28 to 30 hrs to allow them to mature to MII.

2.2.2 Brilliant Cresyl Blue Test

5µg/ml Brilliant Cresyl Blue (BCB) solution was prepared by adding 0.0001g BCB dye to 20ml modified PBS (mPBS). mPBS consists of DPBS supplemented with 75µg/ml Potassium penicillin G, 0.99mg/ml glucose, 0.375mg/ml pyruvate, 4mg/ml BSA and 50µg/ml streptomycin (see Appendix I). Selected immature oocytes were transferred to a dish containing 0.5ml BCB media and incubated for 90 mins at 39ºC, 5% CO_2. After incubation in BCB, the oocytes were washed once in IVM media and separated according to their colour. The blue BCB+ oocytes and the unstained BCB- oocytes were washed separately twice more in IVM media before being transferred to separate culture dishes containing IVM media under mineral oil. Oocytes were then matured for 44 to 46 hrs.

2.2.3 2', 3'-dideoxycytidine treatment

Selected immature oocytes were cultured in IVM media supplemented with 10µM ddC. Being a nucleoside analogue, ddC can be incorporated by PolG into mtDNA molecules being synthesised in place of the normal nucleoside (Feng *et al.*, 2001). However, absence of a 3’ OH group prevents addition of subsequent nucleosides, resulting in termination of the DNA chain (Brinkman & Kakuda, 2000). Replication is further inhibited by failure of the PolG exonuclease to remove the ddC (Feng *et al.*, 2001). Oocytes were treated for the entire maturation period, with one media change after 22 hrs, as for non-treated oocytes.

2.2.4 Mitochondrial extraction and supplementation

Mitochondria were extracted from CCEs obtained from 2 confluent wells of a 6-well plate (SLS) using the mitochondria isolation kit for cultured cells (Pierce Biotechnology Inc, Rockford, IL, USA). 400µl reagent A was added to the cell pellet, which was then incubated for 2 mins on ice. 5µl reagent B was then added and the solution was incubated on ice for a further 5 mins, with a short vortex after each min. After addition of 400µl reagent C, the solution was centrifuged at 700rpm for 10 mins to pellet the nuclei. The supernatant was further centrifuged at 12000rpm for 15 mins to pellet the mitochondria. The mitochondrial pellet was washed once in 250µl reagent C and centrifuged again at 12000rpm for 5 mins to allow reagent C to be removed. The pellet was then resuspended in 20µl oocyte manipulation media and stored on ice until supplementation. During the NT procedure, the mitochondrial suspension was aspirated into the injection pipette, along with a CCE donor cell, and then injected into the cytoplasm of recipient oocytes. Samples of aspirated mitochondrial preparation were saved for mtDNA copy number analysis.

2.2.5 Sperm preparation

Sperm were obtained from purebred boars supplied by JSR (Selby, North Yorkshire, UK). The heavy particles were removed by centrifugation of the whole sperm sample at 1500 rpm for 5 mins. The supernatant was then removed and the pellet resuspended in 3ml of sperm washing media (DPBS supplemented with 1mg/ml BSA, 100µg/ml potassium penicillin G and 75µg/ml streptomycin; see Appendix I) followed by centrifugation at 1200 rpm for 5 mins.

Percoll solutions of 45% and 90% concentrations were prepared by dilution in mTBM. mTBM consists of 113.1mM NaCl, 3mM KCl, 11mM glucose, 7.5mM $CaCl_2.2H_2O$, 20mM tris and 5mM sodium pyruvate supplemented with 0.1% (w/v) BSA, 20µM adenosine and 0.2mM glutathione (see Appendix I). Percoll gradients were produced by layering 2ml 45% Percoll over 2ml 90% Percoll in a 15ml centrifuge tube. The sperm pellet was resuspended in 2ml of sperm washing media and layered onto the 90%: 45% Percoll gradient before centrifugation at 1500 rpm for 25 mins. The 90% fraction was resuspended in 2ml mTBM and finally centrifuged at 1500 rpm for 5 mins. The resulting pellet was resuspended in 1ml mTBM and counted. Sperm counts were performed using a haemocytometer as described previously (see Section 2.1.5) and samples were diluted in mTBM to a concentration of $2x10^6$/ml.

2.2.6 In vitro fertilisation and embryo production

Oocytes were washed three times in mTBM and then transferred in groups of 10 to 15 to 50µl drops of mTBM under mineral oil. Oocytes were inseminated with 50µl of prepared sperm to produce a final concentration of $1x10^6$/ml and were then incubated for 4 hrs to allow fertilisation to take place. After sperm-oocyte co-incubation oocytes were washed 3 times and then transferred to *in vitro* production (IVP) 1 media consisting of North Carolina State University (NCSU) 23 media, with lactate and pyruvate in place of glucose, further supplemented with 0.4% (w/v) BSA, 0.2mM glutathione, 50 IU/ml penicillin and 50µg/ml streptomycin (see Appendix I). After 48 hrs, all cleaved embryos were transferred to IVP2 media consisting of NCSU-23 media (containing glucose, but excluding pyruvate and lactate) supplemented with 0.4% (w/v) BSA, 0.2mM glutathione, 50 IU/ml penicillin and 50µg/ml

streptomycin (see Appendix I). Embryos were cultured for a further 5 days to allow blastocyst formation.

2.2.7 Preparation of pipettes for nuclear transfer

Holding pipettes were flame-pulled from borosilicate glass capillaries of 1mm outer diameter and 0.58 mm internal diameter (Intracel Ltd, Royston, Hertfordshire, UK). The tips were cut to approximately 140 μm using tweezers and melted using a microforge (MF830, Narishige, Japan) on maximum heat to produce an inner diameter of approximately 40 μm. The microforge was also used, at a temperature of 50˚C, to bend the tip to an angle of approximately 35˚. Enucleation/injection pipettes were pulled from borosilicate glass capillaries of 1mm outer diameter and 0.8 mm internal diameter (Intracel Ltd) using a Flaming Brown micropipette puller (P-97, Sutter Instrument Co., USA). The program used was: Temperature- 350˚C; Pull- 200U; Velocity- 50U; Time- 25U. Tweezers were used to cut the tips to 20-25 μm and the pipette was bevelled to an angle of 50° using a microgrinder (EG-400, Narishige) at maximum speed. Pipettes were bevelled under sterile ddH_2O to wash the inside of the pipettes free of glass debris. The ground pipettes were then transferred to the microforge and the glass bead was heated to 50˚C to allow a spike to be produced at the tip of each pipette. Enucleation/injection pipettes were also bent to an angle of approximately 35˚.

2.2.8 Enucleation

Oocytes were transferred to 250μl basic oocyte culture medium in a 1.5ml eppendorf tube and vortexed for 1 min to remove the cumulus cells. Denuded oocytes were then stained with 5μg/ml bisbenzimide (Hoechst) for 30 mins before being transferred to a 50μl droplet of manipulation media consisting of basic oocyte culture media supplemented with 7.5μg/ml

cytochalasin B (see Appendix I) under mineral oil. Oocyte nuclei were visualised under UV light and the oocyte was repositioned if necessary so that the polar body and nuclear material were at the opposite side of the oocyte to the holding pipette. The oocyte nuclei were then aspirated into the enucleation/injection pipette and released into the media.

2.2.9 Injection

MESC and MEF donor cells were detached from plates by treatment with trypsin-EDTA for 2 to 5 mins, and added to the media droplet containing the enucleated oocytes. Donor cells were then individually aspirated into the injection pipette and injected through the ZP into the oocyte cytoplasm. Reconstructed oocytes were incubated at 39ºC in 5% CO_2 for 1 (mESC) or 2 (MEF) hrs.

2.2.10 Activation

Reconstructed oocytes were incubated for 5 mins in manipulation media containing cytochalasin B supplemented with 5µg/ml calcium ionophore for activation. They were then transferred to manipulation media containing 10µg/ml CHX and incubated at 39ºC in 5% CO_2 for 14 to 16 hrs. After activation, oocytes were washed 3 times and transferred to IVP2 media. They were then incubated at 39ºC in 5% CO_2 for 7 days to allow blastocyst formation.

2.3 DNA extraction

DNA was extracted from individual oocytes and embryos and from samples of MEFs, CCEs and extracted mitochondria using the freeze–thaw method as previously described (Lloyd *et al.*, 2006). Briefly, 20µl of autoclaved sterile ddH_2O was added to each sample, which was then frozen at -20ºC, thawed, refrozen and thawed again before use.

2.4 RNA extraction

2.4.1 mESCs, EBs and MEFs

Total RNA was isolated from mESCs or MEFs detached from a confluent well of a 6-well plate or from 30 EBs using the RNAqueous-4-PCR kit (Ambion, Huntingdon, Cambridgeshire, UK). Cells were first lysed using 500µl guanidinium thiocyanate (lysis/binding solution) followed by vigorous vortexing. 500µl 64% ethanol was then added to the cell lysates to allow RNA binding to the silica-based filters inside the spin columns provided with the kit. Spin columns containing the cell lysates were placed inside an RNAse-free tube to collect flow-through from the filters. The cell lysate-ethanol mixture was centrifuged at 13000 rpm for 1min to allow the RNA to bind to the filter. The bound RNA was then washed using a series of 3 ethanol-containing solutions to remove contaminants. 700µl each wash solution was added followed by centrifugation at 13000 rpm for 1 min and removal of the flow-through. The columns were centrifuged once more for 1 min to remove traces of ethanol and the filters were then transferred to a fresh RNAse-free tube. RNA was eluted from the filter using 2 separate additions of 30µl elution solution (nuclease-free water), preheated to 75ºC, followed by centrifugation at 13000 rpm for 1 min. Contaminating genomic DNA was removed from the solution using a DNAse treatment. 10x DNase buffer (100mM Tris, 25mM $MgCl_2$, 1mM $CaCl_2$) and 4 units (2 units/µl) DNase were added to each RNA sample. This mixture was incubated at 37ºC for 2 hrs. 10% volume (6.8µl) DNase inactivation reagent was then added to each tube and incubated for 2 mins at room temperature. The tubes were vortexed briefly once during this time. The inactivation reagent and any contaminating genomic DNA were removed from the mixture by centrifugation of the sample for 1 min at 13000 rpm. The RNA-containing supernatant was transferred to a new RNAse-free tube and stored at -80˚C.

2.4.2 Oocytes and embryos

Oocytes and embryos were stored in groups of 5 and RNA was extracted using the RNAqueous-micro kit (Ambion). The protocol was similar to that used for RNA extraction from mESCs, EBs and MEFs, except that smaller reagent volumes were used, centrifugation times were shorter and filters with a smaller surface area were provided with the kit. 100µl lysis/binding solution was used to disrupt the oocyte/embryo membranes and 50µl 100% ethanol was added to the lysed cells before transferring the solution to the filter. Contaminants were removed using 180µl each wash solution followed by centrifugation for 10s at 13000 rpm. 10µl elution solution was centrifuged through the column twice to elute the RNA. 2µl DNAse buffer and 1µl DNAse enzyme were added to the RNA sample which was incubated for 20 mins at 37°C. 10% volume (2.3µl) DNAse inactivation reagent was then added to the sample before centrifugation at 13000 rpm for 1 min. The supernatant containing the RNA was then transferred to a clean 0.2ml centrifuge tube for storage at –80°C.

2.5 Spectrophotometry

A spectrophotometer (Ultrospec 2000, Pharmacia Biotech, UK) was used to calculate the final concentration of the RNA samples from cells other than oocytes/embryos. 995µl sterile ddH_2O was transferred to a cuvette and placed in the spectrophotometer. The reading was standardised to zero at 260ηm. 5µl of the RNA sample was added to the water and mixed thoroughly. The absorbance value obtained was entered into the following formula (Sambrook *et al.*, 1989) to calculate the final RNA concentration:

$$\frac{\text{Absorbance at 260ηm} \times 40\ (\mu g/ml\ \text{total RNA at absorbance of 1})}{0.005\ (\text{dilution factor})} = \text{Final RNA concentration in } \eta g/\mu l$$

2.6 Reverse Transcription

RNA extracted from mESCs, EBs and MEFs was reverse transcribed into cDNA using the Reverse Transcription System (Promega, Southampton, Hampshire, UK) containing the reverse transcriptase enzyme derived from the Avian Myeloblastosis Virus (AMV). Reactions took place in 20µl volumes consisting of 5mM $MgCl_2$, 1mM dNTPs, 1x buffer, 20 units recombinant RNasin® inhibitor, 0.25µg Oligo$(dT)_{15}$ Primers, 800ηg extracted RNA, and 15 units AMV reverse transcriptase. RNA extracted from oocytes and embryos was reverse transcribed into cDNA using the Absolute 2-step QRT-PCR SYBR Green kit (Abgene, Epsom, UK) containing reverse transcriptase enzymes (QRTase) from both Moloney Murine Leukemia Virus (MMuLV) and AMV. Reactions took place in 20µl volumes consisting of 2mM dNTPs, 1x buffer, 5% (v/v) QRTase enhancer, 0.5µg Oligo $(dT)_{15}$ primers, 5µl extracted RNA (Equivalent to 1.25 oocytes/ embryos) and 5% (v/v) QRTase. The final volume was made up to 20µl with sterile ddH_2O. For each sample, a duplicate reaction was set up containing no reverse transcriptase enzyme. Subsequent PCR amplification would therefore allow confirmation of successful elimination of genomic DNA. A reaction containing no RNA was also set up, allowing for the detection of any contaminated reagents. The reverse transcription reaction was run for 2 hrs at 42ºC (Reverse Transcription System) or 1 hr at 42°C followed by 75°C for 10 mins (Absolute 2-step QRT-PCR kit) in a PCR machine (MJ Research PTC-200 DNA engine, GRI, Braintree, Essex, UK). Samples were then diluted for use in PCR reactions by addition of 80µl sterile ddH_2O.

2.7 The polymerase chain reaction

The polymerase chain reaction (PCR) is a method of amplification of DNA. Double stranded DNA or cDNA is first denatured at temperatures around 95°C. Cooling to around 60°C allows

binding of two short primer sequences complementary to the DNA to be amplified. This is followed by a temperature increase to the optimal temperature of a DNA polymerase, approximately 72°C. The DNA polymerase is then able to produce a copy of the DNA between the two primers. Repeating this process 30 to 50 times allows for production of many copies of the original template DNA that are then detectable on an agarose gel containing a DNA stain such as ethidium bromide. Mouse primers for the genes of interest were designed from published sequences and are listed in Chapter 5. Primers for porcine mitochondrial genes were designed from the published mtDNA sequence (Accession number NC_000845). There were no published porcine sequences for nuclear genes including TFAM, PolGA and PolGB. For these genes, published human and mouse sequences were analysed and primers were designed from homologous regions. In some cases, new primers were designed from sequences obtained from DNA extracted from gel bands. All potential primer sequences were entered into the NCBI BLAST program to search for homology and specificity to the sought-after gene (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Annealing temperatures were calculated using the following formula (Sambrook *et al.*, 1989):

$$T_m = 0.41 \times \%GC + 64.9 - \frac{(600)}{n}$$

Results from this formula were used as a basis for determining the optimal annealing temperature for each primer pair. Mouse product sizes were determined from the published sequences and mouse and human sequences were used to estimate the size of the porcine product. It was assumed that the processivity of the BioTaq Polymerase enzyme was 10bp DNA per second, as stated by the manufacturer. Extension periods were therefore adjusted to

suit each primer pair. Primer sequences, annealing temperatures, PCR reaction conditions and product sizes are shown in the relevant Chapters.

PCR reactions containing 10µl (oocytes/embryos) or 7.5µl (other cell types) diluted cDNA or 2µl DNA were run in 20µl volumes consisting of 1x PCR buffer (Bioline, London, UK), 1.5mM $MgCl_2$ (Bioline), 200µM Nucleotide Mix (Bioline), 0.5µM each primer and 1U BioTaq polymerase (Bioline). Reaction conditions were: initial denaturation at 94°C for 5 mins followed by 35 cycles of denaturation at 94°C, annealing at the appropriate temperature (see individual Chapters) and extension period (see individual Chapters) at 72°C with a final extension of 72°C for 5 mins. The reactions were run in an MJ Research PTC-200 PCR machine (GRI), which was preheated to 94°C before the reaction tubes were added. As this is the temperature of the denaturation phase, the production of primer-dimers is reduced and the efficiency of the reaction is increased.

2.8 Real time PCR

Real time PCR allows quantification of double stranded DNA using the DNA intercalating dye, Sybr Green. Sybr Green fluoresces when bound to double stranded DNA (Ririe *et al.*, 1997), such as during the extension phase of a PCR cycle, but does not fluoresce when no double stranded DNA is present, such as after the denaturation phase of a PCR cycle (see Figure 2.1). During the real time PCR reactions described in this thesis, fluorescence levels were measured at the end of the extension phase of each PCR cycle. These levels increase exponentially with each cycle, correlating to exponential increases in double stranded DNA (Bustin, 2000). Cycle threshold (Ct) values state the cycle number at which the fluorescence detected crosses a threshold level deemed to be significantly higher than background (Gibson

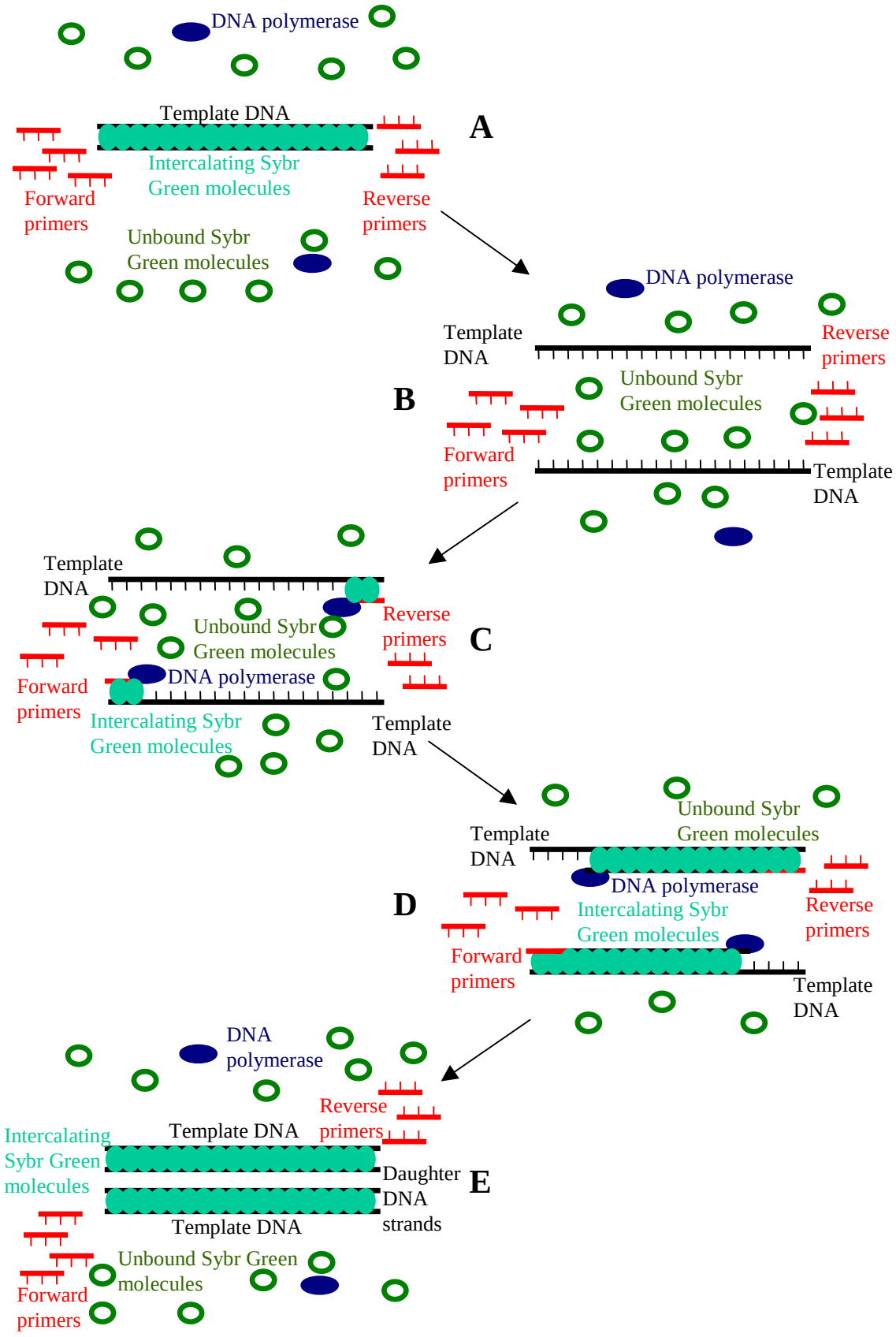
et al., 1996). Samples containing large amounts of template DNA at the start of the real time PCR reaction will exhibit a large exponential increase in fluorescence and will therefore cross the threshold earlier than samples initially containing smaller amounts of template DNA (Bustin, 2000). Ct values obtained for a set of standards of known template DNA concentration can be plotted against the logarithm of the initial template DNA concentration in order to produce a standard curve with the following linear equation:

$$y = mx + c$$

where y is the Ct, m is the gradient of the slope, x is the initial concentration of the template DNA, and c is the required constant that is calculated by the real time PCR machine software (RotorGene; Version 7.0; Corbett Research, Sydney, Australia). This equation can then be used to calculate the initial template concentration of unknown samples from the Ct values obtained.

Figure 2.1 (next page): Sybr Green intercalation into double-stranded DNA during a PCR cycle

A: At the beginning of a PCR cycle, the double stranded DNA template is present and Sybr Green intercalates and fluoresces. B: Template DNA is denatured and Sybr Green is released so no fluorescence can be detected. C: Primers anneal to the template DNA producing a small amount of fluorescence due to Sybr Green intercalation. D: A new copy of the template is synthesised so Sybr Green intercalation and fluorescence levels increase. E: The completed copy of the template DNA is present and Sybr Green intercalation and fluorescence levels have increased two-fold since the beginning of the cycle.


DNA polymerase
Template DNA
A
Intercalating Sybr Green molecules
Forward primers
Reverse primers
Unbound Sybr Green molecules
DNA polymerase
Template DNA
Reverse primers
B
Unbound Sybr Green molecules
Forward primers
Template DNA
Template DNA
Unbound Sybr Green molecules
Reverse primers
C
Forward primers
DNA polymerase
Intercalating Sybr Green molecules
Template DNA
Unbound Sybr Green molecules
Template DNA
DNA polymerase
D
Intercalating Sybr Green molecules
Reverse primers
Forward primers
Template DNA
DNA polymerase
Reverse primers
Intercalating Sybr Green molecules
Template DNA
Daughter DNA strands
E
Template DNA
Unbound Sybr Green molecules
Forward primers

Standards of known concentration were prepared using conventional PCR to amplify the gene of interest. DNA was extracted from resulting agarose gels (see Section 2.9) and used to produce a series of 10-fold dilutions. Real time PCR reactions were run in 15µl volumes containing 2µl of sample or standard, 7.5µl of ABsolute™ QPCR SYBR® Green Mix (Abgene, UK) and 0.1µl of each primer (50pm/µl, Alta Bioscience, Birmingham, UK or Invitrogen). All 15µl reactions were prepared using a CAS-1200 Robotic Liquid Handling System and its associated software (Version 3.08; Corbett Research). Real time PCR amplification was carried out using the RotorGene 3000 real time PCR machine (Corbett Research). The reaction conditions included an initial 15 min hold at 95°C to activate the DNA polymerase enzyme, followed by 50 cycles of denaturation for 10s at 95°C, annealing for 15s at the appropriate temperature (see individual Chapters) and extension and data acquisition on the FAM/Sybr channel (excitation at 470ηm and detection at 510ηm) at 72°C for either 15 or 20s depending on the product size (see individual Chapters). The same aliquots of standards were used for all reactions containing the same primer pair.

At the end of each real time PCR reaction, a melt analysis was performed. This involved denaturation of the PCR products through heating by 1°C at a time, followed by measurement of the fluorescence at each temperature. The temperature at which a PCR product melts is dependent on its length, sequence and GC content and is therefore likely to differ between different products and between an expected product and an unexpected product such as primer-dimers (Ririe *et al.*, 1997). Melting was carried out from 72°C to 99°C in steps of 1°C, waiting 45s after the first step and 5s after each subsequent step. Melt data were acquired on the FAM/Sybr channel and was analysed by the RotorGene software (Version 7.0; Corbett Research), which produced a peak for each product present. It was confirmed that each

sample peak was at the same temperature as the standard peak and that no extra peaks were present before the concentration of the initial template DNA in each sample was quantified.

2.9 Gel electrophoresis

2% agarose gels were produced using 2g agarose (Bioline) in 100ml Tris acetate EDTA (TAE) buffer (see Appendix I). The mixture was heated for approximately 1 min in a microwave to dissolve the agarose. 6µl 10µg/ml ethidium bromide was then added before the gel was poured into a mould containing combs. The gel was then allowed to set before it was transferred to an electrophoresis tank (Biorad, California, USA) and covered with TAE buffer. PCR product samples were prepared by adding 4µl 6x gel loading buffer (see Appendix I) to 20µl of the PCR product. Combs were removed from the gel and the samples were loaded. The loading buffer prevents leakage from the loading wells and, containing a dye, also allows visualisation of the progress of the gel. A 100bp ladder (0.2µg/µl; New England Biolabs, Massachusetts, USA) was also added to one well of each row. Gels were run at 100V for 60 mins to separate the DNA according to size. DNA bands were visualised under UV light using a UV transilluminator (Nucleotech, California, USA) and images were captured using the Nucleovision system and its associated Gel Expert software (Nucleotech, San Mateo, CA, USA).

2.10 DNA extraction from gel bands

Bands were cut from the gel using a clean scalpel and transferred to a 1.5ml centrifuge tube. DNA was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen, Crawley, West Sussex, UK). 300µl buffer QG was added to the gel, which was incubated at 50°C for 10 mins or until the gel had dissolved. 100µl was added to the mixture, which was then

transferred to a spin column placed inside a collection tube, provided with the kit. The column was centrifuged at 13000 rpm for 1 min to allow DNA binding to the silica-based filters and the flow-through was discarded. 0.5ml buffer QG was then washed through the column to remove traces of agarose. 0.75ml buffer PE was washed through the column to remove primers and other impurities and the column was then centrifuged again to remove residual buffer PE. The bound DNA was then eluted into a clean tube with 30µl elution buffer and stored at 4˚C for up to 7 days or at -20˚C for longer periods of time.

A spectrophotometer was used as described above to obtain the DNA concentration. The absorbance value was entered into the following formula (Sambrook *et al.*, 1989) to calculate the final DNA concentration:

$$\frac{\text{Absorbance at 260ηm x 50 (µg/ml total DNA at absorbance of 1)}}{\text{0.005 (dilution factor)}} = \text{final DNA concentration in ηg/µl}$$

2.11 DNA transformation by *E. coli*

The TOPO-TA cloning kit (Invitrogen) was used to ligate extracted DNA from agarose gel into a vector. The vector was then transformed into *E. coli* which replicates and amplifies the ligated DNA. The ligation reaction consisted of: 1µl salt solution (1.2M NaCl and 0.06M $MgCl_2$, Invitrogen) added to approximately 120ηg extracted DNA. The volume was made up to 5µl with sterile water. 1µl TOPO-TA cloning vector was added before incubation at room temperature for 30 mins to allow target DNA ligation into the vector. 2µl of this TOPO ligation reaction was then added to 25µl of One Shot Chemically Competent *E. coli* (Invitrogen) and incubated on ice for 30 mins. The cells were then heat shocked at 42ºC for

30 seconds and added to 300µl SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ 20mM glucose, Invitrogen). The mixture was then shaken horizontally at 37ºC for 1 hr.

LB-Agar plates were prepared by dissolving 4 LB-Agar tablets in 200ml sterile ddH_2O and autoclaving the solution. 400µl 1000x ampicillin (100mg/ml in sterile ddH_2O) was added to produce a final concentration of 200µg/ml. 10ml LB-Agar-ampicillin solution was added to 10cm petri dishes (BD Biosciences) and allowed to set at room temperature for approximately 2 hrs. 100µl transformed *E. coli* was then spread across a selective LB-agar plate and incubated at 37ºC for 8 to 16 hrs to allow colonies to form. LB media was prepared by dissolving 4 LB tablets in 200ml sterile ddH_2O, which was then autoclaved. 200µl 1000x ampicillin (100mg/ml in sterile ddH_2O) was added to produce a final concentration of 100µg/ml. 5µl LB-ampicillin solution was transferred to a sterile 25ml universal tube (SLS). Individual colonies were isolated from the agar plates using a pipette tip and transferred into the LB-ampicillin solution. Lids were screwed on loosely and the tubes were gently shaken horizontally at 37ºC overnight.

2.12 Plasmid extraction from *E. coli*

Universal tubes containing *E. coli* were centrifuged at 2000 rpm for 10 mins to pellet the cells. DNA was extracted using the QIAprep miniprep kit (Qiagen). Pellets were resuspended in 250µl buffer P1 to lyse the cells. 250µl buffer P2 was then added and the tubes were inverted 4 to 6 times to mix the solutions. 350µl buffer N3 was added to neutralise the acidic lysis solutions and the mixture was then centrifuged for 10 mins at 13000 rpm. The resulting supernatant was placed inside a spin column (Qiagen) and centrifuged to bind the plasmid

DNA to the filter. Buffer PB was washed through the filter to remove nucleases followed by buffer PE to remove other impurities. The column was then centrifuged again to remove traces of buffer PE. The DNA was then eluted into a clean tube using 30µl elution buffer. DNA concentrations were calculated as described above (see Section 2.10)

DNA extracted from the transformed *E. coli* was used as a template for PCR using forward (GTAAAACGACGGCCAG) and reverse (CAGGAAACAGCTATGAC) M13 primers, which amplify a region of the TOPO cloning vector containing the inserted sequence. Agarose gel electrophoresis was used to confirm the presence of an insert of the correct size. Conventional PCR with primers specific for the insert sequence was also carried out to confirm insertion of the correct product.

2.13 Sequencing

PCR products from the M13 PCR reactions were used in sequencing reactions to obtain porcine cDNA sequences for the genes of interest. 3.2pmol M13 forward primer was added to approximately 200ηg plasmid DNA and sterile water was used to produce a final volume of 10µl. Sequencing reactions were carried out by the Functional Genomics Laboratory within the School of Biosciences, University of Birmingham. Here, our 10µl sequencing reaction mixes were added to 8µl Terminator Ready Reaction Mix (ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, California, USA) containing optimised concentrations of deoxynucleoside triphosphates, fluorescence-tagged terminating analogues, DNA polymerase, $MgCl_2$ and the appropriate buffer. Sequencing was performed using 25 cycles of 96˚C for 10s, 50˚C for 5s and 60˚C for 4 mins using a GeneAmp 9700 sequencing machine (Applied Biosystems).

2.14 Immunocytochemistry

Immunocytochemistry (ICC) uses antibodies raised against proteins of interest (primary antibodies) in order to determine the localisation of proteins in cultured cells, tissues, oocytes, embryos and many other biological samples. Cells/tissues must first be fixed and also permeabilised if the protein of interest is not on the cell surface. Antibodies often bind non-specifically to proteins other than that which they were raised against. Consequently, samples must be pre-incubated in a blocking solution that contains proteins such as BSA, which bind to these non-specific sites. The primary antibodies may therefore only bind to their specific sites for which they have a higher affinity than the blocking proteins. After primary antibody incubation, cells must be washed in order to remove excess antibody that has not bound to the target protein. Cells are then incubated with a secondary antibody that is raised against the primary antibody and also contains a marker molecule such as a fluorescent tag. After further washing to remove excess secondary antibody, fluorescence microscopy allows visualisation of the localisation of the target protein within a cell.

2.14.1 Fixation

Oocytes/ embryos were pipetted up and down to remove cumulus cells, if necessary, before being placed in 500µl 3.8% formaldehyde (VWR, Lutterworth, Leicestershire, UK; see Appendix I) diluted in PBS, in 4 well dishes (Nunc) for 15 mins at room temperature.

2.14.2 Permeabilisation

The cell membranes were permeabilised to allow antibodies to enter the oocytes/ embryos and bind to the proteins inside. Oocytes/ embryos were transferred to 500µl 0.1% (v/v) Triton X-100 diluted in PBS (see Appendix I) for 5 mins at room temperature for permeabilisation.

2.14.3 Blocking

Oocytes/embryos were placed in 500µl blocking solution (PBS containing 100 µM glycine and 2mg/ml BSA; see Appendix I) for either 30 mins at room temperature or at 4ºC for up to 4 weeks. The BSA in the solution would then bind to any non-specific protein binding sites within the cells to prevent non-specific staining after antibody incubation, as described in Section 2.14.

2.14.4 Antibody staining

Preliminary experiments were performed to determine the optimum incubation times and concentrations of both primary and secondary antibodies, which are listed in Table 2.1. Antibodies were diluted in blocking solution. Negative control experiments were carried out where cells were incubated in blocking solution without primary antibody and then incubated in blocking solution containing secondary antibody. Following each antibody incubation, cells were washed in 0.1% Triton X-100 diluted in PBS (see Appendix I) for 30 mins at room temperature.

2.14.5 Mounting

Oocytes/ embryos were placed in 4µl Vectashield (VectaLabs, California, USA) containing DAPI on a microscope slide with ridges (SLS, Nottingham, Nottinghamshire, UK). The ridges raise the level of the coverslip and therefore prevent the oocyte/embryo from being squashed. 10mm diameter coverslips were gently placed over the Vectashield containing the oocytes/ embryos. Nail varnish was used to seal the coverslips in place. This was allowed to dry for at least 30 mins and then the slides were stored at 4ºC.

Table 2.1: Antibody details and concentrations

Protein	Primary AB	Primary AB concentration	Secondary AB	Secondary AB concentration
COXI	Mouse IgG2a anti COXI (Molecular Probes)	2µg/ml	Alexa Fluor ® 488 anti-mouse IgG (Molecular Probes)	2µg/ml
TFAM	Goat IgG anti TFAM (Santa Cruz)	4µg/ml	Alexa Fluor ® 593 anti-goat IgG (Molecular Probes)	1µg/ml
PolGA (green)	Rabbit IgG anti PolGA (Abcam)	20µg/ml	Alexa Fluor ® 488 anti-rabbit IgG (Molecular Probes)	2µg/ml
PolGA (red)	Rabbit IgG anti PolGA (Abcam)	20µg/ml	Alexa Fluor ® 593 anti-rabbit IgG (Molecular Probes)	1µg/ml

2.15 Mitochondrial staining

Oocytes were cultured in IVM media supplemented with 0.5µg/ ml MitoTracker Green, a mitochondrial-specific fluorescent probe, for 30 mins. Oocytes were then washed three times in PBS.

2.16 Membrane staining

Cells from a confluent well of a 6-well plate were removed from the plate by treatment with trypsin-EDTA for 2 to 5 mins for use in ESC membrane staining experiments using the PKH67-GL labelling kit. 5ml media was added to inactivate the trypsin and the cell suspension was centrifuged for 5 mins at 350 rpm. The supernatant was removed and cells were resuspended in 2ml PBS. Cells were then centrifuged again for 5 mins at 400 rpm. The supernatant was removed from the cell pellet which was resuspended in 1ml diluent C. During the centrifugation, a 2x PKH67 dye solution was prepared by diluting 4µl PKH67 dye to 996µl diluent C, the iso-osmotic solution designed to maximise staining efficiency and maintain cell viability. The cell suspension was then combined with the 2x dye solution and incubated at room temperature for 2 to 5 mins. The staining reaction was terminated by addition of 2ml culture media containing serum followed by incubation at room temperature for 1 min. 4ml media was then added to the cell solution which was centrifuged for 10 mins at 400 rpm. After removal of the supernatant, the cell pellet was transferred to a clean centrifuge tube and resuspended in 1ml culture media. The centrifugation step was then repeated three times to remove any traces of the dye from the media.

2.17 Conventional fluorescent microscopy

Slides were viewed by standard fluorescent microscopy using an Axioplan 2 Imaging System (HBO100, Zeiss, Welwyn Garden City, Hertfordshire, UK) and images were captured on an AxioCam HR Digital Camera (Zeiss). DAPI was excited at 395ηm and detected at 420ηm, FITC excitation was at 488ηm with detection from 515-565ηm and rhodamine excitation was at 450ηm with detection from 580-590ηm. The automatic exposure feature was switched off, allowing the exposure time to remain the same for all samples and negative controls. This prevented the generation of false positive results. The data were collected and merged images were produced using the Axiovision LE Rel 4.2 Programme (Zeiss).

2.18 Confocal microscopy

This technique allows visualisation of fluorescence along one plane, providing a more detailed analysis of protein localisation within cells. Slides were viewed using the Leica DM IRE2 Confocal Microscope with Leica TCS SP2 Scanner (Leica Microsystems Ltd, Milton Keynes, Buckinghamshire, UK) and the fluorescence data were collected using the Leica Confocal Software (Leica). FITC fluorescence was excited at 488ηm and detected at 500-535ηm and rhodamine was excited at 593ηm with detection from 600-700ηm. To allow comparative analysis of protein expression across different oocytes or embryos, the auto gain function was switched off and the same gain and photomultiplier settings were used for each embryo analysed. Each channel was adjusted before the images were taken to remove the effects of bleed into adjacent channels and each image was produced from the average fluorescence data obtained from 25 scans, thereby decreasing the effect of any background fluorescence. When oocytes/ embryos were co-stained with two primary antibodies, FITC and Rhodamine images

were acquired at the same time so that each pair of images corresponded to the same section of the oocyte or embryo, thereby allowing assessment of protein colocalisation.

2.19 Western blotting

Porcine protein extracted from heart tissue and human protein extracted from umbilical vein endothelial cells were run on a 12% polyacrylamide gel, alongside an SDS molecular weight marker (range 6.5 to 175 kDa; New England Biolabs), for 1 hr at 140V. Gels were blotted onto Immobilon P transfer membranes, which were then blocked with 5% Marvel in TBS. Blots were incubated with TFAM primary antibody (0.8μg/ml), either with or without the associated blocking peptide (Santa Cruz), overnight at 4°C. After washing in TBS-T 5 times for 10 mins each, the blot was incubated with the secondary antibody (Rabbit anti-goat IgG conjugated to horseradish peroxidase) for 1 hr before washing a further 5 times for 10 mins each in TBS-T. Visualisation of protein bands was through the ECL Western Blotting System (Pierce Biotechnology Inc.).

CHAPTER 3: mtDNA REPLICATION DURING PRE-IMPLANTATION EMBRYOGENESIS

3.1 Introduction

ATP can be generated through a variety of processes, although the most efficient method is OXPHOS via the ETC (Pfeiffer *et al.*, 2001). It has previously been demonstrated that the ATP content of individual embryonic blastomeres is correlated to their mitochondrial content (Van Blerkom *et al.*, 2000), suggesting that mitochondrially-derived ATP is required during early embryogenesis. Furthermore, aggregation of active mitochondria has been reported around the pronuclei of fertilised oocytes and/or around the nuclei of cleavage stage embryos in many species including the monkey (Squirrell *et al.*, 2003), hamster (Barnett *et al.*, 1996; Bavister & Squirrell, 2000), mouse (Batten *et al.*, 1987) and human (Wilding *et al.*, 2001). This is thought to provide energy for processes such as spindle organisation and chromosome segregation (Van Blerkom, 2004). Mitochondrial structure also changes in early embryos to an active, elongated structure with large numbers of cristae (Piko & Matsumoto, 1976). This is in contrast to the inactive, small, spherical organisation observed in MII oocytes (Van Blerkom *et al.*, 1998) These data indicate an important role for mitochondrial ATP production during embryogenesis.

