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

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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 where 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 predicative 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

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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

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

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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