

Culturing Embryos From the Cleavage to Blastocyst Stage; an Opportunity to Improve Pluripotency and Embryonic Stem Cell Generation Efficiency

Jared M. Campbell, BSc, BHSc (Honours)

Centre for Stem Cell Research

Research Centre for Reproductive Health

Discipline of Obstetrics and Gynaecology

Robinson Institute

The University of Adelaide, Australia

A thesis submitted to the University of Adelaide in total fulfilment of the requirements for the degree of Doctorate of Philosophy in Medical Science

September 2012

Contents

Contents.....	ii
Abstract.....	xiii
Common Abbreviations	xviii
1.0 Literature review.....	1
1.1 Introduction	2
1.2 Embryonic stem cells	3
1.2.1 Definition of an embryonic stem cell.....	3
1.2.1.1 Pluripotency	5
1.2.1.1.1 In vitro differentiation.....	8
1.2.1.1.2 In vivo differentiation.....	9
1.2.1.2 Self renewal.....	9
1.2.2 Derivation of an ESC line	10
1.3 Applications of embryonic stem cells	13
1.3.1 Toxicity	13
1.3.2 Drug discovery.....	15
1.3.3 Developmental biology	16
1.3.4 Regenerative medicine	17
1.3.5 Comparison of embryonic stem cells and induced pluripotent stem cells.....	19
1.4 Current limitations of embryonic stem cells.....	20
1.4.1 Cell line quality	20
1.4.2 Comparison of mouse and human ESCs.....	22
1.5 The preimplantation embryo	24
1.5.1 In vitro fertilisation.....	29
1.5.2 Embryo Culture	30
1.5.2.1 Carbohydrates.....	31
1.5.2.2 Amino acids	32
1.5.2.3 Vitamins	33
1.5.2.4 Chelators	34
1.5.2.5 Macromolecules.....	35
1.5.2.6 Oxygen tension.....	36
1.5.2.7 pH	37
1.5.2.8 Cryopreservation.....	39

1.5.2.9	Sequential and Monoculture systems.....	40
1.6	Improving embryo quality.....	41
1.6.1	Growth Factors.....	41
1.6.1.1	A review of growth factors used to improve embryo health.....	41
1.6.1.2	Insulin, GM-CSF, FGF-4 and LIF	44
1.6.1.2.1	Insulin.....	46
1.6.1.2.2	GM-CSF.....	47
1.6.1.2.3	FGF-4	48
1.6.1.2.4	LIF	48
1.7	Animal model	49
1.8	Conclusion and hypothesis	50
2.0	Materials and Methods.....	54
2.1	Media preparation	55
2.1.1	Preparation of glassware	55
2.1.2	Embryo culture and handling media.....	55
2.1.3	Culture media preparation.....	56
2.1.4	Hyaluronidase preparation	58
2.2	Preparation of pipettes	58
2.3	Animals and induction of ovulation	58
2.3.1	Animals.....	58
2.3.2	Induction of ovulation.....	59
2.4	Embryo culture.....	59
2.4.1	Collection of zygotes	59
2.4.2	Culture of embryos	60
2.4.3	Assessment of embryo morphology	60
2.5	Assessment of blastocyst differentiation.....	63
2.5.1	OCT4 and Nanog co-expression	63
2.6	Blastocyst outgrowth formation	66
2.6.1.	Gelatin coating dishes	66
2.6.2	MEF coating organ well dishes.....	66
2.6.2.1	Derivation of primary MEF lines.....	66
2.6.2.2	Creation of MEF cells for dishes.....	67
2.6.2.3	Creation of MEF coated dishes	68
2.6.3	Blastocyst outgrowth	68

2.7	PCR analysis of outgrowths.....	69
2.7.1	RNA extraction and reverse transcription.....	69
2.7.2	Real time PCR	70
2.8	Assessment of outgrowth differentiation.....	72
2.8.1	Immunocytochemistry	72
2.9	Derivation of primary ESC colonies.....	74
2.9.1	Preparation of dishes	74
2.9.2	Isolation, trypsinisation and replating	74
2.9.3	Identification of pluripotent colonies	75
2.9.3.1	Morphology.....	75
2.9.3.2	Immunocytochemistry	76
2.10	Characterisation of putative ESC lines	77
2.10.1	Passaging.....	77
2.10.2	Cryopreservation.....	77
2.10.3	Expansion of cell lines	78
2.11	Characterisation of an ESC line	78
2.11.1	Alkaline phosphatase staining.....	78
2.11.2	SSEA1 staining	79
2.11.3	Karyotyping	80
2.11.4	Directed differentiation	80
2.11.4.1	Mesoderm differentiation.....	81
2.11.4.2	Definitive endoderm differentiation.....	81
2.11.4.3	Neuroectoderm differentiation	81
2.11.4.4	Differentiation immunocytochemistry	82
3.0	Development of a mouse model for studying the effect of embryo culture on embryonic stem cell derivation.	84
3.1	Introduction	85
3.2	Experimental design.....	86
3.2.1	Statistics	87
3.3	Results	88
3.3.1	Blastocyst Development	88
3.3.2	Pluripotency of ICM Cells in Cultured Blastocysts	90
3.3.3	Gene Expression Levels in Outgrowths.....	93
3.4	Discussion.....	95

4.0	Insulin in embryo culture medium from the cleavage stage increases epiblast cell number and percentage.	98
4.1	Introduction	99
4.2	Experimental design.....	100
4.2.1	Statistics	100
4.3	Results	102
4.3.1	Blastocyst Development	102
4.3.2	Pluripotency of ICM Cells in Cultured Blastocysts	103
4.4	Discussion.....	106
5.0	Insulin increases epiblast cell number of in vitro cultured mouse embryos via the PI3K/GSK3/p53 pathway.....	108
5.1	Introduction	109
5.2	Experimental design.....	111
5.2.1	Culture system	111
5.2.2	Statistics	115
5.3	Results	116
5.3.1	Experiment 1: Effect of PI3K inactivation on epiblast cell number in embryos cultured in the presence or absence of insulin.....	116
5.3.2	Experiment 2: Effect of GSK3 activation/inhibition on epiblast cell number in embryos cultured in the presence or absence of insulin.....	119
5.3.3	Experiment 3: Effect of p53 activation/inhibition on epiblast cell number in embryos cultured in the presence or absence of insulin.....	125
5.3.4	Experiment 4: Interaction of GSK3 and p53 signalling.....	129
5.5	Discussion.....	132
6.0	Epiblast cell number and primary embryonic stem cell colony generation are increased by culture of embryos with insulin from the cleavage stage.....	137
6.1	Introduction	138
6.2	Experimental design.....	139
6.2.1	Culture system	139
6.2.2	Statistics	140
6.3	Results	141
6.3.1	Experiment 1: Effect of insulin on blastocyst development	141
6.3.2	Experiment 2: Effect of insulin on outgrowth formation.....	145
6.3.3	Experiment 3: Effect of insulin on the derivation of primary ESC colonies from day 6 blastocysts	150
6.3.4	Experiment 3: Characterisation of an ESC line.....	155

6.4	Discussion.....	157
7.0	General Discussion	161
7.1	Introduction	162
7.2	Impact of culture conditions on epiblast and pluripotency.....	165
7.3	Insulin stimulation of pluripotency of post-compaction embryos.....	166
7.4	Molecular mechanism of action of insulin on pluripotency in the blastocyst.....	167
7.5	Expression of OCT4 and Nanog in blastocysts	168
7.6	Effect of insulin in embryo culture medium persists in outgrowths.....	168
7.7	Insulin in culture media and the effect of ESC colony generation	169
7.8	Conclusion	171
8.0	Appendix	172
8.1	Mouse embryo bioassay	173
8.2	Insulin dilution.....	175
8.3	Inhibitor dilutions.....	175
8.3.1	LY294002	175
8.3.2	CT99021	175
8.3.3	H-89	176
8.3.4	Pifithrin- α	176
8.3.5	Nicotinamide.....	176
8.4	Hormone preparation	177
8.4.1	Saline solution	177
8.4.2	Equine chorionic gonadotropin (eCG).....	177
8.4.3	Human chorionic gonadotropin	177
8.5	Solutions for immunocytochemistry.....	177
8.5.1	Paraformaldehyde.....	177
8.5.2	Glycine.....	178
8.5.3	Triton-X solution.....	178
8.5.4	Donkey Serum	178
8.5.5	Dapi	178
8.6	Antibody validations	178
8.6.1	OCT4 and Nanog	178
8.6.2	SSEA1.....	179
8.6.3	Antibodies for markers of germ cell lineage	179
9.0	References.....	180

Figures and Tables

FIG. 1.1. Schematic of a blastocyst; the zona pellucida is a glycoprotein membrane that surrounds the blastocyst, the trophoctoderm is a monolayer of cells that forms a sphere around the fluid filled blastocyst cavity and the ICM, the ICM is a group of cells attached to the inner surface of the trophoctoderm which is made up of primitive endoderm and epiblast cells.

..... 3

FIG 1.2. Timeline depicting the first derivation of ESCs from various species. By species sources were mouse [16], hamster [21], mink [22], pig [23], monkey [20], chicken [24], human [1], cow [25], rat [26], and canine [27].

..... 4

FIG. 1.3. Pluripotent epiblast cells can be cultured and retain their pluripotency as ESCs, which can differentiate to the three cell types of the germ layer, ectoderm, endoderm and mesoderm, and give rise to all cell types found in the adult body

..... 5

FIG. 1.4. Brightfield image of a mouse outgrowth attached to a gelatin coated dish. The black arrow indicates the ICM outgrowth, surrounding cells are trophoctoderm. Scale bar is 50µm.

..... 11

FIG. 1.5. Diagram of the derivation of an ESC line from a blastocyst. Pluripotent epiblast/ESCs are shown in green, differentiated primitive endoderm cells are shown in red: The blastocyst is outgrown to isolate ICM cells, the outgrowth is then collected and trypsinised to disaggregate its cells so that when they are replated the epiblast cells are able to form clonal primary ESC colonies, which in turn can be collected, disaggregated, and propagated to an ESC line

..... 12

FIG. 1.6. Diagram depicting the morphological stages of embryo development from fertilisation to the hatched blastocyst stage. The zygote is formed by the fertilisation of the oocyte by the spermatozoa. The embryo then begins to divide by reductive cleavage creating the 2-cell, 4-cell and then 8-cell embryo. After this compaction begins to occur (8-cell for mouse, 10-cell for human) and cells polarise and flatten to maximise cell contacts, creating the morula. Fluid is secreted internally to form the blastocoel and the early blastocyst. The blastocoel continues to expand to form the blastocyst which can begin to herniate from the zona pellucida by hatching, ultimately creating the hatched blastocyst

..... 26

FIG. 1.7. A hatching blastocyst, immunocytochemically stained to differentiate epiblast, primitive endoderm and trophoctoderm nuclei, imaged by confocal microscopy and reconstructed as a 3D image using Z-stack. Yellow nuclei are epiblast cells, they have been stained for OCT4 (red), which is restricted to the ICM and Nanog (green), which is restricted to the epiblast (and occasional trophoctoderm cells), they have also been counter stained with Dapi (blue). Primitive endoderm cells are stained pink (red+blue), as they do not express Nanog, while trophoctoderm cells are stained blue as they do not express OCT4, and in this case none express Nanog

..... 28

FIG. 2.1. A; 8-cell embryo, B; Compacting embryo, C; Morula, D; Early blastocyst, E; Blastocyst, F; Hatching blastocyst, G; Hatched blastocyst. Scale bar 50µm.

..... 62

FIG. 2.2 Representative images of and immunocytochemically stained blastocyst fixed at 115h post culture. Total cell number is stained blue with Dapi, OCT4 positive cell number is stained

red with rhodamine, and Nanog positive cell number is stained green with FITC. In the overlay those nuclei which are stained with Dapi but not OCT4 are trophectoderm cells, those nuclei stained with OCT4 but not Nanog are primitive endoderm (PE) and those nuclei which are stained with both OCT4 and Nanog are epiblast. Scale bar is 50µm. 65

FIG.2.3. Representative images of an immunocytochemically stained outgrowth from a blastocyst plated at 115h after culture and fixed 48h later. Total cell number is stained blue with Dapi, OCT4 positive cell number is stained red with rhodamine, and Nanog positive cell number is stained green with FITC. In the overlay those nuclei which are stained with both OCT4 and Nanog are epiblast cells. Scale bar is 50µm. 73

FIG. 2.4. Representative brightfield image of a primary ESC colony with a typical morphology seen 48h after the plating of the outgrowth, on a MEF feeder layer. Scale bar is 50µm..... 75

FIG. 2.5. Representative images of an immunocytochemically stained primary ESC colony stained 48h after the trypsinisation of the outgrowth from which it was derived and the identification of putative ESC morphology. Total cell number is stained blue with Dapi, OCT4 positive cell number is stained red with rhodamine, and Nanog positive cell number is stained green with FITC. As the cells of the colony are positive for both OCT4 and Nanog they are demonstrated to be pluripotent. Scale bar is 50µm..... 76

FIG. 2.6. Representative bright field image of an ESC colony which has stained positive for alkaline phosphatase (AP) activity, as shown by pink staining. Scale bar is 50µm. 79

FIG.2.7. A brightfield image of ESC colonies and an epifluorescent image of those same colonies stained for SSEA1, shown in green. Scale bar is 50µm. 80

FIG. 2.8. Brightfield and epifluorescent images of differentiated cells from the established commercial D3-ES cell line which have been subjected to the indicated differentiation protocol. Definitive endoderm: green is CXCR4 (fusin), cell surface marker of definitive endoderm and mesoderm; red is SOX17, nuclear marker of endoderm (primitive and definitive). Co-expression shows definitive endoderm. Mesoderm: green is CXCR4 (fusin), cell surface marker of mesoderm and definitive endoderm; red is VEGFR11 (FLK-1), cell surface marker of mesoendoderm and mesoderm. Co-expression shows mesoderm. Neuroectoderm: green is Nestin, cytoskeletal marker of neuroectoderm. Scale bar is 50µm. 83

FIG. 3.1. Representative images of immunocytochemically stained blastocysts. All cell nuclei are stained blue with Dapi, Nanog positive cells are stained green with FITC. Scale bar equal to 50µm. A; Simple / Simple, B; Simple / G2, C; G1/G2, D; Negative control. 92

FIG. 3.2. Gene expression of outgrowths cultured in different culture systems. Data are mean±sem. N=5 samples per treatment. Significantly different from control G1/G2, * (P<0.05). 94

FIG. 4.1. Blastocyst culture in the presence of insulin. A; Total cell number as shown by Dapi staining, B; ICM cell number as shown by OCT4 staining, C; epiblast cell number as shown by OCT4 and Nanog staining, D; percentage of ICM cells that are epiblast cells, E; Trophectoderm cell number as shown by total cell number minus ICM cell number, and F; primitive endoderm cell number as shown by ICM minus epiblast cell number, in the blastocysts of embryos cultured in insulin at the concentrations indicated. Data are mean ± sem. Superscripts a and b are significantly different at P<0.01, N≥ 34 blastocysts per treatment. 104

FIG. 4.2. Representative images of immunocytochemically stained blastocysts after culture in the indicated concentration of insulin. All cell nuclei are stained blue with Dapi, OCT4 positive cells are stained red with rhodamine, Nanog positive cells are stained green with FITC. Scale bar equal to 50µm. A; G2+0pM insulin, B; G2+0.17pM insulin, C; G2+1.7pM insulin, D; G2+1700pM insulin, E; Negative control 105

FIG. 5.1. PI3K inhibition during blastocyst culture in the presence of insulin. A; TCN as shown by Dapi staining, B; ICM cell number as shown by OCT4 staining, C; epiblast cell number as shown by OCT4 and Nanog staining and D; percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in insulin at 1.7pM and/or 50µM of the PI3K inhibitor LY294002 (LY). Data are mean ± sem. Superscripts a and b are significantly different at P<0.01, N≥34 blastocysts per treatment. 118

FIG. 5.2. GSK3 activation during blastocyst culture in the presence of insulin. A; TCN as shown by Dapi staining, B; ICM cell number as shown by OCT4 staining, C; epiblast cell number as shown by OCT4 and Nanog staining and D; percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in insulin at 1.7pM and/or 10µM of the GSK3 activator H-89. Data are mean ± sem. Superscripts a, b and c are significantly different at P<0.05, N≥26 blastocysts per treatment. 121

FIG. 5.3. Results for GSK3 inhibition. A; TCN as shown by Dapi staining, B; ICM cell number as shown by OCT4 staining, C; epiblast cell number as shown by OCT4 and Nanog staining and D; percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in different concentrations of the GSK3 inhibitor CT99021. Data are mean ± sem. Superscripts a, b, c and d are significantly different at P<0.05, N≥46, 3 replicates. 124

FIG. 5.4. p53 activation during blastocyst culture in the presence of insulin. A; TCN as shown by Dapi staining, B; ICM cell number as shown by OCT4 staining, C; epiblast cell number as shown by OCT4 and Nanog staining and D; percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in insulin at 1.7pM and/or 10µM of the p53 activator nicotinamide (Nic). Data are mean ± sem. Superscripts a, b and c are significantly different at P<0.05, N≥38 blastocysts per treatment. 127

FIG. 5.6. Dual inhibition of GSK3 and p53. A; TCN as shown by Dapi staining, B; ICM cell number as shown by OCT4 staining, C; epiblast cell number as shown by OCT4 and Nanog staining and D; percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in 0.3µM of GSK3 inhibitor CT99021 (CT) and/or 30µM of p53 inhibitor pifithrin-α (Pft-α). Data are mean ± sem. Superscripts a and b are significantly different at P<0.05, N≥35 blastocysts per treatment. 131

FIG. 5.7. Schematic of insulin signalling and its relationship to regulating Nanog expression and pluripotency. Green arrows indicate reactions with a stimulatory effect on their target, red closed bars indicate reactions with a retarding effect on their target. P marks reactions where phosphorylation occurs, Ub marks reactions where ubiquitination occurs. Insulin binds the insulin receptor (IR), a tyrosine kinase which is then able to phosphorylate the IRSs. PI3K is able to bind to the phosphorylated IRSs by its SH2 domains, resulting in activation. PI3K phosphorylates the phospholipid PIP2, producing PIP3, which can be bound by the pleckstrin homology domains of PDK-1 and Akt. Results presented in this chapter have shown that activation of PI3K is necessary for insulin to increase the number of Nanog positive epiblast

cells during embryo culture. When PDK-1 and Akt are colocalised to the cell membrane PDK-1 is able to phosphorylate and activate Akt. Active Akt can phosphorylate GSK3, inactivating it. When active GSK3 is able to phosphorylate β -catenin, Hedgehog, and c-Myc; all factors which safeguard pluripotency through interactions with other second messengers. Additionally, active GSK3 phosphorylates and protects the intracellular domain of Notch, promoting differentiation. Further, inactivation of GSK3 is necessary for insulin to increase the number of Nanog positive epiblast cells during embryo culture. Akt is also able to phosphorylate and activate MDM2 which ubiquitinates the proapoptotic factor p53, causing its inactivation and removal from the nucleus, where it would bind to the *Nanog* promoter and suppresses its expression. Inactivation of p53 is necessary for insulin to increase the number of Nanog positive epiblast cells during embryo culture. GSK3 and p53, are able to form a dimer, resulting in the phosphorylation of p53 and the increased activity of both factors. GSK3 is also able to phosphorylate and activate MDM2. However, despite these interactions the interaction of GSK3 and p53 do not have a significant effect on Nanog positive epiblast cell number during embryo culture..... 135

FIG. 6.1. Confocal slices of blastocysts stained for total cell number (TCN, Dapi; blue), OCT4 (Rhodamine; red) and Nanog (FITC; green). Scale bar is 50 μ m. A; Negative control where staining was performed without primary antibodies. B; A day 4 blastocyst fixed as it began cavitation, C; A day 5 hatching blastocyst, D; A day 6 hatched blastocyst. 143

FIG. 6.2 A hatching day 6 blastocyst, immunocytochemically stained for total cell number, OCT4 positive cell number and Nanog positive cell number, imaged by confocal microscopy and reconstructed as a 3D image using Z-stack. Examination of 3D models showed that in day 6 blastocysts all OCT4 positive nuclei were in the ICM as were all cells also positive for Nanog. 144

FIG. 6.3. Confocal slices of outgrowths stained for total cell number (Dapi; blue), OCT4 (Rhodamine; red) and Nanog (FITC; green). Scale bar is 50 μ m. A; Negative control where staining was performed without primary antibodies, B; An outgrowth from a blastocyst plated on day 4, C; An outgrowth from a blastocyst plated on day 5, D. An outgrowth from a blastocyst plated on day 6. 147

FIG. 6.4. The effect insulin at 1.7 μ M in embryo culture media from the compacting stage of development on the outgrowths of early blastocysts plated on day 4, blastocysts plated on day 5 and blastocysts plated on day 6 on outgrowth cell number and differentiation. A; Total cell number of outgrowths plated day 4, B; OCT4 positive cell number of outgrowths plated day 4, C; Epiblast cell number of outgrowths plated day 4, D; Total cell number of outgrowths plated day 5, E; OCT4 positive cell number of outgrowths plated day 5, F; Epiblast cell number of outgrowths plated day 5, G; Total cell number of outgrowths plated day 6, H; OCT4 positive cell number of outgrowths plated day 6, I; Epiblast cell number of outgrowths plated day 6. Data are mean \pm sem. N \geq 27 (3 replicate experiments). Superscripts a and b differ at P<0.05. 148

FIG. 6.5. Confocal slice of a primary ESC colonies stained for TCN (Dapi; blue), OCT4 (Rhodamine; red) and Nanog (FITC; green). A; Negative control where staining was performed without primary antibodies, B; A primary ESC colony which is positive for both OCT4 and Nanog. Scale bar is 50 μ m. 151

FIG. 6.6. The effect of morphological state on days 4, 5 and 6 on the likelihood of blastocysts giving rise to ESC colonies. A; Percentage of blastocysts cultured in control conditions at the indicated morphological states which ultimately gave rise to a primary ESC colony, B; Percentage of blastocysts cultured with insulin at the indicated morphological states which ultimately gave rise to a primary ESC colony. Data are the number of blastocysts which generated ESCs expressed as a percentage of all blastocysts at that morphological stage. N≥44 (9 replicate experiments). Superscripts a and b within paired columns differ at P<0.05..... 153

FIG. 6.7. Characterisation of a mESC line from an embryo cultured with insulin at passage 10. Scale bar is 50µm. A; Alkaline phosphatase activity: red staining indicates alkaline phosphatase activity; B; SSEA1 staining: green indicates cells positive for the cell surface marker SSEA1; C; Definitive endoderm staining: green is CXCR4, cell surface marker of definitive endoderm and mesoderm; red is sox17, nuclear marker of endoderm (primitive and definitive) – co expression shows definitive endoderm; D; Mesoderm staining: green is CXCR4, cell surface marker of mesoderm and definitive endoderm; red is VEGFR11, cell surface marker of mesoendoderm and mesoderm – co expression shows mesoderm; E; Neuroectoderm staining: green is Nestin, cytoskeletal marker of neuroectoderm; F; Normal male mouse karyotype of cell line. 156

FIG. 7.1. Summary of main findings of thesis. D4, D5 and D6 are day 4, day 5 and day 6 respectively. Pluripotent cells are shown in green, differentiated cells are shown in red..... 164

FIG. 7.2 Summary of the culture effects investigated in this thesis and their observed effect on the retention of pluripotency towards ESC derivation. 170

FIG. 8.1 Differential stain of a blastocyst. ICM nuclei stained blue, trophectoderm nuclei stained pink. Scale bar is 50µM 174

TABLE 1.1A. EFFECT OF GROWTH FACTORS ON MOUSE EMBRYONIC CHARACTERISTICS	42
Table 1.1B. EFFECT OF GROWTH FACTORS ON MOUSE EMBRYONIC CHARACTERISTICS	43
TABLE 1.2. MATERNAL REPRODUCTIVE TRACT AND EMBRYO EXPRESSION OF GROWTH FACTORS AND THEIR RECEPTORS IN THE MOUSE AND HUMAN	45
TABLE 2.1. MEDIA FORMULATIONS	57
TABLE 2.2. MARKERS OF THE DIFFERENT CELL TYPES OF THE DEVELOPED BLASTOCYST	63
TABLE 2.3. DETAILS OF PRIMERS USED FOR GENE EXPRESSION ANALYSIS OF INNER CELL MASS OUTGROWTHS	71
TABLE 3.1. EXPERIMENTAL CULTURE SYSTEM	87
TABLE 3.2. THE EFFECT OF CULTURE SYSTEMS ON DEVELOPMENT OF THE BLASTOCYST AT 96h	89
TABLE 3.3. THE EFFECT OF CULTURE SYSTEMS ON DEVELOPMENT OF THE BLASTOCYST AT 115h	89
TABLE 3.4. THE EFFECT OF DIFFERENT CULTURE SYSTEMS ON THE PRESENCE OF NANOG IN THE BLASTOCYST	91

TABLE 4.1 EXPERIMENTAL CULTURE SYSTEM	100
TABLE 4.2. THE EFFECT OF INSULIN ON DEVELOPMENT OF THE BLASTOCYST AT 96h	102
TABLE 4.3. THE EFFECT OF INSULIN ON DEVELOPMENT OF THE BLASTOCYST AT 115h	102
TABLE 5.1. DEVELOPMENTAL MORPHOLOGY AFTER 96 HOURS OF PI3K INHIBITION	117
TABLE 5.2. DEVELOPMENTAL MORPHOLOGY AFTER 115 HOURS OF PI3K INHIBITION	117
TABLE 5.3. DEVELOPMENTAL MORPHOLOGY after 96 HOURS OF GSK3 ACTIVATION	120
TABLE 5.4. DEVELOPMENTAL MORPHOLOGY AFTER 115 HOURS OF GSK3 ACTIVATION	120
TABLE 5.5. DEVELOPMENTAL MORPHOLOGY AFTER 96 HOURS OF GSK3 INHIBITION	123
TABLE 5.6. DEVELOPMENTAL MORPHOLOGY AFTER 115 HOURS OF GSK3 INHIBITION	123
TABLE 5.6. DEVELOPMENTAL MORPHOLOGY after 96 HOURS OF p53 ACTIVATION	126
TABLE 5.7. DEVELOPMENTAL MORPHOLOGY AFTER 115 HOURS OF p53 ACTIVATION	126
TABLE 5.8. DEVELOPMENTAL MORPHOLOGY AFTER 96 HOURS OF p53 INHIBITION	128
TABLE 5.9. DEVELOPMENTAL MORPHOLOGY after 115 HOURS OF p53 INHIBITION	128
TABLE 5.10. DEVELOPMENTAL MORPHOLOGY AFTER 96 HOURS OF BOTH GSK3 and P53 INHIBITION	130
TABLE 5.11. DEVELOPMENTAL MORPHOLOGY AFTER 115 HOURS OF BOTH GSK3 and P53 INHIBITION	130
TABLE 6.1. PRESENCE OF EPIBLAST CELLS IN OUTGROWTHS PLATED AT DIFFERENT TIMES ...	149
TABLE 6.2 EFFICIENCY OF PRIMARY EMBRYONIC STEM CELL COLONY GENERATION	150
TABLE 6.3 EFFECT SIZES FOR THE PREDICTIVE POTENTIAL OF MORPHOLOGICAL MARKERS FOR ESC GENERATION	154

Abstract

Human embryos for embryonic stem cell (ESC) derivation have often been cryopreserved for 5-10 years prior to their donation for research purposes. Many of these embryos will have been cultured in media conditions now known to be perturbing to embryo viability and which support only low levels of blastocyst development, necessitating that cleavage stage transfers be utilised for the majority of IVF cycles performed. As such, embryos for hESC derivation are often donated at the cleavage stage and require further culture to the blastocyst stage before hESC derivation can be attempted. These embryos are normally of poor quality and the efficiency of hESC derivation is low. This thesis investigated the hypothesis that the culture of cleavage stage embryos to the blastocyst stage represents a window of opportunity during which embryo culture conditions can be optimised to produce blastocysts with a greater potential to give rise to ESCs.

Using a mouse model it was demonstrated that the culture of embryos in simple medium, which models the aforementioned historic conditions, perturbs their development and reduces the number of ESC progenitor epiblast cells within the blastocyst. Furthermore, the transfer of embryos exposed to simple medium during the cleavage stage in a modern complex medium purpose designed to support embryo development from the 8-cell stage was insufficient to restore these embryos, despite improving epiblast cell number somewhat. As such, it was shown that additional interventions are necessary to fully utilise the 8-cell to blastocyst period of culture.

The growth factor insulin, despite having previously been shown to increase inner cell mass (ICM) cell number and improve embryo viability, is not routinely included in the majority of embryo culture media commercially available for the culture of human embryos. It was demonstrated in this thesis that supplementation of culture medium from the 8-cell to blastocyst stage with 1.7pM insulin is able to increase the epiblast cell number (as shown by OCT4 and Nanog co-expression) as well as the proportion of the ICM which is made up of epiblast cells. The molecular mechanism of this effect was investigated using small molecule inhibitors, and it was shown that insulin increased epiblast cell number via the activation of phosphoinositide-3-kinase, which subsequently inactivates glycogen synthase kinase 3 and p53, which, when active, inhibit the transcription of pluripotency supporting transcription factor *Nanog* through direct and indirect means.

Culture in the presence of insulin was shown to increase the number of OCT4 and Nanog positive cells in blastocysts on days 4 and five as well as day 6. However, OCT4 and Nanog co-expression was only restricted to the epiblast on day 6. Prior culture of embryos with insulin had no effect on the number of epiblast cells in outgrowths when blastocysts were plated on days 4 or 5. However, when blastocysts were plated on day 6 blastocysts which had been cultured with insulin from the cleavage stage gave rise to outgrowths with more epiblast cells compared with blastocysts cultured in control conditions. Efficiency of attachment and the percent of outgrowths which contained an epiblast were also improved by prior culture with insulin for blastocysts plated on day 6. When blastocysts cultured in control conditions were plated day 6 they were shown to give rise to outgrowths with increased numbers of epiblast cells compared with day 4 and day 5; demonstrating that, as with humans, the optimal time for plating mouse blastocysts is after lineage restriction has occurred.

The culture of embryos from the cleavage stage to the blastocyst stage in the presence of insulin was validated as a strategy for improving their capacity to give rise to ESCs by generating primary ESC colonies from day 6 plated outgrowths and confirming their pluripotency by OCT4 and Nanog staining. Embryos cultured with insulin had a two fold increase in their probability of successfully giving rise to an ESC colony. As embryos were cultured individually embryo morphological development was able to be tracked and compared to ESC generation success. Interestingly, which markers most successfully predicted ESC generation success differed for control and insulin cultured embryos. The most predictive morphological marker of future ESC generation was cavitation on day 4 for blastocysts cultured in control conditions, while for blastocysts cultured with insulin the most predictive marker was being hatched on day 6. The capacity of the model system used to support the derivation of a genuine ESC line was validated by generating a line from a blastocyst cultured in the presence of insulin and characterising it for pluripotency and self renewal by directed differentiation and karyotyping after multiple passages.

These results show that culture of embryos from the cleavage stage with insulin to day 6 increases the epiblast cell number of blastocysts, a property which is conserved through the outgrowth stage and results in an increased capacity to give rise to ESCs which can be serially passaged without losing their pluripotency or self renewal. As such, culture of embryos with insulin may represent a potentially useful strategy to exploit the opportunity created by the donation of human embryos at the cleavage stage for hESC derivation.

Declaration

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

I give consent to this copy of my thesis when deposited in the University Library being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published work contained within this thesis (as listed on the following page) resides with the copyright holder(s) of these works.

I also give permission for the digital version of the thesis to be made available on the web, via the University's digital research registry, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted to the University to restrict access for a period of time.

September 2012

Publications arising from thesis to date

Data presented in Chapter 3.0 have previously been published as follows:

Campbell JM, M Mitchell, MB Nottle and M Lane. (2011). Development of a mouse model for studying the effect of embryo culture on embryonic stem cell derivation. *Stem Cells Dev* 20:1577-86.

Data presented in Chapter 4.0 and Chapter 5.0 have previously been published as follows:

Campbell JM, MB Nottle, I Vassiliev, M Mitchell and M Lane. (2012) Insulin increases epiblast cell number of in vitro cultured mouse embryos via the PI3K/GSK3/p53 pathway. *Stem Cells and Dev* 21:2430-41

Data presented in Chapter 6.0 have been published as follows:

Campbell JM, M Lane, I Vassiliev, and MB Nottle. (2012) Epiblast cell number and primary embryonic stem cell colony generation are increased by culture of cleavage stage embryos in insulin. *J Reprod Dev*

Acknowledgements

'I don't know if it's good, but in hardback it could be used to stun a burglar' Neil Gaiman

I would first like to thank my primary supervisor, Dr Michelle Lane. Michelle, it's been a privilege to work with you all these years during which I have learned so much about being a scientist. You have never failed to impress me with your seemingly endless energy and passion for research. Thank you for all your help, guidance and for setting such a glowing example.

Secondly I would like to thank my co-supervisors A/Prof Mark Nottle and Dr Ivan Vassiliev. Mark, you've been an unfailing point of stability throughout my project, and your tireless efforts in reviewing my writings have not gone unappreciated. Ivan, thanks for showing me the ropes of embryonic stem cell biology!

I would also like to thank all of the post doctoral researchers who have offered me guidance throughout my project; Megan Mitchell, Sean O'Leary, Svetlana Vassilieva, Tod Fullston, Luke Beebe, Maria Teague and Sharon Harrison. The diversity of your perspectives has been enormously useful.

Thanks are also due to all my friends and family; thanks for your support, thanks for your encouragement and thanks for making sure I eat! You've all helped to make a very hard period much more pleasant.

Finally, very special thanks to my wonderful girlfriend Annamaria Quaresima. Your unending support, sympathy and patience have been a blessing. Thank you for listening to me rant when times have been tough, I know that having to deal with your own PhD is hard enough without having to listen to all the problems with someone else's!

Common Abbreviations

AP	Alkaline Phosphatase
ART	Assisted Reproduction Technology
Ct	CT99021
Dapi	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulphur oxide
eCG	Equine Chorionic Gonadotropin
ESC	Embryonic Stem Cell
FCS	Fetal Calf Serum
FGF	Fibroblast Growth Factor
GSK3	Glycogen Synthase Kinase 3
hCG	Human Chorionic Gonadotropin
HSA	Human Serum Albumin
ICM	Inner Cell Mass
ICSI	Intracytoplasmic Sperm Injection
ip	Intraperitoneal
iPSCs	Induced Pluripotent Stem Cells
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
IVF	In Vitro Fertilisation
LIF	Leukaemia Inhibitory Factor
LSD	Least Significant Difference
MAPK	Mitogen Activated Protein Kinase
MDM2	Murine Double Minute 2
MEF	Mouse Embryonic Fibroblasts

NEAA	Non Essential Amino Acid
Nic	Nicotinamide
OCT4	Octamer 4
PBS	Phosphate Buffered Saline
PDK1	Phosphoinositide Dependent Kinase 1
PE	Primitive Endoderm
Pft- α	Pifithrin- α
PI3K	Phosphoinositide 3-kinase
RT	Room Temperature
SH2	Src Homology 2
SIRT	Silent Mating Type Information Regulation 2 Homolog
SSEA	Stage Specific Embryonic Antigen
TCN	Total Cell Number

1.0 Literature review

1.1 Introduction

Since human embryonic stem cells (hESCs) were first derived in 1998 by the Thomson laboratory [1] work has been ongoing to improve the quality of lines, as well as the efficiency of derivation. Maximising the derivation efficiency of hESC lines is a matter of practical and ethical necessity due to the relative scarcity of human embryos available for hESC derivation, as well as the controversial nature of their use for research. To date, the majority of this effort has focused on improving the conditions of initial derivation and subsequent culture i.e. optimising ESC culture medium and culture surface. In comparison little attention has been paid to the embryos from which the hESCs are derived. These are typically embryos at the precompaction stage, created by assisted reproduction technologies (ART), which have been donated due to the limitations many governments place on the time that embryos can be stored [2,3] following culture in differing conditions and prolonged cryopreservation [4], or fresh cultured embryos identified as possessing such poor morphology during culture that they possess no reasonable chance of reproductive success [5,6]. Embryos from either source are generally of poor or reduced quality, which is disadvantageous for ESC research as ESC derivation efficiency is significantly improved by the use of high quality embryos [7].

As mentioned, these embryos are typically donated at the precompaction stage. In the case of cryopreserved embryos this is often because the culture systems used at the time of their initial generation did not support development to the blastocyst stage [8,9], while for rejected embryos this is likely the result of developmental retardation [5,6]. However, hESCs are primarily derived from blastocyst stage embryos, which necessitates that embryos donated for hESC derivation be cultured for a period prior to the derivation attempt, to allow them to develop to the suitable stage. I propose that this period of culture could be utilised as a window of opportunity during which embryo culture could be improved, with the aim of obtaining better quality blastocysts with greater capacity to give rise to hESC lines of a good standard. This outcome would be of benefit to the fields of drug discovery, toxicology, developmental biology and regenerative medicine where hESCs hold great promise to affect significant advances.

1.2 Embryonic stem cells

1.2.1 Definition of an embryonic stem cell

The blastocyst is the final stage of preimplantation embryonic development. It consists of an epithelial monolayer of cells which form an outer sphere, the trophectoderm, with a fluid filled blastocyst cavity and an inner cell mass (ICM), with the ICM containing two subpopulations, the primitive endoderm and the epiblast.

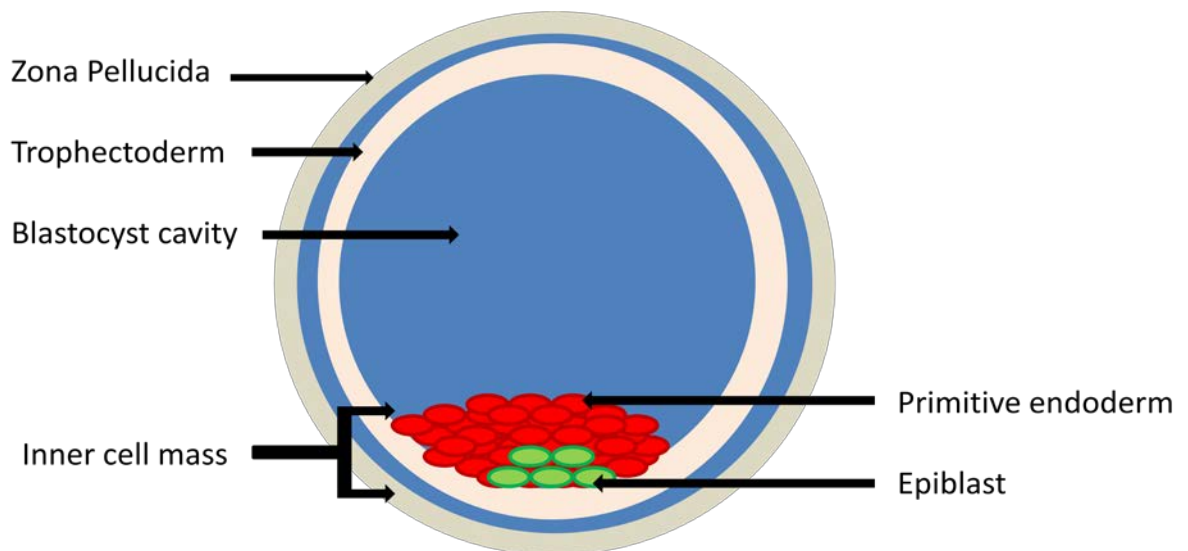


FIG. 1.1. Schematic of a blastocyst; the zona pellucida is a glycoprotein membrane that surrounds the blastocyst, the trophectoderm is a monolayer of cells that forms a sphere around the fluid filled blastocyst cavity and the ICM, the ICM is a group of cells attached to the inner surface of the trophectoderm which is made up of primitive endoderm and epiblast cells.

If the blastocyst implants trophectoderm cells give rise to the diploid trophoblast of the placenta as well as the endoreduplicative trophoblast giant cells [10], primitive endoderm cells give rise to visceral and parietal endoderm of the yolk sack [11], while epiblast cells give rise to the fetus as well as extra-embryonic tissues such as the amnion and extra embryonic mesoderm and ectoderm [12,13]. It is these epiblast cells that can be cultured *in vitro* as embryonic stem cells (ESCs) [14-16].

ESCs are defined by their ability to differentiate to form all cell types of the adult body; a property termed pluripotency (section 1.2.1.1), as well as the theoretical ability to undergo an unlimited number of cell divisions without changing state through differentiation or the acquisition of karyotypic abnormalities; a property termed self renewal [17] (section 1.2.1.2)

The history of pluripotent stem cells begins with embryonic carcinoma cells which are isolated from teratocarcinomas, which are tumours that contain a mixture of cells from the three germ layers (mesoderm, endoderm and ectoderm). These pluripotent cells were discovered in the mouse in 1964 [18], but being cancer stem cells and possessing abnormal karyotypes [19] their therapeutic use was limited. Embryonic carcinoma cells were of use for studying developmental biology as well as toxicology and drug discovery studies, however, again due to their abnormal karyotypes, their use as a model was limited. In 1981 the first ESC lines were derived from the mouse when Evans and Kaufman showed that cells with a similar morphology to embryonic carcinoma cells could be cultured from the ICM cells of blastocysts produced from the mating of 129SvE mice [16]. These cells were maintained for over thirty passages, could differentiate to form embryoid bodies and, if injected into immunodeficient mice, could differentiate to form teratomas [16]. The first non-human primate ESC line was established in 1995 from a rhesus monkey blastocyst. It was able to be passaged for more than a year without losing expression of markers of pluripotency or acquiring an abnormal karyotype, and formed teratomas when injected into immunodeficient mice [20]. In 1998 the first hESC lines were established from blastocysts produced from fresh or frozen embryos donated at the cleavage stage after production by ART [1]. These lines were maintained in culture for 4-5 months, expressed markers of pluripotency and could be differentiated to the three germ layers as evidenced by teratoma and embryoid body formation [1].

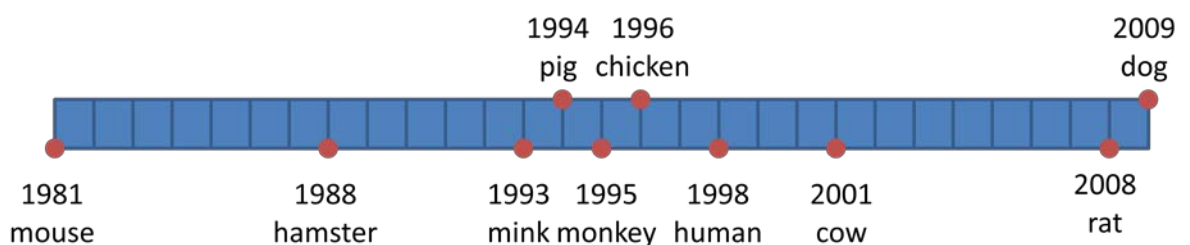


FIG 1.2. Timeline depicting the first derivation of ESCs from various species. By species sources were mouse [16], hamster [21], mink [22], pig [23], monkey [20], chicken [24], human [1], cow [25], rat [26], and canine [27].

1.2.1.1 *Pluripotency*

Pluripotency is an easily compromised cell state requiring a multitude of factors to maintain, which allows ESCs to give rise to any cell type found in the adult body. Pluripotent cell types are distinguished from other cell types by characteristics such as a high ratio of nucleus to cytoplasm, prominent nucleoli, and forming colonies with a smooth morphology [28,29]. The loss of this morphology indicates that a cell line has begun differentiating and is not pluripotent. However, the possession of this morphology is not a guarantee of pluripotency.

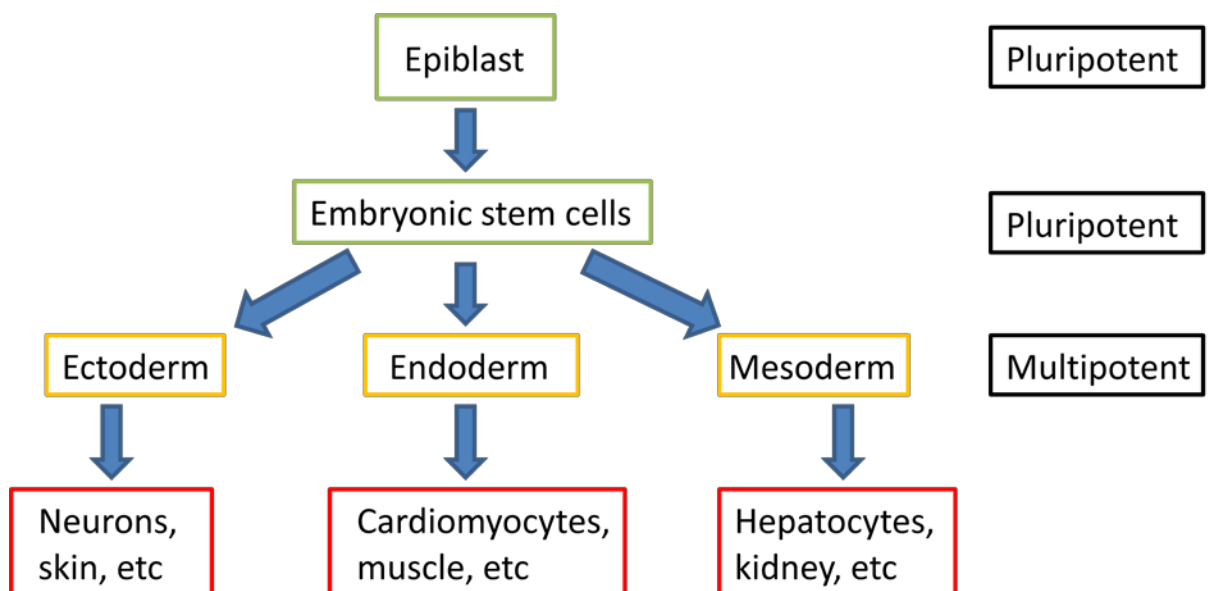


FIG. 1.3. Pluripotent epiblast cells can be cultured and retain their pluripotency as ESCs, which can differentiate to the three cell types of the germ layer, ectoderm, endoderm and mesoderm, and give rise to all cell types found in the adult body

Pluripotent cells express a range of molecular markers that are unique to undifferentiated cells. Amongst the most commonly used of these markers are the stage-specific embryonic antigen (SSEA) family of cell surface markers. SSEAs are cell surface glycolipid antigens with

glycosaminoglycan carbohydrate core structures which were discovered and characterised in embryonic carcinoma cells but have the same patterns of expression in ESCs [30-33]. However, there are species specific differences in the expression of these markers; for example, mouse and human ESCs express SSEAs in opposing ways. Mouse ESCs (mESCs) express SSEA1 and not SSEA3 or 4 while pluripotent, but switch expression upon differentiating, while hESCs express SSEA3 and 4 and not SSEA1 while pluripotent, but switch expression when they differentiate [30,33-37]. In the preimplantation mouse blastocyst the ICM and trophectoderm express SSEA1, but not SSEA3 or 4. While in the human blastocyst the ICM expresses SSEA3 and 4 and not SSEA1, while the trophectoderm expresses SSEA1 but not SSEA3 or 4 [30].

Interestingly hESCs have several characteristics in common with a class of mouse pluripotent stem cells termed epiblast stem cells (EpiSCs). EpiSCs are derived from post-implantation mouse embryos at day 5.5-7.5. Although EpiSCs express Oct4, Nanog and Sox2 and are able to form teratomas when injected into immunocompromised mice, EpiSCs do not contribute efficiently to chimeras when injected into blastocysts. EpiSCs, like hESCs have flat colony morphology, are dependent on bFGF, MAPK and TGF β /Activin/Nodal pathway activity for self renewal and are independent of LIF [38]. As a result mESCs are categorised as naive pluripotent stem cells, while hESCs and EpiSCs are categorised as primed pluripotent stem cells [39].

Two key markers of pluripotency are nuclear transcription factors OCT4 and Nanog, which are expressed in the same pattern in the mouse as human. OCT4 (octamer binding transcription factor) is a POU (PIT/OCT/UNC) homeodomain transcription factor encoded by the gene *Pou5f1*. OCT4 homodimers bind to palindromic Oct regulation elements – extensively characterised regulatory sequences located in many promoters and enhancers– on target genes to cis activate expression [40-42]. OCT4 was first identified in extracts of embryonic carcinoma cells and ESCs as a new protein with octamer binding activity [43-45]. OCT4 is known to activate genes required to maintain ESC self renewal and pluripotency, as well as repress genes whose expression is necessary for differentiation [46-48]. OCT4 is expressed in the ICM, ESCs, and embryonic carcinoma cells [49-51], – which are all pluripotent or contain pluripotent cells. The deletion of *Oct4* by the repression of *Oct4* coding transgenes in an ESC cell line in which *Pou5f1* has been deleted results in cell differentiation [52], however, the over expression of *Oct4* by the induction of *Oct4* coding transgenes in ESC lines in which *Pou5f1* has not been deleted, results in differentiation to a cell lineage expressing trophectoderm cell markers [52]. Further, OCT4 is known to be an activator of *Nanog* transcription in ESCs by

forming heterodimers with molecules including SOX2 [46] and β -CATENIN [53], thereby forming a genetic regulatory network for pluripotency.

Nanog is a divergent homeodomain protein [54], it has three domains with the homeodomain between the 96 amino acids of the serine rich N-terminal and the 150 amino acids of the C-terminal [17]. Nanog was functionally identified by two groups as important for pluripotency and ESC self renewal [17]. In [55] *Nanog* was identified as specifically expressed in mouse ESCs and the preimplantation embryo, and resulting in the differentiation of ESCs to extra-embryonic endoderm when deleted. While in [56], Nanog was shown to be present in all mouse and human ESC lines, and that when over expressed by insertion of an inducible *Nanog* transgene in mouse ESCs it was sufficient to maintain ESCs in an undifferentiated state independently of the LIF/STAT3 signalling pathway, which is normally necessary for undifferentiated mESCs to proliferate [57]. In human cells more than 400 promoter regions have been identified as bound by both OCT4 and Nanog [48], and common downstream targets of the products of the genes affected by OCT4 and Nanog prevent ESC differentiation [58]. As such, evidence suggests the two factors (along with a third, SOX2 [48]) form a regulatory system involving auto regulatory and feed forward loops that support the pluripotent ESC state.

For many years it was believed that Nanog was an absolute requirement for ESCs to maintain their pluripotent state due to the differentiation observed upon its deletion or knock down [59]. However, it has now been shown that ESCs in which *Nanog* has been deleted can continue proliferation in an undifferentiated state, albeit with an increased susceptibility to differentiation, while maintaining the capacity to contribute to all somatic cell types [59]. This was done using ESCs in which a *Nanog* transgene with inducible deletion was the only source of *Nanog* due to prior deletion of wildtype *Nanog* genes via homologous recombination. It has also been shown that even within robustly propagating ESC lines there are Nanog negative cells present [59,60]. These Nanog negative cells retain OCT4 expression and are phenotypically indistinguishable from Nanog positive cells [59,60]. Interestingly, Nanog negative cells are capable of regaining Nanog expression over time, and while the mechanism of the recovery of Nanog expression is not known, the process is mirrored *in vivo* when Nanog is re-expressed at the posterior of the egg cylinder [61] following down regulation in the embryo at implantation [56].

Induced pluripotent stem cells (iPSCs) are somatic cells which have been reprogrammed back to pluripotency. These are created by the transfection of the somatic cells with four select

transcription factors. *Oct4* is one of these transcription factors, however to the surprise of many *Nanog* is not (the remaining factors are *Sox2*, *C-Myc* and *Klf4*) [60]. In (section 1.3.5) iPSCs are examined in greater detail. It appears that the loss of *Nanog* creates a differentiation permissive state and that its expression protects against spontaneous differentiation. Therefore while *Nanog* is not a key factor in the maintenance of pluripotency it can be considered a practical necessity in most situations, demonstrating an element of redundancy in the regulatory network of pluripotency.

While a lack of OCT4 and *Nanog* expression serves well to exclude candidate lines as being pluripotent, expression of these markers does not provide definitive proof. ESCs which are pluripotent are capable of differentiation to form all cell types under a variety of conditions both *in vivo* and *in vitro*.

1.2.1.1.1 *In vitro* differentiation

It is common to differentiate ESCs to the 3 germ layers; ectoderm, endoderm and mesoderm to demonstrate the line's capacity to generate the different cell types, providing evidence of pluripotency. *In vitro*, pluripotent ESCs are differentiated by two key methods; directed differentiation or spontaneous differentiation from embryoid body formation. During directed differentiation the pluripotent cells are cultured under different conditions to induce differentiation to a specific cell fate such as neuroectoderm [62,63], definitive endoderm [64,65] or mesoderm [64]. If ESCs are able to differentiate to cells from the three germ layers it is evidence that they can differentiate to any cell type found in the adult body.

Additionally, cells can be differentiated by embryoid body formation. With this method ESCs are cultured in suspension, which causes them to aggregate, form spheres and spontaneously differentiate to form embryoid bodies will contain a mixture of cells representative of the three germ layers. Using Embryoid bodies contain a wide variety of cell types which gives a broader picture of the line's capacity to differentiate than directed differentiation. As a result embryoid body formation can be used as an early model of germline development [74], which is discussed in greater detail (section 1.3.3).

1.2.1.1.2 *In vivo differentiation*

To examine the capacity of cells to differentiate to any adult cell type *in vivo*, as a key determination of pluripotency, either teratoma formation or chimera generation (the gold standard for pluripotency) can be used. Teratomas, like teratocarcinomas, are tumors that contain a mixture of cell types derived from the three germ layers. However, unlike teratocarcinomas they are not malignant. This assay involves injecting the ESCs cells into an immunodeficient animal, and examining any resultant tumors to determine if cells from the three germ layers have formed [66-68]. Chimeras are animals which incorporate cells from two or more different animals. These are made by injecting the ESCs into a host blastocyst where they can be incorporated into the ICM which develops into the fetus and ultimately a mature animal [67]. This is made possible by the similarities between ESCs and the epiblast cells of the ICM from which the fetus is derived (the epiblast is discussed in detail in section 1.5). Generally morphological differences between animals (such as coat colour) can be used to determine whether chimerism has occurred as well as its extent. Definitive proof of pluripotency is obtained if mating the chimeric animal results in an offspring descended from the introduced cells. This property is termed germline transmission and is extremely useful for the generation of transgenic animals. For ethical reasons chimeras cannot be made from hESCs.

1.2.1.2 *Self renewal*

An essential characteristic of ESCs is the capacity for self-renewal which is the property of undergoing proliferation without losing pluripotency or changing karyotype [69,70]. Self renewal is therefore assessed by passaging the cell line, which is the mechanism where cell colonies are reduced to either to cell clumps or single cells then replated so that they will grow, then examining it for pluripotency as discussed above (section 1.2.1.1), as well as assessing karyotype by cytogenetic imaging [69-71]. To have “self renewal” a cell line should be able to be passaged an indefinite number of times. However, as this is an impossible property to demonstrate, researchers will specify an arbitrary number of passages, usually in the range of seven [72,73] to twenty [74,75], before testing for pluripotency.

A further marker of self renewal is telomerase activity [1,69,70,76]. The level of telomerase activity is important to self renewal as telomerase is a ribonucleotide protein that adds telomere repeats (TTAGGG) to the end of chromosomes in order to maintain telomere length after replication. As such, telomerase and is vital for maintaining replicative lifespan [77,78]. As chromosome shortening leads to a loss of self renewal, high levels of telomerase activity correlates with self renewal in ESCs [79], making it an informative marker..

1.2.2 Derivation of an ESC line

As discussed above (section 1.2.1) ESCs are derived from the epiblast cells of the ICM of the blastocyst. There are several methods in use for isolating these cells for the derivation of ESC lines, but in general, isolation can be accomplished in two ways; attachment and outgrowth, or immunosurgery. In obtaining the ICM by attachment and outgrowth the usual method is to culture the blastocyst on a mitotically inactivated fibroblast feeder layer. The fibroblasts condition the medium which supports the embryonic cells, and provide an extracellular matrix on which the trophectoderm is able to attach and spread, leaving the ICM exposed as a mass of cells known as the outgrowth (Fig. 1.4) [80].



FIG. 1.4. Brightfield image of a mouse outgrowth attached to a gelatin coated dish. The black arrow indicates the ICM outgrowth, surrounding cells are trophectoderm. Scale bar is 50 μ m.

In the human however, it is considered difficult to visualize the ICM, this makes the next step, removing the outgrowth by careful pipetting, technically challenging and introduces the likelihood that trophectoderm will be removed also, introducing contaminating cells [67]. This difficulty can be overcome by using the second method, immunosurgery. In this method the ICM is directly isolated by incubating the blastocyst in the presence of an antibody, then transferring the blastocyst to complement which binds to the antibody on the outer trophectoderm cells, resulting in the destruction of the trophectoderm around the ICM, which will then be plated to allow it grow to an outgrowth [67].

Whichever method of isolation is used, once the outgrowth is obtained it is usually trypsinised to disaggregate the cells. This disaggregation gives each cell of the ICM the opportunity to form a clonal colony, with epiblast cells having the potential to give rise to ESC colonies (Fig. 1.5).

FIG. 1.5. Diagram of the derivation of an ESC line from a blastocyst. Pluripotent epiblast/ESCs are shown in green, differentiated primitive endoderm cells are shown in red: The blastocyst is outgrown to isolate ICM cells, the outgrowth is then collected and trypsinised to disaggregate its cells so that when they are replated the epiblast cells are able to form clonal primary ESC colonies, which in turn can be collected, disaggregated, and propagated to an ESC line.

For this to be possible, however, several factors must be present in order to support ESC derivation and growth. These factors differ slightly between the mouse and human. In both cases an extracellular matrix must be provided in order for the cell lines to proliferate, and cell lines from both mouse and human can be maintained on MEFs [67]. Activation of the LIF/STAT3 signalling pathway with the growth factor leukaemia inhibitory factor (LIF), whether via media supplementation or MEF secretion, is necessary to support undifferentiated growth of mESCs, with BMP4 acting synergistically with LIF to support self renewal [81]. However, LIF does not support undifferentiated growth in hESCs as it does mESCs [82] and BMP4, causes hESCs to differentiate [83].

An additional source of human embryos for hESC derivation are embryos which have been identified as carriers of genetic disorders by preimplantation genetic diagnosis (PGD). PGD is performed at the 8-cell or blastocyst stage when a single cell biopsy is taken from the embryo [84]. The blastomere is then analyzed using fluorescence in situ hybridization (FISH) which

allows the analysis of the chromosomes. If an abnormality is found the embryo is unsuitable for reproductive purposes and is either discarded or donated for research. If donated for research the embryo can be used for hESC derivation. The hESC lines derived from PGD embryos can be very useful as if they carry the mutation which was identified during PGD (which is not guaranteed [85]), they can be used to model the genetic disorder including during the in vitro trialing of potential therapies, and may provide new insights on developmentally regulated events. Additionally, it has been shown that the single cell biopsy can be cultured prior to FISH analysis as such the PGD process has the potential to allow the derivation of genetically normal hESCs, without necessitating the destruction of a human embryo [86].

If successfully derived and cultured, however, ESCs have numerous potential applications.

1.3 Applications of embryonic stem cells

The fields of toxicity study, drug development, developmental biology and regenerative medicine are the main areas where hESCs can be put to practical use [87-89]. However, lines used must be easily and reliably expandable, have similar epigenetic status (no imprinting errors or changes in differentiation status), not have been exposed to animal cells or other products, and possess similar characteristics for successful outcomes to be obtained [87-89].

1.3.1 Toxicity

One of the most problematic stages in bringing a drug to market is when it enters human testing. Many years and much money will have been spent in ensuring that there is the minimum probability of the drug having an unintended toxic side effect when it is administered to humans. However, extensive animal testing has historically proven to be insufficient to ensure that potential therapies do not have a harmful effect on human subjects, with 30% of medicines entering clinical trials being eliminated due to safety concerns, while an additional 30% are removed due to lack of efficacy [90]. What this shows is that the interspecies variation that exists between humans and the animal models used in pre-clinical trials is often too great to produce sufficiently reliable and accurate results. As such, it is

desirable that human cells be exposed to potential medical products *in vitro*, prior to *in vivo* testing [91]. As well as reducing financial and ethical burdens by eliminating ineffective and harmful treatments prior to clinical trials, unnecessary animal trials could also be prevented by early elimination of candidate treatments. Of particular note, the testing of cosmetic products and ingredients on animals will be banned in the EU by 2013 (as will be the marketing of products so tested), creating within the industry a pressing need for *in vitro* tests which can better approximate the human situation [92]. Currently, non ESC human cells are used for the *in vitro* testing of medicines, however, they are either primary cells obtained by taking samples from patients, or transformed cells derived from tumors or otherwise immortalised. Unfortunately primary cells have only limited expandability, may not be harvestable in sufficient quantities, are difficult to standardise and not all cell types are obtainable, and although transformed cells have unlimited expandability, their abnormal genetic status can cause them to react in ways not representative of the system they are intended to model [93,94]. These are significant problems and can prevent primary and transformed cell lines performing as satisfactory models [93,94].

Due to hESC's proliferative potential they are a bountiful source of *in vitro* human material which researchers could use to assess toxicity. Furthermore, due to their pluripotency the effect of the drugs on specific cell types can be examined. Additionally, disease specific cell lines can be created from cells obtained during PGD (prenatal genetic diagnosis) [95] or cells which have been genetically modified to carry the disease of interest [96]. As such, hESCs represent a far more efficient and ethical way of carrying out *in vitro* human toxicity trials.

A specific area where the utilisation of hESCs has been identified as being likely to improve testing is the EST (Embryonic Stem cell Test) which currently evaluates a drug's cytotoxicity and the effect it has on the differentiation of mESCs induced to form embryoid bodies [97] to functional contractile cardiomyocytes. This test aims to evaluate a compound's embryotoxicity using the formation of embryoid bodies from mESCs as a model for embryogenesis. This test has been effective, though not perfect, in identifying embryotoxic compounds –with 100% efficiency for detecting strong embryotoxic compounds and 70% efficiency for weakly embryotoxic compounds out of a panel of 20 compounds [98]. Documented interspecies variations in reaction to some drugs tested [98,99] likely reduce the efficacy of the test, which increases the risk when drugs are later trialled in humans *in vivo*. As such, work has been begun which aims to establish protocols which will allow this test to be performed routinely using hESCs [100,101]. Another area which hESCs have shown significant promise is the

creation of skin derivatives [102,103] which could be used to predict effects such as sensitization, irritation and genotoxicity [88].

Challenges exist, however, for hESCs to be employed to maximum effect in toxicity screening. Existing hESC lines vary widely with regards to quality and characteristics, as such with some lines testing may not produce consistent and useful results (section 1.4.1). This creates an ongoing need for the derivation of more hESCs from high quality embryos, utilising best principle practices to create lines which will ensure reproducibility, relevance and acceptance of tests [88,92,104].