Mitochondrial ETC activity, and therefore ATP production, requires the transcription and replication of mtDNA, with mtDNA copy number determining the OXPHOS capacity of a cell (Moyes *et al.*, 1998). Despite the requirement for mitochondrially derived ATP, mtDNA replication is not thought to occur during pre-implantation embryo development in many species including the frog (el Meziane *et al.*, 1989), mouse (Ebert *et al.*, 1988; Piko & Taylor,

1987) and shrimp (Vallejo *et al.*, 1996). Absence of mtDNA replication until the blastocyst stage was recently confirmed in the mouse (Thundathil *et al.*, 2005) and has also been reported in cattle (May-Panloup *et al.*, 2005b). However, a short burst of mtDNA replication has also been reported recently in murine 2-cell embryos, although this was not associated with an increase in mtDNA copy number (McConnell & Petrie, 2004). In order to support the mitochondrially-derived ATP requirement of early embryos, evidence in many species suggests that mtDNA replication occurs during oocyte development (see Section 1.3.1). Mature oocytes therefore contain high levels of mtDNA to support the ATP requirements of the early embryo. It has also been previously reported that there is a requirement for high levels of mtDNA in porcine oocytes (El Shourbagy, 2004) indicating that mtDNA replication may also not occur during pre-implantation development in larger mammals, such as pigs.

MtDNA replication is regulated by the expression of nuclear-encoded replication factors such as TFAM and PolG (see Sections 1.2.3.1 and 1.2.3.2). These proteins are vital for early post-implantation embryogenesis, as determined by the death of knock-out mice on E8.5 and E10.5, respectively (PolG: Hance *et al.*, 2005; TFAM: Larsson *et al.*, 1998). However, one report suggests that PolG and TFAM are not expressed during early mouse pre-implantation embryogenesis (Thundathil *et al.*, 2005). The expression of nuclear-encoded mtDNA replication factors and the occurrence of mtDNA replication during porcine pre-implantation embryogenesis were therefore investigated.

3.2 Hypothesis

The absence of expression of vital nuclear-encoded replication factors contributes to the strict regulation of mtDNA replication that occurs during porcine pre-implantation embryo development.

3.3 Aims

Using porcine IVF embryos from the 2-cell to the blastocyst stage, this Chapter will determine whether:

1. the genes for key mtDNA replication factors are expressed throughout pre-implantation development.
2. the mRNA derived from genes encoding mtDNA replication factors is translated into protein.
3. mtDNA replication takes place during pre-implantation development.

3.4 Preliminary Experiments

3.4.1 Primer design for mtDNA replication factor genes

As there were no published sequences available for the porcine TFAM, PolGA and PolGB genes, human and mouse cDNA sequences were entered into Clustalw (http://www.ebi.ac.uk/clustalw) in order to identify regions of homology between the two species. Forward and reverse primers were designed in these regions within different exons, thereby eliminating the possibility of genomic DNA amplification. Potential primer sequences were entered into the NCBI BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to confirm specificity to the gene of interest before being used to amplify cDNA extracted from 50 porcine oocytes using conventional PCR. Primers and reaction conditions for conventional PCR are displayed in Table 3.1. PCR products were then run on 2% agarose gels (see Section 2.9). The DNA fragments were excised from the gels and ligated into TOPO-TA cloning vectors for DNA sequencing of porcine TFAM, PolGA and PolGB cDNA (see Sections 2.10-2.13). Finally, the sequences obtained for each gene were entered into Clustalw (http://www.ebi.ac.uk/clustalw) in order to determine the level of homology with the human and mouse sequences.

Table 3.1: Primers and reaction conditions for conventional PCR

Gene	Forward primer	Reverse Primer	Annealing temperature (°C)	Reaction conditions (denaturation; annealing; extension time(s))	Expected product size (bp)
TFAM	GGG AAC TTC CTG ATT CAG AAA	CGA GGT CTT TTT GGT TTT CCA	54	30; 30; 30	211
PolGA	GAG CAT GCA CAT GGC CAT CT	GAG GTT TTC CAG CAG CAG CT	57	30; 30; 50	345/348
PolGB	CTT CTT CAC GGT GCC TTG GA	GAA GCT TCA GTC TTT TCA CCG	54	30; 30; 50	161
ND2	ACC CTA CTC TCA ATA GGA GGC C	TTA GGG ACG AGA GGG CTG GTG	60	30; 30; 45	259

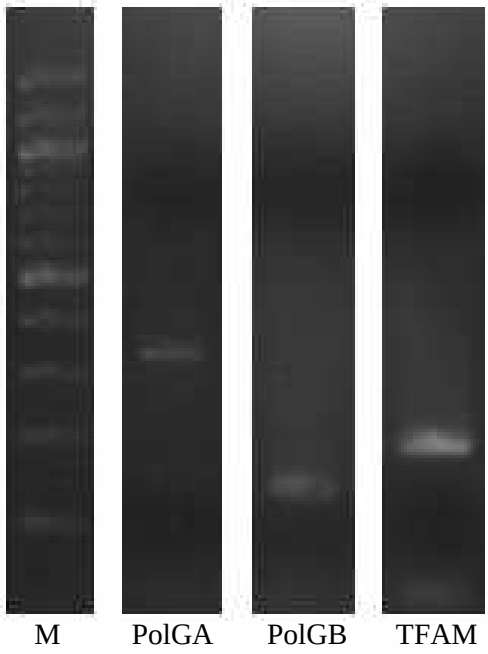


Figure 3.1: Agarose gel of porcine TFAM, PolGA and PolGB PCR products

Primers amplified a PolGA product of approximately 340bp, a PolGB product of approximately 160bp and a TFAM product of approximately 200bp. This is consistent with published human and mouse sequences (see Table 3.1) and those more precise sizes obtained following sequencing of the DNA fragments (see Figure 3.2). NB: Lanes containing other samples have been removed from this gel.

Primers for TFAM, PolGA and PolGB porcine cDNA each amplified a product consistent with the size of the published human and mouse sequences (see Figure 3.1). Sequencing determined that the porcine TFAM fragment was 212bp long and showed 86% homology to the human sequence and 73% homology to the mouse sequence (Figure 3.2A). The porcine PolGA fragment was 341bp long and showed 83% homology to the human sequence and 81% homology to the mouse sequence (Figure 3.2B), whilst the porcine PolGB fragment was 167bp long and showed 88% homology to the human sequence and 82% homology to the mouse sequence. The level of homology of each gene to published sequences from other species, combined with their similar product sizes, suggests that the primers were actually amplifying the appropriate genes.

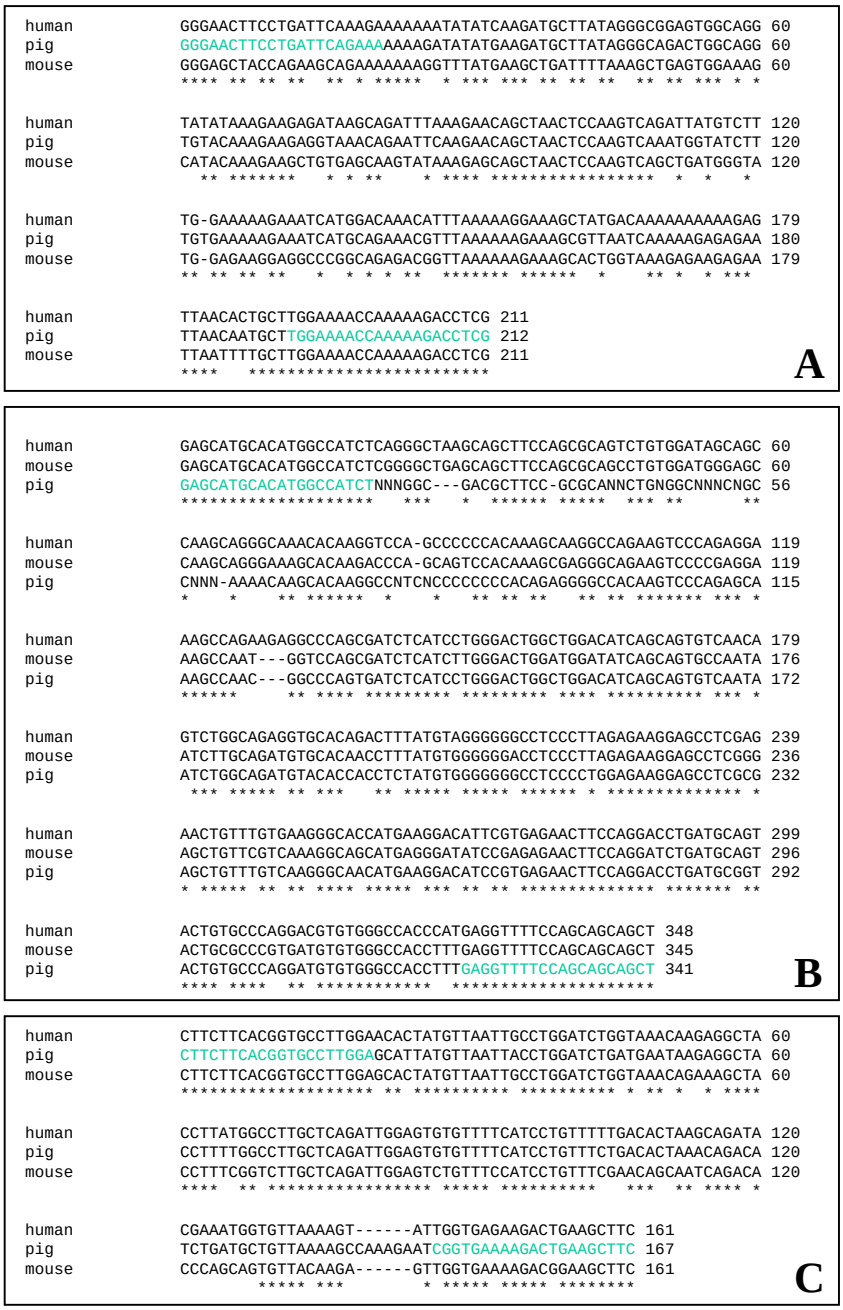


Figure 3.2: Porcine cDNA sequences obtained for TFAM, PolGA and PolGB

Porcine TFAM (A), PolGA (B) and PolGB (C) sequences are aligned with equivalent regions of the published human and mouse sequences. PCR primers are indicated by the turquoise font. TFAM PCR product is 212bp long, PolGA is 345bp long and PolGB is 167bp long, consistent with the gels in Figure 3.1. All porcine sequences show high levels of homology with published human and mouse sequences, as indicated by the asterisks.

3.4.2 Conventional RTPCR embryo analysis

Early embryos are relatively transcriptionally inactive (Newport & Kirschner, 1982; Telford *et al.*, 1990) and lack of mtDNA replication in murine pre-implantation embryos has been reported previously (Piko & Taylor, 1987). This indicates that mtDNA replication factors might be expressed at relatively low levels in porcine pre-implantation embryos. Batches of 5 porcine embryos were therefore used to test whether conventional RTPCR would be sensitive enough for amplification of mtDNA replication factors. RNA extraction and reverse transcription were carried out as described in Sections 2.4.2 and 2.6. Primer details and reaction conditions for PCR are listed in Table 3.1. PCR products were separated on 2% agarose gels and visualised as previously described (see Section 2.9).

Analysis of the mitochondrially encoded ND2 gene was carried out first to confirm that the RNA extraction and reverse transcription procedures had been successful. Agarose gel electrophoresis of ND2 PCR products demonstrated that 6 of the 12 embryo samples and the positive control (cDNA obtained from porcine ovarian follicle cells) produced a product (see Figure 3.3). The presence of product for only some of the samples suggested that the RNA and reverse transcription procedures had been successful but that some embryo samples were not expressing as much ND2 as others. Absence of product in the negative controls lacking the reverse transcriptase enzyme (NEC) for each sample confirmed the elimination of genomic DNA from the RNA samples, whilst absence of a product in the PCR negative control eliminated contamination of the PCR reagents as a source of the product in the sample lanes. Early tests on embryos for TFAM, PolGA and PolGB expression were either negative or produced low levels of product (gels not shown). Furthermore, as described above, only half of the embryo samples produced a product for ND2. These data indicated that the more sensitive

real time RTPCR technique would be required to analyse the expression of TFAM, PolGA and PolGB in embryos.

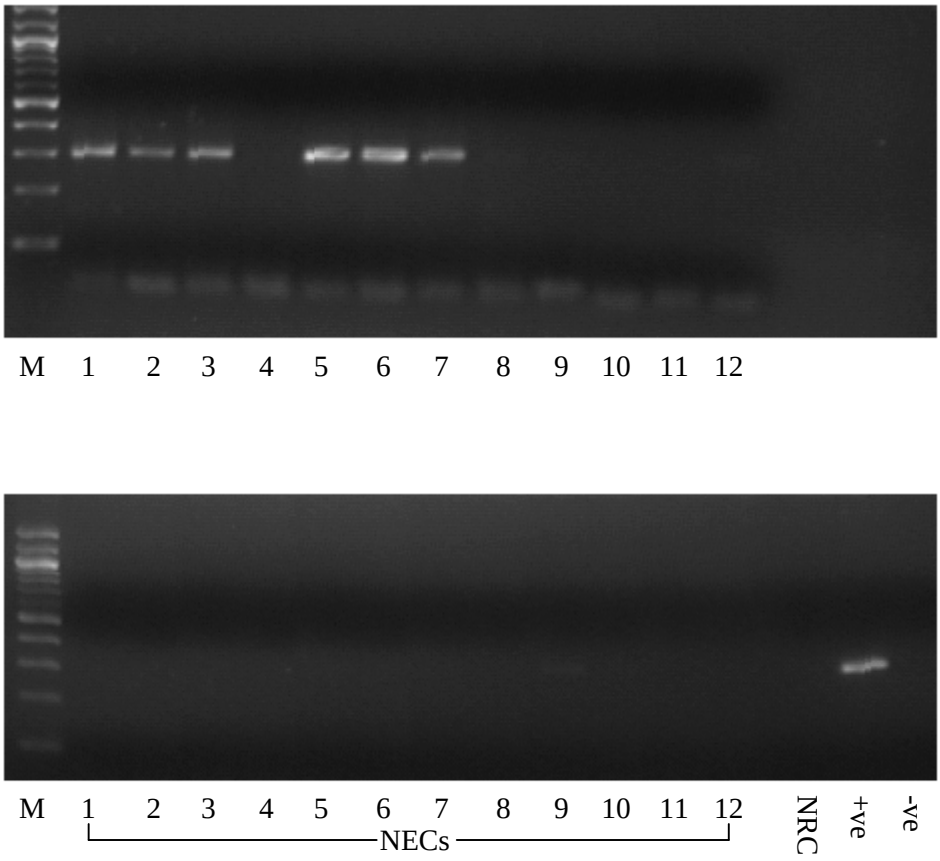


Figure 3.3: Agarose gel electrophoresis analysis of the embryo ND2 PCR products

The top row shows 100bp ladder (M) and 12 embryo samples (1-12), with each embryo containing between one and 12 blastomeres. The bottom row shows 100bp ladder (M), negative controls lacking the reverse transcriptase enzyme for each embryo sample (NECs 1-12), reverse transcription negative control lacking RNA (NRC), PCR positive control (+ve) and PCR negative control (-ve). Bands are present in 6 of the 12 embryo samples and the positive control. None of the negative controls (NECs, NRC, -ve) contain any bands.

3.4.3 Optimisation of real time PCR reaction conditions

Real time PCR allows quantification of the starting level of DNA by comparing fluorescence values of the samples to those obtained for standards of known DNA concentration, as described in Section 2.8. Ten-fold dilutions of conventional PCR product for each gene were prepared as previously described (see Section 2.8) for optimisation of real time PCR primer concentrations, annealing temperatures and/or extension periods for each gene. TFAM and PolGB primers were consistently unable to amplify the lowest concentration standards. As it was expected that the target genes would be transcribed at a low level, this was considered inappropriate. Therefore, a new forward primer was designed for these genes using the newly obtained porcine cDNA sequences (see Figure 3.2). Primer sequences used for real time RTPCR analysis and their optimal reaction conditions are displayed in Table 3.2.

Standard curves were generated for each gene by the RotorGene software (Version 7.0; Corbett Research), using the optimal reaction conditions displayed in Table 3.2 (see Figure 3.4). Each curve had an R^2 value of ≥ 0.99, indicating that the fluorescence results from the standard tubes are consistent with the hypothesis that the standards produce a standard curve suitable for subsequent sample quantification. Each curve also has an efficiency value within the acceptable range of between 0.6 and 1.1 (Pfaffl, 2003), indicating that the fluorescence values increase approximately 2-fold after each cycle. This is consistent with optimal primer, $MgCl_2$ and Sybr Green concentrations.

Table 3.2: Real time RTPCR primers and reaction conditions

Gene	Forward primer	Reverse Primer	Annealing temperature (°C)	Second acquisition temperature (°C)	Reaction conditions (denaturation; annealing; extension time(s))	Expected product size (bp)	Expected number of MtDNA copies in 1st standard
TFAM	CAG ACT GGC AGG TGT ACA	CGA GGT CTT TTT GGT TTT CCA	54	77	10; 15; 15	164	222557195
PolGA	GAG CAT GCA CAT GGC CAT CT	GAG GTT TTC CAG CAG CAG CT	57	84	10; 15; 20	341	107036304
PolGB	GTG CCT TGG AGC ATT ATG TT	GAA GCT TCA GTC TTT TCA CCG	54	79	10; 15; 20	167	232480127

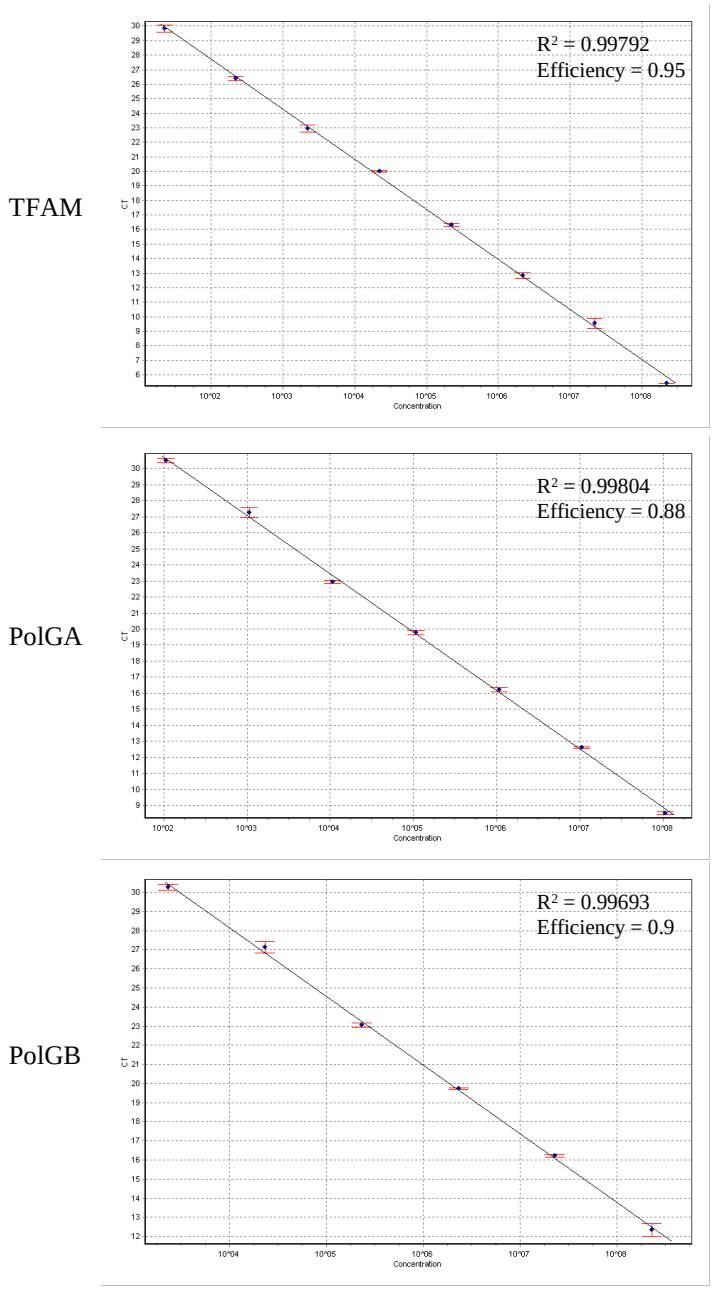


Figure 3.4: Standard curves for TFAM, PolGA and PolGB

Mean ± SEM Ct values are plotted against the log concentration of the standards of 10-fold dilutions. Each curve has an R^2 value ≥ 0.99, due to an equal number of cycles separating standards of 10-fold concentration difference. Each curve also has an efficiency value within the acceptable range of between 0.6 and 1.1 (Pfaffl, 2003).

Melt curves for each reaction were analysed to ensure that only one product was being amplified and then products were separated on agarose gels in order to confirm that the amplified product was of the correct size. TFAM product was 164bp long, and produced a melt peak at 79°C, PolGA product was 341bp long, coinciding with a melt peak at 89°C and PolGB product was 157bp long and produced a melt peak at 83°C (see Figure 3.5). The melt temperatures were used to determine the second acquisition temperatures for each gene (see below). Some of the least concentrated standards produced melt peaks at lower temperatures than the products, as well as the product peak. The negative controls not containing template DNA (NTC) run in each reaction produced only these lower peaks.

Agarose gel separation confirmed that the lower peaks were produced by primer-dimerisation. In the lower standards, primer-dimers were produced as well as the correct gene product, which, if occurring in sample reactions, could cause inaccuracies in subsequent mRNA copy number calculations. An extra step was therefore inserted into each real time PCR reaction, after the extension period. This consisted of a 15s hold at a temperature between the product and the primer-dimer melt temperature (see Table 3.2), which allowed time for the primer-dimers to melt. A second fluorescence acquisition at the end of this hold step therefore included only fluorescence generated by the target product (Pfaffl *et al.*, 2002). Interestingly, the intensities of the bands in the gels presented in Figure 3.5 do not necessarily coincide with the size of the corresponding melt peaks for each sample. This further indicates the reduced accuracy and sensitivity of gel biology and consequently, the need for quantification using real time PCR.

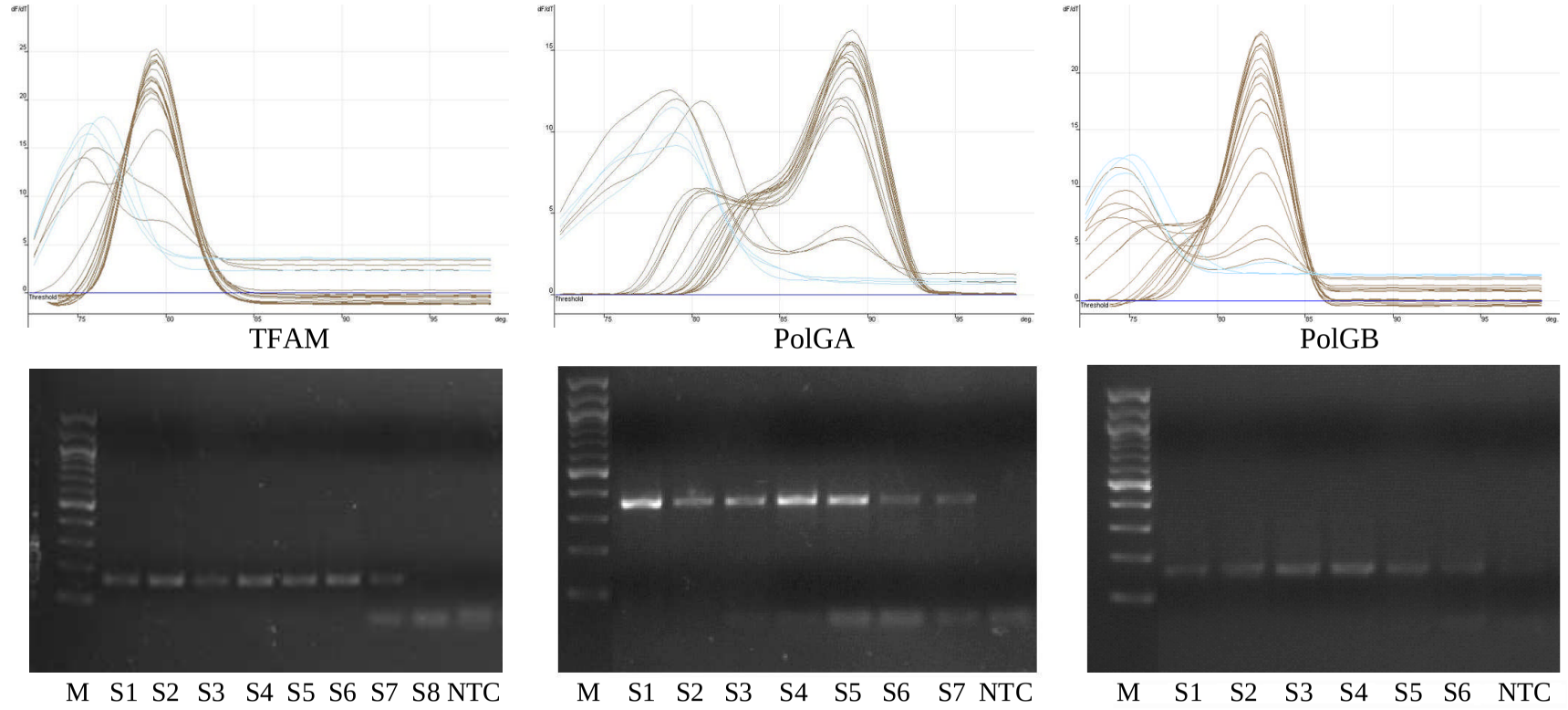


Figure 3.5: Real time PCR product analysis

Top: Standard (brown) and negative control (blue) melt curves from TFAM (left), PolGA (middle) and PolGB (right) reaction tests. Bottom: Agarose gels of PCR products for TFAM (left), PolGA (middle) and PolGB (right). Gels show L-R: 100bp ladder (M) standards (S) of 10-fold decreasing concentration (S1= 0.02ηg DNA/µl) and negative control lacking DNA template (NTC). Increasing levels of primer-dimers form as template levels decrease.

3.4.4 Determination of antibody specificity

Antibodies used in ICC experiments are often able to bind to proteins other than those they target, albeit with reduced affinity (Javois, 1994). This could result in false positive ICC results. As the TFAM and PolGA antibodies had not previously been used with porcine cells, it was necessary to confirm that they did indeed bind specifically to the porcine TFAM and PolGA proteins. Porcine oocytes were fixed, permeabilised and blocked as described in Section 2.14 before being incubated with the TFAM or PolGA antibody and with an antibody against the mitochondrially-encoded COXI protein. COXI was labelled with an Alexa Fluor ® 488 secondary antibody, whilst TFAM and PolGA were labelled with Alexa Fluor ® 593 secondary antibodies. Colocalisation of green and red fluorescent staining would suggest that the TFAM and PolGA antibodies were bound within the mitochondria and were therefore most likely binding to the correct proteins. Antibody incubation conditions are listed in Table 2.1. Oocyte staining patterns for COXI and PolGA and for COXI and TFAM, both merged and individually, are shown in Figure 3.6A. The primarily yellow staining in the merged images suggest that the TFAM and PolGA proteins are indeed colocalised with COXI and that the antibodies are binding to the appropriate proteins.

The availability of a blocking peptide allowed further tests to be performed in order to confirm the specificity of the TFAM antibody. Western blots were carried out both with and without the associated blocking peptide, as described in Section 2.19. A band of approximately 25KDa, as expected for human TFAM (Parisi et al 1993), was observed in the human protein sample (see Figure 3.6B). This was not observed in the sample containing the blocking peptide (see Figure 3.6B). Similarly, a band of approximately 24KDa, as expected for porcine TFAM (Antelman et al 2006), was observed in the porcine protein sample (see Figure 3.6B). Again,

this was not visible in the sample containing the blocking peptide. These data, along with the colocalisation data suggested that the antibodies were indeed binding specifically.

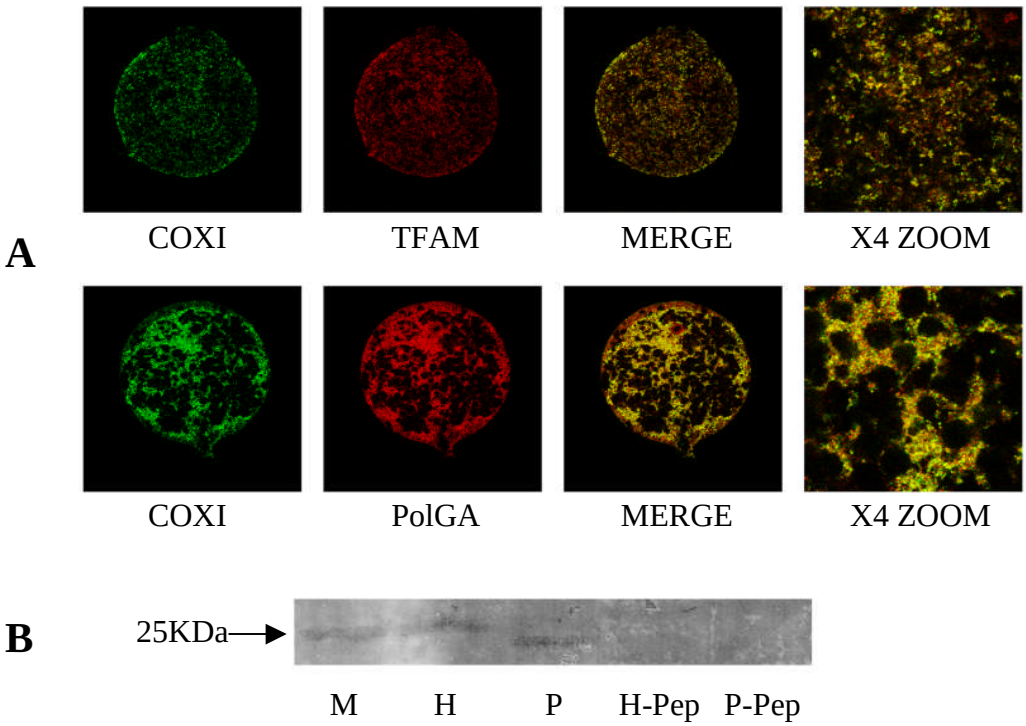


Figure 3.6: PolGA and TFAM antibody specificity

A: Confocal microscopy was used to visualise binding localisation of COXI, TFAM and PolGA antibodies in oocytes. Green staining in the far left panels show COXI staining in oocytes. Adjacent red stained panels show PolGA (top) and TFAM (bottom) staining of the same oocytes. Merged images are also shown, both in full (X 630) and in more detail (including X4 digital zoom, magnification X 2520) demonstrating colocalisation of PolGA and TFAM with COXI.

B: Western blot showing molecular weight marker (M), human (H) and porcine (P) protein incubated with the TFAM antibody, and human (H-Pep) and porcine (P-Pep) protein incubated with the TFAM antibody and its associated blocking peptide.

3.5 Materials and Methods

3.5.1 Preparation of embryo samples for real time PCR analysis

Embryos were derived using IVF, as described in Section 2.2.6. Embryos at the 2-cell, 4-cell, 8-cell, 16-cell, morula and expanded blastocyst stages were transferred in groups of 5 into 0.2ml tubes as each developmental stage was reached and stored at –80°C. RNA was extracted from the embryos and reverse transcribed into cDNA (see Sections 2.4.2 and 2.6). cDNA was then diluted by 1:5 in sterile autoclaved ddH_2O for real time PCR analysis. Embryos at the same stages were also saved individually in 5µl culture media and 20µl sterile ddH_2O for mtDNA copy number analysis. DNA was released using the freeze-thaw method (see Section 2.3) and was also diluted by 1:5 before real time PCR analysis.

3.5.2 Real time RTPCR analysis of TFAM, PolGA and PolGB expression

Standards for real time PCR were prepared as described in Section 2.8. Real time PCR reactions were run as described in Section 2.8 using the primers and reaction conditions listed in Table 3.2. Each standard and sample was run in triplicate and each reaction was run twice, thereby producing 6 results for each sample. The concentration of the standard and the product size for each cDNA molecule were used to calculate the expected number of cDNA molecules for each standard (see Table 3.2) using the following formula (see Section 2.9):

$$\frac{\text{ηg DNA} \times 1515 \times 6.023 \times 10^{11}}{\text{Product size (bp)}}$$

These values were entered into the RotorGene software (Version 7.0; Corbett Research) to produce a standard curve for each reaction. The fluorescence values acquired following melting of primer-dimers were also used to calculate the number of mRNA copies in each

sample from the standard curve. Values obtained were multiplied by the relevant dilution factor in order to give an estimate of the number of copies for each mRNA in a single oocyte. The highest and lowest values for each sample were excluded to adjust for pipetting error (Bustin, 2000). The remaining 4 values were used to calculate each result as a proportion of the mean number of mRNA copies present at the 2-cell stage. The means ± SEM for these proportions are presented. Due to the non-normal distribution of the data, as determined by the one-sample Kolmogorov-Smirnov test, non-parametric Mann Whitney U tests were used to determine the statistical differences between embryo stages (Mann & Whitney, 1947; Petrie, 2005), with $P < 0.05$ considered significant. Melt curves were obtained following each real time reaction and were used to confirm amplification of the correct product (see Section 2.8).

3.5.3 Real time PCR analysis of mtDNA copy number

Primers were designed to amplify a region of the porcine ND1 gene (Accession No.: NC_000845), which is encoded by the mitochondrial genome (Forward: CTC AAC CCT AGC AGA AAC CA, Reverse: TTA GTT GGT CGT ATC GGA ATC G). Standards were prepared from DNA in the same way as they were prepared from cDNA for the real time RTPCR analysis described above. Reactions were run under the same conditions as for the real time RTPCR analysis with an annealing temperature of 56°C and a second acquisition temperature of 79°C to generate a product of 254bp. A standard curve was produced and the mtDNA copy number for each sample was calculated by the RotorGene software (Version 7.0; Corbett Research), as described for the mRNA analysis. These values were multiplied by the relevant dilution factor and, after exclusion of the highest and lowest values, were used to calculate the mean ± SEM mtDNA copy number for each developmental stage.

3.5.4 Immunocytochemistry

Embryos from each stage of development listed above were fixed, permeabilised and blocked as described in Section 2.14 and were then stored at 4°C in blocking solution. Fixed embryos were incubated with TFAM and PolGA antibodies for 2 hrs at the concentrations stated in Table 2.1. Unbound antibody was removed by washing in 0.1% Triton X-100 in PBS for 30 mins and the relevant secondary antibodies (PolGA = green) were added as described in Table 2.1. After removal of unbound secondary antibody using 0.1% Triton X-100 in PBS, embryos were mounted onto slides for imaging. Confocal microscopy was carried out as described in Section 2.18.

3.6 Results

3.6.1 TFAM, PolGA, and PolGB mRNA analysis

In order to determine whether mtDNA replication could take place throughout pre-implantation development, the level of TFAM, PolGA and PolGB mRNA in embryos from the 2-cell stage through to the expanded blastocyst was analysed. Fluorescence data were acquired for TFAM, PolGA and PolGB standards and samples at the end of each cycle after melting of primer-dimers (see Figure 3.7). 4-cell and 8-cell embryos were consistently among the first samples to cross the fluorescence threshold, indicating that embryos at these stages may express mtDNA replication factors at a higher level than embryos at other stages. In contrast, 2-cell embryos were among the last samples to cross the fluorescence threshold indicating that embryos at the 2-cell stage express relatively low levels of each replication factor. Quantitative analysis was undertaken to determine the relative levels of each mRNA at each stage of development and to carry out statistical analysis.

The expression level of each replication factor detected at each of the developmental stages was quantified relative to expression detected at the 2-cell stage (see Figure 3.8). TFAM mRNA was present at relatively low levels in 2-cell embryos. A 43-fold increase was found in 4-cell stage embryos ($P = 0.02$), with similarly high levels remaining in 8-cell embryos (36-fold increase compared to 2-cell). A 2.6-fold drop in TFAM mRNA levels occurred between the 4-cell and 16-cell stages ($P = 0.02$). This was followed by a 1.6-fold increase between the 16-cell and expanded blastocyst stages ($P > 0.05$).

PolGA mRNA was also present at low levels in 2-cell embryos. An 11-fold increase was found in 4-cell embryos ($P > 0.05$). This was followed by a decrease to a level below that of 2-cell

embryos by the 8-cell stage ($P > 0.05$). PolGA mRNA remained at these low levels until the 16-cell stage, after which a significant increase was observed ($P = 0.01$) between the 16-cell and expanded blastocyst stages.

A 48-fold increase in PolGB mRNA levels was observed in 4-cell embryos compared to 2-cell embryos ($P = 0.02$). A further 1.8-fold increase was then observed between 4-cell and 8-cell embryos ($P > 0.05$). This was followed by an 8.9-fold decrease between 8-cell and 16-cell embryos ($P = 0.02$) and a 4-fold increase between 16-cell and morulae ($P = 0.02$). A further increase was observed between morula and expanded blastocyst staged embryos although this was not statistically significant.

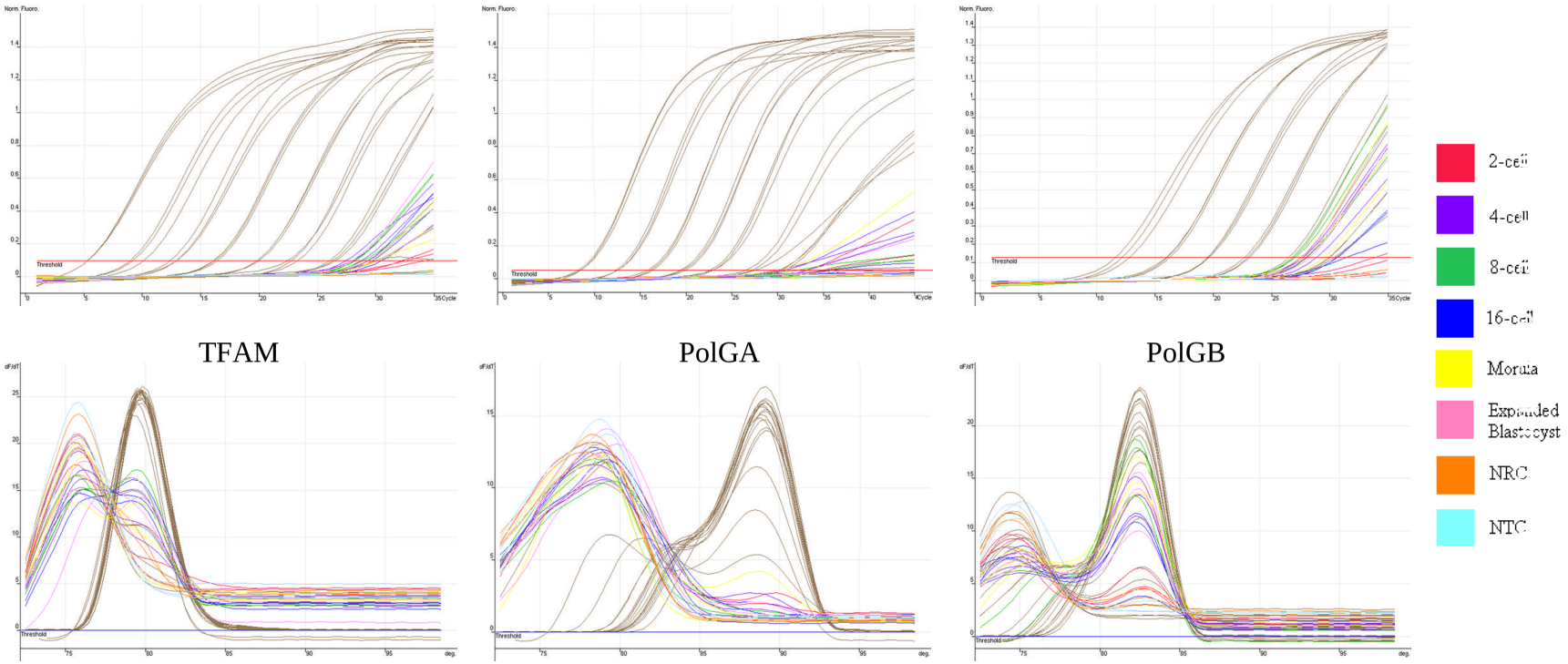


Figure 3.7: Fluorescence and melt data for mtDNA replication factor mRNA

Fluorescence values acquired after melting of primer-dimers (see Table 3.2 for temperatures) are shown (top row) for each standard and sample in real time PCR reactions for TFAM, PolGA and PolGB. Corresponding melt curves are shown below. Standards produced optimal threshold levels of 0.095 for TFAM, 0.0526 for PolGA and 0.1292 for PolGB. Melt curves show that embryo samples contain both product and primer-dimers, demonstrating the need for the second fluorescence acquisition step following melting of primer-dimers.