1.3.2 Drug discovery

For the purpose of drug discovery, hESCs have the potential to enable high throughput *in vitro* screening of small-molecule libraries with high human relevance by providing an unlimited consistent source of human cells of any type [89,105]. This is a significant advantage over the human cells currently available –transformed and primary cells – which are inadequate for the process; transformed cells will often have lost important aspects of their native function – such as the inability of many immortalised pancreatic beta cells to respond to physiological levels of insulin [106] due to permanent changes to their phenotype, and primary cells are difficult to standardise and/or obtain in sufficient quantities as cells must be derived fresh for each trial and can be difficult and/or dangerous to obtain. While mESCs can be and are used in high throughput studies [107], trans-species differences which exist between mouse and human, such as differences in telomere damage signalling [108], have the potential to produce significant confounding effects.

High throughput screening using hESCs which possess a disease specific background, such as Lesch-Nyhan syndrome (created via homologous recombination) [96] or Fragile-X syndrome (created from embryos with the disease genotype) [109], could allow researchers to search for compounds which act on the disease phenotype. To date, no drug screens have been performed using disease specific hESCs [110]. Research is limited by the difficulty of recapitulating disease phenotypes in cultured cells, as well as the small number of diseases for which appropriate lines exist [110]. As such, when embryos with the appropriate backgrounds (as indicated by PGD) become available for hESC derivation, it is critical that they be utilised with maximum efficiency.

Additionally, it has been recognised that natural wound healing occurs via adult stem cells that exist within the body, and that high throughput screens can be used to search for compounds which affect the proliferation or differentiation of these cells, in order to identify novel therapeutic strategies [89]. In cases where a drug has already been identified, hESCs can be used to optimise the drug by screening an array of modified versions of the molecule in structure-activity relationship studies designed to improve properties such as potency and specificity [111,112]. Alternatively, where a good candidate drug has already been found hESCs can be used to examine its effectiveness and dosage on human cells.

Use of hESCs has significant promise to improve the speed of drug discovery while reducing the likelihood that initially promising compounds will prove to be unsuitable due to a lack of action in the human system, potentially dramatically reducing the cost to market of new drugs. Discovering a new drug and bringing it to market is currently estimated to cost around \$900 million US dollars and take 10-15 years [90]. Pharmaceutical companies have already declared their intentions to use hESCs for drug screening [89]. Several studies have provided proof of concept. In one, 1040 compounds were screened using a hESC based assay to identify molecules with an effect on hESC survival and differentiation, resulting in the identification of 17 compounds which promoted differentiation and 5 that promoted survival [107]. Currently high throughput screening using hESCs are generally an attempt to identify compounds that promote survival, self renewal or differentiation [105], but could be expanded to search for therapeutic compounds.

Currently, the divergent nature and quality of different hESC lines (section 1.4.1) confounds the applicability of tests which themselves will require standardisation to improve reproducibility, relevance and acceptance [52,64,65]. This makes the derivation of new hESC lines utilising best practice techniques a necessity. As human embryos are available for hESC derivation in limited numbers, it will be important that they be utilised with the minimum possible wastage.

1.3.3 Developmental biology

ESCs facilitate the study of developmental biology because they allow the *in vitro* replication of differentiation events, which would otherwise only be observed during fetal development *in vivo*. This is of particular value for the study of human developmental biology, where ethical

considerations prevent *in vivo* experimentation. When pluripotent hESCs are induced to a fully differentiated adult cell lineage, observation and study of the process can yield important fundamental knowledge with regards to the cellular mechanism of human development. Examples hESCs being used for the study of human developmental biology are the characterisation of Polycomb group proteins, including OCT4 (section 1.2.1.1) as targeting genes responsible for the maintenance of pluripotency in [113], and the process of hESC directed differentiation (section 1.2.1.1.1) to functional hepatic cells in [114].

Furthermore, when ESCs are used to form embryoid bodies, this can be used as an early model of germline development where the first differentiation events are observed [115]. Treatments can be applied during this process to affect the proportions at which different cell types are formed, to investigate the mechanisms by which the process is controlled. For instance, in [116] hESCs with a constitutively expressed Nodal transgene formed embryoid bodies which consisted of an outer layer of visceral endoderm-like cells and an inner layer of epiblast-like cells, suggesting an inhibitory role for Nodal in differentiation.

Additionally, as discussed above (section 1.3.2) hESCs can be generated from cells known to carry a mutation in order to create *in vitro* models of the disease, which can be used to study its effect on early development with the aim of identifying the mechanism which results in the disease phenotype [95,117], current examples of this are Huntington's disease [118] and cystic fibrosis [117]. Alternatively, hESC lines can be subjected to transgenesis via gene insertion, deletion or mutation, to create undifferentiated cells with a wide variety of disease genotypes to enable similar experiments, or others which study genes whose mutation produces no known clinically relevant phenotype, but are nonetheless of interest to developmental biologists. Examples include the insertion of nonisogenic DNA into the *HPRT* gene via homologous recombination to mutate hESCs to create a model of Lesch-Nyhan syndrome [96], and the retroviral insertion of *HOXB4* into hESCs to cause the gene's overexpression and investigate its influence on haematopoiesis at the earliest stages of development [119].

1.3.4 Regenerative medicine

Although to date there have been no clinical outcomes, the greatest interest shown to hESCs is due to their potential application in the field of regenerative medicine. Regenerative medicine can succinctly be defined as "regenerative medicine replaces or regenerates human cells,

tissue or organs, to restore or establish normal function.” [120]. While adult stem cells have wide spread use in regenerative medicine, with haematopoietic stem cell transplantation for the treatment of diseases including severe combined immunodeficiency [121] and sickle cell anaemia [122], being the most common examples [123,124], the potential of hESCs has so far been largely unrealised. The property of self renewal means hESCs can be expanded *in vitro* to generate the high quantities of identical cells necessary for cell therapies, and as they are pluripotent they can be directed to differentiate into large numbers of a specific cell types. Consequently hESCs are of immense potential value for the treatment of a large number of different disease types.

Currently, application of this therapeutic regime is limited by our incomplete knowledge of how to effect complete differentiation *in vitro*, as such hESC’s ability to proliferate could result in teratocarcinoma formation, and the ability to differentiate could result in undesirable cell types being generated *in vivo*. Furthermore, hESCs suffer from the same problems as all other donor tissues and cells – immune rejection. Even where a suitable match could be found, the patient would have to take immunosuppressants. However, despite these issues, research into the clinical utility of hESCs continues, and has yielded numerous promising results. For example: cardiomyocytes have been derived from hESCs and injected into damaged heart muscle in rats, resulting in an improvement in health [125]. Epithelial cells derived from hESCs and injected into nude mice were able to increase blood oxygen levels following the induction of a model of pulmonary fibrosis by inhalation of silica, demonstrating restoration of lung function [126]. Dopaminergic neurons derived from hESCs have been transplanted to rat models of Parkinson’s disease, and partially ameliorated the symptoms [127]. Finally, injection of hESCs differentiated to oligodendrocyte progenitor cells into a rat model of spinal cord injury results in improved motor function [128]. This last study is especially interesting as its results were part of the basis by which the first clinical trial to be approved by the food and drug administration of the USA that utilises cells derived from hESCs was launched by the biotechnology company Geron Corp [129].

Furthermore, one of the key current challenges is that cells to be introduced to a human must have been cultured in an entirely xeno-free environment. Culture of hESCs on animal cells or in the presence of other animal products (such as serum) can result in the hESCs acquiring the expression of xeno-antigens, as shown in [130], where human cells cultured on MEFs were shown to have mouse cell surface antigens on their plasma membrane. Additionally, use of animal products during culture of human cells for transplantation creates the risk of the

transmission of animal pathogens to the patient. Currently, work to develop alternative xeno-free culture systems [66,131] has generated reports that such conditions reduce cell line quality [132], and many existing lines which have been exposed to animal products are inappropriate for clinical use. As such, there is an ongoing need for the derivation of new hESCs as advances are made that allow the derivation of uncompromised hESC lines.

1.3.5 Comparison of embryonic stem cells and induced pluripotent stem cells

To date ESCs are the gold standard of pluripotent cells [133], being the most thoroughly characterised and worked with pluripotent cell type in regards to the applications discussed above (sections 1.3.1, 1.3.2, 1.3.3, and 1.3.4). However, iPSCs, discussed (section 1.2.1.1) can be created in a patient and disease specific manner from any tissue; giving them an apparent advantage over ESCs, which by definition cannot be created from a patient's own cells, and are derived from material which is extremely limited (human embryos). However, for iPSCs to directly replace ESCs in these areas, they must be demonstrated to be ESCs equivalents, and evidence suggests they are not. Numerous studies in both mouse and human have shown iPSCs to exist in an altered epigenetic state compared with ESCs [134-137]. These differences have been shown to be due to both epigenetic memory of somatic cell lineage, such as iPSCs created from pancreatic β -cells maintaining an open chromatin structure at key β -cell genes and differentiating more readily to insulin producing cells [138], as well as iPSC specific differences in DNA methylation which exist independent of cell lineage [139]. These differences can have an effect on the pluripotency of the cell lines. In [134] dermal fibroblast derived iPSCs were demonstrated to retain DNA methylation at the *Nanog* gene, resulting in reduced *Nanog* transcription. Differences in DNA methylation status between iPSCs and ESCs have been demonstrated to be retained through differentiation [136], and although continuous passaging has been shown to attenuate the differences between iPSCs and ESCs by reducing aberrant DNA methylation, numerous aberrant sites are retained and iPSCs do not become identical to ESCs [137]. These differences have prompted calls for caution in the development of iPSCs for regenerative medicine [133] and have prevented the immediate application of iPSCs to toxicity, drug discovery and developmental biology studies due to the demonstrated propensity of iPSCs to respond to differentiation protocols differently to ESCs

[134,135,138]. As such, iPSCs cannot replace ESCs in most studies, and the derivation of new hESCs remains a research priority.

ESCs can also be derived from parthenotes. Parthenogenesis is the growth and development of embryos that have not been fertilised by a male, and can be induced in vitro in human embryos. Methods to parthogenetically activate mammalian oocytes include electrical stimulation, exposure to Ca^{2+} - Mg^{2+} free medium, exposure to medium containing hyaluronidase, exposure to ethanol, exposure to Ca^{2+} ionophores or chelators and exposure to protein synthesis inhibitors [140]. Human ESCs can be derived from parthenotes - making parthnenogenetic ESCs (pESCs) - bypassing the ethical concerns of destroying a valid human embryo (parthenotes cannot develop into a viable fetus), and creating pluripotent stem cell lines with reduced cell immunogenicity (HLA homozygous pESCs can be made which are histocompatible with significant proportions of the population [141]. However, concerns exist due to pESCs potential for aberrant genomic imprinting and paternal gene expression which, if human pESCs were used for regenerative medicine, could result in pathologies including cancer [142].

1.4 Current limitations of embryonic stem cells

Above I have discussed the potential applications and benefits of hESCs, however, a recurring theme is that there are too few lines of the necessary quality – easily expandable, no epigenetic perturbations, reproducible behaviour between lines, no exposure to xenogenic products, and that there is therefore an ongoing need to derive new ESCs.

1.4.1 Cell line quality

Many hESC lines cannot be passaged enzymatically (as opposed to mechanically) either stably or at all [76,143-146], making their expansion to generate enough cells for use impractical. Furthermore, hESCs grow at a slow rate compared with mESCs (with a population doubling time of 36h compared to 12h [147] with some lines growing more slowly than others [148]. Cell lines will often spontaneously differentiate after prolonged (20-60) passages or lose their normal karyotype thus limiting their expandability and interfering with the development of standardised reproducible tests for toxicity testing and drug discovery, as well as the

generation of the high numbers of hESCs necessary for regenerative medicine [69,132,149-151].

Further, the quality of hESC lines can vary widely from line to line likely as a result of perturbations during embryo culture and hESC derivation. A direct outcome of this is that different lines can respond differently to *in vitro* differentiation protocols, both directed, as shown by [148] where different hESC lines subjected to the same neural directed differentiation protocol responded with different efficiencies of differentiation, and spontaneous, as shown by [132,148] where hESCs induced to form embryoid bodies under the same conditions did so with different efficiencies [132] and produced embryoid bodies with different growth rates [148] and patterns of gene expression [132,148]. Furthermore, teratomas formed by injection of different hESC lines into immunodeficient mice have different structures and patterns of differentiation [149,152]. Application of hESCs to toxicology, drug screening and regenerative medicine is made significantly more complicated by the fact that lines respond differently to differentiation inducing stimuli. Cultured hESCs adapt to the conditions to which they are exposed. Having been derived and cultured in different conditions means that they will have consequently undergone different adaptations to culture [150,153], likely resulting in the differing characteristics discussed above.

These differences in response to induction of differentiation can be explained by hESC lines having undergone some degree of partial differentiation. This is supported by studies which have shown that different hESC lines express variable levels of gene expression for markers of pluripotency such as *OCT4* [148,154], *Nanog* [148,154] and undifferentiated transcription factor 1(*UTF1*) [148] and have different colony morphologies i.e. differences in density of cell compaction and border definition [148]. Additionally, some lines are more difficult to maintain in an undifferentiated state due to an increased tendency to differentiate [148], which suggests an existing level of differentiation. Furthermore, some female lines show X chromosome inactivation, while others do not [152]; a characteristic which has been linked to increased likelihood of spontaneous differentiation in mESCs, which suggests different lines have already undergone some level of differentiation as an adaptation to culture [155,156]. Other hESC characteristics, such as gene expression [76,148,152,154,157] and epigenetic differences [152] have been shown to vary between lines with no apparent effect on their “stemness”.

Variation between hESC lines is by all accounts widespread and results in the reduction of many lines' capacity to proliferate without differentiation and then respond predictably to

standardised methods of differentiation. This impairs the application of hESCs to the fields of toxicity, drug and developmental studies as well as regenerative medicine (section 1.3). One factor which is likely responsible are the different conditions in which hESCs have been derived and cultured. Another potential source of variability is the initial quality of the embryos from which hESC lines are derived – many of which will have been perturbed by the culture conditions to which they were exposed prior to donation (discussed further in section 1.5.2). As such, it will be important to maximise the quality of embryos used for future hESC derivation.

1.4.2 Comparison of mouse and human ESCs

More hESC lines must be derived for them to be put to the uses discussed in (section 1.3), however, the destruction of human embryos for their derivation is a controversial practice and only limited numbers of human embryos are available; as such, they must be used as efficiently as possible.

hESCs divide more slowly [158] and are usually more difficult to passage [143] than mESCs. Additionally, the derivation rate reported for mESCs (38-66%) [159-161] is generally higher than the derivation rate reported for hESCs (1-7%) [73,162]. It may be that a contributing factor to this observation is the difference in how the embryos are generated between the species; human embryos for hESC derivation are created and cultured *in vitro* as a result of ART, while mouse embryos for mESC derivation are primarily created from blastocysts that are developed *in vivo*. In [160] it was shown in the mouse that embryos which develop *in vivo* give rise to mESCs with greater efficiency compared with *in vitro* cultured embryos. Furthermore, *in vitro* cultured embryos are well established as having lower reproductive viability compared to *in vivo* embryos [163]. Additionally, embryos donated for hESC derivation are often of reduced quality [5,6,73,162,164] for two main reasons. Firstly, when embryos are donated fresh without having been cryopreserved, in the majority of cases this will be because they are ART embryos of such low quality as to have no realistic reproductive potential, which consequently prompts couples to discard them [5,6]. Secondly, ART techniques often create more embryos than are required for transfer (especially in clinics which practice single embryo transfer to avoid twinning). These surplus embryos are cryopreserved for use in potential future transfers. However, for a variety of reasons these cryopreserved embryos may never be

needed and are donated for hESC derivation [73]. Many countries set an upper limit on the period that embryos can be cryopreserved, 5-10 years in Australia [2], 10 years in the United Kingdom [3] (cryopreservation in the USA can continue indefinitely [165,166]). A large number of embryos available for hESC derivation are donated when embryos are scheduled to be discarded. Consequently, many years may have passed between the culture of embryos and their donation. As a result of this many embryos donated for hESC derivation will have been cultured in a range of conditions which may contribute to differences in embryo development (section 1.5.1). Interestingly, in part due to the reduced quality of these embryos, they will often be made available during the cleavage stage of their development [167-169]. For fresh donated poor quality embryos this may be because one of the characteristics which marked them as lacking reproductive viability was retarded development, although many clinics do not culture embryos past the cleavage stage in any case [169,170]. For cryopreserved embryos this is likely to be because many of the culture systems used 10 years ago did not support *in vitro* development of human embryos to the blastocyst stage, resulting in routine transfer or cryopreservation during the pre-compaction stage [8,9,167,168]. Consequently, laboratories attempting hESC derivation must often culture cleavage stage human embryos to the blastocyst stage prior to attempting hESC derivation.

The relevance of this is that the efficiency of derivation of hESC lines is highly dependent on the quality of embryos used, with higher quality embryos giving rise to hESC lines at a greater rate [5,6,73]. Therefore, a key window of opportunity to improve hESC quality and derivation efficiency appears to be in improvements to the culture of the preimplantation embryo to improve the quality of the embryo and resultantly ameliorate the perturbations its cells have suffered and improve its ESC generation competence.

Further key differences exist between hESCs and mESCs including hESCs not requiring LIF to maintain pluripotency while mESCs do, hESCs expressing SSEA3 and 4 when pluripotent and switching to SSEA1 when differentiated while mESCs express SSEA1 when pluripotent and lose expression when differentiated, and hESCs forming flat colonies with individual cells being distinguishable while mESCs form rounded masses in which single cells are difficult to identify [171]. In part due to hESCs having much in common with mouse epiblast stem cells (section 1.2.1.1) they are considered more differentiated than mESCs and are termed “primed” stem cells whereas mESCs are considered to be “naive.” Due to these differences caution must be shown in extrapolating findings in mESCs to hESCs.

1.5 The preimplantation embryo

The preimplantation embryo is formed when the haploid oocyte is fertilised by the haploid spermatozoa forming the diploid zygote. The zygote, which is surrounded by a glycoprotein membrane, the zona pellucida, divides several times by a process called reductive cleavage, whereby each division results in smaller cells (termed blastomeres) without changing the overall total size of the embryo. Protein synthesis in the early zygote at first relies on maternal mRNAs as the embryonic genome is inactive. In the mouse genome activation begins between the 1-cell and 2-cell stage [172], while the human embryonic genome begins activation between the 4 and 8-cell stage [173]. In the mouse, after three rounds of cleavage the now 8-cell embryo usually begins the process of compaction. Until compaction blastomeres have very little intracellular adhesion, and require the zona pellucida to prevent dissociation. Compacting embryos increase their intracellular adhesion and flatten against one another, causing an increase in cell-cell contact and a loss of easily discernable cell-cell boundaries. In the human, compaction is more likely to occur around the 10 cell stage [174]. Prior to compaction blastomeres are non-polar. But, with the increase in cell adhesion all cells become polar with cell nuclei [175], endosomes [176], microtubules [177], microvilli [178], and membrane proteins [179-182] segregating apically or basolaterally.

The compacted embryo, or morula, undergoes further cleavage divisions until it reaches the 32-cell stage. From the zygote to the 8-cell stage all blastomeres are outside cells with areas of their cell surface having no contact with any other cells. However, by the 32-cell stage there are a significant number of inside cells which are completely engulfed by the other cells of the embryo. The polar cells of the compacted embryo can divide one of two ways: horizontally i.e. perpendicular to its axis of polarity which will produce two new outside cells, or vertically which will produce an inside and an outside cell. If a blastomere cleaves horizontally then polarity is conserved and the two daughter cells will be essentially the same, however, if the blastomere divides vertically then the outside cell will retain polarity, but the inside cell will be apolar [183,184]. As such, at the 32-cell stage two populations of cells exist, polar outside cells and apolar inside cells. Which population a cell belongs to will determine the cell fate it acquires on the first differentiation event that occurs in the embryo, however, at this point all cells are still totipotent as a cell which is artificially moved, whether inside to outside or vice versa, will change its polarity and its cell fate [185-187].

The next stage in the development of the preimplantation embryo is cavitation. This occurs when water begins to enter the embryo as a result of the accumulated of Na^+ on the inside of the embryo by Na^+/K^+ ATPases [188,189]. The influx of water to the embryo causes a fluid filled cavity, termed the blastocoel, to begin to form within the embryo. The blastocoel is supported by the maintenance of this osmotic gradient as well as the formation of tight junctions which prevent water leakage [190,191]. At this point the embryo is termed a blastocyst and the outside cells have differentiated to trophoctoderm and the inside cells are the ICM. Blastocyst cells will begin to herniate from the zona pellucida, in a process termed hatching, eventually escaping it entirely, creating a hatched blastocyst (Fig. 1.6).

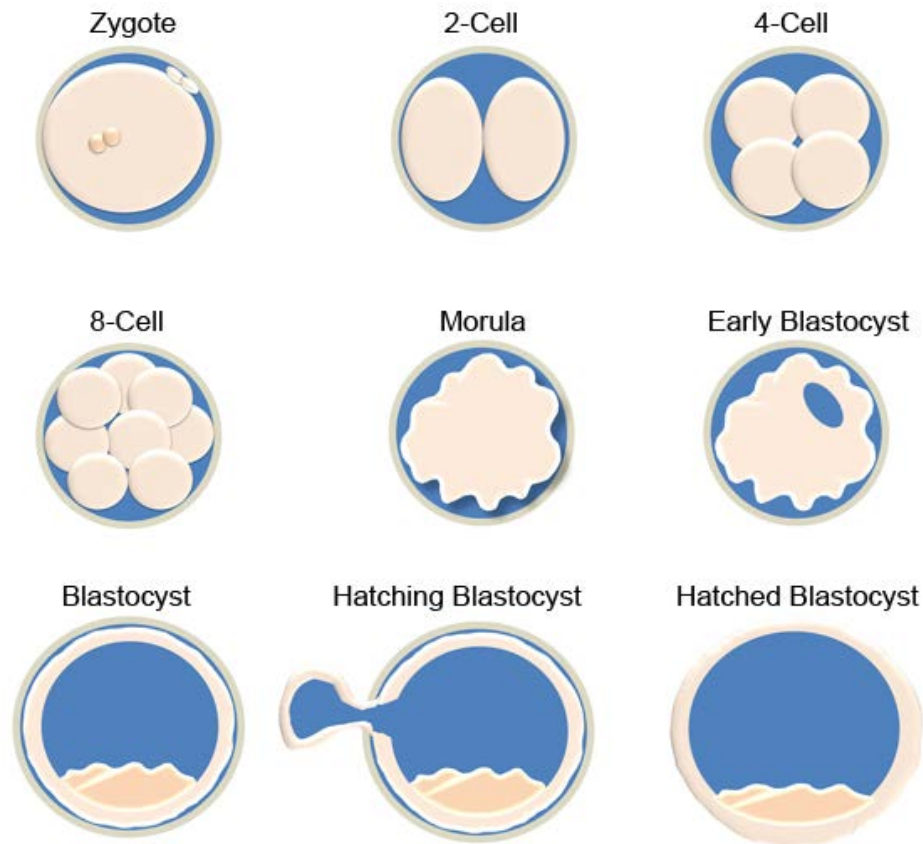


FIG. 1.6. Diagram depicting the morphological stages of embryo development from fertilisation to the hatched blastocyst stage. The zygote is formed by the fertilisation of the oocyte by the spermatozoa. The embryo then begins to divide by reductive cleavage creating the 2-cell, 4-cell and then 8-cell embryo. After this compaction begins to occur (8-cell for mouse, 10-cell for human) and cells polarise and flatten to maximise cell contacts, creating the morula. Fluid is secreted internally to form the blastocoel and the early blastocyst. The blastocoel continues to expand to form the blastocyst which can begin to herniate from the zona pellucida by hatching, ultimately creating the hatched blastocyst.

ICM cells are marked by OCT4 and Nanog, [192,193] , whose relationship with pluripotency was discussed above (section 1.2.1.1), while trophectoderm cells can be distinguished by expression of the transcription factor caudal type homeobox 2 (CDX2), which is required for trophectoderm development [192-195]. OCT4 and Nanog restriction – to the ICM and epiblast, respectively – begins after cavitation [55,196] (and after CDX2 restriction to

trophectoderm [192,196]), and occurs relatively slowly with trophoctoderm cells positive for Nanog being observable long after cavitation has been completed [192] for *in vitro* and *in vivo* cultured embryos.

At this point the blastocyst possesses no totipotent cells. Trophoctoderm cells will ultimately form the fetal contribution to the placenta, while the ICM, which must undergo further preimplantation differentiation, is pluripotent and will form the fetus as well as extra-embryonic tissue such as the yolk sack [12,197].

Whether an ICM cell ultimately contributes to the fetus or extra-embryonic tissue is determined by whether it differentiates to form primitive endoderm or epiblast. In the mouse, these two cell types can be distinguished by their expression of transcription factors. While all ICM cells express OCT4 [198], primitive endoderm specification is controlled by the transcription factor GATA6 [192], whose over expression in ESCs causes differentiation to primitive endoderm [199]. The epiblast is marked by the transcription factor Nanog [55,198] (Fig 1.7).

As the blastocyst begins to cavitate the cells of the ICM will not have differentiated to one lineage or the other and will co-express Nanog and GATA6 [200]. However, as ICM differentiation continues Nanog and GATA6 expression becomes mutually exclusive, with almost no cells co-expressing both factors [192,200]. At this point, in the 32-64 cell early blastocyst (*in vivo* developed), cell fate has largely been determined [200]; GATA6 positive cells will give rise to primitive endoderm, and Nanog positive cells will give rise to epiblast, while a few blastomeres will retain the capacity to give rise to either cell fate [198,201]. Interestingly, although in the late *in vivo* blastocyst primitive endoderm and epiblast are segregated, a cell's position within the ICM does not control whether it gives rise to primitive endoderm or epiblast [192,200,201]. Cells express Nanog and GATA6, seemingly at random in a mixed, 'salt and pepper' pattern [192]. This is followed by the primitive endoderm cells moving to the surface of the ICM, while epiblast cells move from the outside to the inside, resulting in the formation of a monolayer of primitive endoderm over the top of the epiblast [200,201]. Studies report that primitive endoderm cells which are unable to move to the outside surface of the ICM undergo apoptosis [200].

The late *in vivo* blastocyst, immediately before implantation, consists of three cell types, the outer layer of CDX2 positive trophoctoderm cells, the morphologically distinct ICM monolayer of GATA6 positive primitive endoderm cells and the Nanog positive epiblast cells [200]. Above

I have discussed both OCT4 and Nanog as markers of pluripotency in ESCs (section 1.2.1.1). In the late blastocyst Nanog is restricted to the epiblast of the ICM except for a few Nanog positive cells of the trophoctoderm [192]. OCT4, however, is expressed in all cells of the ICM, but no cells of the trophoctoderm [193]. As such the expression of the two factors, OCT4 and Nanog, can be used to distinguish, primitive endoderm (OCT4 positive, Nanog negative), epiblast (OCT4 positive, Nanog positive) and trophoctoderm (OCT4 negative).

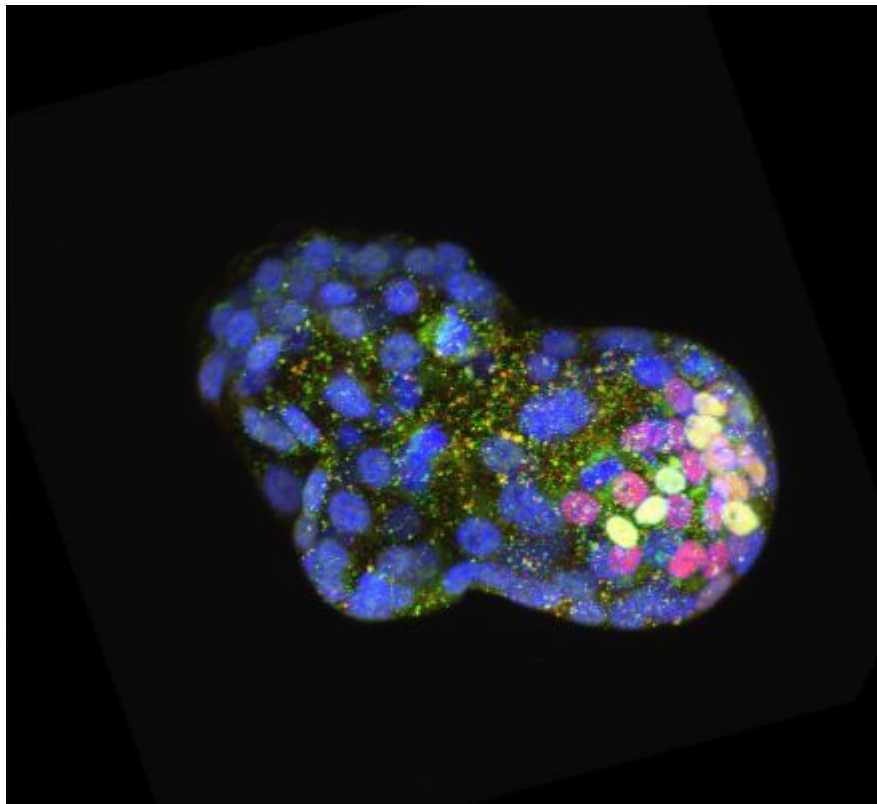


FIG. 1.7. A hatching blastocyst, immunocytochemically stained to differentiate epiblast, primitive endoderm and trophoctoderm nuclei, imaged by confocal microscopy and reconstructed as a 3D image using Z-stack. Yellow nuclei are epiblast cells, they have been stained for OCT4 (red), which is restricted to the ICM and Nanog (green), which is restricted to the epiblast (and occasional trophoctoderm cells), they have also been counter stained with Dapi (blue). Primitive endoderm cells are stained pink (red+blue), as they do not express Nanog, while trophoctoderm cells are stained blue as they do not express OCT4, and in this case none express Nanog.

The above discussion focuses almost solely on the development of the mouse preimplantation embryo, specifically *in vivo* development. This is because although the morphological development of the human embryo is well characterised as a result of observations of human embryos cultured *in vitro* for ART, the study of the molecular mechanics of the lineage segregation of the primitive endoderm and epiblast, as well as the trophoctoderm, has been restricted by the relative scarcity of human material for studying, as well as ethical concerns. Although differences are known to exist between the embryos of the two species, such as CDX2 expression which is not expressed in human embryos until the blastocyst has already formed [202], the mouse remains a good model of human embryology [203] as it can be cultured under similar conditions, under goes the same pattern of morphological development, and the late blastocyst has the same trophoctoderm/primitive endoderm/epiblast structure [204] as the human embryo .

1.5.1 *In vitro* fertilisation

In vitro culture of preimplantation embryos was first achieved when *in vivo* fertilised 8-cell mouse embryos were grown to the blastocyst stage in 1956 [205]. Human embryos were first fertilised *in vitro* in 1969 [206], but it was not until 1978 that the first human, Louise Brown, was born as a result of *in vitro* fertilisation and culture [207]. Since then the *in vitro* fertilisation and culture of human embryos for reproductive purposes has continued such that currently 3.1% of all children born in Australia are a result of ART [208].

IVF technology in general involves a women's ovaries being hyper stimulated to produce multiple follicles by injection of follicle stimulating hormone (FSH). Oocyte maturation *in vivo* is induced by human chorionic gonadotropin (hCG). Oocytes are aspirated 32-36h after injection of hCG [209] with a needle that, guided by ultrasound, pierces the vaginal wall and aspirates the follicles. Cumulus-enclosed oocyte complexes are collected from the follicular fluid. Spermatozoa are typically collected either via ejaculation or by surgical retrieval. Oocytes are either fertilized by co-incubation with sperm overnight or by injecting a single spermatozoa directly into the oocyte via intracytoplasmic sperm injection (ICSI) to achieve fertilisation; reasons for utilising this technique can include especially low sperm motility or numbers.

If fertilisation is successful a zygote is created. This embryo is cultured *in vitro* usually for either 1-6 days discussed below (section 1.5.2) prior to transfer back to the mother to allow implantation to occur.

1.5.2 Embryo Culture

Historically *in vitro* culture of mammalian embryos has been performed in either simple salt solutions (such as Earle's, T6 and HTF [210-212]) or complex tissue culture media (such as Ham's F-10, MEM or TCM-199 [213,214]) [215-218]. Simple media systems were typically composed of balanced salt solutions supplemented with the women's own serum (or fetal cord serum) and with the carbohydrates glucose, pyruvate and lactate [203,211,212,219]. The tissue culture media used were normally developed to support the growth of immortal cell lines, and were supplemented with carbohydrates, amino acids, vitamins, nucleotides and metal ions [213,214]. As knowledge of the requirements of the preimplantation embryo has increased, it has been discovered that components of these culture systems, such as high glucose, divalent metal ions and some growth factors [203], can be harmful to the development and viability of embryos, and specialised embryo culture media systems have been developed which attempt to minimise the perturbation of *in vitro* cultured embryos and preserve reproductive viability.

These culture media usually contain carbohydrates, amino acids, vitamins, chelators, and macromolecules provided in gradients via the utilisation of sequential media systems. Additionally, as part of the culture system specific oxygen tension, temperature, pH and osmolality must be maintained, to minimise perturbation of embryos. Interestingly, as the embryo develops its requirements change with its morphology, as such, conditions which are beneficial at one stage can be harmful at another, creating challenges not encountered with most forms of cell culture. Below is a discussion of the different components of *in vitro* embryo culture systems, and how they relate to present attempts at hESC derivation.

1.5.2.1 Carbohydrates

The early embryo divides repeatedly, but by reductive cleavage. As such it achieves no actual growth. Furthermore, until the embryonic genome is activated the embryo predominantly utilises maternal mRNA, resulting in little metabolic or biosynthetic activity [220-222]. The first cleavage division, from zygote to 2-cell can only be supported by pyruvate [223]. However over the course of the reductive cleavage stage both pyruvate and lactate, which have been shown to be necessary for the 2-cell embryo to reach the blastocyst stage (either suffices, but the presence of both improves yield) [224,225], are the primary metabolic substrates.

Glucose is taken up at low levels during reductive cleavage [226,227], but despite this, high concentrations of glucose have been shown to cause the developmental retardation and arrest of cleavage stage embryos when included in culture media at this stage [228,229].

However, as the embryo compacts metabolic and biosynthetic activity increase greatly, while at genomic activation, discussed above (section 1.5), the embryo's preference in metabolic substrate shifts and glucose becomes the nutrient of choice [226,227], as a result a lack of glucose post-compaction reduces blastocyst development and viability [230,231]. Interestingly, there is a difference in the way ICM and trophoctoderm cells metabolise glucose. ICM cells almost exclusively utilise glycolysis and have very low levels of oxidative metabolism, while trophoctoderm cells primarily metabolise glucose via oxidative metabolism [232,233]. The results of [230] show that the absence of glucose perturbs the ICM, as demonstrated by increased fetal loss after implantation. As ESCs are derived from the epiblast of the ICM, this suggests that the presence of glucose in post-compaction culture medium is likely to be necessary to generate blastocysts able to efficiently give rise to high quality ESC lines.

The changing metabolic requirements of the developing embryo can be predicted by the environment it would be exposed to if it was developing *in vivo*. At the pre-compaction stage the embryo is still within the oviduct and is immersed in an environment with a high concentration of pyruvate and lactate, while the concentration of glucose is relatively low. In the uterus, which the embryo enters later in its development, the concentration of glucose is high, while the concentrations of pyruvate and lactate are lower [234-237].

Such changes in embryo metabolic requirements mean that culture media which support pre-compaction embryo development can be inappropriate for later development, and vice versa. Exposure of embryos to sub-optimal concentrations of carbohydrates can have significant

detrimental effects such as retarded embryo development and fetal loss [228-230]. Little, however, is known of the effect that the carbohydrate concentrations of historic embryo culture medium may have on ESC derivation, an issue of current relevance due to likely delay between embryo culture and donation for ESC derivation.

1.5.2.2 *Amino acids*

Amino acids are known to fulfil various roles in the culture of the preimplantation embryo. As well as serving as biosynthetic precursors [238] specific amino acids can be metabolised for energy [239]. Further, amino acids can act as osmolytes [240] and are able to regulate intracellular pH [241], as well as act as chelators [242], anti-oxidants [243], and regulate metabolism and differentiation [244].

The absolute necessity of amino acids for the healthy development of embryos is shown by the reduced development and blastocyst cell number in embryos with even brief exposure to medium lacking amino acids [230]. That inclusion of amino acids in culture medium improves both blastocyst development and viability has been demonstrated in mouse [230,245-247] as well as human [248,249]. Other species where improvements to blastocyst development have been shown include rabbit [250], hamster [251], rat [252], ovine [253,254], porcine [255] and bovine [256,257]. This beneficial effect is only seen for non-essential amino acids (with glutamine). Initially, if essential amino acids are included in the culture medium of pre-compaction embryos then development is reduced [246]. However, if essential amino acids are added to culture media after compaction, they improve ICM development and blastocyst viability [247]. As such, for optimal results, embryos should be cultured from the zygote to compacting stage in medium containing non-essential amino acids, followed by culture with both non-essential and essential amino acids to the blastocyst stage. When this is done *in vitro*, cultured blastocysts can achieve an implantation rate equivalent to that of *in vivo* cultured embryos [258]. Interestingly, non essential amino acids are present at much higher levels in the oviduct than essential amino acids, which is in keeping with the demonstrated requirements of the precompaction embryo *in vitro*; this observation has been shown in mouse [259], rabbit [259-262], porcine [259], ovine [259], equine [263] and bovine [259].

In mESCs the essential amino acid threonine has been shown to promote self renewal and proliferation when included in culture medium [264,265]. Its inclusion in ESC culture media promotes the activation of the Akt/ PI3K (phosphoinositol-3-kinase) pathway which is known to promote pluripotency in ESCs [266], and increases *Oct4* and *Nanog* transcription [265]. Furthermore, the inhibition of threonine catabolism in mESCs via competitive inhibition with threonine analogue 3-hydroxynorvaline (which produces glycine and propionyl-CoA instead of glycine and acetyl-CoA when catabolised by threonine dehydrogenase [267]) was demonstrated to not only inhibit ESC growth and pluripotency, it also impaired blastocyst formation by preventing cavitation [264]. As such, the ICM cells of the embryo are critically dependent on threonine catabolism, therefore, as ESCs are derived from the pluripotent epiblast cells of the ICM, this suggests that the inclusion of threonine in post-compaction embryo culture medium may be beneficial for epiblast growth and the efficient generation of ESCs.

1.5.2.3 Vitamins

Culture of embryos *in vitro* has the potential to induce stress in the embryo, resulting in perturbation of glycolytic activity and oxidative capacity as well as reduced viability. The addition of vitamins to media has been shown to have a synergistic effect with amino acids in restoring *in vivo* levels of glycolysis, oxidation, and blastocyst viability [268]. Elevated glycolytic activity in *in vitro* cultured embryos has been shown to be associated with embryos failing to develop through the two cell block and reduced fetal development [258,269,270]. This synergy suggests that amino acids and vitamins regulate embryo viability and metabolism and prevent increases in glycolysis through different mechanisms. Complex tissue culture media which have in the past been used for the *in vitro* culture of preimplantation embryos contained the vitamin nicotinamide which has been shown to perturb the development of cleavage stage embryos and their subsequent viability [271]. One potential mechanism of this effect is that nicotinamide inhibits Sirtuin1 (SIRT1) [272], which, when active, has the function of deacetylating and inactivating the pro-apoptotic protein p53 [272], which also binds to the *Nanog* promoter region to prevent transcription [273]. The benefits of vitamins to embryo culture have been predominantly demonstrated post-compaction [268,274]. Although it has been shown that the inhibitory effects of high glucose discussed above (section 1.5.1.1) can be alleviated by the presence of vitamins [203].

As a lack of vitamins in embryo culture medium for the post-compaction embryo results in a loss of embryo metabolic control and developmental competence, it is important that post-compaction embryos cultured *in vitro* be maintained in the presence of vitamins, this is likely to be particularly vital if embryos were exposed to a perturbing environment during precompaction culture, as embryos donated for hESC derivation are likely to have been.

1.5.2.4 Chelators

Chelators are ligands which form two or more bonds with a single central metal atom. In a biological system this has the effect of preventing the metal ion interfering with cellular functions. The presence of metals in *in vitro* embryo culture medium is detrimental to development and can cause embryos to arrest at the cleavage stage [275]. This is believed to be due to the capacity of divalent metal ions to catalyse the Haber-Weis reaction which results in the generation of hydroxy free radicals (OH[•]) from the reactive oxygen species hydrogen peroxide (H₂O₂) [219,276]. The presence of free radicals results in oxidative stress, which damages embryos by causing peroxidation of membrane phospholipids and altering the bonds of lipids, proteins and nucleic acids [277,278]. This damage can result in mitochondrial alterations, adenosine triphosphate deletion, and apoptosis [279-281] and is well characterised as negatively affecting the development of the embryo *in vitro* [219,275,282,283]. *In vitro* cultured embryos have been shown to have increased production of reactive oxygen species [284]. Induction of oxidative stress in mESCs by exposure to H₂O₂ has been shown to impair proliferation [285], increase lipid peroxide formation [286], increase DNA fragmentation [286], induce cell arrest [285] and cause apoptosis [285]. However, the effect appears to be only transitory and to not affect pluripotency, as demonstrated by the removal of H₂O₂ resulting in the restoration of cellular proliferation and pluripotency markers – OCT4, Nanog, SOX2 and alkaline phosphatase activity – to the levels seen for control mESCs not exposed to H₂O₂ [285]. Further, ESCs exposed to H₂O₂ retained the capacity to form embryoid bodies [285].

Chelators can be used to minimise the damage to embryos by sequestering metal ions and preventing them catalysing the reactions which produce free radicals. A trial of different chelators found that the chelator EDTA (Ethylenediaminetetraacetic acid) was the most effective at promoting preimplantation development during the pre-compaction stage, where cleaving cells are prone to arrest [219]. Critically, in inbred mouse strains EDTA has been

shown to be an important component of media for overcoming the “two-cell block” [219,230,287]. Chelator activity has been demonstrated to exist in the oviductal fluid where precompaction embryos develop [288]. Interestingly, the beneficial effects of culture of embryos with EDTA are only seen during pre-compaction development, post-compaction the presence of EDTA becomes inhibitory and results in reduced ICM development and decreased viability [219,230,289]. It is hypothesised that this is a result of EDTA inhibiting the glycolytic pathway [287]. While precompaction embryos uptake and utilise little glucose [226,227], glycolysis is the main method of energy production in the ICM and is critical for development [232]. Further, the inhibition of the glycolytic pathway during the pre-compaction stage has been shown to be beneficial as it prevents premature utilisation of glucose and resultant metabolic perturbations [287]. Further, EDTA has been shown to inhibit the glycolytic enzyme 3-phosphoglycerate kinase which requires metal ions to catalyse its activity. As inhibition of this enzyme by a different inhibitor reproduced EDTA’s ability to overcome the 2-cell block, this suggests that inhibition of glycolysis is the primary mechanism by which EDTA stimulates cleavage [287]. Abnormally high rates of glycolysis, as discussed above (section 1.5.2.1), are known to cause reduced viability [258,269,270].

As such, while the inclusion of EDTA in pre-compaction embryo culture media is beneficial, with its absence resulting in extensive oxidative stress and impaired development, the culture of human embryos to the blastocyst stage after donation pre-compaction for hESC derivation should be undertaken in a culture medium without EDTA. Furthermore, the absence of EDTA is one of the stresses to which many embryos donated for hESC derivation at the cleavage stage may have been exposed.

1.5.2.5 *Macromolecules*

Although it is possible to culture human embryos in a protein free environment [290,291], *in vitro* embryo culture is most often undertaken in media supplemented with a source of protein. This is largely because protein acts as a surfactant and facilitates embryo manipulation *in vitro* [292] while also assisting in the chelation of toxins [293]. Additionally, it has been hypothesised that protein can act as a source of fixed nitrogen [294]. In the past the most commonly used protein source has been serum [203,217], usually the patient’s own serum, but also often fetal cord serum. While these protein sources are attractive by virtue of

their availability, significant drawbacks exist with their use. Human blood products must be tested for the presence of viruses, additionally collection and preparation of serum take considerable time and is expensive [217]. Furthermore, serum is undefined and highly variable; it contains metal ions and pyrogens [295] and has been shown to be detrimental to mammalian embryo development *in vitro*, as it can induce alterations in metabolism, ultrastructure, methylation of imprinted genes, and fetal outgrowth [296]. Furthermore, in the human, the presence of serum in *in vitro* culture medium has been shown to reduce fertilisation, embryo quality and pregnancy success rates [217].

This has led to the replacement of serum as a source of protein with human serum albumin (HSA). Although HSA can be highly variable [290,297,298], compared to serum it is comparatively pure, and its inclusion in culture medium significantly increases the rate of successful outcomes in clinical IVF [217]. Additionally, recombinant HSA is available which eliminates the problems associated with blood derived products and allows embryo culture media to be standardised [299].

Serum, once a standard component of *in vitro* embryo culture, has been shown to be perturbing to embryo development – as discussed above – with batch variation resulting in some lots being more perturbing than others. Importantly, *in vivo* cultured embryos are not exposed to serum [292]. Oviduct and uterine fluids are not comparable to serum; serum is a pathological fluid containing an uncontrolled host of growth factors and other factors shown to be undesirable (i.e. metal ions) to which embryos should never be exposed. However, although human embryos created by ART are now routinely cultured in serum albumin as opposed to serum [203], embryos donated for hESC derivation can have been cryopreserved for up to ten years, and are more likely to have been perturbed by exposure to this condition.

1.5.2.6 Oxygen tension

In vitro cultured embryos must be maintained in a highly controlled environment due to the sensitivity of preimplantation embryo cellular physiology to perturbation [300]. One element of this environment is the gas phase. Oxygen concentration both in oviduct and uterine fluid (approximately 8% in the oviduct, approximately 1.5% in the uterus [203]) is considerably lower than atmospheric oxygen (approximately 20%). Numerous studies have found that *in vitro* culture of preimplantation embryos at physiological concentrations of oxygen,

significantly improves embryo development rate and cell number and viability [230,301-304]. Culture of preimplantation embryos in the presence of high concentrations of oxygen is believed to perturb development due to increased generation of reactive oxygen species, resulting in increased generation of free radicals and oxidative damage. Reduction in oxygen tension has been shown to act cooperatively with chelators to improve embryo development [219]. Interestingly, despite the well characterised negative effects of oxidative damage on the preimplantation embryo, some studies have shown that the benefits of reduced oxygen tension appear to be predominantly evident when embryos are cultured to the blastocyst stage, as when development has been assessed pre-compaction, no difference has been observed [302]. Further, in human studies where embryos were transferred to patients at the cleavage stage reduced oxygen failed to increase pregnancy outcomes [305-308]. This has led to the hypotheses that low oxygen is not necessary for pre-compaction culture, or that damage by high oxygen is progressive and requires a long period to take effect. However, other studies show that transiently high oxygen at the beginning of culture has a significant negative effect on embryo development [230,309]. Interestingly, the oviduct, where the precompaction embryo resides, has a much higher concentration of oxygen than the uterus (8% compared to 1.7%) [203,310-312]. Furthermore, mouse blastocysts cultured in a low oxygen environment had larger ICMs [313], and were more likely to outgrow and produce primary mESC colonies which stained positive for the pluripotency marker alkaline phosphatase [314]. Low oxygen conditions support the long term proliferation of hESCs by safeguarding pluripotency and self renewal, as demonstrated by reduced rates of spontaneous differentiation, chromosomal abnormalities and apoptosis.[315-317]. As such, although reports are conflicting as to whether culture in high oxygen is likely to have perturbed embryos donated for hESC derivation pre-compaction, the available literature suggests culture in low oxygen to the blastocyst stage should improve blastocyst quality, with a concomitant increase in hESC derivation efficiency and quality.

1.5.2.7 pH

Another parameter of the *in vitro* embryo culture environment which must be closely controlled is the pH of the media [300]. The external pH of *in vitro* culture medium is the result of the CO₂ concentration of the gas phase of the incubator, which dissolves in the media and

produces carbonic acid, which reaches equilibrium with the dissolved bicarbonate buffer [300,318].

Intracellular pH is primarily regulated by the Na^+/H^+ antiporter, which regulates against acid loads, and the $\text{HCO}_3^-/\text{Cl}^-$ exchanger, which regulates against alkaline loads, in order to maintain an optimum pH [319-323]; typically 7.2-7.3 [319,323-325], which is important for cellular processes such as enzyme activity, protein synthesis, cell division and cell-cell communication [319,322].

Both the sodium dependent $\text{HCO}_3^-/\text{Cl}^-$ exchanger and the Na^+/H^+ antiporter are active in human embryos [326], but the post-compaction embryo is known to be less sensitive to extracellular pH due to the formation of tight junctions [241,327]. The recently fertilised zygote does not appear to have any means of regulating intracellular pH [325,328].

Culture of embryos in conditions which alter its intracellular pH by ≤ 0.3 pH units can irreversibly disrupt the localisation of mitochondrial and actin microfilaments [329], increase embryo glycolysis, decrease oxidative metabolism [327,330], and cause embryo developmental arrest or delay [327,329]. Short term exposure to reduced pH (<0.2 pH units) has been shown to significantly decrease ICM cell number, fetal weight as well as crown rump length, while longer term exposure decreased not only these factors but implantation as well [331]. These results demonstrate that exposure of embryos to conditions which alter intracellular pH can significantly perturb the ICM, and that the effect is still evident in epiblast descended cells (the fetus). As such, culture of embryos in conditions which alter embryo intracellular pH could reduce ESC derivation efficiency and have long term consequences on ESC quality.

It is essential, therefore, that the external pH of the embryo be maintained at a level which will not result in any alterations to internal pH. Further, it has been hypothesised that even when external pH is not sufficiently aberrant to result in any alterations in internal pH, the stress and resulting adaptations induced in the embryo as it works to maintain its internal pH could be detrimental to future development [318]. This is supported by the findings in [300] where it is shown that culture of embryos in a 6% CO_2 incubator as opposed to a 5% CO_2 incubator significantly improved blastocyst cell number as well as fetal implantation and development rates, despite a difference in external pH of just 0.1 units from 7.3 to 7.4. [300,318].

Recognition of the importance of pH to embryo health and viability as well as the narrow optimal range has been comparatively recent, with some early embryo culture media having

inadequate or no buffering systems [332,333]. Further, due to the vulnerability of the parameter to incubator maintenance and time period embryos are exposed to atmospheric CO₂, embryos are especially vulnerable to the standards, practices and competence of clinics and individuals. As such, many embryos donated for hESC derivation may have had their quality perturbed by culture in media with an aberrant pH.

1.5.2.8 Cryopreservation

Human embryos donated for hESC derivation will frequently have been subjected to a prolonged period of cryopreservation, which can have a significant impact on their quality. Two primary methods exist for the initial freezing of embryos which are to be cryopreserved; slow freezing and vitrification. In both cases frozen embryos are then stored in liquid nitrogen until thawing. Slow freezing utilises low concentrations of cryoprotectants and a slow rate of cooling to dehydrate the cell during freezing and prevent the formation of intracellular crystals [334-336]. These crystals would otherwise damage cellular membranes and organelles and are believed to cause developmental loss from cryopreservation. However, problems exist with slow freezing. Dehydrating the cell too much can increase the concentration of intracellular solutes to toxic levels, the time spent by the embryo at low temperatures before it freezes can cause chilling damage, ice crystals can form during thawing and the rapid influx of water can cause swelling [337,338]. Alternatively vitrification can be used. Vitrification utilises high concentrations of cryoprotectants that can be cooled rapidly, this prevents the formation of ice crystals and chilling damage [339]. Vitrification of human embryos, both at the cleavage and blastocysts stages, has been shown to result in significantly higher rates of cryopreservation survival, and embryos were more likely to implant and result in a live birth compared to slow frozen embryos [340]. Accordingly, vitrification is replacing slow freezing as the method of cryopreservation in many *in vitro* fertilisation clinics [341]. However, as cryopreserved embryos donated for hESC derivation will have often been frozen 5-10 years previously, their quality and capacity to give rise to hESC lines may have been compromised by use of the slow freeze method.

1.5.2.9 Sequential and Monoculture systems

A recurring theme of the above discussion is that pre-compaction embryos which are still dividing by reductive cleavage have different requirements from the media in which they are cultured compared to post-compaction embryos, with some factors which are beneficial at one stage being actively harmful in the other (e.g. essential amino acids, chelators). When embryos are transferred to the recipient or cryopreserved prior to compaction, which is how IVF was performed historically [218], this does not create an issue. However, culture of embryos to the blastocyst stage has been shown to be effective for identifying the highest quality embryos most likely to result in a pregnancy [342], and transfer of precompaction embryos to the uterus places them in an environment for which they are not yet developmentally ready [8]. As such, many *in vitro* fertilisation clinics find it desirable to culture embryos from the zygote to the blastocyst stage [203]. Two alternative culture systems can be used for this; monoculture or sequential culture.

Monoculture systems utilise only one medium formulation for the entirety of culture, and therefore do not accommodate the changing requirements of the developing embryo. In contrast, sequential culture systems utilise two different media formulations for pre and post compaction development. This allows the needs of the developing embryo to be met, resulting in a reduction in stress and relieving the embryo of the necessity to adapt to its environment in order to develop to the blastocyst stage. As a result, although both mono and sequential culture systems have been shown to support embryo development from the zygote to the blastocyst stage, embryos cultured to the blastocyst stage in sequential culture systems have increased viability compared to embryos cultured in monoculture systems [343]. Additionally, mouse embryos cultured in sequential culture medium give rise to ESCs with increased efficiency [71]. As such, although pre-compaction human embryos donated for hESC derivation may have been perturbed by exposure to the harmful conditions discussed above, the use of the second stage of a sequential media culture system, optimised to support post-compaction development during culture to the blastocyst stage for hESC derivation, could serve to rescue final blastocyst quality and increase ESC derivation efficiency and quality.

1.6 Improving embryo quality

As discussed above (section 1.5.2), the inclusion or exclusion of specific factors in embryo culture medium can have significant effects on final blastocyst quality. As ESC derivation occurs at the highest efficiency from good quality blastocysts [7], and human embryos are often donated for hESC derivation at the pre-compaction stage [4-6], this period of culture from the pre-compaction stage to the blastocyst stage could be utilised to improve blastocyst quality with a concomitant increase in ESC derivation efficiency and quality.

1.6.1 Growth Factors

One strategy which may improve the quality of human embryos donated at the cleavage stage for hESC derivation is the supplementation of embryo culture medium with growth factors.

It has been shown that the culture of embryos *in vitro* in the presence of growth factors can improve their development [344,345]. However, the majority of systems used for human embryo culture do not contain growth factors – in a review of culture media only ISM2 [346] was supplemented with a growth factor, while numerous others e.g. G1 [347], G2 [347], ISM1 [348], KSOM [349], Quinn's advantage [350], HTF [351], and Global [352] were not.

Below is a review of the impact many growth factors had been observed to have on the preimplantation development of *in vitro* cultured mouse embryos which was conducted on the literature available at the beginning of this project (Table 1.1A, Table 1.1B).

1.6.1.1 A review of growth factors used to improve embryo health

TABLE 1.1A. EFFECT OF GROWTH FACTORS ON MOUSE EMBRYONIC CHARACTERISTICS

	EGF	HB-EGF	TGF- α	TGF- β	GM-CSF	CSF-1	LIF
Blastocyst formation	Y ^[353]	Y ^[344]	Y ^[354]	-	Y ^[344]	Y ^[355]	Y ^[356]
Blastocyst Cell#	Y ^[354]	Y ^[357]	Y ^[354]	Y ^[358]	Y ^[344]	-	Y ^[359]
Hatching	Y ^[354]	Y ^[360]	Y ^[358]	-	Y ^[344]	-	Y ^[360]
Attaching	Y ^[361]	Y ^[362]	-	-	Y ^[363]	-	Y ^[364]
Glucose transport	-	-	-	-	Y ^[365]	-	-
Decrease apoptosis	Y ^[353]	Y ^[366]	Y ^[344]	Y ^[366]	Y ^[345]	-	-
Blastocoel expansion	Y ^[367]	-	Y ^[367]	-	Y ^[368]	Y ^[355]	-
ICM specific growth	-	-	-	-	Y ^[369]	-	-
TE specific growth	-	-	-	-	-	Y ^[370]	-
Metabolism	-	-	-	-	Y ^[371]	-	-
Protein synthesis	Y ^[344]	-	Y ^[344]	Y ^[372]	-	-	-
Growth rate	Y ^[344]	-	Y ^[358]	-	Y ^[365]	Y ^[370]	-
Zona variation	-	-	Y ^[358]	Y ^[358]	-	-	Y ^[358]
Gene expression	Y ^[361]	Y ^[362]	Y ^[344]	Y ^[344]	Y ^[363]	-	-
Implantation	Y ^[359]	Y ^[362]	-	-	-	-	Y ^[373]
Improve Morphology	Y ^[359]	-	-	-	-	-	Y ^[359]
Stimulates Outgrowth	Y ^[359]	Y ^[362]	Y ^[374]	Y ^[372]	Y ^[344]	-	Y ^[359]
Birth Rate	-	-	-	-	-	-	Y ^[360]

Y = yes, - = No effect yet observed, results may be due to lack of investigation or negative results. TE = trophectoderm, EGF = epidermal growth factor, HB-EGF = Heparin-binding EGF like growth factor, TGF = transforming growth factor, GM-CSF = Granulocyte macrophage colony stimulating factor, CSF-1 = colony stimulating factor 1, LIF = leukaemia inhibitory factor

Table 1.1B. EFFECT OF GROWTH FACTORS ON MOUSE EMBRYONIC CHARACTERISTICS

	Insulin	IGF-I	IGF-II	PAF	FGF4	SCF	IL-6
Blastocyst formation	Y ^[344]	Y ^[344]	Y ^[344]	-	-	Y ^[375]	Y ^[376]
Blastocyst Cell#	Y ^[344]	Y ^[344]	Y ^[377]	Y ^[344]	Y ^[378]	-	Y ^[358]
Hatching	-	Y ^[353]	Y ^[353]	Y ^[379]	-	-	Y ^[358]
Attaching	-	-	-	-	-	-	-
Glucose transport	Y ^[344]	Y ^[365]	Y ^[377]	-	-	-	-
Decrease apoptosis	Y ^[380]	Y ^[344]	Y ^[353]	Y ^[381]	-	-	-
Blastocoel expansion	-	-	-	-	-	-	-
ICM specific growth	Y ^[344]	Y ^[344]	Y ^[344]	-	-	-	-
TE specific growth	-	-	-	-	-	-	-
Metabolism	Y ^[382]	Y ^[382]	-	Y ^[383]	-	-	-
Protein synthesis	Y ^[344]	Y ^[382]	Y ^[344]	-	-	-	-
Growth rate	Y ^[384]	Y ^[385]	Y ^[344]	Y ^[386]	-	Y ^[387]	-
Zona variation	-	Y ^[358]	Y ^[358]	-	-	-	-
Gene expression	Y ^[388]	Y ^[344]	Y ^[344]	-	-	-	-
Implantation	-	-	-	Y ^[386]	-	-	-
Improve Morphology	-	-	-	-	-	-	-
Stimulates Outgrowth	-	-	-	-	Y ^[389]	-	-
Birth Rate	-	-	-	Y ^[390]	-	-	-

IGF = insulin like growth factor, PAF = platelet activating factor, FGF4 = fibroblast growth factor 4, SCF = stem cell factor IL-6 = interleukin 6.

In many instances growth factors have been shown to have no effect on embryo development in one study then to have an effect in another, or for a different effect to be observed. This has been attributed to differences in culture conditions (i.e. simple salt solutions or media systems designed to support blastocyst development) and mouse strains (i.e. inbred, randombred or hybrid mice from strains such as C57Bl/6 or Quackenbush) used between experiments. In many studies embryos were exposed to perturbing environments which might confound the results (i.e. freezing or culture in the presence of apoptotic or oxidative factors).

Growth factors appear to have their most positive effects when embryos have been cultured in sub-optimal environments which perturb their development, suggesting that growth factors have a rescue effect on embryos. Additionally, it was not uncommon for the activity of growth factors to only become apparent when embryos were cultured singly or in large volumes. Evidence suggests that embryos secrete autocrine growth factors which are diluted at low embryo concentrations or large volumes [391-394]. Therefore it appears that the effect of growth factors added to embryo culture medium can be masked by autocrine growth factors. As such, this suggests that experiments to investigate the effects of individual growth factors on embryo development or health are best conducted at a low embryo per volume ratio.

1.6.1.2 *Insulin, GM-CSF, FGF-4 and LIF*

Reviewing the literature, including prior observations of the effects of the growth factors on embryo development, maternal expression of the growth factor, embryo expression of the growth factor's receptor (Table 1.2) and the effect of a null phenotype for the receptor, four growth factors – insulin, GM-CSF, FGF-4 and LIF, were identified as likely candidates for investigation for their ability to improve ESC derivation rates. Each is reviewed below in more detail.

TABLE 1.2. MATERNAL REPRODUCTIVE TRACT AND EMBRYO EXPRESSION OF GROWTH FACTORS AND THEIR RECEPTORS IN THE MOUSE AND HUMAN

	Human	Mouse
Insulin-Expressed maternally	-	Yes ^[389,395]
Insulin-Expressed by the embryo	No ^[344]	No ^[344]
Insulin-Receptor expressed by embryo	mRNA shown at all stages ^[344]	mRNA from the 8-cell onwards ^[344]
GM-CSF-Expressed maternally	Yes ^[363]	Yes ^[365]
GM-CSF-Expressed by the embryo	-	No ^[389]
GM-CSF-Receptor expressed by the embryo	α -chain receptor expressed at all stages ^[371]	α -chain receptor expressed at all stages ^[365]
LIF-Expressed maternally	Yes ^[396]	Yes ^[373]
LIF-Expressed by the embryo	mRNA shown at all stages ^[396]	Transcripts expressed at the blastocyst stage ^[389]
LIF-Receptor expressed by the embryo	mRNA shown at all stages ^[396]	Yes ^[389] (only in the ICM of blastocysts ^[360])
FGF-4-Expressed maternally	-	-
FGF-4-Expressed by the embryo	-	Yes ^[389]
FGF-4-Receptor expressed by the embryo	(Presumed by FGF4 response of ICM derived ES cells ^[397])	Yes ^[398]

- = a review of the literature did not uncover any relevant studies or findings

1.6.1.2.1 Insulin

Insulin has been shown to promote ICM specific growth in the mouse blastocyst when cultured in simple media from the 2-cell to the blastocyst stage [344,369], and promote glucose transport [344,365] which has been shown to increase embryo viability [365]. Additionally, insulin increases blastocyst formation [344]. This demonstrates that inclusion of insulin in embryo culture medium that is known to perturb physiology has the potential to result in the generation of an increased number of higher quality blastocysts. An effect for insulin when included in embryo culture medium from the 2-cell stage or earlier has been observed in murine [344,365,384], bovine [399], and porcine [400]. However, despite this insulin is not routinely included in human embryo culture media.