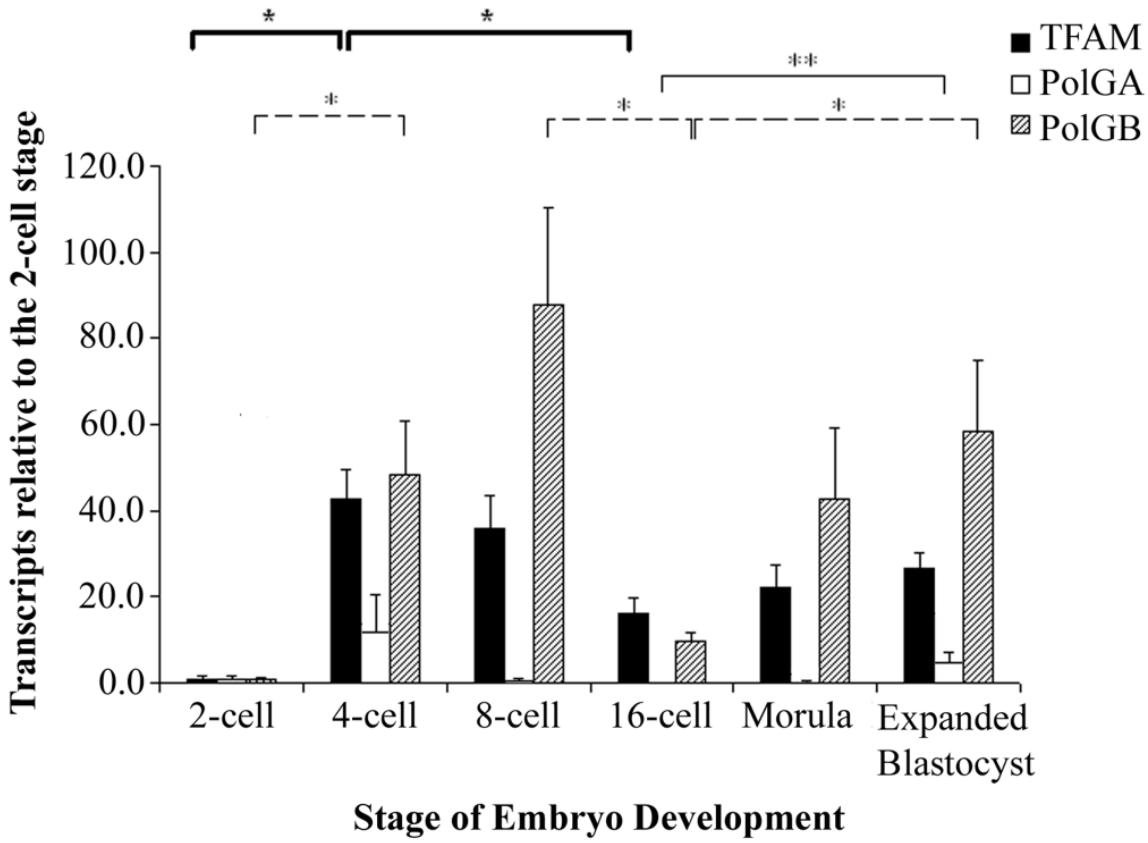


Figure 3.8: Relative expression of mtDNA replication factors during pre-implantation embryo development

Bars represent the number (mean ± SEM) of TFAM, PolGA and PolGB transcripts in 2-cell to expanded blastocyst staged embryos as a proportion of those in 2-cell embryos. Differences between bracketed stages of development are statistically significant (* P = 0.02, ** P = 0.01).

3.6.2 Immunocytochemistry

In order to determine whether the mtDNA replication factor mRNA described above was translated into protein, the localisation and abundance of TFAM and PolGA proteins at each stage of development was determined through ICC (see Figure 3.9). As described in Section 2.18, identical gain and photomultiplier settings were used during confocal microscopy allowing for comparative analysis of protein levels in different embryos. The significant changes in TFAM mRNA levels were mirrored by similar increases or decreases in protein staining intensity. For example, there was low staining intensity in 2-cell embryos, with staining primarily clustered around the nuclei. Much brighter TFAM staining was observed in 4-cell embryos, relatively evenly distributed throughout the cytoplasm. This was followed by reduced staining intensity in 8-cell to blastocyst-staged embryos, although embryos at all stages appeared to contain more TFAM protein than 2-cell embryos. The changes in PolGA mRNA levels also appeared to be mirrored by changes in protein levels. For example, higher staining intensity was observed in 4-cell embryos compared to 2-cell embryos, which appeared to contain little or no PolGA protein. Decreased staining intensity was observed in later staged embryos, with the staining localised progressively towards the outer edges of the blastomeres. A further increase in staining intensity was then observed between the morula and expanded blastocyst stages. More detailed images of PolGA protein staining in early and expanded blastocysts can be seen in Figure 3.10. PolGA protein was concentrated at the outer edges of the embryos in morulae (Figure 3.9) and early blastocysts (Figure 3.10) before high staining intensity was also observed throughout the entire embryo in expanded blastocysts (Figure 3.10).

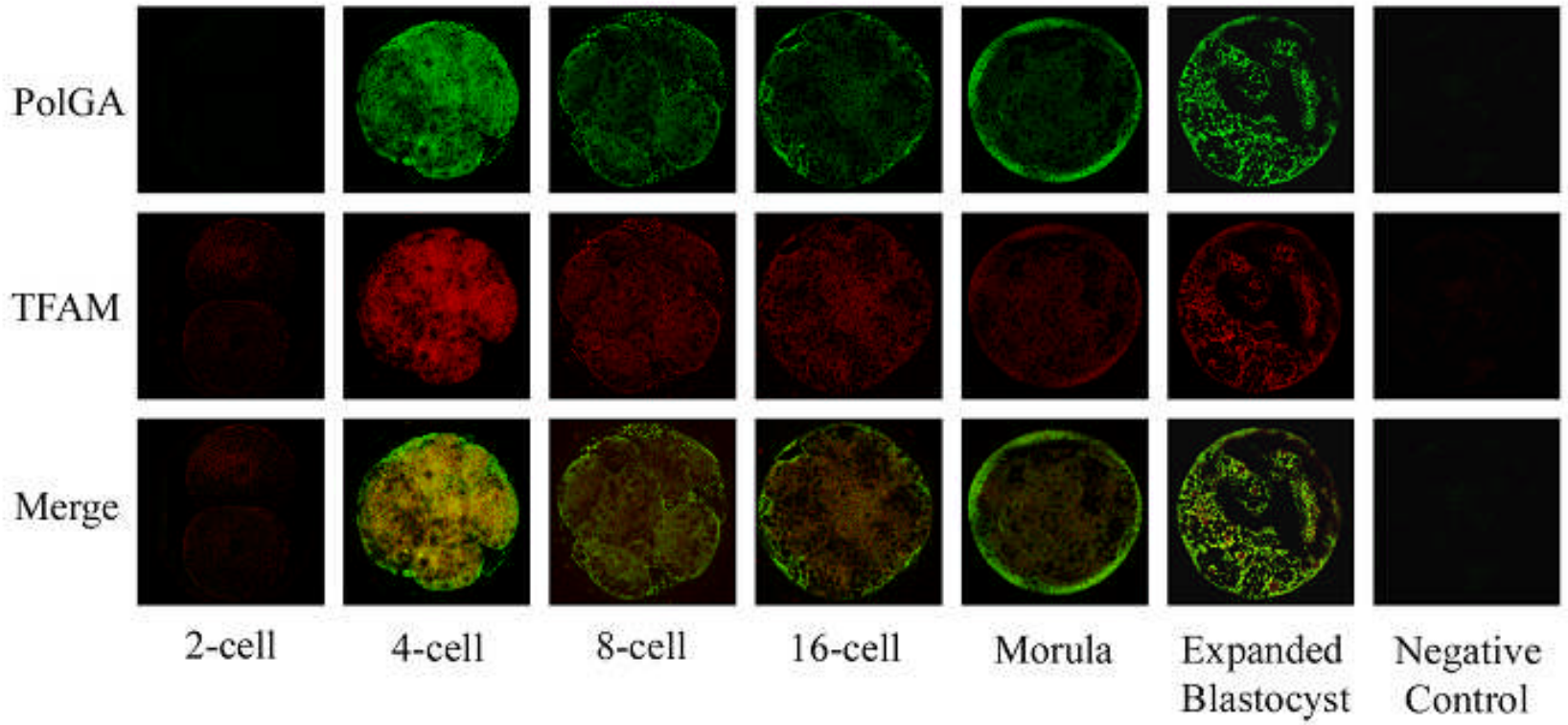


Figure 3.9: Localisation of TFAM and PolGA proteins during pre-implantation development

PolGA and TFAM protein staining patterns in preimplantation embryos from the 2-cell to the expanded blastocyst stage, as determined by ICC and confocal microscopy. Negative controls not incubated with the primary antibody are also shown. Images were captured using the strict criteria detailed in Section 2.18. Magnification: X 630.

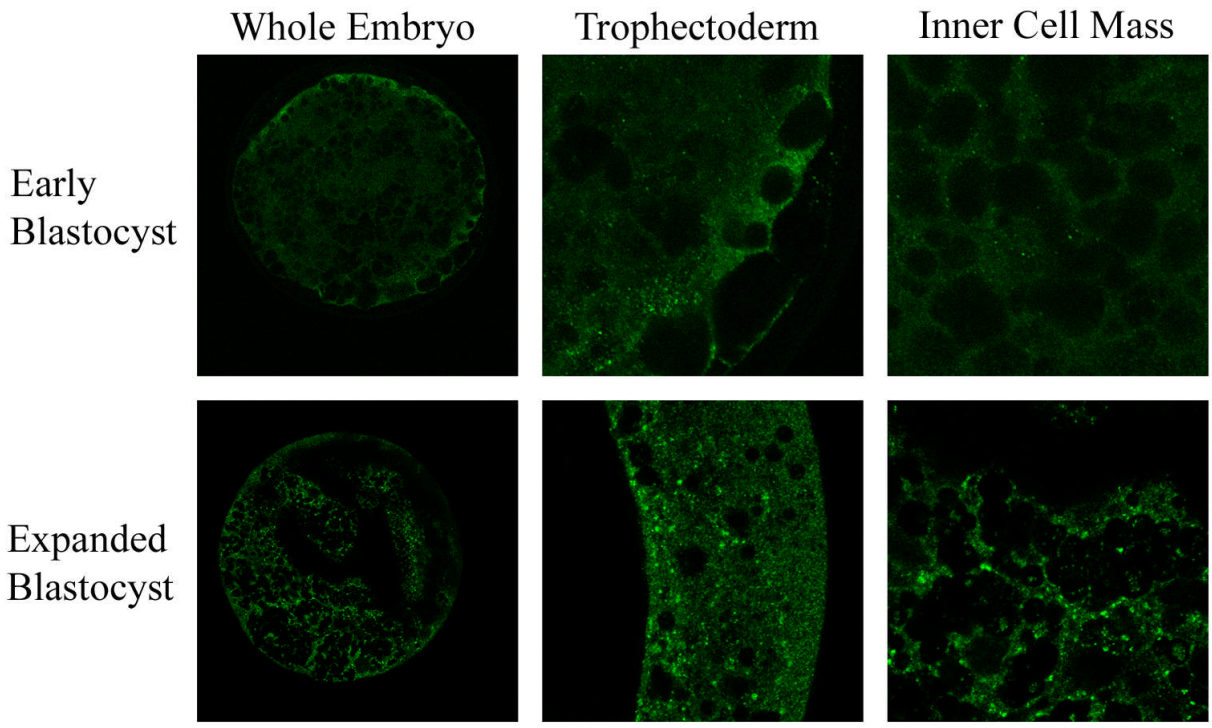


Figure 3.10: Localisation of PolGA in blastocyst TE and ICM

Left panels show localisation of PolGA protein in whole putative early and expanded blastocysts. More detailed (X4 digital zoom, magnification X 2520) images of the trophectoderm (middle panels) and inner cell mass (right panels) are also shown. Images were obtained using confocal microscopy, as described in Section 2.18. Original magnification: X 630. PolGA staining is present primarily in the outer edge of putative early blastocysts, whilst expanded blastocysts show even distribution of PolGA between cells likely to belong to the trophectoderm and those likely to form the inner cell mass.

3.6.3 MtDNA copy number analysis

In order to determine whether the mtDNA replication factors were active when expressed, mtDNA copy number was measured in pre-implantation embryos. A standard curve for mtDNA copy number (see Figure 3.11) was prepared from conventional PCR product that was obtained using primers designed against a region of the ND1 gene contained within mtDNA (see Section 3.5.3). 10-fold dilutions produced Ct values equally distanced from each other. Furthermore, R^2 and efficiency values were within acceptable ranges ($R^2 > 0.98$; Efficiency: 0.6-1.1; Pfaffl, 2003), indicating the suitability of the standards for use in quantification of mtDNA copy number in embryos. Examples of the fluorescence levels detected in embryos of each developmental stage are shown in Figure 3.12. Despite considerable variation between embryos of the same developmental stage, 2-cell embryos and expanded blastocysts consistently appeared earlier than embryos of other stages, indicating higher copy numbers in these embryos. 8-cell embryos were among the later-appearing samples, indicating relatively low mtDNA copy numbers in these embryos. Quantitative analysis was carried out to determine the number of mtDNA molecules present at each developmental stage. Mean ± SEM values for each developmental stage are displayed in Figure 3.13. 564283 ± 253833 mtDNA copies were detected in 2-cell embryos. However, a progressive decrease was then observed resulting in 73561 ± 28106 copies in 4-cell embryos ($P > 0.05$) and 21104 ± 8249 in 8-cell embryos ($P = 0.01$). This was followed by an increase in mtDNA copy number to 175454 ± 55981 in 16-cell embryos ($P = 0.009$ compared to 8-cell embryos) and 131238 ± 34498 in morulae ($P = 0.001$ compared to 8-cell embryos). A further increase then occurred resulting in 1254604 ± 345329 copies in expanded blastocysts ($P = 0.002$ compared to morulae).

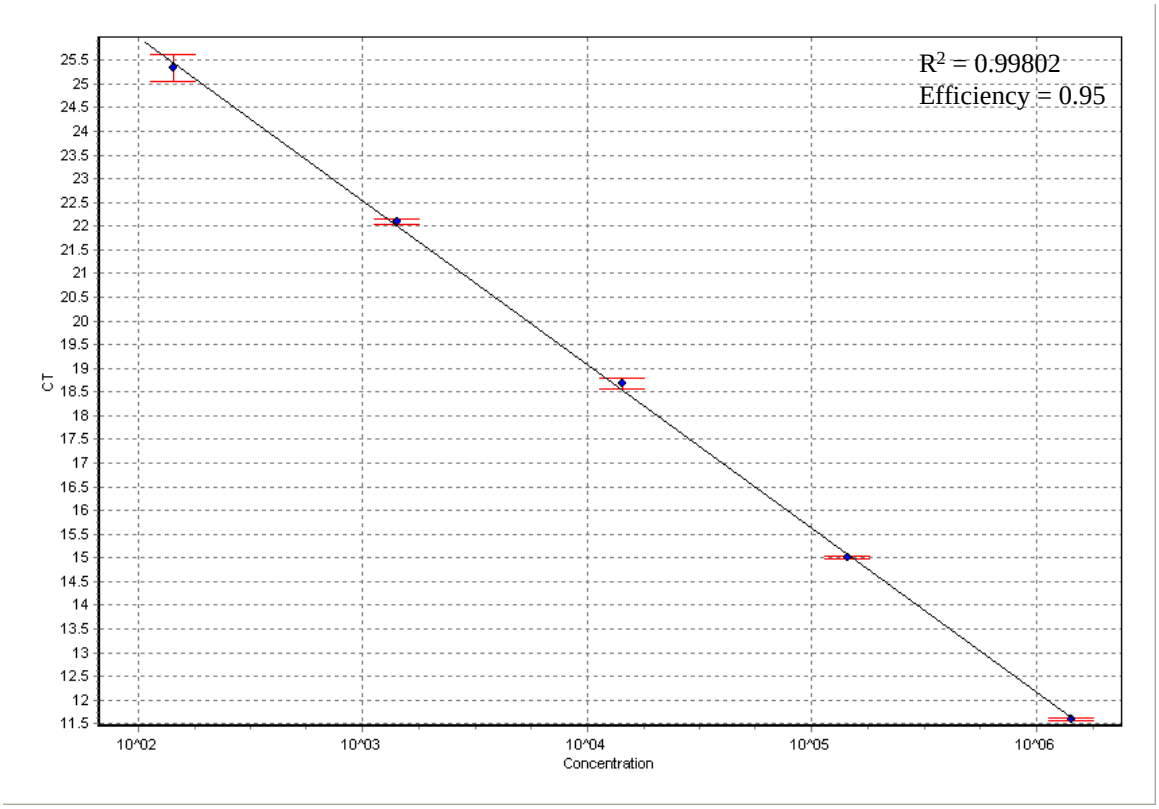


Figure 3.11: Standard curve for mtDNA copy number analysis

Mean + SEM Ct values are plotted against the log concentration of the standards of 10-fold dilutions. Data were obtained using the RotorGene 3000 real time PCR machine, as described in Section 2.8. The curve has an R^2 value ≥ 0.99, due to an equal number of cycles separating standards of 10-fold concentration difference. The curve also has an efficiency value close to the optimal value of 1, obtained when fluorescence levels increase 2-fold each cycle.

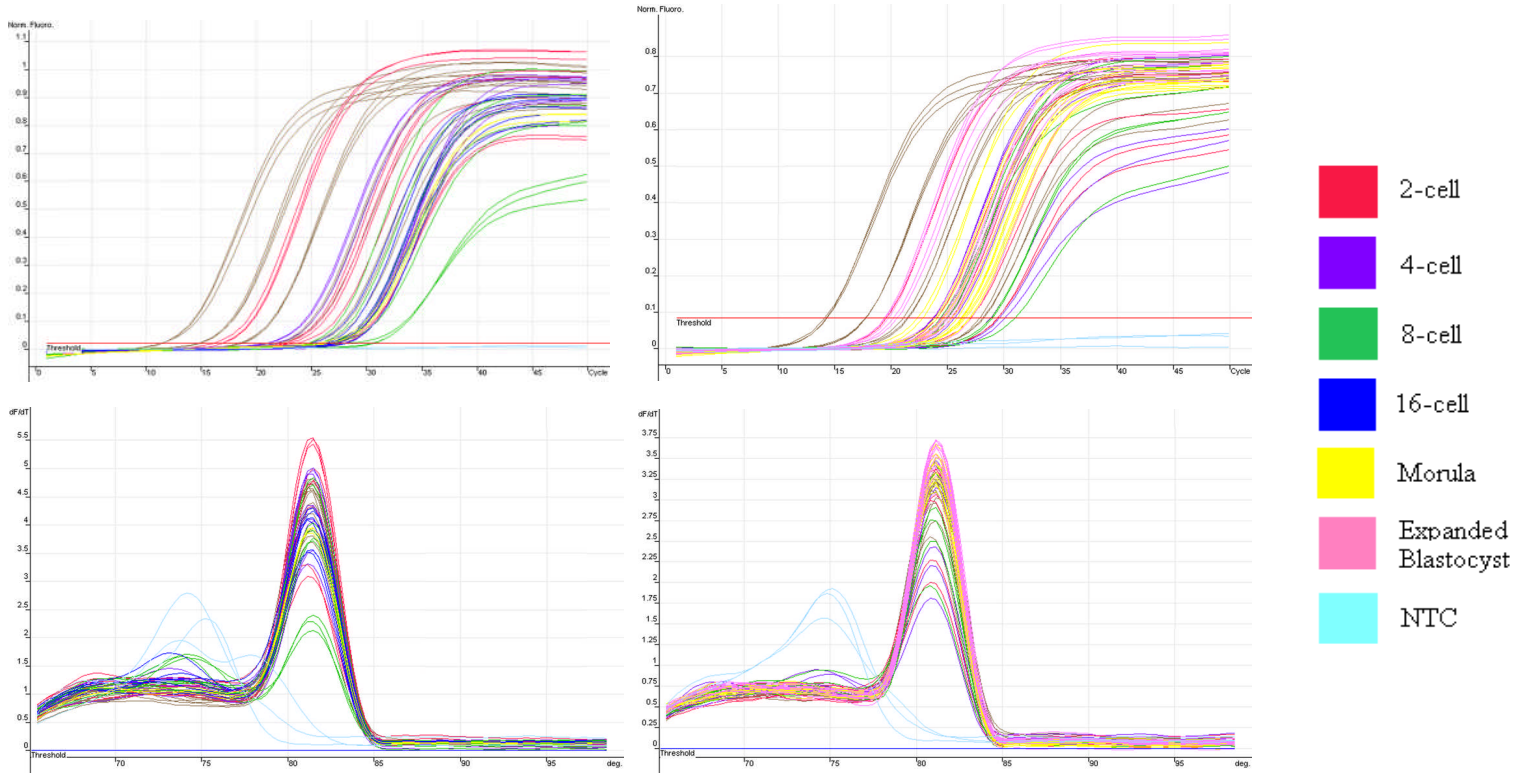


Figure 3.12: Fluorescence and melt data for mtDNA copy number analysis

Fluorescence values obtained after melting of primer-dimers at 79°C are shown (top row) for each standard and sample in real time PCR reactions for mtDNA copy number. Corresponding melt curves are shown below. Melt curves show that some embryo samples contain a small proportion of primer-dimers, although these were eliminated from the quantification through inclusion of the melting of primer-dimers and second fluorescence acquisition steps.

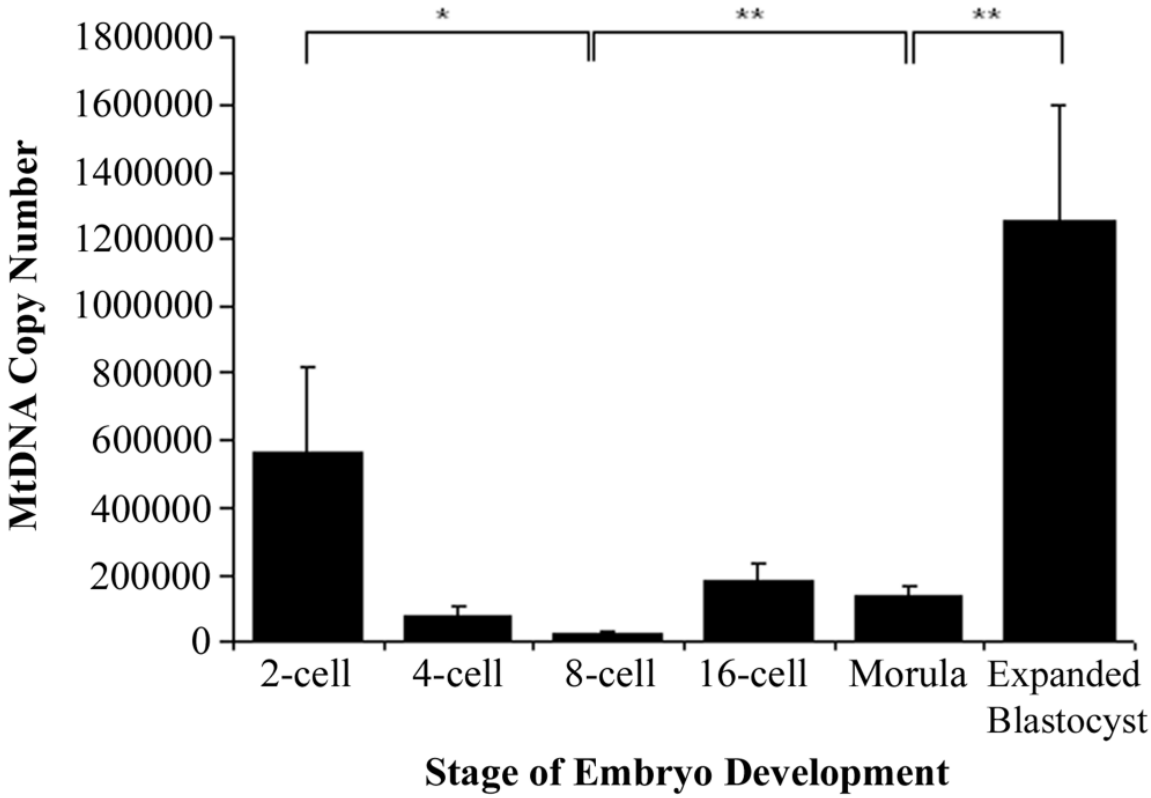


Figure 3.13: MtDNA copy number in pre-implantation embryos

Bars represent mean ± SEM mtDNA copy number in 2-cell to expanded blastocyst staged embryos. Significant differences between developmental stages are indicated (* $P \leq 0.01$, ** $P < 0.005$). Data were obtained using a RotorGene 3000 real time PCR machine and its associated software, as described in Section 2.8.

3.7 Discussion

3.7.1 MtDNA replication occurs at the blastocyst stage

The marked increase in mtDNA copy number observed here in porcine expanded blastocysts is consistent with the increase in mtDNA copy number also detected in bovine blastocysts (May-Panloup *et al.*, 2005b). In the mouse, it was previously reported that mtDNA replication did not occur until post-implantation (Piko & Taylor, 1987). However, a recent study using the more sensitive real time PCR technique reported that some embryos may have begun to replicate their mtDNA at the blastocyst stage (Thundathil *et al.*, 2005). The mtDNA replication observed between the morula and expanded blastocyst stages is likely facilitated by the increased expression of PolG mRNA and PolGA protein also observed at these stages. Increases in PolGA and PolGB mRNAs at the morula and blastocyst stages have also been reported recently in the mouse (Thundathil *et al.*, 2005). Interestingly however, although a small increase in TFAM transcription was observed in morulae and expanded blastocysts, an increase in TFAM protein expression was not observed at the expanded blastocyst stage. This suggests that low levels of TFAM protein were sufficient for mtDNA replication to take place. Unfortunately, studies in the mouse (Thundathil *et al.*, 2005) and cow (May-Panloup *et al.*, 2005b), although consistent with our TFAM mRNA data, did not include analysis of the TFAM protein.

3.7.2 MtDNA replication occurs first in the TE and then in the ICM

The increased PolGA protein observed in morulae and early blastocysts was localised to the outer edges of the embryos, the region likely representing cells destined to become trophectoderm (TE). Increased PolGA protein was not detected in the inner embryo, representing cells of the ICM, until the expanded blastocyst stage. This might suggest that

mtDNA replication occurs first in the TE and then in the ICM. Porcine blastocysts have been reported to contain between 69% (Park *et al.*, 2005) and 83-84% (Machaty *et al.*, 1998) TE, whilst cattle blastocysts have been reported to consist of between 70% (Thouas *et al.*, 2001) and 73-78% (Park *et al.*, 2004) TE. The occurrence of mtDNA replication only in cells of the TE would therefore account for the high copy numbers detected here in porcine expanded blastocysts. This would also be consistent with the low numbers of mitochondria (Sathananthan *et al.*, 2002; St. John *et al.*, 2005b) and low level expression of mtDNA transcription and replication factors (St. John *et al.*, 2005b) detected in hESCs, which are derived from the ICM of blastocysts. The conflicting reports as to whether mtDNA replication in the mouse occurs at the blastocyst stage or later (see Section 3.7.1) could also be partially explained by mtDNA replication occurring only in those cells of the TE, as mouse blastocysts tend to contain slightly lower proportions of TE cells, with reports varying from 25-45% (Cheng *et al.*, 2004) to 62% (Thouas *et al.*, 2001).

3.7.3 MtDNA degradation occurs during early embryogenesis

MtDNA copy number in porcine embryos was observed to decrease by 96% from the 2-cell to the 8-cell stage (see Figure 3.13). A decrease of 60% from the 2-cell to the 4 to 8-cell stage was observed in bovine embryos (May-Panloup *et al.*, 2005b) and a slight (6%), although non-significant, decrease in mean mtDNA copy number was also detected at this stage in the mouse (Thundathil *et al.*, 2005). This suggests that an mtDNA degradation process may be occurring. Indeed, mtDNA degradation has been reported in murine embryos at the 2-cell stage, although this was also accompanied by new mtDNA synthesis (McConnell & Petrie, 2004), providing a possible explanation for the relatively constant mtDNA copy numbers observed in this species (McConnell & Petrie, 2004; Piko & Taylor, 1987; Thundathil *et al.*,

2005). It has been hypothesised that an active mechanism of mtDNA degradation may take place (McConnell & Petrie, 2004), complimenting the ubiquitination mechanism of sperm mtDNA degradation (Sutovsky *et al.*, 2000) and partially explaining the elimination of donor mtDNA observed in some NT embryos and offspring (discussed in St. John *et al.*, 2004). MtDNA degradation could also occur passively through free radical induced accumulation of mtDNA deletions, which is known to occur in various other cell types (reviewed in de Grey, 2005), although there is currently no data to indicate that this occurs in embryos. A further explanation is that an active mtDNA degradation process takes place in order to reduce free radical production through ETC activity, as OXPHOS overactivity during the cleavage stages is hypothesised to contribute to embryo developmental arrest (Van Blerkom, 2004). The mechanism of mtDNA degradation could be as simple as a reduction in TFAM expression, as TFAM has been hypothesised to be required for mtDNA packaging and might therefore contribute to its stability (Alam *et al.*, 2003). Indeed, decreased expression of TFAM has been associated with a variety of mtDNA depletion syndromes (Larsson *et al.*, 1994; Poulton *et al.*, 1994; Siciliano *et al.*, 2000; Spelbrink *et al.*, 1998; Tessa *et al.*, 2000) and both homozygous and heterozygous knock-out mice demonstrate reduced mtDNA copy number (Larsson *et al.*, 1998).

The occurrence of mtDNA degradation during the cleavage stages of embryonic development described here and elsewhere (May-Panloup *et al.*, 2005b), and the lack of mtDNA replication in murine pre-implantation embryogenesis, contribute to the hypothesised mtDNA "bottleneck" (Hauswirth & Laipis, 1982). This restricts segregation of mtDNA to the PGCs, and consequently inheritance to the next generation, in order to maintain homoplasmy (Marchington *et al.*, 1997). However, this also results in a continual dilution of the

mitochondrial genome throughout embryogenesis due to the ever-increasing numbers of blastomeres and the lack of replenishment of mtDNA. It is therefore likely that large numbers of mtDNA molecules are required in the oocyte at fertilisation in order to ensure that sufficient template copies are available in each cell for replication after implantation. Failure to do this may result in offspring harbouring mtDNA disease. This hypothesis is investigated in Chapter 4.

3.7.4 MtDNA copy number is mirrored by OXPHOS activity

As mtDNA encodes subunits of the ETC, the main producer of ATP, it is likely that mtDNA copy number influences mitochondrial ATP production. In mammals, such as the pig (Sturmey & Leese, 2003), cow (Thompson *et al.*, 1996), rat (Brison & Leese, 1991), mouse (Houghton *et al.*, 1996) and human (Leese *et al.*, 1993), the requirement for ATP generated through OXPHOS decreases as the pre-implantation embryo develops. Consequently, there is a greater dependence on glycolysis (Van Blerkom *et al.*, 2002). Once at the blastocyst stage, the requirement for OXPHOS then increases (Trimarchi *et al.*, 2000). This pattern of OXPHOS-derived ATP usage is mirrored by the initial decline followed by an increase in mtDNA copy number observed here in the pig (Figure. 3.10) and that was also reported in cattle (May-Panloup *et al.*, 2005b). Increased mtDNA copy number was also observed in porcine expanded blastocysts (Figure. 3.10). Similarly, somatic cells with reduced mtDNA content, such as aged cells with accumulated mtDNA deletions (Kopsidas *et al.*, 2000), various cancer cells (Lee *et al.*, 2005) and cells not expressing TFAM (Hansson *et al.*, 2004), demonstrate reduced mitochondrial ATP production and increased dependence on glycolysis. This clearly demonstrates the relationship between mtDNA copy number and mitochondrial ATP production, as observed in a variety of specialised somatic cells (Moyes *et al.*, 1998).

3.7.5 TFAM and PolG expression peak at the 4 to 8-cell stage

A short period of mtDNA turnover was reported at the 2-cell stage in murine embryos (McConnell & Petrie, 2004). This was because mtDNA replication was detectable, although no statistically significant increase in mtDNA copy number was observed. Similarly, mRNA levels for TFAM, PolGA and PolGB peaked at the 4 to 8-cell stage in the porcine embryos described in this Chapter. However, mtDNA copy number actually decreased during this period. It is therefore possible that a small amount of mtDNA turnover was also taking place at the 4-cell stage in porcine embryos.

The increase in mtDNA replication factor expression observed here in 4-cell staged porcine embryos and the mtDNA replication observed at the 2-cell stage in murine embryos (McConnell & Petrie, 2004) coincide with EGA, which occurs at the 4-cell stage in pigs (Jarrell *et al.*, 1991) and the 2-cell stage in mice (Bolton *et al.*, 1984). In cattle, upregulation of NRF-1 and TFAM at the 16-cell to morula stages (May-Panloup *et al.*, 2005b) also coincide with EGA taking place at the 16-cell stage (Camous *et al.*, 1986). In the pig, TFAM and PolGB expression were higher at all stages of development compared to the 2-cell stage, just before EGA occurs. This might suggest that basal expression of these factors is initiated at EGA, and may account for the maintained mtDNA copy number seen in early murine embryos that is not seen in the pig or cow where EGA occurs later. On the other hand, it is also possible that mtDNA replication does not occur at EGA and increased expression of mtDNA replication factors described here in the pig is simply due to the generic expression of a whole host of genes at this one particular stage in development (Ma *et al.*, 2001). However, embryos with insufficient mitochondrial activity often arrest at the stage of EGA, suggesting

an essential role for mitochondria in this process (Tarazona *et al.*, 2006). This is supported by reports of a transient increase in mitochondrial membrane potential (Acton *et al.*, 2004) and clustering of mitochondria around the nuclei (Muggleton-Harris & Brown, 1988) in murine 2-cell embryos when the embryonic genome is activated in this species.

3.8 Conclusions

Consistent with previous observations in the cow and mouse, no significant increase in mtDNA copy number was observed until the expanded blastocyst stage of porcine embryonic development. In fact, mtDNA actually decreased during the early pre-implantation stages. Increased replication factor expression suggests that some mtDNA replication may also occur at the 4-cell stage, although this must occur at a lower rate than mtDNA degradation in order to account for the overall decrease in mtDNA copy number. Replication at the 4-cell stage may be linked to EGA, as mtDNA turnover has also been reported at the 2-cell stage in murine embryos, when EGA occurs in this species. Despite limited mtDNA replication and the likely activity of an mtDNA degradation process, previous reports suggest that OXPHOS is active during pre-implantation development in many species. High levels of mtDNA are therefore likely to be required at fertilisation in order to maintain sufficient copies in cleaving blastomeres and to sustain development. The likely requirement for high levels of mtDNA at fertilisation is investigated in Chapter 4.

CHAPTER 4: mtDNA REPLICATION DURING *IN VITRO* OOCYTE MATURATION

4.1 Introduction

As discussed in Chapter 3, little or no mtDNA replication occurs during pre-implantation embryogenesis in many species including the pig. MtDNA molecules are therefore diluted out during the early embryonic cell divisions, with low copy numbers likely to be present in each blastomere just before mtDNA replication resumes at the blastocyst stage. In the pig (Chapter 3) and cow (May-Panloup *et al.*, 2005b), total embryo mtDNA copy number also decreased throughout the pre-implantation stages. It is therefore vital that sufficient mtDNA copies are present within the embryo in order that each cell of the blastocyst contains intact template mtDNA for subsequent amplification. MtDNA is usually maternally inherited (Giles *et al.*, 1980), and evidence in various mammalian species suggests that pre-implantation embryonic mtDNA composition is dependent on the number of oocyte mtDNA copies present at fertilisation, constituting an investment until mtDNA replication is initiated post-implantation. For example, a critical threshold of approximately 100000 copies in the mature oocyte has been proposed for the mouse (Piko & Taylor, 1987), pig (El Shourbagy, 2004) and human (Almeida-Santos *et al.*, 2006; Reynier *et al.*, 2001), suggesting that mtDNA copy number may be an indicator of oocyte competence.

Oocyte competence, the ability of an oocyte to fertilise and develop, is commonly assessed using morphological criteria such as appearance and location of the nucleus and cytoplasmic components, the presence of the polar body and size of perivitelline space and cumulus cell layer (Hyttel *et al.*, 1997). However, after the development of meiotic competence, which is

required for fertilisation to take place, the morphology of oocytes does not alter significantly (Hyttel *et al.*, 1997). It is therefore difficult to assess cytoplasmic development morphologically, although this is required for embryo development to the blastocyst stage. This often results in higher rates of oocyte fertilisation than development of embryos to the blastocyst stage (Hyttel *et al.*, 1997; Marchal *et al.*, 2002). Oocyte developmental competence can also be assessed non-invasively through the activity of glucose-6-phosphate dehydrogenase (G6PD), which breaks down the dye, BCB (Roca *et al.*, 1998; Rodríguez-Gonzáles *et al.*, 2002). Competent oocytes do not express G6PD and therefore fail to enzymatically degrade BCB thus staining positively (BCB+). Oocytes not having reached competency are colourless (BCB-) due to the continued expression of G6PD and the resulting breakdown of BCB. In the pig, competent BCB+ oocytes contain more copies of mtDNA and are more likely to fertilise than incompetent BCB- oocytes (El Shourbagy, 2004). Furthermore, injection of BCB- oocytes with supplementary mitochondria from BCB+ oocytes results in improved fertilisation outcome (El Shourbagy, 2004). This further demonstrates the association between mitochondrial number and fertilisation outcome.