Insulin acts on cells by binding to the insulin receptor; a tyrosine kinase receptor with four components; two extracellular α -subunits and two transmembrane β -subunits. The binding of insulin to the α -subunit causes the tyrosine autophosphorylation of the β -subunit. The activated insulin receptor is then able to phosphorylate its substrates which can activate different signalling pathways including the phosphoinositol-3-kinase (PI3K) pathway, which can increase glucose transport [401], and promote Nanog transcription in ESCs [266]. Insulin has also been shown to cross react with the IGF I receptor, where it binds weakly [402], but this effect is not observed when low concentrations of insulin are used [384]. When the insulin receptor is knocked out (via the mating of two mice heterozygous for the disruption of the insulin receptor gene by deletion or insertional mutation) mice are born with approximately a 10% reduction in size and die shortly after birth, although the null phenotype is obtained at expected Mendelian rates, indicating that the activation of the insulin receptor is dispensable for intrauterine growth [403,404]. These data show that while insulin action may not be an absolute necessity for development of the embryo it has shown benefit, in particular in situations where there are perturbing elements in the culture system.

In conclusion, while neither mouse nor human embryos secrete insulin [344] (removing a potentially confounding factor from experiments), embryos from both species express mRNA for the insulin receptor [344] (Table 1.2). Furthermore, culture of human preimplantation embryos with insulin has previously been shown to increase protein synthesis [344] (an effect also shown for mice), and insulin [405] and the insulin receptor [406] are highly conserved proteins, increasing the likelihood that findings in the mouse will translate to the human. Finally, as a laboratory material, insulin is comparatively inexpensive. As such, if experiments

prove successful and insulin is effective at increasing ESC generation from *in vitro* cultured embryos, it would be practical and easy for researchers to include as a routine component of embryo culture for ESC derivation. As such, for these reasons and those above, insulin was selected as the growth factor of choice for further investigation in this project.

1.6.1.2.2 GM-CSF

Like insulin, GM-CSF is able to increase ICM size [369] and glucose uptake [365] which shows increased viability and suggests embryos cultured in media supplemented with GM-CSF may be able to give rise to quality ESCs with greater efficiency. Furthermore, GM-CSF also increases attachment and outgrowth [363], which, discussed (section 1.2.2), is a primary method of ESC derivation. These effects were demonstrated for embryos cultured from the 2-cell stage or longer in simple salt solutions which did not contain protein.

The GM-CSF receptor normally consists of both an α and β subunit which belong to the superfamily of cytokine receptors typified by the growth hormone receptor [363]. The α -chain has low affinity binding with GM-CSF, while the β -chain does not bind to GM-CSF, instead it forms a high affinity complex when associated with the ligand coupled α -chain [407]. However, in embryos only the α -chain is expressed [408]. The alpha chain has been shown to be capable of transducing signal [408], and is therefore functional in its own right without the β -chain. GM-CSF deficient mice have normal implantation rates and pups are produced. However, they often die due to placental abnormalities [409] and survivors are afflicted with abnormal lungs [410]. Significantly, blastocysts taken from GM-CSF null mutant mice have reduced total cell numbers and retarded development [365].

In conclusion, although GM-CSF was an interesting factor, its positive effects, including stimulating ICM specific growth, were only induced when embryos were cultured in the absence of human serum albumin [369], which is a specifically perturbing condition that would not be a part of the system this project will attempt to model.

1.6.1.2.3 FGF-4

FGF-4 has been shown to be capable of eliciting an increase in blastocyst total cell number, although whether it affects a specific cell type is unknown [378,389]. Furthermore, culture in FGF-4 has been shown to stimulate the outgrowth of blastocysts, which, as has been noted, can form a vital step in ESC derivation [389,411]. The benefit of FGF-4 for ESC derivation currently lies mainly in areas other than its demonstrated effects in improving embryo culture. When the *Fgf-4* gene was knocked out in mice, the embryos were able to develop to the blastocyst stage, but *in vivo* they failed to develop shortly after implantation, and *in vitro* ICM growth was severely impaired while morphology was degenerate [412]. Additionally, mouse embryos that express a non functioning FGF receptor that antagonises the activity of the remaining wildtype receptor fail to develop past the blastocyst stage when cultured *in vitro* [413]. This indicates a vital role for FGF-4 for ICM development and health. In the fibroblast growth factor system there are fifteen fibroblast growth factors and four receptors FGFR1-4. Of the FGFs, FGF-4 is the earliest to act in development [414]. One of the most likely receptors of FGF-4 in the preimplantation embryo is FGFR2, as FGF-4 and FGFR2 possess a mutual binding affinity, are co-expressed, and their knockouts have similar phenotypes [414]. FGFR2 is one of the earliest acting FGF receptors and has been shown to be expressed on the cell membranes of preimplantation mouse blastocysts, while FGF-4 has been shown to be expressed from the 2-cell stage of mouse embryos onwards [414]. Importantly, FGF signalling has been shown to be crucial for maintaining the pluripotency of ESCs in the human [415]. While FGF-4 has been specifically shown to increase their undifferentiated growth [397]. Since the commencement of this project another group has shown FGF-4 signalling (through the FGFR) to be specifically antagonistic to pluripotency in the embryo [416].

1.6.1.2.4 LIF

LIF has traditionally been used for the maintenance of pluripotency in ESCs. However, culture of preimplantation embryos in media supplemented with LIF has been shown to be capable of increasing blastocyst total cell number [359]; although which cell types are unknown. Furthermore, LIF promotes blastocyst formation [356], hatching [360], attaching [364] and outgrowth [359], all of which must occur for ESC derivation. Additionally, LIF improves the viability of mouse embryos as LIF increases both the implantation [373] and birth rate [360] of

embryos cultured in its presence. While LIF null blastocysts are morphologically normal, they fail to implant [417]. Additionally when wildtype embryos are transferred to wildtype mothers whose reproductive tracts have been flushed with LIF antibody to reduce its activity, a decrease in implantation is seen [418], and when wildtype embryos cultured in the presence of LIF are transferred to LIF null mothers they fail to implant [417]. These observations demonstrate how important LIF exposure is for a blastocyst's adhesive potential.

LIF effects its activity by binding to its receptor LIF-R β , this complex is then capable of associating with the membrane bound protein gp-130 which results in its signal transduction [360]. LIF can also act through the MAPK/ERK pathway to promote cell growth [419]. Importantly however, LIF also acts through the JAK/STAT pathway [419]. It is through this pathway that LIF is able to maintain the undifferentiated propagation of mESC lines [364,419]. Only over expression of Nanog has been shown to be capable of making mESCs cells independent of LIF and STAT3 signalling [56]. LIF's crucial activity in the maintenance of pluripotency in mESC lines highlights its role as a candidate for improving ESC derivation from *in vitro* cultured blastocysts.

LIF was not studied here, however, as although the pathway is active, LIF is dispensable and insufficient for pluripotency maintenance for hESC culture [82,420,421], and although in *in vitro* culture of mouse diapause embryos (wherein embryonic development is arrested until the mother weans the previous litter) LIF maintains the epiblast [422] from which ESC lines are derived, human embryos are not subject to diapause. As such any embryological events observed for LIF may not be transferable to human embryology, which this project will be attempting to model.

1.7 Animal model

As a result of legal and ethical complications it is difficult to conduct experimental projects using human embryos. Furthermore, the available embryos are highly variable, with each donating couple having a different genetic background.

Consequently where possible it is advisable to use animal models. Of the mammalian models available, the mouse is the best characterised for embryo development, which can be undertaken under the same conditions as human embryos, progresses along a similar timescale, and produces blastocysts with the same ICM structure (distinguishable primitive

endoderm and epiblast). Initially the most commonly used strain of mouse for mESC derivation was the Sv129 line. However, there is a growing trend for researchers to use mice of the C57Bl/6 background, primarily due to advantages in the generation of transgenic lines. It has been shown that the mouse lines Sv129 and C57Bl/6 have similar efficiencies for the generation of germ line competent ESCs [161,423]. Furthermore hybrid C57Bl/6 mice (crossed with DBA) have been used to produce zygotes which were cultured to the blastocyst stage *in vitro*, before being used for the derivation of mESCs, suggesting that embryos from the C57Bl/6 line will give rise to ESCs after culture *in vitro* [71,160]. It is necessary to supplement outgrowth media with LIF when C57Bl/6 blastocysts are being outgrown to obtain their undifferentiated ICM cells [161]. However, while the Sv129 line is independent of LIF during outgrowth, there is a tendency in male mice to spontaneously grow teratocarcinomas in their testis [424]. Additionally, while the Sv129 line is very well characterised with respect to mESCs, comparatively little is known about the preimplantation development of embryos from the line compared to the C57Bl/6 mouse strain [423]. Significantly, the C57Bl/6 genome has been sequenced [423].

This suggests that the C57Bl/6 mouse is the appropriate line for primary use in any project aimed at improving the derivation of ESC lines from *in vitro* cultured embryos.

1.8 Conclusion and hypothesis

hESC lines are an important potential resource for regenerative medicine [425], as well as the fields of toxicology testing, drug discovery [101] and developmental biology [113,114]. However, current hESC lines have variable characteristics and many are unsuitable for a range of applications due to poor performance with respect to certain characteristics, and the likelihood of their having been derived and cultured in the presence of animal products – raising the risk of contamination by xeno-pathogens [425]. Additionally, high numbers of hESC lines are needed to cover the range of variation amongst humans and avoid rejection in the case of regenerative medicine [426]. As such, it is necessary that more hESCs be derived in the future. The primary source of embryos for hESC derivation is embryos created by ART for treatment of infertility which have been subsequently donated. These embryos are often donated during the pre-compaction stage [167-169]. One reason for this is that embryos are often cryopreserved at this point so that they can be used in future attempts at achieving a pregnancy. As a result often many years will have passed between the initial culture of

embryos and their donation. Therefore, these embryos will have been cultured in media systems now known to be perturbing to embryo development, metabolism and viability [203]. As such, these embryos could be expected to have sustained significant perturbations to their development. Poor quality embryos have been shown to give rise to hESCs with lower efficiency than high quality embryos [5,6,73]. Observation and scoring of embryo morphology has been shown to be an inadequate measure of embryo quality in a reproductive context as many relevant factors are not represented [427].

Most ESC derivation techniques rely on the embryo being at the blastocyst stage. Therefore, donated pre-compaction human embryos must be cultured to the blastocyst stage prior to attempting to derive a new hESC line. The requirements of the precompaction embryo and the post compaction embryo are very different, as discussed above (section 1.5.2.9), and media systems exist which contain culture medium specifically tailored to meet the needs of the post-compaction embryo (specifically, the G1/G2 media system [292]). However, currently these systems are designed for implantation and not specifically around the development of the epiblast from which ESCs are derived, and this may represent a window of opportunity during which attempts can be made to improve embryo quality in order to improve hESC derivation success rates. The inclusion of growth factors in *in vitro* culture media has repeatedly been shown to have the potential to improve embryo quality [344,345]. However, no work has previously been done on the effect of growth factors on the number of ESC progenitor epiblast cells in the ICMs of *in vitro* cultured embryos. In reviewing the literature, insulin was identified as the growth factor whose observed effects and mechanisms of action suggested it was the most likely to have an effect on the epiblast.

It is therefore hypothesised that *in vitro* culture of mouse embryos from the pre-compaction stage in the presence of insulin will result in the development of blastocysts that contain a higher population of epiblast cells.

A large epiblast in the ICM of the blastocyst has previously been shown to correlate to high ESC derivation rates [7]. However, in systems where using a treatment to increase epiblast cell numbers have been shown to directly result in increased ESC derivation rates, the demonstrated increase has been due to treatment with small molecular inhibitors which have caused the ICM to develop to be composed entirely of epiblast cells – a very large increase, and far larger than the increase hypothesised to be shown in our experiments [428]. Nonetheless, each epiblast cell in the ICM of the blastocyst has the potential to proliferate during the outgrowth stage of ESC derivation, and each resultant epiblast cell in the outgrowth

has the potential to give rise to an ESC colony when the cells of the outgrowth are disaggregated by trypsinisation and replated.

It is therefore hypothesised that increasing the number of ICM cells in the blastocyst that are epiblast cells will result in an increased number of cells in the outgrowth that are epiblast cells, which will result in an increased efficiency of ESC colony generation.

In conclusion, the majority of human embryos donated for hESC derivation will be at the precompaction stage and of poor quality, which results in reduced hESC derivation efficiency due to the suboptimal culture environment used at the time of their initial culture. Most prior attempts at improving the efficiency of hESC derivation have focused on optimising the media, techniques and conditions used at the derivation attempt and subsequent culture of the putative lines. However, it is possible that the period during which pre-compaction embryos are cultured to the blastocyst stage can be utilised to increase the number of ESC progenitor epiblast cells in the blastocyst in order to improve the likelihood that a given precompaction embryo will ultimately give rise to an ESC line – a matter of key practical and ethical importance.

It is therefore the hypothesis of this thesis that the culture medium utilised for culture of pre-compaction embryos to the blastocyst stage is a critical component of ESC derivation, and can be optimised to produce blastocysts with a greater potential to give rise to quality ESC lines.

Specific aims

1. To determine, using a mouse model, the effect of culture in a simple *in vitro* embryo culture medium on the quality of blastocysts as measured by epiblast cell number and gene expression in outgrowths.
2. To determine the effect of culturing embryos in a medium purpose designed to support post-compaction development, after culture during the pre-compaction stage in a simple *in vitro* embryo culture medium, on the quality of blastocysts as measured by epiblast cell number and gene expression in outgrowths.
3. To assess the effect of inclusion of different concentrations of insulin in the culture medium of post-compaction mouse embryos on epiblast cell number

4. To investigate the molecular mechanisms by which insulin included in the post-compaction culture medium of mouse embryos affects its influence on epiblast cell number.
5. To demonstrate that increases in epiblast cell number achieved by inclusion of insulin in the culture medium of post-compaction mouse embryos persists through the outgrowing stage and results in an increased efficiency of primary mESC colony generation.
6. To demonstrate that this model supports the generation of mESC lines capable of self renewal and differentiation to cells from the three germ cell layers.

2.0 Materials and Methods

2.1 Media preparation

Unless otherwise stated chemicals used were purchased from Sigma-Aldrich, St Louis, MO.

2.1.1 Preparation of glassware

Glassware used to make embryo handling or culture media was washed with 7X detergent (ICN Biomedicals, Aurora, Ohio, USA) then soaked overnight in RO (reverse osmosis) water. This was followed by 6-8 rinses in RO water and then another overnight RO water soak. Glassware was then rinsed a further 6-8 times in Milli-Q water (18-meg Ω) and placed upside down in an oven for 4h (hours) at 110°C to dry. Openings were then covered by blue absorbent paper overlaid with aluminium foil and sterilised at 130°C for 2h.

2.1.2 Embryo culture and handling media

Media used for embryo culture were G1.2, G2.2 or Simple-G1 [332] (Table. 2.1). MOPS-G1 was used for the collection and handling of embryos [429]. The formulations for all embryo culture and handling media have been included below in (Table. 2.1). G1.2 and G2.2 make up a sequential culture system and were used because they are representative of the modern complex embryo culture media that have been developed to support high rates of human preimplantation embryo development and viable blastocysts [218,430]. Simple-G1 is a simplified version of G1.2 medium, which contains no EDTA or amino acids. Simple-G1 was used to model the simple embryo culture media that have been used historically in human IVF [203,215,218]. MOPS-G1 is a modified version of G1.2 medium which has a reduced concentration of bicarbonate which was replaced with the buffer MOPS (3-(N-morpholino) propanesulfonic acid). MOPS-G1 was adjusted to pH 7.3 \pm 0.05 with NaOH. As such, unlike the embryo culture media MOPS-G1 did not need to be gassed with CO₂ to reach a physiological pH. Confocal microscopy used a modified version of MOPS-G1 – confocal loading media – which contained no phenol red (Table 2.1). All media were supplemented with serum albumin at 5mg/ml.

2.1.3 Culture media preparation

All chemicals and consumables used for standard embryo culture were tested for compatibility with embryo development in a 1-cell mouse embryo assay [300] (Appendix 8.1). For 1L of culture media, working in a clean area, chemicals (Table 2.1) were weighed out into a sterilised volumetric flask (with the exception of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, which was weighed out into a 14ml tube (BD Biosciences, BD Falcon, North Ryde, NSW, Australia). These factors were then dissolved in approximately 500ml of Milli-Q water, which was added to the flask and swirled. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was dissolved by the addition of 10ml of Milli-Q water, followed by agitation. The contents of the tube were then added to the volumetric flask. The volume of the flask was then increased to 950ml by the addition of further Milli-Q water, with the 50ml difference excluded to allow the later addition of HSA (human serum albumin). Media were then sterile filtered using an Acrodisc 0.2 μm filter (Pall Corporation, Ann Arbor, MI, USA) and stored at 4°C in glass bottles or plastic flasks (Nalgen Nunc International, Rochester, NY, USA). Culture medium with HSA was made by adding 500 μl of HSA (Vitrolife, Kungsbacka, Sweden) to 9.5ml of G1.2, G2.2 or Simple-G1 culture medium in a 10ml tube (BD Biosciences, BD Falcon). Media were only stored for 4 weeks from preparation.

TABLE 2.1. MEDIA FORMULATIONS

Component	Simple-G1 (mM)	G1.2 (mM)	G2.2 (mM)	MOPS-G1 (mM)	Confocal Medium (mM)
NaCl	90.1	90.1	90.1	90.1	90.1
KCl	5.5	5.5	5.5	5.5	5.5
NaH ₂ PO ₄	0.25	0.25	0.25	0.25	0.25
MgSO ₄	1.0	1.0	1.0	1.0	1.0
NaHCO ₃	25.0	25.0	25.0	5.0	5.0
CaCl ₂	1.8	1.8	1.8	1.8	1.8
Glucose	0.5	0.5	3.15	0.5	0.5
L-Lactate	10.5	10.5	5.87	10.5	10.5
Pyruvate	0.32	0.32	0.1	0.32	0.32
EDTA	-	0.01	-	-	-
Glycyl-glutamine	-	0.5	1.0	0.5	0.5
Alanine	-	0.1	0.1	0.1	0.1
Arginine	-	-	0.6	-	-
Asparagine	-	0.1	0.1	0.1	0.1
Aspartate	-	0.1	0.1	0.1	0.1
Cystine	-	-	0.1	-	-
Glutamate	-	0.1	0.1	0.1	0.1
Glycine	-	0.1	0.1	0.1	0.1
Histidine	-	-	0.2	-	-
Isoleucine	-	-	0.4	-	-
Leucine	-	-	0.4	-	-
Lysine	-	-	0.4	-	-
Methionine	-	-	0.1	-	-
Phenylalanine	-	-	0.2	-	-
Proline	-	0.1	0.1	0.1	0.1
Serine	-	0.1	0.1	0.1	0.1
Taurine	-	0.1	-	-	-
Threonine	-	-	0.4	-	-
Tryptophan	-	-	0.5	-	-
Tyrosine	-	-	0.2	-	-
Valine	-	-	0.4	-	-
Choline chloride	-	-	0.0072	-	-
Folic Acid	-	-	0.0023	-	-
Inositol	-	-	0.01	-	-
Nicotinamide	-	-	0.0082	-	-
Pantothenate	-	-	0.0042	-	-
Pyridoxine	-	-	0.0049	-	-
Riboflavin	-	-	0.00027	-	-
Thiamine	-	-	0.00296	-	-
MOPS	-	-	-	20.0	20.0
Phenol Red	0.005g/L	0.005g/L	0.005g/L	0.005g/L	-
Albumin	5mg/ml	5mg/ml	5mg/ml	5mg/ml	-

Culture media were made to be at pH 7.3±0.05 at 6%CO₂, osmolarity is 255±5, 255±5, 261±5 and 255±5 for simple-G1, G1.2, G2.2 and MOPS-G1 respectively.

2.1.4 Hyaluronidase preparation

Bovine testes hyaluronidase was prepared at 1mg/ml in MOPS-G1 by adding 30mg of hyaluronidase to a sterile glass beaker and dissolving it in 30ml of MOPS-G1. This solution was then filtered through a 0.2 μ M filter and kept for a maximum of one month at -20°C in 0.5ml aliquots. Hyaluronidase was added to MOPS-G1 medium which contained zygote-cumulus cell complexes and pipetting was used to dissociate cumulus cells and denude the zygotes (discussed in further detail section 2.4.1).

2.2 Preparation of pipettes

Pipettes used for the handling of embryos throughout this project were Pasteur pipettes pulled to a diameter of approximately 100 μ m, which is slightly larger than the diameter of an embryo (80 μ m). This was done by placing the neck of a sterilised Pasteur pipette in a Bunsen burner's flame and rotating it until the glass was softened. At this point the pipette was removed from the heat and its two ends were pulled in opposite directions to stretch the glass until the required thickness was achieved. Pipettes were then snapped at the desired length. Care was taken that the thin end of the pipette which the embryo culture medium was exposed to never came into contact with any potential source of contamination, and a stand was used to hold them upright.

2.3 Animals and induction of ovulation

2.3.1 Animals

The mice used in this study were bred at the Central Animal house, Waite institute, Adelaide, or the Animal Resource Centre, Perth, and were housed at Laboratory Animal services at the University of Adelaide. Animals were caged, fed and watered ad-libitum and kept on a 14:10h light:dark cycle at 21°C. Approval for all procedures was obtained from the University of

Adelaide Animal Ethics Committee, in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.3.2 Induction of ovulation

C57BL/6 or CBA×C57BL/6 female mice aged 3-4 weeks were injected intraperitoneally (ip) with 5 IU equine chorionic gonadotropin (eCG; Folligon, Intervet Australia Pty Ltd, Bendigo, Victoria), followed by 5 IU human chorionic gonadotropin (hCG; Pregnyl, Organon, Sydney, Australia) 48h later, inducing ovulation. Ovulating females were placed with a male mouse of the same strain, and mating was assessed the following morning by the presence of a vaginal plug.

2.4 Embryo culture

2.4.1 Collection of zygotes

Female mice were killed 22h post hCG by cervical dislocation. Their abdomens were sterilised with 70% ethanol and the skin was split and retracted to expose the peritoneum. The peritoneum was then cut using surgical scissors and the visceral organs were pushed aside to allow access to the reproductive organs. Needle point forceps were used to dissect the mesentery from the uterus and the oviduct. The uterus was then pulled taught by gripping the organ at the utero-tubal junction with forceps, and a cut was made between the oviduct and the ovary. The oviduct was then removed by a second cut below the utero-tubal junction immediately adjacent to the forceps, and placed in a small vial of MOPS-G1 which was maintained at 37°C.

Zygotes could be found in the distended ampulla of the oviduct, which was held to the bottom of the dish in 0.5ml MOPS-G1 by a pair of fine forceps and a second pair were used to tear the wall of the ampulla and release the zygotes and cumulus mass. The oviduct was then discarded, which left the zygotes and cumulus mass in the MOPS-G1 medium.

In order to denude zygotes from surrounding cumulus cells hyaluronidase (0.5ml of 1mg/ml) (section 2.1.4) was added to the MOPS-G1 medium, which contained the zygotes and cumulus mass. After approximately 1 min cumulus cells began to disperse and any remaining cells were dissociated by repeatedly pipetting zygotes up and down. Isolated zygotes were then washed through three drops of MOPS-G1, then one drop of G1.2 to remove all traces of hyaluronidase, and placed in their G1.2 culture drops.

2.4.2 Culture of embryos

Embryo culture dishes were prepared by placing 20µl drops of the applicable embryo culture medium (Table 2.1) in 35mm petri dish (BD Biosciences, BD Falcon™) overlaid with 2.5ml light weight paraffin oil (Vitrolife, Kungsbacka, Sweden). Dishes were pre-equilibrated at 37°C in 6% CO₂ (Sanyo CO₂ Incubator: MCO15AC or Sanyo O₂/CO₂ incubator MCO-18M) at least 3h prior to the introduction of embryos.

Zygotes from five female mice were collected and randomly distributed to culture drops. For all experiments embryos were cultured in groups of ten in a tri-gas incubator at 37°C in 6% CO₂, 5% O₂, 89% N₂ (Sanyo O₂/CO₂ incubator MCO-18M or MCO-5M). On day 3 after 48h in culture embryos were transferred, either in groups of ten or singly, to fresh culture media (simple-G1.2 or G2.2). Time outside of incubators was kept to a minimum and embryo manipulation was performed at 37°C to minimise stress.

2.4.3 Assessment of embryo morphology

Embryo morphology was assessed using phase contrast microscopy at 37°C.

Embryos were categorised as:

- 8-cell: Cleavage stage embryos in which 8-cells could be seen. (Fig.2.1A)
- Compacting: Embryos in which some cells had begun the process of compaction; where they showed signs of polarity, flattening and increased cell to cell contact. (Fig.2.1B)
- Morula: Embryos which had completed compaction. (Fig.2.1C)

- Early blastocyst: Embryos where a blastocyst cavity had begun to form but was $<2/3$ the volume of the embryo (Fig.2.1D).
- Blastocyst: Embryos where the cavity was $>2/3$ of the volume of the embryo (Fig.2.1E).
- Hatching blastocyst: Embryos where cells had begun to herniate from the zona pellucida (Fig.2.1F).
- Hatched blastocyst: Embryos which were completely free of the zona pellucida (Fig.2.1G).

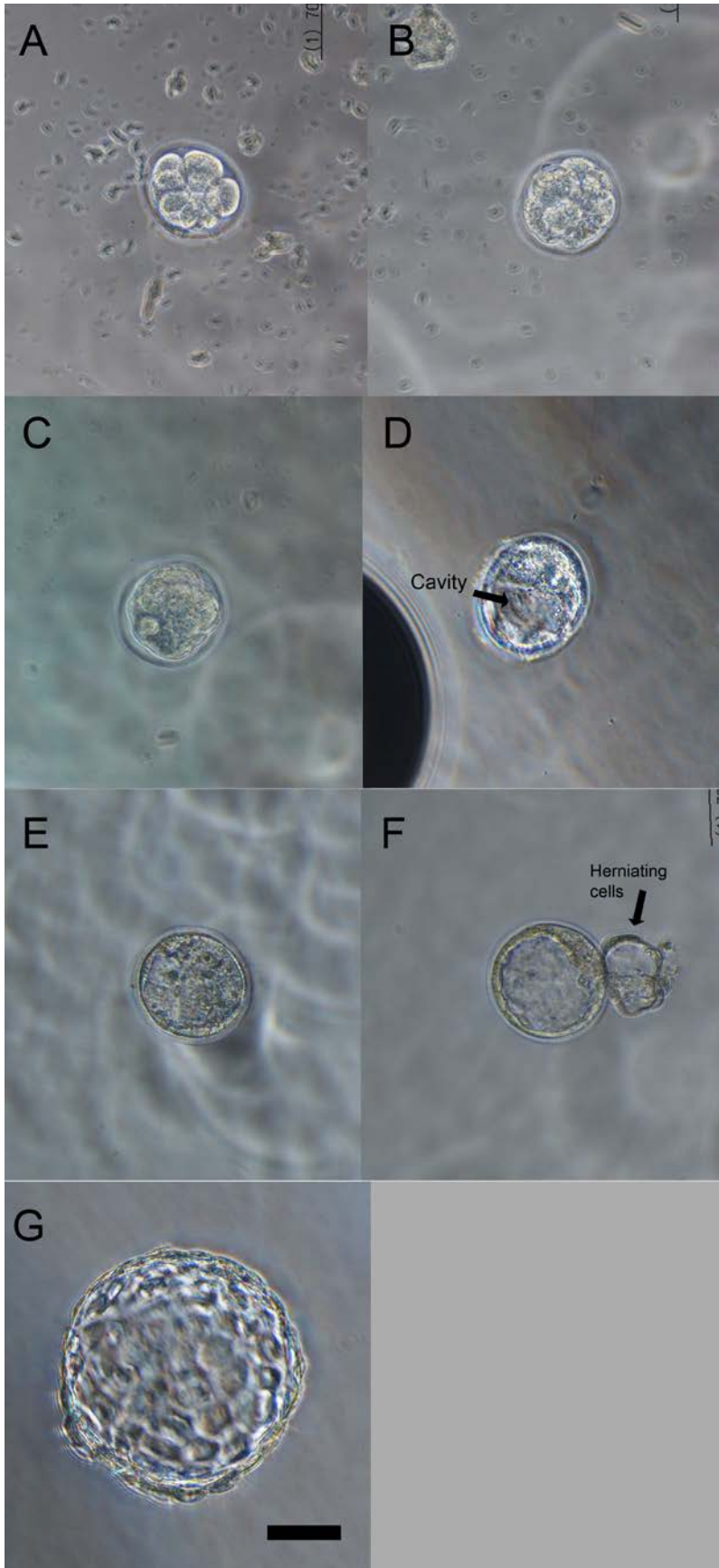


FIG. 2.1. A; 8-cell embryo, B; Compacting embryo, C; Morula, D; Early blastocyst, E; Blastocyst, F; Hatching blastocyst, G; Hatched blastocyst. Scale bar 50 μ m.

2.5 Assessment of blastocyst differentiation

To determine the differentiation of blastocysts into the epiblast, primitive endoderm, ICM and trophoctoderm an immunocytochemical method was employed.

TABLE 2.2. MARKERS OF THE DIFFERENT CELL TYPES OF THE DEVELOPED BLASTOCYST

Cell type	OCT4	Nanog	Dapi
Trophoctoderm	-	±	+
ICM	+	±	+
Primitive Endoderm	+	-	+
Epiblast	+	+	+

Markers which must be present to indicate a particular cell type in the developed blastocyst are shown with +, markers which must not be present are shown with -, markers which may be present or absent are shown with ±

2.5.1 OCT4 and Nanog co-expression

OCT4 and Nanog, whose co-expression marks epiblast cells, were assessed in blastocysts using immunocytochemistry and confocal microscopy. Blastocysts were fixed overnight in 4% paraformaldehyde at 4°C. Immunocytochemistry was performed in 96 well plates (BD Biosciences BD Falcon™, North Ryde, NSW, Australia), with each well containing 50µl of reagent. Blastocysts were transferred between wells by mouth pipetting, with care taken to minimise the quantity of reagent transferred. Following fixation embryos were incubated for 5 min in 0.1M glycine in PBS at room temperature (RT) to effect antigen unmasking, due to the possibility of epitopes being made inaccessible to the antibodies used due to the paraformaldehyde fixation. Blastocysts were permeabilised in PBS with 0.25% TritonX-100 (PBS-TX) for 30 min at RT, then blocked in 10% Normal Donkey Serum (Sapphire Bioscience, Redfern, New South Wales, Australia) for 1h at RT. Embryos were incubated with Nanog rabbit anti mouse polyclonal antibody (Sapphire, Cat#120-21603 or Cosmo Bio, Tokyo, Japan, Cat#REC-RCAB0002P-F) at 1:200 and Oct-3/4 goat anti mouse polyclonal antibody (Santa Cruz

Biotechnology inc, Santa Cruz, CA, sc-8628) at 1:100 overnight in PBS-TX at 4°C (only Nanog antibody was applied in Chapter 3.0). Blastocysts were then washed in PBS-TX, and incubated with Donkey anti rabbit secondary antibody (1:100) conjugated to FITC (Australian Laboratory Services, Homebush New South Wales, Australia) and donkey anti goat secondary antibody (1:100) conjugated to Rhodamine (Jackson immunoResearch, West Grove, PA, 705-025-003) for 2h at 37°C. Embryos were then incubated with 3nM of 4'-6-Diamidino-2-phenylindole (Dapi), a nuclear stain, for 2-3 min at RT, before being loaded between two coverslips in loading solution (500µl glycerol, 1000µl confocal loading media with 1 drop of Prolong Gold antifade reagent (Invitrogen)) and being examined by confocal microscopy (Nikon, EZ-C1 software or Calcium Lecia SP5, Lecia SP5 software). The laser wavelengths which excited the different fluorophores were 405, 488 and 561nm for Dapi, FITC and Rhodamine, respectively. The number of cell nuclei stained blue by Dapi gave total cell number, ICM cell number was the number of cells whose nuclei were stained red by Rhodamine; indicating the presence of OCT4, and epiblast cell number was the number of red ICM nuclei which were also stained green by FITC; indicating the presence of both OCT4 and Nanog, trophectoderm cell number could be calculated by subtracting ICM cell number from total cell number, and primitive endoderm cell number could be calculated by subtracting epiblast cell number from ICM cell number Table 2.2 (Fig. 2.2). A cell was counted as positive for the fluorophore when the intensity of the stain was sufficient for the nucleus to be clearly distinguished from the background. A negative control was assessed where the primary antibody was not applied. A positive control was performed where embryos were allowed to develop to the blastocyst stage *in vivo* before being flushed and stained to demonstrate that the observation of zero epiblast cells in blastocysts was not a technical artefact. In this study a total of 16 flushed blastocysts were examined and all contained an epiblast.

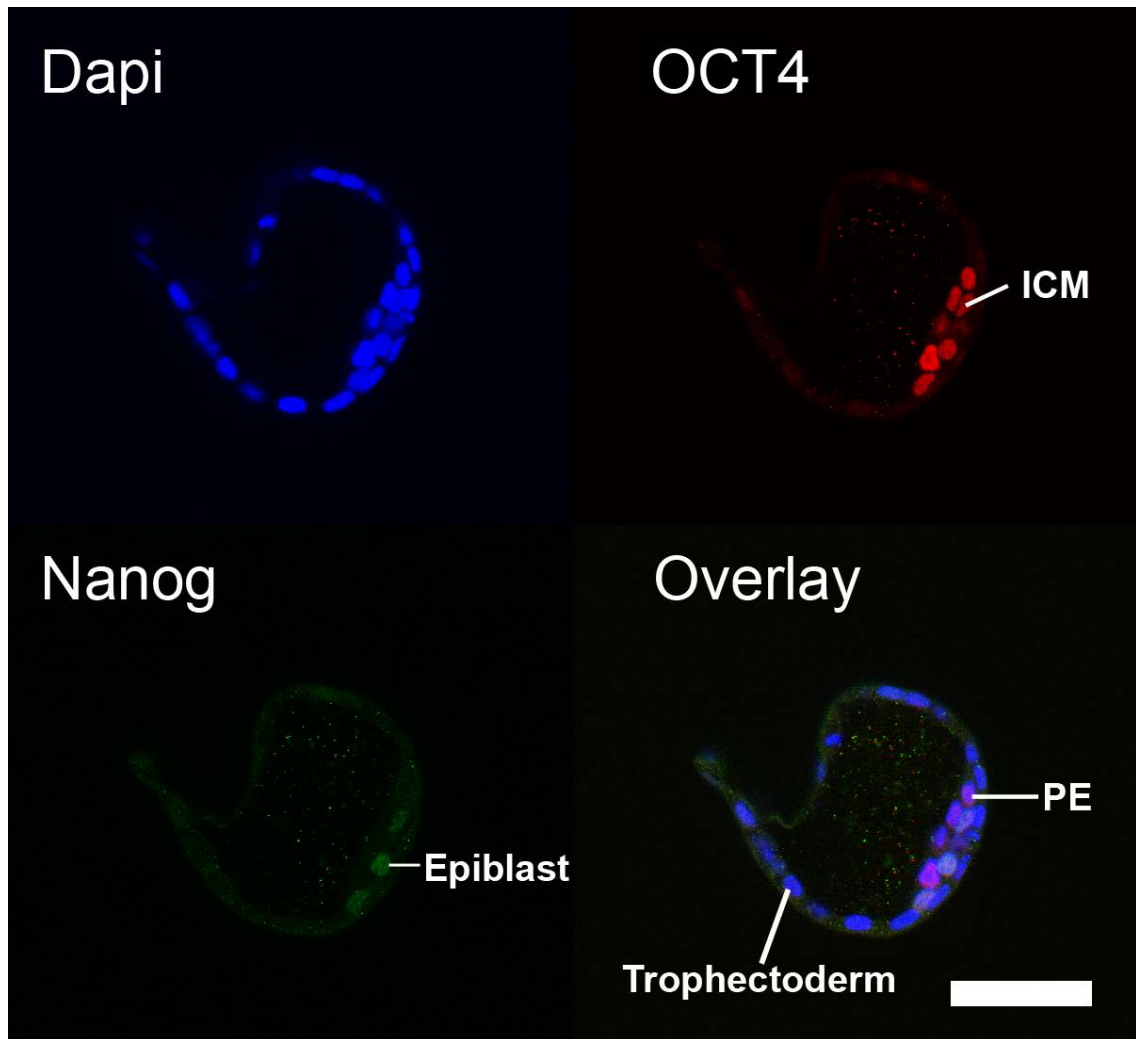


FIG. 2.2 Representative images of and immunocytochemically stained blastocyst fixed at 115h post culture. Total cell number is stained blue with Dapi, OCT4 positive cell number is stained red with rhodamine, and Nanog positive cell number is stained green with FITC. In the overlay those nuclei which are stained with Dapi but not OCT4 are trophoctoderm cells, those nuclei stained with OCT4 but not Nanog are primitive endoderm (PE) and those nuclei which are stained with both OCT4 and Nanog are epiblast. Scale bar is 50 μ m.

2.6 Blastocyst outgrowth formation

Blastocyst outgrowth refers to the process wherein blastocysts which are cultured on a surface which facilitates cell adhesion will attach to that surface via the trophectoderm, resulting in the extrusion and exposure of the ICM (the 'outgrowth'). This is one method by which ICM cells can be isolated for ESC derivation.

Two methodologies have been used over the course of this project, in one gelatin coated dishes (BD Biosciences BD Falcon™) were used as the surface for blastocysts to attach to (Chapter 3.0), in the other mouse embryonic fibroblast cell (MEF) coated organ well dishes (BD Biosciences BD Falcon™) were used (Chapter 6.0).

2.6.1. Gelatin coating dishes

For gelatin coated dishes, 1ml of 0.01% sterile solution (0.5g gelatin/50ml Milli-Q water) was placed in each dish intended for use, which were then incubated for 1h at 37°C. Dishes were then washed 3 times with outgrowth culture medium (section 2.6.3) and equilibrated for at least 3h at 37°C in a 6% CO₂ environment (Sanyo CO₂ Incubator: MCO15AC or Sanyo O₂/CO₂ incubator MCO-18M). Gelatin coated dishes were also used for ESC differentiation (section 2.11.4).

2.6.2 MEF coating organ well dishes

2.6.2.1 Derivation of primary MEF lines

The preparation of organ well dishes coated with a feeder layer of MEFs began with the derivation of primary MEFs. For this 12-14 day pregnant Sv129 mice were sacrificed by cervical dislocation. Their abdomens were sterilised with 70% ethanol before the peritoneum was exposed by splitting and retracting the skin. The peritoneum was then cut using surgical scissors and the visceral organs were pushed aside to allow access to the reproductive organs.

Needle point forceps were used to dissect the mesentery from the uterus which was detached by cutting below each oviduct and at its very base[332]. Uteri were then placed in PBS with penicillin/streptomycin/ fungazol (PSF) and maintained at 37°C. Uteri were then transferred to 10mm petri dishes where fetuses were extracted by dissection using surgical scissors and fine forceps. The internal organs and heads of the fetuses were removed. Fetuses were then placed in a small volume of 0.25% Trypsin (Invitrogen) at 37°C and finely diced using a sterile razor blade. This was followed by repeated pipetting up and down using a 30G needle until the fetuses were reduced to a slurry. The volume of the cell solution was then increased to 10ml using MEF culture medium (DMEM (Invitrogen)+non essential amino acids (NEAAs) (Invitrogen)+PSF) at 37°C and the concentration was calculated using a haemocytometer. Haemocytometers were loaded with 10µl of cell solution per counting chamber. The full 25 squares were counted in all cases, with cells touching the topmost and leftmost borders of the grid being included in the count, and those touching the bottommost and rightmost borders being excluded. The average number of cells was calculated for the two grids which, if multiplied by 10^4 gave number of cells per ml. Cells were then plated in 75cm² falcon flasks with filters (BD Biosciences BD Falcon™) and grown until confluent. Once confluence was reached (approximately 3 days), MEF culture medium was aspirated and cells were washed with PBS before being trypsinised using 10ml 0.25% trypsin at 37°C. Trypsin was inactivated using 20ml of MEF culture medium and concentration of cells was again calculated. Cells were then spun in a centrifuge (Rotofix 32A, Hettich, Tuttlingen, Germany) at 1800 rpm for 5 min and the supernatant was removed via vacuum suction. Cells were then resuspended in freezing medium (92% FCS, 8% DMSO which was kept on ice) so that 0.5ml contained 5×10^6 cells. Cryotube vials (Nalgene Nunc International, Rochester, NY, USA) were filled with 0.5ml of cell solution each and frozen via the 'slow freeze' method wherein cells were cooled to -80°C at -1°C per minute by being placed in a Cryo 1°C freezing container (Nalgene Nunc International) which was filled with isopropanol which was pre-cooled to 4°C then placed in a -80°C freezer. Frozen vials were kept in liquid nitrogen until thawing.

2.6.2.2 *Creation of MEF cells for dishes*

Vials were thawed in a water bath at 37°C (Ratek, Boronia, VIC, Australia) for 3 min. The volume of the thawed solution was then slowly increased to 10ml in a falcon tube (BD Biosciences BD Falcon™) with 37°C MEF culture medium. This solution was then centrifuged at

1800 rpm for 5 min in a Rotofix 32A centrifuge (Hettich) to remove freezing medium. Following aspiration the cell pellet was slowly resuspended in 10ml of 37°C MEF medium (section 2.6.3) and split between three 75cm² Falcon flasks which already contained 25ml of MEF culture medium (7×10⁴ cells/ml) warmed to 37°C. Cells were grown to confluence (generally around four days) then either passaged further, refrozen following the same protocol discussed above for primary cells, or used to create MEF coated dishes.

2.6.2.3 *Creation of MEF coated dishes*

Confluent flasks were aspirated and washed with PBS+PSF. Cells were then trypsinised with 10ml 0.25% trypsin (Invitrogen) warmed to 37°C. The trypsin was then inactivated by the addition of 20ml of MEF culture medium warmed to 37°C. The cell solution was then centrifuged at 1800rpm for 5 min to pellet the cells so that the supernatant with the trypsin could be removed. At the same time the number of cells was calculated using a haemocytometer (section 2.6.2.1). Cells were then slowly resuspended in 10ml MEF culture medium, and enough cells to seed wells at 145×10³ cells/well were placed on ice. Cells for use were mitotically inactivated by exposure to 30Gy radiation with an IBL 437C blood irradiator at the IMVS transfusion medicine centre in the Royal Adelaide Hospital. The cell solution was then increased in volume to 0.7ml of MEF culture medium per 145×10³ cells (concentration of 102×10³ cells/ml). Each organ well had its outer reservoir filled with 2ml Milli-Q water, the inner reservoir was then filled with 0.7ml of the cell solution (which was regularly agitated to avoid cells settling). Dishes were left on the bench for thirty minutes to allow cells to homogeneously settle to the bottom of the dish. Following this period cells were incubated at 37°C in 6%CO₂, 5%O₂, 89%N₂. Cells were matured for a maximum of 14 days before use.

2.6.3 *Blastocyst outgrowth*

For Chapter 3.0 blastocysts were placed in gelatin coated dishes (section 2.6.1) in outgrowth medium (Glasgow MEM supplemented with 10% FCS (Invitrogen, Carlsbad, CA)). Blastocysts were incubated at 37°C in 6%CO₂, 5%O₂, 89%N₂ for 96h before assessment of attachment and outgrowth. For chapter 6.0 ,for derivation of cell lines MEF coated organ wells were used with

outgrowth culture medium; α -MEM with glutamax, ribo- and deoxyribonucleosides, supplemented with 10% knockout serum replacement (Invitrogen, Carlsbad, CA), 1 \times non-essential amino acids (Invitrogen, Carlsbad, CA), 55 μ M β -mercaptoethanol (Invitrogen, Carlsbad, CA), 1 \times insulin-transferrin-selenium (Invitrogen, Carlsbad, CA), 20ng/ml bFGF (Chemicon, Temecula CA), 20ng/ml Activin-A (R and D systems, Minneapolis, MN), 20ng/ml human recombinant EGF (Invitrogen, Carlsbad, CA), 100ng/ml Vitronectin and 10ng/ml mouse LIF, which was pre-equilibrated to 37°C in gas phase of 6% CO₂, 5% O₂, 89% N₂. Blastocysts were washed in 20 μ l drops of MOPS-G1 handling medium with no protein under oil, pre-warmed for at least 3h, before incubation in 20 μ l drops Tyrode's Acidic solution to remove the zona pellucida. Once the zona pellucida was removed, blastocysts were then washed through three drops of MOPS-G1. Blastocysts were then placed in the outgrowth medium in the MEF coated organ wells. Attachment was assisted using the 'pressing method' wherein a 30G needle was used to gently flatten the blastocysts into the surface of the MEFs [431]. As well as potentially increasing the efficiency of attachment (not all blastocysts spontaneously attach and generate outgrowths) this process allowed tracking of individual blastocysts. Outgrowths were maintained at 37°C in a gas phase of 6% CO₂, 5% O₂, 89% N₂ for 48h. At the end of this period attachment and outgrowing were assessed as successful if there was a raised colony of cells, distinguishable from the MEFs, at the site of pressing.

2.7 PCR analysis of outgrowths

2.7.1 *RNA extraction and reverse transcription*

Individual outgrowths were snap frozen in 0.2ml tubes in liquid nitrogen. RNA was extracted from individual frozen outgrowth samples following the manufacturer's directions for the Qiagen RNeasy Micro extraction kit (Qiagen, Doncaster, Victoria, Australia). Briefly, outgrowths were placed in buffer RLT with β -mercaptoethanol to lyse cells, carrier RNA was then added, cells were vortexed, then 70% ethanol was added to homogenise the lysate. The solution was placed in a MiniElute column and spun. The flowthrough was discarded, RW1 buffer was added and the column was spun again, then the flowthrough was discarded again. DNase was placed in the column and incubated at RT, then RW1 buffer was added, the column was spun and flowthrough discarded. RPE buffer was added to the column which was spun

and flowthrough discarded. 80% ethanol was placed in column which was spun, then removed and placed in new collection tube and spun again. RNase free water was then added to the tube for 1 min, which was then spun to collect the RNA. The RNA extract was then stored at -80°C.

For reverse transcription extracted RNA (6µl) was placed on ice and made up to 14µl with 8µl of PCR grade water. The RNA was then heated at 65°C, placed on ice, spun down and placed back on ice. 6µl of a master mix of 2µl Buffer (Qiagen), 2µl dNTPs (Qiagen), 1µl Random primers (Invitrogen, Carlsbad, CA) and 1µl Sensiscript (Qiagen) was added to the RNA sample which was then incubated at 37°C for 1h during which reverse transcription took place. cDNA samples were then stored at -20°C.

2.7.2 Real time PCR

Real Time Polymerase Chain Reaction was performed using a Rotor Gene 6000 PCR machine (Corbett Life Science, Sydney, NSW) to determine the expression levels of *Atrx*, *Nanog*, *Lsh*, *Nrf-1*, and *Sirt-1*. All oligonucleotide primers were designed using Primer Express (Applied Biosystems, Victoria, Australia) and synthesised by Geneworks (Adelaide, Australia). The thermal cycling program was as follows; 5°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1min. The reaction was performed in 20µl volumes using a master mix which contained 10µl SYBR Green Mix (Applied Biosystems, Foster City, California), 5pM of each forward and reverse primer (Table 2.3), and the cDNA equivalent of '0.1' outgrowths per reaction. For the housekeeping gene, cDNA from the equivalent of '0.1' outgrowths per reaction was analysed. Delta-delta ct analysis was performed using a threshold value of 0.05 [432] as measured by Rotor Gene 6000 analysis software. The gene *18S* was used as a housekeeper. All samples were run in triplicate. The presence of only one amplified product was established by assessing melt curves.

TABLE 2.3. DETAILS OF PRIMERS USED FOR GENE EXPRESSION ANALYSIS OF INNER CELL MASS OUTGROWTHS

Gene	Accession Number	Primer Sequence	Amplicon (bp)
<i>18S</i>	AF176811	5'- AGAAACGGCTACCACATCCAA 3'- CCTGTATTGTTATTTTTCGTCACTACCT	91
<i>Atrx</i>	NM_009530	5'- TGCATGGGCCTTGTAAGAC 3'- CCTCCTGCCACTTCTCAAATTC	148
<i>Nanog</i>	NM_028016.1	5'- ATGCTGCTCCGCTCCATAAC 3'- CCCTAGTGGCTTCCAAATTCAC	108
<i>Lsh</i>	NM_008234	5'- AGGACCGTCGGAAATTAGTAAAGAA 3'- TCGCATAGCGATCTCGAATG	95
<i>Nrf-1</i>	NM_010938.3	5'- GTCCGCACAGAAGAGCAAAAAC 3'- TCCTCCCGCCCATGTTG	92
<i>Sirt-1</i>	NM_019812	5'- CGCTGAGGTATACTCAGACTCTGAAG 3'- GCATGTGCCACTGTCACTGTT	79

Atrx=ATP-dependent heterochromatin remodelling protein, *Nanog*= marker of pluripotency, *Lsh*= member of SNF2 family of chromatin remodelling proteins, nuclear respiratory factor-1, *Nrf-1*= regulator of expression of respiratory subunits and mitochondrial transcription factors, *Sirt-1*= sirtuin 1 NAD⁺ dependent deacetylase.

2.8 Assessment of outgrowth differentiation

2.8.1 Immunocytochemistry

The procedure for staining outgrowths was performed as described for blastocysts above (section 2.5.1), but with the following alterations; permeabilisation was reduced to 5 min in 0.05% TX-100, primary antibodies were applied with blocking solution, the wash step between primary and secondary antibody application was extended to 3h, the wash step between the secondary antibodies and Dapi was increased to 3 rinses at 5 min each and Dapi staining was increased to 5 min.

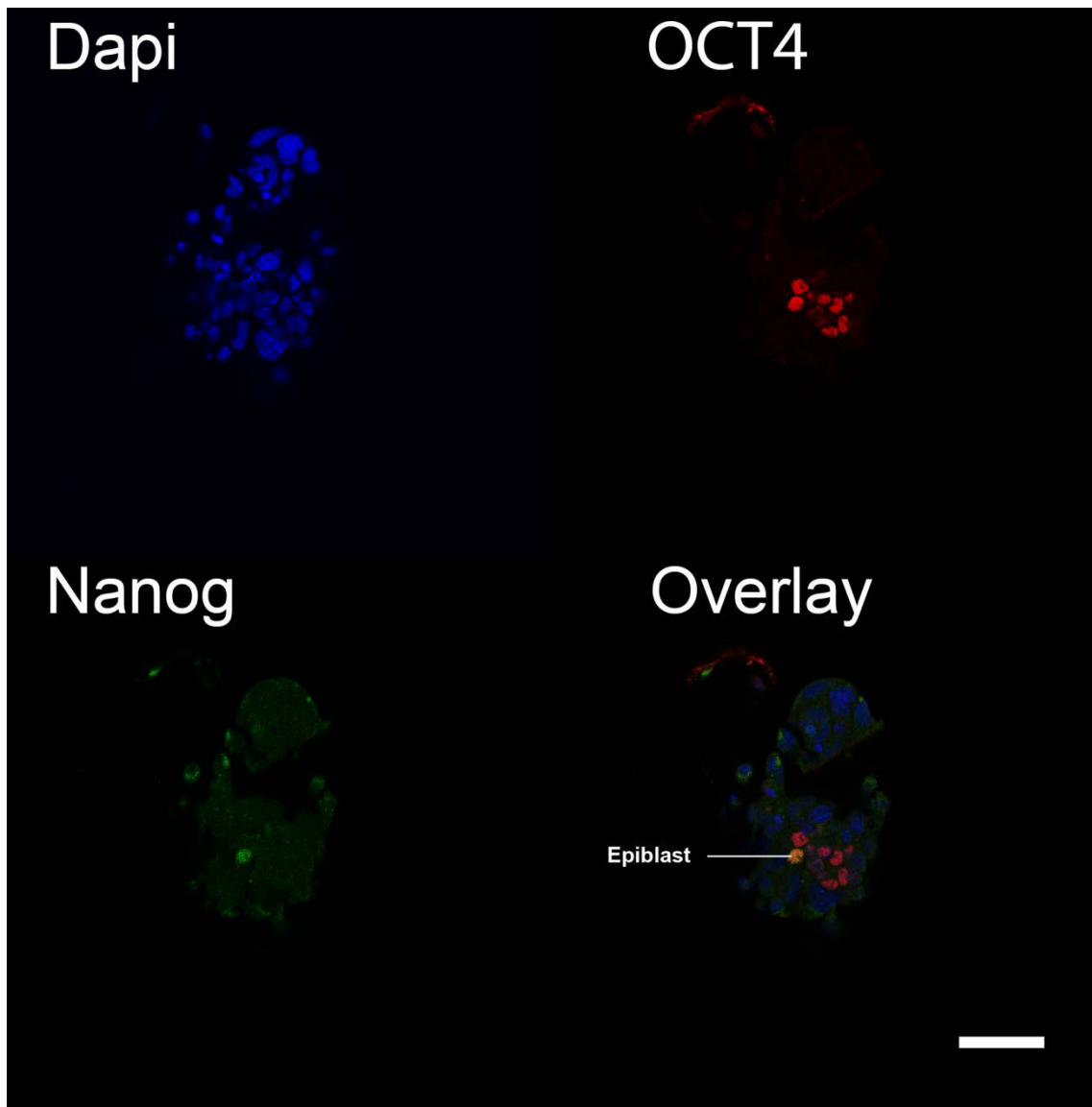


FIG.2.3. Representative images of an immunocytochemically stained outgrowth from a blastocyst plated at 115h after culture and fixed 48h later. Total cell number is stained blue with Dapi, OCT4 positive cell number is stained red with rhodamine, and Nanog positive cell number is stained green with FITC. In the overlay those nuclei which are stained with both OCT4 and Nanog are epiblast cells. Scale bar is 50 μ m.

2.9 Derivation of primary ESC colonies

For ESCs to be isolated, outgrowths were trypsinised so that those cells within with the capacity to form ESC colonies (epiblast cells) were able to grow and form primary ESC colonies.

2.9.1 Preparation of dishes

ESC colonies were derived in MEF coated 12 well dishes (BD Biosciences, BD Falcon), prepared as per (section 2.6.2.3) with the modification of seeding with 1.7×10^5 cells per well. MEF culture medium was aspirated from the 12 well dishes and replaced with 800 μ l ESC culture medium (the same as outgrowth culture medium, section 2.6.3) which had been pre-equilibrated at 37°C in a gas phase of 6% CO₂, 5% O₂, 89% N₂.

2.9.2 Isolation, trypsinisation and replating

Day 6 (115h in culture) blastocysts were used to generate primary ESC colonies. Blastocysts were plated in rows of five, with the top left corner of the grid marked by the needle used for pressing in order to provide an orientation point; in this way multiple blastocysts could be outgrown in the same dish and still be linked to their development profile. After 48h outgrowths were picked by pipetting and trypsinised in 20 μ l drops of 0.05% trypsin (Invitrogen, Carlsbad, CA) under paraffin oil (Merek KGaA, Darmstadt, Germany), pre-equilibrated for 3h at 37°C in the gas phase 6% CO₂, 5% O₂, 89% N₂. Cells were then replated onto MEFs in the 12 well dishes with ESC culture medium, with care taken that the minimum amount of trypsin solution was transferred. Cells were cultured at 37°C in the gas phase 6% CO₂, 5% O₂, 89% N₂ for 48h.

2.9.3 Identification of pluripotent colonies

2.9.3.1 Morphology

ESC colonies were initially identified by their morphology. Pluripotent colonies are multilayered with a domed shape and have a distinctive smooth appearance which easily distinguishes them from MEFs and colonies of differentiated cells [16] (Fig.2.4). Colonies which were identified as having this morphology were fixed in 4% paraformaldehyde overnight at 4°C and subjected to immunocytochemistry.

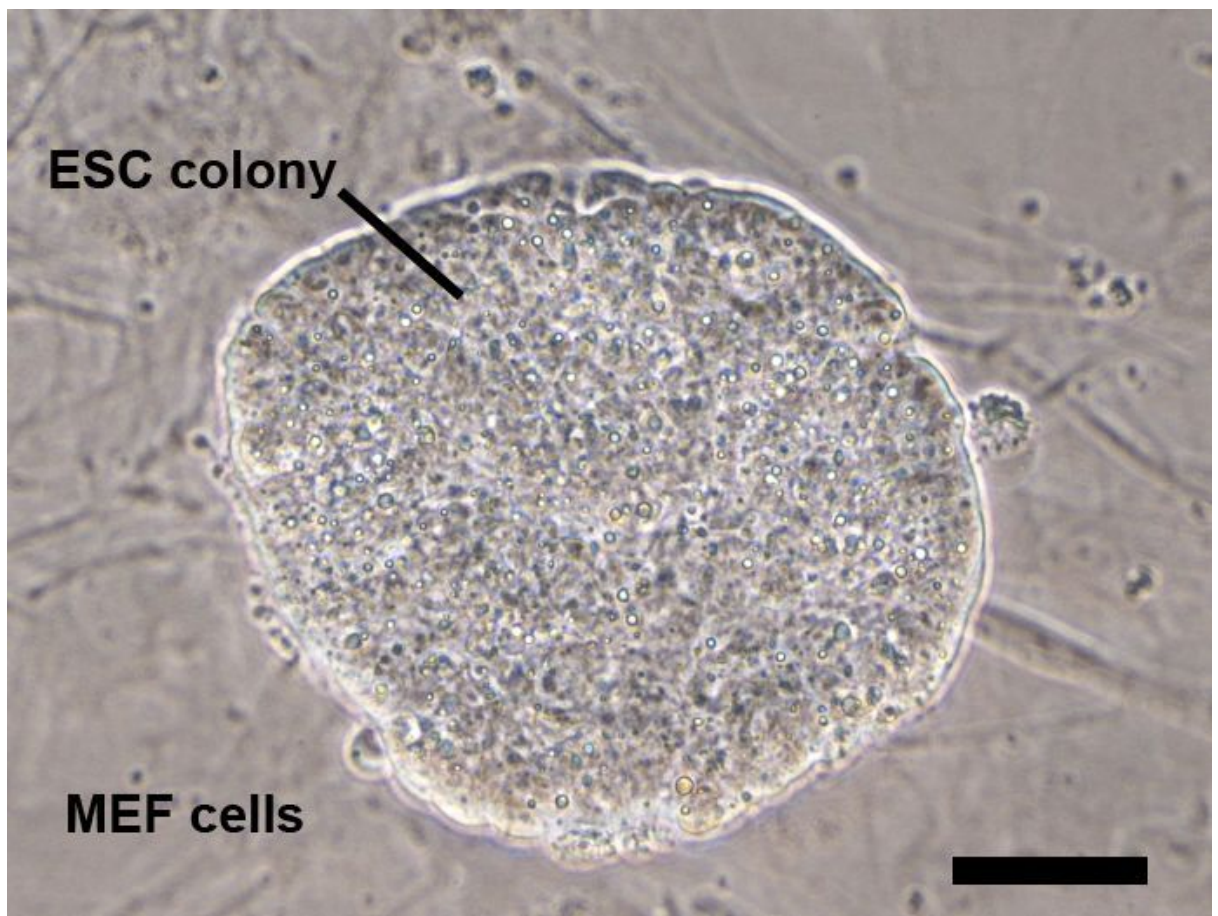


FIG. 2.4. Representative brightfield image of a primary ESC colony with a typical morphology seen 48h after the plating of the outgrowth, on a MEF feeder layer. Scale bar is 50µm.

2.9.3.2 Immunocytochemistry

Although undifferentiated colonies can be selected by their morphology, the pluripotency of primary ESC lines was confirmed by co-expression of pluripotency markers OCT4 and Nanog. Staining was performed as per (section 2.5.1). This stain was performed individually to preserve the ability to track individual outgrowths.

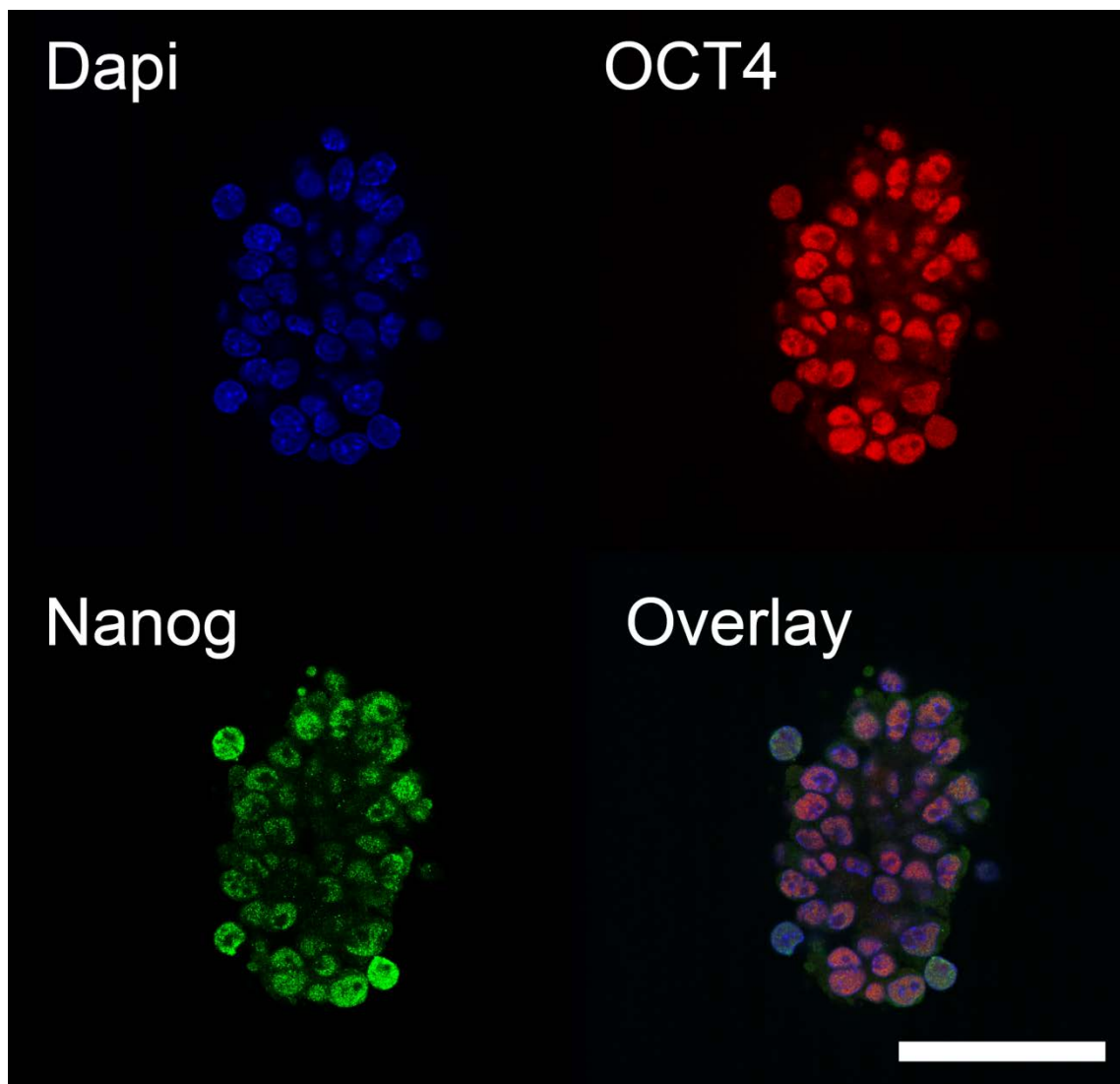


FIG. 2.5. Representative images of an immunocytochemically stained primary ESC colony stained 48h after the trypsinisation of the outgrowth from which it was derived and the identification of putative ESC morphology. Total cell number is stained blue with Dapi, OCT4 positive cell number is stained red with rhodamine, and Nanog positive cell number is stained

green with FITC. As the cells of the colony are positive for both OCT4 and Nanog they are demonstrated to be pluripotent. Scale bar is 50 μ m.