In order to produce mature oocytes with large numbers of mtDNA molecules, mtDNA must be replicated from the 10 or so copies thought to be present in PGCs (Jansen & de Boer, 1998). Indeed, a 45-fold increase has been reported in cattle from the PGC stage to pre-ovulating oocytes (Smith & Alcivar, 1993) with values ranging up to 314000 (Lin *et al.*, 2004) or even 700000 (Almeida-Santos *et al.*, 2006) having been reported for mature human oocytes. Few data are available regarding the stage of oocyte development in which mtDNA replication occurs or how replication is regulated, although inhibition of mtDNA replication may allow some of these data to be generated. MtDNA replication can be inhibited through

use of the drug, ddC, which is a nucleoside analogue that can therefore be incorporated into mtDNA by PolG in place of the normal nucleoside (Feng *et al.*, 2001). However, as described in Section 2.2.3, ddC incorporation results in termination of the mtDNA chain (Brinkman & Kakuda, 2000). Replication of mtDNA is further inhibited by failure of the PolG exonuclease to remove ddC (Feng *et al.*, 2001). Treatment of oocytes with ddC may therefore allow assessment of the importance of mtDNA replication during *in vitro* maturation through analysis of the effects of its absence.

4.2 Hypothesis

Developmentally competent oocytes replicate their mtDNA earlier and/ or contain more mtDNA molecules than less competent oocytes due to the synchronised regulation by nuclear-encoded mtDNA replication factors.

4.3 Aims

Using porcine oocytes from various stages of *in vitro* maturation that have been separated following incubation with BCB, the proposed experiments will determine whether:

1. mtDNA replication factors are expressed during *in vitro* oocyte maturation
2. mtDNA replication occurs during *in vitro* oocyte maturation
3. expression of mtDNA replication factors and therefore occurrence of mtDNA replication differs between developmentally competent and incompetent oocytes
4. inhibition of mtDNA replication during *in vitro* oocyte maturation affects oocyte competence and embryo developmental ability

4.4 Preliminary Experiments

4.4.1 Improvement of maturation media

The standard laboratory oocyte maturation protocol included the use of 0.1mg/ml cysteine for the first 22 hrs of maturation, after which oocytes were matured for a further 22 hrs in media without cysteine. However, it has been reported that cysteine is required during the later stages of porcine oocyte maturation (Sawai *et al.*, 1997). Cysteine is also used by the oocyte in order to produce glutathione, required for formation of the male pronucleus after sperm penetration (Yoshida, 1993). In order to determine whether fertilisation rates could be improved by including cysteine in the media for the entire maturation period, oocytes were matured in the standard maturation media (see Section 2.2.1) for 22 hrs and then for a further 22 hrs in the standard maturation media containing either: 1) 10ηg/ml EGF, the standard hormonal supplement known to mediate glutathione production, 2) 0.1mg/ml cysteine in place of EGF, or 3) both 10ηg/ml EGF and 0.1mg/ml cysteine. Oocytes were then inseminated and fertilisation rates were compared.

Supplementation of maturation media with EGF alone produced a fertilisation rate of 12.8%. However supplementation of maturation media with cysteine in place of EGF increased fertilisation rates to 25.4% ($P = 0.003$). A further increase in fertilisation rate to 37.5% was also observed when cysteine was used in combination with EGF ($P = 0.02$). In subsequent experiments, the maturation media was therefore supplemented with both EGF and cysteine.

4.4.2 Determination of BCB toxicity

The BCB test is commonly used to assess an oocyte's ability to successfully undergo *in vitro* maturation, fertilisation and further embryo development and takes place prior to *in vitro* maturation (see Section 2.2.2). It had previously been demonstrated that exposure to 13μM BCB for 90 mins before *in vitro* maturation was not toxic to oocytes. For example, BCB+ oocytes produced higher fertilisation rates than control oocytes not incubated with BCB in both goats (Rodríguez-Gonzáles *et al.*, 2002) and cows (Pujol *et al.*, 2004). In order to confirm that BCB was also not toxic to porcine oocytes, oocytes were either incubated with BCB before being classified as BCB+ or BCB- or they were incubated under the same conditions (see Section 2.2.2) in media not containing BCB (control). BCB+, BCB- and control oocytes were then matured and inseminated *in vitro* (see Section 2.2) and fertilisation and blastocyst rates were compared 2 and 7 days after insemination, respectively.

Of 220 inseminated control oocytes, 71 cleaved and 13 developed to the blastocyst stage, producing a fertilisation rate of 32.3% and a blastocyst rate of 5.9%. BCB+ oocytes (N = 294) produced significantly higher fertilisation (46.6%; $P < 0.0001$) and blastocyst (9.9%; $P < 0.001$) rates than control oocytes and BCB- oocytes (N = 242) produced significantly lower fertilisation (22.7%; $P < 0.01$) and blastocyst (2.9%; $P = 0.05$) rates than control oocytes. Furthermore, fertilisation (35.8%) and blastocyst (6.7%) rates for all oocytes incubated with BCB were comparable to those obtained from control oocytes not exposed to BCB ($P > 0.05$). These data indicate that BCB is also not toxic to porcine oocytes.

4.4.3 Preparation of new standards for PolGA and PolGB

Real time RTPCR to measure the number of TFAM, PolGA and PolGB mRNA molecules in maturing oocytes was carried out using the same primers and standards that were used for the embryo analysis described in Chapter 3. Interestingly, melt curve analysis for PolGA and PolGB demonstrated that PCR product for some samples melted at a slightly lower temperature to the PCR product for the standards and other samples (see Figure 4.1). This indicates that these two products might not have been identical. Sequencing of these products determined that these samples produced a PolGA product 2bp longer than the PolGA standards and produced a PolGB product 3bp shorter than the PolGB standards. In order to ensure that the standards and samples were amplifying the same product, thereby enabling accurate quantification of relative mRNA levels in these samples, new standards were prepared for PolGA and PolGB. The new standard curves and their respective melt curves with only one peak for each product are shown in Figure 4.2. The number of mRNA molecules contained within each new standard was calculated as described previously (see Section 2.8) using the new product lengths. Quantification of relative mRNA levels in the oocyte samples was therefore as accurate as for the embryo samples described in Chapter 3.

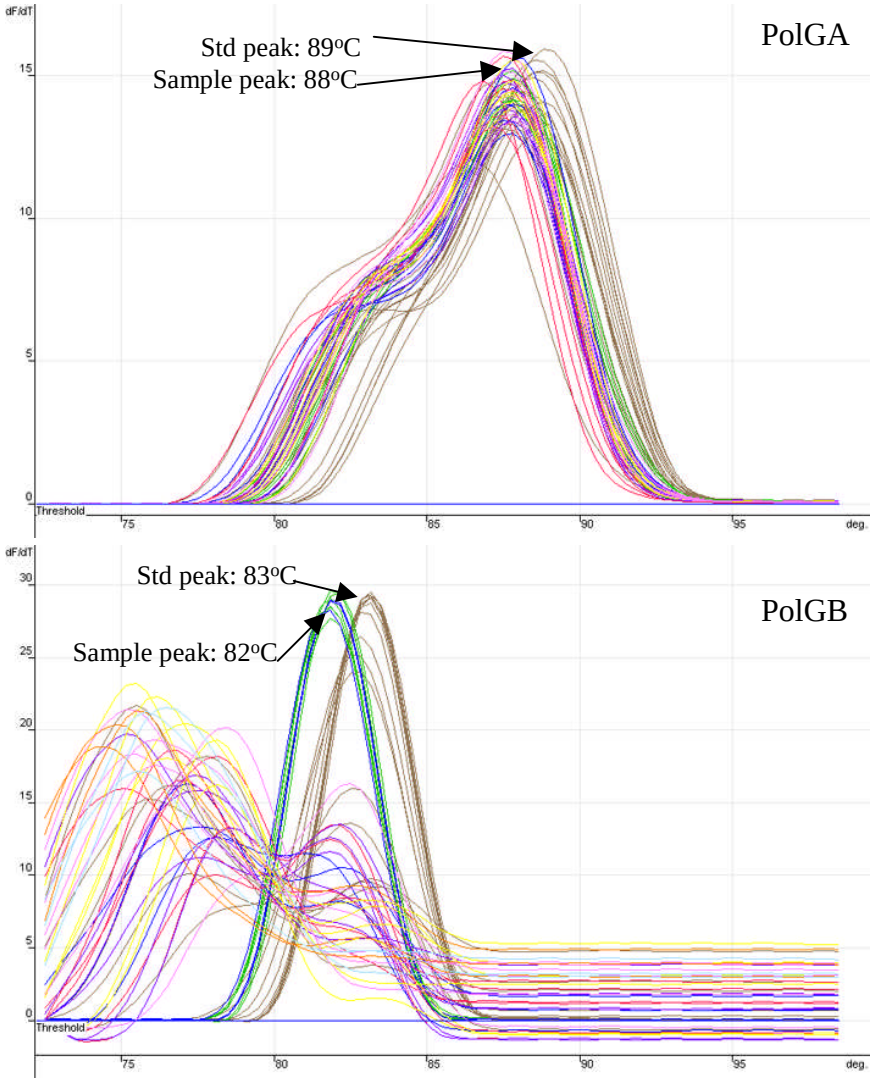


Figure 4.1: Melt curve analysis for PolGA and PolGB

PolGA standards (std) melted at 89°C, whilst some of the samples produced a peak at 88°C, representing a product 2bp longer than the stds. PolGB standards melted at 83°C and some samples melting at 82°C. In this case, the samples produced a product 3bp shorter than the standards. New standards were therefore prepared in order to enable accurate quantification of mRNA levels.

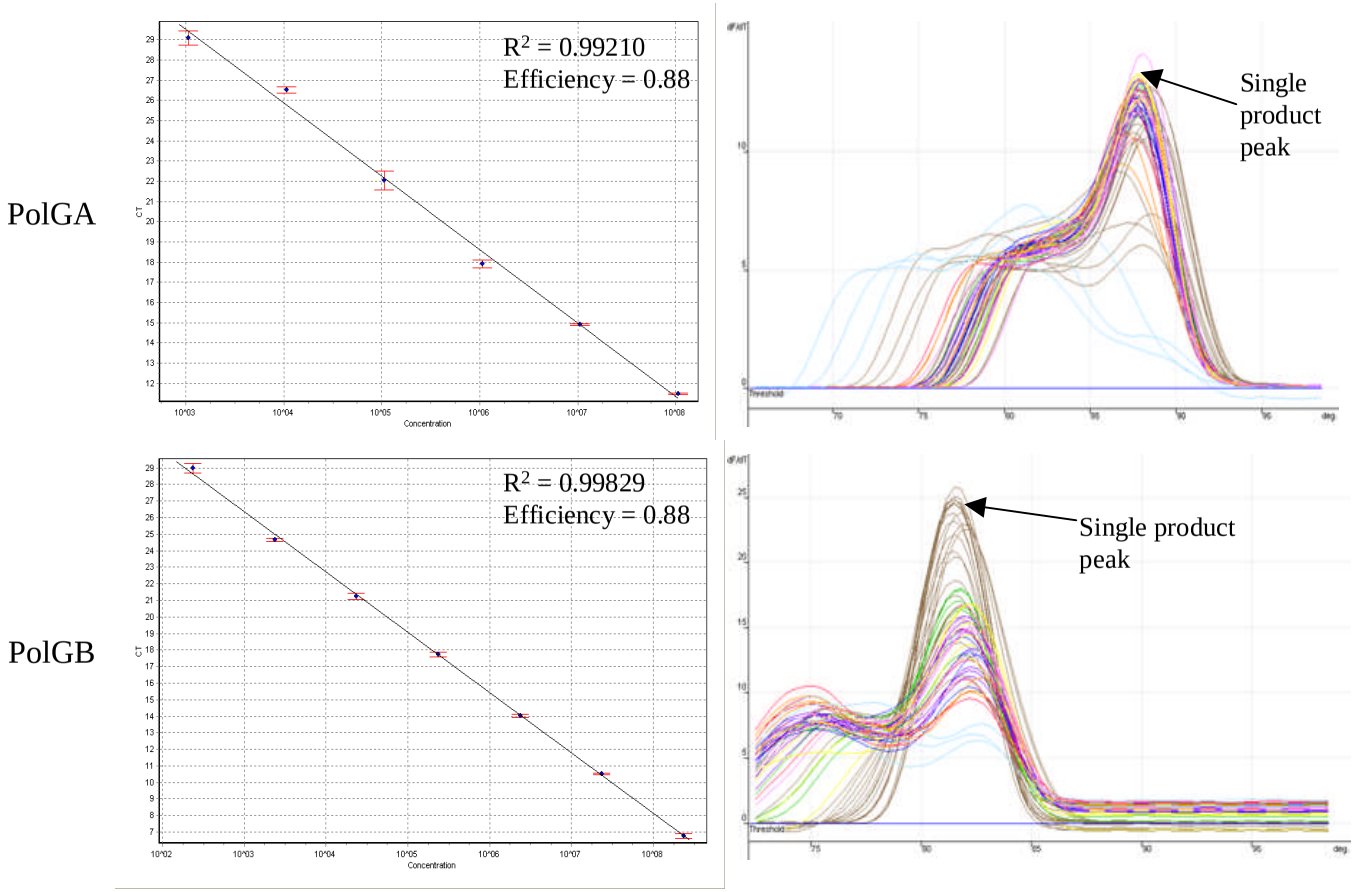


Figure 4.2: New standard and melt curves generated for PolGA and PolGB

New standards were generated for PolGA and PolGB and Mean ± SEM CT values were plotted against the log concentration of the standards. Each curve has an R^2 value ≥ 0.99 and an efficiency value within the accepted range of between 0.6 and 1.1 . Melt curves with only one peak per product (see arrows) confirmed that all of the standards and samples were amplifying the same product.

4.5 Materials and Methods

4.5.1 Preparation of oocyte samples for analysis

Ovaries were obtained from a slaughterhouse and used to aspirate oocytes, as described in Section 2.2. Oocytes were incubated with 13µM BCB for 90 mins, washed and then classified as BCB+ or BCB-, as described in Section 2.2.2. BCB+ and BCB- oocytes were matured *in vitro* (see Section 2.2.1) either with or without 10µM ddC, for 44 to 46 hrs, with a media change after 22 hrs. BCB+ ddC-treated and untreated oocytes and BCB- ddC-treated and untreated oocytes were saved for analysis after 0, 22 and 44 hrs of *in vitro* maturation (See Figure 4.3). Oocytes saved for real time RTPCR mRNA analysis were transferred to 0.2ml tubes in groups of 5 and stored at –80°C. Oocytes saved for ICC analysis were fixed, permeabilised and blocked as described in Section 2.14, before being stored in blocking solution at 4°C. Oocytes saved for mtDNA copy number analysis by real time PCR were transferred individually in volumes of 25µl to 0.2ml tubes for storage at –20°C.

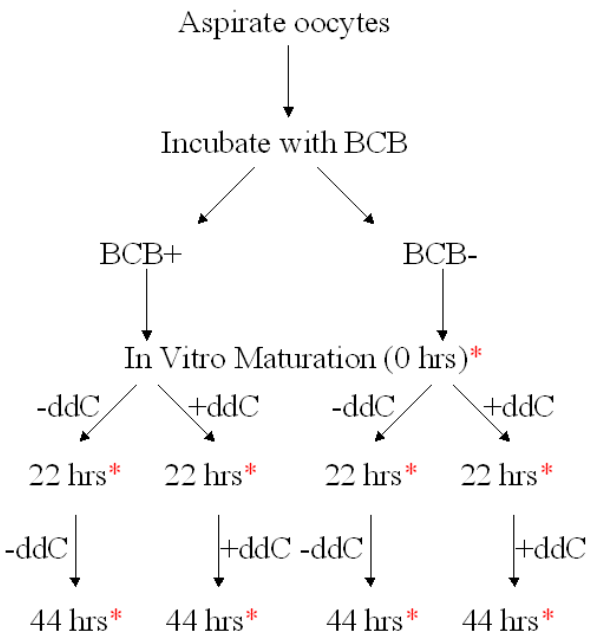


Figure 4.3: Protocol for BCB staining and ddC treatment during oocyte maturation

Oocytes were separated according to their BCB status before being matured either with or without ddC for 44 to 46 hrs. Oocytes were saved for analysis (*) after 0, 22 and 44 hrs of *in vitro* maturation.

4.5.2 Real time RTPCR

RNA was extracted from groups of 5 oocytes and reverse transcribed into cDNA as described in Sections 2.4.2 and 2.6. Primer pairs and real time PCR reaction conditions were optimised as described in Chapter 3 to produce standard curves suitable for quantification of samples. Primers and reaction conditions used in this Chapter were as for Chapter 3 (Table 3.2). Due to the large number of samples, experiments were run in 2 batches and samples were therefore separated into two groups. These consisted of 1) BCB+ and BCB- untreated oocytes at 22 and 44 hrs run in parallel with BCB+ and BCB- oocytes from 0 hrs, and 2) BCB+ and BCB- ddC treated oocytes run with a different set of 0 hrs oocytes. As previously described, each standard and sample was run in triplicate and each reaction was run twice, producing 6 results for each sample. Values obtained were multiplied by the relevant dilution factor in order to give an estimate of the number of copies of each mRNA in a single oocyte. All results are presented as a proportion of mRNA molecules detected in BCB+ oocytes at 0 hrs. However, due to the variation in absolute values obtained between the two groups of samples, mRNA numbers in each sample were presented as a proportion of the mean value for BCB+ 0 hrs oocytes run in the same sample group. The highest and lowest values for each sample were excluded to adjust for pipetting error (Bustin, 2000) and the remaining 4 values were used to calculate the means and standard errors presented.

4.5.3 Immunocytochemistry

BCB+ and BCB- oocytes from each day of maturation, either ddC treated or untreated, were incubated with TFAM, PolGA and COXI antibodies for 2 hrs at the concentrations listed in Table 2.1. Unbound antibody was removed by washing in 0.1% Triton X-100 in PBS for 30 mins and the relevant secondary antibodies were added as described in Table 2.1 and Section

2.14.1. After removal of unbound secondary antibody using 0.1% Triton X-100 in PBS, oocytes were mounted onto slides for imaging. Confocal microscopy was carried out as previously described (see Section 2.18).

4.5.4 Analysis of mtDNA copy number

DNA was extracted from individual oocytes from each group using the freeze-thaw method as described in Section 2.3. Primers designed to amplify a region of the ND1 gene were used as described in Chapter 3. Each sample was run in triplicate twice and mtDNA copy number for ddC treated and untreated BCB+ and BCB- oocytes on each stage of maturation was calculated, as described in Section 2.8.

4.5.5 Fertilisation analysis

BCB+ oocytes treated with ddC and non-treated BCB+ oocytes were inseminated with boar sperm prepared as previously described (see Sections 2.2.5 and 2.2.6). Fertilisation rates were determined 48 hrs after insemination. This experiment was carried out 3 times and data were combined before statistical analysis.

4.5.6 Statistical analysis

The one-sample Kolmogorov-Smirnov test determined that none of the real time PCR data were normally distributed ($P < 0.05$). Differences in mRNA levels and mtDNA copy number between BCB+ and BCB- oocytes, between different stages of maturation and between ddC treated and non-treated oocytes were therefore analysed using the Mann Whitney U test (Mann & Whitney, 1947; May-Panloup *et al.*, 2005b). For comparisons of mRNA levels, statistical analysis was carried out on the data, expressed as a proportion, to avoid any bias resulting

from the variation in absolute values between sample groups. Fertilisation rates of BCB+ ddC treated and untreated oocytes were used to create a 2x2 contingency table, which was analysed using the Fisher Exact test to determine whether or not the differences were significant (Agresti, 1992; Lloyd *et al.*, 2006).

4.6 Results

4.6.1 TFAM, PolGA and PolGB mRNA analysis

In order to investigate the mechanisms that ensure sufficient mtDNA copy numbers are present prior to fertilisation, the expression of TFAM, PolGA and PolGB genes was analysed throughout *in vitro* maturation in untreated BCB+ and BCB- oocytes and also in those treated with the mtDNA replication inhibitor, ddC. BCB+ oocytes at 0 hrs contain relatively high levels of TFAM mRNA (see Figure 4.4A). These were significantly reduced throughout the maturation process, with 33% remaining at 22 hrs ($P = 0.004$) and 11% remaining at 44 hrs ($P = 0.004$). On the other hand, BCB- oocytes began the maturation process with relatively low levels of TFAM mRNA, only 19% of those compared to BCB+ oocytes ($P < 0.001$). TFAM expression was then increased throughout maturation by 1.2 fold at 22 hrs ($P > 0.05$) and by 2.8 fold at 44 hrs ($P = 0.02$). BCB+ oocytes treated with ddC showed a similar pattern of TFAM expression to untreated BCB+ oocytes. However, the decrease throughout maturation was much sharper, with a decrease to 13% of 0 hrs levels by 22 hrs ($P < 0.001$) and to undetectable levels by 44 hrs ($P < 0.001$). These oocytes therefore contained significantly fewer TFAM mRNA molecules than equivalent untreated oocytes after both 22 hrs ($P = 0.006$) and 44 hrs ($P = 0.004$). BCB- oocytes treated with ddC also expressed TFAM at significantly lower levels than untreated BCB- oocytes at 22 hrs ($P = 0.008$) and 44 hrs ($P = 0.005$).

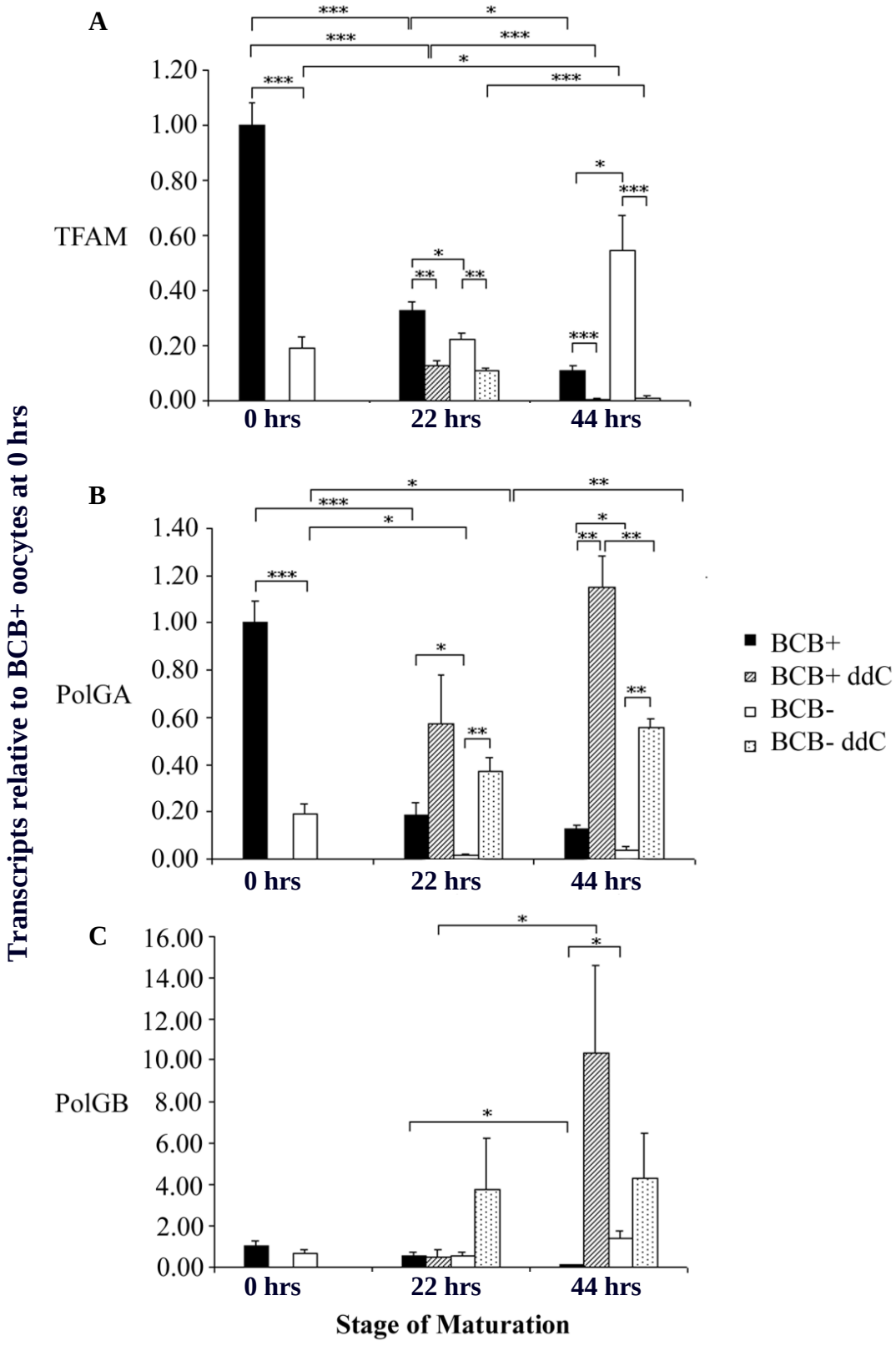
Like TFAM, PolGA expression was relatively high in BCB+ oocytes at 0 hrs and decreased throughout maturation to 18% at 22 hrs ($P = 0.004$) and 13% at 44 hrs ($P = 0.004$; see Figure 4.4B). BCB- oocytes contained significantly fewer PolGA mRNA molecules than BCB+ oocytes at 0 hrs ($P < 0.001$). These initially decreased 19 fold by 22 hrs ($P = 0.02$) and then

increased 4-fold by 44 hrs ($P > 0.05$). All ddC treated oocytes expressed considerably more PolGA than equivalent untreated oocytes. For example, BCB+ ddC treated oocytes contained >3 times the number of PolGA mRNA molecules than untreated oocytes at 22 hrs ($P > 0.05$) and almost 9 times more at 44 hrs ($P = 0.007$). BCB- oocytes treated with ddC contained 37 times more PolGA mRNA molecules than untreated oocytes at 22 hrs ($P = 0.006$) and 14 times more at 44 hrs ($P = 0.006$).

As with the other mtDNA replication factors, untreated BCB+ oocytes contained the highest levels of PolGB at 0 hrs and expression then decreased by 42% at 22 hrs ($P > 0.05$) and by a further 83% at 44 hrs ($P = 0.02$; see Figure 4.4C). BCB- oocytes contained slightly lower levels of PolGB expression than BCB+ oocytes at 0 hrs ($P > 0.05$) and as with PolGA, transcription of PolGB initially decreased slightly at 22 hrs ($P > 0.05$) and then increased by 2.4 fold at 44 hrs ($P > 0.05$), resulting in BCB- oocytes containing significantly more PolGB mRNA molecules than BCB+ oocytes at 44 hrs ($P = 0.02$). BCB+ oocytes treated with ddC did not decrease expression of PolGB during maturation. Instead, these oocytes significantly upregulated PolGB transcription between 22 hrs and 44 hrs ($P = 0.04$) and contained 100 times more mRNA molecules than untreated oocytes at 44 hrs ($P > 0.05$). BCB- oocytes treated with ddC also upregulated PolGB transcription 16-fold from 0 hrs to 44 hrs ($P > 0.05$).

Figure 4.4 (next page): Expression of mtDNA replication factors in maturing oocytes

Bars represent mean ± SEM levels of TFAM (A), PolGA (B) and PolGB (C) mRNA in BCB+ and BCB- oocytes matured with and without ddC at 0 hrs, 22 hrs and 44 hrs. Significant differences between groups are represented using asterisks. * $P<0.05$, ** $P<0.01$, *** $P \leq 0.005$.


A
B
C
TFAM
PolGA
PolGB
Transcripts relative to BCB+ oocytes at 0 hrs
0 hrs
22 hrs
44 hrs
Stage of Maturation
BCB+
BCB+ ddC
BCB-
BCB- ddC

4.6.2 Replication factor and COXI protein in maturing BCB+ and BCB- oocytes

In order to determine whether expression of mtDNA replication factor genes resulted in protein production, ddC treated and untreated BCB+ and BCB- oocytes were analysed throughout *in vitro* maturation for TFAM and PolGA protein expression and localisation using ICC. ICC for the mitochondrially encoded COXI protein was also carried out to determine whether mtDNA replication factor expression influenced the expression of mtDNA encoded subunits of the ETC. As described in Chapter 2 (see Section 2.18), confocal imaging of oocytes at all stages was carried out using the same gain and photomultiplier settings, allowing for comparative analysis of staining intensity between oocytes. In untreated BCB+ oocytes, staining for COXI protein was less diffuse and of increased intensity at 44 hrs compared to 0 hrs and 22 hrs. BCB- oocytes, however, expressed COXI protein at relatively constant levels throughout maturation and the protein was less evenly distributed throughout the oocyte cytoplasm (see Figure 4.5). BCB+ oocytes treated with ddC showed evenly distributed COXI protein with a stronger staining intensity than untreated oocytes at both 22 hrs and 44 hrs. BCB- oocytes treated with ddC also showed evenly distributed COXI protein with a stronger staining intensity than untreated oocytes, although this was only observed at 44 hrs. At 22 hrs, similar staining patterns and intensity were observed between BCB- oocytes treated with ddC and equivalent untreated oocytes.

In BCB+ oocytes, PolGA was expressed at high levels at 0 hrs, distributed evenly throughout the cytoplasm (see Figure 4.5). A similar distribution pattern was seen in BCB+ oocytes at 22 hrs although the staining intensity was slightly reduced. By 44 hrs, PolGA protein appeared to be localised predominantly to the outer edge of the oocyte cytoplasm, with almost no staining in the remainder of the oocyte. BCB- oocytes appeared to contain very low levels of PolGA

protein at 0 hrs as the staining was barely visible. At 22 hrs, BCB- oocytes contained low levels of the protein distributed evenly throughout the cytoplasm. At 44 hrs, however, BCB- oocytes demonstrated an even distribution of the PolGA protein with high staining intensity present throughout the oocyte. In ddC treated BCB+ oocytes, PolGA staining was not localised to the outer edges of the oocyte at 44 hrs, as observed for untreated oocytes, but was maintained throughout the oocyte cytoplasm (see Figure 4.5). Unexpectedly, ddC treated BCB- oocytes at 44 hrs did not demonstrate an increase in PolGA protein to the extent observed in the untreated oocytes, despite the high numbers of mRNA molecules present.

TFAM protein was present in BCB+ oocytes at 0 hrs and staining was of similar intensity throughout the entire cytoplasm of the oocyte (see Figure 4.5). The protein remained evenly distributed in the oocyte during the entire *in vitro* maturation process, although the staining intensity in BCB+ oocytes at both 22 hrs and particularly 44 hrs appeared to be decreased compared to BCB+ oocytes at 0 hrs. TFAM protein in BCB- oocytes behaved in a similar manner to PolGA, with apparent increases in expression throughout oocyte maturation. For example, BCB- oocytes at 22 hrs show slightly increased staining intensity compared to BCB+ oocytes at 22 hrs, with the difference becoming more marked by 44 hrs. BCB+ oocytes treated with ddC showed similar staining patterns for TFAM protein as untreated BCB+ oocytes, although the staining intensity may have been slightly increased at 44 hrs. BCB- oocytes however did not increase TFAM staining intensity throughout maturation as did their untreated equivalents.

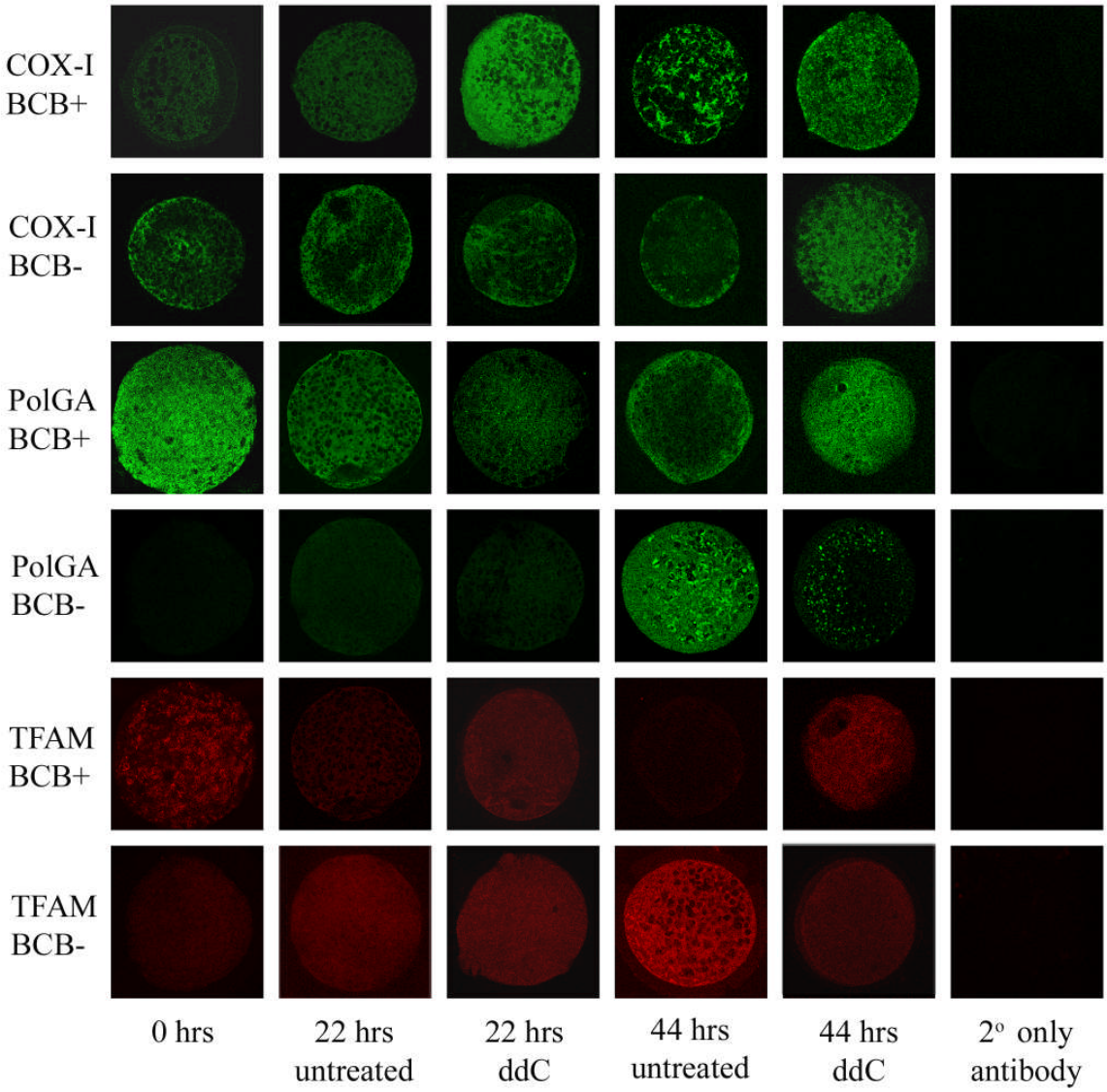


Figure 4.5: Localisation of COXI, TFAM and PolGA proteins during oocyte maturation

Each row shows the expression and localisation of COXI, TFAM or PolGA protein in either BCB+ or BCB- oocytes at 0 hrs (before addition of ddC), 22 hrs (untreated and ddC treated) and 44 hrs (untreated and ddC treated). Negative controls lacking primary antibody but including the appropriate secondary antibody are also shown. Magnification: X 630.

4.6.3 MtDNA copy number in maturing BCB+ and BCB- oocytes

In order to determine whether the expression of these proteins had a functional role, mtDNA copy number was analysed in each group of oocytes. Fluorescence data from a selection of untreated and ddC treated oocytes in mtDNA copy number reactions indicated that some oocytes did not increase fluorescence sufficiently to cross the threshold (see Figure 4.6). The majority of these were ddC treated oocytes, indicating that these oocytes were likely to contain fewer mtDNA copies than equivalent untreated oocytes. This was also reflected in the melt curves which demonstrated slightly increased production of primer-dimers in these samples (see Figure 4.6). In order to accurately quantify mtDNA copy number in these oocytes, fluorescence data were acquired following melting of primer-dimers.

Mean ± SEM mtDNA copy numbers for each group of oocytes are shown in Figure 4.7. BCB+ oocytes contained 403113 ± 80314 mtDNA copies at 0 hrs which decreased to 165362 ± 30461 at 22 hrs ($P > 0.05$) followed by an increase to 323038 ± 71650 at 44 hrs ($P > 0.05$). BCB- oocytes contained significantly fewer mtDNA copies at 0 hrs (66781 ± 8587, $P < 0.001$) and 22 hrs (68406 ± 13733, $P = 0.001$). However, a 7-fold increase resulted in 503263 ± 193295 copies being present at 44 hrs ($P = 0.02$). BCB+ oocytes treated with ddC decreased their mtDNA copy number at 22 hrs to 92857 ± 20160 ($P = 0.04$) and then to 20160 ± 4625 at 44 hrs ($P < 0.001$) resulting in these oocytes containing significantly fewer mtDNA molecules than the untreated oocytes ($P < 0.001$). BCB- oocytes treated with ddC also decreased their mtDNA copy number during maturation, with 9925 ± 2949 copies present at 22 hrs ($P < 0.001$) and 35848 ± 15803 at 44 hrs ($P < 0.001$), again with treated oocytes at 44 hrs containing significantly fewer mtDNA copies than the untreated oocytes ($P < 0.001$).

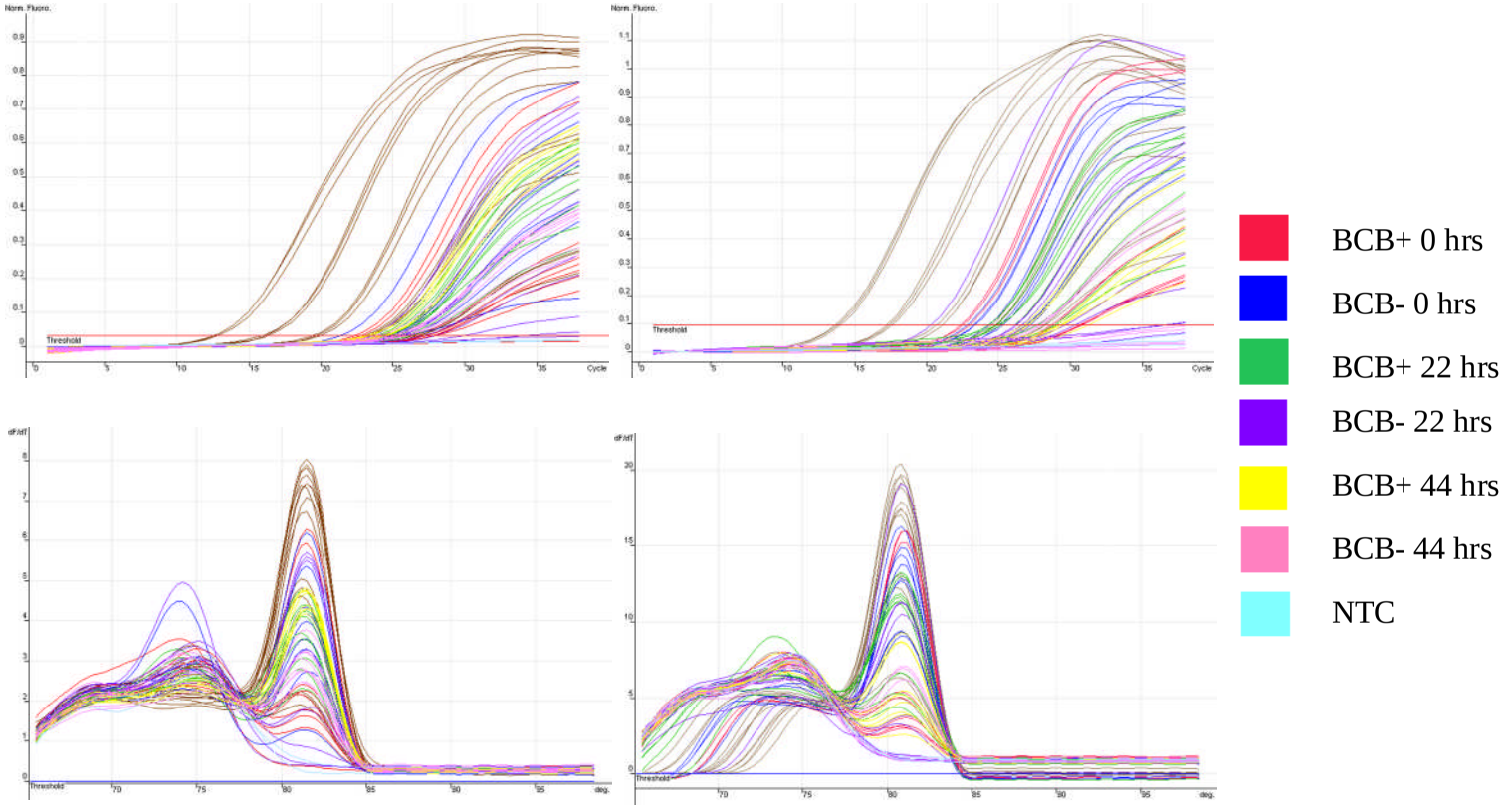


Figure 4.6: Fluorescence and melt data from mtDNA copy number reactions

Fluorescence (top) and melt (bottom) data are presented from a selection of untreated (left) and ddC treated (right) oocytes. More ddC treated oocytes than untreated oocytes did not increase fluorescence enough to cross the threshold, with the majority of these being ddC treated oocytes at 44 hrs. These oocytes are therefore likely to contain relatively few copies of mtDNA. In order to exclude the fluorescence generated by primer-dimers, mtDNA copy number was calculated from fluorescence data acquired after melting of primer-dimers.

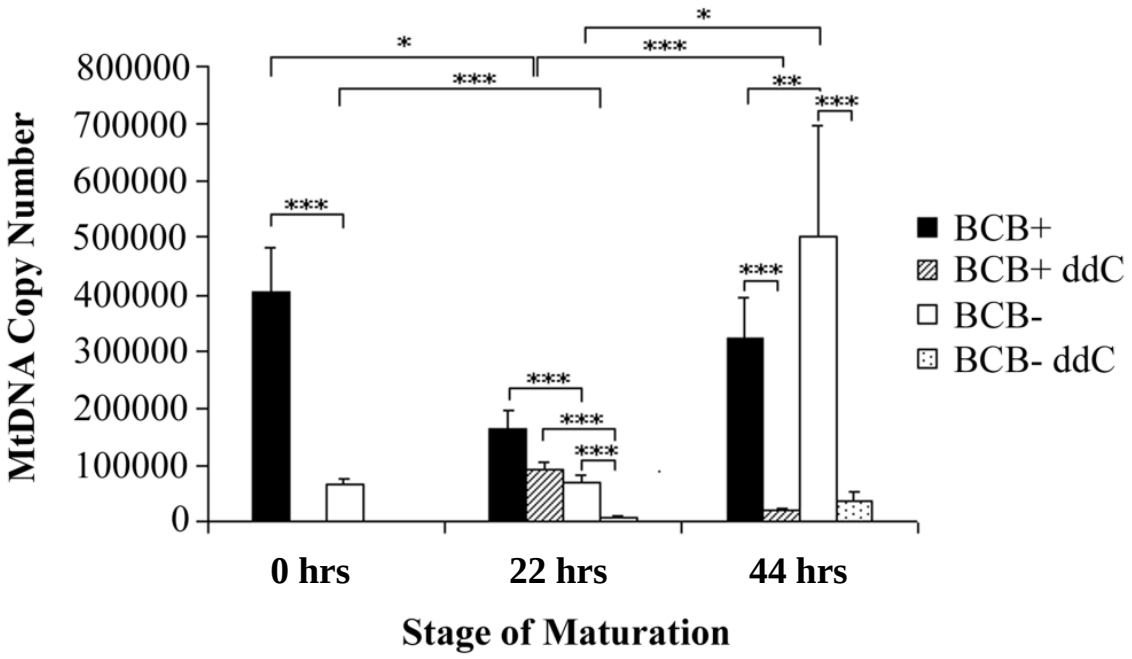


Figure 4.7: MtDNA copy number in maturing oocytes

Bars represent mean ± SEM mtDNA copy number in BCB+ and BCB- oocytes matured with and without ddC 0 hrs, 22 hrs and 44 hrs. Asterisks represent significant difference in mtDNA copy number between the two bracketed groups. * $P < 0.05$, ** $P < 0.01$, *** $P \leq 0.001$. Data were obtained using a RotoGene 3000 real time PCR machine and its associated software, as described in Section 2.8.

4.6.4 Effect of ddC treatment on fertilisation ability of BCB+ oocytes

There was no significant difference in fertilisation outcome between ddC treated (20.7%) and untreated (28.8%) BCB+ oocytes ($P > 0.05$). However, those ddC treated oocytes that were able to fertilise did not develop beyond the 4 to 6-cell stage. MtDNA copy number was therefore analysed in these early embryos in order to determine whether an insufficient number of mtDNA copies was responsible for their developmental arrest. 2-cell embryos derived from ddC treated oocytes contained 185234 ± 49874 copies of mtDNA, only slightly less than 2-cell embryos derived from untreated oocytes ($P > 0.05$, Chapter 3). However, this was significantly higher than in oocytes at 44 hrs treated with ddC ($P < 0.001$), indicating that mtDNA replication had occurred at fertilisation. As with embryos derived from untreated oocytes (see Chapter 3), mtDNA copy number then decreased to 106692 ± 28497 for 4-cell embryos ($P > 0.05$) and 4804 ± 1092 for 6-cell embryos ($P < 0.001$). These values were not significantly different from their respective untreated 4-cell and 8-cell counterparts (Chapter 3).

4.7 Discussion

4.7.1 High oocyte mtDNA copy numbers improve fertilisation outcomes

The data presented in Chapter 3 demonstrate a decrease in mtDNA copy number during the early stages of porcine pre-implantation development. A decrease in mtDNA copy number during pre-implantation development has also been reported in the cow (May-Panloup *et al.*, 2005b), whilst in the mouse, there is limited or no mtDNA replication during this time (McConnell & Petrie, 2004; Piko & Taylor, 1987; Thundathil *et al.*, 2005). These data indicate the likely requirement for mtDNA replication in the oocyte, allowing high mtDNA copy numbers to be present at fertilisation. Data presented in this Chapter are consistent with this hypothesis. In this instance, the mean mtDNA copy number for porcine BCB+ oocytes, those most likely to fertilise (El Shourbagy, 2004; Rodríguez-Gonzáles *et al.*, 2002), was relatively high at 323038 at 44 hrs. Furthermore, it has previously been demonstrated that injection of BCB- oocytes with supplementary mitochondria can improve fertilisation outcome (El Shourbagy, 2004). This is likely due to the increase in mtDNA copy number from below the ~100000 threshold to above the threshold thought to be required for fertilisation success (El Shourbagy, 2004).

Dramatic increases in mtDNA copy number were detected in maturing BCB- oocytes and in ddC treated oocytes following removal of ddC. For example, BCB- oocytes increased mtDNA copy number from 66781 ± 8587 at 0 hrs to 503263 ± 193295 at 44 hrs and ddC treated BCB+ oocytes increased mtDNA copy number from 20160 ± 4625 at 44 hrs to 185234 ± 49874 in 2-cell embryos. These increases in mtDNA copy number, although delayed compared to BCB+ oocytes, provide further evidence for a requirement for high levels of mtDNA. In the cow, a 45-fold increase in mtDNA copy number has been reported (Smith & Alcivar, 1993) and in the

human, a 10000-fold increase in mtDNA copy number has been suggested (Jansen & de Boer, 1998) from PGCs to mature oocytes. As described in this Chapter, approximately 10-fold only increases in mtDNA copy number were observed during porcine *in vitro* maturation, (66781 ± 8587 at 0 hrs to 503263 ± 193295 at 44 hrs for BCB- oocytes and 20160 ± 4625 at 44 hrs to 185234 ± 49874 in 2-cell embryos for ddC treated oocytes). It is therefore likely that mtDNA replication is also required before *in vitro* maturation begins, perhaps even occurring predominantly before *in vitro* maturation begins. This hypothesis is consistent with the high mtDNA copy numbers that were present throughout the entire maturation process in the BCB+ oocytes described in this Chapter. Mitochondria adopt an inactive morphology in mature oocytes and early embryos, being relatively spherical in shape and containing very few cristae (Sathananthan & Trounson, 2000). Completion of mtDNA replication before *in vitro* maturation begins may therefore be necessary in order to allow time for the mitochondria to reduce activity and prepare for fertilisation.

4.7.2 Delayed mtDNA replication occurs in BCB-oocytes due to delayed expression of nuclear-encoded mtDNA replication factors

Previous studies have indicated that BCB+ oocytes tend to fertilise more readily (El Shourbagy, 2004; Rodríguez-Gonzáles *et al.*, 2002) and also have higher numbers of mtDNA copies at 22 hrs of maturation (El Shourbagy, 2004) than BCB- oocytes. Here, it has been shown that BCB+ oocytes contain high numbers of mtDNA copies at 0 hrs and 22 hrs, significantly higher than BCB- oocytes. These high levels are also maintained at 44 hrs. However, BCB- oocytes show increased mtDNA copy number at 44 hrs, consistent with patterns of mtDNA replication factor expression. For example, mtDNA replication factor expression decreased during *in vitro* maturation in the BCB+ oocytes, suggesting that the

major increase in mtDNA copy number had already occurred by this time. However, BCB- oocytes increased their expression of mtDNA replication factors during oocyte maturation, suggesting that the majority of mtDNA replication in these oocytes was occurring during the *in vitro* maturation period. Data obtained from this Chapter therefore suggest that less competent BCB- oocytes exhibited a delay in mtDNA replication due to the delayed onset of expression of their nuclear-encoded mtDNA replication factors. Subsequent delays in downregulating mitochondrial activity and adopting an inactive morphology may therefore have contributed to the reduced fertilisation outcomes observed for these oocytes (El Shourbagy, 2004).

4.7.3 Continued mtDNA replication occurs following the major increase in mtDNA copy number

Although it was determined that BCB+ oocytes completed the majority of their mtDNA replication before *in vitro* maturation, slight fluctuations in mtDNA copy number were observed throughout the maturation process. This may be due to variation in mtDNA copy number between the individual oocytes analysed. However, an alternative explanation is that mtDNA levels are maintained at high levels through continual degradation and replication. To determine whether an active replication process was taking place, oocytes were treated with ddC, the antiretroviral nucleoside analogue that inhibits mtDNA replication (Feng *et al.*, 2001). The initial decline in mtDNA copy number between 0 hrs and 22 hrs was more severe than for untreated oocytes, and the tendency for an increase in mtDNA copy number between 22 hrs and 44 hrs was lost. This resulted in a further 75% decrease in mtDNA copy number over the maturation period in those oocytes treated with ddC. This would suggest that, in untreated oocytes, mtDNA replication was still taking place during the later stages of maturation in order to replenish degraded mtDNA molecules. Indeed, accumulation of mtDNA deletions has also

been demonstrated during oocyte maturation in the rhesus macaque (Gibson *et al.*, 2005). As mtDNA replication would have to take place for the mutation to be incorporated into the genome, these data further support the concept of discreet levels of mtDNA replication taking place, facilitated by the expression of PolGA and PolGB (Figure 4.4). MtDNA deletions have also been associated with aged oocytes from older women, which led to the development of cytoplasmic transfer, where aged oocytes are supplemented with ooplasmic material from younger women during the ICSI process (Cohen *et al.*, 1998). Increased levels of non-deleted mtDNA molecules in these oocytes may therefore have contributed to the improved fertilisation and developmental outcomes obtained using this technique (Barritt *et al.*, 2000) and those obtained following oocyte donation in the fertility treatment of older women (Yaron *et al.*, 1995).

4.7.4 MtDNA levels are increased or maintained via a feedback mechanism

The further 75% loss of mtDNA copies following the ddC treatment is comparable with human MOLT T-lymphoblastic cells which, following 48 hrs of ddC treatment, decreased their mtDNA copy number 5-fold (Chen & Cheng, 1989). To compensate, these cells upregulated glycolysis following mtDNA loss, producing increased levels of lactate after 72 hrs. Increased lactate production was also noted in rat PC12 cells after 48 hrs of ddC treatment (Keilbaugh *et al.*, 1997). However, at the transcriptional and translational level, two further responses to ddC inhibition of mtDNA replication were noted. Firstly, increased transcription of the mitochondrially encoded COXI protein was observed at 22 hrs and 44 hrs in BCB+ oocytes and only at 44 hrs in BCB- oocytes. This may have been an attempt by the oocyte to maintain ETC activity when mtDNA copy number was low. Secondly, increased transcription of the PolGA and PolGB subunits and also maintenance of the PolGA protein were observed in

BCB+ oocytes. This likely facilitated the vastly increased rates of mtDNA replication that occurred following removal of ddC at insemination. Similarly, BCB- oocytes increased expression of TFAM and PolG during *in vitro* maturation, possibly triggered by the low levels of mtDNA present at 0 hrs and 22 hrs and, as a consequence, increased mtDNA copy number late in the maturation process. MtDNA depletion due to ddC has also been reported to initiate a feedback mechanism whereby TFAM expression is increased (Poulton *et al.*, 1994), although effects on PolG expression were not analysed. These data suggest that a feedback mechanism may be responsible for maintaining sufficient mtDNA copies to support fertilisation. This may be similar to other feedback mechanisms regulating the expression of genes such as smad7 by myostatin (Forbes *et al.*, 2006) and nitric oxide synthase by nitric oxide (Grumbach *et al.*, 2005).

4.7.5 Correct regulation and timing of the major burst of mtDNA replication is essential for successful embryonic development

Due to insufficient mtDNA levels at 44 hrs, it was anticipated that ddC treated oocytes would have reduced fertilisation rates. However, fertilisation rates for ddC treated and untreated oocytes were similar ($P > 0.05$). Furthermore, analysis of mtDNA copy number in early embryos showed similar copy numbers to embryos derived from untreated BCB+ oocytes. As the ddC treated oocytes contained significantly fewer mtDNA copies than untreated oocytes at insemination, significantly increased rates of mtDNA replication must have occurred just prior to fertilisation, when the ddC was removed. This was most likely facilitated by the increased production of mRNA for PolGA and PolGB in ddC treated oocytes during the later stages of maturation. Interestingly, an increase in TFAM mRNA was not observed following ddC treatment, again suggesting that minimum levels of TFAM were sufficient for increased

mtDNA replication to take place. However, despite the apparently normal fertilisation ability of ddC treated oocytes, the resulting embryos did not develop beyond the 4 to 6-cell stage. Similarly, BCB- oocytes showed delayed expression of mtDNA replication factors which resulted in delayed mtDNA replication. These oocytes were also shown to have reduced developmental competence, though this was reflected in decreased fertilisation rates (El Shourbagy, 2004; Rodríguez-Gonzáles *et al.*, 2002) rather than developmental arrest. Delayed mtDNA replication, whether naturally occurring or chemically induced, is therefore detrimental to oocyte developmental success.

4.8 Conclusions

Consistent with previously published work (Almeida-Santos *et al.*, 2006; El Shourbagy, 2004; Reynier *et al.*, 2001) and data obtained in Chapter 3, data presented in this Chapter lead to the conclusion that mtDNA is replicated during *in vitro* oocyte maturation to provide sufficient copies for pre-implantation development when mtDNA replication does not occur. More importantly, failure of mtDNA replication to take place during *in vitro* maturation can result in developmental arrest. These data support the rationale behind the cytoplasmic supplementation procedures that were developed to attempt to overcome fertilisation failure and developmental arrest associated with insufficient or damaged cytoplasmic components, such as mitochondria and mtDNA. However, the cause of the foetal abnormalities that occurred following the use of this technique has not yet been determined. It is not only essential that mtDNA replication takes place during oocyte development, but also that it is correctly regulated in order to take place at the correct time. Failure to do this significantly affects the developmental potential of these oocytes, indicating the importance of regulated mtDNA replication for successful differentiation of specialised cells.

CHAPTER 5: mtDNA REPLICATION DURING DIFFERENTIATION OF EMBRYONIC STEM CELLS

5.1 Introduction

On reaching the blastocyst stage, the embryo consists of two types of cells, cells of the TE that will develop into the placenta and extraembryonic tissues, and cells of the ICM that will form the various tissues of the developing offspring (Gardner & Johnson, 1972). *In vitro*, ICM cells can be used to create ESC lines, which can be maintained in culture indefinitely and which express various markers of pluripotency, including OCT-4, DPPA5, PrameL7 and NDP52L1 (Bortvin *et al.*, 2003). Failure to express these markers at sufficient levels results in differentiation of the ESCs. For example, siRNA knockdown of OCT-4 or NANOG results in differentiation of both mouse (Hay *et al.*, 2004; Hough *et al.*, 2006) and human (Hay *et al.*, 2004; Hyslop *et al.*, 2005) ESCs. Pluripotent ESCs can be induced to differentiate into EBs in a similar way to the differentiation that occurs *in vivo* following implantation of a blastocyst. Cellular differentiation either *in vitro* or *in vivo* gives rise to 3 embryonic germ layers known as the endoderm, mesoderm and ectoderm (Tam & Beddington, 1992). Further differentiation along specific lineages then occurs before cells become committed to a particular fate. For example, the endoderm eventually forms the digestive tract (Grapin-Botton & Melton, 2000), the ectoderm forms tissues of the skin and central nervous system (Tam & Zhou, 1996), whilst the cardiovascular and respiratory systems originate from cells of the mesoderm (Tam & Beddington, 1987).

During differentiation of an oocyte, mtDNA copy number is significantly amplified (see Chapter 4) in order to prepare for the pre-implantation embryo development period when

mtDNA replication is downregulated (see Chapter 3). MtDNA replication is not resumed until either the blastocyst stage in the pig (see Chapter 3) and the cow (May-Panloup *et al.*, 2005b), or until after implantation of the blastocyst in the mouse (Piko & Taylor, 1987). Downregulation of mtDNA replication during pre-implantation embryogenesis results in decreasing numbers of mtDNA molecules per cell as the embryo develops. For example, human PGCs, which are formed shortly after implantation (Ginsburg *et al.*, 1990), are reported to contain just 10 mtDNA molecules (Jansen & de Boer, 1998). Data presented in Chapter 3 indicate that the downregulation of mtDNA replication during pre-implantation embryo development is due to decreased expression of nuclear-encoded mtDNA replication factors. Similarly, hESCs cultured *in vitro* have been reported to possess low numbers of mitochondria and express low levels of mtDNA replication factors, indicative of minimal mitochondrial metabolism (St. John *et al.*, 2005b).

Unlike ESCs, fully differentiated cells types within mammals vary considerably in their metabolic activity, due to the significant differences in size, shape and function of specialised cell types. It is therefore not surprising that each cell type can be characterised by its number of mitochondria. This has been especially highlighted in muscle (see Moyes *et al.*, 1998) for review). Furthermore, the number of mtDNA genomes per mitochondrion is also specific to cell type. For example, there is a significant difference between skeletal and cardiac muscle, with 3,650 ± 620 and 6,790 ± 920 mtDNA copies per diploid nuclear genome, respectively ($P = 0.006$; Miller *et al.*, 2003). Similar differences have been observed in, for example, peripheral blood mononuclear cells and subcutaneous fat (Gahan *et al.*, 2001); cultured fibroblasts (Zhang *et al.*, 1994); bovine oocytes and bovine foetal heart fibroblasts (Michaels *et al.*, 1982). Despite this variation, the vast majority of differentiated cell types have

significantly more mtDNA molecules than the ten copies reported in the relatively undifferentiated PGCs (Jansen & de Boer, 1998). In order to achieve these higher numbers, expression of nuclear-encoded mtDNA replication factors is likely to increase as embryonic blastomeres differentiate during post-implantation embryo development. Consequently, the relationship between expression of mtDNA replication factors and cellular differentiation was investigated.

5.2 Hypothesis

During early post-implantation development, embryos increase expression of nuclear-encoded mtDNA replication factors as the blastomeres lose their pluripotency during differentiation into specialised cell types.

5.3 Aims

Using mESCs and progressively differentiating EBs, the experiments described in this Chapter aim to determine whether:

1. mtDNA replication factor expression is increased during mESC differentiation into EBs
2. pluripotency associated gene expression is decreased during mESC differentiation into EBs
3. an increase in mtDNA replication factor expression and a decrease in pluripotency associated gene expression occur concurrently

5.4 Preliminary Experiment

5.4.1 RTPCR analysis of spontaneously formed EBs

An initial study was carried out using a protocol for generating EBs spontaneously from R1 ESCs (see Section 2.1.4). Undifferentiated and migratory stem cell colonies, floating and attached EBs, and fully differentiated MEFs (which were not derived through differentiation of ESCs) were saved individually for RNA extraction and cDNA synthesis as described in Sections 2.4.1 and 2.6. RTPCR was carried out as previously described (see Sections 2.6 and 2.7) using primers for TFAM, TFB1M, TFB2M, NRF1, NRF2, PolGA, PolGB, CytB and the pluripotency related genes, OCT-4, DPPA2, DPPA3, DPPA4, DPPA5, PrameL6, PrameL7 and NDP52L1. Samples were also analysed for expression of the "housekeeping gene", GAPDH. Primer details are listed in Table 5.1. PCR products were separated by agarose gel electrophoresis and products were visualised as described previously. Each band was graded according to its intensity as strong, weak or absent, corresponding to a high or low level of gene expression or no gene expression (see Figure 5.1 for examples). The most common level of expression for each gene at each stage of differentiation is listed in Table 5.2.

A progressive increase in mRNA levels for mitochondrial-related genes was observed from the undifferentiated mESCs to the fully differentiated MEF stage. Undifferentiated mESCs contained mRNA molecules for only TFAM, NRF1 and NRF2α. These mRNA molecules were also present in differentiating migratory stem cells, along with additional mRNA molecules for CytB, NRF2β, TFB1M, TFB2M and also low levels of PolGA. At the attached EB stage, PolGB mRNA molecules were detected for the first time and in MEFs, mRNA molecules for all analysed mitochondrial-related genes were present at their highest levels (see Table 5.2). In contrast to the mtDNA replication factor analysis, a general decrease in

mRNA levels for all pluripotency genes was observed from the undifferentiated mESC to the fully differentiated MEF stage. Specifically, OCT-4, DPPA2, DPPA3, DPPA4, PrameL6 and NDP52L1 had mRNA present in the undifferentiated mESCs and EBs at all stages but not in the MEFs, while mRNA for the other two pluripotency-associated genes, DPPA5 and PrameL7, were found to be absent or present at decreased levels by the EB stages (see Table 5.2). The most marked changes in mRNA levels occurred during the EB stages and specifically, upregulation of the PolG subunits coincided with DPPA5 and PrameL7 downregulation (see Figure 5.1). The TFAM, PolGA, PolGB, DPPA5, PrameL7 and NDP52L1 genes were selected for further analysis by real time RTPCR.

Table 5.1: Primer details and PCR reaction conditions

Gene	Accession number	Forward Primer	Reverse Primer	Annealing temp (°C)	Product size (bp)
GAPDH	NM_001001303	GGGAAGCCCATCACCATCTTC	AGAGGGGCCATCCACAGTCT	60	366
TFAM	NM_009360	TGGAAAACCAAAAAGACCTCG	TCTTCCCAAGACTTGATTTCATT	55	161
PolGA	NM_017462	GGACCTCCCTTAGAGAGGGA	AGCATGCCAGCCAGAGTCACT	60	188
PolGB	NM_015810	GACAGTGCCTTCAGGTTAGT	ACCAGAAATCAAGCCACTGG	54	342
TFB1M	BC032930	CCTCTGCAATGTTGAACACCTC	ACT CAT CTG TGG GGA TGC AC	57	498
TFB2M	NM_008249	AAAAGCAGTTCCTTGGTCAG	AATGGCGTATTATTGGGGCA	53	483
NRF1	NM_010938	CCACGTTGGATGAGTACACG	CTGAGCCTGGGTCATTTTGT	61	263
NRF2α	BC052448	AAGGATGCTCGAGACTGTA	ATGACCAGACGGTTCAGTT	53	259
NRF2β	BC013558	GGGCATTTCTCTACCACAGAG	TGCGTGTGTACATCAGCACC	58	240
CytB	NC_005089	GGGTTAAACTCAGATGCAGAT	TAGGTTAGCTACTAGGATTC	56	431
OCT-4	NT_082375	AGTATGAGGCTACAGGGACA	CAAAGCTCCAGGTTCTCTTG	55	260
DPPA2	NM_028615	CCTGGCTGTCAGTCATCAGA	GTTAAAATGCAACGGGCTGT	60	107
DPPA3	NM_139218	CTTTCCCAAGAGAAGGGTCC	TGCAGAGACATCTGAATGGC	60	149
DPPA4	BC064754	TTCTGGATGAGAAAGGCACC	TGCCCCAAGTGTGTTCATAA	60	186
DPPA5	NM_025274	GCTTGATCTCGTCTTCCCTG	TCCATTTAGCCCGAATCTTG	60	293
PrameL6	NM_178249	ACTTTCTTGGGCTGCCTTTT	AGATTCCAGGGCTCATCCTT	56	334
PrameL7	NM_178250	AGAGAACCCACATGGCTTTG	GGATTTGGCTTGGCATACAT	60	336
NDP52L1	G73531	TTGATGCTCTTGCACAGGAC	TCACTGTTAGCACTGCCTG	60	475

Table 5.2: Presence of pluripotency and mitochondria-related gene mRNA molecules at five stages of differentiation.

Gene	Undifferentiated	Migratory	EB	Attached EB	MEFs
GAPDH	High	High	High	High	High
CytB	None	High	High	High	High
NRF1	High	High	High	High	High
NRF2α	High	High	High	High	High
NRF2β	None	High	Low	High	High
TFAM	High	High	High	High	High
TFB1M	None	High	Low	High	High
TFB2M	None	High	None	High	High
PolGA	None	Low	Low	Low	High
PolGB	None	None	None	High	High
OCT-4	High	High	High	High	None
DPPA2	High	High	High	High	Low
DPPA3	High	High	Low	High	Low
DPPA4	High	High	High	High	Low
DPPA5	High	High	High	Low	None
PrameL6	High	Low	High	High	None
PrameL7	High	High	Low	None	None
NDP52L1	High	High	Low	High	None

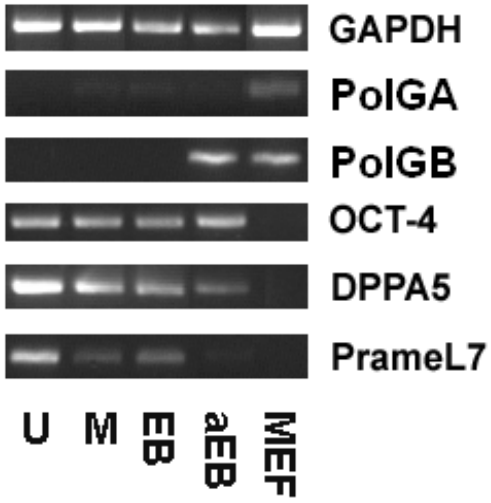


Figure 5.1: Presence of pluripotency and mitochondria-related gene mRNA molecules at five stages of differentiation

Undifferentiated (U) and differentiating migratory (M) stem cells, floating EBs (EB), attached EBs (aEB) and fully differentiated mouse embryonic fibroblasts (MEF) were analysed by semi-quantitative RTPCR for the catalytic (PolGA) and accessory (PolGB) subunits of the mitochondrial DNA polymerase and for the pluripotency related genes OCT-4, DPPA5 and PrameL7. GAPDH was used as a housekeeping gene.

The intensity of each agarose gel band was used to assign the level of gene expression, relative to the differentiation stage at which each gene is expressed most. The most common level of expression for each gene at each stage of differentiation is listed in Table 5.2. For example, PolGA had the most intensity in MEFs, corresponding to a high level of expression (Table 5.2). Expression was at a lower level in migratory mESCs, floating and attached EBs and there was no detectable expression in undifferentiated mESCs. On the other hand, DPPA5 was expressed most in undifferentiated mESCs, migratory mESCs and floating EBs and to a lesser extent in attached EBs, as demonstrated by the less intense agarose gel band. DPPA5 was not expressed at all in MEFs.

5.5 Materials and Methods

5.5.1 EB formation using the hanging drop method

EBs were produced using the "hanging drop" method as described previously (see Section 2.1.5). This method was chosen because it allowed synchronised production of EBs. This is in contrast to the spontaneous method which resulted in EBs being formed over varying periods of time. The hanging drop method also resulted in EBs being produced within two days rather than up to 4 weeks. Briefly, this method involved ESCs being cultured upside down for 2 days in the absence of LIF, cultured for a further 6 days in suspension and then plated on to culture plates for attachment, migration and further differentiation (Keller, 1995). This tissue culture work was carried out in collaboration with my colleague, João Facucho-Oliveira.

5.5.2 Real time RTPCR analysis of EBs formed using the hanging drop method

EBs were harvested for analysis every 24 hrs during the hanging drop (Day 1 and Day 2) and further suspension (Days 3-8) stages of EB formation (see Section 2.1.5). RNA was extracted from 30 EBs harvested on each of the 8 days and 800ηg RNA from each sample was reverse transcribed into cDNA (see Sections 2.4.1 and 2.6). Data from the preliminary experiment described above (Section 5.4.1) indicated that expression of TFAM, PolGA, PolGB, DPPA5, PrameL7 and NDP52L1 should be analysed further. The real time PCR reactions for TFAM, PolGA and PolGB were carried out by my colleague, João Facucho-Oliveira. Difficulties in generating good standard curves resulted in the design of new primer pairs for TFAM and PolGB. Details of these are listed in Table 5.3. Conventional PCR using these new primers was carried out to generate cDNA for each gene of interest and the products were separated using agarose gel electrophoresis. cDNA extracted from the agarose gel bands was then serially diluted 10-fold to produce standards of known target cDNA concentration.

Table 5.3: Murine TFAM and PolGB primers and reaction conditions for real time RTPCR

Gene	Forward	Reverse	Tm (°C)	Size (bp)
TFAM	GCA TAC AAA GAA GCT GTG AG	GTT ATA TGC TGA ACG AGG TC	53	165
PolGB	ACA GTG CCT TCA GGT TAG TC	ACT CCA ATC TGA GCA AGA CC	55	214

Expression levels per cell of each gene at each stage of EB derivation were calculated relative to the housekeeping gene, GAPDH, as an unknown number of cells were present in each sample and relative to a control sample of undifferentiated R1 ESCs. Relative expression in this experiment was calculated using the Pfaffl method (Pfaffl, 2001). This allowed the real time PCR reaction for the gene of interest to be carried out separately from that of the housekeeping gene, thereby allowing use of optimal reaction conditions for each primer set. This method also takes into account any variation in reaction efficiency between different primer sets and allows gene expression levels to be calculated relative to an internal reference sample such as the undifferentiated R1 ESCs used in this experiment, facilitating detection of alterations in expression levels. Reaction efficiency for each gene was calculated by the RotorGene software (Version 7.0; Corbett Research) using the Ct values generated from the standards of known cDNA concentration. Reaction efficiencies and sample Ct values were entered into the Pfaffl equation to calculate relative expression:

$$\frac{\text{Target Gene Reaction Efficiency}^{\Delta \text{ Target Gene Ct (Control Ct- Sample Ct)}}}{\text{Housekeeping Gene Reaction Efficiency}^{\Delta \text{ Housekeeping Gene Ct (Control Ct- Sample Ct)}}}$$

where “control” is the undifferentiated mESC internal reference sample.

5.5.3 Statistical analysis of expression levels

As for experiments described in Chapters 3 and 4, each real time reaction was carried out twice in triplicate thereby producing 6 values per sample. The highest and lowest values for each sample were excluded from further analysis to adjust for pipetting errors (Bustin, 2000). The remaining 4 values for each sample were used to calculate mean ± SEM levels of expression of each gene and to determine whether the data were normally distributed using the one-sample Kolmogorov-Smirnov test. TFAM data were determined to be normally distributed and comparisons between TFAM expression in EBs on different days were therefore made using independent samples *t*-tests. Data for all other genes of interest were not normally distributed, as determined by the one-sample Kolmogorov-Smirnov test. Expression levels for these genes were therefore compared using the non-parametric Mann Whitney U tests. Values of $P < 0.05$ were considered significant.

5.6 Results

5.6.1 Calculation of reaction efficiency

In order to use the Pfaffl method of gene expression quantification, the efficiency of each real time PCR reaction had to be calculated. A standard curve was therefore produced for each gene following real time PCR amplification of a set of standards of known cDNA concentration (see Figure 5.2). The slope values obtained from the standard curves were then used to calculate the reaction efficiencies for each primer set (RotorGene software; Version 7.0; Corbett Research). Using the Pfaffl equation (see Section 5.5.2), these efficiency values allowed determination of the level of mRNA per cell for each gene of interest during EB formation. Although the R^2 values for all genes were close to the optimal value of 1, the efficiency values for DPPA5 and for NDP52L1 were outside the normal range (0.6- 1.1; (Pfaffl, 2003). This needed to be taken into account during the calculation of relative expression levels. The Pfaffl method of relative quantification was therefore ideal, as it allowed the variable efficiency values between different set of primers to be incorporated into the calculations, as described in Section 5.5.2.

Figure 5.2A

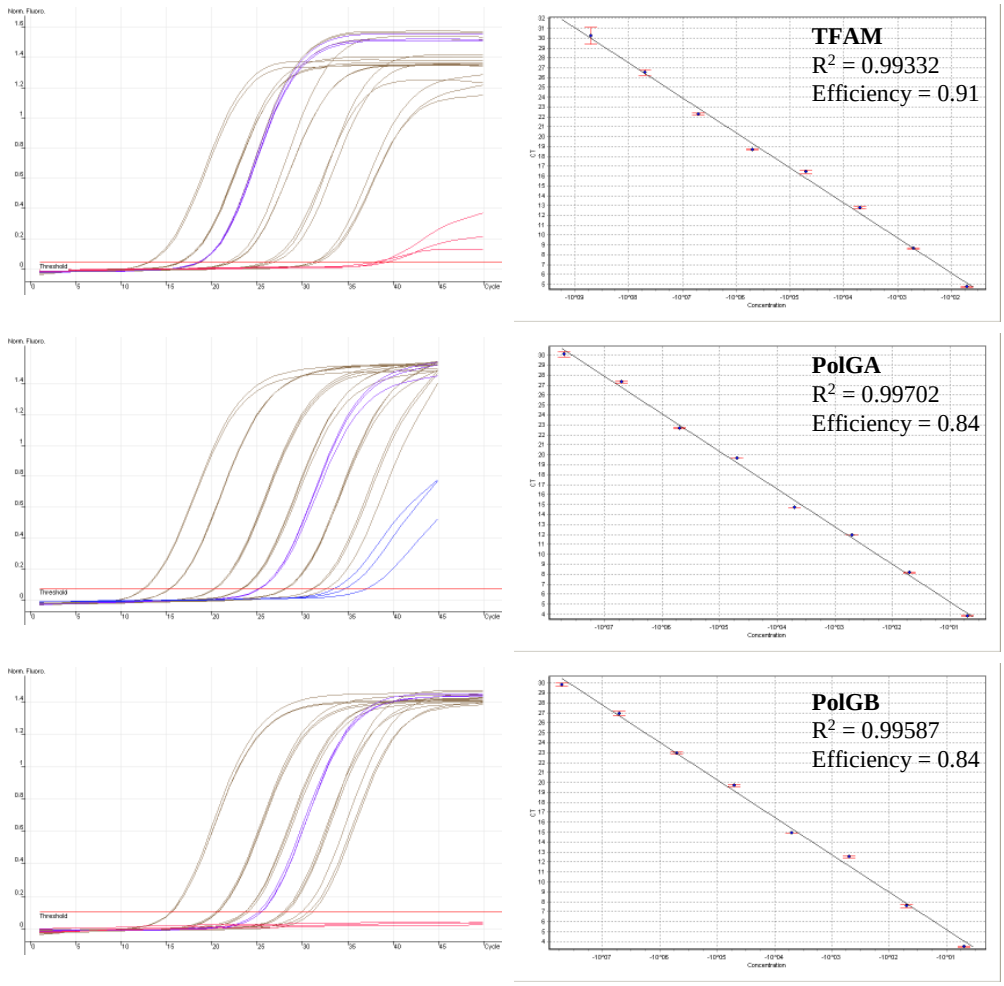
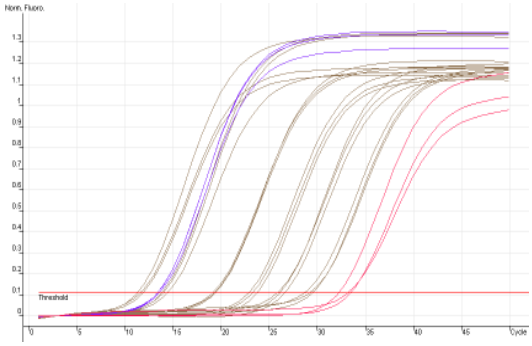
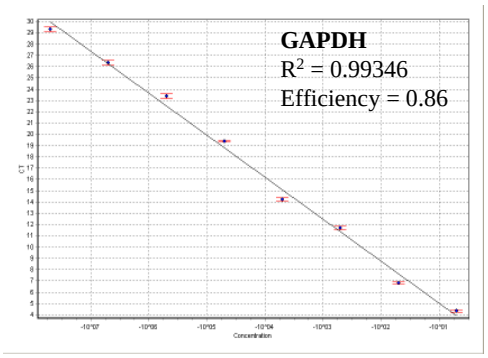


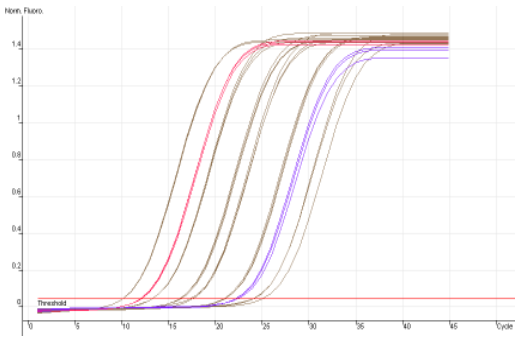
Figure 5.2: Example of fluorescence measurements and standard curves

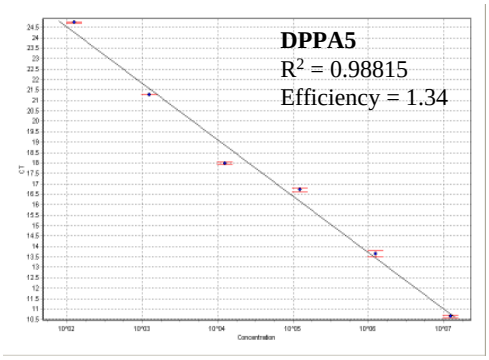
A (this page): Examples of fluorescence measurements (left) and standard curves (right) obtained for TFAM, PolGA and PolGB. B (next page): Examples of fluorescence measurements (left) and standard curves (right) obtained for GAPDH, DPPA5, PrameL7 and NDP52L1. Fluorescence graphs show fluorescence measurements obtained for standards (brown) of 10-fold dilutions and some examples of samples (red, blue and purple). The efficiency values that were generated were used for the relative quantification of gene expression during EB production, as described in Section 5.5.2.