2.10 Characterisation of putative ESC lines

2.10.1 *Passaging*

To confirm that the system used was capable of generating and supporting genuine ESC lines it was necessary to expand and characterise at least one primary ESC colony as an ESC line. As such, several colonies were trypsinised in 0.05% trypsin, when they reached a diameter of approximately 100 μ m, and replated on MEFs prepared as discussed (section 2.6.2.3) in ESC culture medium in order to expand them.

This process was repeated until there were sufficient cells for cryopreservation. When lines became composed of too many colonies for “picking” to be practicable, ESC culture medium was aspirated and 0.05% trypsin (Invitrogen, Carlsbad, CA) pre-equilibrated to 37°C in the gas phase 6% CO₂, 5% O₂, 89% N₂ at least 3h prior to experimentation was used to detach colonies from MEFs. The resultant cell suspension was then mixed 1:2 with ESC culture medium supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KA, USA) to inactivate the trypsin and spun at 1300rpm for 2 min. The supernatant was then discarded and the cell pellet was resuspended in equilibrated ESC culture medium and replated.

2.10.2 *Cryopreservation*

When there were approximately 2.5 \times 10⁶ cells in culture (normally passage five) colonies were trypsinised and pelleted (as discussed above). The cell pellet was resuspended in cryopreservation medium (92% FCS with 8% DMSO) with an estimate of 0.5 \times 10⁶ cells per 1/2ml. Cryovials (Nalgene Nunc International) were filled with 0.5ml of cell solution and cooled to -80° C at a rate of -1°C per minute by being placed in a Cryo 1°C freezing container (Nalgene Nunc International), filled with isopropanol, which was pre-cooled to 4°C and placed in a -80°C freezer, then stored in liquid nitrogen.

2.10.3 *Expansion of cell lines*

For cell line expansion cryovials were removed from liquid nitrogen (at passage 5) and warmed in a 37°C water bath with constant monitoring for 3 min. When the solutions were fully defrosted their volume was increased from 0.5ml to 10ml with ESC culture medium (without LIF) after which the cell suspension was spun at 1300rpm for 2 min. The cell pellet was then resuspended in ESC culture medium and cells were plated onto MEFs. Cell lines were expanded to passage ten using the methods discussed above, transitioning from 12 well plates and organ wells to 100mm² plates (BD Biosciences, BD Falcon) and 25cm² falcon flasks (BD Biosciences, BD Falcon) as colony densities neared confluence.

2.11 Characterisation of an ESC line

2.11.1 *Alkaline phosphatase staining*

Alkaline phosphatase (a marker of pluripotency) staining was performed on a sample of cell colonies before freezing, immediately after freezing and at passage ten. Alkaline phosphatase staining was performed using a kit (Millipore, Bedford, MA, USA) and was performed according to manufacturer's instructions. Briefly, cells were fixed at RT in 4% paraformaldehyde for 1 min, then rinsed with rinse buffer and stained with the staining solution (Fast red violet stain with Naphthol AS-BI) for 15 min in the dark. Colonies were then rinsed with rinse buffer and covered with PBS. Colonies were imaged by brightfield microscopy on a TS 100 (Nikon, Tokyo, Japan). Total number of colonies positive for alkaline phosphatase activity and total number of colonies negative for alkaline phosphatase activity were counted.

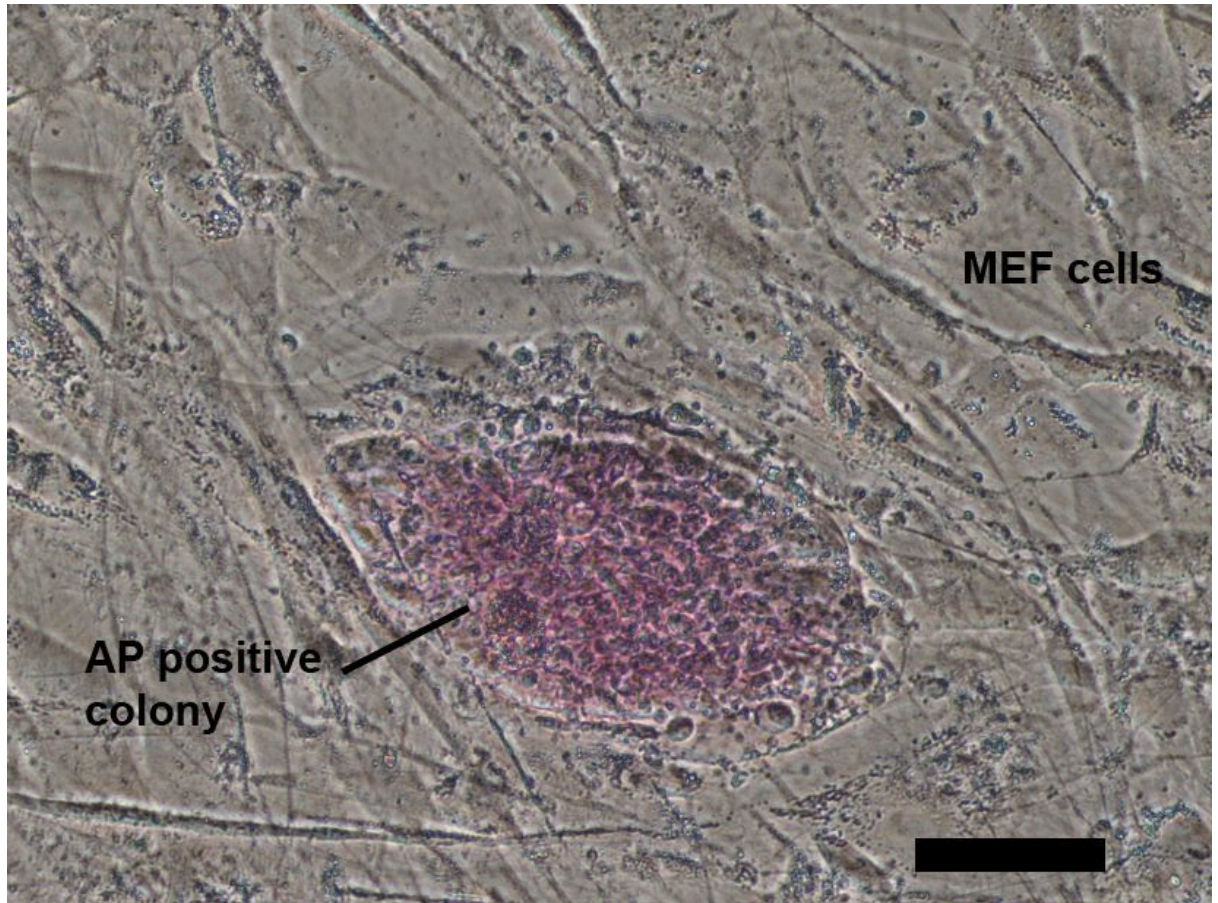


FIG. 2.6. Representative bright field image of an ESC colony which has stained positive for alkaline phosphatase (AP) activity, as shown by pink staining. Scale bar is 50 μ m.

2.11.2 ***SSEA1 staining***

SSEA1 (a marker of pluripotency [31]) staining was performed on a sample of cell colonies before freezing, immediately after freezing, and at passage ten. Staining was performed with anti-mouse SSEA1 antibody conjugated to FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-101462) which was diluted to 1:200 in ESC culture medium which colonies were then exposed to for 1h at 37°C in a gas phase of 6% CO₂, 5% O₂, 89% N₂. Colonies were then imaged using epifluorescence at wavelength ex: 460-500nm. Total number of colonies positive for SSEA1 expression (at least half of the colony's area fluorescing) and total number of colonies negative for SSEA1 expression were counted.

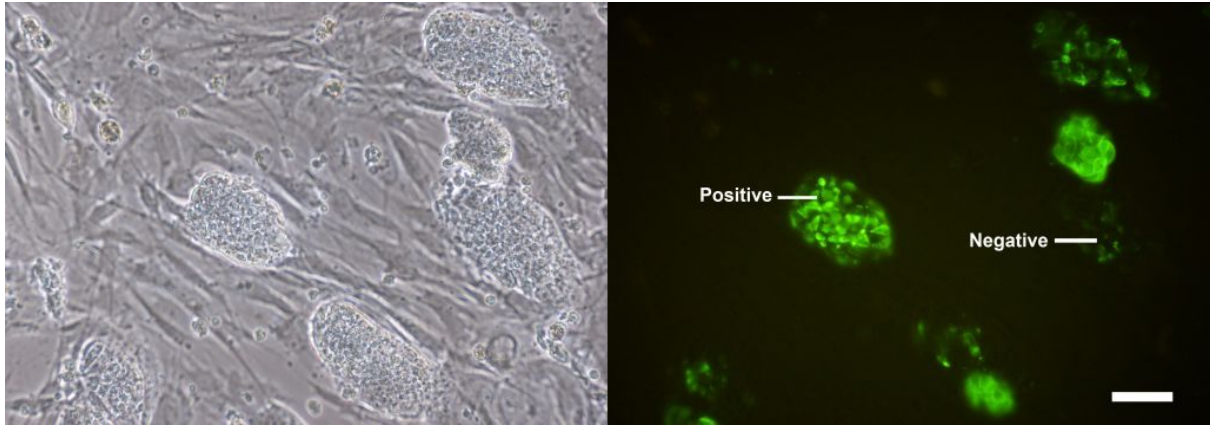


FIG.2.7. A brightfield image of ESC colonies and an epifluorescent image of those same colonies stained for SSEA1, shown in green. Scale bar is 50 μ m.

2.11.3 ***Karyotyping***

In order to demonstrate that colonies could be propagated through ten passages with freezing and thawing and maintain a normal karyotype, karyotype analysis was performed by the cytogenetics laboratory at the Women's and Children's Hospital, Adelaide on passage ten cells that had grown to near confluence on a 25cm² flask. Ten cells were fully karyotyped by G-banding, while twenty were counted and found to be XX and to have the normal 40 chromosomes.

2.11.4 ***Directed differentiation***

To directly confirm the pluripotency of the cell line, the directed differentiation protocols discussed below were applied to direct the pluripotent cells to differentiate and give rise to cells descended from the three germ layers; mesoderm, endoderm and ectoderm. Cells from the established commercial D3-ES cell line which is known to be pluripotent were used as positive controls to confirm the efficacy of the differentiation and staining protocols (Fig.2.8).

2.13.4.1 Mesoderm differentiation

For differentiation towards a mesoderm cell fate ESCs which reached passage ten were plated at 2000 cells/cm² on gelatin coated dishes (section 2.6.1) in mesoderm differentiation medium: KO-DMEM (Invitrogen) supplemented with N-2 (Invitrogen), B27 (Invitrogen), NEAAs (Gibco, Gaithersburg, MD, USA), glutamax (Gibco), PSF, 0.1mM 2-mercaptoethanol (Gibco) and 10ng/ml BMP4 (MBL international corporation, Woburn, MA, USA) [64]. Cells were cultured for five days with medium changed every second day, at which point the culture was fixed and stained for markers of mesoderm differentiation (section 2.13.4.4).

2.11.4.2 Definitive endoderm differentiation

For differentiation towards a definitive endoderm cell fate ESCs which reached passage ten were plated at 2000 cells/cm² on gelatin coated dishes (section 2.6.1) in definitive endoderm differentiation medium: KO-DMEM supplemented with N-2 supplement, B27 supplement, NEAAs, glutamax, PSF, 0.1mM 2-mercaptoethanol and 100ng/ml Activin-A (R and D systems, Minneapolis, MN) [64,65]. Cells were cultured for five days with medium changed every second day, at which point they were fixed and stained for markers of definitive endoderm differentiation (section 2.13.4.4).

2.13.4.3 Neuroectoderm differentiation

For differentiation towards a neuroectoderm cell fate ESCs which reached passage ten were plated at 5000 cells/cm² on gelatin coated dishes (section 2.6.1) in neuroectoderm differentiation medium: 1:1 KO-DMEM/F12(Gibco) supplemented with N-2 supplement; and neurobasal medium (Invitrogen) supplemented with B27 supplement [62,63]. Cells were cultured for seven days with medium changed every second day, at which point they were fixed and stained for markers of neuroectoderm differentiation (section 2.13.4.4).

2.13.4.4 Differentiation immunocytochemistry

Differentiated cells were fixed on the dish overnight at 4°C in 4% paraformaldehyde after washing in PBS. Cells were then rinsed twice with PBS and incubated for 10 min in 0.1M Glycine at RT followed by a further two PBS rinses. Then cells were permeabilised with Triton-X at 0.1% in PBS for 5 min at RT and washed twice with PBS. Blocking was performed with 10% donkey serum (Sapphire Bioscience, Redfern, New South Wales, Australia) for 1h at RT, after which cells were washed with PBS and incubated with their primary antibody over night at 4°C.

Cells from the mesoderm directed differentiation treatment were stained for VEGFR2 and CXCR4. VEGFR2 is a cell surface marker expressed by mesoderm and mesendoderm [433] and was stained using a goat polyclonal antibody (Santa Cruz Biotechnology, sc-48161) at 1:100 in PBS. CXCR4 is a cell surface marker of definitive endoderm and mesoderm [433] and was stained using a rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-9046) at 1:100 in PBS. Co-expression of these two markers indicates a mesoderm cell fate [433].

Cells from the definitive endoderm directed differentiation treatment were stained for SOX17 and CXCR4. SOX17 is a nuclear marker of visceral and definitive endoderm [434] and was stained using a goat polyclonal antibody (Santa Cruz Biotechnology, sc-17356) at 1:100 in PBS. CXCR4, as discussed above, marks definitive endoderm and mesoderm [433] and was stained as above. Co-expression of these two markers indicates a mesoderm cell fate [434].

Cells from the neuroectoderm directed differentiation treatment were stained for Nestin. Nestin is a cytoskeletal marker of neuroectoderm and was stained for using a rabbit polyclonal antibody (Santa Cruz Biotechnology, sc-20978) at 1:100 [435] in PBS.

After incubation with the primary antibodies cells were rinsed twice in PBS and incubated for 2h at 37°C with polyclonal anti-goat antibody conjugated to Texas red (Sapphire Bioscience, Redfern, New South Wales, Australia) at 1:100 and/or polyclonal anti-rabbit antibody conjugated to Alexa Fluor 488 (Invitrogen, Mulgrave Victoria, Australia). After a final two PBS washes cells were imaged on a TS 100 (Nikon, Japan) microscope using epifluorescence. Cells stained with the secondary antibodies without the primary antibodies were used as a negative control. Cells from the established commercial D3-ES cell line which is known to be pluripotent were used as positive controls (Fig. 2.8).

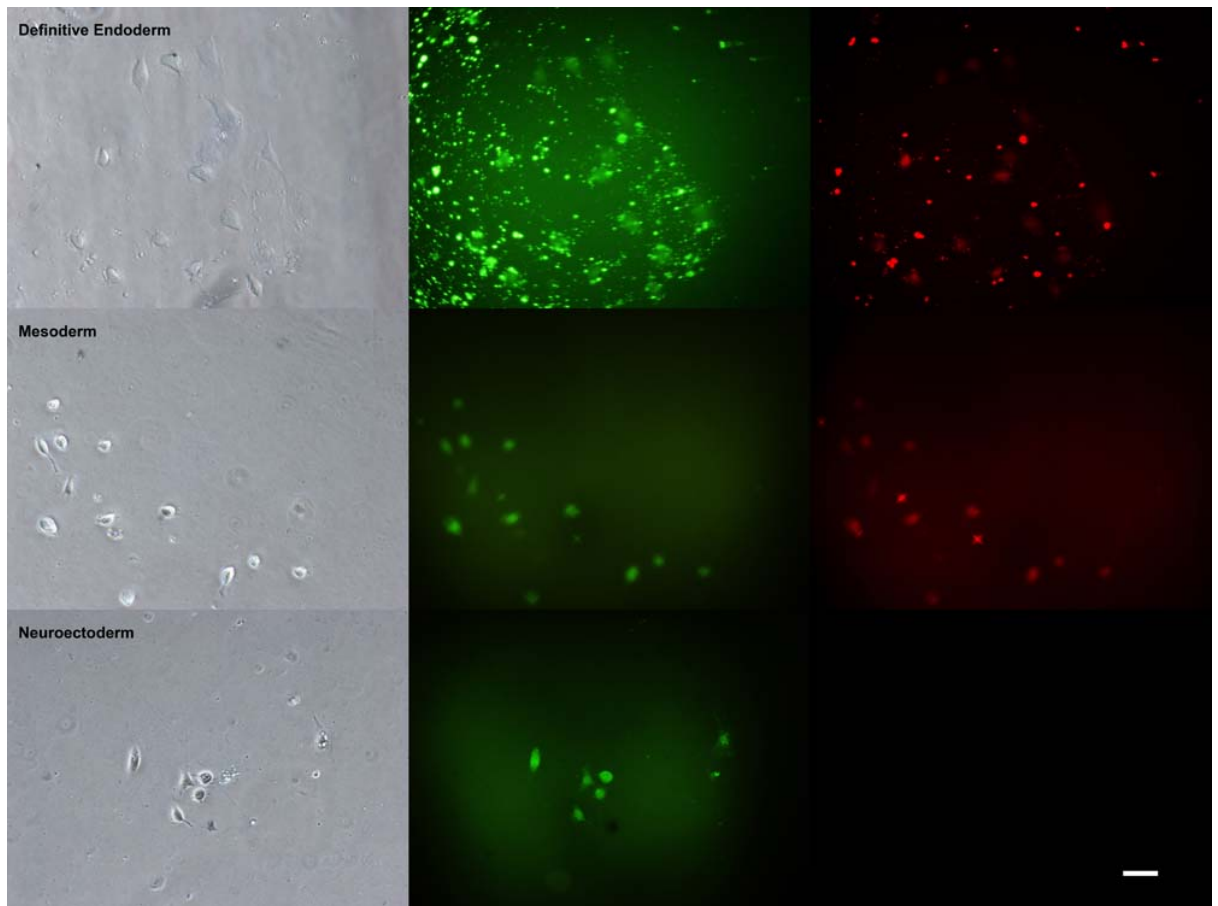


FIG. 2.8. Brightfield and epifluorescent images of differentiated cells from the established commercial D3-ES cell line which have been subjected to the indicated differentiation protocol. Definitive endoderm: green is CXCR4 (fusin), cell surface marker of definitive endoderm and mesoderm; red is SOX17, nuclear marker of endoderm (primitive and definitive). Co-expression shows definitive endoderm. Mesoderm: green is CXCR4 (fusin), cell surface marker of mesoderm and definitive endoderm; red is VEGFR2 (FLK-1), cell surface marker of mesoendoderm and mesoderm. Co-expression shows mesoderm. Neuroectoderm: green is Nestin, cytoskeletal marker of neuroectoderm. Scale bar is 50 μ m.

3.0 Development of a mouse model for studying the effect of embryo culture on embryonic stem cell derivation.

The work presented in this chapter has been published in the following journal:

Campbell JM, M Mitchell, MB Nottle and M Lane. (2011). Development of a mouse model for studying the effect of embryo culture on embryonic stem cell derivation. *Stem Cells Dev* 20:1577-86.

Statement of authorship contributions

Jared M. Campbell: Designed and performed all experiments, interpreted and analysed data and wrote manuscript

Megan Mitchell: Provided intellectual input, assisted with experimental design, data interpretation and manuscript preparation.

Mark B. Nottle: Provided intellectual input, assisted with experimental design and manuscript preparation.

Michelle Lane: Principal project supervisor, assisted with experimental design, data analysis, data interpretation, manuscript preparation and is corresponding author.

Development of a Mouse Model for Studying the Effect of Embryo Culture on Embryonic Stem Cell Derivation

Jared M. Campbell,^{1,2} Megan Mitchell,¹ Mark B. Nottle,^{2,3} and Michelle Lane¹

For most of the derived human embryonic stem cell (ESC) lines thus far, the majority of human embryos used have been frozen in liquid nitrogen at or prior to the compacting stage for up to 10 years before human ESC derivation. As such they were grown in media that were relatively simple in their formulation compared with those used today. Here we report that culture of mouse embryos in media similar to these produces blastocysts in which both the inner cell mass cell number and the number of ESC progenitor cells (epiblast cells) in the inner cell mass are reduced compared with blastocysts cultured in a purpose-designed sequential (G1/G2) system commonly used today. Embryos cultured in a simple medium were less likely to attach and generate outgrowths. Further, these outgrowths had increased metabolic activity, which has been linked to differentiation, and altered gene expression. Culture of embryos in a simple medium to the compacting stage followed by culture in G2 to the blastocyst stage reduced some of these effects. However, none were improved to the level seen for culture in G1/G2. These results highlight the influence of embryo culture on embryo quality and pluripotency, which is a key factor in determining ESC isolation efficiencies.

Introduction

EMBRYONIC STEM CELLS (ESCs) are derived from ESC progenitor epiblast cells found in the inner cell mass (ICM) cells of expanded blastocysts [1–3]. Since human ESCs (hESCs) were first isolated [4,5], numerous lines have been produced. However, these vary in quality and frequently display aneuploidy, slow growth, and spontaneous differentiation [6–9]. In general, there is more reported variation in cell quality compared with mouse ESC (mESC) models [7]. mESCs are routinely isolated from blastocysts that develop *in vivo*, whereas hESCs are derived from excess embryos from assisted reproductive technologies such as *in vitro* fertilization or intracytoplasmic sperm injection [10]. The period that human embryos can legally be cryopreserved varies; 5–10 years in Australia [11], 10 years in the United Kingdom [12], and indefinitely in the United States [13,14]. Consequently, before their cryopreservation donated embryos have frequently been cultured in relatively simple culture systems compared with those used today [15–18], and are likely to have been exposed to a range of conditions subsequently shown to perturb development, including high oxygen [19,20], too high pH or inadequate buffering [21], no amino acids (or the wrong amino acids for the developmental stage) [16,22,23], no chelators [24,25], and serum as a source of protein [17,25]. These conditions are known to affect embryo

development and many do not adequately support growth to the blastocyst stage [26,27]; this resulted in the original establishment of transfer protocols, dictating that human embryos be transferred to the recipient or cryopreserved at the cleavage stage [28,29]. The aim of the present study was to develop a mouse model that mimicked these culture conditions to determine what effect culture media have on ESC progenitor cell number and primary outgrowth formation. As well, we determined whether this can be improved for embryos cultured in a simple medium to the compacting stage by culturing them subsequently in G2 medium, a medium purpose-designed for the physiological requirements of the embryo from postcompaction to the blastocyst stage.

In our investigation we have cultured embryos in the purpose-designed culture media system G1/G2 (Table 1), which contain different concentrations of carbohydrates, amino acids, vitamins, and the chelator ethylenediaminetetraacetic acid (EDTA) for pre- and postcompaction culture [16,22–25], or a simple medium modeled after historic culture conditions lacking amino acids, vitamins, EDTA, and with the same concentrations of carbohydrates throughout culture (Table 1). We hypothesized that culture in the simple medium would affect the number of ESC progenitor cells in embryos, as well as other factors relating to ESC derivation, compared with culture in the purpose-designed system.

¹School of Paediatrics and Reproductive Health, ²Centre for Stem Cell Research, and ³Robinson Institute, University of Adelaide, Adelaide, South Australia.

TABLE 1. MEDIA FORMULATIONS

Component	Simple culture media (mM)	G1 culture media (mM)	G2 culture media (mM)
NaCl	90.1	90.1	90.1
KCl	5.5	5.5	5.5
NaH ₂ PO ₄	0.25	0.25	0.25
MgSO ₄	1.0	1.0	1.0
NaHCO ₃	25.0	25.0	25.0
CaCl ₂	1.8	1.8	1.8
Glucose	0.5	0.5	3.15
L-Lactate	10.5	10.5	5.87
Pyruvate	0.32	0.32	0.1
EDTA	—	0.01	—
Alanyl-glutamine	—	0.5	1.0
Alanine	—	0.1	0.1
Arginine	—	—	0.6
Asparagine	—	0.1	0.1
Aspartate	—	0.1	0.1
Cystine	—	—	0.1
Glutamate	—	0.1	0.1
Glycine	—	0.1	0.1
Histidine	—	—	0.2
Isoleucine	—	—	0.4
Leucine	—	—	0.4
Lysine	—	—	0.4
Methionine	—	—	0.1
Phenylalanine	—	—	0.2
Proline	—	0.1	0.1
Serine	—	0.1	0.1
Taurine	—	0.1	—
Threonine	—	—	0.4
Tryptophan	—	—	0.5
Tyrosine	—	—	0.2
Valine	—	—	0.4
Choline chloride	—	—	0.0072
Folic acid	—	—	0.0023
Inositol	—	—	0.01
Nicotinamide	—	—	0.0082
Pantothenate	—	—	0.0042
Pyridoxine	—	—	0.0049
Riboflavin	—	—	0.00027
Thiamine	—	—	0.00296
Albumin	5 mg/mL	5 mg/mL	5 mg/mL

Media were made to be at pH 7.3±0.05 at 6% CO₂; osmolarity was 255±5, 255±5, and 261±5 for simple, G1, and G2. Media were prepared as per [34].

EDTA, ethylenediaminetetraacetic acid.

The primary difference between the simple medium and G1, the first stage of the G1/G2 sequential culture system, is the presence of amino acids and EDTA in G1 (Table 1). The absence of amino acids or EDTA has been shown to reduce the size of the ICM in humans [22] and bovines [30], respectively. Therefore, we have investigated the effect of culturing embryos in a simple medium during cleavage before transfer to G2 at compaction. In this way we have modeled the culture of human embryos used for hESC derivation and investigated whether the subsequent culture of embryos, perturbed by simple media during their most sensitive period of development [15,31,32], in a purpose-designed medium tailored to the physiological requirements of a postcompaction embryo, influences factors related to ESC derivation.

Methods

Experimental design

The MOPS-G1 handling medium was used for zygote collection and manipulation [33], and cleavage-stage embryos were cultured in G1 or simple medium for 48 h (Table 1) (0–48 h, precompaction). Embryos were then washed and further cultured in G2 (Table 1) or simple culture medium to the blastocyst stage (48–96 h, postcompaction). At the blastocyst stage differential or Nanog immunocytochemical staining was performed, or blastocysts were plated on gelatin-coated dishes to attach and outgrow. Outgrowths were subjected to reverse transcription (RT)-polymerase chain reaction (PCR) or JC1 staining.

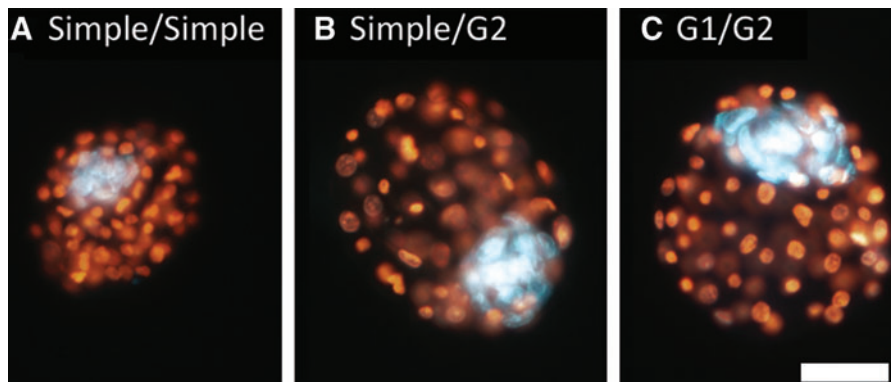


FIG. 1. Representative images of differentially stained blastocysts: trophoblast cell nuclei are stained with both stains, whereas inner cell mass (ICM) cell nuclei are stained with bisbenzimidazole. Scale bar = 50 μ m. (A) Simple/simple, (B) simple/G2, and (C) G1/G2. Color images available online at www.liebertonline.com/scd

Media and chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. All chemicals and consumables used in this study were screened for their compatibility with embryo development in a one-cell mouse embryo assay [34].

Embryo collection, culture, and assessment

Approval for all procedures was obtained from The University of Adelaide Animal Ethics Committee, in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. All mice were kept in a 14:10 light:dark cycle, and fed *ad libitum*. Female mice (C57BL/6 \times CBA F1 hybrid), aged 3–4 weeks, were given an intraperitoneal injection of 5 IU equine chorionic gonadotropin (Folligon; Intervet Australia Pty Ltd.), followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Pregnyl; Organon) to induce ovulation. Immediately after hCG injection female mice were placed with a male mouse of the same strain and mating was assessed the following morning by the presence of a vaginal plug. Zygotes were flushed from the oviducts 22 h post-hCG into MOPS-G1 and denuded of surrounding cumulus cells using 50 IU/mL hyaluronidase for up to 2 min. Denuded zygotes were then washed in MOPS-G1 and cultured to the blastocyst stage. The 3 treatment groups used for this study were (1) simple medium (precompaction) followed by simple medium (postcompaction), (2) simple medium followed by G2 medium, and (3) G1 medium followed by G2 medium. The simple medium (prepared inhouse) was a model of historic media containing no chelators, vitamins, or amino acids and possessing the same concentration of carbohydrates for pre- and postcompaction development (Table 1), and is known to perturb embryo development [21], whereas the G1/G2 sequential media system (prepared inhouse) was selected as it is a currently used culture system known to support mouse and human embryo development [35–37]. All embryos were cultured in groups of 10 in 20 μ L drops of medium overlaid with paraffin oil (Merek KGaA) at 37°C in 6% CO₂, 5% O₂, 89% N₂. After 48 h of culture all embryos were transferred to a fresh medium for a further 48 h of culture at 37°C, 5% O₂, 6% CO₂, 89% N₂.

Assessment of ICM and trophoblast cell number

Assessment of the number of ICM and trophoblast (TE) cells in blastocysts was performed using a previously described method [38]. Briefly, blastocysts were placed in

0.5% pronase to dissolve the zona pellucida. Embryos were then incubated in 10% TNBS (picrylsulfonic acid) for 10 min at 4°C before being washed and incubated in 10% anti-DNP at 37°C for 10 min. Blastocysts were again washed and incubated in guinea pig serum (Gibco) in propidium iodide (25 μ g/ μ L) solution for 5 min. Embryos were then stained in 6 μ g/mL bisbenzimidazole in ethanol overnight and washed in 100% ethanol before mounting in glycerol on a microscope slide the following day. Blastocysts were observed using a fluorescent microscope, with TE cells appearing pink and ICM cells appearing blue under a ultraviolet filter (Fig. 1). Cell number was counted independently for each cell type. *In vivo*-derived blastocysts were used to validate the methodology.

Immunocytochemistry

The presence of Nanog protein in blastocysts was measured using immunocytochemical staining. Blastocysts were fixed in 4% paraformaldehyde overnight at 4°C, followed by incubation in 0.1 M glycine in phosphate-buffered saline (PBS) for 5 min at room temperature. Blastocysts were permeabilised in fresh PBS with 0.25% Triton X-100 (PBS-TX) for 30 min at room temperature, and then blocked in 10% Normal Donkey Serum (Sapphire Bioscience) for 30 min at room temperature. Embryos were incubated with Nanog rabbit anti mouse polyclonal antibody (Sapphire, Cat#120-21603) at 1:200 in PBS-TX overnight at 4°C, washed the next day in PBS, and incubated with Donkey anti rabbit secondary antibody (1:100) conjugated to fluorescein isothiocyanate (Australian Laboratory Services) for 2 h at 37°C. Finally, embryos were incubated with 3 nM of nuclear stain 4'-6-diamidino-2-phenylindole at room temperature for 2–3 min, before imaging using confocal microscopy (Nikon, EZ-C1 software). A negative control was performed where primary antibody was not applied (Fig. 2). A positive control of *in vivo*-cultured blastocysts was included to validate the methodology.

Blastocyst attachment and outgrowth

Blastocysts were placed in culture dishes coated with gelatin (0.1%) [39,40] and cultured in Glasgow minimal essential medium (MEM) supplemented with 10% fetal calf serum (Invitrogen) for 96 h. Attachment of embryos to the dish was assessed, and outgrowth morphology determined using criteria from [41], and classified as small (20 μ m), medium (40 μ m), or large (60 μ m) outgrowths (Fig. 3). After morphological

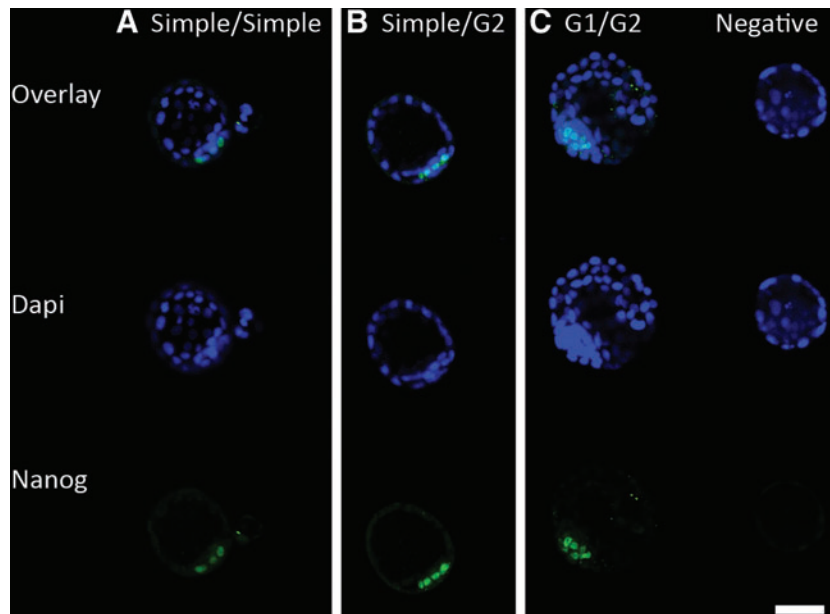


FIG. 2. Representative images of immunocytochemically stained blastocysts: all cell nuclei are stained with 4'-6-diamidino-2-phenylindole (DAPI), and Nanog-positive cells are stained with fluorescein isothiocyanate. Scale bar=50 μ m. (A) Simple/simple, (B) simple/G2, and (C) G1/G2. Color images available online at www.liebertonline.com/scd

assessment, individual ICM cell outgrowths were removed from the TE stalks and assessed for mitochondrial parameters or frozen at -80°C for analysis of gene expression.

Measurement of mitochondrial membrane potential

The ratiometric dye JC1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide; Molecular Probes) was used to determine mitochondrial membrane potential (MMP) [42]. ICM outgrowths were incubated in 1.5 μM JC1 in MOPS-G1 for 15 min [43]. The outgrowths were then immediately imaged using confocal microscopy (Nikon, EZ-C1 software) and the MMP assessed using IP Lab (Scanalytics V3.61) as described previously [38]. TE cells were clearly distinguishable and excluded from analysis.

RNA extraction, reverse transcription, and real-time RT-PCR

RNA was extracted from individual frozen outgrowth samples following the manufacturer's directions for the Qiagen RNeasy Micro extraction kit (Qiagen). Reverse transcription was carried out using Sensiscript (Qiagen) and samples were stored at -20°C before PCR. Real-time PCR was performed using a Rotor Gene 6000 PCR machine (Corbett Life Science) to determine the expression levels of *Atrx*, *Nanog*, *Lsh*, *Nrf-1*, and *Sirt-1*. The thermal cycling program was as follows: 5°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and 60°C for 1 min. The reaction was performed in 20 μL volumes using a master mix containing 10 μL SYBR Green Mix (Applied Biosystems), 5 pM of each

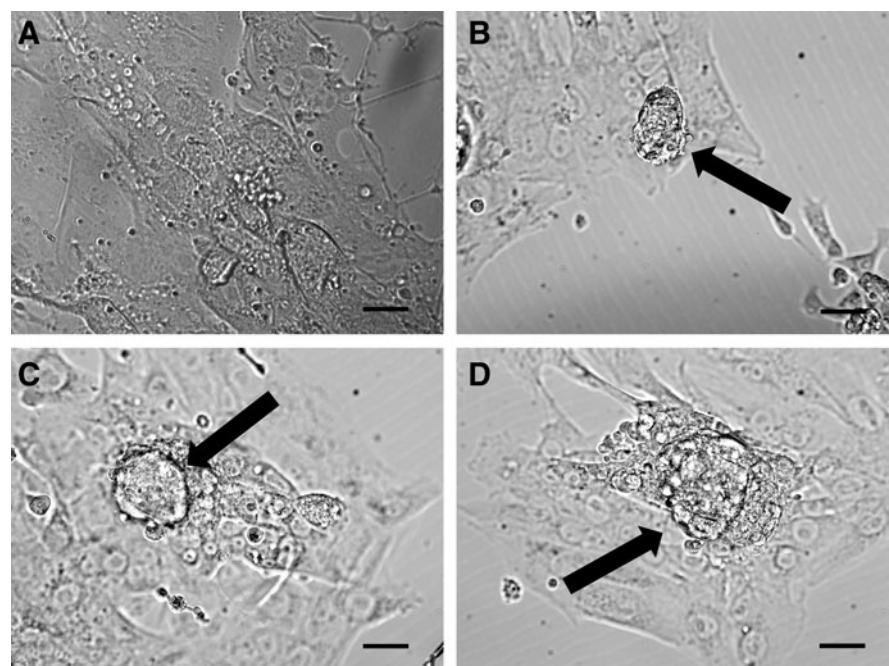


FIG. 3. Representative images of outgrowths for each morphological category. Outgrowth morphology was assessed on day 4 after plating. Scale bar=50 μ m. Arrows indicate outgrowths. (A) Absent outgrowth, (B) small outgrowth, (C) medium outgrowth, and (D) large outgrowth.

TABLE 2. DETAILS OF PRIMERS USED FOR GENE EXPRESSION ANALYSIS OF INNER CELL MASS OUTGROWTHS

Gene	Accession no.	Primer sequence	Amplicon (bp)
18S	AF176811	5'-AGAAACGGCTACCACATCCAA 3'-CCTGTATTGTTATTTTCGTCACCT	91
<i>Atrx</i>	NM_009530	5'-TGCATGGGCCTTGGTAAGAC 3'-CCTCCTGCCACTTCTCAAATC	148
<i>Nanog</i>	NM_028016.1	5'-ATGCTGCTCCGCTCCATAAC 3'-CCCTAGTGGCTTCCAAATTCAC	108
<i>Lsh</i>	NM_008234	5'-AGGACCGTCGGAAATTAGTAAAGAA 3'-TCGCATAGCGATCTCGAATG	95
<i>Nrf-1</i>	NM_010938.3	5'-GTCCGCACAGAAGAGCAAAAAC 3'-TCCTCCCGCCCATGTTG	92
<i>Sirt-1</i>	NM_019812	5'-CGCTGAGGTATACTCAGACTCTGAAG 3'-GCATGTGCCACTGTCACCTGTT	79

Atrx, ATP-dependent heterochromatin remodeling protein; *Nanog*, marker of pluripotency; *Lsh*, member of SNF2 family of chromatin remodeling proteins, nuclear respiratory factor-1; *Nrf-1*, regulator of expression of respiratory subunits and mitochondrial transcription factors; *Sirt-1*, sirtuin 1 NAD⁺-dependent deacetylase.

forward and reverse primer (Table 2), and the cDNA equivalent of "0.1" outgrowths per reaction. For the housekeeping gene, cDNA from the equivalent of "0.1" outgrowths per reaction was analyzed. Delta-delta ct analysis was performed using a threshold value of 0.05 [44]. The gene *18S* was used as a housekeeper. The presence of only one amplified product was established by assessing melt curves and by running the PCR products on a gel.

Statistics

All data are expressed as mean ± standard error of the mean. Culture environment was fitted as a fixed factor and replicate was fitted as a cofactor in all analyses. Data were analyzed using Univariate General Linear Model using SPSS 15.0 or chi-square tests. Between treatment differences were assessed using the least significant difference method. Values of $P < 0.05$ were considered significant.

Results

Embryo development

The proportion of embryos that reached the expanded or hatched blastocyst stage after 96 h of culture was significantly less when embryos were cultured in simple/simple medium compared with culture in G1/G2 ($P < 0.01$, Table 3). Culture in simple/G2 medium did not increase development compared with culture in simple/simple medium (Table 3).

Assessment of blastocyst ICM and TE cell numbers

Blastocysts cultured in G1/G2 medium had significantly more total cells than those cultured in simple/simple medium ($P < 0.05$, Fig. 4A). However, TE cell number did not change (Fig. 4B). ICM development was higher in embryos that were cultured in the G1/G2 system than in those cultured in the simple/simple or simple/G2 systems. Culture in simple/G2 medium increased the number of ICM cells in the blastocyst ($P < 0.05$, Fig. 4C) compared with those cultured in simple/simple media. Culture in G1/G2 media resulted in a higher number of ICM cells as a proportion of the total cells in the blastocyst compared with embryos cultured in simple/simple or simple/G2. However, culture in simple/

G2 media increased this proportion compared with culture in simple/simple ($P < 0.05$, Fig. 4D).

Pluripotency of ICM cells in cultured blastocysts

The number of pluripotent cells in blastocysts was investigated using staining for Nanog protein, a marker of pluripotency that, in the blastocyst, is expressed in epiblast cells—the progenitors of ESCs [45]. Culture in the G1/G2 system produced blastocysts that had significantly more Nanog-positive cells than those cultured in the simple/simple system ($P < 0.01$; Table 4). Culture in simple/G2 resulted in an increased number of Nanog-positive cells relative to the simple/simple treatment ($P < 0.01$, Table 4). Not all blastocysts contained Nanog-positive cells. The G1/G2 system produced the largest proportion of blastocysts possessing Nanog-positive cells; however, there was no difference between the simple/simple and simple/G2 systems ($P < 0.05$, Table 4). This indicates that culture in G2 after culture in simple does not increase the proportion of blastocysts with the capacity to give rise to ESC lines. Our findings were validated by staining in vivo-cultured blastocysts for Nanog. As all in vivo blastocysts contained cells that were positive for Nanog, we can conclude that among in vitro-cultured blastocysts a finding of zero Nanog-positive cells was not a technical artefact.

Attachment and outgrowth size

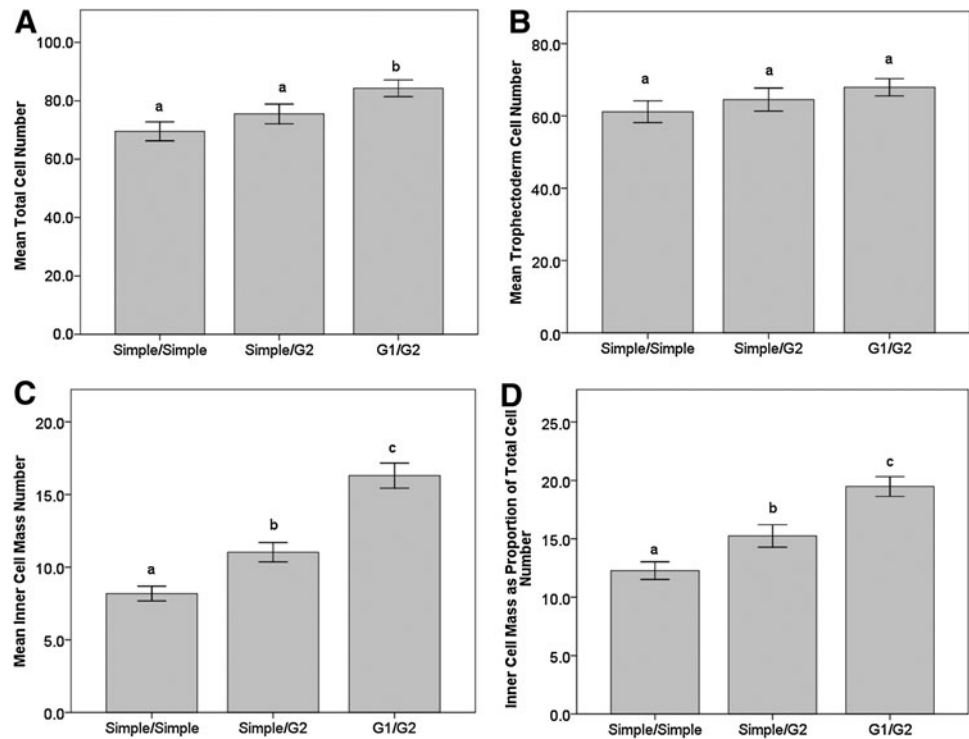
Blastocysts cultured in simple/simple medium had significantly lower rates of attachment compared with those

TABLE 3. THE EFFECT OF DIFFERENT CULTURE SYSTEMS ON MOUSE EMBRYO DEVELOPMENT TO THE BLASTOCYST STAGE

Culture media 0–48 h	Culture media 48–96 h	Total blastocyst (%) ^A
Simple	Simple	71 ^a
Simple	G2	75 ^{ab}
G1	G2	80 ^b

^ATotal number of blastocysts as proportion of embryos cleaved after 20 h of culture shown as a percentage. $n \geq 411$ embryos per treatment (15 replicate experiments). Different superscripts are significantly different: a, b ($P < 0.01$).

FIG. 4. The effect of culture system on mouse blastocyst cell number and differentiation. **(A)** Total cell number. **(B)** Trophectoderm cell number. **(C)** ICM cell number. **(D)** Number of ICM cells as a proportion of total cell number. Data are mean \pm standard error of the mean. $n \geq 37$ (8 replicate experiments). Different superscripts are significantly different: a, b, c ($P < 0.05$).



cultured in G1/G2 ($P < 0.001$, Table 5). Further, in the G1/G2 and simple/G2 treatment, the size of the ICM outgrowths was significantly larger than outgrown embryos cultured in simple/simple ($P < 0.05$, Table 5). Conversely, culture in the simple/simple medium significantly increased the number of small outgrowths compared with G1/G2 ($P < 0.05$, Table 5).

Mitochondrial membrane potential

The average overall MMP within outgrowths was significantly lower for outgrowths obtained from blastocysts cultured in the G1/G2 culture system compared with outgrowths from blastocysts cultured in simple/simple or simple/G2 ($P < 0.05$, Table 6). Further, when the mean highest and lowest regions of MMP for outgrowths from the different treatments were compared, outgrowths from simple/simple or simple/G2 cultured embryos were found to have a higher maximum MMP compared with G1/G2, while G1/G2 embryos produced outgrowths with a lower minimum than simple/simple or simple/G2 ($P < 0.05$, Table 6).

Gene expression levels in outgrowths

RT-PCR was performed to determine the expression levels of *Atrx*, *Nanog*, *Lsh*, *Nrf-1*, and *Sirt-1*. *Atrx* is an ATP-dependent centromeric protein that is involved in chromatin remodeling and methylation [46]. *Nanog* is a transcription factor whose expression is necessary for the maintenance of pluripotency and is used as a primary marker thereof [47]. *Lsh* is an ATP-dependent histone methylation protein of the same family as *Atrx* (SNF2) [48]. *Nrf-1* is a nuclear-encoded mitochondrial transcription factor [49] with target genes that influence respiration, mitochondrial protein import, and mtRNA transcription and replication [50,51]. *Sirt-1* is an NAD^+ -dependent histone deacetylase also capable of deacetylating and inactivating proapoptotic protein p53 [52,53], which causes differentiation in its active acetylated form [54]. *Atrx*, *Lsh*, *Nrf-1*, and *Sirt-1* were examined to investigate whether embryo culture had a permanent effect on the epigenetic state of later cells, as the differences observed between hESC lines [6–9] are likely to have some basis in epigenetics [55].

TABLE 4. THE EFFECT OF DIFFERENT CULTURE SYSTEMS ON THE PRESENCE OF NANOG IN THE BLASTOCYST

Culture media 0–48 h	Culture media 48–96 h	Nanog cell no. ^A	Nanog present (%) ^B
Simple	Simple	2.9 \pm 0.4 ^a	78 ^a
Simple	G2	4.5 \pm 0.4 ^b	83 ^a
G1	G2	8.1 \pm 0.5 ^c	92 ^b

^AThe number of cells positive for Nanog in the blastocyst stage embryo. Data are mean \pm SEM. $n \geq 38$ (3 replicate experiments). Different superscripts are significantly different: a, b, c ($P < 0.01$).

^BProportion of blastocysts with at least one Nanog-positive cell shown as a percentage. $n \geq 38$ embryos per treatment (3 replicate experiments). Different superscripts are significantly different: a, b ($P < 0.05$).

SEM, standard error of the mean.

TABLE 5. THE EFFECT OF DIFFERENT CULTURE SYSTEMS ON MOUSE OUTGROWTH MORPHOLOGY

Culture media 0–48 h	Culture media 48–96 h	Attachment (%) ^A	Absent (%) ^B	Small (%) ^B	Medium (%) ^B	Large (%) ^B
Simple	Simple	58 ^a	30 ^a	28 ^a	30 ^a	12 ^a
Simple	G2	57 ^a	21 ^a	18 ^{ab}	34 ^a	27 ^b
G1	G2	85 ^b	18 ^a	13 ^b	35 ^a	34 ^b

^AAttachment was scored on day 2 after plating and is expressed as number of attached embryos per number of blastocysts plated, shown as a percentage. $n \geq 65$ embryos per treatment (8 replicate experiments). Different superscripts are significantly different: a, b ($P < 0.001$).

^BOutgrowth morphology was scored on day 4 after plating and is expressed as a percentage of successful attachments. Outgrowths were scored as absent (no inner cell mass clump observed), small (20 μ m), medium (40 μ m), or large (60 μ m). Data represent mean \pm SEM. $n \geq 50$ embryos per treatment (8 replicate experiments). Different superscripts within columns are significantly different: a, b ($P < 0.05$).

Nanog levels in outgrowths were significantly reduced in outgrowth from embryos cultured in simple/simple or simple/G2 compared with G1/G2 outgrowths ($P < 0.05$) as were levels of *Atrx* ($P < 0.05$). There was no significant difference in the expression of mRNA transcripts for *Lsh*, *Nrf-1*, or *Sirt-1* between the different culture media (Fig. 5).

Discussion

Sequential culture systems are designed to mimic the changing environment of the reproductive tract and concomitant changes in the embryo’s requirements as it develops. They contain altered concentrations of carbohydrates, amino acids, chelators, and vitamins within the 2 sequential media [16,36,56,57]. Use of these media in clinical in vitro fertilization treatment has enabled extended culture in vitro with higher pregnancy rates [18,27]. In contrast, many embryos now used for hESC derivation were cultured in simple media systems before cryopreservation at the compacting stage, whose compositions are now known to adversely affect development [15,18,58–60]. The aim of present study was to develop a mouse model to determine the effect of culture in simple media on ESC progenitor cells compared with a purpose-designed embryo culture system in a mouse model.

Similar to previous findings [18,37,58], culture in G1/G2 increased the proportion of embryos that reached the blastocyst stage compared with those cultured in simple/simple. Culture in the simple medium during cleavage followed by culture in G2 did not increase development. However, ICM cell number was higher in blastocysts cultured in simple/G2 media compared with simple/simple, although it was still lower than for embryos cultured in G1/G2. Similar effects of culture were seen on ESC progenitor cell number as determined by staining for *Nanog* protein. Culture in G1/G2 produced embryos with the largest number of *Nanog*-positive cells, whereas culture in simple/simple produced embryos with the lowest number of *Nanog*-positive cells.

Culture in G2 after culture in simple during cleavage increased the number of *Nanog*-positive cells, which indicates that the purpose-designed medium enabled the negative effects on pluripotency of simple medium for the first 48 h of culture to be partially overcome. However, it did not increase the proportion of embryos possessing *Nanog*-positive ESC progenitor cells. High numbers of *Nanog*-positive cells in the ICMs of blastocysts [61] or plated ICMs [62] have previously correlated with high rates of mESC derivation, whereas the absence of *Nanog* has been shown to prevent mESC derivation [47]. Therefore, while culture in a purpose-designed medium that addresses the physiological requirements of postcompaction embryos may increase the capacity of embryos cultured in a simple historic medium before cryopreservation to give rise ESCs, the number which lack the capability due to a complete lack of ESC progenitor cells may remain the same.

Embryos cultured in G1/G2 attached at a significantly greater rate than embryos cultured in the simple media system, which may indicate a greater propensity to give rise to ESC lines as many methods of ESC derivation rely on outgrowing blastocysts to obtain the ICM. However, culture in G2 after culture in the simple medium had no effect on attachment efficiency. These results demonstrate that there are perturbations in culture that may affect ESC isolation induced by culture in the simple medium during the pre-compaction stage that are not reversed by subsequent culture in purpose-designed media.

In embryos, mitochondrial dysfunction is a common perturbation that occurs after exposure to an in vitro-induced stress. This mitochondrial dysfunction is linked to a reduction in development and subsequent viability [33]. Additionally, high mitochondrial activity indicates differentiation and a lack of pluripotency in ESC lines [63,64]. Therefore, we have used high metabolic activity as an indicator of differentiation and a reduced capacity to give rise to ESCs in outgrowths. Blastocysts cultured in the simple medium throughout development produced outgrowths that

TABLE 6. THE EFFECT OF DIFFERENT CULTURE SYSTEMS ON MITOCHONDRIAL MEMBRANE POTENTIAL OF MOUSE EMBRYO

Culture media 0–48 h	Culture media 48–96 h	Average MMP ^A	Highest MMP ^A	Lowest MMP ^A
Simple	Simple	1.86 \pm 0.07 ^a	2.77 \pm 0.19 ^a	1.41 \pm 0.05 ^a
Simple	G2	1.87 \pm 0.06 ^a	2.63 \pm 0.16 ^a	1.43 \pm 0.04 ^a
G1	G2	1.61 \pm 0.07 ^b	2.09 \pm 0.19 ^b	1.27 \pm 0.05 ^b

^AThe average, highest, and lowest fluorescence reading (MMP) for each culture system. Data represent mean \pm SEM. $n \geq 12$ outgrowths per culture system (8 replicate experiments). Different superscripts within columns are significantly different: a, b ($P < 0.05$).

MMP, mitochondrial membrane potential.

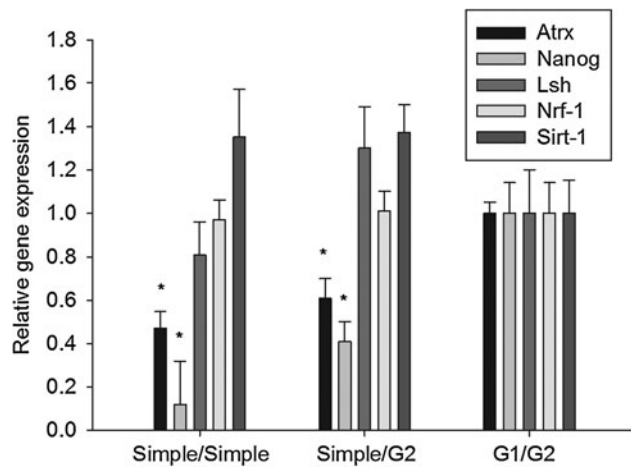


FIG. 5. Gene expression of outgrowths cultured in different culture systems. Data are mean \pm standard error of the mean. $n=5$ samples per treatment. Significantly different from control G1/G2, * $P < 0.05$.

were significantly more hyperpolarized, as shown by increased MMP, compared with blastocysts cultured in G1/G2. This was also true for blastocysts cultured in the simple medium during cleavage before transfer to G2, indicating that there is a permanent change to the mitochondria during this time that persists to the outgrowth stage, possibly reducing their pluripotency.

Although we found no evidence of perturbed expression of the genes *Lsh*, *Nrf-1*, or *Sirt-1* in outgrowths due to culture conditions, expression of *Nanog* and *Atrx* transcripts were reduced for both culture in the simple system and culture in simple/G2 compared with embryos cultured in G1/G2. Expression of *Nanog* is essential for the maintenance of pluripotency; it is a nuclear transcription factor expressed in pluripotent ESCs whose loss both accompanies and causes differentiation [47]. Therefore, the reduction in *Nanog* expression in these outgrowths is suggestive of an increased number of cells that are in the stages of differentiation. This supports the conclusions we have made from our metabolic findings as it suggests a reduced capacity to give rise to ESCs. It is of interest that gene expression of ATP-dependent chromatin remodeling protein, ATRX, is also reduced in the outgrowths derived from embryos cultured in a simple medium. ATRX is a centromeric protein that is involved in chromatin remodeling and methylation [46]. Further, in oocytes it has been shown to be involved in chromosome alignment during meiosis [46]. The reduced levels of *Atrx* in the outgrowths suggests that there is reduced activity of this protein that may impact on heterochromatin remodeling and normal function. This observation warrants further investigation.

The pluripotency and overall quality of hESC lines is of utmost importance if they are to be used in the field of regenerative medicine. However, embryos now used for hESC derivation have often been cultured in relatively simple media that have been subsequently shown to perturb development. In [65], it is reported that embryo quality is a top priority for successful hESC derivation. After selecting only good-quality cryopreserved cleavage-stage embryos for thawing and culture to the blastocyst stage, a derivation ef-

iciency of 18.6% was obtained. In [66], the quality of cryopreserved cleavage stage embryos was not used as a selection parameter and a derivation efficiency of 1.7% was obtained (the rate is higher, 4.9% when embryos cryopreserved at the blastocyst stage are included). These studies demonstrate the importance of embryo quality for hESC derivation. We have shown, using our mouse model, that culture in the simple medium decreases the quality of embryos with regard to several parameters important for ESC derivation, highlighting the importance of embryo culture. Although a number of these parameters were improved by subsequent culture in a purpose-designed culture media, some, such as MMP, proportion of blastocysts expressing *Nanog* and blastocyst attachment were not. Moreover, in our study none were improved to the level seen for embryos cultured in the purpose-designed system throughout culture. This is likely due to the precompaction stage being the most sensitive stage of embryo development [15,31,32] and the point at which the embryonic genome is activated and a wave of demethylation occurs [67,68]. From our findings we conclude that for embryos previously cultured in a simple culture system during cleavage, full embryo health and pluripotency will not be fully restored by culture in a system optimized for postcompaction development, to the blastocyst stage; however, partial improvements were seen.

Our findings highlight the necessity of careful embryo culture to retain pluripotency and suggest that further investigation into culture medium is important so that culture conditions from compaction to the expanded blastocyst stage can be used to optimize the efficiency of stem cell derivation and the long-term viability of the cell lines.

Acknowledgments

The authors acknowledge the support of the NHMRC program grant for funding. Dr. Michelle Lane and A/Prof. Mark B. Nottle are recipients of NHMRC Senior Research Fellowships. The authors thank Dr. Ivan Vassiliev for his useful discussions and Alicia Filby for technical assistance.

Author Disclosure Statement

No authors have commercial associations that might create a conflict of interest in connection with this article.

References

1. Brook FA and RL Gardner. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci U S A* 94:5709–5712.
2. Martin GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78: 7634–7638.
3. Evans MJ and MH Kaufman. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154–156.
4. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
5. Bongso A, CY Fong, SC Ng and S Ratnam. (1994). Isolation and culture of inner cell mass cells from human blastocysts. *Hum Reprod* 9:2110–2117.