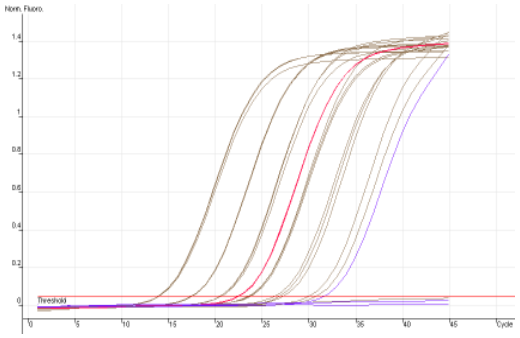
Figure 5.2B

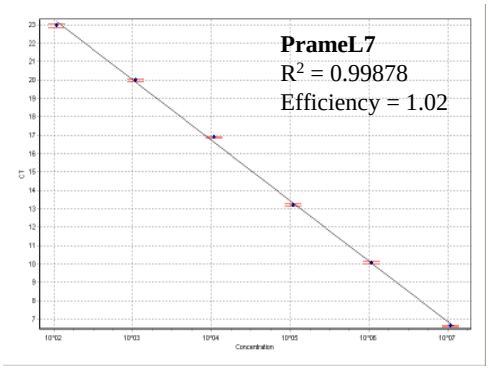


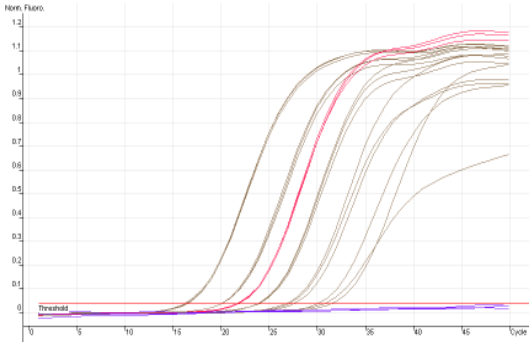

GAPDH
R2 = 0.99346
Efficiency = 0.86

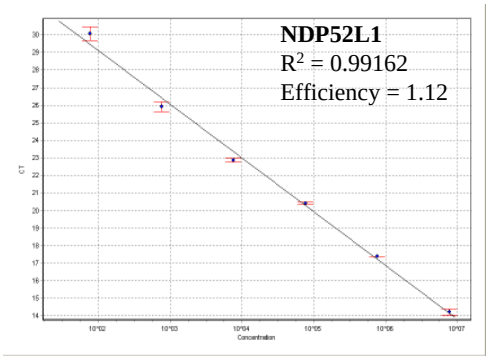



DPPA5
R2 = 0.98815
Efficiency = 1.34




PrameL7
R2 = 0.99878
Efficiency = 1.02




NDP52L1
R2 = 0.99162
Efficiency = 1.12

5.6.2 Real time PCR analysis mtDNA replication factor expression

In order to investigate the timing of the onset of mtDNA replication during early post-implantation embryogenesis, expression levels of the mtDNA replication factors, TFAM, PolGA and PolGB in EBs on Days 1 to 8 were determined relative to undifferentiated R1 mESCs using real time RTPCR. TFAM expression was relatively constant in undifferentiated R1 ESCs and Day 1-5 EBs although a slight decrease was detected (see Figure 5.3). However, by Day 6, expression of TFAM was significantly reduced ($P < 0.001$ compared to undifferentiated R1 ESCs) and remained at this low level on Day 7 and Day 8 (see Figure 5.3). PolGA expression increased 2.7-fold in Day 1 EBs ($P = 0.02$) before mRNA levels decreased to undetectable levels by Day 3 ($P = 0.01$; see Figure 5.3). A significant increase in PolGA expression was observed on Day 5 ($P = 0.01$), although no mRNA molecules were detected on Day 6 (see Figure 5.3). Significant increases in PolGA expression were then observed on Day 7 ($P = 0.01$) and again on Day 8 ($P = 0.02$) (see Figure 5.3). In contrast to the pattern observed for PolGA, PolGB expression did not increase on Day 1 of EB formation but instead decreased, with expression not detectable by Day 3 ($P = 0.01$; see Figure 5.3). PolGB expression remained at this low level until Day 6. A significant increase in PolGB expression was then observed between Day 6 and Day 8 ($P = 0.01$), coinciding with the increase observed for PolGA (see Figure 5.3).

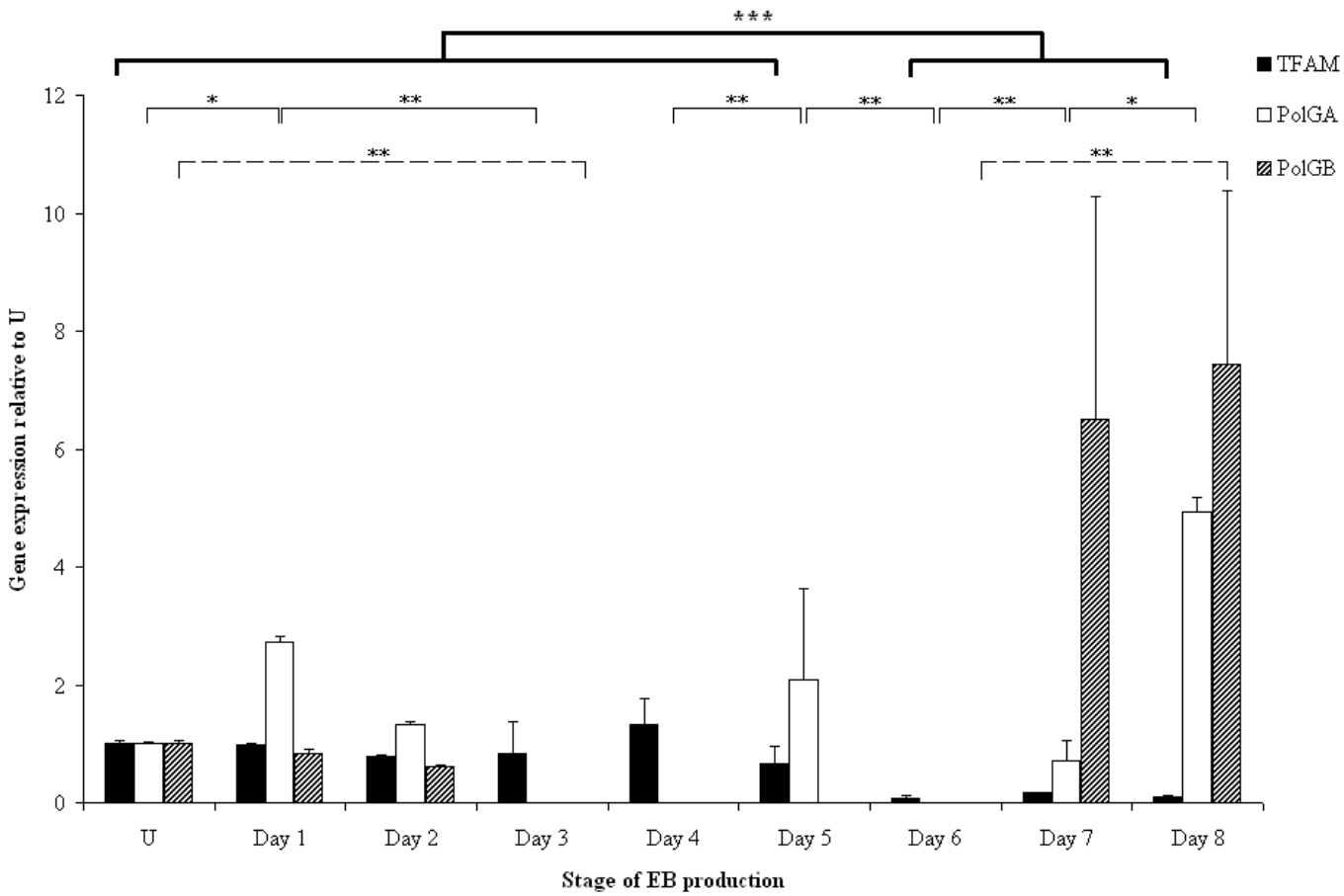


Figure 5.3: Expression of mtDNA replication factors during EB formation

Expression levels of TFAM, PolGA and PolGB are shown over 8 days of EB formation relative to the expression level for each gene in undifferentiated R1 mESCs (U). TFAM expression levels were relatively constant until Day 5 after which expression significantly decreased. PolGA and PolGB expression decreased early on in EB formation, despite some brief increases in PolGA expression, before significantly increasing between Days 6 and 8. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

5.6.3 Real time PCR analysis of pluripotency related gene expression

In order to investigate the timing of the decrease in pluripotency of the ICM cells during early post-implantation embryo development, mRNA levels of the pluripotency associated genes, DPPA5, PrameL7 and NDP52L1, in EBs on Days 1 to 8 were determined relative to undifferentiated R1 mESCs. Interestingly, significant increases in expression of all three pluripotency genes were observed over the first 2 days of EB production (see Figure 5.4). For example, DPPA5 increased expression by 25% ($P = 0.02$), PrameL7 expression 3-fold ($P = 0.02$) and NDP52L1 21-fold ($P = 0.006$). However, expression levels for each gene then decreased between Day 2 and Day 4 (see Figure 5.4). For example, DPPA5 expression decreased to 8% of that in undifferentiated R1 mESCs ($P = 0.02$) and both PrameL7 and NDP52L1 expression decreased to undetectable levels ($P = 0.01$ and $P = 0.01$, respectively). Expression levels of PrameL7 and NDP52L1 remained low for the remainder of the suspension culture period. However, a significant increase in DPPA5 expression was detected on Day 6 ($P = 0.02$), although this was significantly decreased again by Day 7 ($P = 0.02$; see Figure 5.4).

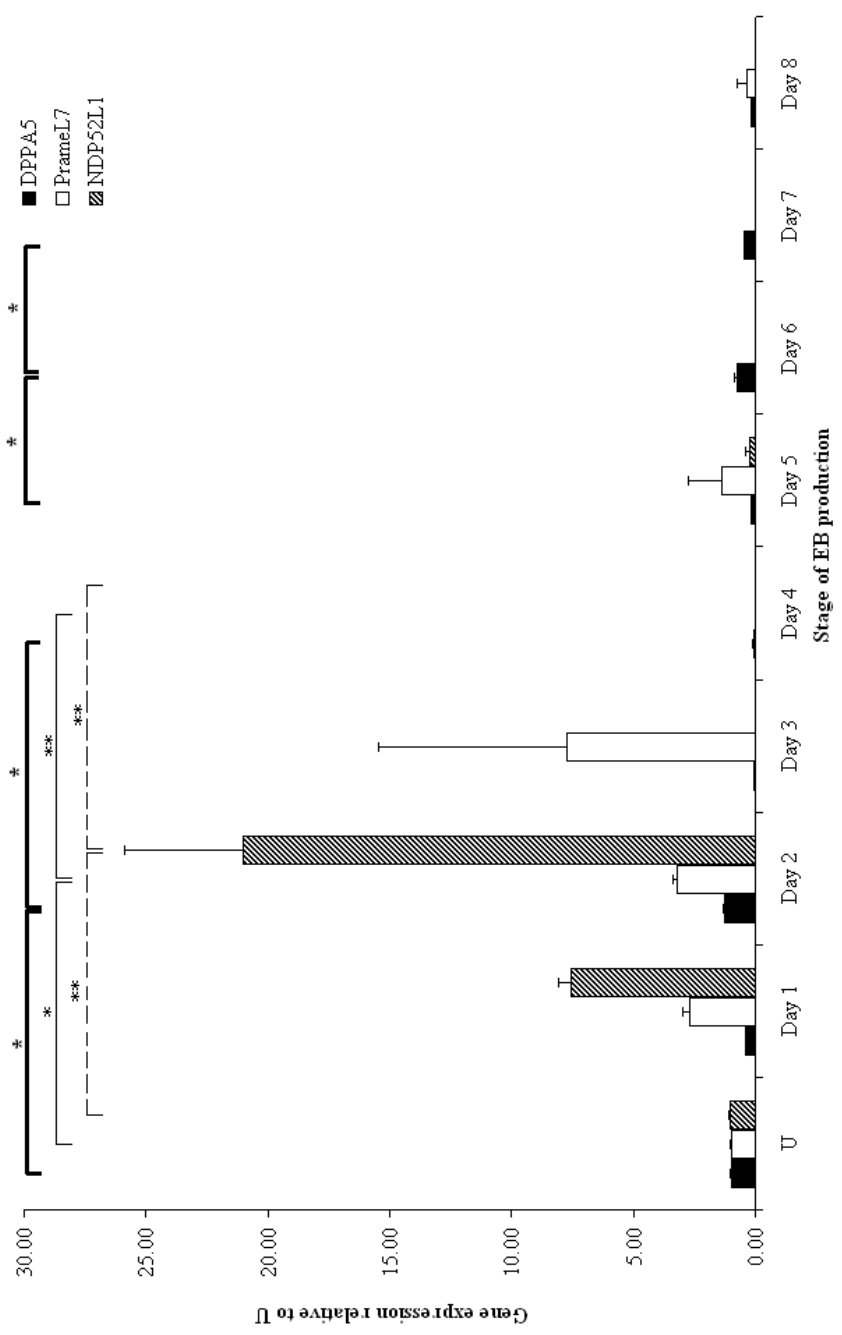


Figure 5.4: Expression of pluripotency related genes during EB formation

Expression levels of DPPA5, PrameL7 and NDP52L1 are shown over 8 days of EB formation relative to the expression level for each gene in undifferentiated R1 mESCs (U). Expression levels for all three genes increased significantly in the first two days of EB formation, before falling to very low levels for the remainder of the culture period. DPPA5 expression also increased briefly on Day 6. * $P < 0.05$, ** $P < 0.01$.

5.7 Discussion

5.7.1 MtDNA replication factor expression increases during EB formation

ESCs are reported to contain low numbers of mitochondria and be relatively inactive metabolically (Sathananthan *et al.*, 2002; St. John *et al.*, 2005b). In contrast, fully differentiated cells tend to have larger numbers of mitochondria and mtDNA molecules, although these vary considerably depending on the needs of different cell types (Moyes *et al.*, 1998). It was therefore hypothesised that nuclear-encoded mtDNA replication factors would be expressed at relatively low levels in undifferentiated ESCs and that expression of these factors would increase during the formation of EBs. Consistent with this hypothesis, both PolGA and PolGB expression increased significantly during EB formation after Day 6, following an initial decrease to undetectable levels between Day 1 and Day 3. The similar expression patterns between PolGA and PolGB are consistent with the fact that neither subunit plays an important role in mtDNA replication in the absence of the other (Yakubovskaya *et al.*, 2006). However, on both Day 1 and Day 5, PolGA expression is increased whilst PolGB expression is not, and on Day 5, PolGB expression is not even detectable. This may reflect the alternative but associated role of PolGA as an exonuclease, which is required for mtDNA repair (Pinz & Bogenhagen, 2006).

By Day 8 of EB formation, PolGA expression was 5-fold higher and PolGB expression was 7.5-fold higher than in undifferentiated ESCs. As mRNA molecules for neither of these genes were present at detectable levels on Day 3, Day 4 or Day 6, this represents a considerable increase and suggests that EBs beyond Day 6 are preparing for mtDNA replication by increasing expression of these mtDNA replication factors. Interestingly, TFAM expression is not increased between Day 6 and Day 8. In fact, although TFAM expression levels are

relatively constant in undifferentiated ESCs and throughout the first 5 days of EB formation, expression of TFAM is significantly reduced on Day 6 and remains so on Day 7 and Day 8. As TFAM is essential for transcription of the primer required for mtDNA replication (Clayton, 1982), these data indicate that mtDNA replication may not have yet been initiated. However, data described in Chapter 3 and Chapter 4 suggest that mtDNA replication can indeed occur without increased expression of TFAM. These seemingly conflicting observations may be explained by a recently published report indicating that, through its roles in both mtDNA transcription initiation and in mtDNA packaging, TFAM is able to subtly control both the level and the mechanism of mtDNA replication in order to meet the needs of specific cell types (Pohjoismaki *et al.*, 2006). In particular, it was reported that cells overexpressing TFAM behaved, in terms of the mechanism of mtDNA replication, in a similar manner to cells treated with ddC, indicating that excess TFAM may inhibit mtDNA replication and subsequent increases in mtDNA copy number (Pohjoismaki *et al.*, 2006). This may explain why TFAM expression was observed to decrease significantly during EB formation just before the increase in PolGA and PolGB expression.

5.7.2 Pluripotency associated gene expression is lost soon after EB formation

Mammalian ESCs are initially pluripotent but commit to a particular cell fate during the differentiation process, thereby losing their pluripotency (Tiedemann *et al.*, 2001). However, the timing of the onset of this process is not clearly understood. Real time RTPCR was used to quantify the expression of three genes associated with pluripotency during the process of EB formation. DPPA5, PrameL7 and NDP52L1 expression all increased during the first stages of EB formation, with significantly higher levels of expression detected in Day 2 EBs when compared to undifferentiated ESCs. This was intriguing as these genes were expected to

demonstrate decreases in expression, as observed in the preliminary experiment described in this Chapter. However, after Day 2, expression levels of the three pluripotency associated genes significantly decreased to below the level present in undifferentiated ESCs and remained low for the remainder of the culture period. These data are comparable to another study using R1s where expression of OCT-4 and SSEA-1, which are other pluripotent stem cell markers, decreased considerably by 3 to 4 days after LIF removal following an initial increase in expression of these genes (Palmqvist *et al.*, 2005).

Despite the low levels of pluripotency associated gene expression described here in EBs beyond Day 3 to 4, small increases in Pramel7 and DPPA5 expression were detected on Day 5 and Day 6, respectively. Similarly, cultured EBs derived from R1 ESCs were also reported to exhibit persistent patchy expression of OCT-4 and FGF-5, other markers associated with pluripotency (Leahy *et al.*, 1999). These authors suggested that some cells contained within EBs maintain their pluripotency. This is not surprising as EBs are thought to mimic the differentiation that occurs during early post-implantation embryogenesis (Desbaillets *et al.*, 2000). It is well known that mammalian embryos and even adults possess a small number of stem cells among the many differentiated cells they possess (Beresford, 1989; McKay, 1997; Zuk *et al.*, 2002). EBs are also likely to include cells destined to become germ cells which also express pluripotency markers such as DPPA3 (Bortvin *et al.*, 2004) and NANOG (Yamaguchi *et al.*, 2005). The overall decrease in expression of pluripotency-associated genes described here is consistent with the early differentiation of ESCs into EBs containing precursors to a variety of cell types, the vast majority of which are non-pluripotent.

5.7.3 Timing of pluripotency associated and mtDNA replication factor gene expression corresponds to the requirements of early post-implantation embryos

It was hypothesised that mitochondrial differentiation is accompanied by loss of pluripotency. Consistent with this hypothesis, mtDNA replication factor expression increased significantly and expression of pluripotency markers also decreased significantly during EB formation. However, these events did not take place concurrently as the pluripotency markers showed decreased expression levels during the first 2 days of EB formation, whilst mtDNA replication factor expression was not increased until after Day 6. The patterns of gene expression observed here can therefore be divided into 3 groups i.e. Days 1 and 2 (early), when pluripotency markers were highly expressed, Days 3-5 (intermediate), when pluripotency marker expression was reduced and Days 6-8 (late), when mtDNA replication factor expression began to increase. The differentiation of cells in EBs derived from R1 ESCs has previously been correlated to that which occurs in the mouse embryo *in vivo* (Leahy *et al.*, 1999). These authors reported that Day 1-2 EBs were equivalent to E 4.5 to E 6.5, representing the early post-implantation embryo that has yet to undergo gastrulation. As pre-gastrulation embryos are still relatively undifferentiated, it is not surprising that high expression levels of pluripotency genes were detected in cells of early EBs or that mtDNA replication factor expression in these cells was equivalent to that in undifferentiated ESCs.

Day 3-5 EBs were determined to be equivalent to E 6.5 to E 7, which is when gastrulation takes place (Leahy *et al.*, 1999). Gastrulation involves the movement of cells committed to one of the three germ cell layers to positions within the embryo associated with either the endoderm, mesoderm or ectoderm (Tam & Behringer, 1997). As these cells are now committed to a specific germ layer, they are no longer pluripotent. This is reflected in the

decreased expression of pluripotency-associated genes in the Day 3 EBs described in this Chapter. However, no increase in mtDNA replication factor expression was observed in Day 3-5 EBs, which might suggest that the mtDNA was not being replicated at this time. This is consistent with a requirement for non-mitochondrial metabolism such as glycolysis during the gastrulation event (Kelly & West, 1996). This is also consistent with an emphasis on the migration of cells throughout the embryo rather than rapid cell division that requires amplification of cytoplasmic contents. Indeed, xenopus gastrulation has been reported to occur in the absence of DNA replication and cell division (Takagi *et al.*, 2005), whilst downregulation of cell proliferation precedes gastrulation in rabbits (Viebahn *et al.*, 2002).

Day 6 EBs were determined to be equivalent to E 7.5, which is when gastrulation is complete and organogenesis is initiated (Leahy *et al.*, 1999). Consistent with early organogenesis and further cell differentiation, significant increases in PolGA and PolGB expression were observed between Day 6 and Day 8 EBs. The importance of PolG activity at this stage is further highlighted by the death of homozygous PolGA knock-out murine embryos, which occurs on E 8.5 (Hance *et al.*, 2005). Despite an overall increase in PolGA and PolGB expression, relatively large SEM values were obtained for PolGB expression in Day 7 and Day 8 EBs (3.6 and 2.8, respectively, compared to 0.06 on Day 1). This might be due to variation in mtDNA replication requirements in different cells within and between EBs, consistent with the varied OXPHOS requirements of different specialised cell types (Moyes *et al.*, 1998). However, mean PolGA and PolGB mRNA levels on Day 7 and Day 8 were significantly higher than in undifferentiated mESCs, indicating that the majority of cells at this stage have higher OXPHOS requirements than undifferentiated stem cells. The increase in PolGA and PolGB expression in Day 7 and Day 8 EBs followed a decrease in TFAM expression on Day 6.

This lack of requirement for TFAM in EBs between Day 6 and Day 8 is consistent with the survival of homozygous TFAM knock-out mice beyond this stage of development (Larsson *et al.*, 1998).

5.8 Conclusions

Consistent with the original hypothesis, undifferentiated ESCs acquired the ability to replicate their mtDNA through increased expression of mtDNA replication factors and lost their pluripotent status during the formation of EBs. However, these events did not occur concurrently as, although pluripotency associated gene expression decreased by Day 3, PolGA and PolGB expression was not increased until Day 6-8. Significantly, these time points correlate well with important events that occur in equivalent staged early post-implantation embryos *in vivo*. For example, loss of pluripotency associated mRNA on Day 3 correlates with the beginning of gastrulation and the formation of the three, non-pluripotent, embryonic germ layers, whilst increasing expression of mtDNA replication factors from Day 6 correlates with the completion of gastrulation and the subsequent initiation of organogenesis. The data described in this Chapter, as well as the reported early death of mice lacking PolGA, indicate that mitochondrial differentiation is an essential component of early cellular differentiation and organogenesis.

CHAPTER 6: mtDNA REPLICATION IN EMBRYOS DERIVED BY NUCLEAR TRANSFER

6.1 Introduction

NT is associated with low developmental rates and a variety of abnormalities have been reported in embryos and offspring generated using this procedure (Cibelli *et al.*, 2002). These include epigenetic abnormalities (Chung *et al.*, 2003) as well as altered patterns of gene expression (Humpherys *et al.*, 2002). In particular, failure to correctly reprogram the nucleus has been demonstrated through incomplete reactivation of pluripotency associated genes including OCT-4 (Bortvin *et al.*, 2003). These genetic and epigenetic abnormalities result in detrimental phenotypic outcomes such as cardiovascular (Lanza *et al.*, 2001), respiratory (Ono *et al.*, 2001; Wakayama & Yanagimachi, 1999), renal (Denning *et al.*, 2001; McCreath *et al.*, 2000) and hepatic (Denning *et al.*, 2001; McCreath *et al.*, 2000) defects.

Due to the requirement for interaction between the nucleus and mitochondria, mitochondrial activity could also be abnormal in NT embryos. This may explain why many cloned animals exhibit symptoms similar to those present in mitochondrial disease such as dilated cardiomyopathy (Lanza *et al.*, 2001). Mitochondrial activity must be highly regulated in order to meet the requirements of early embryos. For example, somatic, i.e. non-embryonic, mitochondria have been demonstrated to negatively affect the development of parthenogenetically activated murine oocytes (Takeda *et al.*, 2005). This is likely to be because somatic mitochondria are tailored to meet the specific needs of the somatic cells from which they are derived (Moyes *et al.*, 1998). NT embryos derived using somatic cells may therefore exhibit mitochondrial characteristics associated with the donor somatic cell, which

may not be consistent with the needs of an embryo. Indeed, overexpression of TFAM and PolG has been reported in sheep 4-cell SCNT embryos when compared to those generated through IVF (Lloyd *et al.*, 2006).

Successful embryogenesis also requires a sufficient number of mtDNA molecules, which are usually inherited from the oocyte. For example, oocytes with higher copy numbers of mtDNA are known to be associated with improved fertilisation rates (Almeida-Santos *et al.*, 2006; El Shourbagy, 2004; May-Panloup *et al.*, 2005a; Reynier *et al.*, 2001). Furthermore, a critical threshold of approximately 100000 copies in the mature oocyte has been proposed for the mouse (Piko & Taylor, 1987) and human (Almeida-Santos *et al.*, 2006; Reynier *et al.*, 2001). Supplementation of oocytes containing insufficient mtDNA copies with further oocyte-derived mitochondria resulted in sufficient mtDNA copy number and also increased developmental outcomes in the pig (El Shourbagy, 2004). This further demonstrates the importance of mtDNA copy number for successful embryogenesis and supports the rationale behind CT, where donor cytoplasm containing supplementary, often younger, non-mutated mtDNA is injected into the oocyte during the ICSI procedure to compensate for the defective cytoplasm in the oocyte (Cohen *et al.*, 1997).

For successful embryogenesis, NT embryos must not only contain sufficient of the appropriate mitochondria but must exhibit sufficient nucleo-mitochondrial interaction. This is necessary for the mtDNA transcription that is reported to occur in early murine embryos (Piko & Taylor, 1987) and that is necessary to generate the primer for mtDNA replication, which is initiated at or shortly after the blastocyst stage (Chapter 3; May-Panloup *et al.*, 2005b; Piko & Taylor, 1987). Successful mtDNA replication and formation of a functional ETC require

interaction between nuclear-encoded mtDNA replication factors and the mitochondrial D-loop and between ETC subunits encoded by the nuclear and mitochondrial genomes (see Section 1.2). If the nucleus and mitochondria are derived from a different source, which is usually the case for NT, mtDNA replication and/or formation of the ETC may be compromised, potentially resulting in mitochondrial dysfunction in the offspring. This is particularly likely for inter-species and cross-species NT, as cybrid studies have indicated that decreased ATP output arises with increasing genetic diversity (Kenyon & Moraes, 1997; McKenzie *et al.*, 2003).

It is possible that incompatibility between the nucleus and mitochondria could be overcome by the preferential replication of donor cell-derived mtDNA. This has been reported in some cases of cross-species NT (Chen *et al.*, 2002). Preferential replication of donor cell-derived mtDNA might be induced if donor nuclei are programmed to express high levels of the mtDNA replication factors. These may bind to the donor mtDNA D-loop with increased affinity compared to the recipient oocyte mtDNA and therefore replicate their own mtDNA with increased efficiency. Replication of donor cell-derived mtDNA might also be induced if the donor nucleus is unable to detect sufficient mtDNA copies for embryo development. Although sufficient copies may be present in an oocyte, differences between donor and recipient mtDNA molecules may result in the detection of only those molecules derived from the donor cell. Preferential replication of donor cell-derived mtDNA may therefore be initiated in order to increase donor cell-derived mtDNA copy number above the threshold requirement, thereby improving interactions between the nucleus and mitochondria and consequently, developmental outcomes. This would be similar to the correlation observed between the level of OXPHOS defect and the evolutionary distance between contributing

partners of both mouse (McKenzie *et al.*, 2003) and primate (Kenyon & Moraes, 1997) intra-species cybrid crosses. However, it is not normal for mtDNA replication to occur during early embryogenesis (Piko & Taylor, 1987; Chapter 3). Therefore, donor cell-derived mtDNA replication during early embryogenesis could have the same detrimental effects on development as observed in embryos derived from the ddC treated oocytes described in Chapter 4, which replicated their mtDNA shortly after fertilisation. Experiments described in this Chapter investigate donor cell-derived mtDNA replication and developmental outcome in cross-species NT embryos reconstructed with a variety of mitochondrial sources.

6.2 Hypothesis

Evolutionary diverse partners contributing to NT embryos will only recognise mtDNA derived from the nuclear donor cell and therefore induce mtDNA replication during early pre-implantation development in order to increase donor cell-derived mtDNA copy number to the threshold required for embryogenesis. However, the induction of mtDNA replication is detrimental to embryo development.

6.3 Aims

Through the use of cross-species NT embryos generated using differentiated and undifferentiated donor cells and undifferentiated donor cells with supplementary mitochondria, these experiments aim to determine whether:

1. the levels of expression of mtDNA replication factors by the donor cell nucleus influences the amount of donor cell-derived mtDNA replication in cross-species NT embryos.

2. supplementation of undifferentiated donor cell-derived NT embryos with fewer mitochondria than are thought to be required for embryogenesis induces donor cell-derived mtDNA replication.
3. donor cell-derived mtDNA replication influences development of cross-species NT embryos.

6.4 Preliminary Experiments

6.4.1 Choice of donor cells

Murine donor cells were selected due to the considerable genetic diversity between the mouse and the pig. This increases the likelihood of preferential replication of the donor cell-derived mtDNA due to the murine-encoded mtDNA replication factors being unable to interact with the porcine mitochondrial genome. For example, preferential replication of panda donor cell-derived mtDNA was observed following cross-species NT with rabbit oocytes (Chen *et al.*, 2002), but was not observed following bovine inter-species NT with *Bos taurus* oocytes and *Bos indicus* donor cells (Meirelles *et al.*, 2001). The 73% sequence diversity between the two species, as determined by Clustalw analysis of the murine (NC_005089) and porcine (NC_000845) mitochondrial genomes (http://www.ebi.ac.uk/clustalw), will also permit species-specific detection of mtDNA to take place. The mouse is also one of the few species in which mtDNA replication patterns during pre-implantation (McConnell & Petrie, 2004; Piko & Taylor, 1987; Thundathil *et al.*, 2005) and post-implantation (Chapter 5) embryogenesis have been investigated. In order to determine the effect of donor cell mtDNA replication factor expression on cross-species NT outcome, donor cells with relatively low and high mtDNA replication factor expression were selected for use as donor cells. Undifferentiated (mESCs) and differentiated (MEFs) were selected due to their respective low and high mtDNA replication factor expression, as determined in Chapter 5.

The analysis of the expression of mtDNA replication factors in differentiating mESCs described in Chapter 5 was carried out using the R1 cell line. This cell line requires growth in the presence of MEF feeder layers in order to maintain pluripotency. As MEFs could contaminate the suspension of R1 mESCs and they were also to be used in this experiment, it

was decided to use a mESC line that did not require a feeder layer for maintenance of pluripotency. The CCE mESC line was therefore selected, as these cells can attach to gelatin in place of feeder cells. The cells were cultured on gelatin-coated plates in media containing LIF to allow maintenance of pluripotency.

In order to confirm that CCE mESCs had similar patterns of expression for mtDNA replication factors to R1 mESCs, undifferentiated cells from both of these mESC lines were analysed by ICC using antibodies against TFAM, PolGA, TFB2M and COXI. An OCT-4 antibody was also used to determine or confirm pluripotency. Staining was carried out as described in Section 2.14 and was viewed using conventional fluorescent microscopy (see Section 2.17). OCT-4 staining was present and was localised only to the nucleus in both R1s and CCEs (see Figure 6.1). OCT-4 is a nuclear transcription factor and would therefore be expected to be present in the nucleus when active. All other proteins analysed were detected only in the cell cytoplasm (see Figure 6.2) in both R1s and CCEs, as would be expected for mitochondrial proteins. In both cell lines, TFAM appeared to be expressed more widely throughout the cytoplasm than PolGA, COXI and TFB2M, which localised closer to the cell nuclei (see Figure 6.2). This could indicate that the small amount of mtDNA transcription and replication thought to occur in ESCs occurs in close proximity to the nucleus. In summary, for each of the proteins analysed, staining for the CCE line was comparable to the R1 line, indicating their suitability as an alternative to R1s as undifferentiated donor cells for NT.

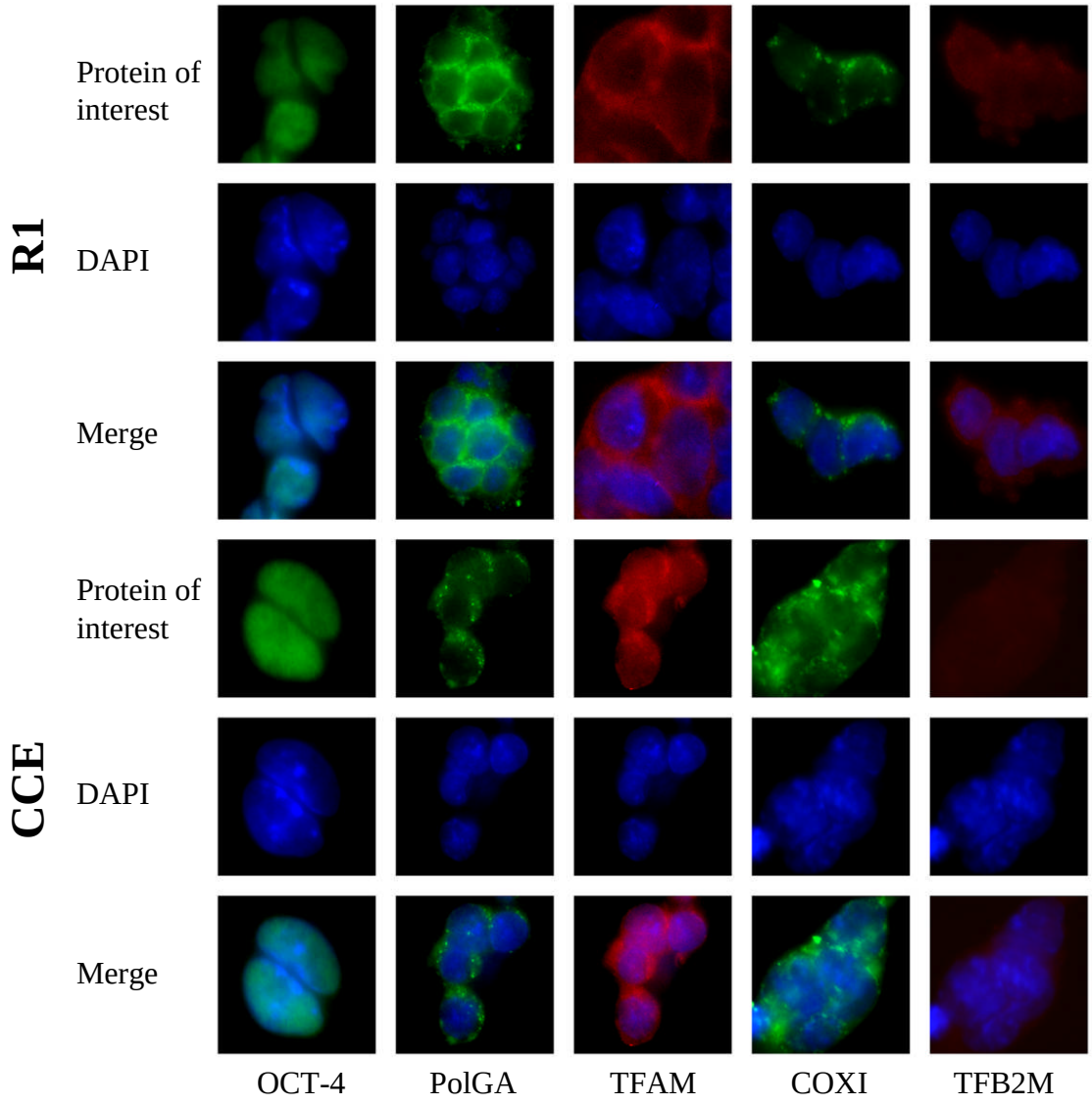


Figure 6.1: Comparison of R1 and CCE mESC lines

Expression and localisation of OCT-4, PolGA, TFAM, COXI and TFB2M proteins in undifferentiated cells from the R1 (top three rows) and CCE (bottom three rows) mESC lines, as visualised using conventional fluorescent microscopy. Each protein is shown individually (top row of each group) and after co-staining with DAPI (bottom row of each group). DAPI staining is also shown separately (middle row of each group). Expression and localisation of each of the proteins in the CCE mESC line is comparable to the R1 mESC line.

Magnification: X 400

6.4.2 Donor cell membrane breakdown

In order to confirm that the porcine oocyte cytoplasm was capable of breaking down the murine donor cell membrane, MEFs and CCE mESCs were stained with the PKH67 cell membrane marker as described in Section 2.16. The PKH67 marker molecules consist of lipophilic tails that diffuse into and bind to the cell membrane, leaving the fluorescent tag outside the cell. They were then visualised at 30 min intervals by conventional fluorescence microscopy (see Section 2.17) to determine whether the donor cell membrane would completely break down.

MEF membrane components formed a spherical shape 5 mins after injection, likely representing an intact donor cell membrane (see Figure 6.2A). Similar staining patterns have also been observed with the use of porcine fibroblasts as donor cells (Lee *et al.*, 2003). The spherical shape was no longer observed 30 mins after injection (see Figure 6.2B). The membrane components appeared to have spread from the original injection site towards the outer edge of the reconstructed oocyte, although the staining was still more intense at the point of injection. This suggests that the donor cell membrane had begun to break down. At 60 mins past injection, the membrane components appeared to have spread further from the site of injection and were localised relatively consistently throughout the ooplasm (see Figure 6.2C). This suggests that by this time, the donor cell membrane was no longer intact and that the cell contents had been released into the ooplasm. Others have also reported this in reconstructed pig oocytes 6 hrs after injection of porcine fibroblasts, but unfortunately earlier time points were not investigated (Lee *et al.*, 2003).