6. Mantel C, Y Guo, MR Lee, MK Kim, MK Han, H Shibayama, S Fukuda, MC Yoder, LM Pelus, KS Kim and HE Broxmeyer. (2007). Checkpoint-apoptosis uncoupling in human and mouse embryonic stem cells: a source of karyotypic instability. *Blood* 109:4518–4527.
7. Rippon HJ and AE Bishop. (2004). Embryonic stem cells. *Cell Prolif* 37:23–34.
8. Bongso A, CY Fong and K Gauthaman. (2008). Taking stem cells to the clinic: major challenges. *J Cell Biochem* 105:1352–1360.
9. Spits C, I Mateizel, M Geens, A Mertzaniidou, C Staessen, Y Vandekelde, J Van der Elst, I Liebaers and K Sermon. (2008). Recurrent chromosomal abnormalities in human embryonic stem cells. *Nat Biotechnol* 26:1361–1363.
10. Hoffman DI, GL Zellman, CC Fair, JF Mayer, JG Zeitz, WE Gibbons and TG Turner Jr. (2003). Cryopreserved embryos in the United States and their availability for research. *Fertil Steril* 79:1063–1069.
11. National Health And Medical Research Council. (2007). Ethical guidelines on the use of assisted reproductive technology in clinical practice and research. Australian Government Canberra, p1–104.
12. Human Fertilisation Embryology Authority (2010). Code of Practice. Government of the United Kingdom, London. p1–230.
13. Bankowski BJ, AD Lyerly, RR Faden and EE Wallach. (2005). The social implications of embryo cryopreservation. *Fertil Steril* 84:823–832.
14. Klock SC. (2004). Embryo disposition: the forgotten “child” of in vitro fertilization. *Int J Fertil Womens Med* 49:19–23.
15. Gardner DK. (2008). Dissection of culture media for embryos: the most important and less important components and characteristics. *Reprod Fertil Dev* 20:9–18.
16. Lane M and DK Gardner. (2007). Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol* 21:83–100.
17. Laverge H, P De Sutter, R Desmet, J Van der Elst and M Dhont. (1997). Prospective randomized study comparing human serum albumin with fetal cord serum as protein supplement in culture medium for in-vitro fertilization. *Hum Reprod* 12:2263–2266.
18. Gardner DK and M Lane. (2003). Towards a single embryo transfer. *Reprod Biomed Online* 6:470–481.
19. Kovacic B and V Vlasisavljevic. (2008). Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts in vitro: a prospective study on sibling oocytes. *Reprod Biomed Online* 17:229–236.
20. Waldenstrom U, AB Engstrom, D Hellberg and S Nilsson. (2009). Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril* 91:2461–2465.
21. Gardner DK, M Lane and AJ Watson. (2004). *A Laboratory Guide to the Mammalian Embryo*. Oxford University Press, New York.
22. Devreker F, K Hardy, M Van den Bergh, AS Vannin, S Emiliani and Y Englert. (2001). Amino acids promote human blastocyst development in vitro. *Hum Reprod* 16:749–756.
23. Devreker F, RM Winston and K Hardy. (1998). Glutamine improves human preimplantation development in vitro. *Fertil Steril* 69:293–299.
24. Lane M and DK Gardner. (2001). Inhibiting 3-phosphoglycerate kinase by EDTA stimulates the development of the cleavage stage mouse embryo. *Mol Reprod Dev* 60:233–240.
25. Lane M and DK Gardner. (2007). Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol* 21: 83–100.
26. Gardner DK and M Lane. (1998). Culture of viable human blastocysts in defined sequential serum-free media. *Hum Reprod* 13 (Suppl. 3):148–159; discussion 160.
27. Gardner DK. (2000). Blastocyst culture: toward single embryo transfers. *Hum Fertil (Camb)* 3:229–237.
28. Van der Elst J, E Van den Abbeel, M Camus, J Smits, P Devroey and A Van Steirteghem. (1996). Long-term evaluation of implantation of fresh and cryopreserved human embryos following ovarian stimulation with busserelin acetate-human menopausal gonadotrophin (HMG) or clomiphene citrate-HMG. *Hum Reprod* 11:2097–2106.
29. Oehninger S, J Mayer and S Muasher. (2000). Impact of different clinical variables on pregnancy outcome following embryo cryopreservation. *Mol Cell Endocrinol* 169: 73–77.
30. Gardner DK, MW Lane and M Lane. (2000). EDTA stimulates cleavage stage bovine embryo development in culture but inhibits blastocyst development and differentiation. *Mol Reprod Dev* 57:256–261.
31. Lane M and DK Gardner. (1998). Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 13:991–997.
32. Zander DL, JG Thompson and M Lane. (2006). Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod* 74:288–294.
33. Mitchell M, KS Cashman, DK Gardner, JG Thompson and M Lane. (2009). Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod* 80:295–301.
34. Lane M, M Mitchell, KS Cashman, D Feil, S Wakefield and DL Zander-Fox. (2008). To QC or not to QC: the key to a consistent laboratory? *Reprod Fertil Dev* 20:23–32.
35. Gardner DK, M Lane and WB Schoolcraft. (2000). Culture and transfer of viable blastocysts: a feasible proposition for human IVF. *Hum Reprod* 15 (Suppl. 6):9–23.
36. Van Langendonck A, D Demyelle, C Wyns, M Nisolle and J Donnez. (2001). Comparison of G1.2/G2.2 and Sydney IVF cleavage/blastocyst sequential media for the culture of human embryos: a prospective, randomized, comparative study. *Fertil Steril* 76:1023–1031.
37. Gardner DK and M Lane. (2002). *Development of Viable Mammalian Embryos In Vitro: Evolution of a Sequential Media*. Academic Press, New York.
38. Mitchell M, SL Schulz, DT Armstrong and M Lane. (2009). Metabolic and mitochondrial dysfunction in early mouse embryos following maternal dietary protein intervention. *Biol Reprod* 80:622–630.
39. Li Y, CS Lin, L Wang, Y Liu, XN Mu, Y Ma and LS Li. (2009). Maintenance of human embryonic stem cells on gelatin. *Chin Sci Bull* 54:4214–4220.
40. Turksen K, ed. (2002). *Embryonic Stem Cells Methods and Protocols*. Humana Press, Totowa.
41. Spindle AI and RA Pedersen. (1973). Hatching, attachment, and outgrowth of mouse blastocysts in vitro: fixed nitrogen requirements. *J Exp Zool* 186:305–318.
42. Van Blerkom J, H Cox and P Davis. (2006). Regulatory roles for mitochondria in the peri-implantation mouse blastocyst: possible origins and developmental significance of differential DeltaPsim. *Reproduction* 131:961–976.
43. Wakefield SL, M Lane, SJ Schulz, ML Hebart, JG Thompson and M Mitchell. (2008). Maternal supply of omega-3 polyunsaturated fatty acids alter mechanisms involved in oocyte

- and early embryo development in the mouse. *Am J Physiol Endocrinol Metab* 294:E425–E434.
44. Nolan T, RE Hands and SA Bustin. (2006). Quantification of mRNA using real-time RT-PCR. *Nat Protoc* 1:1559–1582.
 45. Silva J, J Nichols, TW Theunissen, G Guo, AL van Oosten, O Barrandon, J Wray, S Yamanaka, I Chambers and A Smith. (2009). Nanog is the gateway to the pluripotent ground state. *Cell* 138:722–737.
 46. De La Fuente R, MM Viveiros, K Wigglesworth and JJ Eppig. (2004). ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes. *Dev Biol* 272:1–14.
 47. Mitsui K, Y Tokuzawa, H Itoh, K Segawa, M Murakami, K Takahashi, M Maruyama, M Maeda and S Yamanaka. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113:631–642.
 48. Yan Q, J Huang, T Fan, H Zhu and K Muegge. (2003). Lsh, a modulator of CpG methylation, is crucial for normal histone methylation. *EMBO J* 22:5154–5162.
 49. Chau CM, MJ Evans and RC Scarpulla. (1992). Nuclear respiratory factor 1 activation sites in genes encoding the gamma-subunit of ATP synthase, eukaryotic initiation factor 2 alpha, and tyrosine aminotransferase. Specific interaction of purified NRF-1 with multiple target genes. *J Biol Chem* 267:6999–7006.
 50. Virbasius CA, JV Virbasius and RC Scarpulla. (1993). NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. *Genes Dev* 7:2431–2445.
 51. Kelly DP and RC Scarpulla. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* 18:357–368.
 52. Luo J, AY Nikolaev, S Imai, D Chen, F Su, A Shiloh, L Guarante and W Gu. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 107:137–148.
 53. Motta MC, N Divecha, M Lemieux, C Kamel, D Chen, W Gu, Y Bultsma, M McBurney and L Guarante. (2004). Mammalian SIRT1 represses forkhead transcription factors. *Cell* 116:551–563.
 54. Lin T, C Chao, S Saito, SJ Mazur, ME Murphy, E Appella and Y Xu. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 7:165–171.
 55. Adewumi O, B Aflatoonian, L Ahrlund-Richter, M Amit, PW Andrews, G Beighton, PA Bello, N Benvenisty, LS Berry, S Bevan, B Blum, J Brooking, KG Chen, AB Choo, GA Churchill, M Corbel, I Damjanov, JS Draper, P Dvorak, K Emanuelsson, RA Fleck, A Ford, K Gertow, M Gertsenstein, PJ Gokhale, RS Hamilton, A Hampl, LE Healy, O Hovatta, J Hyllner, MP Imreh, J Itskovitz-Eldor, J Jackson, JL Johnson, M Jones, K Kee, BL King, BB Knowles, M Lako, F Lebrin, BS Mallon, D Manning, Y Mayshar, RD McKay, AE Michalska, M Mikkola, M Mileikovsky, SL Minger, HD Moore, CL Mummery, A Nagy, N Nakatsuji, CM O'Brien, SK Oh, C Olsson, T Otonkoski, KY Park, R Passier, H Patel, M Patel, R Pedersen, MF Pera, MS Piekarczyk, RA Pera, BE Reubinoff, AJ Robins, J Rossant, P Rugg-Gunn, TC Schulz, H Semb, ES Sherrer, H Siemen, GN Stacey, M Stojkovic, H Suemori, J Szatkiewicz, T Turetsky, T Tuuri, S van den Brink, K Vintersten, S Vuoristo, D Ward, TA Weaver, LA Young and W Zhang. (2007). Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 25:803–816.
 56. Lane M, DK Gardner, MJ Hasler and JF Hasler. (2003). Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. *Theriogenology* 60:407–419.
 57. Pool TB. (2005). An update on embryo culture for human assisted reproductive technology: media, performance, and safety. *Semin Reprod Med* 23:309–318.
 58. Reed L, M Lane and DK Gardner. (2003). In vivo rates of mouse embryo development can be obtained in vitro. *Theriogenology* 59:349.
 59. Reed ML, M Lane, DK Gardner, NL Jensen and J Thompson. (2002). Vitriification of human blastocysts using the cryoloop method: successful clinical application and birth of offspring. *J Assist Reprod Genet* 19:304–306.
 60. Lane M and DK Gardner. (2005). Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev* 17:371–378.
 61. Nichols J, J Silva, M Roode and A Smith. (2009). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* 136:3215–3222.
 62. Battle-Morera L, A Smith and J Nichols. (2008). Parameters influencing derivation of embryonic stem cells from murine embryos. *Genesis* 46:758–767.
 63. Babcock DF, J Herrington, PC Goodwin, YB Park and B Hille. (1997). Mitochondrial participation in the intracellular Ca²⁺ network. *J Cell Biol* 136:833–844.
 64. Cho YM, S Kwon, YK Pak, HW Seol, YM Choi, J Park do, KS Park and HK Lee. (2006). Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun* 348:1472–1478.
 65. Bongso A and EH Lee, eds. (2005). *Stem Cells: From Bench to Bedside*. World Scientific Press, Singapore.
 66. Cowan CA, I Klimanskaya, J McMahon, J Atienza, J Witmyer, JP Zucker, S Wang, CC Morton, AP McMahon, D Powers and DA Melton. (2004). Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 350:1353–1356.
 67. Weaver JR, M Susiarjo and MS Bartolomei. (2009). Imprinting and epigenetic changes in the early embryo. *Mamm Genome* 20:532–543.
 68. Braude P, V Bolton and S Moore. (1988). Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332:459–461.

Address correspondence to:

Dr. Michelle Lane
 School of Paediatrics and Reproductive Health
 University of Adelaide
 Medical School Nth, Level 6 Frome Road
 Adelaide 5005
 South Australia

E-mail: michelle.lane@adelaide.edu.au

Received for publication August 22, 2010

Accepted after revision November 24, 2010

Prepublished on Liebert Instant Online November 24, 2010

3.1 Introduction

Since hESCs were first isolated [1,436], numerous lines have been produced. However, these vary in quality and frequently display aneuploidy, slow growth and spontaneous differentiation [437-440]. In general there is more reported variation in cell quality compared with mESC models [438]. mESCs are routinely isolated from blastocysts that develop *in vivo*, while hESCs are derived from excess embryos from assisted reproductive technologies such as IVF or ICSI [4]. The period that human embryos can legally be cryopreserved varies; 5-10 years in Australia [2], 10 years in the United Kingdom [3] and indefinitely in the United States of America [165,166]. Consequently, prior to their cryopreservation donated embryos have frequently been cultured in relatively simple culture systems compared with those used today [215-218], and are likely to have been exposed to a range of conditions subsequently shown to perturb development including high oxygen [304,441], too high pH or inadequate buffering [332], no amino acids (or the wrong amino acids for the developmental stage) [216,248,249], no chelators [203,287] and serum as a source of protein [203,217] (section 1.5.2). These conditions are known to affect embryo development and many do not adequately support growth to the blastocyst stage [8,9], this resulted in the original establishment of transfer protocols dictating that human embryos be transferred to the recipient or cryopreserved at the cleavage stage [167,168]. The aim of this chapter was to develop a mouse model which mimicked these culture conditions to determine what effect embryo culture media has on epiblast cell number and primary outgrowth formation. As well, it investigated whether these factors can be improved for embryos cultured in simple medium to the compacting stage by culturing them subsequently in G2 medium, a medium purpose designed for the physiological requirements of the embryo from post-compaction to the blastocyst stage.

3.2 Experimental design

In this chapter the embryos used were from prepubertal (3-4 weeks) F1 CBA×C57Bl/6 mice as embryos from inbred C57Bl/6 mice do not develop in Simple-G1. Embryos were collected at the zygote stage (section 2.4.1) 22h after induction of ovulation by hCG (section 2.3.2). They were cultured in groups of 10 under paraffin oil at 37°C in 6% CO₂, 5% O₂, 89% N₂. Embryos were randomly allocated to different culture media (Simple-G1 medium or G1.2 culture medium) and cultured for 48h before those which passed the 2-cell block were transferred to the second stage of the culture system (Simple-G1 culture medium or G2.2 culture medium, (Table 3.1)).

Embryos were cultured in the purpose designed culture media system G1.2/G2.2, which contain different concentrations of carbohydrates, amino acids, vitamins and the chelator EDTA for pre and post-compaction culture [203,216,248,249,287], or a simple medium modelled after historic culture conditions lacking amino acids, vitamins, EDTA and with the same concentrations of carbohydrates throughout culture (Table 2.1). I hypothesised that culture in the simple medium would affect the number of epiblast cells in embryos, as well as other factors relevant to ESC derivation, compared with culture in the purpose designed G1.2/G2.2 system. This chapter also investigated the effect of culturing embryos in simple medium during cleavage prior to transfer to G2.2 at compaction to determine whether G2.2 could be used to reduce the negative effects of culture in simple media on embryo development. The treatment groups used in this study are detailed in Table 3.1

TABLE 3.1. EXPERIMENTAL CULTURE SYSTEM

First stage of culture system	Second stage of culture system
Simple-G1	Simple-G1
Simple-G1	G2.2
G1.2	G2.2

Media compositions in Table 2.1

Blastocyst development was scored at 96h and 115h (as per section 2.4.3). Blastocysts were either fixed and stained for total cell number and Nanog expression (as per section 2.5.1), or outgrown and subjected to real time RT-PCR for *Atrx*, *Nanog*, *Lsh*, *Nrf-1* and *Sirt-1* (as per sections 2.6.3 and 2.7).

3.2.1 Statistics

Data was expressed as mean \pm SEM and analysed using Univariate General Linear Model or an independent samples t-test using PASW Statistics 17. Replicate was fitted as a cofactor and between treatment differences were assessed using the Least Significant Difference (LSD) method. Gene expression data was normalised against the G1/G2 treatment group and differences were assessed using a Student's t test. Levene's test of equality was used to confirm equal variance. Categorical data was analysed by chi square tests. Values of $P < 0.05$ were considered significant.

3.3 Results

3.3.1 Blastocyst Development

The developmental stage which embryos had reached was assessed at 96h and 115h of culture. Culture system had no significant effect on the percentage of embryos which were arrested, at the early blastocyst stage, or at the blastocyst stage at 96h. However, culture in simple/simple significantly reduced the percentage of embryos which had begun hatching compared to culture in simple/G2 and G1/G2 ($P < 0.05$, Table 3.2). The developmental stage which embryos had reached was also assessed at 115h. No significant difference was seen for the percentage of embryos whose development had arrested before cavitation or otherwise degenerated. Culture in simple/simple retarded development, resulting in an increased percentage of embryos which were at the blastocyst stage compared with embryos cultured in simple/G2 or G1/G2 ($P < 0.05$, Table 3.3). Culture system had no significant effect on the percentage of embryos which had begun hatching by 115h, however, the percentage which had completed hatching was significantly decreased by culture in simple/simple compared to simple/G2 and G1/G2 ($P < 0.05$, Table 3.3).

TABLE 3.2. THE EFFECT OF CULTURE SYSTEMS ON DEVELOPMENT OF THE BLASTOCYST AT 96h

Culture media	Culture media	Arrested(%)	Early blastocyst(%)	Blastocyst(%)	Hatching blastocyst(%)
0-48h	48-96h				
Simple	Simple	21	10	46	23 ^a
Simple	G2	13	10	37	40 ^b
G1	G2	10	7	38	45 ^b

The total number of embryos which had reached the specified morphological stage after 96h of culture, shown as a percentage. N≥60, (3 replicate experiments). Different superscripts are significantly different; a and b (P<0.05).

TABLE 3.3. THE EFFECT OF CULTURE SYSTEMS ON DEVELOPMENT OF THE BLASTOCYST AT 115h

Culture media	Culture media	Arrested(%)	Blastocyst(%)	Hatching blastocyst(%)	Hatched blastocyst(%)
0-48h	48-115h				
Simple	Simple	24	23 ^a	33	20 ^a
Simple	G2	15	10 ^b	37	38 ^b
G1	G2	13	5 ^b	42	40 ^b

The total number of embryos which had reached the specified morphological stage after 115h of culture, shown as a percentage. N≥60, (3 replicate experiments). Different superscripts are significantly different; a and b (P<0.05).

3.3.2 Pluripotency of ICM Cells in Cultured Blastocysts

The total cell number of blastocysts was assessed using Dapi nuclear staining (Fig. 3.1). Culture in the G1/G2 system significantly increased blastocyst cell number compared to culture in simple/simple ($P<0.05$; Table 3.4). Culture in simple/G2 also significantly increased total cell number compared to simple/simple ($P<0.05$; Table 3.4), but there was no significant difference between simple/G2 and G1/G2. The number of pluripotent cells in blastocysts was investigated using immunocytochemical staining for Nanog protein (Fig. 3.1). Nanog is a marker of pluripotency which, in the blastocyst, is expressed in epiblast cells, which are the progenitors of embryonic stem cells [442]. Culture in G1/G2 produced blastocysts which had significantly more Nanog positive cells compared with those cultured in simple/simple or simple/G2 ($P<0.01$; Table 3.4). Culture in simple/G2 resulted in an increased number of Nanog positive cells compared with the simple/simple treatment ($P<0.01$, Table 3.4). Not all blastocysts contained Nanog positive cells. The G1/G2 system produced the largest proportion of blastocysts possessing Nanog positive cells., However, there was no difference between the simple/simple and simple/G2 systems ($P<0.05$, Table 3.4). This indicates that culture in G2 after culture in simple medium does not increase the proportion of blastocysts with the capacity to give rise to ESC lines. Negative staining was validated by staining *in vivo* cultured blastocysts for Nanog, with all *in vivo* blastocysts containing cells that were positive for Nanog (section 2.5.1).

TABLE 3.4. THE EFFECT OF DIFFERENT CULTURE SYSTEMS ON THE PRESENCE OF NANOG IN THE BLASTOCYST

Culture media	Culture media	^A Total cell number	^B Nanog cell no.	^C Nanog present (%)
0-48h	48-115h			
Simple	Simple	65.6±3 ^a	2.9±0.4 ^a	78 ^a
Simple	G2	71.1±3 ^b	4.5±0.4 ^b	83 ^a
G1	G2	74.9±3 ^b	8.1±0.5 ^c	92 ^b

^A The total number of cells in the blastocyst stage embryo. N≥38 (3 replicate experiments). Different superscripts are significantly different; a, b (P<0.05). ^B The number of cells positive for Nanog in the blastocyst stage embryo. N≥38 (3 replicate experiments). Different superscripts are significantly different; a, b, c (P<0.01). ^C Proportion of blastocysts with at least one Nanog positive cell shown as a percentage. N≥38 embryos per treatment (3 replicate experiments). Different superscripts are significantly different; a, b (P<0.05).

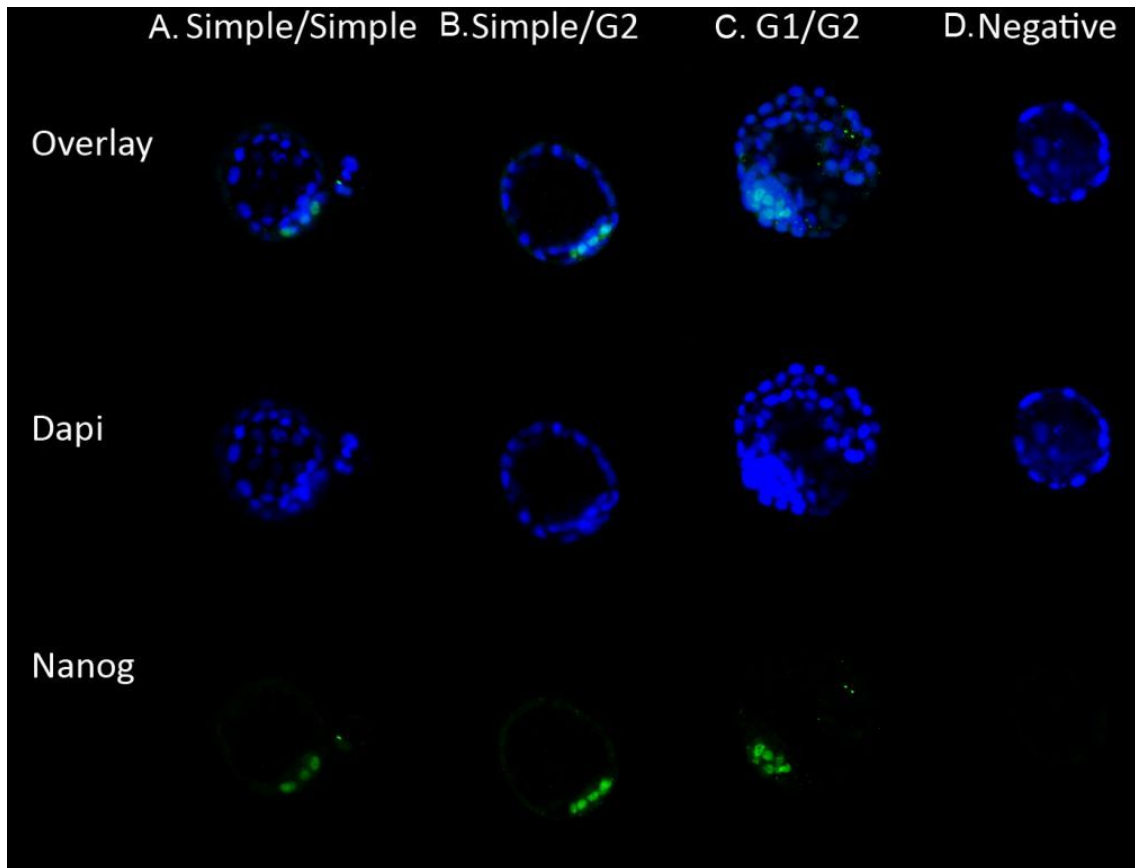


FIG. 3.1. Representative images of immunocytochemically stained blastocysts. All cell nuclei are stained blue with Dapi, Nanog positive cells are stained green with FITC. Scale bar equal to 50 μ m. A; Simple / Simple, B; Simple / G2, C; G1/G2, D; Negative control.

3.3.3 Gene Expression Levels in Outgrowths

RT-PCR was performed to determine the expression levels of *Atrx*, *Nanog*, *Lsh*, *Nrf-1*, and *Sirt-1* (Table 3.5). ATRX is an ATP dependent centromeric protein that is involved in chromatin remodelling and methylation [443]. Nanog is a transcription factor whose expression is necessary for the maintenance of pluripotency and is used as a primary marker thereof [55]. LSH is an ATP dependent histone methylation protein of the same family as ATRX (SNF2) [444]. NRF-1 is a nuclear encoded mitochondrial transcription factor [445] with target genes that influence respiration, mitochondrial protein import and mtRNA transcription and replication [446,447]. SIRT-1 is a NAD⁺ dependent histone deacetylase also capable of deacetylating and inactivating pro-apoptotic protein p53 [448,449] which causes differentiation in its active acetylated form [273]. *Atrx*, *Lsh*, *Nrf-1* and *Sirt-1* were examined to investigate whether embryo culture had a permanent effect on the epigenetic state of later cells, as the differences observed between hESC lines [437-440] are likely to have some basis in epigenetics [152]. *Nanog* levels in outgrowths were significantly reduced in outgrowths from embryos cultured in simple/simple or simple/G2 compared with G1/G2 outgrowths ($P < 0.05$) as were levels of *Atrx* ($P < 0.05$). There was no significant difference in the expression of mRNA transcripts for *Lsh*, *Nrf-1*, or *Sirt-1* between outgrowths derived from embryos grown in the different culture media (Fig. 3.2).

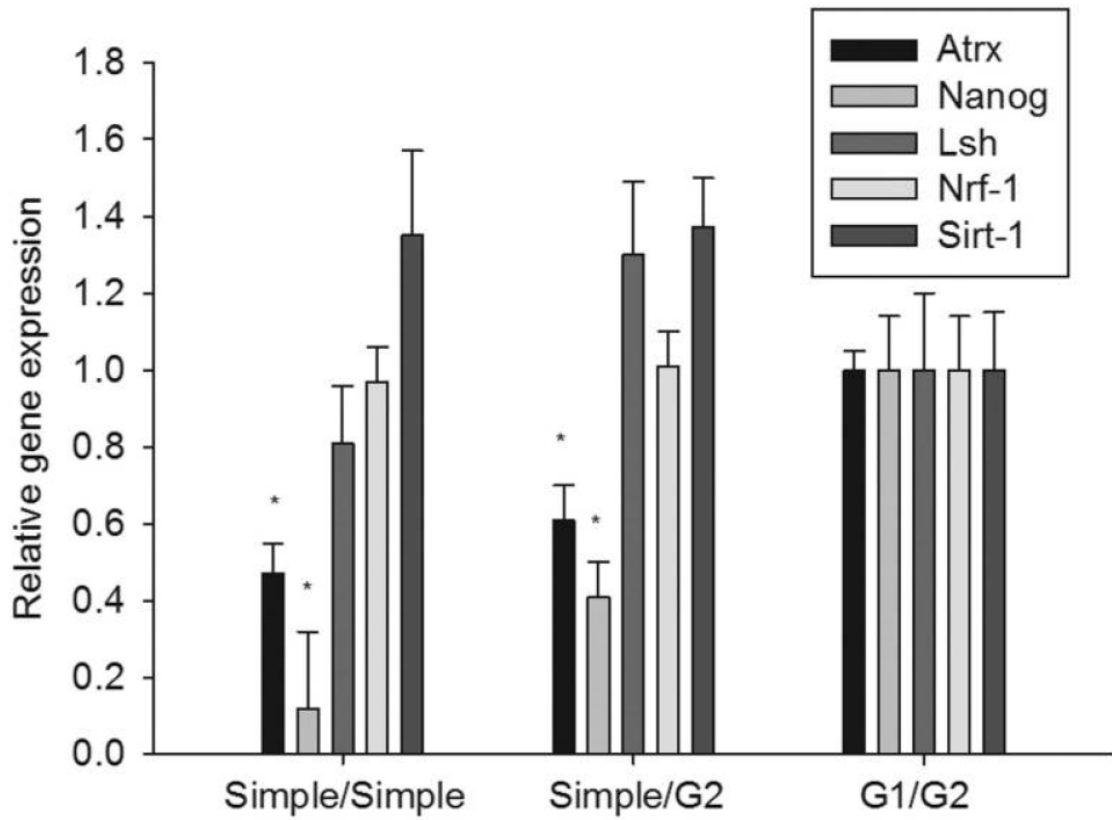


FIG. 3.2. Gene expression of outgrowths cultured in different culture systems. Data are mean±sem. N=5 samples per treatment. Significantly different from control G1/G2, * (P<0.05).

3.4 Discussion

Sequential culture systems are designed to mimic the changing environment of the reproductive tract and concomitant changes in the embryo's requirements as it develops. They contain altered concentrations of carbohydrates, amino acids, chelators, and vitamins within the two sequential media [216,347,450,451]. Use of these media in clinical IVF treatment has enabled extended culture *in vitro* with higher pregnancy rates [9,218]. In contrast, prior to their cryopreservation at the compacting stage many embryos now used for hESC derivation were cultured in simple media systems, which lacked key regulators such as amino acids and vitamins, whose compositions are now known to adversely affect development [215,218,452-454]. The aim of this chapter was to develop a mouse model to determine the effect of culture in simple media on the number of ESC progenitor cells compared with a purpose designed embryo culture system. Furthermore the present chapter aimed to determine if these effects could be ameliorated by post-compaction media.

Similar to previous findings [218,292,452], this chapter has shown that culture in G1/G2 increased the proportion of embryos which reached the hatching blastocyst stage after 96h of culture and the proportion which had completely hatched after 115h compared with embryos cultured in simple/simple. Culture in G2 following culture in simple medium during cleavage significantly increased the rate of hatching at 96h and completed hatching at 115h, demonstrating that culture in G2 is able to exert a rescue effect on the negative effects of culture in a simple medium. This finding was further supported by data for total cell number which was significantly higher in blastocysts from simple/G2 and G1/G2 compared with blastocysts cultured in simple/simple. However, in a previous work I found that ICM cell number was higher in blastocysts cultured in simple/G2 media compared with simple/simple, but that it was still lower than for embryos cultured in G1/G2 [455].

Similar effects of culture were seen on epiblast cell number as determined by staining for Nanog protein. Culture in G1/G2 produced embryos with the largest number of Nanog positive cells, whereas culture in simple/simple produced embryos with the lowest number of Nanog positive cells. Culture in G2 after culture in simple medium during cleavage increased the number of Nanog positive cells, which indicates that the purpose designed medium enabled the negative effects of simple medium for the first 48h of culture on pluripotency to be partially overcome. However, it did not increase the proportion of embryos possessing Nanog positive cells and the increase in number of Nanog positive cells was significantly lower than

that achieved by culture in G1/G2. High numbers of Nanog positive cells in the ICMs of blastocysts [428] or plated ICMs [72] have previously correlated with high rates of mESC derivation. While the absence of Nanog has been shown to prevent mESC derivation [55]. Therefore, while culture in a purpose designed medium that addresses the physiological requirements of post-compaction embryos may increase the capacity of embryos cultured in a simple historic medium prior to cryopreservation to give rise ESCs, the number which lack this capability due to a complete lack of ESC progenitor epiblast cells remains the same. However, this does suggest that this could be a novel window of intervention to improve ESC derivation.

Previous work showed that embryos cultured in G1/G2 were more likely to form outgrowths than embryos cultured in simple/simple or simple/G2 [455], a significant observation as outgrowth formation is a necessary step in ESC isolation. Furthermore, although this chapter found no evidence of perturbed expression of the genes *Lsh*, *Nrf-1*, or *Sirt-1* in outgrowths due to embryo culture conditions, expression of *Nanog* and *Atrx* transcripts was reduced for both culture in the simple system and in simple/G2 compared with embryos cultured in G1/G2. Expression of Nanog protein is essential for the maintenance of pluripotency; it is a nuclear transcription factor expressed in pluripotent ESCs whose loss both accompanies and causes differentiation [55]. Therefore, the reduction in *Nanog* expression in these outgrowths is suggestive of an increased number of cells that are in the stages of differentiation. Previous work on outgrowth metabolism has shown that outgrowths cultured in the simple system or simple followed by G2 both had higher metabolic activity than outgrowths from embryos cultured in G1/G2, which is a sign of differentiation [456,457]. Together these findings suggest that the efficiency with which ESCs can be derived from outgrowths produced from blastocysts cultured in simple/simple or simple/G2 is likely to be lower than that for those cultured in G1/G2. Furthermore the data suggests that culture in purpose designed G2 cannot ameliorate all detrimental effects arising from initial culture in simple medium.

Interestingly gene expression of ATP-dependent chromatin remodelling protein, ATRX, is also reduced in the outgrowths derived from embryos cultured in a simple medium. ATRX is a centromeric protein that is involved in chromatin remodelling and methylation [443]. Furthermore, in oocytes it has been shown to be involved in chromosome alignment during meiosis [443]. The reduced levels of *Atrx* seen in the outgrowths suggest that there is reduced activity of this protein that may impact on heterochromatin remodelling and normal function.

The pluripotency and overall quality of hESC lines is of utmost importance if they are to be used in the field of regenerative medicine. However, embryos now used for hESC derivation

have often been cultured in relatively simple media which have been subsequently shown to perturb development. In [458] it is reported that embryo quality is a top priority for successful hESC derivation. After selecting only good quality cryopreserved cleavage stage embryos for thawing and culture to the blastocyst stage a derivation efficiency of 18.6% was obtained. In [73] the quality of cryopreserved cleavage stage embryos was not used as a selection parameter and a derivation efficiency of 1.7% was obtained (the rate is higher, 4.9% when embryos cryopreserved at the blastocyst stage are included). These studies demonstrate the importance of embryo quality for hESC derivation.

Data presented in this chapter shows that culture in simple medium decreases the quality of embryos which consequently possess fewer pluripotent cells and produce outgrowths with altered gene expression. Although some measures, such as blastocyst development and epiblast cell number, were improved by subsequent culture in a purpose designed culture media others, such as gene expression and proportion of blastocysts expressing Nanog, were not. Moreover, only rate of development, which has been demonstrated to be an unreliable metric of embryo quality [427], was improved to the level seen for embryos cultured in the purpose designed system throughout culture. This is likely due to the pre-compaction stage being the most sensitive stage of embryo development [215,268,459] and the point at which the embryonic genome is activated and a wave of demethylation occurs [173,460]. From the findings in this chapter it can be concluded that for embryos previously cultured in a simple culture system during cleavage, subsequent culture in G2, which has been optimised for post-compaction development to the blastocyst stage, can aid retention of pluripotency. However, full embryo health and pluripotency are not completely restored by culture in this medium despite partial improvements. As such, it is necessary to investigate other strategies for increasing the number of pluripotent epiblast cells in the blastocyst to take advantage of culture from compaction to the expanded blastocyst stage, and optimise the efficiency of stem cell derivation and the long term viability of cell lines.

4.0 Insulin in embryo culture medium from the cleavage stage increases epiblast cell number and percentage.

The work presented in this chapter has been published in the following journal:

Campbell JM, MB Nottle, I Vassiliev, M Mitchell and M Lane. (2012) Insulin increases epiblast cell number of *in vitro* cultured mouse embryos via the PI3K/GSK3/p53 pathway. Stem Cells and Dev21:2430-41

Statement of authorship contributions

Jared M. Campbell: Designed and performed all experiments, interpreted and analysed data, wrote manuscript and is corresponding author.

Mark B. Nottle: Provided intellectual input, assisted with experimental design, data interpretation and manuscript preparation.

Ivan Vassiliev: Provided intellectual input, assisted with data interpretation and manuscript preparation.

Megan Mitchell: Provided intellectual input, assisted with experimental design and manuscript preparation.

Michelle Lane: Principal project supervisor, assisted with experimental design, data interpretation and manuscript preparation.

Insulin Increases Epiblast Cell Number of In Vitro Cultured Mouse Embryos via the PI3K/GSK3/p53 Pathway

Jared M. Campbell,¹ Mark B. Nottle,² Ivan Vassiliev,² Megan Mitchell,³ and Michelle Lane³

High-quality embryos give rise to embryonic stem cells (ESCs) at greater efficiencies than poor-quality embryos. However, most embryos available for human ESC derivation are of a reduced quality as a result of culture in relatively simple media up to 10 years earlier, before cryopreservation, or before compaction. In the present study, we used a mouse model to determine whether a culture with insulin from the 8-cell stage could increase the number of ESC progenitor epiblast cells in blastocysts, as well as endeavor to determine the molecular mechanism of the insulin's effect. Culture in media containing 1.7 μ M insulin increased epiblast cell number (determined by Oct4 and Nanog co-expression), and proportion in day 6 blastocysts. The inhibition of phosphoinositide 3 kinase (PI3K) (via LY294002), an early second messenger of the insulin receptor, blocked this effect. The inhibition of glycogen synthase kinase 3 (GSK3) or p53, 2s messengers inactivated by insulin signaling (via CT99021 or pifithrin- α , respectively), increased epiblast cell numbers. When active, GSK3 and p53 block the transcription of Nanog, which is important for maintaining pluripotency. A simultaneous inhibition of GSK3 and p53 had no synergistic effects on epiblast cell number. The induced activation of GSK3 and p53, via the inhibition of proteins responsible for their inactivation (PKA via H-89 and SIRT-1 via nicotinamide, respectively), blocked the insulin's effect on the epiblast. From our findings, we conclude that insulin increases epiblast cell number via the activation of PI3K, which ultimately inactivates GSK3 and p53. Furthermore, we suggest that the inclusion of insulin in culture media could be used as a strategy for increasing the efficiency with which the ESC lines can be derived from cultured embryos.

Introduction

WHILE MOUSE EMBRYONIC STEM cells (mESCs) are primarily isolated from in vivo derived blastocysts, human ESCs (hESCs) are typically isolated from frozen-assisted reproduction technology embryos donated for research after the completion of treatment, often up to 5–10 years after the embryos were produced [1,2]. As a consequence, many embryos donated for hESC derivation have been cultured under a range of conditions, including relatively simple media, subsequently shown to compromise development [3–6]. The quality of an embryo and the number of epiblast cells, identified as cells that co-express the pluripotency markers Oct4 and Nanog [7], is known to be of key importance to that embryo's capacity to give rise to an hESC line [8]. Therefore, the efficiency and quality of the ESC lines will likely be improved by optimizing conditions for the 8-cell embryo (the

stage around which human embryos typically become available for ESC derivation) to increase blastocyst quality and the number of pluripotent cells. A significant number of stored human embryos for donation have been frozen at the cleavage stage before compaction [9–11], and in a previous study using a mouse model, we reported that subsequent culture from the 8-cell stage to the expanded blastocyst stage can influence the number of epiblast cells [12], which are the progenitor cells that give rise to ESC lines [13–15].

The addition of insulin to embryo culture media for development from the zygote or 2-cell stage has been previously shown as having beneficial effects, with an increased blastocyst formation rate [16], accelerated development [16,17], increased blastocyst cell number [16], stimulated protein synthesis [18–20], reduced protein degradation [19], and increased protein uptake [19]. While the capacity of insulin or any other growth factor—many of which have

¹Centre for Stem Cell Research, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, South Australia.

²Centre for Stem Cell Research, Robinson Institute, University of Adelaide, Adelaide, South Australia.

³School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, South Australia.

similar effects to those just described [21,22]—to increase the number of epiblast cells is unknown, insulin differs from most in that it increases total cell number (TCN) by increasing inner cell mass (ICM) cell number [16]. The aim of the present study, therefore, was to determine whether the addition of insulin from the 8-cell stage increases the number of epiblast cells in the expanded blastocyst. We then undertook a series of experiments to investigate the involvement of phosphoinositide 3 kinase (PI3K), glycogen synthase kinase 3 (GSK3), and p53 in insulin's effect on epiblast cell number, as these are known pathways for insulin stimulation in other tissues [23–26].

Materials and Methods

Experimental design

To determine the effect of insulin on epiblast cell number, an embryo culture medium was supplemented with 0, 0.17, 1.7, and 1,700 μM insulin from the 8-cell stage in Experiment 1. In Experiment 2 the effect of LY294002 [27], an inhibitor of PI3K—a second messenger present in the mouse preimplantation embryo involved in the maintenance of pluripotency [25,28]—was examined in the presence or absence of insulin to determine whether insulin was acting via PI3K. Since the PI3K second messenger pathway involves the subsequent phosphorylation and inactivation of GSK3 [29], which has been shown to be beneficial for maintaining cells in a pluripotent state in outgrown ICMs [30], we hypothesized that the activation of GSK3 would inhibit the positive response of insulin, while inactivation would mimic the effects of insulin. This hypothesis was tested in Experiment 3 using H-89 [31] in the presence or absence of insulin and CT99021 [32] for GSK3 activation and inhibition, respectively. Stimulation of the PI3K second messenger pathway can also result in the ubiquitination, inactivation, and degradation of pro-apoptotic protein p53 [23,33,34]. In the nucleus, p53 is known to directly inhibit the transcription of Nanog [35] and, therefore, inhibit pluripotency. To establish whether the positive effects of insulin on the epiblast were mediated via p53, Experiment 4 was performed where p53 was indirectly activated via acetylation due to nicotinamide [36,37] in the presence or absence of insulin and inhibited by pifithrin- α [38]. Finally, p53 and GSK3 are known to have significant cross talk, with GSK3 inactivation leading to p53 build up [39] and resultant apoptosis [40], or loss of p53 activity in different circumstances [41–43]. In Experiment 5, we inhibited both factors at once to determine whether there was synergy.

Insulin and inhibitors

Chemicals used were purchased from Sigma-Aldrich, (St. Louis, MO) except where otherwise noted. In this study, all the chemicals and consumables used for standard embryo culture were tested for compatibility with embryo development in a one-cell mouse embryo assay [44]. Bovine insulin was used at concentrations of 0, 0.17, 1.7, and 1,700 μM . LY294002 was used at 50 μM to inhibit PI3K; this concentration has been shown to reduce the phosphorylation of Akt and P70^{S6k} in mESCs, which are phosphorylated as a result of PI3K activity (in the same experiment, LY294002 was shown to have the same effect on blastocyst cells as ESCs

(induce apoptosis)) [27]. LY294002 has also been directly shown to be effective in decreasing PI3K activity in blastocysts, as evidenced by the decreased Akt phosphorylation at 250 μM [45]. Additionally, the treatment of ESCs with LY294002 reduced the phosphorylation of Akt at 5–30 μM , as well as S6 and GSK3 at 10–60 μM , which are also phosphorylated as a result of PI3K activity [46]. In an in vitro assay, LY294002 was shown to completely abolish PI3K activity at 100 μM [47]. CT99021 (Axon Medchem, Groningen, The Netherlands) was used to inhibit GSK3 (the concentrations studied were 0.04, 0.3, 3.0, and 15 μM ; initial experiments using CT99021 at 30 μM showed a significant negative effect on the development of embryos). In [48], CT99021 at 3 μM was shown to decrease the phosphorylation of β -catenin, a GSK3 target in ESCs. Furthermore, a culture of ESCs in which GSK3 α and β had been deleted with CT99021 did not produce the effects seen when wild-type ESCs were cultured with CT99021 [48]. This decrease in β -catenin phosphorylation was also observed in embryos (bovine 2-cell) cultured with CT99021 at 3 μM , an effect that was the same as LiCl, another recognized inhibitor of GSK3 [49]. CT99021 at 1 μM was shown to reduce GSK3 β activity to 1% in an in vitro assay in which the inhibitor was shown to be highly specific [32]. H-89 at 10 μM was used to activate GSK3, as it is a robust activator of GSK3 whose inclusion in culture medium has been shown to prevent or inhibit the phosphorylation of GSK3 (which would inactivate GSK3) in embryonic kidney cells at 10 μM [31,50], muscle cells at 50 μM [51], spermatozoa at 100 μM [52], and glioma cells at 10 μM [53]. Pifithrin- α was used to inhibit p53 at 30 μM [38], as it has been established as an effective inhibitor of p53 that blocks the activation of p53 responsive *LacZ* in ConA cells, and inhibits p53-mediated apoptosis at 10–20 μM [54]. Pifithrin- α at 10–20 μM is also able to affect p53-dependent cell-cycle checkpoint control, as it prevents gamma irradiation-induced arrest, but not in cells with no functional p53 [54]. Embryos cultured in the presence of pifithrin- α at 10–30 μM reproduced the effect of *p53* deletion, and reversed the effect of culture in conditions known to induce increased p53 activity [38]. Pifithrin- α lowers the level of nuclear p53 at 10–20 μM in vitro and 2.2 mg/mL when injected in vivo [54,55]. Nicotinamide at 10 μM was used to activate p53; our group has previously used this inhibitor in embryo culture [36], as it has been shown to increase the levels of acetylated p53 (active p53) in lung, breast, and bone cancer cells [37] and induce apoptosis in a p53-dependent manner in chronic lymphocytic leukemia cells as well as increase p53 acetylation [56]. Where dimethyl sulfoxide (DMSO) was used to dissolve inhibitors, a vehicle control of an equal quantity of DMSO was included.

Embryo collection, culture, and assessment

Approval for all procedures was obtained from The University of Adelaide Animal Ethics Committee, in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Mice were fed ad libitum, and kept in a 14:10 light:dark cycle. C57BL/6 female mice aged 3–4 weeks were injected intraperitoneally with 5 IU equine chorionic gonadotropin (Folligon, Intervet Australia Pty Ltd, Bendigo, Victoria), followed by 5 IU human chorionic gonadotropin (hCG; Pregnyl,

Organon, Sydney, Australia) 48 h later, inducing ovulation. The ovulating females were placed with a male mouse of the same strain, and mating was assessed the next morning by the presence of a vaginal plug. Zygotes were collected 22 h post hCG in MOPS-G1 [57], and cumulus cells were removed with 50 IU/mL hyaluronidase. Zygotes were cultured in 20 μ L of G1.2 medium [44], in groups of 10 at 37°C in 6% CO₂, 5% O₂, and 89% N₂ for 48 h. Embryos that reached the 8-cell stage after 48 h of culture were transferred to 20 μ L drops of G2.2 supplemented with the relevant treatment, and cultured individually at 37°C in 6% CO₂, 5% O₂, and 89% N₂ from 48 to 115 h. Since embryos secrete exogenous factors, which affect development when they are cultured in groups [58,59], individual culture was used to minimize the potentially confounding paracrine growth-factor effect.

Embryo development was assessed at 94 and 115 h. Embryos were scored as being in the following stages of development: arrested (embryos that failed to develop from the 8-cell stage), early blastocysts (embryos where the blastocoel cavity was <2/3rds the volume), blastocysts (embryos with a blastocoel cavity >2/3rds the volume), hatching blastocysts (embryos with cells herniating from the zona pellucida), and hatched blastocysts (embryos that had hatched completely from the zona pellucida).

Immunohistochemistry

Oct4 and Nanog, whose co-expression marks epiblast cells, were assessed in blastocysts at 115 h of culture using immunohistochemistry and confocal microscopy. Blastocysts were fixed overnight in 4% paraformaldehyde at 4°C. After fixation, the embryos were incubated for 5 min in 0.1M glycine in phosphate-buffered saline (PBS) at room temperature (RT). Blastocysts were permeabilized in PBS with 0.25% TritonX-100 (PBS-TX) for 30 min at RT, then blocked in 10% Normal Donkey Serum (Sapphire Bioscience, Redfern, New South Wales, Australia) for 1 h at RT. Embryos were incubated with Nanog rabbit anti-mouse polyclonal antibody (Sapphire, Cat#120-21603 or Cosmo Bio, Tokyo, Japan, Cat#REC-RCAB0002P-F) at 1:200 and Oct-3/4 goat anti mouse polyclonal antibody (Santa Cruz Biotechnology inc, Santa Cruz, CA; sc-8628) at 1:100 overnight in PBS-TX at 4°C. Blastocysts were then washed in PBS-TX, and incubated with donkey anti-rabbit secondary antibody (1:100) conjugated to FITC (Australian Laboratory Services, Homebush New South Wales, Australia) and donkey anti-goat secondary antibody (1:100) conjugated to Rhodamine (Jackson ImmunoResearch, West Grove, PA; 705-025-003) for 2 h at 37°C. Embryos were then incubated with 3 nM of 4'-6-diamidino-2-phenylindole (DAPI), a nuclear stain, for 2–3 min at RT, before being examined by confocal microscopy (Nikon, EZ-C1 software or Calcium Lacia SP5, Lacia SP5 software). The number of cell nuclei stained blue by DAPI gave TCN; ICM cell number was the number of cells whose nuclei were stained red by rhodamine, indicating the presence of Oct4, and epiblast cell number was the number of red ICM nuclei that were also stained green by FITC, indicating the presence of both Oct4 and Nanog. Trophoblast cell number was TCN minus ICM cell number, and primitive endoderm cell number was ICM cell number minus epi-

blast cell number. A cell was counted as being positive for the fluorophore in question when the intensity of the stain was sufficient for the nucleus to be clearly distinguished from the background. A negative control was assessed where the primary antibody was not applied.

Statistics

All data are expressed as mean \pm sem. The treatment group was fitted as a fixed factor, and the replicate was fitted as a cofactor in all analyses. Data were analyzed using Univariate General Linear Model using PASW Statistics 17 or chi-square tests. Between-treatment differences were assessed using the Least Significant Difference method. Values such as $P < 0.05$ were considered significant.

Results

Experiment 1: effect of insulin on epiblast cell number

The supplementation of culture medium with insulin (0, 0.17, 1.7, and 1,700 pM) from the 8-cell stage had no effect on the development at 94 h (blastocyst or hatching blastocysts for all treatments, range 81%–87%) or 115 h (hatching blastocyst or hatched blastocyst for all treatments, range 77%–86%). At 115 h of culture, there was no effect of the insulin on TCN, ICM, trophoblast, or primitive endoderm cell numbers (Fig. 1A, B, E, and F). However, culture in the presence of 1.7 pM insulin significantly increased epiblast cell number ($P < 0.05$, Fig. 1C). Since the epiblast exists as a subpopulation of the ICM, this meant that there was an increase in the proportion of ICM cells which were ESC progenitor epiblast cells in the presence of 1.7 pM insulin ($P < 0.05$, Fig. 1D). Representative images of blastocysts cultured in the control treatment or 1.7 pM of insulin and stained for Oct4, Nanog, and TCN are supplied in Fig. 2.

Experiment 2: effect of PI3K inactivation on epiblast cell number in embryos cultured in the presence or absence of insulin

To determine whether the effect of insulin was a result of PI3K stimulation, PI3K was inactivated with LY294002 at 50 μ M [27] in the presence or absence of insulin. At 94 h of culture, LY294002 prevented hatching in either the presence or absence of insulin, and significantly increased the number of arrested or degenerate embryos in the presence of insulin ($P < 0.01$, Table 1). At 115 h, the inhibition of PI3K in the presence of insulin significantly reduced the percentage of embryos that developed in the blastocyst stage, with hatching reduced in either the presence or absence of insulin ($P < 0.001$, Table 2).

PI3K inhibition significantly reduced TCN and ICM cell number, irrespective of the presence of insulin ($P < 0.01$, Fig. 3A, B). However, while the inhibition of PI3K in the absence of insulin did not affect epiblast cell number compared with the control, PI3K inhibition prevented the stimulatory effect of insulin on epiblast cell number ($P < 0.01$, Fig. 3C).

Interestingly, insulin in the presence of PI3K inhibition increased ICM size compared with embryos cultured in LY294002 alone ($P < 0.01$, Fig. 3B), suggesting that insulin is able to affect ICM size through signaling pathways other than PI3K.

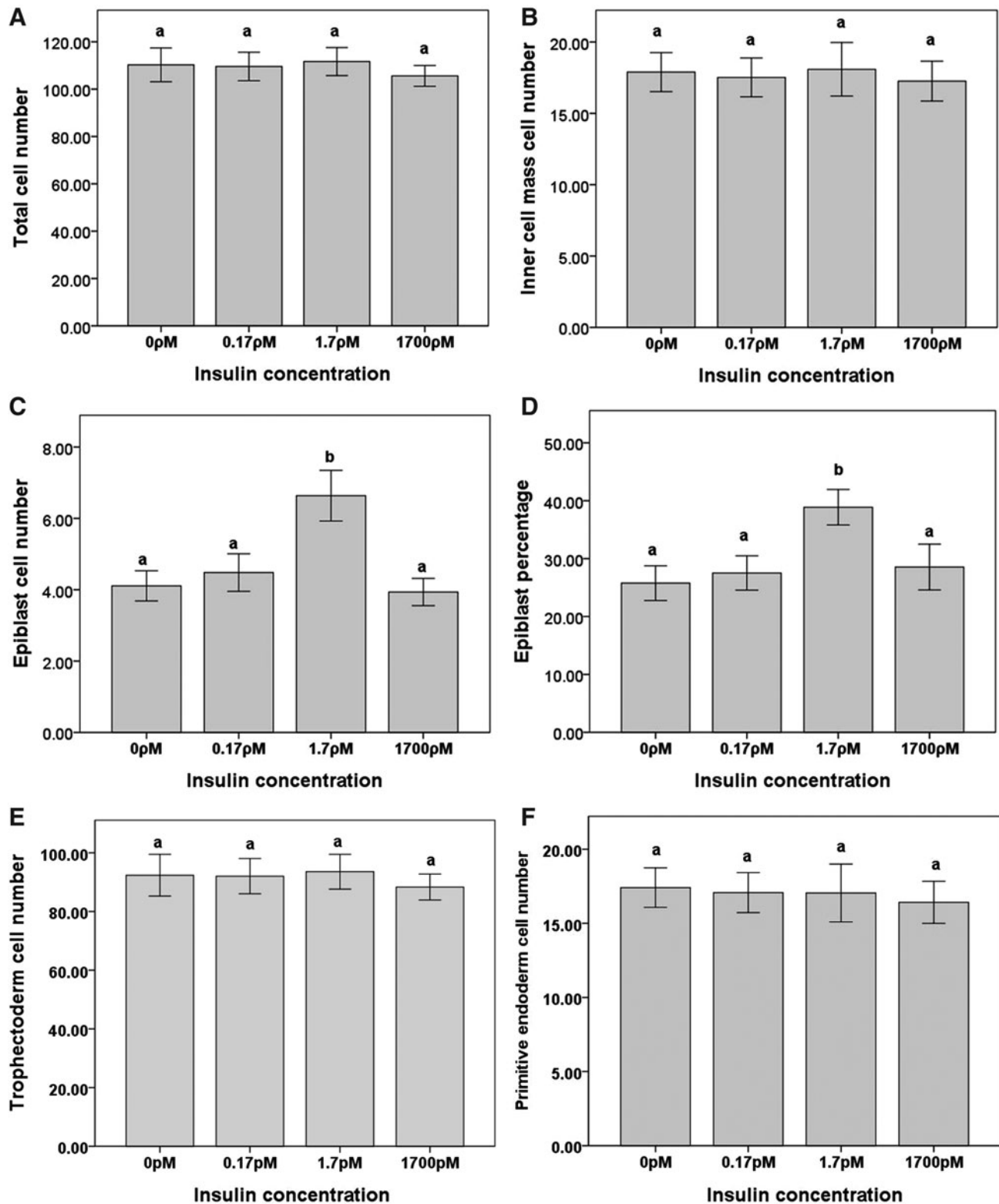
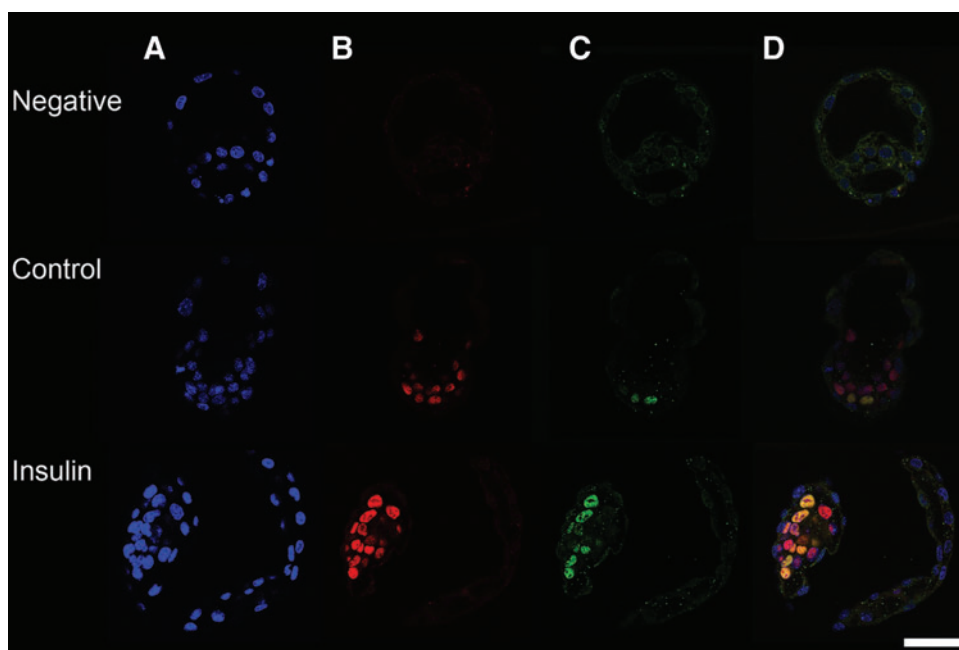


FIG. 1. Blastocyst culture in the presence of insulin. (A) TCN as shown by DAPI staining, (B) ICM cell number as shown by Oct4 staining, (C) epiblast cell number as shown by Oct4 and Nanog staining, (D) percentage of ICM cells that are epiblast cells, (E) Trophectoderm cell number as shown by TCN minus ICM cell number, and (F) primitive endoderm cell number as shown by ICM minus epiblast cell number in the blastocysts of embryos cultured in insulin at the concentrations indicated. Data are mean \pm sem. Superscripts a and b are significantly different at $P < 0.01$, $n \geq 34$ blastocysts per treatment. TCN, total cell number; ICM, inner cell mass; DAPI, 4'-6-diamidino-2-phenylindole.

FIG. 2. Representative images of blastocysts stained for Oct4, Nanog, and TCN after culture in control medium and medium supplemented with 1.7 μ M insulin. (A) DAPI staining all cell nuclei blue, (B) rhodamine staining Oct4 positive ICM nuclei red, (C) FITC staining Nanog positive nuclei green, (D) an overlay of all stains. Scale bar is 50 μ M. Color images available online at www.liebertonline.com/scd



Experiment 3: effect of GSK3 activation/inhibition on epiblast cell number in embryos cultured in the presence or absence of insulin

GSK3 activation by the PKA inhibitor H-89 [31] had no effect on the development at 94 h of culture (blastocyst or hatching blastocysts for all treatments, range 84%–94%); however, it significantly reduced the number of blastocysts that had hatched in the presence or absence of insulin after 115 h of culture ($P < 0.01$, Table 3). Furthermore, GSK3 activation did not alter TCN (Fig. 4A), but significantly decreased the ICM cell number with or without insulin ($P < 0.05$, Fig. 4B). The activation of GSK3 in the absence of insulin did not affect epiblast cell numbers relative to the control, but insulin's stimulation of epiblast cell number was prevented by the activation of GSK3 ($P < 0.05$, Fig. 4C). The percentage of the ICM that was epiblast was unchanged with GSK3 activation with or without insulin (Fig. 4D).

The inactivation of GSK3 with CT99021 had no effect on the development at 94 h (blastocyst or hatching blastocyst for all treatments, range 75%–85%) or 115 h (hatching blastocyst

or hatched blastocyst for all treatments, range 54%–67%) of the culture. However, GSK3 inhibition significantly increased ICM cell number (20.1 ± 1.2 compared with control 15.9 ± 1.2 $P < 0.01$; $n \geq 50$) and epiblast cell number (5.1 ± 0.3 compared with control 3.2 ± 0.3 $P < 0.001$; $n \geq 50$), although the epiblast proportion (23.2 ± 2.5 compared with control 23.8 ± 2.5 NS; $n \geq 50$) and TCN (111.7 ± 4.4 compared with control 107.2 ± 4.4 NS; $n \geq 50$) were not affected.

Experiment 4: effect of p53 activation/inhibition on epiblast cell number in embryos cultured in the presence or absence of insulin

The activation of p53 by nicotinamide in the presence or absence of insulin had no effect on embryo development at 94 h (blastocyst or hatching blastocyst for all treatments, range 88%–98%) or 115 h (hatching blastocyst or hatched blastocyst for all treatments, range 69%–84%), nor TCN (Fig. 5A). However, the activation of p53 significantly reduced ICM cell number both with and without insulin ($P < 0.05$, Fig. 5B). The activation of p53 did not affect epiblast cell numbers compared with control-treated embryos; however, insulin's

TABLE 1. DEVELOPMENTAL MORPHOLOGY AFTER 94 H OF PHOSPHOINOSITIDE 3 KINASE INHIBITION

	Arrested (%)	Early blastocyst (%)	Blastocyst (%)	Hatching blastocyst (%)
G2 (control)	7.0	8.8	68.4	15.8 ^a
Insulin	1.7 ^a	3.4	75.9	19.0 ^b
LY294002	12.7	18.2	69.1	0.0 ^a
Insulin + LY294002	21.8 ^a	21.8	56.4	0.0 ^b

Mean percentage of embryos at stage of development after 94 h of culture in the specified treatment. $n \geq 55$ per treatment.

Like pairs, ^a and ^b, are significantly different ($P < 0.01$).

TABLE 2. DEVELOPMENTAL MORPHOLOGY AFTER 115 H OF PHOSPHOINOSITIDE 3 KINASE INHIBITION

Total	Arrested (%)	Total blastocyst (%)	Hatching blastocyst (%)	Hatched blastocyst (%)
G2	3.5	96.5	26.3 ^a	42.1 ^a
Insulin	5.2	94.8	27.6 ^b	32.8 ^b
LY294002	16.4	83.6	83.6 ^a	0.0 ^a
Insulin + LY294002	20.0	80	72.7 ^b	5.5 ^b

Mean percentage of embryos at stage of development after 115 h of culture in the specified treatment. $n \geq 55$ per treatment.

Like pairs, ^a and ^b are significantly different ($P < 0.001$).

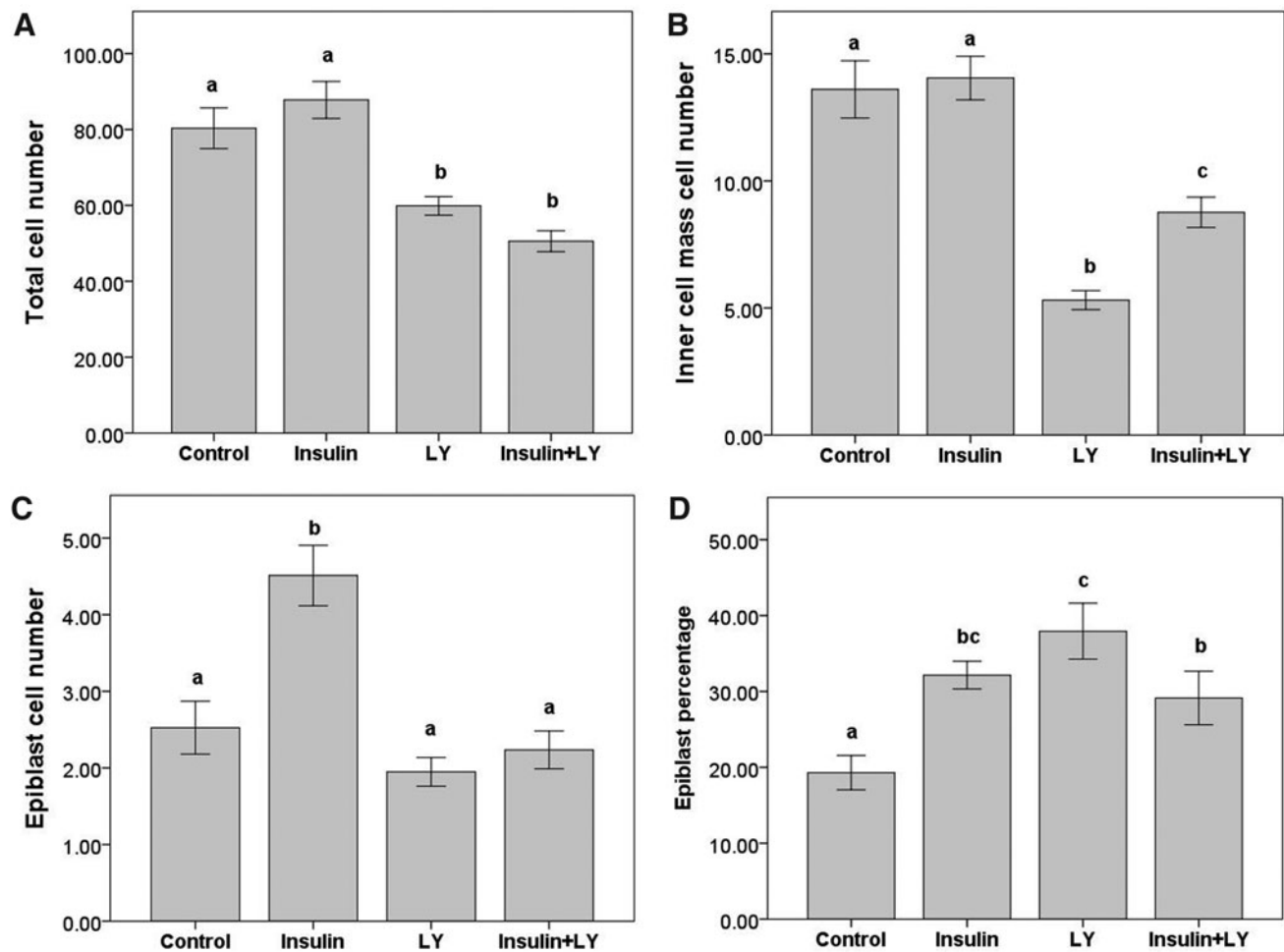


FIG. 3. PI3K inhibition during blastocyst culture in the presence of insulin. (A) TCN as shown by DAPI staining, (B) ICM cell number as shown by Oct4 staining, (C) epiblast cell number as shown by Oct4 and Nanog staining, and (D) percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in insulin at 1.7 μ M and/or 50 μ M of the PI3K inhibitor LY294002 (LY). Data are mean \pm sem. Superscripts a, b, and c are significantly different at $P < 0.01$, $n \geq 34$ blastocysts per treatment. PI3K, phosphoinositide 3 kinase.

increase of epiblast cell numbers was prevented ($P < 0.05$, Fig. 5C). The inactivation of p53 had no significant effect on the percentage of the ICM that was epiblast (Fig. 5D).

The inhibition of p53, with pifithrin- α (30 μ M) [38], was able to mimic the effects of insulin with no effect on blasto-

cyst development at 94 h (blastocyst or hatching blastocyst for all treatments, range 85%–86%) or 115 h (hatching blastocyst or hatched blastocyst for all treatments, range 75%–80%). However, TCN was increased (132 ± 7.0 compared with control 111.7 ± 6.6 $P < 0.05$; $n \geq 24$) as was ICM (30.5 ± 2.4 compared with control 18.1 ± 2.2 $P < 0.001$; $n \geq 24$) and epiblast cell number (7.1 ± 0.6 compared with control 4.1 ± 0.6 $P < 0.01$; $n \geq 24$) that were increased by p53 inhibition, while the percentage of ICM that was epiblast was unchanged (14.8 ± 3.7 compared with control 13.6 ± 3.4 NS; $n \geq 24$).

Experiment 5: interaction of GSK3 and p53 signaling

Our results show that insulin acts on the epiblast via the inactivation of GSK3 and p53. The inactivation of one factor may reduce the other’s activity [41–43], but the inactivation of GSK3 can also lead to p53 accumulation [39] and apoptosis [40]. If the inactivation of GSK3 causes p53 accumulation, then inactivation of both GSK3 and p53 may have a synergistic effect.

TABLE 3. DEVELOPMENTAL MORPHOLOGY AFTER 115 H OF GLYCOGEN SYNTHASE KINASE 3 ACTIVATION

	Total Arrested (%)	Total Blastocyst (%)	Hatching blastocyst (%)	Hatched blastocyst (%)
G2	13.5	86.5	16.2	43.2
Insulin	2.8	97.2	13.9	30.6 ^a
H89	6.9	93.1	24.1	69.0
Insulin + H89	2.6	97.4	17.9	74.4 ^a

Mean percentage of embryos at stage of development after 115 h of culture in the specified treatment. $n \geq 29$ per treatment.

Like pairs, ^a and ^b are significantly different ($P < 0.01$).

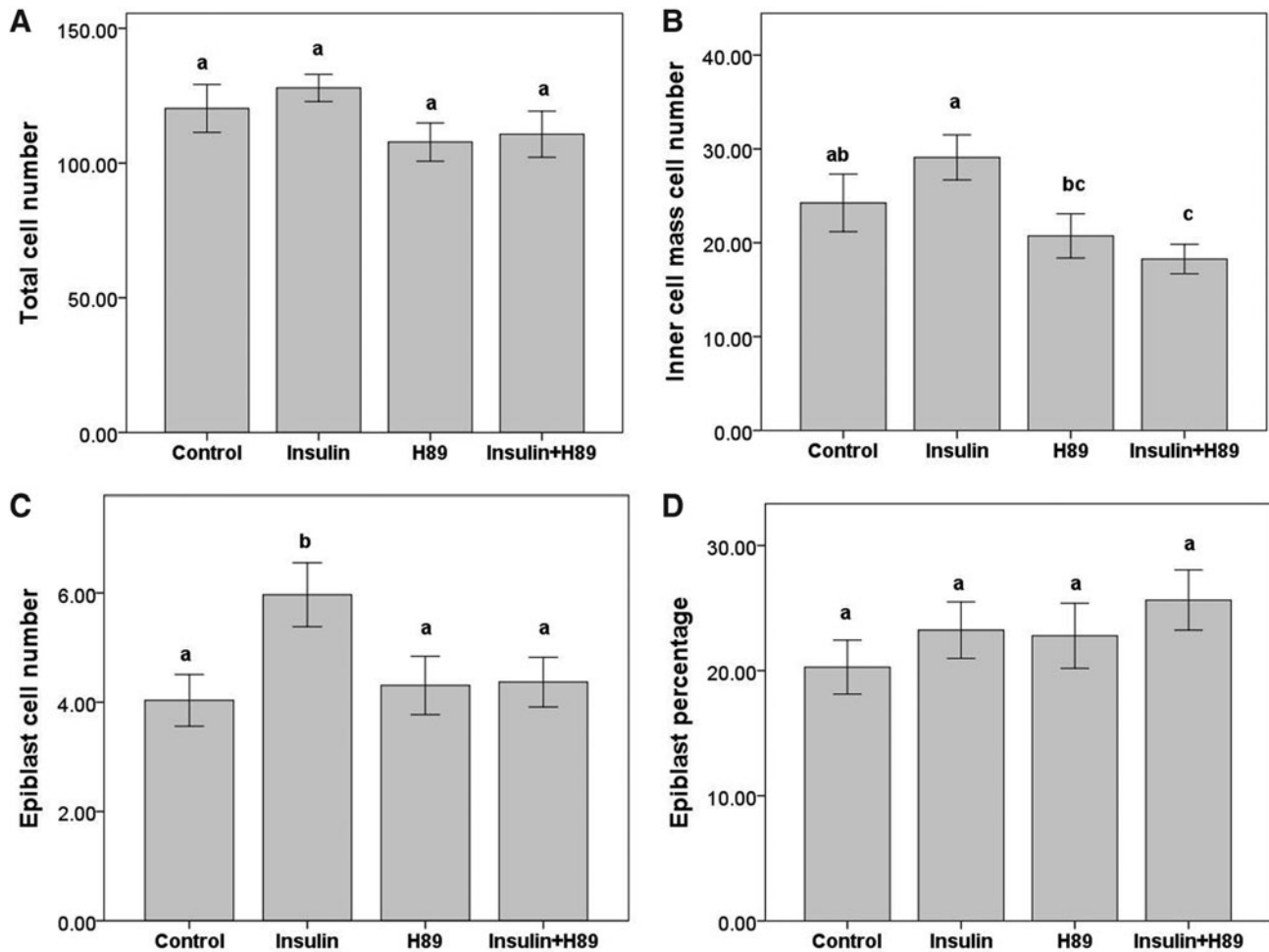


FIG. 4. GSK3 activation during blastocyst culture in the presence of insulin. **(A)** TCN as shown by DAPI staining, **(B)** ICM cell number as shown by Oct4 staining, **(C)** epiblast cell number as shown by Oct4 and Nanog staining, and **(D)** percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in insulin at 1.7 μ M and/or 10 μ M of the GSK3 activator H-89. Data are mean \pm sem. Superscripts a, b, and c are significantly different at $P < 0.05$, $n \geq 26$ blastocysts per treatment. GSK3, glycogen synthase kinase 3.

The inactivation of GSK3 together with p53 had no effect on the development at 94 h (blastocyst hatching blastocyst for all treatments, range 89%–97%), but significantly increased the number of hatched blastocysts relative to the culture with either inhibitor alone ($P < 0.05$, Table 4). However, there were no further synergistic effects on TCN, ICM, or proportion of epiblast compared with either inhibitor alone (Fig. 6).

Discussion

The present study demonstrated that the culture of 8-cell embryos with 1.7 μ M insulin increases the number of epiblast cells in the ICM without affecting the size of the ICM itself and without increasing trophectoderm, primitive endoderm, or TCN. This suggests that rather than acting as a nonspecific mitogenic factor, insulin is specifically acting on the ICM to shift the ratio of epiblast and primitive endoderm toward a larger population of pluripotent cells. Since ESCs are derived from the pluripotent epiblast [13,60], the number of pluripotent cells in a blastocyst is a key determinant of the capacity

of a blastocyst to give rise to an ESC line [7,48,61]. Therefore, it is desirable to have conditions for the development of the blastocyst that maximize the number of epiblast cells. Although treatments using inhibitors can produce larger epiblasts [7,62], inhibitors frequently have nonspecific activities [32,63], while insulin is present in vivo and a component of routine ESC culture, although not routine in embryo culture. As such, culture in insulin could be a useful strategy for improving the pluripotential of embryos cultured in vitro, such as human embryos that are often donated for hESC derivation at the cleavage stage after 5–10 years of cryopreservation and culture in media now known to be perturbing [12]—without the use of inhibitors.

Insulin binds to the insulin receptor (IR), which phosphorylates tyrosine residues on the insulin receptor substrates (IRS-1, IRS-2 and IRS-3), thus enabling the IRSs to activate PI3K via its SRC homology 2 domains [64,65]. PI3K is a second messenger known to be present in the mouse preimplantation embryo [25]. Previous studies have demonstrated that PI3K inhibition impairs embryo development and reduces TCN [27,45], a result that we reproduced in this

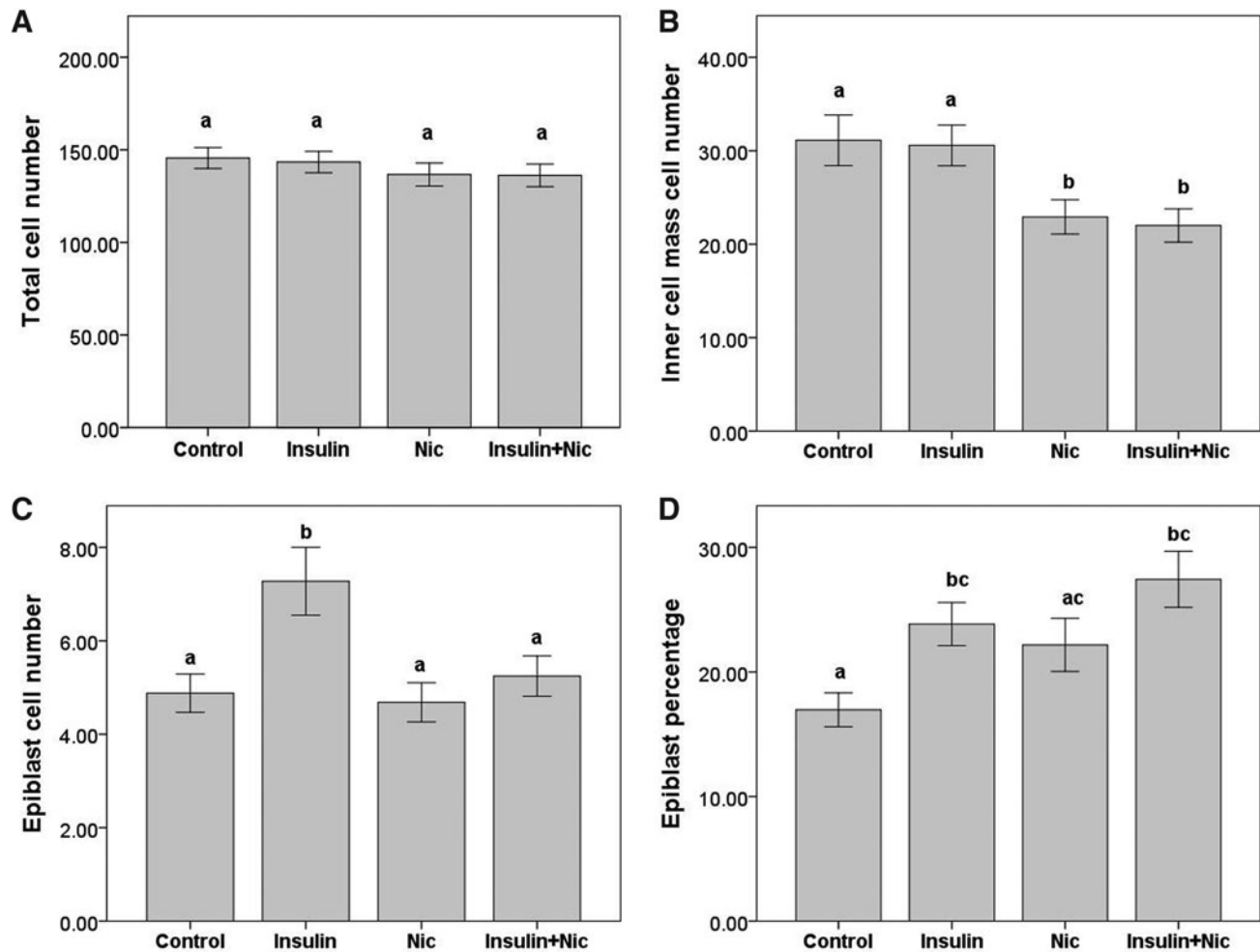


FIG. 5. p53 activation during blastocyst culture in the presence of insulin. (A) TCN as shown by DAPI staining, (B) ICM cell number as shown by Oct4 staining, (C) epiblast cell number as shown by Oct4 and Nanog staining, and (D) percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in insulin at 1.7 μ M and/or 10 μ M of the p53 activator nicotinamide (Nic). Data are mean \pm sem. Superscripts a, b, and c are significantly different at $P < 0.05$, $n \geq 38$ blastocysts per treatment.

study. Importantly, in ESCs, PI3K has been shown to play a role in maintaining pluripotency [28,46], and its inhibition in ESCs decreases Nanog protein levels [28]. Our results suggest that the activation of PI3K in the later-stage embryo is a part of a signaling pathway involved in insulin's stimulation of epiblast cell numbers.

An intermediate of the PI3K signaling pathway, GSK3, has been shown to decrease Nanog transcription and retention of

pluripotency via inactivation of β -catenin [66–68], Hedgehog [69,70], and c-Myc [71,72]. Additionally, active GSK3 protects the intracellular domain of Notch from degradation [73], increasing differentiation [74]. The indirect activation of GSK3 by H-89 [31]—which inhibits PKA whose activity would otherwise prevent GSK3 activation [75–77]—was sufficient to block insulin's ability to increase epiblast cell number. We hypothesized that the inhibition of GSK3 would

TABLE 4. DEVELOPMENTAL MORPHOLOGY AFTER 115 H OF BOTH GLYCOGEN SYNTHASE KINASE 3 AND p53 INHIBITION

Total	Arrested (%)	Total blastocyst (%)	Blastocyst (%)	Hatching blastocyst (%)	Hatched blastocyst (%)
Control	4.5	95.5	13.6	45.5	36.4
CT99021	2.3	97.7	20.5	43.2 ^a	34.1 ^a
Pft- α (pifithrin- α)	5.1	94.9	15.4	56.4 ^b	23.1 ^b
CT99021 + Pft- α	0	100	23.8	16.7 ^{a,b}	59.5 ^{a,b}

Mean percentage of embryos at stage of development after 115 h of culture in the specified treatment. $n \geq 39$ per treatment.

Like pairs, ^a and ^b are significantly different ($P < 0.05$).

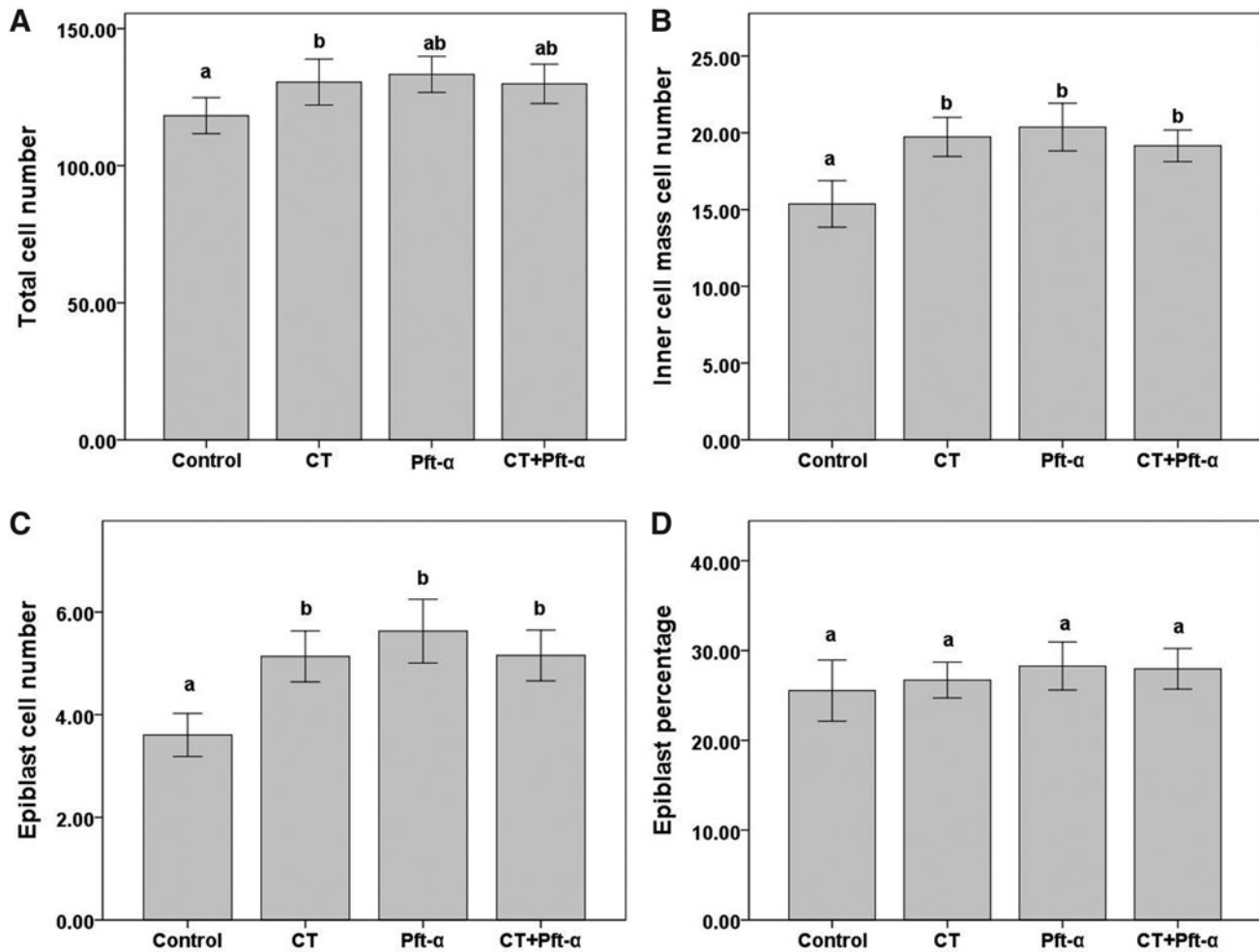


FIG. 6. Dual inhibition of GSK3 and p53. (A) TCN as shown by DAPI staining, (B) ICM cell number as shown by Oct4 staining, (C) epiblast cell number as shown by Oct4 and Nanog staining, and (D) percentage of ICM cells that are epiblast cells in the blastocysts of embryos cultured in 0.3 μ M of GSK3 inhibitor CT99021 (CT) and/or 30 μ M of p53 inhibitor pifithrin- α (Pft- α). Data are mean \pm sem. Superscripts a and b are significantly different at $P < 0.05$, $n \geq 35$ blastocysts per treatment.

have the opposite outcome and replicate the effects of insulin on the epiblast. GSK3 inhibition increased epiblast cell number, similar to that seen with insulin supplementation. Taken together, these data suggest that insulin increases the proportion of epiblast cells in the ICM through mechanisms which involve the inactivation of GSK3.

This is in keeping with previous studies where GSK3 inactivation via BIO increased the percentage of epiblast in outgrown ICMs and increased ESC derivation efficiency [30]. Additionally, the GSK3 inhibitor CT99021 is one of the 3 inhibitors that make up the 3i culture system which improves ESC culture and derivation as well as increasing epiblast proportion of the ICM to close to 100% (the other 2 inhibitors inhibit FGFR and MEK signaling) [7]. However, embryos cultured with GSK3 inhibitor LiCl have reduced hatching and attachment rates [78] and often fail to develop past the 2-cell stage [79]. In our own results, we saw an increase in epiblast cell number at 0.3 μ M CT99021, but this increase was reduced at 3 μ M and lost at 15 μ M (data not shown). Additionally, at 94 h, 15 μ M reduced hatching, reproducing previous findings [78] but with a different inhibitor, thereby suggesting that negative

effects are not due to nonspecific activity by the inhibitor at high concentrations, but that GSK3's broad influence [80,81] makes moderate inhibition the key to a successful embryo culture.

The activation of PI3K is also able to exert significant effects on p53 availability and activity. As a pro-apoptotic protein, active p53 causes cell death and differentiation [35,82]. Specifically, active p53 has been shown to cause differentiation in ESCs by binding to the *Nanog* promoter region and repressing expression [35]. Additionally, the in vitro culture of embryos has been shown to increase p53 activity, with p53-dependant negative effects on embryo development and viability [83], while culture with pifithrin- α , an inhibitor of p53, has been shown to improve the proportion of embryos that develop to the blastocyst stage [38]. When p53 was indirectly activated by inhibiting SIRT-1 [37] with nicotinamide, insulin's ability to increase epiblast cell number was, as with GSK3 activation and PI3K inhibition, ameliorated. Furthermore, the inhibition of p53 had the opposing effect of increasing TCN, ICM, and epiblast cell numbers. This shows that the inhibition of p53 can mimic insulin's effect on the epiblast, suggesting that p53

inactivation is involved in insulin-mediated increase of epiblast cell number.

The beta isoform of GSK3 (GSK3 β) can phosphorylate and form a complex with p53, which increases the activity of both GSK3 β and p53 [41–43]; however, active GSK3 activates MDM2 [39], which targets p53 for degradation. Therefore, the inactivation of GSK3 can cause the accumulation of p53 [39] and apoptosis [40]. This makes it difficult to say whether the effect of GSK3's inhibition on epiblast cell number is helped or hindered by its role in p53 regulation, and suggested to us that GSK3 inactivation coupled with p53 inactivation may be necessary for optimal epiblast increases.

However, the inhibition of both GSK3 and p53 produced no additional synergistic effect on epiblast cell number, suggesting that the effect of GSK3 inhibition at 0.3 μ M CT99021 on epiblast cell number is not being limited by an increase in p53 due to the inactive GSK3's inability to phosphorylate and activate MDM2.

Our results show that the culture of embryos from the 8-cell stage with insulin increases the number and percentage of epiblast cells in the ICM of blastocysts via the activation of PI3K, which, in turn, inactivates GSK3 and p53. While being involved in many signaling pathways with many different targets, in their active forms, both GSK3 and p53 inhibit *Nanog* transcription [28,35], making it likely that insulin's apparent inactivation of these factors increases *Nanog* transcription, resulting in more *Nanog*-positive epiblast cells. Since epiblast cells are the progenitor cells that give rise to ESCs, a culture with insulin offers a potential strategy, effective from the cleavage stage, for increasing the derivation efficiency of ESCs from *in vitro* cultured embryos, though this will require validation by the derivation of ESCs. This is of particular importance for hESC derivation, which often utilizes embryos cryopreserved at the cleavage stage after culture 5–10 years ago in relatively simple media.

Acknowledgments

The authors acknowledge the support of the NHMRC program grant for funding. Dr. Michelle Lane is a recipient of an NHMRC Senior Research Fellowship. The authors thank Alicia Filby, Sarah L. Wakefield, and Nicole Palmer for their technical assistance.

Author Disclosure Statement

No competing financial interests exist.

References

1. Bankowski BJ, AD Lyerly, RR Faden and EE Wallach. (2005). The social implications of embryo cryopreservation. *Fertil Steril* 84:823–832.
2. Klock SC. (2004). Embryo disposition: the forgotten "child" of *in vitro* fertilization. *Int J Fertil Womens Med* 49:19–23.
3. Gardner DK. (2008). Dissection of culture media for embryos: the most important and less important components and characteristics. *Reprod Fertil Dev* 20:9–18.
4. Lane M and DK Gardner. (2007). Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol* 21:83–100.
5. Laverge H, P De Sutter, R Desmet, J Van der Elst and M Dhont. (1997). Prospective randomized study comparing human serum albumin with fetal cord serum as protein supplement in culture medium for *in-vitro* fertilization. *Hum Reprod* 12:2263–2266.
6. Gardner DK and M Lane. (2003). Towards a single embryo transfer. *Reprod Biomed Online* 6:470–481.
7. Nichols J, J Silva, M Roode and A Smith. (2009). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* 136:3215–3222.
8. Bongso A and EH Lee, eds. (2005). *Stem Cells: From Bench to Bedside*. World Sc Press, Singapore.
9. Van der Elst J, E Van den Abbeel, M Camus, J Smits, P Devroey and A Van Steirteghem. (1996). Long-term evaluation of implantation of fresh and cryopreserved human embryos following ovarian stimulation with buserelin acetate-human menopausal gonadotrophin (HMG) or clomiphene citrate-HMG. *Hum Reprod* 11:2097–2106.
10. Oehninger S, J Mayer and S Muasher. (2000). Impact of different clinical variables on pregnancy outcome following embryo cryopreservation. *Mol Cell Endocrinol* 169:73–77.
11. Wang YA, GM Chambers, M Dieng and EA Sullivan. (2009). Assisted reproductive technology in Australia and New Zealand 2007. *Assist Reprod Technol Ser* 13:1–56.
12. Campbell JM, M Mitchell, M Nottle and M Lane. (2011). Development of a mouse model for studying the effect of embryo culture on embryonic stem cell derivation. *Stem Cells Dev* 20:1577–1586.
13. Brook FA and RL Gardner. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci U S A* 94:5709–5712.
14. Martin GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78:7634–7638.
15. Evans MJ and MH Kaufman. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154–156.
16. Harvey MB and PL Kaye. (1990). Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts *in vitro*. *Development* 110:963–967.
17. Gardner HG and PL Kaye. (1991). Insulin increases cell numbers and morphological development in mouse pre-implantation embryos *in vitro*. *Reprod Fertil Dev* 3:79–91.
18. Harvey MB and PL Kaye. (1988). Insulin stimulates protein synthesis in compacted mouse embryos. *Endocrinology* 122:1182–1184.
19. Dungleon GF and PL Kaye. (1993). Insulin regulates protein metabolism in mouse blastocysts. *Mol Reprod Dev* 36:42–48.
20. Harvey MB and PL Kaye. (1991). Mouse blastocysts respond metabolically to short-term stimulation by insulin and IGF-1 through the insulin receptor. *Mol Reprod Dev* 29:253–258.
21. Hardy K and S Spanos. (2002). Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol* 172:221–236.
22. Sharkey A. (1998). Cytokines and implantation. *Rev Reprod* 3:52–61.
23. Mayo LD and DB Donner. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* 98:11598–11603.
24. Sabbatini P and F McCormick. (1999). Phosphoinositide 3-OH kinase (PI3K) and PKB/Akt delay the onset of p53-mediated, transcriptionally dependent apoptosis. *J Biol Chem* 274:24263–24269.

25. Riley JK, MO Carayannopoulos, AH Wyman, M Chi, CK Ratajczak and KH Moley. (2005). The PI3K/Akt pathway is present and functional in the preimplantation mouse embryo. *Dev Biol* 284:377–386.
26. Ding VW, RH Chen and F McCormick. (2000). Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. *J Biol Chem* 275:32475–32481.
27. Gross VS, M Hess and GM Cooper. (2005). Mouse embryonic stem cells and preimplantation embryos require signaling through the phosphatidylinositol 3-kinase pathway to suppress apoptosis. *Mol Reprod Dev* 70:324–332.
28. Storm MP, HK Bone, CG Beck, PY Bourillot, V Schreiber, T Damiano, A Nelson, P Savatier and MJ Welham. (2007). Regulation of Nanog expression by phosphoinositide 3-kinase-dependent signaling in murine embryonic stem cells. *J Biol Chem* 282:6265–6273.
29. Cross DA, DR Alessi, P Cohen, M Andjelkovich and BA Hemmings. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785–789.
30. Umehara H, T Kimura, S Ohtsuka, T Nakamura, K Kitajima, M Ikawa, M Okabe, H Niwa and T Nakano. (2007). Efficient derivation of embryonic stem cells by inhibition of glycogen synthase kinase-3. *Stem Cells* 25:2705–2711.
31. Yan W and HH Tai. (2006). Glycogen synthase kinase-3 phosphorylation, T-cell factor signaling activation, and cell morphology change following stimulation of thromboxane receptor alpha. *J Pharmacol Exp Ther* 317:267–274.
32. Bain J, L Plater, M Elliott, N Shpiro, CJ Hastie, H McLauclan, I Klevernic, JS Arthur, DR Alessi and P Cohen. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408:297–315.
33. Yu ZK, RK Geyer and CG Maki. (2000). MDM2-dependent ubiquitination of nuclear and cytoplasmic P53. *Oncogene* 19:5892–5897.
34. Ogawara Y, S Kishishita, T Obata, Y Isazawa, T Suzuki, K Tanaka, N Masuyama and Y Gotoh. (2002). Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *J Biol Chem* 277:21843–21850.
35. Lin T, C Chao, S Saito, SJ Mazur, ME Murphy, E Appella and Y Xu. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 7:165–171.
36. Mitchell M, A Georgiou, K Cashman, A Stump and M Lane. (2008). Do sirtuin proteins function in the developing mammalian embryo? *Biol Reprod* 78:134.
37. Solomon JM, R Pasupuleti, L Xu, T McDonagh, R Curtis, PS DiStefano and LJ Huber. (2006). Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol Cell Biol* 26:28–38.
38. Jin XL, V Chandrakanthan, HD Morgan and C O'Neill. (2009). Preimplantation embryo development in the mouse requires the latency of TRP53 expression, which is induced by a ligand-activated PI3 kinase/AKT/MDM2-mediated signaling pathway. *Biol Reprod* 80:286–294.
39. Kulikov R, KA Boehme and C Blattner. (2005). Glycogen synthase kinase 3-dependent phosphorylation of Mdm2 regulates p53 abundance. *Mol Cell Biol* 25:7170–7180.
40. Ghosh JC and DC Altieri. (2005). Activation of p53-dependent apoptosis by acute ablation of glycogen synthase kinase-3beta in colorectal cancer cells. *Clin Cancer Res* 11:4580–4588.
41. Watcharasi P, GN Bijur, L Song, J Zhu, X Chen and RS Jope. (2003). Glycogen synthase kinase-3beta (GSK3beta) binds to and promotes the actions of p53. *J Biol Chem* 278:48872–48879.
42. Watcharasi P, GN Bijur, JW Zmijewski, L Song, A Zmijewska, X Chen, GV Johnson and RS Jope. (2002). Direct, activating interaction between glycogen synthase kinase-3beta and p53 after DNA damage. *Proc Natl Acad Sci U S A* 99:7951–7955.
43. Turenne GA and BD Price. (2001). Glycogen synthase kinase3 beta phosphorylates serine 33 of p53 and activates p53's transcriptional activity. *BMC Cell Biol* 2:12.
44. Lane M, M Mitchell, KS Cashman, D Feil, S Wakefield and DL Zander-Fox. (2008). To QC or not to QC: the key to a consistent laboratory? *Reprod Fertil Dev* 20:23–32.
45. Riley JK, MO Carayannopoulos, AH Wyman, M Chi and KH Moley. (2006). Phosphatidylinositol 3-kinase activity is critical for glucose metabolism and embryo survival in murine blastocysts. *J Biol Chem* 281:6010–6019.
46. Paling NR, H Wheadon, HK Bone and MJ Welham. (2004). Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. *J Biol Chem* 279:48063–48070.
47. Vlahos CJ, WF Matter, KY Hui and RF Brown. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 269:5241–5248.
48. Ying QL, J Wray, J Nichols, L Battle-Morera, B Doble, J Woodgett, P Cohen and A Smith. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453:519–523.
49. Aparicio IM, M Garcia-Herreros, T Fair and P Lonergan. (2010). Identification and regulation of glycogen synthase kinase-3 during bovine embryo development. *Reproduction* 140:83–92.
50. Fujino H, KA West and JW Regan. (2002). Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostanoicid receptors by prostaglandin E2. *J Biol Chem* 277:2614–2619.
51. Jensen J, EO Brennesvik, YC Lai and PR Shepherd. (2007). GSK-3beta regulation in skeletal muscles by adrenaline and insulin: evidence that PKA and PKB regulate different pools of GSK-3. *Cell Signal* 19:204–210.
52. Bragado MJ, IM Aparicio, MC Gil and LJ Garcia-Marin. (2010). Protein kinases A and C and phosphatidylinositol 3 kinase regulate glycogen synthase kinase-3A serine 21 phosphorylation in boar spermatozoa. *J Cell Biochem* 109:65–73.
53. Ku BM, YK Lee, JY Jeong, J Ryu, J Choi, JS Kim, YW Cho, GS Roh, HJ Kim, et al. (2011). Caffeine inhibits cell proliferation and regulates PKA/GSK3beta pathways in U87MG human glioma cells. *Mol Cells* 31:275–279.
54. Komarov PG, EA Komarova, RV Kondratov, K Christov-Tselkov, JS Coon, MV Chernov and AV Gudkov. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 285:1733–1737.
55. Schafer T, C Scheuer, K Roemer, MD Menger and B Vollmar. (2003). Inhibition of p53 protects liver tissue against endotoxin-induced apoptotic and necrotic cell death. *FASEB J* 17:660–667.
56. Audrito V, T Vaisitti, D Rossi, D Gottardi, G D'Arena, L Laurenti, G Gaidano, F Malavasi and S Deaglio. (2011). Nicotinamide blocks proliferation and induces apoptosis of chronic lymphocytic leukemia cells through activation of the p53/miR-34a/SIRT1 tumor suppressor network. *Cancer Res* 71:4473–4483.

57. Mitchell M, KS Cashman, DK Gardner, JG Thompson and M Lane. (2009). Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod* 80:295–301.
58. Lane M and DK Gardner. (1992). Effect of incubation volume and embryo density on the development and viability of mouse embryos *in vitro*. *Hum Reprod* 7:558–562.
59. O'Neill C. (1997). Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos *in vitro*. *Biol Reprod* 56:229–237.
60. Silva J, J Nichols, TW Theunissen, G Guo, AL van Oosten, O Barrandon, J Wray, S Yamanaka, I Chambers and A Smith. (2009). Nanog is the gateway to the pluripotent ground state. *Cell* 138:722–737.
61. Batlle-Morera L, A Smith and J Nichols. (2008). Parameters influencing derivation of embryonic stem cells from murine embryos. *Genesis* 46:758–767.
62. Yamanaka Y, F Lanner and J Rossant. (2010). FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. *Development* 137:715–724.
63. Davies SP, H Reddy, M Caivano and P Cohen. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95–105.
64. White MF. (1998). The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* 182:3–11.
65. Ogawa W, T Matozaki and M Kasuga. (1998). Role of binding proteins to IRS-1 in insulin signalling. *Mol Cell Biochem* 182:13–22.
66. Takao Y, T Yokota and H Koide. (2007). Beta-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. *Biochem Biophys Res Commun* 353:699–705.
67. Sineva GS and VA Pospelov. (2010). Inhibition of GSK3 β enhances both adhesive and signaling activities of beta-catenin in mouse embryonic stem cells. *Biol Cell* 102:549–560.
68. He X, M Semenov, K Tamai and X Zeng. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* 131:1663–1677.
69. Po A, E Ferretti, E Miele, E De Smaele, A Paganelli, G Canetti, S Coni, L Di Marcotullio, M Biffoni, et al. (2010). Hedgehog controls neural stem cells through p53-independent regulation of Nanog. *EMBO J* 29:2646–2658.
70. Jia J, K Amanai, G Wang, J Tang, B Wang and J Jiang. (2002). Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. *Nature* 416:548–552.
71. Cartwright P, C McLean, A Sheppard, D Rivett, K Jones and S Dalton. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* 132:885–896.
72. Gregory MA, Y Qi and SR Hann. (2003). Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *J Biol Chem* 278:51606–51612.
73. Foltz DR, MC Santiago, BE Berechid and JS Nye. (2002). Glycogen synthase kinase-3 β modulates notch signaling and stability. *Curr Biol* 12:1006–1011.
74. Lowell S, A Benchoua, B Heavey and AG Smith. (2006). Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol* 4:e121.
75. Aitken A, T Bilham and P Cohen. (1982). Complete primary structure of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *Eur J Biochem* 126:235–246.
76. Endo S, X Zhou, J Connor, B Wang and S Shenolikar. (1996). Multiple structural elements define the specificity of recombinant human inhibitor-1 as a protein phosphatase-1 inhibitor. *Biochemistry* 35:5220–5228.
77. Zhang F, CJ Phiel, L Spece, N Gurvich and PS Klein. (2003). Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *J Biol Chem* 278:33067–33077.
78. Li J, JV Zhang, YJ Cao, JX Zhou, WM Liu, XJ Fan and EK Duan. (2005). Inhibition of the beta-catenin signaling pathway in blastocyst and uterus during the window of implantation in mice. *Biol Reprod* 72:700–706.
79. Acevedo N, X Wang, RL Dunn and GD Smith. (2007). Glycogen synthase kinase-3 regulation of chromatin segregation and cytokinesis in mouse preimplantation embryos. *Mol Reprod Dev* 74:178–188.
80. Doble BW and JR Woodgett. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116:1175–1186.
81. Jope RS and GV Johnson. (2004). The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 29:95–102.
82. Qin H, T Yu, T Qing, Y Liu, Y Zhao, J Cai, J Li, Z Song, X Qu, et al. (2007). Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. *J Biol Chem* 282:5842–5852.
83. Li A, V Chandrakanthan, O Chami and C O'Neill. (2007). Culture of zygotes increases TRP53 [corrected] expression in B6 mouse embryos, which reduces embryo viability. *Biol Reprod* 76:362–367.

Address correspondence to:

Jared M. Campbell

Centre for Stem Cell Research

School of Paediatrics and Reproductive Health

University of Adelaide

Medical School Nth

Level 6 Frome Road

Adelaide 5005

South Australia

E-mail: jared.campbell@adelaide.edu.au

Received for publication October 21, 2011

Accepted after revision February 16, 2012

Prepublished on Liebert Instant Online February 16, 2012

4.1 Introduction

The previous chapter demonstrated that the addition of embryo regulators such as amino acids and vitamins to culture medium for the development of post-compaction embryos increased their epiblast cell number irrespective of medium used for the culture during the cleavage stage. This demonstrated that optimisation of conditions for embryo culture will be important for increasing hESC derivation from donated cleavage stage embryos which were cultured in suboptimal conditions or which are of reduced quality. As discussed earlier (section 1.6.1) growth factors are not commonly present in culture for mouse or human embryos. However, the addition of different growth factors has been shown to improve embryo quality with respect to markers of quality as well as reproductive viability [344,345,353], particularly when embryos have been cultured in suboptimal conditions such as low protein, lack of amino acids etc. The effects of different growth factors on *in vitro* culture are discussed and summarised (section 1.6.1). Several are capable of increasing blastocyst development and ICM development and therefore may provide a novel method to improve embryo development, and in particular epiblast cell number which would ultimately increase ESC derivation efficiency and quality.

One of these growth factors, insulin (section 1.6.1.2.1), is an interesting candidate as the addition of insulin to embryo culture media for culture from the zygote or 2-cell stage has been shown previously to result in increased blastocyst formation rate [384], accelerated development [384,461], increased blastocyst cell number [384], stimulated protein synthesis [382,462,463], reduced protein degradation [463], and increased protein uptake [463]. While the capacity of insulin or any other growth factor – many of which have similar effects to those described above [344,360] – to increase the number of epiblast cells is unknown, insulin differs from most growth factors in that it increases total cell number by increasing inner cell mass cell number [384]. Additionally, the insulin receptor is present on both mouse and human embryos [344], while insulin is found in the fluid of the maternal reproductive tract of both species [389,464]. Moreover, as insulin is a highly conserved proteins [405,406], any observations made in the mouse will likely translate to the human.

The aim of the present chapter was therefore to determine whether the addition of insulin to embryo culture media from the 8-cell stage increases both embryo development and formation of the epiblast.

4.2 Experimental design

In this chapter the embryos used were from prepubertal C57Bl/6 mice. Embryos were collected at the zygote stage 22h after induction of ovulation by hCG. They were cultured in groups of 10 under paraffin oil at 37°C in 6% CO₂, 5% O₂, 89% N₂ in G1.2 medium. At 48h of culture embryos were transferred to 20µl drops of G2.2 supplemented with the relevant treatment (Table 4.1), and cultured individually at 37°C in 6% CO₂, 5% O₂, 89% N₂ from 48-115h.

TABLE 4.1 EXPERIMENTAL CULTURE SYSTEM

First stage of culture system	Second stage of culture system
G1.2	G2.2
G1.2	G2.2 + 0.17pM insulin
G1.2	G2.2 + 1.7pM insulin
G1.2	G2.2 + 1700pM insulin

Concentrations of insulin were based on the work in [384], dilution protocol (Appendix 8.2).

Blastocyst development was assessed at 96h and 115h (section 2.4.3). Blastocysts were then fixed and stained to determine total, ICM and epiblast cell number. In the previous chapter the expression of Nanog alone was used to identify epiblast cells. Here, in order to establish ICM, epiblast and primitive endoderm cell number in the same embryo, staining included OCT4 as well as Nanog.

4.2.1 Statistics

Data was expressed as mean±SEM and analysed using Univariate General Linear Model or an independent samples t-test using PASW Statistics 17. For quantitative analyses treatment group was fitted as a fixed factor and replicate was fitted as a cofactor. Between treatment differences were assessed using the Least Significant Difference (LSD) method. Levene's test of equality was used to

confirm equal variance. Categorical data was analysed by chi square tests. Values of $P < 0.05$ were considered significant.

4.3 Results

4.3.1 Blastocyst Development

The supplementation of culture medium with insulin (0, 0.17, 1.7 and 1700pM) from the 8-cell stage had no significant effect on blastocyst development at 96h (Table 4.2) or 115h (Table 4.3).

TABLE 4.2. THE EFFECT OF INSULIN ON DEVELOPMENT OF THE BLASTOCYST AT 96h

Treatment	Arrested(%)	Early blastocyst(%)	Blastocyst(%)	Hatching blastocyst(%)
G2 + 0pM insulin	3	12	46	39
G2 + 0.17pM insulin	3	10	40	48
G2 + 1.7pM insulin	9	9	34	47
G2 + 1700pM insulin	3	3	54	33

The total number of embryos which had reached the specified morphological stage after 96h of culture, shown as a percentage. N≥69, (4 replicate experiments). No significant differences between treatments

TABLE 4.3. THE EFFECT OF INSULIN ON DEVELOPMENT OF THE BLASTOCYST AT 115h

Treatment	Arrested(%)	Blastocyst(%)	Hatching blastocyst(%)	Hatched blastocyst(%)
G2 + 0pM insulin	4	12	43	41
G2 + 0.17pM insulin	9	14	40	36
G2 + 1.7pM insulin	10	7	41	42
G2 + 1700pM insulin	4	10	54	31

The total number of embryos which had reached the specified morphological stage after 115h of culture, shown as a percentage. N≥69, (4 replicate experiments). No significant differences between treatments

4.3.2 Pluripotency of ICM Cells in Cultured Blastocysts

At 115h of culture there was no effect of insulin on total cell number or ICM cell numbers (Fig. 4.1A and B). However, culture in the presence of 1.7pM insulin, significantly increased epiblast cell number ($P < 0.01$, Fig. 4.1C). As the epiblast exists as a subpopulation of the ICM, this meant that there was an increase in the proportion of ICM cells that were ESC progenitor epiblast cells in the presence of 1.7pM insulin ($P < 0.01$, Fig. 4.1D). Trophectoderm cell number was calculated by subtracting ICM cell number from TCN (Table 2.2) and primitive endoderm cell number was calculated by subtracting epiblast cell number from ICM cell number (Table 2.2), with no difference seen for either (Fig. 4.1E and F). No difference was observed in the percentage of blastocysts at 115h which possessed an epiblast (percent with epiblast, range 96-100%). Representative images are shown in (Fig. 4.2).

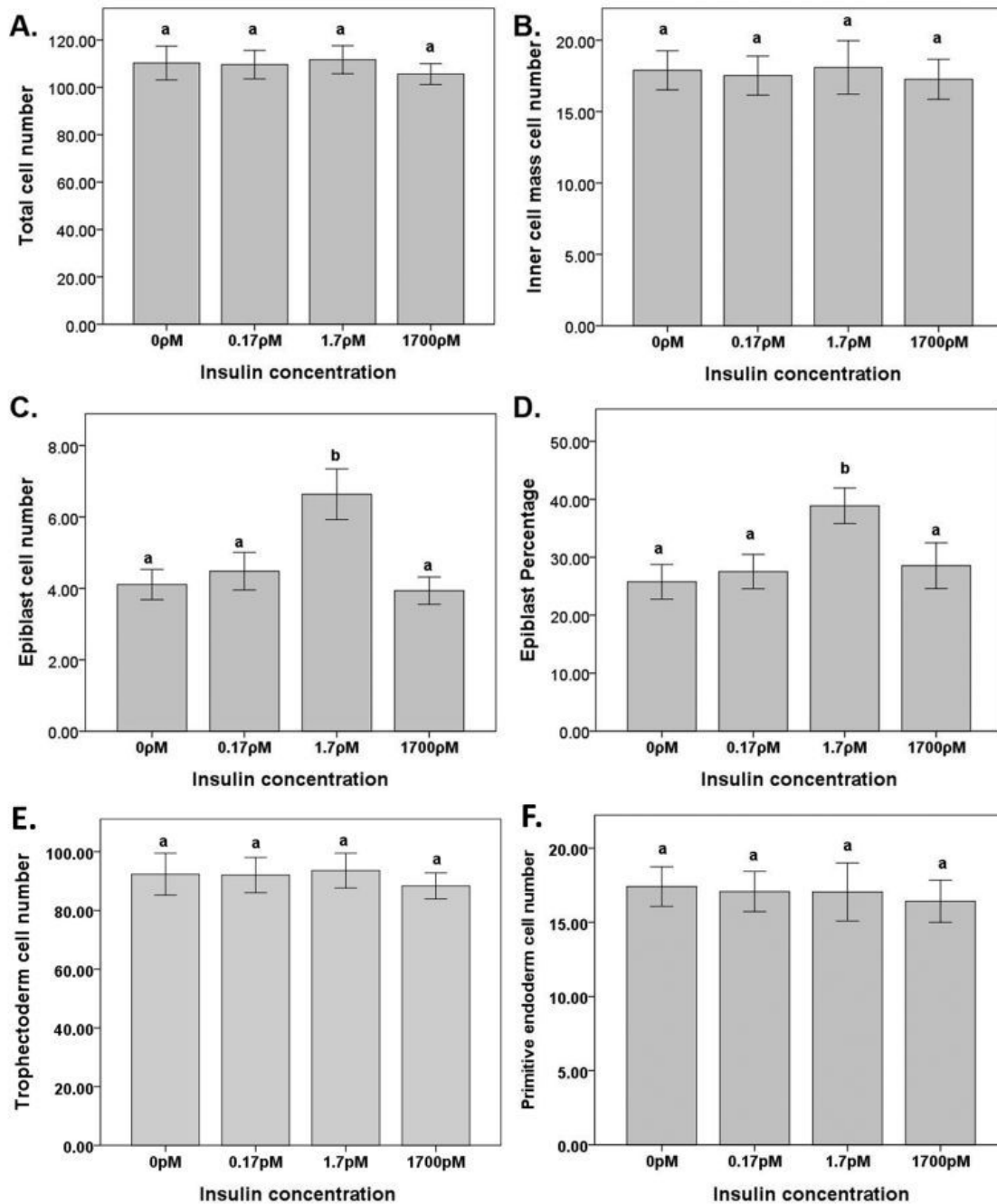


FIG. 4.1. Blastocyst culture in the presence of insulin. A; Total cell number as shown by Dapi staining, B; ICM cell number as shown by OCT4 staining, C; epiblast cell number as shown by OCT4 and Nanog staining, D; percentage of ICM cells that are epiblast cells, E; Trophectoderm cell number as shown by total cell number minus ICM cell number, and F; primitive endoderm cell number as shown by ICM minus epiblast cell number, in the blastocysts of embryos cultured in insulin at the concentrations indicated. Data are mean \pm sem. Superscripts a and b are significantly different at $P < 0.01$, $N \geq 34$ blastocysts per treatment.

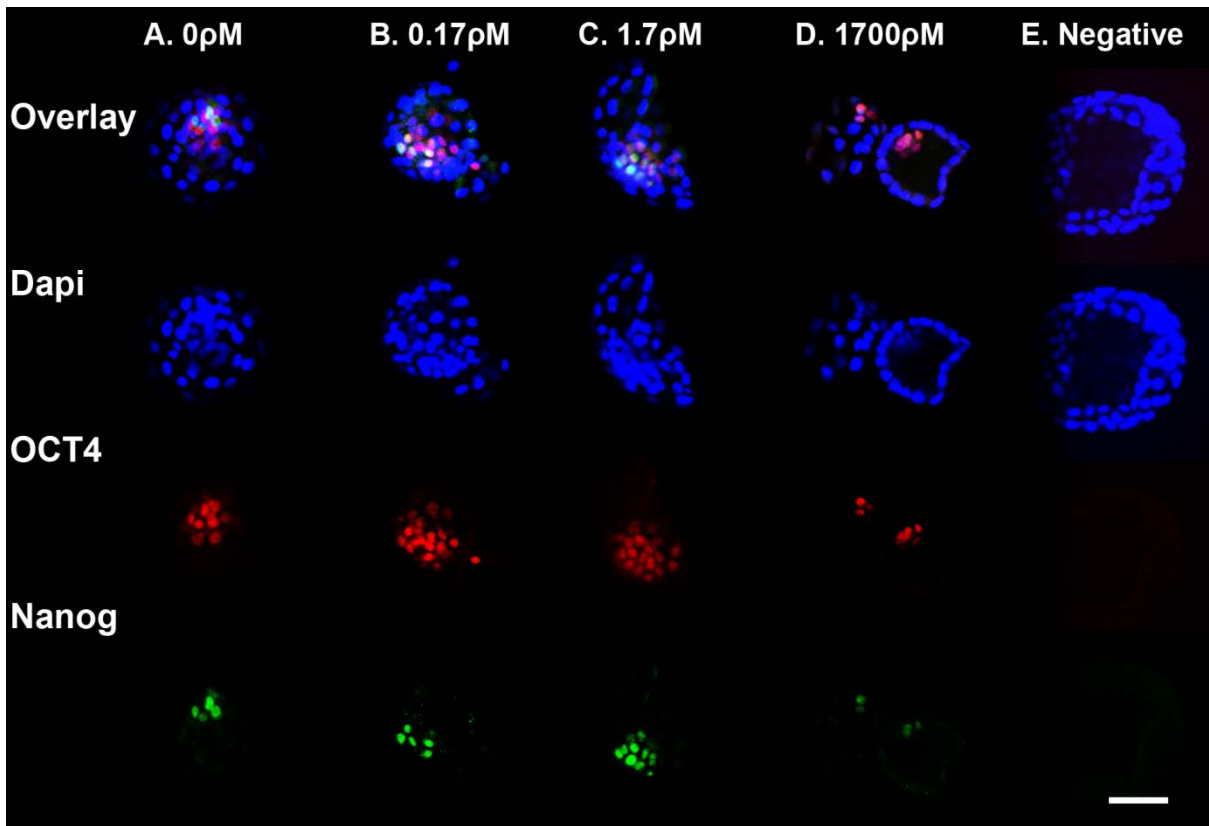


FIG. 4.2. Representative images of immunocytochemically stained blastocysts after culture in the indicated concentration of insulin. All cell nuclei are stained blue with Dapi, OCT4 positive cells are stained red with rhodamine, Nanog positive cells are stained green with FITC. Scale bar equal to 50 μ m. A; G2+0pM insulin, B; G2+0.17pM insulin, C; G2+1.7pM insulin, D; G2+1700pM insulin, E; Negative control

4.4 Discussion

The aim of the present chapter was to determine investigate the effect of addition of insulin to embryo culture media from the 8-cell stage on embryo development and the formation of the epiblast. Culture of 8-cell embryos with 1.7pM insulin increased the number of epiblast cells in the ICM without affecting the size of the ICM itself, and without increasing primitive endoderm, trophoctoderm or total cell number. This is an interesting finding as it suggests that rather than acting as a non-specific mitogenic factor, insulin is specifically acting on the ICM to shift the ratio of epiblast and primitive endoderm towards a larger population of pluripotent cells. As ESCs are derived from the pluripotent epiblast [14,442], the number of epiblast cells in a blastocyst is a key determinant of the capacity of a blastocyst to give rise to an ESC line [72,428,465]. Therefore, it is desirable to have conditions for the development of the blastocyst that maximise the number of epiblast cells. Although treatments using inhibitors can produce larger epiblasts [416,428], inhibitors frequently have non-specific activities [466,467], while insulin is present *in vivo* and a component in the propagation of ESC lines – although not routine in embryo culture. As such, culture in insulin could be a useful strategy for increasing pluripotency in embryos cultured *in vitro* – such as human embryos which are often donated for hESC derivation at the cleavage stage after 5-10 years of cryopreservation and culture in media now known to be perturbing [455] or upon identification of poor quality – without the use of inhibitors.

The finding that culture with 1.7pM insulin did not increase the number of ICM cells in the blastocyst was an unexpected result as it appears to contradict the work of Harvey and Kaye [384] who found a significant increase in ICM cell number in the mouse blastocyst at this concentration, which was the key finding which prompted the selection of insulin for investigation of its effect on the epiblast. However, this variation may be attributed to differences in the models used. In the study by Harvey and Kaye embryos were cultured in modified BMOC2; a simple culture medium as modelled by simple-G1 in chapter 3.0. Previous work using simple-G1 has shown that culture of embryos in simple media reduces their ICM cell number [468]. Furthermore, as discussed (section 1.6.1.1), the effects of growth factors are often most evident when embryos are cultured in perturbing conditions. As such, a possible explanation for this difference is that the increase in ICM cell number caused by insulin in the study by Harvey and Kaye is the result of insulin's amelioration of the reduction caused by culture in simple medium, whereas no increase in ICM cell number is observed in this chapter due to the use of complex culture media. A review of the literature found no previous studies which demonstrated a positive effect of insulin in a physiological based medium. Furthermore, relative to the culture system used in this chapter, ICM cell number in [384] was

examined at approximately 80h (embryos were flushed at the 2-cell rather than zygote stage, which adds a confounding factor with regards to timing, but not insulin exposure as mouse embryos do not express the insulin receptor until the 8-cell stage [344]), whereas in this chapter assessment was carried out at 115h. This raises the question as to whether the differences observed could be the result of the time point at which ICM cell number was examined. Similarly, the results of Harvey and Kaye showed an improved rate of blastocyst development, whereas the results of the present chapter, where blastocyst development was assessed at different time points, showed no effect. The progression of insulin's effect on blastocyst development and differentiation over time is investigated further in Chapter 6.0.

An additional difference is that in [384] the ICM increase was maximal at 1.7pM insulin, and that when the insulin concentration was increased to 1700pM a plateau effect was demonstrated, whereas in this chapter the beneficial effects of insulin at 1.7pM on epiblast cell number and percentage were lost when insulin concentration was increased to 1700pM. One explanation for the loss of insulin's positive effects is that at higher concentrations of insulin, the insulin receptor could stimulate different second messenger pathways to different degrees. Notably, the MAP kinase (MAPK) pathway has been shown to be activated by the insulin receptor [401], and to be antagonistic to *Nanog* transcription and pluripotency despite its ability to stimulate cell growth [428]. High levels of insulin (700nM) have been shown to cause apoptosis in cultured mouse embryos [469], and in human lymphocytes high levels of insulin (10nM) have been shown to reduce the number of insulin receptors on the cell surface [470]. Moreover, at high concentrations insulin can bind to and activate the IGF 1 receptor [402] whereas at lower concentrations insulin is only able to activate the insulin receptor [384] – which is relevant as mouse embryos cultured in high levels of IGF-1 (130nM) have reduced viability as a result of increased resorption following transfer to pseudopregnant females [471], demonstrating that IGF-1 receptor activation could have a negative effect on embryo development.

Insulin is a growth factor whose activity results in numerous downstream effects, many of which act in apparent antagonism of one another. This raises the question of exactly how insulin exerts its effect on *Nanog* transcription, ICM differentiation and epiblast cell number which is examined in the following chapter.

5.0 Insulin increases epiblast cell number of in vitro cultured mouse embryos via the PI3K/GSK3/p53 pathway

The work presented in this chapter has been published in the following journal:

Campbell JM, MB Nottle, I Vassiliev, M Mitchell and M Lane. (2012) Insulin increases epiblast cell number of in vitro cultured mouse embryos via the PI3K/GSK3/p53 pathway. *Stem Cells and Dev* 21:2430-41

Statement of authorship contributions

Jared M. Campbell: Designed and performed all experiments, interpreted and analysed data, wrote manuscript and is corresponding author.

Mark B. Nottle: Provided intellectual input, assisted with experimental design, data interpretation and manuscript preparation.

Ivan Vassiliev: Provided intellectual input, assisted with data interpretation and manuscript preparation.

Megan Mitchell: Provided intellectual input, assisted with experimental design and manuscript preparation.

Michelle Lane: Principal project supervisor, assisted with experimental design, data interpretation and manuscript preparation.

Campbell, J.M., Nottle, M.B., Vassiliev, I, Mitchell, M. & Lane, M. (2012) Insulin increases epiblast cell number of on vitro cultured mouse embryos via the P13K/GSK3/p53 pathway.
Stem Cells and Development, v. 21(13), pp. 2430-2441

NOTE:

This publication is included on pages 109-136 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://doi.org/10.1089/scd.2011.0598>

6.0 Epiblast cell number and primary embryonic stem cell colony generation are increased by culture of embryos with insulin from the cleavage stage

The work presented in this chapter has been published in the following journal:

Campbell JM, M Lane, I Vassiliev, and M Nottle. (2012). Epiblast cell number and primary embryonic stem cell colony generation are increased by culture of cleavage stage embryos in insulin. *J Reprod Dev*

Statement of authorship contributions

Jared M. Campbell: Designed and performed all experiments, interpreted and analysed data, wrote manuscript and is corresponding author

Michelle Lane: Provided intellectual input, assisted with experimental design, data interpretation and manuscript preparation.

Ivan Vassiliev: Provided intellectual input, assisted with experimental design, data interpretation and manuscript preparation.

Mark B. Nottle: Principal project supervisor, assisted with experimental design, data interpretation and manuscript preparation.

Campbell, J.M., Lane, M., Vassiliev, I. & Nottle, M. (2012) Epiblast cell number and primary embryonic stem cell colony generation are increased by culture of cleavage stage embryos in insulin.
Journal of Reproduction and Development, v. 59(2), pp. 131-138

NOTE:

This publication is included after page 137 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

<http://doi.org/10.1262/jrd.2012-103>

7.0 General Discussion

7.1 Introduction

The embryo begins as a single totipotent cell, it then undergoes multiple rounds of division coupled with differentiation until it forms a blastocyst and has the potential to implant in the uterus. The pluripotent epiblast of the ICM then undergoes further division and differentiation to develop into the fetus, and eventually a fully developed organism. Epiblast cells can be isolated and cultured in conditions which allow ESC lines to be derived. The efficiency with which these lines can be derived is related to the number of epiblast cells in the ICM [72,428]. ESC lines, especially hESC lines, hold great potential value in the fields of drug toxicity studies, drug discovery studies, developmental biology and regenerative medicine. However, the efficiency of hESC derivation is low. The aim of my thesis was to use a mouse model to identify and develop a strategy which improved the efficiency of ESC derivation. In particular, given that most embryos donated for human ESC derivation are of poor quality and/or have been cultured in relatively simple media now known to perturb embryo development prior to being frozen at the precompaction stage up to 10 years earlier, the hypothesis that the period where they are cultured to the blastocyst stage could be exploited to improve their quality and ability to give rise to hESCs by the addition of insulin was examined.

The results from chapter 3 showed that *in vitro* culture of embryos during the precompaction stage in simple medium, which model the historic culture conditions that human embryos available for hESC derivation were exposed to, reduces the quality of those embryos as evidenced by a range of criteria including retarded developmental rates, decreased epiblast cell number and altered gene expression in outgrowths. Some aspects of this reduction in quality could be restored post-compaction by culture in a medium designed to support post-compaction embryo development *in vitro* (G2), including epiblast cell number. However, epiblast cell number was only partially improved. Later results suggest that increased epiblast cell number correlates with an increased capacity to give rise to ESCs. Culture in G2 medium post compaction also increased the proportion of embryos which reached the hatched blastocyst stage which was subsequently shown in Chapter 6.0 to be correlated with an increased capacity to give rise to ESCs. Together these findings highlight that subsequent culture in improved culture media may improve the efficiency of ESC derivation from embryos initially cultured in relatively simple media.

One of the components not routinely included in culture media for human embryos, but which is nevertheless known to influence development, is growth factors. However, a role for growth factors is well established in the ESC field with LIF being vital to the retention of pluripotency of mESC. This led to the hypothesis that addition of growth factor could further improve blastocyst development, pluripotency, outgrowth and ESC derivation. To this end, insulin was chosen for investigation because it has previously been shown to increase ICM cell number when included in embryo culture media [344,369].

The inclusion of insulin in post-compaction culture medium was able to improve the number of pluripotent cells in blastocysts, working through the PI3K, GSK3, p53 pathway to shift the balance of differentiation versus pluripotency within the ICM to increase epiblast number and proportion. This resulted in a permanent increase in the quality of embryos as demonstrated by their capacity to give rise to outgrowths with more pluripotent cells. These increases in blastocyst quality translated into an increase in capacity to give rise to primary ESC colonies (Fig.7.1). These results suggest that the inclusion of insulin in embryo culture medium post-compaction could be used to improve the quality of cryopreserved or fresh human embryos donated at or near compaction.

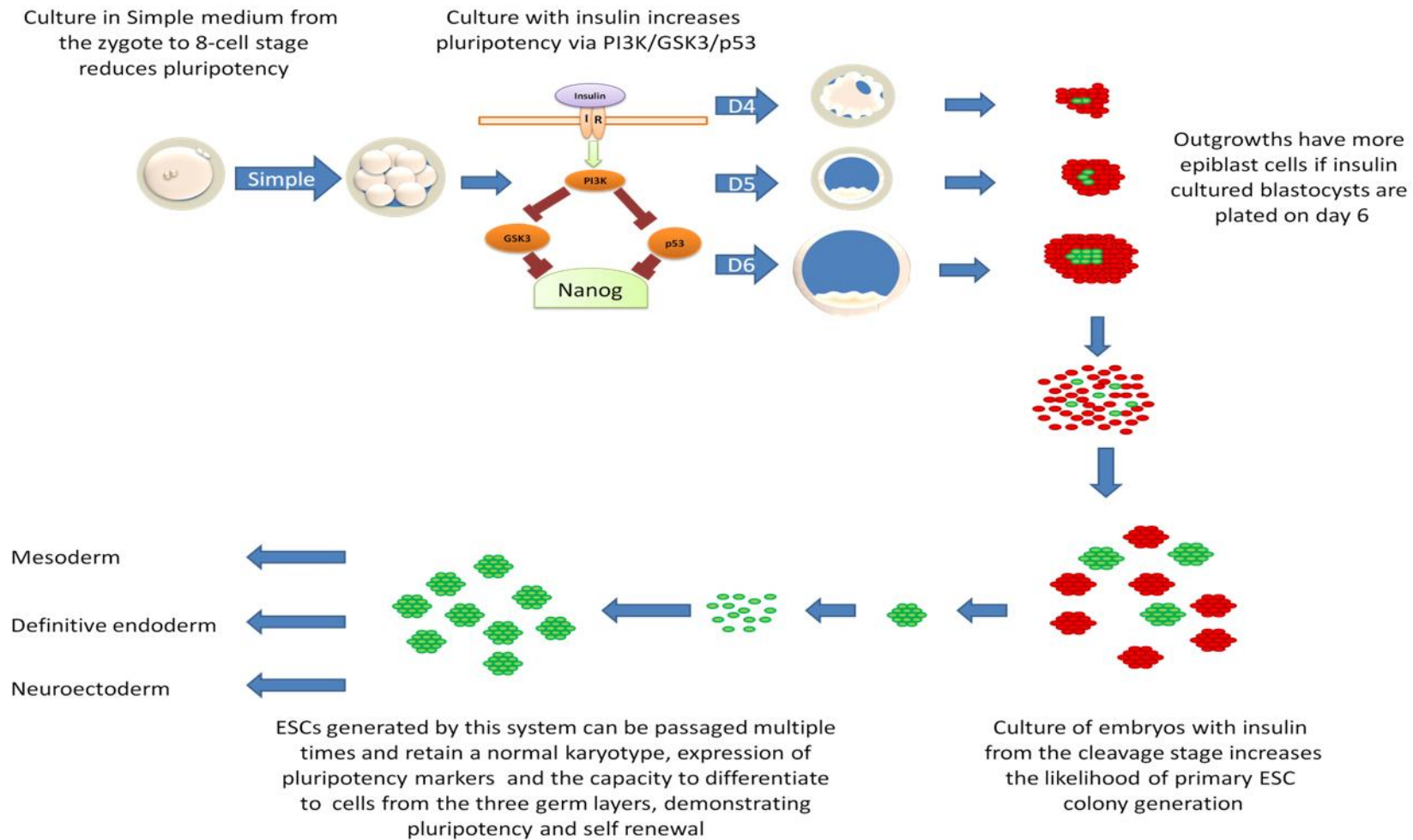


FIG. 7.1. Summary of main findings of thesis. D4, D5 and D6 are day 4, day 5 and day 6 respectively. Pluripotent cells are shown in green, differentiated cells are shown in red.