Staining of the cell membrane for CCE cells was not visible inside reconstructed oocytes using conventional fluorescent microscopy. Enucleated oocytes containing CCE donor cells were therefore fixed in 3.8% formaldehyde (see Section 2.14.1) and viewed using a confocal microscope (see Section 2.18). A small patch of green fluorescent staining for the CCE donor cell was visible in oocytes fixed 5 mins after injection, although this was not clearly spherical (see Figure 6.2D). This suggests that only 5 mins was required before the breakdown of CCE membranes was initiated. Oocytes fixed 30 mins after injection of the CCE donor cell were observed to contain faint green staining throughout the ooplasm (see Figure 2E). However, this was no longer observed in oocytes fixed 60 mins after injection (see Figure 6.2F). The even distribution of the CCE membranes 30 mins after injection suggests that the CCE cytoplasm was released by degradation of the cell membrane very shortly after injection and that the donor cell membranes had been totally degraded by the ooplasm by 60 mins after injection. These results therefore suggest that the porcine cytoplasm is indeed capable of degrading both somatic and embryonic murine cell membrane components. However, this is dependent on the cell type as MEF cell membranes required 30 to 60 mins longer to degrade than CCE mESC membranes.

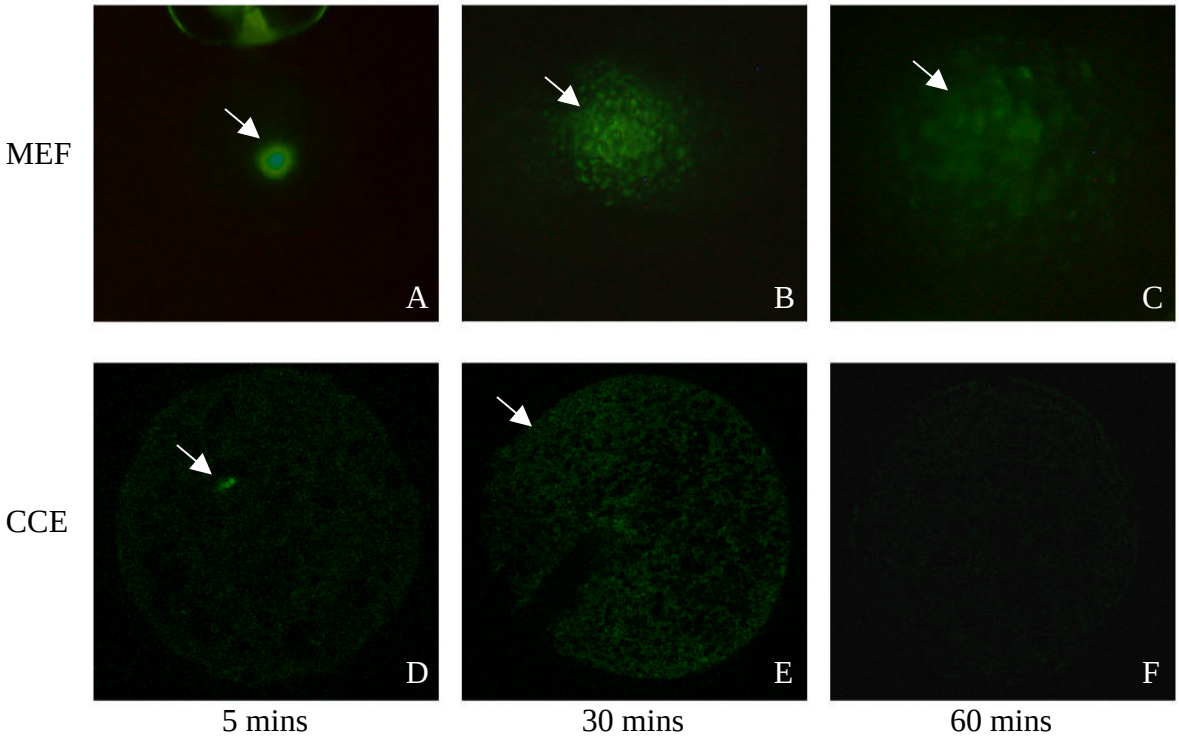


Figure 6.2: Donor cell membrane breakdown

Localisation of MEF (top row) and CCE (bottom row) membrane components following injection into porcine oocytes, as determined by PKH67 staining. The MEF membrane appeared intact 5 mins after injection (A), as indicated by the arrow, but had begun to break down and disperse throughout the oocyte cytoplasm after 30mins (B; see arrow). The membrane components had dispersed even further after 60 mins as indicated by the arrow (C). The CCE membrane had partially broken down just 5 mins after injection, resulting in a non-circular staining pattern, as indicated by the arrow (D). Membrane components had dispersed throughout the entire cytoplasm after 30 mins (E; see arrow) and by 60 mins after injection, the membrane components had been broken down completely, as indicated by the total lack of staining (F). Staining was visualised using either conventional fluorescence (MEFs) or confocal (CCEs) microscopy. Magnification: X 400 (MEFs) or X 630 (CCEs).

6.4.3 Mitochondrial extraction and supplementation

In order to determine whether NT embryos recognise and preferentially replicate mtDNA derived from the donor cell to generate sufficient donor cell-derived mtDNA for successful embryogenesis, NT embryos were created using CCE donor cells, which were not primed to drive mtDNA replication. These embryos were supplemented with CCE-derived mitochondria containing fewer than the 100000 mtDNA copies thought to be required for successful embryogenesis to determine whether mtDNA replication could be induced. CCEs being prepared for mitochondrial extraction were stained with the mitochondrial marker, MitoTracker Green to enable successful supplementation to be confirmed. CCEs were detached from one confluent well of a 6-cell plate using trypsin-EDTA. Trypsin was then removed by centrifugation and cells were resuspended in CCE culture media supplemented with 0.5µg/ml MitoTracker Green. Cells were then incubated at 37°C for 30 mins before undergoing the mitochondrial extraction procedure as described in Section 2.2.4. Briefly, this involved cell lysis followed by a series of centrifugation steps to separate the cytosolic compartments. Fluorescent microscopy of the extracted mitochondria was carried out (see Section 2.17) to confirm successful mitochondrial extraction. However, staining was extremely faint and was not detectable using conventional fluorescence microscopy. Further confirmation was therefore obtained by confocal microscopy of fixed NT embryos containing the supplementary mitochondria (see Figure 6.3) and by quantification with real time PCR of the number of mtDNA molecules contained within supplemented samples. Additionally, quantification confirmed that fewer than 100000 mtDNA copies were present in each supplemented sample. Similar analysis of the NT embryos derived using mitochondrial supplementation then allowed determination of whether replication of the donor cell-derived mtDNA had taken place.

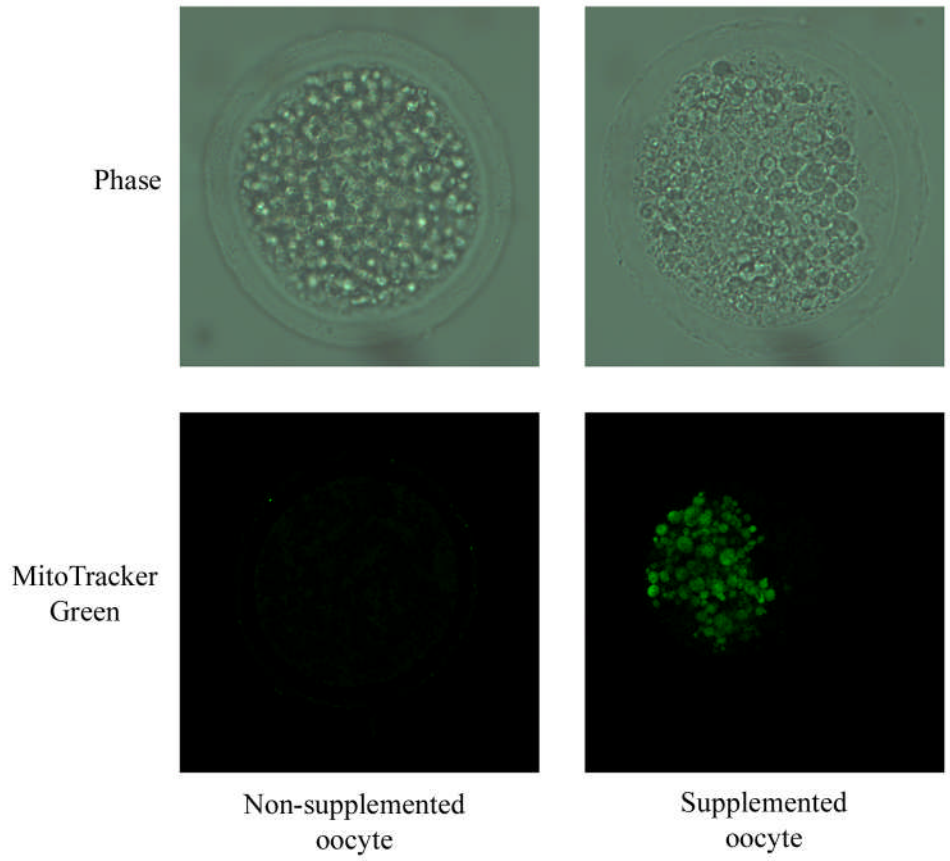


Figure 6.3: Visualisation of supplementary mitochondria in NT embryos

Supplementary CCE-derived mitochondria were stained with MitoTracker green and visualised following injection into enucleated oocytes (Top: Phase images of oocytes taken using a conventional fluorescent microscope (HBO100, Zeiss); Bottom; Fluorescent images taken using a confocal microscope (DM IRE2, Leica)). Non-supplemented oocytes were imaged as negative controls (left panels). Green staining in a supplemented oocyte (right panels) indicates successful mitochondrial extraction and supplementation. Original magnification: X 400 (Phase images) or X 630 (Fluorescent images).

6.5 Materials and Methods

6.5.1 Reconstruction of porcine oocytes using murine donor cells

Oocytes were obtained from slaughterhouse ovaries and matured *in vitro* for 44 to 46 hrs as described in (see Section 2.2.1). MEF and CCE donor cells were cultured as described previously (see Section 2.1) until they required passaging. Oocyte cumulus cells were removed by gently vortexing oocytes for 30 s. Oocytes were then stained with bisbenzimide in order to visualise the metaphase plate before being transferred to manipulation media for enucleation (see Section 2.2.8). Batches of 5 to 8 oocytes were enucleated and then injected with a donor cell (see below), before being activated (see Section 2.2.10) after allowing some time for nuclear reprogramming. For MEF donor cells, a 2 hr period was used, which has been reported to be optimal following SCNT with bovine fibroblasts (Aston *et al.*, 2006) and also suitable for porcine fibroblasts (De Sousa *et al.*, 2002). As the MEF membrane required 30 to 60 mins to break down, approximately only 1 hr remained for nuclear reprogramming. However, CCE membrane break down occurred within 5 mins after injection, allowing reprogramming to begin up to 1 hr earlier than for MEFs. Oocytes reconstructed using CCE donor cells were therefore incubated for just 1 hr before activation. NT embryos were then cultured in NCSU-23 media, as previously described (see Section 2.2.6).

6.5.2 Real time PCR determination of mtDNA copy number

Samples of donor cells, mitochondrial supplements and NT embryos were diluted in sterile ddH_2O and DNA was extracted as described in Section 2.3 for determination of mtDNA copy number. Primers were designed to amplify a 211bp region of the murine mitochondrial genome (Accession No.: NC_005089; Forward: CAG TCT AAT GCT TAC TCA GC, Reverse: GGG CAG TTA CGA TAA CAT TG). In order to ensure that these primers did not

also amplify porcine mtDNA, DNA extracted from a non-reconstructed porcine oocyte was included in each real time PCR reaction as a negative control. These non-reconstructed porcine oocytes had previously been determined, using porcine mtDNA primers, to contain large amounts of mtDNA (see Chapter 4). Standards were prepared from murine mtDNA as described in Section 2.8. Real time PCR conditions consisted of 10 mins enzyme activation at 95°C followed by 45 cycles of denaturation at 95°C for 10s, annealing at 53°C for 15s and extension at 72°C for 15s, with data being acquired after melting of primer-dimers at 77°C for 15s. A standard curve was produced and the mtDNA copy number in each sample was calculated by the RotorGene software (Version 7.0; Corbett Research), as described in Section 2.8. These values were multiplied by the relevant dilution factor and, after exclusion of the highest and lowest values (Bustin, 2000), were used to calculate the mean and SEM values for mtDNA copy number for each sample.

6.5.3 Statistical analysis

Comparisons between the proportions of embryos from one donor cell type reaching specific developmental stages were made using the one-sample *t*-test between proportions (StatPac Statistics Calculator, StatPac Inc, Minnesota, USA). Due to the low frequency of embryo development obtained in the experiments described in this Chapter, Fisher Exact tests were used to compare development at each embryonic stage between NT embryos derived using different donor cells (StatPac Statistics Calculator).

MtDNA copy number results for donor cells and subsequently derived embryos were subjected to the one sample Kolmogorov-Smirnov test to determine whether the data were normally distributed. Data for CCEs and MEFs and the associated NT embryos were

determined to be normally distributed and were therefore analysed using *t*-tests. Data for extracted mitochondria and supplemented embryos were not normally distributed and were therefore analysed using non-parametric Mann Whitney U tests.

6.6 Results

In order to determine whether donor cell-derived mtDNA would be preferentially replicated following NT and whether this would affect development, embryos were generated using murine donor cells transplanted into enucleated porcine oocytes. The first set of embryos were generated using fully differentiated MEF donor cells, whilst the second set of embryos were generated using undifferentiated CCE donor cells. Finally, a set of embryos was generated using CCE donor cells supplemented further with CCE-derived mitochondria. The resulting embryos were firstly analysed for rates of development and then for mtDNA content similar to the donor cell at various stages of development.

6.6.1 Development of NT embryos generated using a differentiated donor cell

In order to determine whether donor cell expression of mtDNA replication factors would result in donor cell-derived mtDNA replication following NT, 153 reconstructed oocytes were created using differentiated MEF donor cells, a common choice of somatic donor cell for NT experiments. Of these, a total of 10 (6.5%) cleaved. The majority of these (7, 4.6%) arrested at the 2-cell stage, with 1 (0.7%) continuing to the 3-cell stage, another 1 (0.7%) continuing to the 4-cell stage and 1 (0.7%) progressing to the 8 to 12-cell stage (see Figure 6.4). The proportions of embryos arresting at each developmental stage were compared using a one-sample *t*-test between proportions. Significantly more embryos arrested at the 2-cell stage than any other stage of development ($P < 0.04$ for comparisons with embryos arresting at 3-cell, 4-cell and 8 to 12-cell stages). There was no significant difference between the number of embryos that reached the 4-cell stage and those that did not. However, significantly more embryos arrested prior to the 8-cell stage than developed to the 8-cell stage or further ($P = 0.01$).

6.6.2 Development of NT embryos generated using an undifferentiated donor cell

In order to determine whether using a less differentiated donor cell, that is less likely to drive mtDNA replication, could improve NT embryo development, 246 reconstructed oocytes were created using undifferentiated CCE donor cells. Of these, 20 (8.1%) cleaved. This is a higher cleavage rate than was obtained when MEFs were used as donor cells, although this is not a statistically significant increase ($P > 0.05$). 5 (2%) embryos arrested at the 2-cell stage, 6 (2.4%) embryos arrested at the 3-cell stage and 3 (1.2%) embryos arrested at the 4-cell stage (see Figure 6.4). One other (0.4%) embryo arrested containing 5 to 7 blastomeres, while a further 5 (2%) embryos progressed to the 8 to 12-cell stage (see Figure 6.4). Interestingly, fewer CCE derived NT embryos than MEF derived NT embryos arrested at the 2-cell stage ($P > 0.05$), which resulted in significantly more CCE derived NT embryos than MEF derived NT embryos arresting after the 2-cell stage ($P = 0.03$).

6.6.3 Development of NT embryos generated using an undifferentiated donor cell and mitochondrial supplementation

In order to test whether the introduction of an insufficient number of compatible mtDNA molecules would initiate replication of donor cell-derived mtDNA, 151 NT embryos were produced containing a CCE donor cell and 48997 ± 4774 supplementary CCE-derived mitochondria. 16 (10.6%) of these cleaved, which is a higher percentage than obtained with non-supplemented CCE derived embryos, though this increase is not statistically significant ($P > 0.05$). 13 (8.6%) embryos arrested at the 2-cell stage, significantly more than arrested at any other developmental stage ($P = 0.01$). 3 (2%) embryos continued development beyond the 2-cell stage with 1 (0.7%) arresting at each of the 3-cell, 4-cell and 5 to7-cell stages (see

Figure 6.4). Therefore, significantly more embryos failed to reach the 4-cell stage than developed to the 4-cell stage or further ($P = 0.02$). Furthermore, significantly more supplemented embryos arrested at the 2-cell stage than non-supplemented CCE derived embryos ($P = 0.002$).

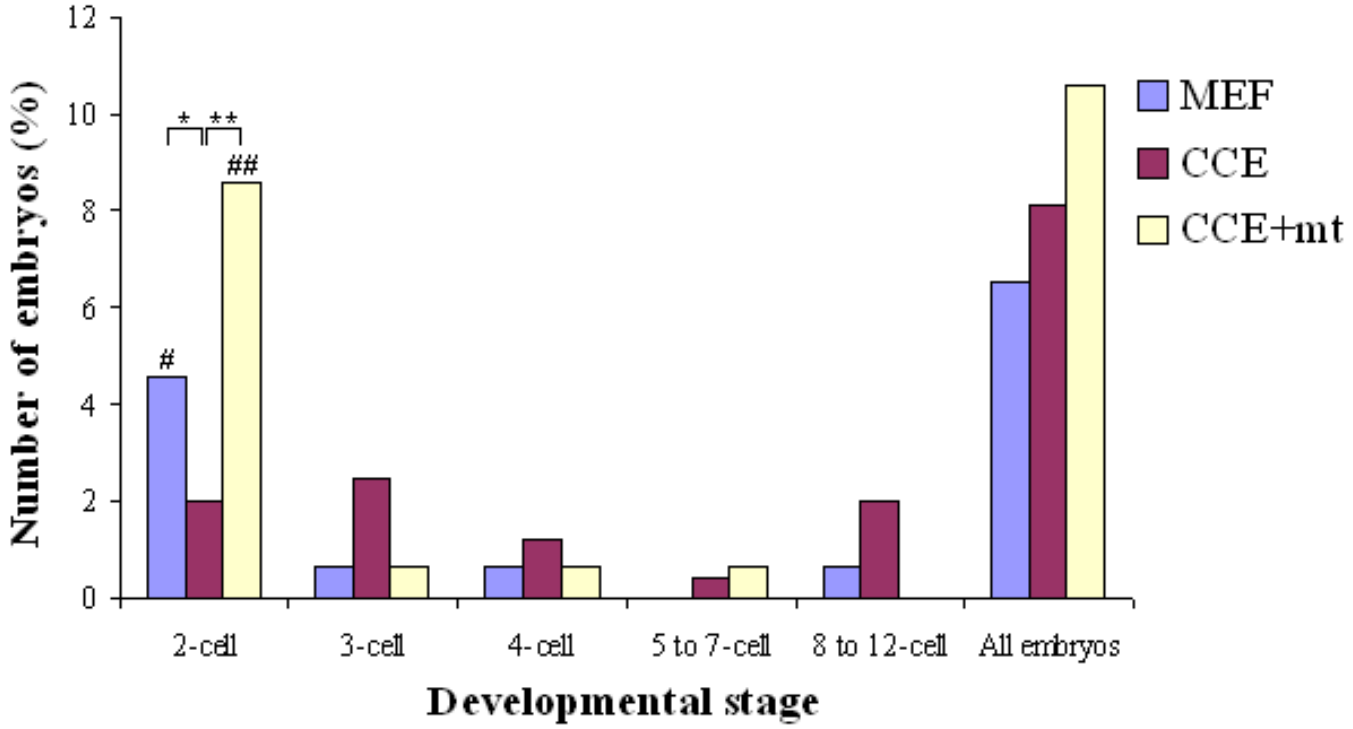


Figure 6.4: Cleavage and development rates of NT embryos

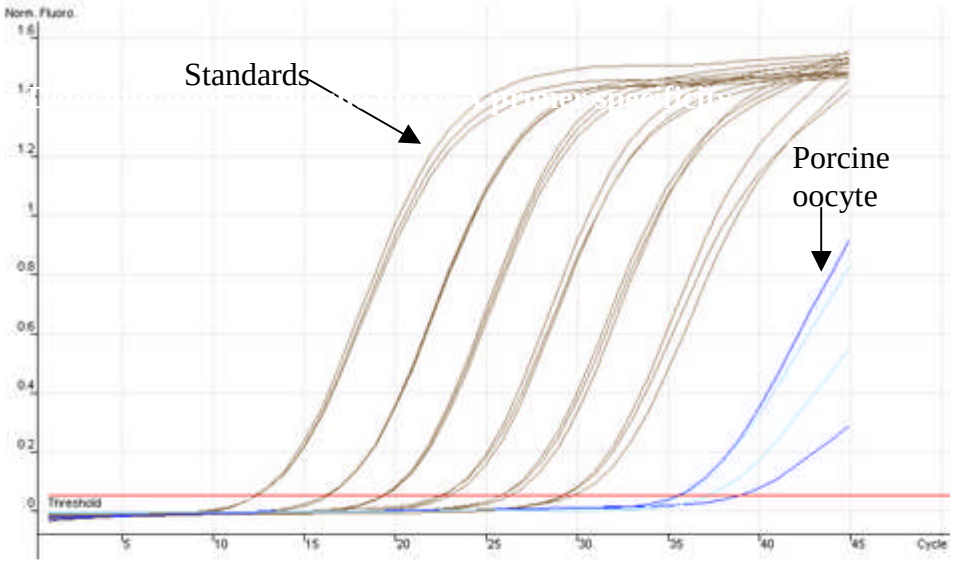
Development to each of the early embryonic stages and total cleavage rates are shown for MEF-derived NT embryos (MEF), CCE-derived NT embryos (CCE) and CCE-derived NT embryos supplemented with CCE-derived mitochondria (CCE+mt). Asterisks indicate significant differences in development to the indicated stages between bracketed NT embryo groups (* $P < 0.05$; ** $P < 0.001$). Hash symbols indicate that NT embryos from the indicated group were significantly more likely to cease development at the 2-cell stage than at later stages (# $P < 0.05$; ## $P < 0.005$).

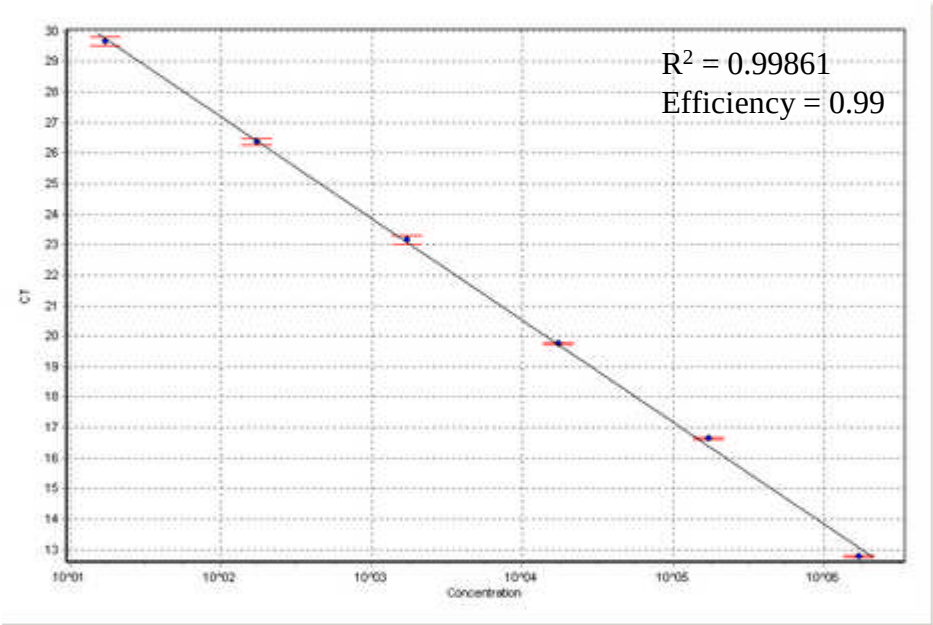
6.6.4 MtDNA replication analysis

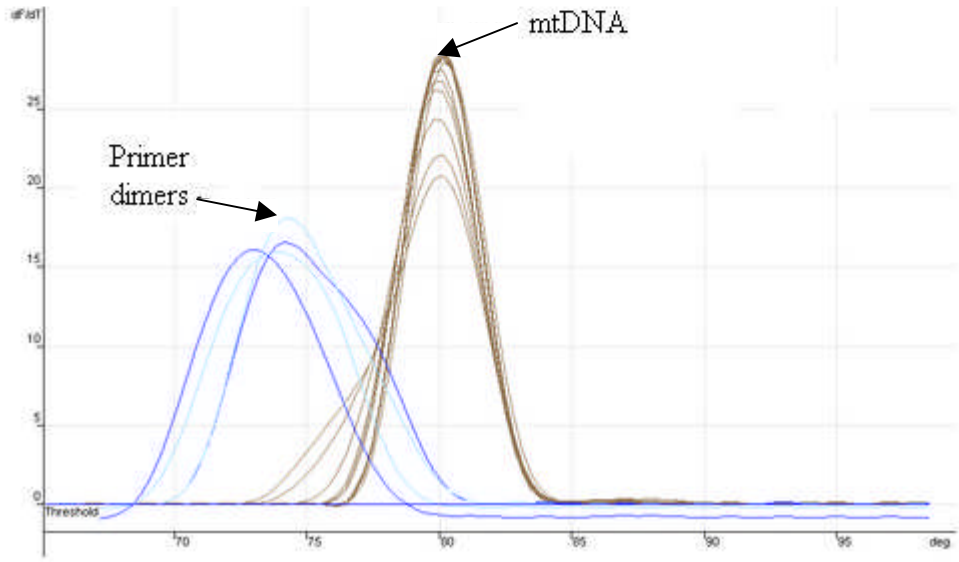
Before samples were analysed, it was confirmed using real time PCR that murine mtDNA primers (see Section 6.5.2) did not amplify porcine mtDNA, as this could result in overestimation of murine mtDNA copy number in the NT embryos. Murine mtDNA standards generated increased fluorescence with increased concentration and formed a standard curve suitable for mtDNA copy number analysis (see Figure 6.5). DNA extracted from a porcine oocyte generated only low levels of fluorescence, which was determined by melt curve analysis to be due to primer-dimerisation and not amplification of porcine mtDNA (see Figure 6.5). The standards were therefore deemed suitable for use in real time PCR reactions for calculation of murine mtDNA copy number in donor cells, mitochondrial supplements and NT embryos. Examples of fluorescence data and melt curves for these samples are shown in Figure 6.6.

Figure 6.5 (next page): Determination of murine mtDNA primer specificity

10-fold dilutions of mouse mtDNA (standards; brown), a porcine oocyte (blue) and a negative control that did not contain any template DNA (turquoise) were analysed for mouse mtDNA copy number by real time PCR. Fluorescence values for standards crossed the threshold level earlier in the reaction with increasing standard concentration (top panel) and formed a suitable standard curve (middle panel). Fluorescence values for the porcine oocyte and the negative control that did not contain any template DNA both crossed the threshold level later in the reaction (top panel). However, melt curve analysis (bottom panel) determined that this was due to primer-dimerisation and not due to amplification of porcine mtDNA or reagent contamination, as indicated by the arrows.


Norm. Fluoro
Standards
Porcine
oocyte
Threshold
Cycle


R² = 0.99861
Efficiency = 0.99
CT
Concentration


mtDNA
Primer
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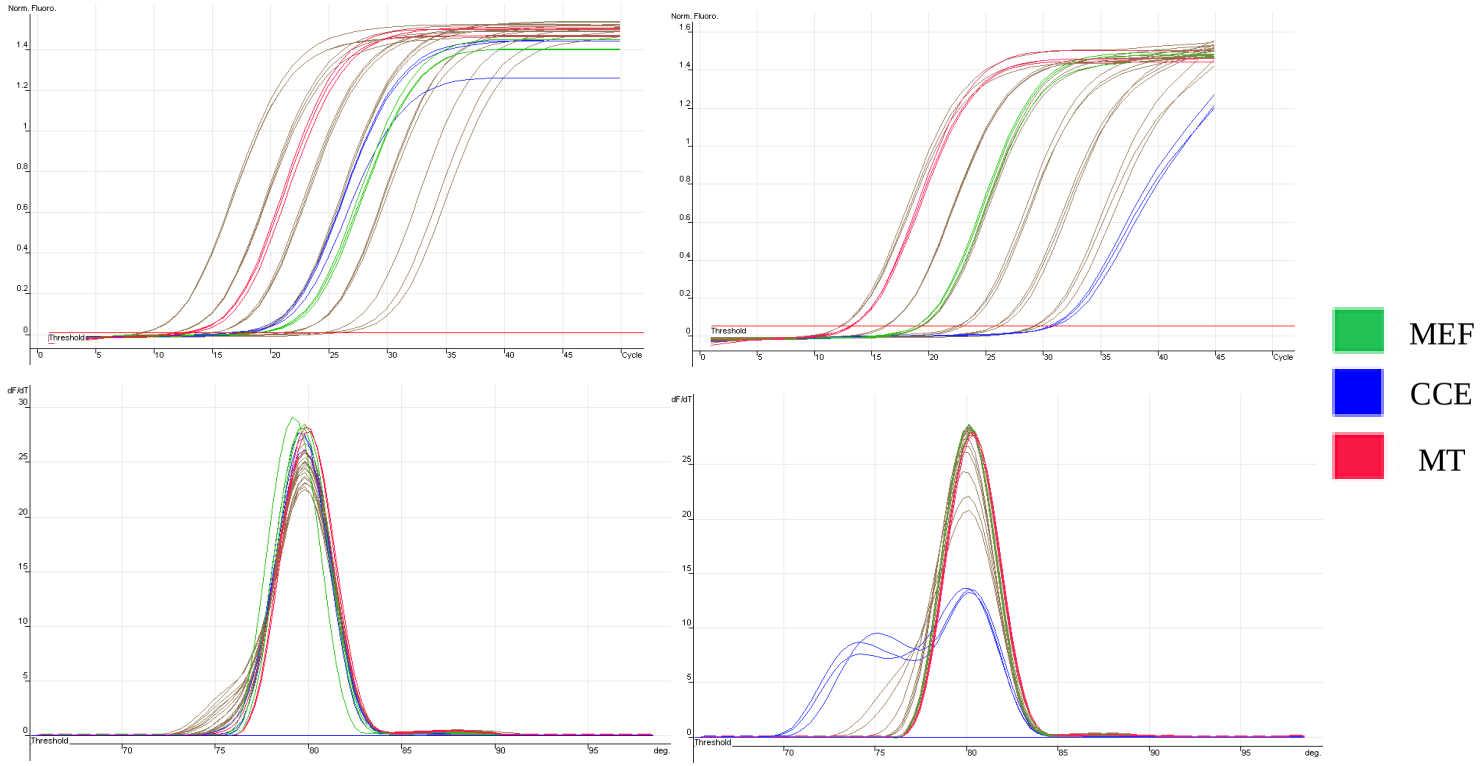


Figure 6.6: Real time PCR determination of mtDNA copy number in donor cells and NT embryos

Examples of fluorescence data (top) and melt curves (bottom) obtained during real time PCR determination of mtDNA copy number of the following samples. The left panels show fluorescence and melt data for MEF and CCE donor cells and mitochondrial supplements (MT) and the right panels show the same information for the resulting embryos. Samples producing the data shown in the left panels contained the equivalent amount of mtDNA from one MEF or mitochondrial supplement or from 80 CCE mESCs, whilst samples producing the data shown in the right panels contained the equivalent amount of DNA from one tenth of one embryo.

6.6.5 Donor cell-derived mtDNA replication in NT embryos generated using differentiated donor cells

In order to determine whether mtDNA replication factor expression in donor cells results in donor cell-derived mtDNA replication in resulting NT embryos, mtDNA copy number was measured in MEFs and in MEF-derived NT embryos. Using the standard curve displayed in Figure 6.5, MEFs were calculated to contain 663 ± 143 copies of mtDNA. However, MEF-derived NT embryos contained 231297 ± 13932 (all cleaved embryos) or 102766 ± 14718 (all uncleaved embryos) murine mtDNA copies, representing 350-fold ($P < 0.001$) and 155-fold ($P = 0.006$) increases respectively (see Figure 6.7). This suggests that mtDNA replication occurred in both MEF-derived cleaved NT embryos and in those MEF-derived NT embryos that failed to cleave. No significant differences in mtDNA copy number were detected between all the cleaved embryos of various stages ($P > 0.05$). For example, embryos at the 2-cell stage contained 212734 ± 29563 mtDNA copies, whilst embryos at the 3-cell stage contained 249896 ± 11276 copies, 4-cell embryos contained 244744 ± 31170 copies and 8-cell embryos contained 236377 ± 23574 copies.

6.6.6 Donor cell-derived mtDNA replication in NT embryos generated using undifferentiated donor cells

In order to determine whether lack of mtDNA replication factor expression in donor cells results in no donor cell-derived mtDNA replication occurring in resulting NT embryos, mtDNA copy number was measured in CCEs and in CCE-derived NT embryos. CCEs were determined to contain 44 ± 12 copies of mtDNA. Unlike MEF-derived NT embryos, CCE-derived NT embryos contained fewer mtDNA copies than were injected, with 38 ± 16 copies

detected in cleaved CCE-derived NT embryos and even fewer (24 ± 12) copies detected in uncleaved CCE-derived NT embryos (see Figure 6.7).

6.6.7 Donor cell-derived mtDNA replication in NT embryos generated using undifferentiated donor cells and mitochondrial supplementation

In order to determine whether supplementation of NT embryos with fewer than the required number of donor cell-derived mtDNA could induce donor cell derived mtDNA replication, mtDNA copy number was measured in mitochondrial supplements and in supplemented NT embryos. CCE-derived NT embryos injected with supplementary CCE-derived mitochondria were supplemented with a further 48997 ± 4774 mtDNA copies. However, all cleaved embryos contained 8663184 ± 1498834 mtDNA copies whilst all embryos that failed to cleave contained 666630 ± 67278, representing significant increases in mtDNA copy number (177-fold, $P = 0.02$ and 14-fold, $P = 0.004$, respectively; see Figure 6.7). This suggests that mtDNA replication occurred in both cleaved and uncleaved CCE-derived NT embryos supplemented with CCE-derived mtDNA, as was the case for MEF-derived NT embryos. No significant difference was detected between all cleaved and all uncleaved embryos ($P > 0.05$). However, a trend towards increased mtDNA copy number in later staged embryos was noted. For example, embryos at the 2-cell stage contained 3919814 ± 1625361 copies, whilst embryos at the 3-cell stage contained 12188491 ± 939824 copies and embryos with more than four blastomeres contained 14624616 ± 854342 copies. Significantly more mtDNA copies were detected in cleaved embryos with more than 2 blastomeres than was detected in 2-cell embryos ($P = 0.006$).

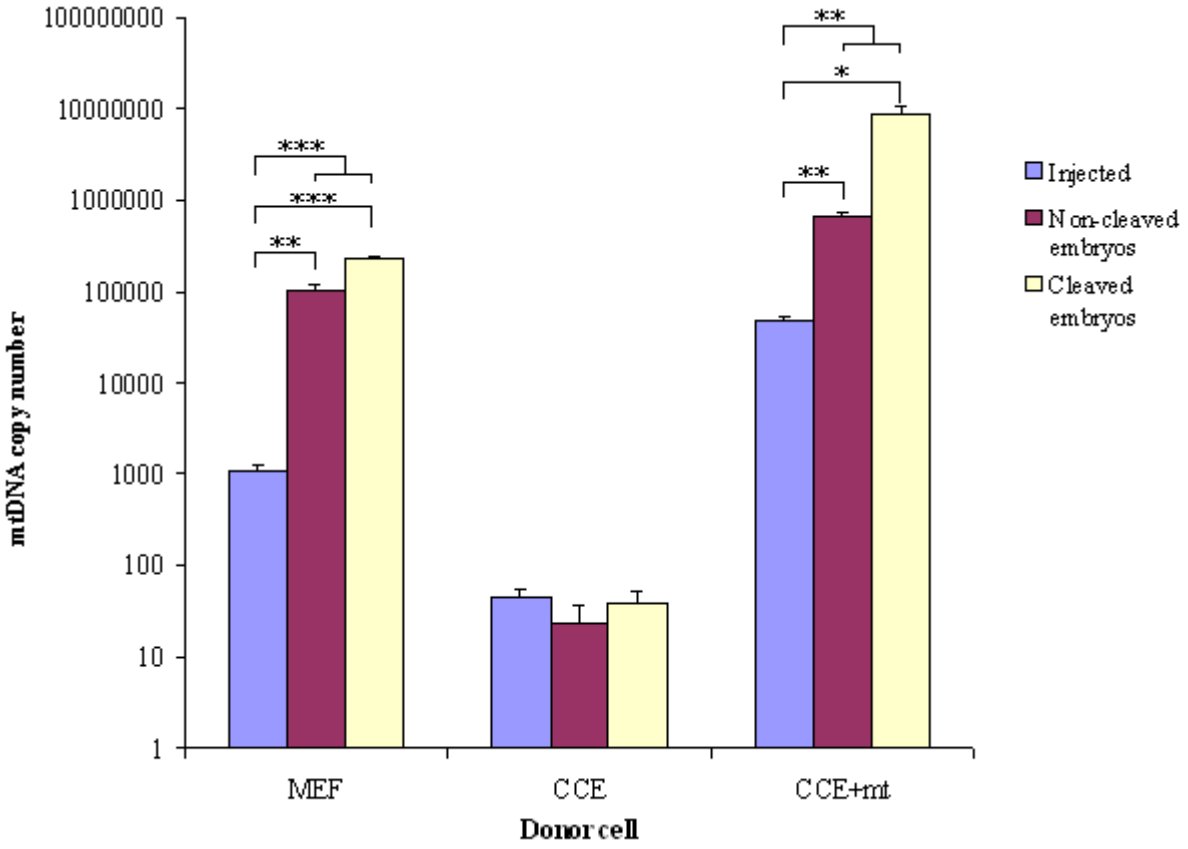


Figure 6.7: Murine mtDNA replication in NT embryos

The number of mouse mtDNA copies injected into each NT embryo was determined by analysing mtDNA copy number in donor cell and mitochondrial supplemented samples by real time PCR (blue bars). Analysis of mouse mtDNA copy number in resulting NT embryos allowed determination of mtDNA replication activity in MEF-derived NT embryos (MEF), CCE-derived NT embryos (CCE) and CCE-derived NT embryos injected with supplementary CCE-derived mitochondria (CCE+mt). Significant mouse mtDNA replication occurred in both MEF-derived NT embryos and CCE-derived NT embryos injected with supplementary CCE-derived mitochondria. In contrast, a slight decrease in mtDNA copy number was observed in CCE-derived NT embryos, indicating that mtDNA replication had not occurred. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

6.7 Discussion

6.7.1 MtDNA replication factor expression in donor cells is reflected in the amount of donor cell-derived mtDNA replication in resulting NT embryos

The data described in Chapter 5 indicate that increased mtDNA replication factor expression occurs during cellular differentiation. For example, increased levels of PolGA and PolGB transcripts were detected as undifferentiated mESCs were allowed to differentiate and form EBs and conventional PCR determined that PolGA and PolGB transcripts were present at high levels in fully differentiated MEFs but were not detected in undifferentiated mESCs. Experiments described in this Chapter investigated whether this variation in mtDNA replication factor expression in the donor cell was reflected in the amount of donor cell-derived mtDNA replication that occurred in the NT embryos. NT embryos generated using fully differentiated MEF donor cells replicated MEF mtDNA up to 350-fold following oocyte reconstruction. In contrast, undifferentiated CCEs were determined to contain 44 ± 12 copies of mtDNA before injection and no increase was detected following oocyte reconstruction, indicating that mtDNA replication did not take place.