7.2 Impact of culture conditions on epiblast and pluripotency

The low derivation efficiency of hESCs has been attributed to the reduced quality of human embryos available for derivation attempts [5,6,73]. *In vitro* culture of embryos is typically associated with reductions in embryo quality and viability [163]. Human embryos for hESC derivation have often been cryopreserved for 5-10 years prior to their donation for research purposes [165,166], as a result many have been cultured in media now known to be perturbing to embryo viability and which support only low levels of blastocyst development, necessitating cleavage stage transfers for the majority of IVF cycles performed.

To model this system for human embryos, experiments in Chapter 3.0 cultured mouse embryos in a simple medium for the first 48h to the 8-cell stage to approximate the culture period commonly used in human IVF. These experiments determined that culture of embryos in simple medium retarded the development of blastocysts and significantly reduced the average number of epiblast cells per blastocyst, which was consistent with previous studies in human and mouse [8,9,215-218]. However, these experiments did establish that there was some capacity to improve blastocyst development and epiblast cell number by transferring embryos to G2 medium from the 8-cell stage. Despite this there was a lasting negative impact of the initial culture period on development. Furthermore, an additional novel finding was made that embryos cultured in simple medium were less likely to contain an epiblast irrespective of the culture medium for the second 48h and therefore lack the capacity to generate an ESC line. Additionally, assessment of outgrowths generated from these blastocysts showed that the perturbing conditions of a simple medium had lasting effects on the gene expression of the outgrowths, with altered gene expression of *Atrx* and *Nanog*.

Human embryos donated for hESC derivation are likely to have been exposed to conditions such as simple style culture medium, examples of which include HTF, Earle's, and T6, which were widely used in IVF and can still be found in use. Collectively, the data generated in Chapter 3.0 therefore indicate that these embryos are likely to have significantly reduced capacities to give rise to hESCs. This in turn suggests that the characteristics of hESC lines could be being affected by the culture conditions to which the embryos from which they were derived were exposed to prior to donation.

As many human embryos are cryopreserved at the cleavage stage [167-169] and therefore donated for ESC generation at the cleavage stage, they must be further cultured to the blastocyst stage before ESC derivation. This additional culture period represents a window where the pluripotency of embryos which have previously been exposed to perturbing culture conditions can be improved.

The results of chapter three demonstrated that while the quality of mouse embryos could be improved by culturing them in modern complex medium purpose designed to support embryo development from the 8-cell stage, additional interventions would likely be necessary to fully exploit the cleavage to blastocyst culture period. In future work these findings would be reinforced by experiments where these embryos were used to establish mESCs and the relative efficiencies compared.

7.3 Insulin stimulation of pluripotency of post-compactation embryos

The inclusion of select growth factors in embryo culture media has previously been shown to be capable of improving embryo development and viability [344,345], however, growth factors are not routinely included in culture media commercially available for human embryo culture [347,349,527-529]. To further examine how interventions to the culture medium for the post-compactation stage embryo may effect epiblast cells and pluripotency, the growth factor insulin was added to the culture medium. Insulin has previously been shown to increase the ICM cell number of embryos and was selected as the most promising candidate from a large panel of growth factors previously used to improve embryo culture.

The data in Chapter 4.0 demonstrated that 1.7pM insulin increased epiblast cell number without affecting ICM cell number. This resulted in a significant increase in the proportion of ICM cells which were epiblast as opposed to primitive endoderm. This novel finding suggested that insulin was acting to shift the balance of differentiation within the ICM towards more pluripotent cells, rather than acting as a general mitogenic factor and stimulating overall cell growth. This was further highlighted by the finding that total cell number and trophoctoderm cell number were also unaffected by the presence of insulin. However, as in previous studies there was a threshold concentration where the effect of insulin was maximal, above which further increases in concentration led to the loss of the increase in epiblast cell number. If this strategy is implemented in the human it may be necessary to repeat dose response experiments to establish the optimal dose for maximal epiblast cell number increases. Although the work presented has shown that insulin as an additive to culture media is able to maintain pluripotency in the ICM and direct differentiation towards epiblast, other growth factors, such as those discussed in the literature review (section 1.6.1, specifically LIF and GM-CSF) may also have beneficial effects. It is likely that a combination of growth factors may produce a

synergistic effect and improve blastocyst quality and pluripotency beyond what can be achieved by insulin alone and this should be the subject of further systematic experiments.

7.4 Molecular mechanism of action of insulin on pluripotency in the blastocyst

Having demonstrated that the culture of post-compact stage embryos with insulin increases pluripotency in the blastocyst, experiments in Chapter 5.0 investigated the signalling pathways behind this effect. At the concentration identified as increasing epiblast cell number and proportion in Chapter 4.0 (1.7pM), insulin is known to activate the insulin receptor [384]. One of the primary second messengers of the insulin receptor is PI3K, which has previously been shown to be integral for maintaining pluripotency in ESCs [266]. Using inhibitors it was demonstrated that PI3K activity was necessary for insulin to increase epiblast cell number (Fig. 7.1). One target of PI3K is GSK3, which is phosphorylated by active PI3K, inactivating it. When active, GSK3 is capable of phosphorylating many second messengers which converge to reduce *Nanog* transcription, which is important for the retention of pluripotency [53,478-483]. Inhibiting GSK3, theoretically reproducing the effect of culture with insulin and active PI3K, increased epiblast cell number. Additionally, activating GSK3 blocked insulin's ability to increase epiblast cell number without affecting the epiblast cell number of embryos cultured without insulin – replicating the effect of PI3K inhibition. These results strongly suggest that the inactivation of GSK3 is an important component of the insulin signalling pathway with regards to increasing epiblast cell number. Additionally, the pro-apoptotic protein p53, which is also regulated by PI3K, specifically via PI3K activated ubiquitinase MDM2, causes cell death and differentiation when active, and binds to the *Nanog* promoter region to repress *Nanog* transcription. As with GSK3, inhibition of p53 increased epiblast cell number, while activation blocked insulin mediated epiblast increases, strongly suggesting that p53 is involved in insulin mediated increases to epiblast cell number. Interestingly there are multiple points of cross reactivity between GSK3 and p53 [491-495], however, no additional epiblast increases were found for co-inhibition of the two factors. This suggests that the potential of GSK3 inhibition to cause the accumulation of p53 [494,495] did not create a confounding effect in these experiments. Therefore, the results of Chapter 5.0 demonstrated that insulin increased the epiblast cell number via the activation of PI3K (most likely via its interaction with the insulin receptor), which subsequently inactivates the second messengers GSK3 and p53, to increase *Nanog* transcription and therefore promote pluripotency and the epiblast. Further work in this area could include analysis PI3K

localisation as well as levels of phosphorylated GSK3 and ubiquitinated p53 in blastocysts cultured in control and insulin supplemented media to directly confirm the mechanism of insulin.

7.5 Expression of OCT4 and Nanog in blastocysts

Studies in Chapter 6.0 determined the localisation of OCT4 and Nanog in blastocysts on day 4 (early blastocysts) compared to those which develop to day 5 (predominantly expanded blastocyst) and day 6 (hatching blastocysts). Of note, it was found that at the stage of development where literature sources suggested that OCT4 and Nanog would be restricted to the ICM and epiblast respectively [198,416], both were still widely expressed. A comparison of methodologies suggests that this difference is likely the result of collecting and beginning *in vitro* embryo culture at the zygote stage in this study rather than at the 2-cell stage or later.

Human embryos are ubiquitously cultured from the zygote stage following *in vitro* fertilisation. As such, this finding suggests that future researchers who use the mouse blastocyst to model the *in vitro* development of human embryos, particularly with regards to epiblast development, culture embryos from the zygote stage, as the discrepancy appears to produce a meaningful difference in the progression of epiblast development. Future work in this area should include the direct comparison of OCT4 and Nanog expression of *in vitro* and *in vivo* grown mouse embryos as well as the characterisation of Oct4 and Nanog expression in human blastocysts.

7.6 Effect of insulin in embryo culture medium persists in outgrowths

Despite the increase in Nanog positive cell number due to culture with insulin, I found that when blastocysts were plated before the transcription factor was restricted to the epiblast, outgrowths from insulin treated embryos contained no more epiblast cells than outgrowths from control embryos. Further, my results showed that despite earlier stage blastocysts possessing more OCT4 and Nanog positive cells than later stage blastocysts, they gave rise to outgrowths with significantly fewer epiblast cells. This finding supports the use of mouse blastocysts in modelling human embryo development and hESC derivation, as hESCs have been shown to be most efficiently derived from blastocyst where OCT4 has been restricted to the ICM [202], an event I observed at the same time point where Nanog was restricted to the epiblast. The important finding from these experiments,

however, was that when embryos were allowed to develop until Nanog was restricted to the epiblast and OCT4 was restricted to the ICM before plating, culture of embryos in insulin post compaction resulted in the generation of outgrowths which were more likely to contain an epiblast and which contained a larger number of epiblast cells.

Future work in this area should include a more precise investigation of the optimal time point for plating on day 6 as well studies to assess whether outgrowth quality could be improved by plating blastocysts based on spatial rather than temporal development.

7.7 Insulin in culture media and the effect of ESC colony generation

Ultimately epiblast cell number is a marker of blastocyst competence for ESC derivation, and as such there is a need to generate ESCs to validate the conclusions made based on epiblast findings. Blastocysts were twice as likely to give rise to primary ESC colonies if they were cultured with insulin for the post-compaction stage. Interestingly, examining how the inclusion of insulin interacted with this process led to the novel observation that culture of embryos with insulin increased the proportion of embryos which, at the point of plating, were at the most advanced morphological stage (hatched) and also increased the proportion of those hatched blastocysts which gave rise to ESCs. As such, hatched insulin cultured blastocysts are more plentiful, and more likely to give rise to ESCs than hatched control cultured blastocysts. This result demonstrates that insulin improves ESC isolation through mechanisms beyond simply improving morphology, which has previously been linked to increased ESC derivation rates. It is likely that the improved capacity of insulin cultured blastocysts with the highest morphological quality to give rise to primary ESC colonies is the result of the increased epiblast cell numbers demonstrated in Chapters 4.0 and 6.0.

Modelling of the experimental outcomes enabled conclusions to be made around the most significant characteristics that an embryo must contain to generate a primary ESC colony; the greatest predictor of a control cultured blastocyst giving rise to a primary ESC colony was it cavitating on day 4, whereas for blastocysts cultured with insulin the greatest predictor was being hatched on day 6. For day 4 this observation is likely the result of insulin increasing the rate of cavitation and thereby making the marker less selective, the finding on day 6 is suggestive that in the control group hatched blastocysts, which have shown the best development, have no more epiblast cells than their more slowly developing counterparts. Both of these observations warrant further investigation.

The results of this work demonstrate that the addition of insulin to embryo culture medium from the cleavage stage, which is the stage at which human embryos are most often donated, to the blastocyst stage, which is the stage at which ESC derivation is most often attempted, improves the efficiency with which ESCs can be generated from these embryos. As such, application of this strategy may have the potential to improve hESC derivation efficiency, a matter of key importance due to the limited availability of human embryos for ESC derivation.

Future work which would be necessary to validate these findings is the expansion of mESC colonies from control and insulin cultured embryos to fully characterised mESC lines and the reproduction of these experiments in the human. Further, my work has shown that the effect of insulin persists beyond embryo culture through the outgrowth phase and into ESC derivation. This suggests that during embryo culture insulin may have a permanent positive effect on cell properties and that ESC lines derived from embryos cultured with insulin may have altered characteristics. As such, future work should include not just the characterisation of ESC lines from control and insulin cultured embryos for pluripotency and self renewal, but also more in depth characterisation including, metabolic profile, an assessment of DNA methylation and acetylation, and gene expression, to provide a more detailed and precise picture of the quality and differentiation status of ESC lines, with a view towards investigating whether the culture of embryos with insulin results in the derivation of higher quality ESC lines.

Embryonic stem cell derivation and embryo culture



FIG. 7.2 Summary of the culture effects investigated in this thesis and their observed effect on the retention of pluripotency towards ESC derivation.

7.8 Conclusion

In conclusion, the data presented in this thesis show that while culture in simple medium during the cleavage stage decreases pluripotency, the inclusion of insulin in embryo culture medium from the compaction stage stimulates pluripotency supporting pathways to increase the number of epiblast cells in the fully developed blastocyst, resulting in an increased capacity to generate ESCs (Fig. 7.2). This strategy is ultimately intended to be applied to hESC derivation where embryos are most often donated at the cleavage stage and of reduced quality.

8.0 Appendix

8.1 Mouse embryo bioassay

For consistent *in vitro* embryo development and quality, research has shown that it is necessary that consumables used undergo quality control [300]. As such, plasticware and all chemicals used for embryo culture and handling were pre-screened using a 1-cell mouse bioassay. For this test mouse zygotes were collected (section 2.4) from prepubescent F1 hybrid female mice (C57Bl/6×CBA) crossed with Swiss males. These zygotes were grown in simple G1.2 with 5% HSA to the 2-cell stage under test conditions. These embryos were then transferred to simple G1.2 without albumin, under test conditions and cultured to the blastocyst stage. HSA was excluded in the second stage of culture because it is a chelator and could mask the negative effects of the test conditions. Embryo culture was scored at 74h and 91hr, and the result was compared to the development of control embryos grown alongside the test embryos which were cultured using consumables which had previously passed the bio assay. A minimum of thirty embryos were used for each test condition and consumables had to have greater than 50% early blastocyst development (cavity <2/3rds the volume of the blastocyst) at 74h and greater than 75% expanded blastocyst (cavity >2/3rds the volume of the blastocyst) at 91hr.

Where culture dishes were tested embryos were grown in them for the test's duration as the test condition. To assay test tubes culture media were stored in them at 4°C for a week and embryos were then cultured in that medium. When amino acids were tested blastocyst trophectoderm and ICM cell number were assessed by differential staining. Differential staining was performed at 37°C under mineral oil in dishes which were prewarmed for a minimum of one hour prior to the experiment. Embryos were placed in 0.5% pronase in MOPS medium with no HSA to dissolve the zona pellucida. Zona dissolution took 2-5 minutes during which embryos were observed by a microscope. When the process was judged complete, embryos were removed from the pronase and washed in MOPS without HSA. Embryos were then incubated in 10mM 2,4,6-trinitrobenzenesulfonic acid (TNBS) at 4°C in the dark for 10min. They were then washed 3 times to remove any trace of TNBS and placed in 0.1mg/ml anti-DNP (anti-dinitrophenol-BSA) for 10 min. During this period tight junctions between trophectoderm cells prevented ICM cells being exposed to the anti-DNP. Embryos were then placed in compliment (guinea pig serum and 50µg/µl propidium iodide (PI)) for 5-10 minutes, resulting in trophectoderm cells, which had been exposed to the anti-DNP, partially lysing due to the binding of the compliment, which allowed the PI to enter the trophectoderm cells and stain their nuclei. Following this, embryos were transferred to 25µg/ml bisbenzamide (Hoechst

33258) in ethanol and incubated overnight at 4°C in the dark. As bisbenzamide stains all cell nuclei, the trophoctoderm is stained with both PI and bisbenzamide, while the ICM is stained with bisbenzamide only.

The next day embryos were transferred to 100% ethanol and kept for a maximum of one week prior to counting. In order to count the cells of the blastocysts, embryos were loaded in a small drop of glycerol on a siliconised slide (with minimal transfer of medium). A cover slip was then gently placed over the drop while still watching down the microscope, and small dots were made near each embryo with a red permanent marker to aid in their subsequent location. The cover slip was then gently compressed to flatten the blastocysts and put all cells on a single plane. Blastocysts were then visualised by a fluorescent microscope with a UV filter (ex: 330-380nm) which causes the nuclei of the ICM to fluoresce blue and the nuclei of the trophoctoderm cells to fluoresce pink. Due to photo bleaching of light sensitive PI the most accurate way to assess cell numbers is to first count both pink and blue nuclei under the UV filter to obtain total cell number, then count trophoctoderm cells under a green filter (ex: 530nm em 615nm). ICM cell nuclei number could then be calculated by subtracting trophoctoderm cell number from total cell number.

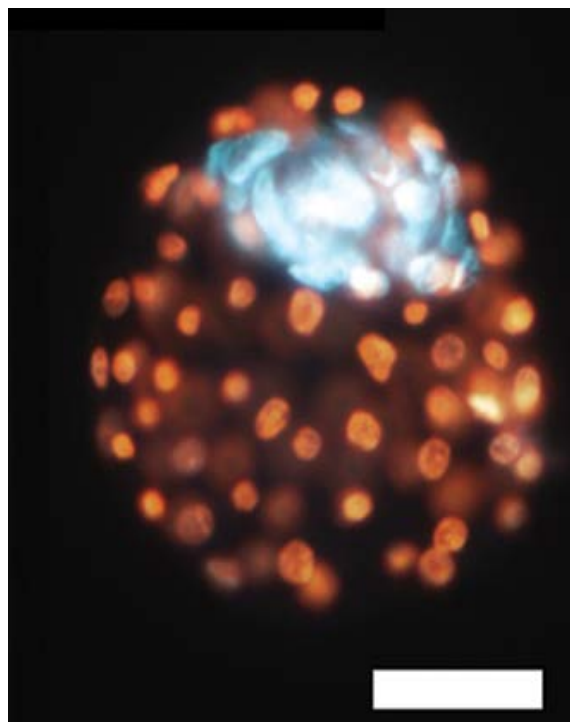


FIG. 8.1 Differential stain of a blastocyst. ICM nuclei stained blue, trophoctoderm nuclei stained pink. Scale bar is 50µM

8.2 Insulin dilution

In Chapter 4.0 embryos were cultured in G2 medium supplemented with 0.17, 1.7 and 1700pM insulin prepared as per the following protocol.

Working in sterile conditions 9.75mg of insulin powder was dissolved in 10ml Milli Q water reduced to pH 2.0-3.0 with 1M HCl (insulin will not dissolve at neutral pH) (solution α).

10 μ l of solution α was added to 10ml Milli Q water which was then sterile filtered with a 0.2 μ m filter (solution β).

10 μ l of solution β in 990 μ l of G2 made the 1700pM insulin medium.

10 μ l of solution β was added to 10ml Milli Q water which was then sterile filtered with a 0.2 μ m filter (solution γ).

10 μ l of solution γ in 990 μ l of G2 made the 1.7pM insulin medium.

1 μ l of solution γ in 990 μ l G2 (with 9 μ l Milli Q water sterile filtered with a 0.2 μ m filter) made the 0.17pM insulin medium.

8.3 Inhibitor dilutions

8.3.1 LY294002

In Chapter 5.0 embryos were cultured in media supplemented with 50 μ M LY294002.

Working in sterile conditions LY294002 powder was dissolved in DMSO at 20mg/ml (solution α) and kept stored at -20°C.

10 μ l of solution α was diluted with 120 μ l G2 (solution β).

10 μ l of solution β in 990 μ l G2 made 50 μ M LY294002 medium.

8.3.2 CT99021

In Chapter 5.0 embryos were cultured in media supplemented with 0.04, 0.3, 3 and 15 μ M CT99021.

Working in sterile conditions CT99021 powder was dissolved in DMSO at 20mg/ml (solution α) and kept stored at -20°C.

10 μ l of solution α was diluted with 276.5 μ l G2 (solution β)

10 μ l of solution β in 990 μ l G2 made 15 μ M CT99021 medium

2 μ l of solution β with 8 μ l DMSO in 990 μ l G2 made 3 μ M CT99021 medium

10 μ l solution β was diluted with 90 μ l G2 (solution γ)

2 μ l of solution γ with 9 μ l DMSO in 989 μ l G2 made 0.3 μ M CT99021 medium

10 μ l of the 15 μ M solution was diluted with 27.5 μ l G2 (solution δ)

10 μ l of solution δ with 10 μ l DMSO in 980 G2 made 0.04 μ M CT99021 medium

8.3.3 H-89

In Chapter 5.0 embryos were cultured in media supplemented with 10 μ M H-89.

Working in sterile conditions H-89 powder was dissolved in DMSO at 10mM (solution α), and kept stored at -20°C.

10 μ l of solution α was diluted with 90 μ l G2 (solution β)

10 μ l of solution β in 990 μ l G2 made 10 μ M H-89 medium

8.3.4 Pifithrin- α

In Chapter 5.0 embryos were cultured in media supplemented with 30 μ M pifithrin- α

Working in sterile conditions pifithrin- α powder was dissolved in DMSO at 20mg/ml (solution α), and kept stored at -20°C.

20 μ l of solution α was diluted with 343 μ l G2 medium (solution β)

10 μ l of solution β in 990 μ l G2 made 30 μ M pifithrin- α medium

8.3.5 Nicotinamide

In Chapter 5.0 embryos were cultured in medium supplemented with 10 μ M Nicotinamide

Working in sterile conditions 122.2mg of Nicotinamide powder was dissolved in 1ml Milli Q water and sterile filtered with a 0.2 μ m filter (solution α)

10 μ l of solution α in 990 μ l G2 made 10 μ M Nicotinamide medium

8.4 Hormone preparation

8.4.1 Saline solution

Working in sterile conditions 0.9g of ultra pure (99.5%) sodium chloride was dissolved in 100ml Milli-Q water and then sterile filtered with a 0.2 μ m filter, making 0.9% sterile saline solution. Saline solution was filtered into a sterile container for immediate use.

8.4.2 Equine chorionic gonadotropin (eCG)

Working under sterile conditions eCG lyophilized powder was dissolved in 0.9% sterile saline solution to a concentration of 50IU/ml. eCG solution was stored for up to 1 week at 2-4°C or 3 weeks at -20°C.

8.4.3 Human chorionic gonadotropin

Working under sterile conditions hCG lyophilized powder was dissolved in 0.9% sterile saline solution to a concentration of 50IU/ml. hCG solution was stored for up to 1 week at 2-4°C or 3 weeks at -20°C.

8.5 Solutions for immunocytochemistry

8.5.1 Paraformaldehyde

1g of paraformaldehyde powder was suspended in 25ml PBS and one drop of 1M NaOH. The powder was then dissolved by heating to 60°C with occasional agitation, making 4% w/v paraformaldehyde solution for the fixation of cells. Paraformaldehyde solution was stored at 4°C for a maximum of 1 week.

8.5.2 Glycine

37.5mg Glycine powder was dissolved in 5ml PBS, making 0.1M Glycine for the unmasking of fixed cells. Glycine solution was stored at 4°C.

8.5.3 Triton-X solution

5ml of PBS was added to 12.5µl of Triton-X which was dissolved by incubation for 1h at 37°C, making 0.25% PBS-TX solution for the permeabilisation of cells. PBS-TX was made fresh for each use.

8.5.4 Donkey Serum

50µl donkey serum was diluted with 950µl PBS, making 5% donkey serum solution for the blocking of cells. 5% donkey serum solution was made fresh for each use.

8.5.5 Dapi

5mg Dapi was dissolved in 1.428ml Milli-Q water making 10mM stock (solution α) which was kept at -20°C and protected from light.

2µl of solution α was diluted with 198µl PBS (solution β)

5µl solution β was diluted with 495µl PBS making 3µM Dapi solution for counter staining cell nuclei.

8.6 Antibody validations

8.6.1 OCT4 and Nanog

OCT4 and Nanog antibodies used for immunocytochemistry were validated by negative controls where secondary antibodies were applied without primary antibodies first being applied. To confirm the specificity of the stain the negative controls were imaged by confocal

microscopy and gain was adjusted down to the level at which no fluorescence could be seen, experimental samples were then imaged at the same setting with the presence of fluorescence being taken as positive.

8.6.2 SSEA1

The SSEA1 mouse monoclonal antibody was validated using the differentiated MEFS on which the ESC colonies it stained were cultured as negative controls. As viewing these differentiated cells under epifluorescence showed no staining, the specificity of the SSEA1 antibody to pluripotent ESCs was confirmed.

8.6.3 *Antibodies for markers of germ cell lineage*

Antibodies for VEGFR2, CXCR4, SOX17 and Nestin were validated with both positive and negative controls. Differentiated cells from the established commercial D3-ES cell line, which are known to be capable of differentiating to the desired cell types, were used as positive controls (section 2.13.4.4). For the negative control secondary antibodies were applied without primary antibodies first being applied, with the lack of fluorescence under epifluorescent microscopy confirming specificity. As the techniques used to direct differentiation are known to not be 100% efficient, further evidence of the specificity of the primary antibodies was provided by the anticipated presence of a subpopulation of cells which did not express the stained for markers within the differentiated cell populations.

9.0 References

1. Thomson JA, J Itskovitz-Eldor, SS Shapiro, MA Waknitz, JJ Swiergiel, VS Marshall and JM Jones. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145-7.
2. National Health And Medical Research Council. (2007). Ethical guidelines on the use of assisted reproductive technology in clinical practice and research. Australian Government, Canberra. p 1-104.
3. Department of Health. (2008). Human Fertilisation and Embryology Act 2008. Government of the United Kingdom.
4. Hoffman DI, GL Zellman, CC Fair, JF Mayer, JG Zeitz, WE Gibbons and TG Turner, Jr. (2003). Cryopreserved embryos in the United States and their availability for research. *Fertil Steril* 79:1063-9.
5. Lerou PH, A Yabuuchi, H Huo, A Takeuchi, J Shea, T Cimini, TA Ince, E Ginsburg, C Racowsky and GQ Daley. (2008). Human embryonic stem cell derivation from poor-quality embryos. *Nat Biotechnol* 26:212-4.
6. Mitalipova M, J Calhoun, S Shin, D Wininger, T Schulz, S Noggle, A Venable, I Lyons, A Robins and S Stice. (2003). Human embryonic stem cell lines derived from discarded embryos. *Stem Cells* 21:521-6.
7. Bongso A and EH Lee, eds. (2005). *Stem Cells: From Bench to Bedside*. World Sc Press, Singapore.
8. Gardner DK and M Lane. (1998). Culture of viable human blastocysts in defined sequential serum-free media. *Hum Reprod* 13 Suppl 3:148-59; discussion 160.
9. Gardner DK. (2000). Blastocyst culture: toward single embryo transfers. *Hum Fertil (Camb)* 3:229-237.
10. Rossant J. (2001). Stem cells from the Mammalian blastocyst. *Stem Cells* 19:477-82.
11. Bielinska M, N Narita and DB Wilson. (1999). Distinct roles for visceral endoderm during embryonic mouse development. *Int J Dev Biol* 43:183-205.
12. Gardner RL and J Rossant. (1979). Investigation of the fate of 4-5 day post-coitum mouse inner cell mass cells by blastocyst injection. *J Embryol Exp Morphol* 52:141-52.
13. Pereira PN, MP Dobрева, L Graham, D Huylebroeck, KA Lawson and AN Zwijsen. (2011). Amnion formation in the mouse embryo: the single amniochorionic fold model. *BMC Dev Biol* 11:48.
14. Brook FA and RL Gardner. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci U S A* 94:5709-12.
15. Martin GR. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* 78:7634-8.
16. Evans MJ and MH Kaufman. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature* 292:154-6.
17. Yates A and I Chambers. (2005). The homeodomain protein Nanog and pluripotency in mouse embryonic stem cells. *Biochem Soc Trans* 33:1518-21.
18. Kleinsmith LJ and GB Pierce, Jr. (1964). Multipotentiality of Single Embryonal Carcinoma Cells. *Cancer Res* 24:1544-51.
19. Martin GR. (1980). Teratocarcinomas and mammalian embryogenesis. *Science* 209:768-76.

20. Thomson JA, J Kalishman, TG Golos, M Durning, CP Harris, RA Becker and JP Hearn. (1995). Isolation of a primate embryonic stem cell line. *Proc Natl Acad Sci U S A* 92:7844-8.
21. Doetschman T, P Williams and N Maeda. (1988). Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev Biol* 127:224-7.
22. Sukoyan MA, SY Vatolin, AN Golubitsa, AI Zhelezova, LA Semenova and OL Serov. (1993). Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: comparisons of their pluripotencies. *Mol Reprod Dev* 36:148-58.
23. Wheeler MB. (1994). Development and validation of swine embryonic stem cells: a review. *Reprod Fertil Dev* 6:563-8.
24. Pain B, ME Clark, M Shen, H Nakazawa, M Sakurai, J Samarut and RJ Etches. (1996). Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities. *Development* 122:2339-48.
25. Mitalipova M, Z Beyhan and NL First. (2001). Pluripotency of bovine embryonic cell line derived from precompacting embryos. *Cloning* 3:59-67.
26. Buehr M, S Meek, K Blair, J Yang, J Ure, J Silva, R McLay, J Hall, QL Ying and A Smith. (2008). Capture of authentic embryonic stem cells from rat blastocysts. *Cell* 135:1287-98.
27. Vaags AK, S Rosic-Kablar, CJ Gartley, YZ Zheng, A Chesney, DA Villagomez, SA Kruth and MR Hough. (2009). Derivation and characterization of canine embryonic stem cell lines with in vitro and in vivo differentiation potential. *Stem Cells* 27:329-40.
28. Solter D. (2006). From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. *Nat Rev Genet* 7:319-27.
29. Friel R, S van der Sar and PJ Mee. (2005). Embryonic stem cells: understanding their history, cell biology and signalling. *Adv Drug Deliv Rev* 57:1894-903.
30. Henderson JK, JS Draper, HS Baillie, S Fishel, JA Thomson, H Moore and PW Andrews. (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. *Stem Cells* 20:329-37.
31. Solter D and BB Knowles. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc Natl Acad Sci U S A* 75:5565-9.
32. Gooi HC, T Feizi, A Kapadia, BB Knowles, D Solter and MJ Evans. (1981). Stage-specific embryonic antigen involves alpha 1 goes to 3 fucosylated type 2 blood group chains. *Nature* 292:156-8.
33. Shevinsky LH, BB Knowles, I Damjanov and D Solter. (1982). Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. *Cell* 30:697-705.
34. Kannagi R, NA Cochran, F Ishigami, S Hakomori, PW Andrews, BB Knowles and D Solter. (1983). Stage-specific embryonic antigens (SSEA-3 and -4) are epitopes of a unique globo-series ganglioside isolated from human teratocarcinoma cells. *Embo J* 2:2355-61.
35. Fenderson BA, PW Andrews, E Nudelman, H Clausen and S Hakomori. (1987). Glycolipid core structure switching from globo- to lacto- and ganglio-series during retinoic acid-induced differentiation of TERA-2-derived human embryonal carcinoma cells. *Dev Biol* 122:21-34.
36. Kannagi R, SB Levery, F Ishigami, S Hakomori, LH Shevinsky, BB Knowles and D Solter. (1983). New globoseries glycosphingolipids in human teratocarcinoma reactive with the monoclonal antibody directed to a developmentally regulated antigen, stage-specific embryonic antigen 3. *J Biol Chem* 258:8934-42.
37. Draper JS, C Pigott, JA Thomson and PW Andrews. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. *J Anat* 200:249-58.

38. Takehara T, Teramura Y, Onodera C, Hamanishi H and Fukuda K. (2012). Reduced oxygen concentration enhances conversion of embryonic stem cells to epiblast stem cells. *Stem Cells Dev* 21:1239-49.
39. Ohtsuka S, Nishikawa-Torikai H and Niwa H. (2012). E-cadherin promotes incorporation of mouse epiblast stem cells into normal development. *PLoS One* 7:e45220.
40. Landolfi NF, Capra JD and Tucker PW. (1986). Interaction of cell-type-specific nuclear proteins with immunoglobulin VH promoter region sequences. *Nature* 323:548-51.
41. Botquin V, Hess H, Fuhrmann G, Anastassiadis MK, Gross G, Vriend G and Scholer HR. (1998). New POU dimer configuration mediates antagonistic control of an osteopontin preimplantation enhancer by Oct-4 and Sox-2. *Genes Dev* 12:2073-90.
42. Nordhoff V, Hubner K, Bauer A, Orlova I, Malapetsa A and Scholer HR. (2001). Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences. *Mamm Genome* 12:309-17.
43. Scholer HR, Hatzopoulos AK, Balling R, Suzuki N and Gruss P. (1989). A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. *EMBO J* 8:2543-50.
44. Lenardo MJ, Staudt L, Robbins P, Kuang A, Mulligan RC and Baltimore D. (1989). Repression of the IgH enhancer in teratocarcinoma cells associated with a novel octamer factor. *Science* 243:544-6.
45. Okamoto K, Okazawa H, Okuda M, Sakai M, Muramatsu M and Hamada H. (1990). A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell* 60:461-72.
46. Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH and Robson P. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. *J Biol Chem* 280:24731-7.
47. Campbell PA, Perez-Iratxeta C, Andrade-Navarro MA and Rudnicki MA. (2007). Oct4 targets regulatory nodes to modulate stem cell function. *PLoS ONE* 2:e553.
48. Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R and Young RA. (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. *Cell* 122:947-56.
49. Hattori N, Imao Y, Nishino K, Ohgane J, Yagi S, Tanaka S and Shiota K. (2007). Epigenetic regulation of Nanog gene in embryonic stem and trophoblast stem cells. *Genes Cells*. 12:387-96.
50. Jones TD, Ulbright TM, Eble JN, Baldrige LA and Cheng L. (2004). OCT4 staining in testicular tumors: a sensitive and specific marker for seminoma and embryonal carcinoma. *Am J Surg Pathol* 28:935-40.
51. Pesce M and Scholer HR. (2001). Oct-4: gatekeeper in the beginnings of mammalian development. *Stem Cells* 19:271-8.
52. Niwa H, Miyazaki J and Smith AG. (2000). Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Genet* 24:372-6.
53. Takao Y, Yokota T and Koide H. (2007). Beta-catenin up-regulates Nanog expression through interaction with Oct-3/4 in embryonic stem cells. *Biochem Biophys Res Commun* 353:699-705.
54. Silva J, Chambers I, Pollard S and Smith A. (2006). Nanog promotes transfer of pluripotency after cell fusion. *Nature* 441:997-1001.
55. Mitsui K, Tokuzawa Y, Itoh H, Segawa M, Murakami K, Takahashi M, Maruyama M, Maeda S and Yamanaka S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* 113:631-42.
56. Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S and Smith A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* 113:643-55.

57. Williams RL, DJ Hilton, S Pease, TA Willson, CL Stewart, DP Gearing, EF Wagner, D Metcalf, NA Nicola and NM Gough. (1988). Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* 336:684-7.
58. Loh YH, Q Wu, JL Chew, VB Vega, W Zhang, X Chen, G Bourque, J George, B Leong, J Liu, KY Wong, KW Sung, CW Lee, XD Zhao, KP Chiu, L Lipovich, VA Kuznetsov, P Robson, LW Stanton, CL Wei, Y Ruan, B Lim and HH Ng. (2006). The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. *Nat Genet* 38:431-40.
59. Chambers I, J Silva, D Colby, J Nichols, B Nijmeijer, M Robertson, J Vrana, K Jones, L Grotewold and A Smith. (2007). Nanog safeguards pluripotency and mediates germline development. *Nature* 450:1230-4.
60. Hatano SY, M Tada, H Kimura, S Yamaguchi, T Kono, T Nakano, H Suemori, N Nakatsuji and T Tada. (2005). Pluripotential competence of cells associated with Nanog activity. *Mech Dev* 122:67-79.
61. Hart AH, L Hartley, M Ibrahim and L Robb. (2004). Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human. *Dev Dyn* 230:187-98.
62. Ying QL and AG Smith. (2003). Defined conditions for neural commitment and differentiation. *Methods Enzymol* 365:327-41.
63. Parekkadan B, Y Berdichevsky, D Irimia, A Leeder, G Yarmush, M Toner, JB Levine and ML Yarmush. (2008). Cell-cell interaction modulates neuroectodermal specification of embryonic stem cells. *Neurosci Lett* 438:190-5.
64. Hansson M, DR Olesen, JM Peterslund, N Engberg, M Kahn, M Winzi, T Klein, P Maddox-Hyttel and P Serup. (2009). A late requirement for Wnt and FGF signaling during activin-induced formation of foregut endoderm from mouse embryonic stem cells. *Dev Biol* 330:286-304.
65. Tada S, T Era, C Furusawa, H Sakurai, S Nishikawa, M Kinoshita, K Nakao and T Chiba. (2005). Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. *Development* 132:4363-74.
66. Klimanskaya I, Y Chung, L Meisner, J Johnson, MD West and R Lanza. (2005). Human embryonic stem cells derived without feeder cells. *Lancet* 365:1636-41.
67. Lanzendorf SE, C Boyd and D Wright. (2004). Human Embryonic Stem Cells. In: *Laboratory Guide to Mammalian Embryos*. Oxford University Press, New York. p 313-333.
68. Shao H, Z Wei, L Wang, L Wen, B Duan, L Mang and S Bou. (2007). Generation and characterization of mouse parthenogenetic embryonic stem cells containing genomes from non-growing and fully grown oocytes. *Cell Biol Int* 31:1336-44.
69. Stojkovic M, M Lako, T Strachan and A Murdoch. (2004). Derivation, growth and applications of human embryonic stem cells. *Reproduction* 128:259-67.
70. Heins N, MC Englund, C Sjoblom, U Dahl, A Tønning, C Bergh, A Lindahl, C Hanson and H Semb. (2004). Derivation, characterization, and differentiation of human embryonic stem cells. *Stem Cells* 22:367-76.
71. Liu J, L Schoonjans, S Tielens, F Speleman, M Cornelissen, P De Sutter, M Dhont and J Van der Elst. (2006). Culturing in vitro produced blastocysts in sequential media promotes ES cell derivation. *Mol Reprod Dev* 73:1017-21.
72. Battle-Morera L, A Smith and J Nichols. (2008). Parameters influencing derivation of embryonic stem cells from murine embryos. *Genesis* 46:758-67.
73. Cowan CA, I Klimanskaya, J McMahon, J Atienza, J Witmyer, JP Zucker, S Wang, CC Morton, AP McMahon, D Powers and DA Melton. (2004). Derivation of embryonic stem-cell lines from human blastocysts. *N Engl J Med* 350:1353-6.

74. Bryja V, S Bonilla, L Cajanek, CL Parish, CM Schwartz, Y Luo, MS Rao and E Arenas. (2006). An efficient method for the derivation of mouse embryonic stem cells. *Stem Cells* 24:844-9.
75. Rodin S, A Domogatskaya, S Strom, EM Hansson, KR Chien, J Inzunza, O Hovatta and K Tryggvason. (2010). Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. *Nat Biotechnol* 28:611-5.
76. Rosler ES, GJ Fisk, X Ares, J Irving, T Miura, MS Rao and MK Carpenter. (2004). Long-term culture of human embryonic stem cells in feeder-free conditions. *Dev Dyn* 229:259-74.
77. Harley CB. (1991). Telomere loss: mitotic clock or genetic time bomb? *Mutat Res* 256:271-82.
78. Harley CB, H Vaziri, CM Counter and RC Allsopp. (1992). The telomere hypothesis of cellular aging. *Exp Gerontol* 27:375-82.
79. Thomson JA and VS Marshall. (1998). Primate embryonic stem cells. *Curr Top Dev Biol* 38:133-65.
80. Roach M and J McNeish. (2002). Methods for the Isolation and Maintenance of Murine Embryonic Stem Cells. In: *Embryonic Stem Cells: Methods and Protocols*. Humana Press Inc, Totowa. p 1-16.
81. Ying QL, J Nichols, I Chambers and A Smith. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* 115:281-92.
82. Humphrey RK, GM Beattie, AD Lopez, N Bucay, CC King, MT Firpo, S Rose-John and A Hayek. (2004). Maintenance of pluripotency in human embryonic stem cells is STAT3 independent. *Stem Cells* 22:522-30.
83. Xu RH, X Chen, DS Li, R Li, GC Addicks, C Glennon, TP Zwaka and JA Thomson. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* 20:1261-4.
84. Taei A, H Gourabi, A Seifinejad, M Totonchi, E Shahbazi, MR Valojerdi, P Eftekhari, L Karimian and H Baharvand. (2010). Derivation of new human embryonic stem cell lines from preimplantation genetic screening and diagnosis-analyzed embryos. *In Vitro Cell Dev Biol Anim* 46:395-402.
85. Peura T, A Bosman, O Chami, RP Jansen, K Texlova and T Stojanov. (2008). Karyotypically normal and abnormal human embryonic stem cell lines derived from PGD-analyzed embryos. *Cloning Stem Cells* 10:203-16.
86. Klimanskaya I, Y Chung, S Becker, SJ Lu and R Lanza. (2006). Human embryonic stem cell lines derived from single blastomeres. *Nature* 444:481-5.
87. Watt FM and RR Driskell. (2010). The therapeutic potential of stem cells. *Philos Trans R Soc Lond B Biol Sci* 365:155-63.
88. Laustriat D, J Gide and M Peschanski. (2010). Human pluripotent stem cells in drug discovery and predictive toxicology. *Biochem Soc Trans* 38:1051-7.
89. Lou YJ and XG Liang. (2011). Embryonic stem cell application in drug discovery. *Acta Pharmacol Sin* 32:152-9.
90. Kola I and J Landis. (2004). Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* 3:711-5.
91. Kramer JA, JE Sagartz and DL Morris. (2007). The application of discovery toxicology and pathology towards the design of safer pharmaceutical lead candidates. *Nat Rev Drug Discov* 6:636-49.
92. Vojnits K and S Bremer. (2010). Challenges of using pluripotent stem cells for safety assessments of substances. *Toxicology* 270:10-7.
93. Sartipy P, P Bjorquist, R Strehl and J Hyllner. (2007). The application of human embryonic stem cell technologies to drug discovery. *Drug Discov Today* 12:688-99.

94. McNeish JD. (2007). Stem cells as screening tools in drug discovery. *Curr Opin Pharmacol* 7:515-20.
95. Ben-Yosef D, M Malcov and R Eiges. (2008). PGD-derived human embryonic stem cell lines as a powerful tool for the study of human genetic disorders. *Mol Cell Endocrinol* 282:153-8.
96. Urbach A, M Schuldiner and N Benvenisty. (2004). Modeling for Lesch-Nyhan disease by gene targeting in human embryonic stem cells. *Stem Cells* 22:635-41.
97. Genschow E, H Spielmann, G Scholz, A Seiler, N Brown, A Piersma, M Brady, N Clemann, H Huuskonen, F Paillard, S Bremer and K Becker. (2002). The ECVAM international validation study on in vitro embryotoxicity tests: results of the definitive phase and evaluation of prediction models. *European Centre for the Validation of Alternative Methods. Altern Lab Anim* 30:151-76.
98. Genschow E, H Spielmann, G Scholz, I Pohl, A Seiler, N Clemann, S Bremer and K Becker. (2004). Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests. *Altern Lab Anim* 32:209-44.
99. Nau H. (1986). Species differences in pharmacokinetics and drug teratogenesis. *Environ Health Perspect* 70:113-29.
100. Adler S, C Pellizzer, L Hareng, T Hartung and S Bremer. (2008). First steps in establishing a developmental toxicity test method based on human embryonic stem cells. *Toxicol In Vitro* 22:200-11.
101. Mehta A, VB Konala, A Khanna and AS Majumdar. (2008). Assessment of drug induced developmental toxicity using human embryonic stem cells. *Cell Biol Int* 32:1412-24.
102. Guenou H, X Nissan, F Larcher, J Feteira, G Lemaitre, M Saidani, M Del Rio, CC Barrault, FX Bernard, M Peschanski, C Baldeschi and G Waksman. (2009). Human embryonic stem-cell derivatives for full reconstruction of the pluristratified epidermis: a preclinical study. *Lancet* 374:1745-53.
103. Green H, K Easley and S Iuchi. (2003). Marker succession during the development of keratinocytes from cultured human embryonic stem cells. *Proc Natl Acad Sci U S A* 100:15625-30.
104. Loring JF and MS Rao. (2006). Establishing standards for the characterization of human embryonic stem cell lines. *Stem Cells* 24:145-50.
105. Barbaric I, PJ Gokhale, M Jones, A Glen, D Baker and PW Andrews. (2010). Novel regulators of stem cell fates identified by a multivariate phenotype screen of small compounds on human embryonic stem cell colonies. *Stem Cell Res* 5:104-19.
106. Skelin M, M Rupnik and A Cencic. (2010). Pancreatic beta cell lines and their applications in diabetes mellitus research. *ALTEX* 27:105-13.
107. McNeish J, M Roach, J Hambor, RJ Mather, L Weibley, J Lazzaro, J Gazard, J Schwarz, R Volkman, D Machacek, S Stice, L Zawadzke, C O'Donnell and R Hurst. (2010). High-throughput screening in embryonic stem cell-derived neurons identifies potentiators of alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate-type glutamate receptors. *J Biol Chem* 285:17209-17.
108. Smogorzewska A and T de Lange. (2002). Different telomere damage signaling pathways in human and mouse cells. *EMBO J* 21:4338-48.
109. Eiges R, A Urbach, M Malcov, T Frumkin, T Schwartz, A Amit, Y Yaron, A Eden, O Yanuka, N Benvenisty and D Ben-Yosef. (2007). Developmental study of fragile X syndrome using human embryonic stem cells derived from preimplantation genetically diagnosed embryos. *Cell Stem Cell* 1:568-77.
110. Maury Y, M Gauthier, M Peschanski and C Martinat. (2012). Human pluripotent stem cells for disease modelling and drug screening. *Bioessays* 34:61-71.
111. Xu Y, Y Shi and S Ding. (2008). A chemical approach to stem-cell biology and regenerative medicine. *Nature* 453:338-44.

112. Desbordes SC, DG Placantonakis, A Ciro, ND Socci, G Lee, H Djaballah and L Studer. (2008). High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. *Cell Stem Cell* 2:602-12.
113. Lee TI, RG Jenner, LA Boyer, MG Guenther, SS Levine, RM Kumar, B Chevalier, SE Johnstone, MF Cole, K Isono, H Koseki, T Fuchikami, K Abe, HL Murray, JP Zucker, B Yuan, GW Bell, E Herbolsheimer, NM Hannett, K Sun, DT Odom, AP Otte, TL Volkert, DP Bartel, DA Melton, DK Gifford, R Jaenisch and RA Young. (2006). Control of developmental regulators by Polycomb in human embryonic stem cells. *Cell* 125:301-13.
114. Cai J, Y Zhao, Y Liu, F Ye, Z Song, H Qin, S Meng, Y Chen, R Zhou, X Song, Y Guo, M Ding and H Deng. (2007). Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* 45:1229-39.
115. Itskovitz-Eldor J, M Schuldiner, D Karsenti, A Eden, O Yanuka, M Amit, H Soreq and N Benvenisty. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. *Mol Med* 6:88-95.
116. Vallier L, D Reynolds and RA Pedersen. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. *Dev Biol* 275:403-21.
117. Mateizel I, N De Temmerman, U Ullmann, G Cauffman, K Sermon, H Van de Velde, M De Rycke, E Degreef, P Devroey, I Liebaers and A Van Steirteghem. (2006). Derivation of human embryonic stem cell lines from embryos obtained after IVF and after PGD for monogenic disorders. *Hum Reprod* 21:503-11.
118. Tropel P, J Tournois, J Come, C Varela, C Moutou, P Fragner, M Cailleret, Y Laabi, M Peschanski and S Viville. (2010). High-efficiency derivation of human embryonic stem cell lines following pre-implantation genetic diagnosis. *In Vitro Cell Dev Biol Anim* 46:376-85.
119. Helgason CD, G Sauvageau, HJ Lawrence, C Largman and RK Humphries. (1996). Overexpression of HOXB4 enhances the hematopoietic potential of embryonic stem cells differentiated in vitro. *Blood* 87:2740-9.
120. Mason C and P Dunnill. (2008). A brief definition of regenerative medicine. *Regen Med* 3:1-5.
121. Buckley RH, SE Schiff, RI Schiff, L Markert, LW Williams, JL Roberts, LA Myers and FE Ward. (1999). Hematopoietic stem-cell transplantation for the treatment of severe combined immunodeficiency. *N Engl J Med* 340:508-16.
122. Johnson FL, AT Look, J Gockerman, MR Ruggiero, L Dalla-Pozza and FT Billings, 3rd. (1984). Bone-marrow transplantation in a patient with sickle-cell anemia. *N Engl J Med* 311:780-3.
123. Perry AR and DC Linch. (1996). The history of bone-marrow transplantation. *Blood Rev* 10:215-9.
124. Austin EB, M Guttridge, D Pamphilon and SM Watt. (2008). The role of blood services and regulatory bodies in stem cell transplantation. *Vox Sang* 94:6-17.
125. Laflamme MA, KY Chen, AV Naumova, V Muskheli, JA Fugate, SK Dupras, H Reinecke, C Xu, M Hassanipour, S Police, C O'Sullivan, L Collins, Y Chen, E Minami, EA Gill, S Ueno, C Yuan, J Gold and CE Murry. (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol* 25:1015-24.
126. Spitalieri P, MC Quitadamo, A Orlandi, L Guerra, E Giardina, V Casavola, G Novelli, C Saltini and F Sangiuolo. (2011). Rescue of murine silica-induced lung injury and fibrosis by human embryonic stem cells. *Eur Respir J*.

127. Cho MS, YE Lee, JY Kim, S Chung, YH Cho, DS Kim, SM Kang, H Lee, MH Kim, JH Kim, JW Leem, SK Oh, YM Choi, DY Hwang, JW Chang and DW Kim. (2008). Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A* 105:3392-7.
128. Keirstead HS, G Nistor, G Bernal, M Totoiu, F Cloutier, K Sharp and O Steward. (2005). Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants remyelinate and restore locomotion after spinal cord injury. *J Neurosci* 25:4694-705.
129. Strauss S. (2010). Geron trial resumes, but standards for stem cell trials remain elusive. *Nat Biotechnol* 28:989-90.
130. Martin MJ, A Muotri, F Gage and A Varki. (2005). Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 11:228-32.
131. Nichols J and QL Ying. (2006). Derivation and propagation of embryonic stem cells in serum- and feeder-free culture. *Methods Mol Biol* 329:91-8.
132. Richards M, CY Fong, WK Chan, PC Wong and A Bongso. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nat Biotechnol* 20:933-6.
133. Jandial R and ML Levy. (2011). Cellular alchemy: induced pluripotent stem cells retain epigenetic memory. *World Neurosurg* 75:5-6.
134. Kim K, A Doi, B Wen, K Ng, R Zhao, P Cahan, J Kim, MJ Aryee, H Ji, LI Ehrlich, A Yabuuchi, A Takeuchi, KC Cunniff, H Hongguang, S McKinney-Freeman, O Naveiras, TJ Yoon, RA Irizarry, N Jung, J Seita, J Hanna, P Murakami, R Jaenisch, R Weissleder, SH Orkin, IL Weissman, AP Feinberg and GQ Daley. (2010). Epigenetic memory in induced pluripotent stem cells. *Nature* 467:285-90.
135. Polo JM, S Liu, ME Figueroa, W Kulalart, S Eminli, KY Tan, E Apostolou, M Stadtfeld, Y Li, T Shioda, S Natesan, AJ Wagers, A Melnick, T Evans and K Hochedlinger. (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotechnol* 28:848-55.
136. Lister R, M Pelizzola, YS Kida, RD Hawkins, JR Nery, G Hon, J Antosiewicz-Bourget, R O'Malley, R Castanon, S Klugman, M Downes, R Yu, R Stewart, B Ren, JA Thomson, RM Evans and JR Ecker. (2011). Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. *Nature* 471:68-73.
137. Nishino K, M Toyoda, M Yamazaki-Inoue, Y Fukawatase, E Chikazawa, H Sakaguchi, H Akutsu and A Umezawa. (2011). DNA methylation dynamics in human induced pluripotent stem cells over time. *PLoS Genet* 7:e1002085.
138. Bar-Nur O, HA Russ, S Efrat and N Benvenisty. (2011). Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. *Cell Stem Cell* 9:17-23.
139. Sullivan GJ, Y Bai, J Fletcher and I Wilmut. (2010). Induced pluripotent stem cells: epigenetic memories and practical implications. *Mol Hum Reprod* 16:880-5.
140. Taupin P. (2011). Parthenogenetically activated human oocytes and parthenogenetic embryonic stem cells: US20100233143. *Expert Opin Ther Pat* 21:1281-3.
141. Harness JV, NA Turovets, MJ Seiler, G Nistor, G Altun, LS Agapova, D Ferguson, LC Laurent, JF Loring and HS Keirstead. (2011). Equivalence of conventionally-derived and parthenote-derived human embryonic stem cells. *PLoS One* 6:e14499.
142. Liu W, L Guo, W He, Q Li and X Sun. (2012). Higher copy number variation and diverse X chromosome inactivation in parthenote-derived human embryonic stem cells. *J Reprod Dev* 58:642-8.
143. Thomson A, D Wojtacha, Z Hewitt, H Priddle, V Sottile, A Di Domenico, J Fletcher, M Waterfall, NL Corrales, R Ansell and J McWhir. (2008). Human embryonic stem cells passaged using enzymatic methods retain a normal karyotype and express CD30. *Cloning Stem Cells* 10:89-106.

144. Mitalipova MM, RR Rao, DM Hoyer, JA Johnson, LF Meisner, KL Jones, S Dalton and SL Stice. (2005). Preserving the genetic integrity of human embryonic stem cells. *Nat Biotechnol* 23:19-20.
145. Brimble SN, X Zeng, DA Weiler, Y Luo, Y Liu, IG Lyons, WJ Freed, AJ Robins, MS Rao and TC Schulz. (2004). Karyotypic stability, genotyping, differentiation, feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. *Stem Cells Dev* 13:585-97.
146. Pera MF, B Reubinoff and A Trounson. (2000). Human embryonic stem cells. *J Cell Sci* 113 (Pt 1):5-10.
147. Gepstein L. (2002). Derivation and potential applications of human embryonic stem cells. *Circ Res* 91:866-76.
148. Tavakoli T, X Xu, E Derby, Y Serebryakova, Y Reid, MS Rao, MP Mattson and W Ma. (2009). Self-renewal and differentiation capabilities are variable between human embryonic stem cell lines I3, I6 and BG01V. *BMC Cell Biol* 10:44.
149. Mikkola M, C Olsson, J Palgi, J Ustinov, T Palomaki, N Horelli-Kuitunen, S Knuutila, K Lundin, T Otonkoski and T Tuuri. (2006). Distinct differentiation characteristics of individual human embryonic stem cell lines. *BMC Dev Biol* 6:40.
150. Baker DE, NJ Harrison, E Maltby, K Smith, HD Moore, PJ Shaw, PR Heath, H Holden and PW Andrews. (2007). Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. *Nat Biotechnol* 25:207-15.
151. Xie X, A Hiona, AS Lee, F Cao, M Huang, Z Li, A Cherry, X Pei and JC Wu. (2011). Effects of long-term culture on human embryonic stem cell aging. *Stem Cells Dev* 20:127-38.
152. Adewumi O, B Aflatoonian, L Ahrlund-Richter, M Amit, PW Andrews, G Beighton, PA Bello, N Benvenisty, LS Berry, S Bevan, B Blum, J Brooking, KG Chen, AB Choo, GA Churchill, M Corbel, I Damjanov, JS Draper, P Dvorak, K Emanuelsson, RA Fleck, A Ford, K Gertow, M Gertsenstein, PJ Gokhale, RS Hamilton, A Hampl, LE Healy, O Hovatta, J Hyllner, MP Imreh, J Itskovitz-Eldor, J Jackson, JL Johnson, M Jones, K Kee, BL King, BB Knowles, M Lako, F Lebrin, BS Mallon, D Manning, Y Mayshar, RD McKay, AE Michalska, M Mikkola, M Mileikovsky, SL Minger, HD Moore, CL Mummery, A Nagy, N Nakatsuji, CM O'Brien, SK Oh, C Olsson, T Otonkoski, KY Park, R Passier, H Patel, M Patel, R Pedersen, MF Pera, MS Piekarczyk, RA Pera, BE Reubinoff, AJ Robins, J Rossant, P Rugg-Gunn, TC Schulz, H Semb, ES Sherrer, H Siemen, GN Stacey, M Stojkovic, H Suemori, J Szatkiewicz, T Turetsky, T Tuuri, S van den Brink, K Vintersten, S Vuoristo, D Ward, TA Weaver, LA Young and W Zhang. (2007). Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol* 25:803-16.
153. Harrison NJ, D Baker and PW Andrews. (2007). Culture adaptation of embryonic stem cells echoes germ cell malignancy. *Int J Androl* 30:275-81; discussion 281.
154. Skottman H, M Mikkola, K Lundin, C Olsson, AM Stromberg, T Tuuri, T Otonkoski, O Hovatta and R Lahesmaa. (2005). Gene expression signatures of seven individual human embryonic stem cell lines. *Stem Cells* 23:1343-56.
155. Enver T, S Soneji, C Joshi, J Brown, F Iborra, T Orntoft, T Thykjaer, E Maltby, K Smith, R Abu Dawud, M Jones, M Matin, P Gokhale, J Draper and PW Andrews. (2005). Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. *Hum Mol Genet* 14:3129-40.
156. Dhara SK and N Benvenisty. (2004). Gene trap as a tool for genome annotation and analysis of X chromosome inactivation in human embryonic stem cells. *Nucleic Acids Res* 32:3995-4002.
157. Abeyta MJ, AT Clark, RT Rodriguez, MS Bodnar, RA Pera and MT Firpo. (2004). Unique gene expression signatures of independently-derived human embryonic stem cell lines. *Hum Mol Genet* 13:601-8.

158. Laslett AL, AA Filipczyk and MF Pera. (2003). Characterization and culture of human embryonic stem cells. *Trends Cardiovasc Med* 13:295-301.
159. Bryja V, S Bonilla and E Arenas. (2006). Derivation of mouse embryonic stem cells. *Nat Protoc* 1:2082-7.
160. Tielens S, B Verhasselt, J Liu, M Dhont, J Van Der Elst and M Cornelissen. (2006). Generation of embryonic stem cell lines from mouse blastocysts developed in vivo and in vitro: relation to Oct-4 expression. *Reproduction* 132:59-66.
161. Schoonjans L, V Kreemers, S Danloy, RW Moreadith, Y Laroche and D Collen. (2003). Improved generation of germline-competent embryonic stem cell lines from inbred mouse strains. *Stem Cells* 21:90-7.
162. Fan Y, Y Luo, X Chen and X Sun. (2010). A Modified Culture Medium Increases Blastocyst Formation and the Efficiency of Human Embryonic Stem Cell Derivation from Poor-Quality Embryos. *J Reprod Dev*.
163. Giritharan G, S Talbi, A Donjacour, F Di Sebastiano, AT Dobson and PF Rinaudo. (2007). Effect of in vitro fertilization on gene expression and development of mouse preimplantation embryos. *Reproduction* 134:63-72.
164. Liu W, Y Yin, X Long, Y Luo, Y Jiang, W Zhang, H Du, S Li, Y Zheng, Q Li, X Chen, B Liao, G Xiao, W Wang and X Sun. (2009). Derivation and characterization of human embryonic stem cell lines from poor quality embryos. *J Genet Genomics* 36:229-39.
165. Bankowski BJ, AD Lysterly, RR Faden and EE Wallach. (2005). The social implications of embryo cryopreservation. *Fertil Steril* 84:823-32.
166. Klock SC. (2004). Embryo disposition: the forgotten "child" of in vitro fertilization. *Int J Fertil Womens Med* 49:19-23.
167. Van der Elst J, E Van den Abbeel, M Camus, J Smits, P Devroey and A Van Steirteghem. (1996). Long-term evaluation of implantation of fresh and cryopreserved human embryos following ovarian stimulation with busarelin acetate-human menopausal gonadotrophin (HMG) or clomiphene citrate-HMG. *Hum Reprod* 11:2097-106.
168. Oehninger S, J Mayer and S Muasher. (2000). Impact of different clinical variables on pregnancy outcome following embryo cryopreservation. *Mol Cell Endocrinol* 169:73-7.
169. Wang YA, GM Chambers, M Dieng and EA Sullivan. (2009). Assisted reproductive technology in Australia and New Zealand 2007. *Assisted reproduction technology series* 13:1-56.
170. Farquhar CM, YA Wang and EA Sullivan. (2010). A comparative analysis of assisted reproductive technology cycles in Australia and New Zealand 2004-2007. *Hum Reprod* 25:2281-9.
171. Koestenbauer S, NH Zech, H Juch, P Vanderzwalmen, L Schoonjans and G Dohr. (2006). Embryonic stem cells: similarities and differences between human and murine embryonic stem cells. *Am J Reprod Immunol* 55:169-80.
172. Aoki F, DM Worrad and RM Schultz. (1997). Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev Biol* 181:296-307.
173. Braude P, V Bolton and S Moore. (1988). Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332:459-61.
174. Nikas G, A Ao, RM Winston and AH Handyside. (1996). Compaction and surface polarity in the human embryo in vitro. *Biol Reprod* 55:32-7.
175. Reeve WJ and FP Kelly. (1983). Nuclear position in the cells of the mouse early embryo. *J Embryol Exp Morphol* 75:117-39.
176. Fleming TP and SJ Pickering. (1985). Maturation and polarization of the endocytotic system in outside blastomeres during mouse preimplantation development. *J Embryol Exp Morphol* 89:175-208.

177. Houliston E and B Maro. (1989). Posttranslational modification of distinct microtubule subpopulations during cell polarization and differentiation in the mouse preimplantation embryo. *J Cell Biol* 108:543-51.
178. Fleming TP, SJ Pickering, F Qasim and B Maro. (1986). The generation of cell surface polarity in mouse 8-cell blastomeres: the role of cortical microfilaments analysed using cytochalasin D. *J Embryol Exp Morphol* 95:169-91.
179. Louvet S, J Aghion, A Santa-Maria, P Mangeat and B Maro. (1996). Ezrin becomes restricted to outer cells following asymmetrical division in the preimplantation mouse embryo. *Dev Biol* 177:568-79.
180. Plusa B, S Frankenberg, A Chalmers, AK Hadjantonakis, CA Moore, N Papaioannou, DM Glover and M Zernicka-Goetz. (2005). Downregulation of Par3 and aPKC function directs cells towards the ICM in the preimplantation mouse embryo. *J Cell Sci* 118:505-15.
181. Vinot S, T Le, S Ohno, T Pawson, B Maro and S Louvet-Vallee. (2005). Asymmetric distribution of PAR proteins in the mouse embryo begins at the 8-cell stage during compaction. *Dev Biol* 282:307-19.
182. Pauken CM and DG Capco. (2000). The expression and stage-specific localization of protein kinase C isoforms during mouse preimplantation development. *Dev Biol* 223:411-21.
183. Johnson MH and CA Ziomek. (1981). The foundation of two distinct cell lineages within the mouse morula. *Cell* 24:71-80.
184. Sutherland AE, TP Speed and PG Calarco. (1990). Inner cell allocation in the mouse morula: the role of oriented division during fourth cleavage. *Dev Biol* 137:13-25.
185. Rossant J and WT Lis. (1979). Potential of isolated mouse inner cell masses to form trophoblast derivatives in vivo. *Dev Biol* 70:255-61.
186. Spindle AI. (1978). Trophoblast regeneration by inner cell masses isolated from cultured mouse embryos. *J Exp Zool* 203:483-9.
187. Handyside AH. (1978). Time of commitment of inside cells isolated from preimplantation mouse embryos. *J Embryol Exp Morphol* 45:37-53.
188. Watson AJ, DR Natale and LC Barcroft. (2004). Molecular regulation of blastocyst formation. *Anim Reprod Sci* 82-83:583-92.
189. Watson AJ and LC Barcroft. (2001). Regulation of blastocyst formation. *Front Biosci* 6:D708-30.
190. Sheth B, JJ Fontaine, E Ponza, A McCallum, A Page, S Citi, D Louvard, A Zahraoui and TP Fleming. (2000). Differentiation of the epithelial apical junctional complex during mouse preimplantation development: a role for rab13 in the early maturation of the tight junction. *Mech Dev* 97:93-104.
191. Sheth B, I Fesenko, JE Collins, B Moran, AE Wild, JM Anderson and TP Fleming. (1997). Tight junction assembly during mouse blastocyst formation is regulated by late expression of ZO-1 alpha+ isoform. *Development* 124:2027-37.
192. Dietrich JE and T Hiiragi. (2007). Stochastic patterning in the mouse pre-implantation embryo. *Development* 134:4219-31.
193. Dietrich JE and T Hiiragi. (2008). Stochastic Processes during Mouse Blastocyst Patterning. *Cells Tissues Organs*.
194. Jedrusik A, DE Parfitt, G Guo, M Skamagki, JB Grabarek, MH Johnson, P Robson and M Zernicka-Goetz. (2008). Role of Cdx2 and cell polarity in cell allocation and specification of trophoblast and inner cell mass in the mouse embryo. *Genes Dev* 22:2692-706.
195. Ralston A and J Rossant. (2008). Cdx2 acts downstream of cell polarization to cell-autonomously promote trophoblast fate in the early mouse embryo. *Dev Biol* 313:614-29.

196. Strumpf D, CA Mao, Y Yamanaka, A Ralston, K Chawengsaksophak, F Beck and J Rossant. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development* 132:2093-102.
197. Cockburn K and J Rossant. (2010). Making the blastocyst: lessons from the mouse. *J Clin Invest* 120:995-1003.
198. Chazaud C, Y Yamanaka, T Pawson and J Rossant. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. *Dev Cell* 10:615-24.
199. Fujikura J, E Yamato, S Yonemura, K Hosoda, S Masui, K Nakao, J Miyazaki Ji and H Niwa. (2002). Differentiation of embryonic stem cells is induced by GATA factors. *Genes Dev* 16:784-9.
200. Plusa B, A Piliszek, S Frankenberg, J Artus and AK Hadjantonakis. (2008). Distinct sequential cell behaviours direct primitive endoderm formation in the mouse blastocyst. *Development* 135:3081-91.
201. Meilhac SM, RJ Adams, SA Morris, A Danckaert, JF Le Garrec and M Zernicka-Goetz. (2009). Active cell movements coupled to positional induction are involved in lineage segregation in the mouse blastocyst. *Dev Biol* 331:210-21.
202. Chen AE, D Egli, K Niakan, J Deng, H Akutsu, M Yamaki, C Cowan, C Fitz-Gerald, K Zhang, DA Melton and K Eggan. (2009). Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. *Cell Stem Cell* 4:103-6.
203. Lane M and DK Gardner. (2007). Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol* 21:83-100.
204. Cauffman G, M De Rycke, K Sermon, I Liebaers and H Van de Velde. (2009). Markers that define stemness in ESC are unable to identify the totipotent cells in human preimplantation embryos. *Hum Reprod* 24:63-70.
205. Whitten WK. (1956). Culture of tubal mouse ova. *Nature* 177:96.
206. Edwards RG, BD Bavister and PC Steptoe. (1969). Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature* 221:632-5.
207. Steptoe PC and RG Edwards. (1978). Birth after the reimplantation of a human embryo. *Lancet* 2:366.
208. Laws P and E Sullivan. (2009). Australia's mothers and babies. AIHW National Perinatal Statistics Unit, Sydney.
209. Lass A, D Peat, S Avery and P Brinsden. (1998). Histological evaluation of endometrium on the day of oocyte retrieval after gonadotrophin-releasing hormone agonist-follicle stimulating hormone ovulation induction for in-vitro fertilization. *Hum Reprod* 13:3203-5.
210. Gardner D and M Lane. (1999). Embryo culture systems. In: *Handbook of In Vitro Fertilisation*. CRC Press, New York. p 123-150.
211. Quinn P, JF Kerin and GM Warnes. (1985). Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril* 44:493-8.
212. Whittingham DG. (1971). Culture of mouse ova. *J Reprod Fertil Suppl* 14:7-21.
213. Eagle H. (1959). Amino acid metabolism in mammalian cell cultures. *Science* 130:432-7.
214. Ham RG. (1963). An improved nutrient solution for diploid Chinese hamster and human cell lines. *Exp Cell Res* 29:515-26.
215. Gardner DK. (2008). Dissection of culture media for embryos: the most important and less important components and characteristics. *Reprod Fertil Dev* 20:9-18.
216. Lane M and DK Gardner. (2006). Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol*.

217. Laverge H, P De Sutter, R Desmet, J Van der Elst and M Dhont. (1997). Prospective randomized study comparing human serum albumin with fetal cord serum as protein supplement in culture medium for in-vitro fertilization. *Hum Reprod* 12:2263-6.
218. Gardner DK and M Lane. (2003). Towards a single embryo transfer. *Reprod Biomed Online* 6:470-81.
219. Orsi NM and HJ Leese. (2001). Protection against reactive oxygen species during mouse preimplantation embryo development: role of EDTA, oxygen tension, catalase, superoxide dismutase and pyruvate. *Mol Reprod Dev* 59:44-53.
220. Leese HJ. (1991). Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol* 13:35-72.
221. Leese HJ. (1995). Metabolic control during preimplantation mammalian development. *Hum Reprod Update* 1:63-72.
222. Brinster RL. (1967). Protein content of the mouse embryo during the first five days of development. *J Reprod Fertil* 13:413-20.
223. Whittingham DG and JD Biggers. (1967). Fallopian tube and early cleavage in the mouse. *Nature* 213:942-3.
224. Brinster RL. (1963). A Method for in Vitro Cultivation of Mouse Ova from Two-Cell to Blastocyst. *Exp Cell Res* 32:205-8.
225. Brinster RL. (1965). Studies on the development of mouse embryos in vitro. IV. Interaction of energy sources. *J Reprod Fertil* 10:227-40.
226. Gott AL, K Hardy, RM Winston and HJ Leese. (1990). Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod* 5:104-8.
227. Shumak SL, M Gulan, B Zinman and IS Gottesman. (1989). Determination and kinetic analysis of non-insulin mediated glucose uptake in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 32:28-33.
228. Quinn P. (1995). Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. *J Assist Reprod Genet* 12:97-105.
229. Chatot CL, CA Ziomek, BD Bavister, JL Lewis and I Torres. (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J Reprod Fertil* 86:679-88.
230. Gardner DK and M Lane. (1996). Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. *Hum Reprod* 11:2703-12.
231. Martin KL and HJ Leese. (1995). Role of glucose in mouse preimplantation embryo development. *Mol Reprod Dev* 40:436-43.
232. Hewitson LC and HJ Leese. (1993). Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. *J Exp Zool* 267:337-43.
233. Hewitson LC, KL Martin and HJ Leese. (1996). Effects of metabolic inhibitors on mouse preimplantation embryo development and the energy metabolism of isolated inner cell masses. *Mol Reprod Dev* 43:323-30.
234. Gardner DK, M Lane, I Calderon and J Leeton. (1996). Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 65:349-53.
235. Gardner DK and HJ Leese. (1990). Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil* 88:361-8.
236. Leese HJ and AM Barton. (1984). Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil* 72:9-13.

237. Leese HJ, Ji Tay, J Reischl and SJ Downing. (2001). Formation of Fallopian tubal fluid: role of a neglected epithelium. *Reproduction* 121:339-46.
238. Crosby IM, F Gandolfi and RM Moor. (1988). Control of protein synthesis during early cleavage of sheep embryos. *J Reprod Fertil* 82:769-75.
239. Lane M and DK Gardner. (2005). Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem* 280:18361-7.
240. Van Winkle LJ, N Haghighat and AL Campione. (1990). Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *J Exp Zool* 253:215-9.
241. Edwards LJ, DA Williams and DK Gardner. (1998). Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 13:3441-8.
242. Lindenbaum A. (1973). A survey of naturally occurring chelating ligands. *Adv Exp Med Biol* 40:67-77.
243. Liu Z and RH Foote. (1995). Effects of amino acids on the development of in-vitro matured/in-vitro fertilization bovine embryos in a simple protein-free medium. *Hum Reprod* 10:2985-91.
244. Martin PM and AE Sutherland. (2001). Exogenous amino acids regulate trophoblast differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol* 240:182-93.
245. Lane M and DK Gardner. (1997). Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compaction of mouse zygotes in vitro. *J Assist Reprod Genet* 14:398-403.
246. Gardner DK and M Lane. (1993). Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 48:377-85.
247. Lane M and DK Gardner. (1994). Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil* 102:305-12.
248. Devreker F, K Hardy, M Van den Bergh, AS Vannin, S Emiliani and Y Englert. (2001). Amino acids promote human blastocyst development in vitro. *Hum Reprod* 16:749-56.
249. Devreker F, RM Winston and K Hardy. (1998). Glutamine improves human preimplantation development in vitro. *Fertil Steril* 69:293-9.
250. Daniel JC, Jr. and RS Krishnan. (1967). Amino acid requirements for growth of the rabbit blastocyst in vitro. *J Cell Physiol* 70:155-60.
251. Kane MT and BD Bavister. (1988). Protein-free culture medium containing polyvinylalcohol, vitamins, and amino acids supports development of eight-cell hamster embryos to hatching blastocysts. *J Exp Zool* 247:183-7.
252. Zhang X and DT Armstrong. (1990). Presence of amino acids and insulin in a chemically defined medium improves development of 8-cell rat embryos in vitro and subsequent implantation in vivo. *Biol Reprod* 42:662-8.
253. Walker SK, JL Hill, DO Kleemann and CD Nancarrow. (1996). Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. *Biol Reprod* 55:703-8.
254. Gardner DK, M Lane, A Spitzer and PA Batt. (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod* 50:390-400.
255. Rosenkrans CF, Jr., DL Davis and G Milliken. (1989). Pig blastocyst development in vitro is affected by amino acids. *J Anim Sci* 67:1503-8.

256. Takahashi Y and NL First. (1992). In vitro development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* 37:963-78.
257. Rosenkrans CF, Jr. and NL First. (1994). Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. *J Anim Sci* 72:434-7.
258. Lane M and DK Gardner. (1997). Differential regulation of mouse embryo development and viability by amino acids. *J Reprod Fertil* 109:153-64.
259. Gu erin P, E Gallois, S Croteau, N Revol, F Maurin and J Guillaud. (1995). Techniques de recolte et aminogrammes des liquides tudaire et folliculaire chez les femelles domestiques. *Rev Med Vet* 146:805-814.
260. Iritani A, Y Nishikawa, WR Gomes and NL VanDemark. (1971). Secretion rates and chemical composition of oviduct and uterine fluids in rabbits. *J Anim Sci* 33:829-35.
261. Miller JG and GA Schultz. (1987). Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol Reprod* 36:125-9.
262. Menezo Y and P Laviolette. (1972). [Amino constituents of tubal secretions in the rabbit. Zymogram--proteins--free amino acids]. *Ann Biol Anim Biochim Biophys* 12:383-96.
263. Engle CC, ED Foley, PE D. and DM Witherspoon. (1984). Free amino acids and protein concentrations in reproductive tract fluids of the mare. *Theriogenology* 21:919-930.
264. Wang J, P Alexander, L Wu, R Hammer, O Cleaver and SL McKnight. (2009). Dependence of mouse embryonic stem cells on threonine catabolism. *Science* 325:435-9.
265. Ryu JM and HJ Han. (2011). L-threonine regulates G1/S phase transition of mouse embryonic stem cells via PI3K/Akt, MAPKs, and mTORC pathways. *J Biol Chem* 286:23667-78.
266. Storm MP, HK Bone, CG Beck, PY Bourillot, V Schreiber, T Damiano, A Nelson, P Savatier and MJ Welham. (2007). Regulation of Nanog expression by phosphoinositide 3-kinase-dependent signaling in murine embryonic stem cells. *J Biol Chem* 282:6265-73.
267. Kazuoka T, S Takigawa, N Arakawa, Y Hizukuri, I Muraoka, T Oikawa and K Soda. (2003). Novel psychrophilic and thermolabile L-threonine dehydrogenase from psychrophilic *Cytophaga* sp. strain KUC-1. *J Bacteriol* 185:4483-9.
268. Lane M and DK Gardner. (1998). Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 13:991-7.
269. Gardner DK. (1998). Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 49:83-102.
270. Lane M and DK Gardner. (1996). Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod* 11:1975-8.
271. Tsai FC and DK Gardner. (1994). Nicotinamide, a component of complex culture media, inhibits mouse embryo development in vitro and reduces subsequent developmental potential after transfer. *Fertil Steril* 61:376-82.
272. Solomon JM, R Pasupuleti, L Xu, T McDonagh, R Curtis, PS DiStefano and LJ Huber. (2006). Inhibition of SIRT1 catalytic activity increases p53 acetylation but does not alter cell survival following DNA damage. *Mol Cell Biol* 26:28-38.
273. Lin T, C Chao, S Saito, SJ Mazur, ME Murphy, E Appella and Y Xu. (2005). p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat Cell Biol* 7:165-71.
274. Kane MT and BD Bavister. (1988). Vitamin requirements for development of eight-cell hamster embryos to hatching blastocysts in vitro. *Biol Reprod* 39:1137-43.

275. Nasr-Esfahani M, MH Johnson and RJ Aitken. (1990). The effect of iron and iron chelators on the in-vitro block to development of the mouse preimplantation embryo: BAT6 a new medium for improved culture of mouse embryos in vitro. *Hum Reprod* 5:997-1003.
276. Gutteridge JM and B Halliwell. (1989). Iron toxicity and oxygen radicals. *Baillieres Clin Haematol* 2:195-256.
277. Wang X, T Falcone, M Attaran, JM Goldberg, A Agarwal and RK Sharma. (2002). Vitamin C and vitamin E supplementation reduce oxidative stress-induced embryo toxicity and improve the blastocyst development rate. *Fertil Steril* 78:1272-7.
278. Nasr-Esfahani MH, JR Aitken and MH Johnson. (1990). Hydrogen peroxide levels in mouse oocytes and early cleavage stage embryos developed in vitro or in vivo. *Development* 109:501-7.
279. Kowaltowski AJ and AE Vercesi. (1999). Mitochondrial damage induced by conditions of oxidative stress. *Free Radic Biol Med* 26:463-71.
280. Hyslop PA, DB Hinshaw, WA Halsey, Jr., IU Schraufstatter, RD Sauerheber, RG Spragg, JH Jackson and CG Cochrane. (1988). Mechanisms of oxidant-mediated cell injury. The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. *J Biol Chem* 263:1665-75.
281. Yang HW, KJ Hwang, HC Kwon, HS Kim, KW Choi and KS Oh. (1998). Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Hum Reprod* 13:998-1002.
282. Dennerly PA. (2007). Effects of oxidative stress on embryonic development. *Birth Defects Res C Embryo Today* 81:155-62.
283. Bedaiwy MA, T Falcone, MS Mohamed, AA Aleem, RK Sharma, SE Worley, J Thornton and A Agarwal. (2004). Differential growth of human embryos in vitro: role of reactive oxygen species. *Fertil Steril* 82:593-600.
284. Goto Y, Y Noda, T Mori and M Nakano. (1993). Increased generation of reactive oxygen species in embryos cultured in vitro. *Free Radic Biol Med* 15:69-75.
285. Guo YL, S Chakraborty, SS Rajan, R Wang and F Huang. (2010). Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal. *Stem Cells Dev* 19:1321-31.
286. Lee MN, SH Lee, MY Lee, YH Kim, JH Park, JM Ryu, SP Yun, YJ Lee, MO Kim, K Park and HJ Han. (2008). Effect of dihydrotestosterone on mouse embryonic stem cells exposed to H₂O₂-induced oxidative stress. *J Vet Sci* 9:247-56.
287. Lane M and DK Gardner. (2001). Inhibiting 3-phosphoglycerate kinase by EDTA stimulates the development of the cleavage stage mouse embryo. *Mol Reprod Dev* 60:233-40.
288. Noda Y, H Matsumoto, Y Umaoka, K Tatsumi, J Kishi and T Mori. (1991). Involvement of superoxide radicals in the mouse two-cell block. *Mol Reprod Dev* 28:356-60.
289. Gardner DK, MW Lane and M Lane. (2000). EDTA stimulates cleavage stage bovine embryo development in culture but inhibits blastocyst development and differentiation. *Mol Reprod Dev* 57:256-61.
290. Bavister BD. (1995). Culture of preimplantation embryos: facts and artifacts. *Hum Reprod Update* 1:91-148.
291. Gardner DK, H Rodriegez-Martinez and M Lane. (1999). Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. *Hum Reprod* 14:2575-80.
292. Gardner DK and M Lane. (2002). Development of viable mammalian embryos in vitro: evolution of a sequential media. Academic Press, New York.
293. Flood LP and B Shirley. (1991). Reduction of embryotoxicity by protein in embryo culture media. *Mol Reprod Dev* 30:226-31.

294. Fissore RA, KV Jackson and AA Kiessling. (1989). Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. *Biol Reprod* 41:835-41.
295. Barnes D and G Sato. (1980). Methods for growth of cultured cells in serum-free medium. *Anal Biochem* 102:255-70.
296. Thompson JG, DK Gardner, PA Pugh, WH McMillan and HR Tervit. (1995). Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol Reprod* 53:1385-91.
297. Valeyre D, P Soler, G Basset, P Loiseau, J Pre, P Turbie, JP Battesti and R Georges. (1991). Glucose, K⁺, and albumin concentrations in the alveolar milieu of normal humans and pulmonary sarcoidosis patients. *Am Rev Respir Dis* 143:1096-101.
298. McKiernan SH and BD Bavister. (1992). Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. *In Vitro Cell Dev Biol* 28A:154-6.
299. Bungum M, L Bungum, P Humaidan and C Yding Andersen. (2003). Day 3 versus day 5 embryo transfer: a prospective randomized study. *Reprod Biomed Online* 7:98-104.
300. Lane M, M Mitchell, KS Cashman, D Feil, S Wakefield and DL Zander-Fox. (2008). To QC or not to QC: the key to a consistent laboratory? *Reprod Fertil Dev* 20:23-32.
301. Umaoka Y, Y Noda, K Narimoto and T Mori. (1992). Effects of oxygen toxicity on early development of mouse embryos. *Mol Reprod Dev* 31:28-33.
302. Quinn P and GM Harlow. (1978). The effect of oxygen on the development of preimplantation mouse embryos in vitro. *J Exp Zool* 206:73-80.
303. Booth PJ, P Holm and H Callesen. (2005). The effect of oxygen tension on porcine embryonic development is dependent on embryo type. *Theriogenology* 63:2040-52.
304. Waldenstrom U, AB Engstrom, D Hellberg and S Nilsson. (2009). Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril* 91:2461-5.
305. Feichtinger W, P Kemeter and S Szalay. (1983). The Vienna program of in vitro fertilization and embryo-transfer--a successful clinical treatment. *Eur J Obstet Gynecol Reprod Biol* 15:63-70.
306. Dumoulin JC, CJ Meijers, M Bras, E Coonen, JP Geraedts and JL Evers. (1999). Effect of oxygen concentration on human in-vitro fertilization and embryo culture. *Hum Reprod* 14:465-9.
307. Dumoulin JC, RC Vanvuchelen, JA Land, MH Pieters, JP Geraedts and JL Evers. (1995). Effect of oxygen concentration on in vitro fertilization and embryo culture in the human and the mouse. *Fertil Steril* 63:115-9.
308. Behr B, TB Pool, AA Milki, D Moore, J Gebhardt and D Dasig. (1999). Preliminary clinical experience with human blastocyst development in vitro without co-culture. *Hum Reprod* 14:454-7.
309. Pabon JE, Jr., WE Findley and WE Gibbons. (1989). The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. *Fertil Steril* 51:896-900.
310. Mastroianni L, Jr. and R Jones. (1965). Oxygen Tension within the Rabbit Fallopian Tube. *J Reprod Fertil* 9:99-102.
311. Maas DH, BT Storey and L Mastroianni, Jr. (1976). Oxygen tension in the oviduct of the rhesus monkey (*Macaca mulatta*). *Fertil Steril* 27:1312-7.
312. Fischer B and BD Bavister. (1993). Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil* 99:673-9.
313. Karagenc L, Z Sertkaya, N Ciray, U Ulug and M Bahceci. (2004). Impact of oxygen concentration on embryonic development of mouse zygotes. *Reprod Biomed Online* 9:409-17.

314. Gibbons J, Hewitt, E, Gardner, DK. (2006). Effects of oxygen tension on the establishment and lactate dehydrogenase activity of murine embryonic stem cells. *Cloning and stem cells* 8:117-22.
315. Zachar V, SM Prasad, SC Weli, A Gabrielsen, K Petersen, MB Petersen and T Fink. (2010). The effect of human embryonic stem cells (hESCs) long-term normoxic and hypoxic cultures on the maintenance of pluripotency. *In Vitro Cell Dev Biol Anim* 46:276-83.
316. Lim HJ, J Han, DH Woo, SE Kim, SK Kim, HG Kang and JH Kim. (2011). Biochemical and morphological effects of hypoxic environment on human embryonic stem cells in long-term culture and differentiating embryoid bodies. *Mol Cells* 31:123-32.
317. Prasad SM, M Czepiel, C Cetinkaya, K Smigielska, SC Weli, H Lysdahl, A Gabrielsen, K Petersen, N Ehlers, T Fink, SL Minger and V Zachar. (2009). Continuous hypoxic culturing maintains activation of Notch and allows long-term propagation of human embryonic stem cells without spontaneous differentiation. *Cell Prolif* 42:63-74.
318. Swain JE. (2010). Optimizing the culture environment in the IVF laboratory: impact of pH and buffer capacity on gamete and embryo quality. *Reprod Biomed Online* 21:6-16.
319. Lane M, JM Baltz and BD Bavister. (1998). Regulation of intracellular pH in hamster preimplantation embryos by the sodium hydrogen (Na⁺/H⁺) antiporter. *Biol Reprod* 59:1483-90.
320. Lane M, JM Baltz and BD Bavister. (1999). Bicarbonate/chloride exchange regulates intracellular pH of embryos but not oocytes of the hamster. *Biol Reprod* 61:452-7.
321. Roos A and WF Boron. (1981). Intracellular pH. *Physiol Rev* 61:296-434.
322. Baltz JM. (1993). Intracellular pH regulation in the early embryo. *Bioessays* 15:523-30.
323. Baltz JM, JD Biggers and C Lechene. (1991). Two-cell stage mouse embryos appear to lack mechanisms for alleviating intracellular acid loads. *J Biol Chem* 266:6052-7.
324. Lane M and BD Bavister. (1999). Regulation of intracellular pH in bovine oocytes and cleavage stage embryos. *Mol Reprod Dev* 54:396-401.
325. Dale B, Y Menezo, J Cohen, L DiMatteo and M Wilding. (1998). Intracellular pH regulation in the human oocyte. *Hum Reprod* 13:964-70.
326. Phillips KP, MC Leveille, P Claman and JM Baltz. (2000). Intracellular pH regulation in human preimplantation embryos. *Hum Reprod* 15:896-904.
327. Edwards LJ, DA Williams and DK Gardner. (1998). Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. *Mol Reprod Dev* 50:434-42.
328. Lane M, JM Baltz and BD Bavister. (1999). Na⁺/H⁺ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 208:244-52.
329. Squirrell JM, M Lane and BD Bavister. (2001). Altering intracellular pH disrupts development and cellular organization in preimplantation hamster embryos. *Biol Reprod* 64:1845-54.
330. Lane M, EA Lyons and BD Bavister. (2000). Cryopreservation reduces the ability of hamster 2-cell embryos to regulate intracellular pH. *Hum Reprod* 15:389-94.
331. Zander-Fox DL, M Mitchell, JG Thompson and M Lane. (2010). Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. *Reprod Biomed Online* 21:219-29.
332. Gardner DK, M Lane and AJ Watson. (2004). *A Laboratory Guide to the Mammalian Embryo*. Oxford University Press, New York.
333. Vijayakumar R, J Simoni, B Ndubisi, F DeLeon and W Heine. (1987). Mouse embryo growth in different culture media: selection of a medium for quality control cross-testing of human in vitro fertilization conditions. *Arch Androl* 19:149-58.
334. Leibo SP, P Mazur and SC Jackowski. (1974). Factors affecting survival of mouse embryos during freezing and thawing. *Exp Cell Res* 89:79-88.

335. Leibo SP, JJ McGrath and EG Cravalho. (1978). Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. *Cryobiology* 15:257-71.
336. Rall WF. (1987). Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology* 24:387-402.
337. Martino A, JW Pollard and SP Leibo. (1996). Effect of chilling bovine oocytes on their developmental competence. *Mol Reprod Dev* 45:503-12.
338. Lane M, WB Schoolcraft and DK Gardner. (1999). Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 72:1073-8.
339. Rall WF and GM Fahy. (1985). Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification. *Nature* 313:573-5.
340. Wong JY and AY Wong. (2011). Phasing-in of vitrification into routine practice: why, how, and what. *Hong Kong Med J* 17:119-26.
341. Herrero L, M Martinez and JA Garcia-Velasco. (2011). Current status of human oocyte and embryo cryopreservation. *Curr Opin Obstet Gynecol* 23:245-50.
342. Gardner DK, M Lane, J Stevens, T Schlenker and WB Schoolcraft. (2000). Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 73:1155-8.
343. Blake DA, M Proctor and NP Johnson. (2004). The merits of blastocyst versus cleavage stage embryo transfer: a Cochrane review. *Hum Reprod* 19:795-807.
344. Hardy K and S Spanos. (2002). Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol* 172:221-36.
345. Huppertz B and A Herrler. (2005). Regulation of proliferation and apoptosis during development of the preimplantation embryo and the placenta. *Birth Defects Res C Embryo Today* 75:249-61.
346. (2012). ISM2TM. Origio.
347. Lane M, DK Gardner, MJ Hasler and JF Hasler. (2003). Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. *Theriogenology* 60:407-19.
348. (2012). ISM1TM. Origio.
349. Ho Y, K Wigglesworth, JJ Eppig and RM Schultz. (1995). Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 41:232-8.
350. (2012). Quinn's advantage blastocyst medium. Cooper Surgical, Inc.
351. (2012). HTF Medium with Gentamicin. Irvine Scientific.
352. (2004). Global. LifeGlobal.
353. Kurzawa R, W Glabowski and L Wenda-Rozewicka. (2001). Evaluation of mouse preimplantation embryos cultured in media enriched with insulin-like growth factors I and II, epidermal growth factor and tumor necrosis factor alpha. *Folia Histochem Cytobiol* 39:245-51.
354. Paria BC and SK Dey. (1990). Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. *Proc Natl Acad Sci U S A* 87:4756-60.
355. Bhatnagar P, VE Papaioannou and JD Biggers. (1995). CSF-1 and mouse preimplantation development in vitro. *Development* 121:1333-9.
356. Mezhevnikina LM, VV Fedorova, IV Kapralova and EE Fesenko. (2006). [Increased survival of preimplantation mouse embryos in medium with recombinant cytokine LIF]. *Ontogenez* 37:55-62.
357. Martin KL, DH Barlow and IL Sargent. (1998). Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum Reprod* 13:1645-52.

358. Desai N, J Lawson and J Goldfarb. (2000). Assessment of growth factor effects on post-thaw development of cryopreserved mouse morulae to the blastocyst stage. *Hum Reprod* 15:410-8.
359. Cheung LP, HY Leung and A Bongso. (2003). Effect of supplementation of leukemia inhibitory factor and epidermal growth factor on murine embryonic development in vitro, implantation, and outcome of offspring. *Fertil Steril* 80 Suppl 2:727-35.
360. Sharkey A. (1998). Cytokines and implantation. *Rev Reprod* 3:52-61.
361. Kim CH, HD Chae, YP Cheon, BM Kang, YS Chang and JE Mok. (1999). The effect of epidermal growth factor on the preimplantation development, implantation and its receptor expression in mouse embryos. *J Obstet Gynaecol Res* 25:87-93.
362. Lim JJ, DR Lee, HS Song, KS Kim, TK Yoon, MC Gye and MK Kim. (2006). Heparin-binding epidermal growth factor (HB-EGF) may improve embryonic development and implantation by increasing vitronectin receptor (integrin α 5 β 3) expression in peri-implantation mouse embryos. *J Assist Reprod Genet* 23:111-9.
363. Behr B, S Mooney, Y Wen, ML Polan and H Wang. (2005). Preliminary experience with low concentration of granulocyte-macrophage colony-stimulating factor: a potential regulator in preimplantation mouse embryo development and apoptosis. *J Assist Reprod Genet* 22:25-32.
364. Lavranos TC, PD Rathjen and RF Seamark. (1995). Trophic effects of myeloid leukaemia inhibitory factor (LIF) on mouse embryos. *J Reprod Fertil* 105:331-8.
365. Robertson SA, C Sjoblom, MJ Jasper, RJ Norman and RF Seamark. (2001). Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos. *Biol Reprod* 64:1206-15.
366. Kilburn BA, PJ Chiang, J Wang, GR Flentke, SM Smith and DR Armant. (2006). Rapid induction of apoptosis in gastrulating mouse embryos by ethanol and its prevention by HB-EGF. *Alcohol Clin Exp Res* 30:127-34.
367. Dardik A and RM Schultz. (1991). Blastocoel expansion in the preimplantation mouse embryo: stimulatory effect of TGF- α and EGF. *Development* 113:919-30.
368. Papayannis M, V Eyheremendy, C Sanjurjo, J Blaquier and FG Raffo. (2007). Effect of granulocyte-macrophage colony stimulating factor on growth, resistance to freezing and thawing and re-expansion of murine blastocysts. *Reprod Biomed Online* 14:96-101.
369. Karagenc L, M Lane and DK Gardner. (2005). Granulocyte-macrophage colony-stimulating factor stimulates mouse blastocyst inner cell mass development only when media lack human serum albumin. *Reprod Biomed Online* 10:511-8.
370. Pollard JW. (1997). Role of colony-stimulating factor-1 in reproduction and development. *Mol Reprod Dev* 46:54-60; discussion 60-1.
371. Sjoblom C, M Wikland and SA Robertson. (2002). Granulocyte-macrophage colony-stimulating factor (GM-CSF) acts independently of the beta common subunit of the GM-CSF receptor to prevent inner cell mass apoptosis in human embryos. *Biol Reprod* 67:1817-23.
372. Nowak RA, F Haimovici, JD Biggers and GT Erbach. (1999). Transforming growth factor-beta stimulates mouse blastocyst outgrowth through a mechanism involving parathyroid hormone-related protein. *Biol Reprod* 60:85-93.
373. Sheikholislami B, M Salehnia, MR Valojerdi and M Ramezanzadeh. (2008). Developmental potential of isolated blastomeres from early mouse embryos in the presence and absence of LIF and GM-CSF. *J Assist Reprod Genet* 25:7-12.
374. Chen S, Y Cao, G Zeng and E Duan. (2001). Transforming growth factor- α promotes mouse blastocyst outgrowth and secretion of matrix metalloproteinases. *Chin Med J (Engl)* 114:1300-4.

375. Glabowski W, R Kurzawa, B Wiszniewska, T Baczkowski, M Marchlewicz and P Brelik. (2005). Growth factors effects on preimplantation development of mouse embryos exposed to tumor necrosis factor alpha. *Reprod Biol* 5:83-99.
376. Desai N, M Scarrow, J Lawson, D Kinzer and J Goldfarb. (1999). Evaluation of the effect of interleukin-6 and human extracellular matrix on embryonic development. *Hum Reprod* 14:1588-92.
377. Pantaleon M, H Jericho, G Rabnott and PL Kaye. (2003). The role of insulin-like growth factor II and its receptor in mouse preimplantation development. *Reprod Fertil Dev* 15:37-45.
378. Kim DH, BC Yang, SK Lee, SB Park and WK Chang. (2004). The effect of fibroblast growth factor-4 on the development of nuclear transfer bovine embryos. *Reproduction, fertility and development* 16.
379. Stoddart NR, AE Wild and TP Fleming. (1996). Stimulation of development in vitro by platelet-activating factor receptor ligands released by mouse preimplantation embryos. *J Reprod Fertil* 108:47-53.
380. Byrne AT, J Southgate, DR Brison and HJ Leese. (2002). Effects of insulin-like growth factors I and II on tumour-necrosis-factor-alpha-induced apoptosis in early murine embryos. *Reprod Fertil Dev* 14:79-83.
381. Desai N, N Kattal, FF AbdelHafez, J Szeptycki-Lawson and J Goldfarb. (2007). Granulocyte-macrophage colony stimulating factor (GM-CSF) and co-culture can affect post-thaw development and apoptosis in cryopreserved embryos. *J Assist Reprod Genet* 24:215-22.
382. Harvey MB and PL Kaye. (1991). Mouse blastocysts respond metabolically to short-term stimulation by insulin and IGF-1 through the insulin receptor. *Mol Reprod Dev* 29:253-8.
383. Ryan JP, C O'Neill and RG Wales. (1990). Oxidative metabolism of energy substrates by preimplantation mouse embryos in the presence of platelet-activating factor. *J Reprod Fertil* 89:301-7.
384. Harvey MB and PL Kaye. (1990). Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts in vitro. *Development* 110:963-7.
385. Kurzawa R, W Glabowski, T Baczkowski and P Brelik. (2002). Evaluation of mouse preimplantation embryos exposed to oxidative stress cultured with insulin-like growth factor I and II, epidermal growth factor, insulin, transferrin and selenium. *Reprod Biol* 2:143-62.
386. Ryan JP, C O'Neill, AJ Ammit and CG Roberts. (1992). Metabolic and developmental responses of preimplantation embryos to platelet activating factor (PAF). *Reprod Fertil Dev* 4:387-98.
387. Glabowski W. (2005). [The protective effect of stem cell factor (SCF) on in vitro development of preimplantation mouse embryos]. *Ann Acad Med Stetin* 51:83-93.
388. Shao WJ, LY Tao, JY Xie, C Gao, JH Hu and RQ Zhao. (2007). Exposure of preimplantation embryos to insulin alters expression of imprinted genes. *Comp Med* 57:482-6.
389. Diaz-Cueto L and GL Gerton. (2001). The influence of growth factors on the development of preimplantation mammalian embryos. *Arch Med Res* 32:619-26.
390. Roudebush WE, JB Massey, HI Kort, CW Elsner, AA Toledo, D Mitchell-Leef and DB Shapiro. (2004). Exposure of preimplantation embryos to platelet-activating factor increases birth rate. *J Assist Reprod Genet* 21:297-300.
391. Lane M and DK Gardner. (1992). Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. *Hum Reprod* 7:558-62.

392. Salahuddin S, S Ookutsu, K Goto, Y Nakanishi and Y Nagata. (1995). Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of in-vitro fertilized mouse embryos. *Hum Reprod* 10:2382-5.
393. O'Neill C. (1997). Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. *Biol Reprod* 56:229-37.
394. O'Neill C. (1998). Autocrine mediators are required to act on the embryo by the 2-cell stage to promote normal development and survival of mouse preimplantation embryos in vitro. *Biol Reprod* 58:1303-9.
395. Heyner S, LV Rao, L Jarett and RM Smith. (1989). Preimplantation mouse embryos internalize maternal insulin via receptor-mediated endocytosis: pattern of uptake and functional correlations. *Dev Biol* 134:48-58.
396. Chen HF, JY Shew, HN Ho, WL Hsu and YS Yang. (1999). Expression of leukemia inhibitory factor and its receptor in preimplantation embryos. *Fertil Steril* 72:713-9.
397. Mayshar Y, E Rom, I Chumakov, A Kronman, A Yaron and N Benvenisty. (2008). Fibroblast growth factor 4 and its novel splice isoform have opposing effects on the maintenance of human embryonic stem cell self-renewal. *Stem Cells* 26:767-74.
398. Harvey MB, MY Arcellana-Panlilio, X Zhang, GA Schultz and AJ Watson. (1995). Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo coculture. *Biol Reprod* 53:532-40.
399. Augustin R, P Pocar, C Wrenzycki, H Niemann and B Fischer. (2003). Mitogenic and anti-apoptotic activity of insulin on bovine embryos produced in vitro. *Reproduction* 126:91-9.
400. Lee MS, SK Kang, BC Lee and WS Hwang. (2005). The beneficial effects of insulin and metformin on in vitro developmental potential of porcine oocytes and embryos. *Biol Reprod* 73:1264-8.
401. Saltiel AR and CR Kahn. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799-806.
402. Nissley SP, JF Haskell, N Sasaki, MA De Vroede and MM Rechler. (1985). Insulin-like growth factor receptors. *J Cell Sci Suppl* 3:39-51.
403. Accili D, J Drago, EJ Lee, MD Johnson, MH Cool, P Salvatore, LD Asico, PA Jose, SI Taylor and H Westphal. (1996). Early neonatal death in mice homozygous for a null allele of the insulin receptor gene. *Nat Genet* 12:106-9.
404. Joshi RL, B Lamothe, N Cordonnier, K Mesbah, E Monthieux, J Jami and D Bucchini. (1996). Targeted disruption of the insulin receptor gene in the mouse results in neonatal lethality. *Embo J* 15:1542-7.
405. Chan SJ and DF Steiner. (2000). Insulin through the ages: Phylogeny of a growth promoting and metabolic regulatory hormone. *American Zoologist* 40:213-222.
406. Hernandez-Sanchez C, A Mansilla, F de Pablo and R Zardoya. (2008). Evolution of the insulin receptor family and receptor isoform expression in vertebrates. *Mol Biol Evol* 25:1043-53.
407. Lopez AF, MF Shannon, T Hercus, NA Nicola, B Cambareri, M Dottore, MJ Layton, L Eglinton and MA Vadas. (1992). Residue 21 of human granulocyte-macrophage colony-stimulating factor is critical for biological activity and for high but not low affinity binding. *Embo J* 11:909-16.
408. Sjoblom C, M Wikland and SA Robertson. (1999). Granulocyte-macrophage colony-stimulating factor promotes human blastocyst development in vitro. *Hum Reprod* 14:3069-76.
409. Robertson SA, CT Roberts, KL Farr, AR Dunn and RF Seamark. (1999). Fertility impairment in granulocyte-macrophage colony-stimulating factor-deficient mice. *Biol Reprod* 60:251-61.

410. Stanley E, GJ Lieschke, D Grail, D Metcalf, G Hodgson, JA Gall, DW Maher, J Cebon, V Sinickas and AR Dunn. (1994). Granulocyte/macrophage colony-stimulating factor-deficient mice show no major perturbation of hematopoiesis but develop a characteristic pulmonary pathology. *Proc Natl Acad Sci U S A* 91:5592-6.
411. Menino ARJ, JS Williams, CS Gardiner and TL John. (1989). Mouse embryo development in media containing growth factors. *Animal reproductive science* 18.
412. Feldman B, W Poueymirou, VE Papaioannou, TM DeChiara and M Goldfarb. (1995). Requirement of FGF-4 for postimplantation mouse development. *Science* 267:246-9.
413. Rappolee DA, C Basilico, Y Patel and Z Werb. (1994). Expression and function of FGF-4 in peri-implantation development in mouse embryos. *Development* 120:2259-69.
414. Arman E, R Haffner-Krausz, Y Chen, JK Heath and P Lonai. (1998). Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. *Proc Natl Acad Sci U S A* 95:5082-7.
415. Greber B, H Lehrach and J Adjaye. (2007). Silencing of core transcription factors in human EC cells highlights the importance of autocrine FGF signaling for self-renewal. *BMC Dev Biol* 7:46.
416. Yamanaka Y, F Lanner and J Rossant. (2010). FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. *Development* 137:715-24.
417. Stewart CL, P Kaspar, LJ Brunet, H Bhatt, I Gadi, F Kontgen and SJ Abbondanzo. (1992). Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. *Nature* 359:76-9.
418. Mitchell MH, RJ Swanson and S Oehninger. (2002). In vivo effect of leukemia inhibitory factor (LIF) and an anti-LIF polyclonal antibody on murine embryo and fetal development following exposure at the time of transcervical blastocyst transfer. *Biol Reprod* 67:460-4.
419. Suzuki S, T Yamashita, K Tanaka, H Hattori, K Sawamoto, H Okano and N Suzuki. (2005). Activation of cytokine signaling through leukemia inhibitory factor receptor (LIFR)/gp130 attenuates ischemic brain injury in rats. *J Cereb Blood Flow Metab* 25:685-93.
420. Dameron L, SL Opitz, H Zaehres, MW Lensch, PW Andrews, J Itskovitz-Eldor and GQ Daley. (2004). LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. *Stem Cells* 22:770-8.
421. Sumi T, Y Fujimoto, N Nakatsuji and H Suemori. (2004). STAT3 is dispensable for maintenance of self-renewal in nonhuman primate embryonic stem cells. *Stem Cells* 22:861-72.
422. Nichols J, I Chambers, T Taga and A Smith. (2001). Physiological rationale for responsiveness of mouse embryonic stem cells to gp130 cytokines. *Development* 128:2333-9.
423. Keskinetepe L, K Norris, G Pacholczyk, SM Dederscheck and A Eroglu. (2007). Derivation and comparison of C57BL/6 embryonic stem cells to a widely used 129 embryonic stem cell line. *Transgenic Res* 16:751-8.
424. Stevens LC and CC Little. (1954). Spontaneous Testicular Teratomas in an Inbred Strain of Mice. *Proc Natl Acad Sci U S A* 40:1080-7.
425. Malgieri A, G Novelli and F Sangiuolo. (2011). Potential clinical applications of embryonic stem cells. In: *Embryonic Stem Cells - Recent Advances in Pluripotent Stem Cell-Based Regenerative Medicine*. InTech. p 21-48.
426. Taylor CJ, EM Bolton, S Pocock, LD Sharples, RA Pedersen and JA Bradley. (2005). Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. *Lancet* 366:2019-25.
427. Lane M. (2001). Mechanisms for managing cellular and homeostatic stress in vitro. *Theriogenology* 55:225-36.

428. Nichols J, J Silva, M Roode and A Smith. (2009). Suppression of Erk signalling promotes ground state pluripotency in the mouse embryo. *Development* 136:3215-22.
429. Mitchell M, KS Cashman, DK Gardner, JG Thompson and M Lane. (2009). Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod* 80:295-301.
430. Pool TB. (2002). Recent advances in the production of viable human embryos in vitro. *Reprod Biomed Online* 4:294-302.
431. Vassiliev I, S Vassilieva, LF Beebe, SJ Harrison, SM McIlpatrick and MB Nottle. (2010). In vitro and in vivo characterization of putative porcine embryonic stem cells. *Cell Reprogram* 12:223-30.
432. Nolan T, RE Hands and SA Bustin. (2006). Quantification of mRNA using real-time RT-PCR. *Nat Protoc* 1:1559-82.
433. Nelson TJ, RS Faustino, A Chiriack, R Crespo-Diaz, A Behfar and A Terzic. (2008). CXCR4+/FLK-1+ biomarkers select a cardiopoietic lineage from embryonic stem cells. *Stem Cells* 26:1464-73.
434. Yasunaga M, S Tada, S Torikai-Nishikawa, Y Nakano, M Okada, LM Jakt, S Nishikawa, T Chiba and T Era. (2005). Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. *Nat Biotechnol* 23:1542-50.
435. Ying QL, M Stavridis, D Griffiths, M Li and A Smith. (2003). Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotechnol* 21:183-6.
436. Bongso A, CY Fong, SC Ng and S Ratnam. (1994). Isolation and culture of inner cell mass cells from human blastocysts. *Hum Reprod* 9:2110-7.
437. Mantel C, Y Guo, MR Lee, MK Kim, MK Han, H Shibayama, S Fukuda, MC Yoder, LM Pelus, KS Kim and HE Broxmeyer. (2007). Checkpoint-apoptosis uncoupling in human and mouse embryonic stem cells: a source of karyotypic instability. *Blood* 109:4518-27.
438. Rippon HJ and AE Bishop. (2004). Embryonic stem cells. *Cell Prolif* 37:23-34.
439. Bongso A, CY Fong and K Gauthaman. (2008). Taking stem cells to the clinic: Major challenges. *J Cell Biochem* 105:1352-60.
440. Spits C, I Mateizel, M Geens, A Mertzaniidou, C Staessen, Y Vandesselde, J Van der Elst, I Liebaers and K Sermon. (2008). Recurrent chromosomal abnormalities in human embryonic stem cells. *Nat Biotechnol* 26:1361-3.
441. Kovacic B and V Vlaisavljevic. (2008). Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts in vitro: a prospective study on sibling oocytes. *Reprod Biomed Online* 17:229-36.
442. Silva J, J Nichols, TW Theunissen, G Guo, AL van Oosten, O Barrandon, J Wray, S Yamanaka, I Chambers and A Smith. (2009). Nanog is the gateway to the pluripotent ground state. *Cell* 138:722-37.
443. De La Fuente R, MM Viveiros, K Wigglesworth and JJ Eppig. (2004). ATRX, a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes. *Dev Biol* 272:1-14.
444. Yan Q, J Huang, T Fan, H Zhu and K Muegge. (2003). Lsh, a modulator of CpG methylation, is crucial for normal histone methylation. *Embo J* 22:5154-62.
445. Chau CM, MJ Evans and RC Scarpulla. (1992). Nuclear respiratory factor 1 activation sites in genes encoding the gamma-subunit of ATP synthase, eukaryotic initiation factor 2 alpha, and tyrosine aminotransferase. Specific interaction of purified NRF-1 with multiple target genes. *J Biol Chem* 267:6999-7006.
446. Virbasius CA, JV Virbasius and RC Scarpulla. (1993). NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. *Genes Dev* 7:2431-45.

447. Kelly DP and RC Scarpulla. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* 18:357-68.
448. Luo J, AY Nikolaev, S Imai, D Chen, F Su, A Shiloh, L Guarente and W Gu. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 107:137-48.
449. Motta MC, N Divecha, M Lemieux, C Kamel, D Chen, W Gu, Y Bultsma, M McBurney and L Guarente. (2004). Mammalian SIRT1 represses forkhead transcription factors. *Cell* 116:551-63.
450. Van Langendonck A, D Demylle, C Wyns, M Nisolle and J Donnez. (2001). Comparison of G1.2/G2.2 and Sydney IVF cleavage/blastocyst sequential media for the culture of human embryos: a prospective, randomized, comparative study. *Fertil Steril* 76:1023-31.
451. Pool TB. (2005). An update on embryo culture for human assisted reproductive technology: media, performance, and safety. *Semin Reprod Med* 23:309-18.
452. Reed L, M Lane and G DK. (2003). In vivo rates of mouse embryo development can be obtained in vitro. *Theriogenology* 59:349.
453. Reed ML, M Lane, DK Gardner, NL Jensen and J Thompson. (2002). Vitrification of human blastocysts using the cryoloop method: successful clinical application and birth of offspring. *J Assist Reprod Genet* 19:304-6.
454. Lane M and DK Gardner. (2005). Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev* 17:371-8.
455. Campbell JM, M Mitchell, M Nottle and M Lane. (2010). Development of a mouse model for studying the effect of embryo culture on embryonic stem cell derivation. *Stem Cells Dev*.
456. Babcock DF, J Herrington, PC Goodwin, YB Park and B Hille. (1997). Mitochondrial participation in the intracellular Ca²⁺ network. *J Cell Biol* 136:833-44.
457. Cho YM, S Kwon, YK Pak, HW Seol, YM Choi, J Park do, KS Park and HK Lee. (2006). Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. *Biochem Biophys Res Commun* 348:1472-8.
458. Bongso A, M Richards and B Fong. (2005). From Human Embryos to Clinically Compliant Embryonic Stem Cells: Blastocyst Culture, Xeno-free Derivation and Cryopreservation, Properties and Applications of Embryonic Stem Cell Lines. In: *Stem cells: from bench to bedside*. World Scientific Publishing Co. Pte. Ltd., Singapore. p 14-38.
459. Zander DL, JG Thompson and M Lane. (2006). Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod* 74:288-94.
460. Weaver JR, M Susiarjo and MS Bartolomei. (2009). Imprinting and epigenetic changes in the early embryo. *Mamm Genome* 20:532-43.
461. Gardner HG and PL Kaye. (1991). Insulin increases cell numbers and morphological development in mouse pre-implantation embryos in vitro. *Reprod Fertil Dev* 3:79-91.
462. Harvey MB and PL Kaye. (1988). Insulin stimulates protein synthesis in compacted mouse embryos. *Endocrinology* 122:1182-4.
463. Dunglison GF and PL Kaye. (1993). Insulin regulates protein metabolism in mouse blastocysts. *Mol Reprod Dev* 36:42-8.
464. Onat A, G Hergenc, D Dursunoglu, Z Kucukdurmaz, S Bulur and G Can. (2008). Relatively high levels of serum adiponectin in obese women, a potential indicator of anti-inflammatory dysfunction: relation to sex hormone-binding globulin. *Int J Biol Sci* 4:208-14.

465. Ying QL, J Wray, J Nichols, L Batlle-Morera, B Doble, J Woodgett, P Cohen and A Smith. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453:519-23.
466. Davies SP, H Reddy, M Caivano and P Cohen. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 351:95-105.
467. Bain J, L Plater, M Elliott, N Shpiro, CJ Hastie, H McLauchlan, I Klevernic, JS Arthur, DR Alessi and P Cohen. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochem J* 408:297-315.
468. Campbell JM, M Mitchell, MB Nottle and M Lane. (2011). Development of a mouse model for studying the effect of embryo culture on embryonic stem cell derivation. *Stem Cells Dev* 20:1577-86.
469. Chi MM, AL Schlein and KH Moley. (2000). High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* 141:4784-92.
470. Kosmakos FC and J Roth. (1980). Insulin-induced loss of the insulin receptor in IM-9 lymphocytes. A biological process mediated through the insulin receptor. *J Biol Chem* 255:9860-9.
471. Pinto AB, AL Schlein and KH Moley. (2002). Preimplantation exposure to high insulin-like growth factor I concentrations results in increased resorption rates in vivo. *Hum Reprod* 17:457-62.
472. Ogawa W, T Matozaki and M Kasuga. (1998). Role of binding proteins to IRS-1 in insulin signalling. *Mol Cell Biochem* 182:13-22.
473. White MF. (1998). The IRS-signalling system: a network of docking proteins that mediate insulin action. *Mol Cell Biochem* 182:3-11.
474. Riley JK, MO Carayannopoulos, AH Wyman, M Chi, CK Ratajczak and KH Moley. (2005). The PI3K/Akt pathway is present and functional in the preimplantation mouse embryo. *Dev Biol* 284:377-86.
475. Scheid MP and JR Woodgett. (2003). Unravelling the activation mechanisms of protein kinase B/Akt. *FEBS Lett* 546:108-12.
476. Cross DA, DR Alessi, P Cohen, M Andjelkovich and BA Hemmings. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785-9.
477. Umehara H, T Kimura, S Ohtsuka, T Nakamura, K Kitajima, M Ikawa, M Okabe, H Niwa and T Nakano. (2007). Efficient derivation of embryonic stem cells by inhibition of glycogen synthase kinase-3. *Stem Cells* 25:2705-11.
478. Sineva GS and VA Pospelov. (2010). Inhibition of GSK3beta enhances both adhesive and signaling activities of beta-catenin in mouse embryonic stem cells. *Biol Cell*.
479. He X, M Semenov, K Tamai and X Zeng. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* 131:1663-77.
480. Po A, E Ferretti, E Miele, E De Smaele, A Paganelli, G Canettieri, S Coni, L Di Marcotullio, M Biffoni, L Massimi, C Di Rocco, I Screpanti and A Gulino. (2010). Hedgehog controls neural stem cells through p53-independent regulation of Nanog. *Embo J* 29:2646-58.
481. Jia J, K Amanai, G Wang, J Tang, B Wang and J Jiang. (2002). Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. *Nature* 416:548-52.
482. Cartwright P, C McLean, A Sheppard, D Rivett, K Jones and S Dalton. (2005). LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* 132:885-96.
483. Gregory MA, Y Qi and SR Hann. (2003). Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. *J Biol Chem* 278:51606-12.
484. Foltz DR, MC Santiago, BE Berechid and JS Nye. (2002). Glycogen synthase kinase-3beta modulates notch signaling and stability. *Curr Biol* 12:1006-11.

485. Lowell S, A Benchoua, B Heavey and AG Smith. (2006). Notch promotes neural lineage entry by pluripotent embryonic stem cells. *PLoS Biol* 4:e121.
486. Mayo LD, JE Dixon, DL Durden, NK Tonks and DB Donner. (2002). PTEN protects p53 from Mdm2 and sensitizes cancer cells to chemotherapy. *J Biol Chem* 277:5484-9.
487. Gottlieb TM, JF Leal, R Seger, Y Taya and M Oren. (2002). Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis. *Oncogene* 21:1299-303.
488. Ogawara Y, S Kishishita, T Obata, Y Isazawa, T Suzuki, K Tanaka, N Masuyama and Y Gotoh. (2002). Akt enhances Mdm2-mediated ubiquitination and degradation of p53. *J Biol Chem* 277:21843-50.
489. Yu ZK, RK Geyer and CG Maki. (2000). MDM2-dependent ubiquitination of nuclear and cytoplasmic P53. *Oncogene* 19:5892-7.
490. Sabbatini P and F McCormick. (1999). Phosphoinositide 3-OH kinase (PI3K) and PKB/Akt delay the onset of p53-mediated, transcriptionally dependent apoptosis. *J Biol Chem* 274:24263-9.
491. Turenne GA and BD Price. (2001). Glycogen synthase kinase3 beta phosphorylates serine 33 of p53 and activates p53's transcriptional activity. *BMC Cell Biol* 2:12.
492. Watcharasit P, GN Bijur, L Song, J Zhu, X Chen and RS Jope. (2003). Glycogen synthase kinase-3beta (GSK3beta) binds to and promotes the actions of p53. *J Biol Chem* 278:48872-9.
493. Watcharasit P, GN Bijur, JW Zmijewski, L Song, A Zmijewska, X Chen, GV Johnson and RS Jope. (2002). Direct, activating interaction between glycogen synthase kinase-3beta and p53 after DNA damage. *Proc Natl Acad Sci U S A* 99:7951-5.
494. Kulikov R, KA Boehme and C Blattner. (2005). Glycogen synthase kinase 3-dependent phosphorylation of Mdm2 regulates p53 abundance. *Mol Cell Biol* 25:7170-80.
495. Ghosh JC and DC Altieri. (2005). Activation of p53-dependent apoptosis by acute ablation of glycogen synthase kinase-3beta in colorectal cancer cells. *Clin Cancer Res* 11:4580-8.
496. Mayo LD and DB Donner. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* 98:11598-603.
497. Ding VW, RH Chen and F McCormick. (2000). Differential regulation of glycogen synthase kinase 3beta by insulin and Wnt signaling. *J Biol Chem* 275:32475-81.
498. Gross VS, M Hess and GM Cooper. (2005). Mouse embryonic stem cells and preimplantation embryos require signaling through the phosphatidylinositol 3-kinase pathway to suppress apoptosis. *Mol Reprod Dev* 70:324-32.
499. Riley JK, MO Carayannopoulos, AH Wyman, M Chi and KH Moley. (2006). Phosphatidylinositol 3-kinase activity is critical for glucose metabolism and embryo survival in murine blastocysts. *J Biol Chem* 281:6010-9.
500. Paling NR, H Wheadon, HK Bone and MJ Welham. (2004). Regulation of embryonic stem cell self-renewal by phosphoinositide 3-kinase-dependent signaling. *J Biol Chem* 279:48063-70.
501. Vlahos CJ, WF Matter, KY Hui and RF Brown. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* 269:5241-8.
502. Yan W and HH Tai. (2006). Glycogen synthase kinase-3 phosphorylation, T-cell factor signaling activation, and cell morphology change following stimulation of thromboxane receptor alpha. *J Pharmacol Exp Ther* 317:267-74.
503. Fujino H, KA West and JW Regan. (2002). Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP2 and EP4 prostanoid receptors by prostaglandin E2. *J Biol Chem* 277:2614-9.

504. Jensen J, EO Brennesvik, YC Lai and PR Shepherd. (2007). GSK-3 β regulation in skeletal muscles by adrenaline and insulin: evidence that PKA and PKB regulate different pools of GSK-3. *Cell Signal* 19:204-10.
505. Bragado MJ, IM Aparicio, MC Gil and LJ Garcia-Marin. (2010). Protein kinases A and C and phosphatidylinositol 3 kinase regulate glycogen synthase kinase-3A serine 21 phosphorylation in boar spermatozoa. *J Cell Biochem* 109:65-73.
506. Ku BM, YK Lee, JY Jeong, J Ryu, J Choi, JS Kim, YW Cho, GS Roh, HJ Kim, GJ Cho, WS Choi and SS Kang. (2011). Caffeine inhibits cell proliferation and regulates PKA/GSK3 β pathways in U87MG human glioma cells. *Mol Cells* 31:275-9.
507. Aitken A, T Bilham and P Cohen. (1982). Complete primary structure of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *Eur J Biochem* 126:235-46.
508. Endo S, X Zhou, J Connor, B Wang and S Shenolikar. (1996). Multiple structural elements define the specificity of recombinant human inhibitor-1 as a protein phosphatase-1 inhibitor. *Biochemistry* 35:5220-8.
509. Zhang F, CJ Phiel, L Spece, N Gurvich and PS Klein. (2003). Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium. Evidence for autoregulation of GSK-3. *J Biol Chem* 278:33067-77.
510. Aparicio IM, M Garcia-Herreros, T Fair and P Lonergan. (2010). Identification and regulation of glycogen synthase kinase-3 during bovine embryo development. *Reproduction* 140:83-92.
511. Mitchell M, A Georgiou, K Cashman, A Stump and M Lane. (2008). Do Sirtuin Proteins Function in the Developing Mammalian Embryo? . *Biology of Reproduction* 78:134.
512. Jin XL, V Chandrakanthan, HD Morgan and C O'Neill. (2009). Preimplantation embryo development in the mouse requires the latency of TRP53 expression, which is induced by a ligand-activated PI3 kinase/AKT/MDM2-mediated signaling pathway. *Biol Reprod* 80:286-94.
513. Mitchell M, A Georgio, K Kashman, A Stump and M Lane. (2008). Do Sirtuin Proteins Function in the Developing Mammalian Embryo? *Biology of Reproduction* 78:134.
514. Audrito V, T Vaisitti, D Rossi, D Gottardi, G D'Arena, L Laurenti, G Gaidano, F Malavasi and S Deaglio. (2011). Nicotinamide blocks proliferation and induces apoptosis of chronic lymphocytic leukemia cells through activation of the p53/miR-34a/SIRT1 tumor suppressor network. *Cancer Res* 71:4473-83.
515. Komarov PG, EA Komarova, RV Kondratov, K Christov-Tselkov, JS Coon, MV Chernov and AV Gudkov. (1999). A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 285:1733-7.
516. Hoagland MS, EM Hoagland and HI Swanson. (2005). The p53 inhibitor pifithrin- α is a potent agonist of the aryl hydrocarbon receptor. *J Pharmacol Exp Ther* 314:603-10.
517. Schafer T, C Scheuer, K Roemer, MD Menger and B Vollmar. (2003). Inhibition of p53 protects liver tissue against endotoxin-induced apoptotic and necrotic cell death. *FASEB J* 17:660-7.
518. Li J, JV Zhang, YJ Cao, JX Zhou, WM Liu, XJ Fan and EK Duan. (2005). Inhibition of the beta-catenin signaling pathway in blastocyst and uterus during the window of implantation in mice. *Biol Reprod* 72:700-6.
519. Acevedo N, X Wang, RL Dunn and GD Smith. (2007). Glycogen synthase kinase-3 regulation of chromatin segregation and cytokinesis in mouse preimplantation embryos. *Mol Reprod Dev* 74:178-88.
520. Doble BW and JR Woodgett. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 116:1175-86.
521. Jope RS and GV Johnson. (2004). The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 29:95-102.

522. Qin H, T Yu, T Qing, Y Liu, Y Zhao, J Cai, J Li, Z Song, X Qu, P Zhou, J Wu, M Ding and H Deng. (2007). Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. *J Biol Chem* 282:5842-52.
523. Li A, V Chandrakanthan, O Chami and C O'Neill. (2007). Culture of zygotes increases TRP53 [corrected] expression in B6 mouse embryos, which reduces embryo viability. *Biol Reprod* 76:362-7.
524. Ferguson CJ. (2009). An Effect Size Primer: A Guide for Clinicians and Researchers. *Professional Psychology: Research and Practice* 40:532-538.
525. O'Leary T, B Heindryckx, S Lierman, M Van der Jeught, B Menten, D Deforce, R Cornelissen, SC de Sousa Lopes and P De Sutter. (2011). The influence of early embryo traits on human embryonic stem cell derivation efficiency. *Stem Cells Dev* 20:785-93.
526. Harlow GM and P Quinn. (1982). Development of preimplantation mouse embryos in vivo and in vitro. *Aust J Biol Sci* 35:187-93.
527. Greenblutt E, T Di Berardino, P Chronis-Brown, D Holt and A Lains. (2005). Comparison of Global Medium and G1/G2 cleavage/blastocyst sequential media for culture of human embryos after IVF. In: *ESHRE*, Copenhagen.
528. Xella S, T Marsella, D Tagliasacchi, S Giulini, A La Marca, A Tirelli and A Volpe. (2010). Embryo quality and implantation rate in two different culture media: ISM1 versus Universal IVF Medium. *Fertil Steril* 93:1859-63.
529. Baltz JM. (2012). Media composition: salts and osmolality. *Methods Mol Biol* 912:61-80.