Data obtained in Chapter 5 indicate that fully differentiated MEFs express higher levels of PolGA and PolGB than undifferentiated mESCs. Failure of the recipient oocyte cytoplasm to correctly reprogram the donor cell nucleus to an embryonic state may therefore have resulted in continued expression of mtDNA replication factors such as PolGA and PolGB. Continued expression of donor cell-derived mtDNA replication factors has been reported in sheep intra-species 4-cell NT embryos derived using sheep foetal fibroblasts (Lloyd *et al.*, 2006). Similar expression of MEF-derived mtDNA replication factors could have caused the replication of MEF-derived mtDNA observed in the NT embryos generated using MEF donor cells. Cloned

embryos have also been reported to harbour other genetic markers associated with the donor cell type. For example, GLUT-4, a gene normally active in myoblasts but not embryos, was expressed in murine NT embryos generated using myoblast nuclei (Gao *et al.*, 2003). This indicates that the donor cell choice may be a significant contributor to the phenotype and possibly developmental potential of cloned embryos.

6.7.2 Donor cell mtDNA copy number may influence donor cell-derived mtDNA replication in NT embryos

The CCE-derived NT embryos described in this Chapter either did not replicate the donor cell-derived mtDNA or its replication was at a similar rate to mtDNA degradation. However, CCE-derived NT embryos with mitochondrial supplementation did replicate the donor cell-derived mtDNA up to 177-fold. As these two groups of NT embryos were generated using donor nuclei from the same cell type, the different mtDNA replication patterns could not be due to different patterns of expression of nuclear-encoded mtDNA replication factors. The only difference in content between the two groups of NT embryos was the supplementary mitochondria. Therefore, the number of mitochondria and/or mtDNA copy number of the donor cells may influence the amount of donor cell-derived mtDNA replication that occurs in cross-species NT embryos. Indeed, analysis of sheep intra-species NT embryos demonstrated an increase in the mean level of persistence of donor cell-derived mtDNA when fibroblast donor cells were used compared to when fibroblast donor cells from the same source were depleted of their mtDNA before oocyte reconstruction (Lloyd *et al.*, 2006). In this case, both types of donor cell expressed TFAM and PolG, indicating that the difference in donor cell-derived mtDNA replication was not due to differences in nuclear control of mtDNA replication. However, use of blastomeres from more advanced embryonic stages, thought to

contain reduced levels of mtDNA due to lack of mtDNA replication during embryogenesis, as donor cells for bovine NT did not always result in decreased donor cell-derived mtDNA content in resulting offspring (Steinborn *et al.*, 1998). This could have been due to similarities between donor and recipient mtDNA sequences that allowed efficient replication of oocyte-derived mtDNA in some NT embryos, or due to differences in donor cell-derived mtDNA segregation to the tissue analysed between different animals, although neither of these hypotheses were investigated. These data indicate that the number of donor cell-derived mtDNA copies present at oocyte reconstruction may influence the amount of donor cell-derived mtDNA replication, but that there are also other as yet unidentified factors to consider.

6.7.3 Donor cell-derived mtDNA may be preferentially replicated in cross-species NT embryos

Both mtDNA transcription and replication and subsequent formation of ETC complexes require interaction between the nucleus and mitochondria. Failure of this interaction is likely to cause decreased efficiency of the ETC and/or mtDNA replication and transcription levels that are not tailored to specific cell types. In order to overcome this problem, donor cell-derived mtDNA could be preferentially replicated in NT embryos to improve the likelihood of the resulting offspring containing homoplasmic mtDNA with the donor cell sequence. Here, MEF-derived NT embryos replicated MEF-derived mtDNA between oocyte reconstruction and the first cleavage division, after which no further mtDNA replication took place. 2-cell MEF derived NT embryos contained 212734 ± 29563 murine mtDNA molecules, a level consistent with previous estimates of mtDNA copy number in early murine embryos (Piko & Taylor, 1987; Thundathil *et al.*, 2005), whereas MEFs contained only 663 ± 143 mtDNA

copies, significantly fewer than the predicted requirement for embryogenesis (Piko & Taylor, 1987). It is therefore possible that only murine mtDNA copies were detected by the reconstructed oocyte, through a similar mechanism to that which resulted in mtDNA replication in the BCB- and ddC-treated oocytes described in Chapter 4, and that these numbers were considered to be insufficient for embryogenesis. Donor cell-derived mtDNA replication was then initiated until sufficient donor cell-derived mtDNA was present. However, it is also possible that this mtDNA replication occurred simply due to the continued expression of mtDNA replication factors by the donor nucleus. NT embryos were therefore created using CCE donor cells that were supplemented with an insufficient number of CCE-derived mtDNA molecules in order to mimic the BCB- oocytes described in Chapter 4, which initially expressed low levels of mtDNA replication factors and possessed insufficient copies of mtDNA. As occurred in both BCB- oocytes and ddC treated oocytes, introduction of fewer than the required number of mtDNA molecules into CCE-derived NT embryos also resulted in the initiation of mtDNA replication. This may have been facilitated by the mtDNA replication factors that may have remained within the supplementary mitochondria, or upregulated expression of mtDNA replication factors may have been induced in these embryos.

A number of other analyses of mtDNA transmission following inter- and cross-species NT have been carried out, with varied levels of donor cell-derived mtDNA transmission having been reported. For example, there are two reports of apparently healthy heteroplasmic calves following bovine inter-species NT (Han *et al.*, 2004; Steinborn *et al.*, 2002), although cross-species NT-derived foetuses generated using panda donor cells and rabbit oocytes demonstrated preferential replication of the nuclear donor cell-derived mtDNA (Chen *et al.*,

2002). This suggests that some combinations of inter- or cross-species NT, where the donor and recipient mtDNA sequences are similar, may result in formation of functional ETC complexes. However, crosses between more diverse species, including those described in this Chapter, may require preferential replication of the donor cell-derived mtDNA in order to form functional ETC complexes.

6.7.4 The amount of mtDNA replication in cross-species NT embryos may influence embryo development

It is evident that the differentiation status of the donor cell can influence how far inter-species NT-derived embryos/foetuses will develop. For example, bovine intra-species NT embryos generated using differentiated granulosa cells produced similar cleavage rates but significantly fewer blastocysts than embryos generated using undifferentiated blastomere donor cells (Lavoir *et al.*, 1997). ICM-derived donor cells have also been reported to result in live bovine offspring when granulosa cell-derived NT embryos did not develop to term (Collas & Barnes, 1994). As well as differences in the pluripotent status of the nucleus between these donor cells, differences in the mitochondria would also be present. For example, low levels of mitochondria have been reported in undifferentiated hESCs and these contained low numbers of cristae (Cho *et al.*, 2006). However, hESCs differentiated for just one week showed increased numbers of mitochondria and with more cristae and an electron dense matrix (Cho *et al.*, 2006). Injection of somatic mitochondria into parthenogenetically activated bovine oocytes has been demonstrated to have a negative effect on embryo development (Takeda *et al.*, 2005). This may have been due to excess mitochondrial ATP production as more differentiated mitochondria tend to generate more ATP than embryonic mitochondria (Cho *et al.*, 2006; Houghton, 2006) and addition of OXPHOS inhibitors to early

embryo culture media appears to improve developmental outcomes (Machaty *et al.*, 2001). Reduced mitochondrial ATP production may therefore have contributed to the increased developmental outcomes observed following NT with undifferentiated donor cells. The likely increase in mitochondrial ATP production following mitochondrial supplementation may also have contributed to the decreased developmental outcomes observed for these embryos.

As mitochondrial ATP production capacity is correlated to mtDNA copy number (Moyes *et al.*, 1998) which in turn is dependent on mtDNA replication, it was hypothesised that donor cell-derived mtDNA replication in NT embryos could influence embryo development. Consistent with the hypothesis, decreased donor cell-derived mtDNA replication in undifferentiated CCE-derived NT embryos improved development compared to fully differentiated MEF-derived NT embryos which replicated the donor cell-derived mtDNA. For example, cleavage rates for CCE-derived NT embryos were slightly improved when compared to MEF-derived NT embryos, although the increase was not statistically significant. More importantly, significantly fewer CCE-derived NT embryos than MEF-derived NT embryos arrested at the 2-cell stage, with many continuing until the 8 to 12-cell stage. MtDNA replication does not normally occur during pre-implantation embryogenesis in either the mouse (Piko & Taylor, 1987; Thundathil *et al.*, 2005) or pig (Chapter 3) and embryos derived from ddC-treated oocytes that did replicate their mtDNA during early embryogenesis arrested at the 4 to 6-cell stage (Chapter 4). Similarly, the mtDNA replication that occurred in MEF-derived NT embryos and that was induced in CCE-derived NT embryos with mitochondrial supplementation could have caused the reduced developmental outcomes compared to non-supplemented CCE-derived NT embryos. It is also interesting to note that the majority of MEF-derived and CCE derived NT embryos with mitochondrial

supplementation arrested at the 2-cell stage, whilst the embryos derived from porcine ddC treated oocytes arrested at the 4 to 6-cell stage, when EGA occurs in the mouse and pig, respectively. It is therefore possible that mtDNA replication is specifically detrimental to EGA. This could be a result of upregulation of nuclear-encoded mtDNA replication factor expression and subsequent disruption of nuclear DNA packaging, although this hypothesis remains to be verified.

6.8 Conclusions

The mtDNA replication factor expression in donor cells is reflected in the amount of donor cell-derived mtDNA replication that takes place within resulting NT embryos. The large genetic diversity between the nucleus and donor cell could result in recognition by the nucleus of only the donor cell-derived mtDNA. As the donor cell-derived mtDNA content is below the level required for successful fertilisation and embryo development, mtDNA replication is initiated. However, mtDNA replication during embryogenesis has detrimental effects on developmental outcomes, and this attempt by the embryo to increase donor cell-derived mtDNA content consequently contributes to developmental arrest. Future cross-species NT experiments should therefore investigate methods of generating sufficient donor cell-derived mtDNA by the time of oocyte reconstruction, or perhaps earlier, in order to prevent initiation of mtDNA replication and reduce subsequent developmental arrest.

CHAPTER 7: GENERAL DISCUSSION

7.1 MtDNA replication is required during oocyte growth and throughout oocyte maturation in order to support embryogenesis

The downregulation of mtDNA replication initially reported during early mammalian embryogenesis (Piko & Taylor, 1987) indicates a requirement for large numbers of mitochondrial genomes to be present in the oocyte at fertilisation. Indeed, a number of reports have indicated that high mtDNA copy numbers are present in mature oocytes and that those oocytes with the highest levels of mtDNA were associated with increased fertilisation outcomes (Almeida-Santos *et al.*, 2006; El Shourbagy, 2004; May-Panloup *et al.*, 2005a; Reynier *et al.*, 2001). However, many of those oocytes with fewer copies of mtDNA successfully fertilised (Almeida-Santos *et al.*, 2006; El Shourbagy, 2004; May-Panloup *et al.*, 2005a; Reynier *et al.*, 2001), indicating the need for further investigation.

Chapter 4 describes the first analysis of mtDNA replication throughout the entire *in vitro* maturation period. Consistent with previous reports for other species (see above), high levels of mtDNA were detected in both BCB+ and BCB- porcine oocytes at 44 hrs. However, those oocytes least likely to fertilise, such as BCB- oocytes, were observed to significantly increase their mtDNA copy number during *in vitro* maturation, whilst the most competent BCB+ oocytes already contained a high level of mtDNA at the beginning of the maturation period. This indicates that mtDNA replication was delayed in those oocytes least likely to fertilise. Data presented in Chapter 4 further indicated that mtDNA replication was delayed in those oocytes less likely to fertilise due to reduced expression of nuclear-encoded mtDNA replication factors such as PolG and TFAM. It is likely that a checkpoint and feedback

mechanism occurs at some point during the *in vitro* maturation process in order to confirm that a sufficient number of mtDNA copies are present (see Figure 7.1). This could be similar to the checkpoint controls that ensure nuclear DNA replication is completed before somatic cell division takes place (Roberge, 1992). The presence of a checkpoint and feedback mechanism would explain the increase in expression of mtDNA replication factors that was initiated in the least competent oocytes and the subsequent increase in mtDNA copy number to sufficient levels. Significantly, analysis of ddC treated oocytes, in which mtDNA replication was inhibited, indicated that mtDNA turnover had taken place. This was probably due to concurrent mtDNA degradation and replication, similar to that which commonly occurs in somatic cells (Kai *et al.*, 2006). Continuation of mtDNA replication throughout the *in vitro* maturation period is therefore necessary in order to maintain the appropriate number of mtDNA copies and consequently, oocyte fertilisation and developmental ability.

7.2 Limited mtDNA replication occurs during early mammalian embryogenesis

In 1987, it was determined through dot hybridisation experiments that mtDNA content remained relatively constant throughout murine pre-implantation embryo development, indicating an absence of mtDNA replication until after implantation (Piko & Taylor, 1987). Since then, there were no reports on the subject until 2004 when it was confirmed that there was no change in mtDNA copy number during this period (McConnell & Petrie, 2004). Consistent with these reports and with the original hypothesis, no increase in mtDNA copy number was detected in early porcine embryos (see Chapter 3). In fact, mtDNA copy number actually decreased by 96% between the 2-cell and 8-cell stages. Similarly, a 60% decrease in mtDNA copy number was recently reported between the 2-cell and 8-cell stages in bovine

embryos (May-Panloup *et al.*, 2005b). This indicates that absence of mtDNA replication is likely to be a common feature of early mammalian pre-implantation embryogenesis.

However, despite the absence of an increase in mtDNA copy number during murine pre-implantation development (Piko & Taylor, 1987), a short period of mtDNA replication was reported in murine embryos at the 2-cell stage (McConnell & Petrie, 2004). The authors suggested that a small amount of mtDNA turnover might occur at this stage. Consistent with this hypothesis, data presented in Chapter 3 indicated that mtDNA replication factor expression was upregulated at the 4 to 8-cell stage in porcine embryos, although this also did not result in an increase in mtDNA copy number. Interestingly, the 2-cell and 4-cell stages correspond to activation of the embryonic genome in mouse (Bolton *et al.*, 1984) and pig (Jarrell *et al.*, 1991) embryos, respectively. It is therefore possible that a small amount of mtDNA replication may occur when the embryonic genome is activated. This hypothesis is further supported by the increased expression of TFAM and NRF1 reported at the 16-cell and morula stages in cattle embryos (May-Panloup *et al.*, 2005b), when EGA occurs in this species (Camous *et al.*, 1986). However, as discussed in Chapter 3, it is as yet unclear whether this might be the result of a general increase in transcription of nuclear genes that occurs with EGA (Ma *et al.*, 2001), or whether mitochondria have a specific role to play in this process.

Regardless of whether mtDNA replication occurs when the embryonic genome is activated, mtDNA copy number was not increased above the level present in the mature oocyte until at least the blastocyst stage in pig (Chapter 3), cow (May-Panloup *et al.*, 2005b) and mouse (Piko & Taylor, 1987) embryos. The decrease in mtDNA copy number observed between the

2-cell and 8-cell stages in pig (Chapter 3) and cow (May-Panloup *et al.*, 2005b) embryos is consistent with a decreased requirement for OXPHOS and therefore an increased dependence on glycolysis during this time (Sturmey & Leese, 2003; Thompson *et al.*, 1996). It is possible that OXPHOS is downregulated in order to avoid DNA damage that might result from the associated production of reactive oxygen species (Richter *et al.*, 1988), which could in turn hinder embryo development (Karja *et al.*, 2006; Kitagawa *et al.*, 2004). This hypothesis is supported by the reduced embryo development that was obtained when mtDNA replication was induced to occur in both IVF (Chapter 4) and NT-derived (Chapter 6) early embryos. For example, ddC treated oocytes, which could not maintain mtDNA copy number but produced PolGA and PolGB transcripts for use after the removal of ddC at fertilisation, generated similar fertilisation rates to untreated oocytes, which were able to maintain high mtDNA copy numbers throughout *in vitro* maturation. However, the embryos derived from these oocytes arrested at the 4 to 6-cell stage (Chapter 4). Similarly, mtDNA replication occurred in NT embryos generated using murine donor cells that contained mtDNA replication factors (Chapter 6). This also resulted in increased developmental arrest at the 2-cell stage. Further work could however be carried out in order to test this hypothesis directly. This could include analysis of BrdU incorporation into mtDNA (McConnell & Petrie, 2004), reactive oxygen species production and embryo development.

Interestingly, the increases in mtDNA copy number detected in early IVF and NT-derived embryos occurred at a time when the nuclear material would have been undergoing a significant amount of remodelling and would therefore be unlikely to be transcriptionally active (Borsuk & Milik, 2005; Telford *et al.*, 1990). This indicates the possible presence of a second checkpoint (see Figure 7.1) regulating mtDNA copy number shortly after

fertilisation/activation. This might result in feedback responses that induce further mtDNA replication if necessary, but only if PolGA and PolGB transcripts and/or protein are already available, as was likely the case for embryos derived from ddC treated oocytes and for MEF-derived NT embryos but not for CCE-derived NT embryos. This might involve post-translational modifications such as phosphorylation, which is a key factor in the nuclear DNA replication checkpoints (Roberge, 1992). However, further investigation into the regulation of mtDNA replication during the fertilisation and/or activation events would be required to verify this hypothesis.

7.3 MtDNA replication is re-initiated following differentiation of embryonic cells

Data presented in Chapter 3 describe an increase in PolGA and PolGB transcripts at the morula stage and, subsequently, an increase in mtDNA copy number in porcine expanded blastocysts. These data are consistent with the increases in PolGA and PolGB transcripts in murine morulae and blastocysts that were reported recently (Thundathil *et al.*, 2005) and also with the increases in mtDNA copy number reported in murine (Thundathil *et al.*, 2005) and bovine (May-Panloup *et al.*, 2005b) blastocysts. ICC analysis of the localisation of PolGA in porcine blastocysts (Chapter 3) and metabolic analysis of dissected murine blastocysts (Houghton, 2006) indicated that the increase in mtDNA copy number might occur primarily in cells destined to become TE. Furthermore, porcine and bovine blastocysts were reported to increase their mtDNA copy number slightly earlier than murine blastocysts and were also reported to have increased TE:ICM ratios, as discussed in Chapter 3 (see Section 3.7.2). These data would support the hypothesis that mtDNA replication is upregulated following cellular differentiation.

Chapter 5 describes the differentiation of mESCs into EBs, which was associated with a decrease in pluripotency associated gene transcripts, followed by an increase in PolGA and PolGB expression. The time points at which these changes occurred coincided with particular stages of early post-implantation embryogenesis and their predicted mtDNA replication requirements. Specifically, the increase in PolGA and PolGB transcripts (Chapter 5) occurred at an equivalent time point to the death of PolGA knock-out mice (Hance *et al.*, 2005). This indicates that mtDNA replication not only increases with cellular differentiation but also that it is essential for embryo survival.

7.4 MtDNA replication driven by differentiated cell nuclei can be problematic in SCNT embryos

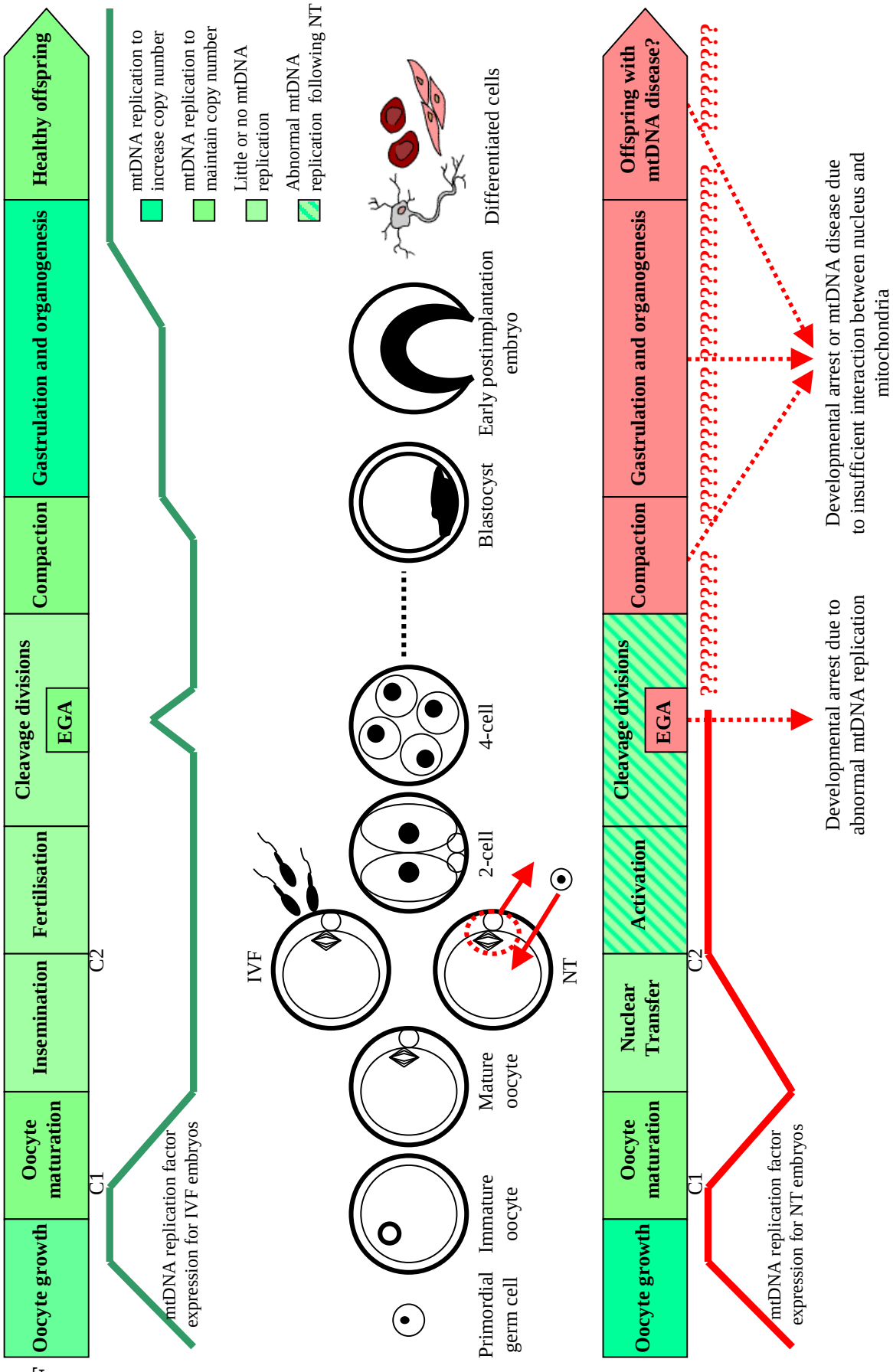
MtDNA replication is required in many situations such as during oocyte maturation (Chapter 4), post-implantation gastrulation (Chapter 5) and in regenerating livers (Koyama *et al.*, 1998) and cardiac tissue following myocardial infarction (Ikeuchi *et al.*, 2005). However, replication of mtDNA in early pre-implantation embryos can result in developmental arrest (Chapter 4; Chapter 6). Its occurrence must therefore be strictly regulated. The mechanisms through which this regulation normally occurs are now beginning to be understood, with many external factors, such as temperature, indirectly influencing mtDNA replication (see Scarpulla, 2006 for review).

However, mtDNA replication is more directly controlled by the level of expression of nuclear-encoded transcription and replication factors, as described throughout this thesis and in the literature cited. Experiments described in Chapter 5 indicate that these factors are

expressed at varying levels in different cell types. This is likely to ensure that mtDNA copy number is tailored to meet the differing OXPHOS requirements of specialised cell types (Moyes *et al.*, 1998). However, varying patterns of mtDNA replication factor expression in different cell types could be problematic for techniques such as SCNT, which involves the transfer of a nucleus from a specialised cell type into an embryonic cytoplasm (see Figure 7.1). The donor nucleus must therefore be reprogrammed to an embryonic state if it is to be capable of supporting embryo development (Hiiragi & Solter, 2005). However, the continued expression in NT embryos of genes associated with the donor cell type (Gao *et al.*, 2003) indicates that reprogramming is not always successfully completed. This could result in overexpression of PolG and TFAM, as was reported in sheep 4-cell SCNT embryos (Lloyd *et al.*, 2006). Consequently, abnormal mtDNA replication could occur, as was observed in the cross-species NT embryos derived from differentiated MEFs (see Chapter 6). However, it remains to be investigated precisely how the premature replication of mtDNA in these embryos may have contributed to their decreased developmental outcomes compared to control embryos that were not generated using a differentiated nucleus.

Figure 7.1 (next page): Model of mtDNA replication following IVF and NT

MtDNA replication factor expression is upregulated during oocyte growth and mtDNA copy number subsequently increases. At the end of the growth phase, a checkpoint (C1) mechanism ensures that sufficient mtDNA copies are present and mtDNA replication factor expression is downregulated. The high mtDNA copy number is then maintained throughout oocyte maturation through small amounts of concurrent mtDNA degradation and replication. Oocytes with insufficient mtDNA increase mtDNA replication factor expression in order to generate sufficient copies of the genome. Following IVF (top), a second mtDNA copy number checkpoint (C2) at fertilisation is passed and mtDNA replication factor expression remains low. MtDNA copy number therefore either decreases or remains constant until the morula or blastocyst stages when mtDNA replication factor expression in individual blastomeres is upregulated. MtDNA copy number can then increase as cells differentiate in order to meet the requirements of specialised cell types. Following NT (bottom), mtDNA replication could be upregulated due to the continued expression of mtDNA replication factors by the donor nucleus. MtDNA replication could also be actively induced if the reconstructed oocyte recognises only the donor cell-derived mtDNA, fails the second checkpoint and consequently upregulates expression of mtDNA replication factors. This abnormal mtDNA replication is likely to result in developmental arrest at EGA. Should the embryo develop beyond this stage, abnormal regulation of mtDNA replication is likely to result in mtDNA dysfunction in subsequent offspring.


IVF
Oocyte growth
Oocyte maturation
Insemination
Fertilisation
Cleavage divisions
EGA
Compaction
Gastrulation and organogenesis
Healthy offspring
C1
C2
mtDNA replication factor expression for IVF embryos
mtDNA replication to increase copy number
mtDNA replication to maintain copy number
Little or no mtDNA replication
Abnormal mtDNA replication following NT
IVF
Primordial germ cell
Immature oocyte
Mature oocyte
2-cell
4-cell
Blastocyst
Early postimplantation embryo
Differentiated cells
NT
Oocyte growth
Oocyte maturation
Nuclear Transfer
Activation
Cleavage divisions
EGA
Compaction
Gastrulation and organogenesis
Offspring with mtDNA disease?
C1
C2
mtDNA replication factor expression for NT embryos
Developmental arrest due to abnormal mtDNA replication
Developmental arrest or mtDNA disease due to insufficient interaction between nucleus and mitochondria

7.5 Nucleocytoplasmic compatibility is essential for correct regulation of mtDNA replication and viability of NT-derived embryos and offspring

Premature mtDNA replication might be induced during early embryo development if the proposed mtDNA copy number checkpoint and feedback mechanisms that were mentioned previously fail to recognise the mtDNA present and subsequently initiate replication of mtDNA in error. This could occur if more than one haplotype is present, such as might take place following inter- or cross-species NT (St. John *et al.*, 2005a; Steinborn *et al.*, 2002). For example, failure to recognise mtDNA from another sub-species resulted in the transmission of paternal mtDNA following inter-specific murine NT (Kaneda *et al.*, 1995). Variation in mtDNA sequence may prevent mtDNA binding and/or recognition by the, as yet, unidentified factors involved in these proposed checkpoints. Although the mature porcine oocytes described in Chapter 4 were determined to contain large numbers of mtDNA copies, replication of donor cell-derived mtDNA occurred in NT embryos generated using oocytes from the same source. For MEF-derived NT embryos, this may have occurred due to continued expression of PolG after oocyte reconstruction, as observed for sheep SCNT embryos (Lloyd *et al.*, 2006). However, this does not account for the mtDNA replication that occurred in CCE-derived NT embryos with mitochondrial supplementation, as these embryos contained nuclear material that was not primed to drive mtDNA replication (Chapter 5).

The presence of a checkpoint that only detected the approximately 50000 (see Chapter 6) murine mtDNA copies that were present in the reconstructed oocyte may provide an explanation for the donor-cell derived mtDNA replication that was initiated in these embryos. However, further investigation to identify the proteins involved in the recognition of mtDNA would be required to confirm this hypothesis. If confirmed, it would also be interesting to

investigate whether the feedback increase in mtDNA replication was facilitated by PolG protein already present within the supplementary mitochondria, or whether new transcription of the PolGA and PolGB genes was initiated. If mtDNA replication requires new transcription of PolGA and PolGB genes, packaging of the nuclear genetic material would then be disrupted (Reeves, 1984). This could provide an explanation for the observed developmental arrest at the 2-cell stage, when EGA occurs in the mouse (Bolton *et al.*, 1984).

In order to successfully produce cross-species NT embryos, the nuclear and mitochondrial genomes must be compatible. This would ensure adequate interaction between nuclear-encoded mtDNA replication factors and the mitochondrial D-loop and also between nuclear and mitochondrially encoded subunits of ETC complexes, thereby decreasing the occurrence of mtDNA disease symptoms (see Figure 7.1). Experiments described in Chapter 6 indicate that donor cell-derived mtDNA replication is initiated when insufficient copies of mtDNA of the donor cell sequence are present in the reconstructed oocyte. Future experiments could therefore investigate methods of ensuring that sufficient donor cell-derived mtDNA copies are present at oocyte reconstruction so that mtDNA replication is not initiated in the early embryo. This could include supplementation of at least 100000 mitochondria shortly before oocyte reconstruction. However, this would also include functional ETC complexes as well as mtDNA. In order to prevent a potential increase in ETC activity and subsequent damage to DNA by reactive oxygen species, it might be more appropriate to supplement oocytes with smaller numbers of mitochondria earlier, perhaps during *in vitro* maturation. Although, in this case, additional supplementation with exogenous PolG protein and perhaps other mtDNA replication factors might also be required in order to facilitate maintenance and preferential replication of the donor cell-derived mtDNA.

7.6 Conclusions

MtDNA replication is downregulated during mammalian pre-implantation embryo development and its premature occurrence can result in developmental arrest. A large number of mtDNA molecules are therefore required in mature oocytes in order to meet the mitochondrially-derived ATP requirements of the pre-implantation embryo. Various mechanisms exist to ensure that sufficient mtDNA replication takes place before fertilisation. However, if insufficient mtDNA copies are present, mtDNA replication may be initiated in the early embryo. This is highly likely following NT, as the donor nuclei may not interact sufficiently well with the recipient oocyte cytoplasm. This could result in mtDNA not being detected. In order to prevent subsequent premature replication of embryonic mtDNA, methods of creating mature oocytes that contain sufficient copies of donor cell-derived mtDNA need to be investigated. Should this be achievable, it may ultimately become possible to create NT offspring and/or NT-derived ESCs that are truly genetically identical to the donor cell source. This would significantly reduce the risk of insufficient or unregulated mtDNA replication, and potentially mtDNA disease, for both offspring produced by NT and for human patients receiving treatment through therapeutic cloning.

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APPENDIX I: MEDIA AND STOCK SOLUTIONS

MEF media
450ml High Glucose DMEM
50ml FBS
5ml 200mM L-Glutamine
5ml non-essential amino acids
Stored at 4°C

R1 mESC media
425ml Knock-out DMEM
75ml Serum Replacement
5ml Penicillin/Streptomycin solution
5ml 200mM L-Glutamine
5ml NEAA
0.5ml β-Mercaptoethanol stock
500µl LIF
Stored at 4°C

β-Mercaptoethanol stock
60µl β-Mercaptoethanol
10ml knock-out DMEM
Stored at 4°C

CCE mESC media
425ml High glucose DMEM
75ml Hyclone FBS
5ml NEAA
5ml 200mM L-Glutamine
5ml Penicillin/Streptomycin solution
0.5ml β- Mercaptoethanol stock
500µl LIF
Stored at 4°C

Freeze media
41ml Hyclone FBS
9ml DMSO
Stored at 4°C

Basic Oocyte Culture Media
60ml TCM-199 with Earle's salts and sodium bicarbonate
1.5ml 25mM hepes
900µl 200mM L-glutamine
20mg BSA
60µl 0.57M cysteine stock
300µl Penicillin/Streptomycin solution
Stored at 4°C

***In vitro* maturation (IVM) media**
20ml Basic Oocyte Culture Medium without hepes
20µl 10µg/ml EGF stock
20µl 0.2mg/ml LH stock
20µl 50µg/ml FSH stock
Prepared fresh for each use

EGF stock
0.01g EGF
10ml Embryo tested water
Diluted 1:100 to make 1000x stock
Stored at –20°C in 25µl aliquots

LH stock
0.002g LH
10ml Embryo tested water
Stored at –20°C in 25µl aliquots

FSH stock
0.05g FSH
10ml Embryo tested water
Diluted 1:100 to make 1000x stock
Stored at –20°C in 25µl aliquots

Modified phosphate buffered saline (mPBS)
200ml Ca^{2+} and Mg^{2+} free Dulbecco PBS
15mg Potassium penicillin G
10mg Streptomycin sulphate
198mg Glucose
75mg Sodium pyruvate
0.8g BSA
Stored at 4°C

BCB media
0.0001g BCB
20ml mPBS
Prepared fresh for each use

Sperm Washing Media
200ml Ca^{2+} and Mg^{2+} free Dulbecco PBS
0.2g BSA
20mg Potassium penicillin G
15mg Streptomycin sulphate
Stored at 4°C

Modified tris buffered media (mTBM)
1.6524g NaCl
0.559g KCl
0.5g Glucose
0.2756g $CaCl_2.2H_2O$
0.25g BSA
0.6057g Tris
0.1375g Pyruvate
250ml Embryo tested water
Stored at 4°C
Supplement 20ml on day of use with 80µl adenosine stock and 40µl glutathione stock

Adenosine stock
13.4mg Adenosine
10ml Embryo tested water
Stored in 100µl aliquots at -20°C

Glutathione stock
15.4mg Glutathione
0.5ml PBS
Stored in 50µl aliquots at -20°C

IVP1 media
1.5875g NaCl
0.089g KCl
0.0405g KH_2PO_4
0.0735g $MgSO_4.7H_2O$
0.5265g $NaHCO_3$
0.0365g Glutamine
0.126g Lactate
0.008g Pyruvate
0.219g Taurine
0.1363g Hypotaurine
0.0625g $CaCl_2.2H_2O$
1g BSA
250ml Embryo tested water
Stored at 4°C
Supplement 20ml on day of use with 40µl glutathione stock and 100µl penicillin/streptomycin solution

IVP2 media
1.5875g NaCl
0.089g KCl
0.0405g KH_2PO_4
0.0735g $MgSO_4.7H_2O$
0.5265g $NaHCO_3$
0.0365g Glutamine
0.25g Glucose

0.219g Taurine
0.1363g Hypotaurine
0.0625g $CaCl_2.2H_2O$
1g BSA
250ml Embryo tested water
Stored at 4°C
Supplement 20ml on day of use with 40µl glutathione stock and 100µl penicillin/streptomycin solution

Manipulation media
20ml basic oocyte culture media
20µl 7.5mg/ml cytochalasin B stock
Stored at 4°C

Cytochalasin B stock
0.075g Cytochalasin B
10ml Embryo tested water
Stored in 25µl aliquots at –20°C

Hoechst 33342 stock
25mg Hoechst 33342
5ml sterile ddH_2O
Stock solution was dispensed in 10µl aliquots, and stored at -20ºC.

Ca^{2+} Ionophore (A23187) stock
5mg Ca^{2+} Ionophore (A23187)
1.91ml DMSO
Stock solution (5mM) was dispensed in 10µl aliquots, and stored at -20ºC.

Cycloheximide stock
50mg Cycloheximide
5ml Ca^{2+} and Mg^{2+} free DPBS
Stored in 25µl aliquots at –20°C.

50X TAE buffer
242g Tris base
57.1ml glacial acetic acid
100ml 0.5M EDTA (pH8.0)
842.9ml sterile ddH_2O
A 1x working solution of 0.04M Tris-acetate and 0.001M EDTA was produced by diluting 20ml 50X stock into 980ml sterile ddH_2O. Storage was at room temperature.

0.5M EDTA
186.1g disodium ethylene diamine tetraacetate.$2H_20$
800ml sterile ddH_2O
The pH was adjusted to 8.0 using NaOH and the solution was stored at 4°C.

6X Loading buffer
0.25g Bromophenol blue
0.25g Xylene cyanol
40g sucrose
100ml sterile ddH_2O
The 1x final solution was produced by 6x dilution into PCR products before loading into the gel. Storage of the 6x solution was at 4ºC.

Fixation solution: 3.8% formaldehyde
450µl Ca^{2+} and Mg^{2+} free Dulbecco PBS
50µl 38% formaldehyde
Prepared fresh for each experiment

Permeabilisation/Wash solution: 0.1% Triton
500ml Ca^{2+} and Mg^{2+} free Dulbecco PBS
500µl Triton-X 100
Stored at 4°C

Blocking solution
500ml Ca^{2+} and Mg^{2+} free Dulbecco PBS
100 µM glycine
1g BSA
Stored at 4°C

APPENDIX II: LIST OF PUBLICATIONS

Research Articles

Emma C Spikings, Jon Alderson and Justin C St. John (2007) Regulated mtDNA replication during oocyte maturation is essential for successful porcine embryonic development. Biology of Reproduction 76, 327-335.

El Shourbagy SH*, **Spikings EC***, Freitas M, St John JC (2006) Mitochondria directly influence fertilisation outcome in the pig. Reproduction 131, 233-245.

Reviews

EC Spikings, J Alderson and JC St. John (2006) Transmission of mitochondrial DNA following assisted reproduction and nuclear transfer. Human Reproduction Update 12, 401-415.

St John JC, Lloyd RE, Bowles EJ, **Thomas EC**, El Shourbagy S (2004) The consequences of nuclear transfer for mammalian foetal development and offspring survival. A mitochondrial DNA perspective. Reproduction 127, 631-41.

Book contributions

Justin C. St. John, Alexandra Amaral, Emma Bowles, João Facucho, Rhiannon Lloyd, Mariana Freitas, Heather L. Gray, Christopher S. Navara, Gisela Oliveira, Gerald P. Schatten, **Emma Spikings** and João Ramalho-Santos (2006) The analysis of mitochondria and mitochondrial DNA in human embryonic stem cells. Methods in Molecular Biology vol. 331 chapter 21, Humana Press. ISBN: 1-58829-497-8.

Abstracts

Emma Spikings and Justin St. John (2006) Delayed TFAM and PolG activity reduces oocyte competence. Society for Reproduction and Fertility: Annual Conference 2006.

João Facucho, **Emma Spikings**, Emma Bowles, Jon Alderson and Justin St. John (2006) Regulation of mitochondrial DNA replication during murine Embryonic Stem Cells differentiation. Society for Reproduction and Fertility: Annual Conference 2006.

Emma Spikings, João Facucho and Justin St. John (2005) Loss of pluripotency triggers mtDNA differentiation. Society for Reproduction and Fertility: Annual Conference 2005.