Deriving Bovine Embryonic Stem-Like Cells in Defined Conditions

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Abstract

The first embryonic stem ES cell line was isolated from mouse in 1981 (Evans and Kaufman 1981; Martin1981). However, ESCs are only available in rodent species. Although research has been carried out on bovine embryos for more than two decades, there is no evidence of ESCs (Shanbo, Fang et al. 2009). Reasons for this are unknown developmental aspects associated with bovine embryology.

Most attempts to culture bovine ES-like cells have been to isolate them from the ICM of day seven blastocysts, which have been generated by *in vitro* production (IVP). Here I report an alternative method to derive bES-like cells using dissociated blastomeres from early stages embryos (8 and 16-cell) and day 5 and 7 embryos. These bovine outgrowths were cultured in order to investigate which developmental stage associated with better blastomere attachment. Also, to examine their pluripotency, as it was hypothesised that early stages of development up to the blastocyst stage are pluripotent and that the naive state of bovine ESCs exist. In addition, instead of using animal material as feeder layers such as mouse embryonic fibroblasts (MEFs), which provides leukemic inhibitory factor (LIF) to maintain pluripotency, defined extra cellular matrixes (ECM) were used in order to derive bES-like cells in defined conditions, avoid contamination by reagents used in cell culture and to find the substrate associated with better attachment and proliferation.

2i media was applied to the development of bovine embryos in order to determine if the use of 2i media with different numbers of blastomeres would encourage bES-like cell development, proliferation and to introduce cell uniformity. Blastomeres were cultured singularly, and in groups in 96 well plates and in tissue culture plates in order to produce short term cell lines. Development rate, attachment and outgrowth production were measured, for example, the proportion of blastomeres attached and proliferated. Markers of ESCs, epiblast stem cells (epiESCs) and trophectoderm were investigated in produced bovine outgrowths in order to examine their pluripotency, and their karyotyping was examined.

In this study, from the different developmental stages used, outgrowths were produced from all stages used, but the inner cell mass (ICM) was associated with better outgrowth production. Different ECMs promoted attachment, however, with variable efficiency, in which gelatin was associated with better attachment and proliferation. The use of 2i media resulted in attachment, but with poor proliferation. cDNA isolated from bovine outgrowths expressed some of pluripotency markers, but with a lack of uniformity. In addition, metaphase spreads from outgrowths showed an abnormal karyotype.

From this study, we increased our understanding of the factors that enhance the derivation of bES-like cells such as the culture media and the substrates. In addition, we have gained more information on bovine gene expression in blastomere derived outgrowths.

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Table of Contents

List of Tables

Table of Figures

List of Abbreviations

10-c	ten cells
16b	16 blastomeres
16-c	16 cells
1b	One blastomere
20b	20 blastomeres
2b	two blastomeres
2-c	two cells
2i	SU5402 & PD184352
4b	four blastomeres
4-c	four cells
8b	eight blastomeres
8-c	eight cells
B1-2	Blastocycts Grade 1-2
B1-3	Blastocycts Grade 1-3
B199	Bicarbonate-buffered M199
bES-like cells	BOVINE EMBRYONIC LIKE STEM CELLS
BMP	bone morphogenetic protein
BSA	Bovine Serum Albumin
bp	base pairs
CB	Cytochalasin B
cDNA	Complementry Dexyribose Nucleic Acid
COC	Cumulus-Oocyte Complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
Dnase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
EB	embryoid body
ECCs	embryonic carcinoma cells
ECM	extra cellular matrix
EDTA	Ethylene-diamine-tetra-acid
ESC	Embryonic stem cell
ESOF	Early Synthetic Oviduct Fluid
F12	Nutrient Mixture F-12
FAK	focal adhesion kinase
FCS	Fetal Calf Serum
FGF4	fibroblast growth factor 4
g	Relative Gravitation Force
GSCs	germ line stem cells
h	Hour

H199	Hepes-buffered M19
hESCs	Human Embryonic stem cells
HLA	human leukocyte antigen
HSOF	Hepes-buffered Synthetic Oviduct Fluid
ILK	integrin-liked kinase
iPSCs	induced pluripotent stem cells
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
LCMM	LightCycler Master Mix
LIF	leukemia inhibitry factor
LOS	large offspring syndrome
LSOF	Late Synthetic Fluid
M199	Medium 199
MEF	embryonic feeder cells
mESCs	MOUSE EMBRYONIC STEM CELLS
MOET	for multiple ovulation embryo transfer
mRNA	Messenger Ribonucleic Acid
MQ water	milli-Q (Millipore) filter-purified water
OPU	ovum pick up
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pES	parthenogenetic stem cells
PGCs	primordial germ cells
PGD	pre-implantation genetic diagnosis
PPHH	Pyruvate, Penicillamine, Hypotaurine, Heparin
PVA	Polyvinyl Alcohol
RNA	Ribonucleic Acid
Rnase	Ribonuclease
rpm	Revolutions per Minute
RT	room tempreture
SCNT	somatic cell nuclear transfer
SOF	Synthetic Oviduct Fluid
SSCs	Spermatogonia stem cells
STO	Ouabain-resistant mouse fibroblast
TE	Trophectoderm
U.V	Ultra Violet
V	Volts

Chapter One

Introduction and literature review

1.1 Introduction

The improvement in productive and reproductive traits in cattle requires advanced genetic technologies. Working on embryos in farm animals, especially dairy and beef cattle is necessary as is work on genetically modified animals in order to improve animals' traits to match the increasing demand for agricultural products worldwide and avoid the adult mortality that is associated with somatic cloning (Wells, Oback et al. 2003). In addition, farm animal can be a valuable source that can be used as research models for human biomedical research (Maherali and Hochedlinger 2008). For example, genetically modified organs and tissues can provide the solution for some human diseases (Keefer, Pant et al. 2007).

The first embryonic stem cells (ESCs) were isolated in the mouse over twenty five years ago (Evans and Kaufman 1981). Since that time, there has been a huge amount of research in order to generate these ESCs in other species including domestic animals due to their potential for genetic generation (Oback 2008). ESCs are classified depending on their ability to maintain an undifferentiated state, self renew, and with the ability to express pluripotency *in vitro* by the ability to give rise to all tissues and organs, and *in vivo* by teratoma formation and the contribution to the germ cells of a developing foetus and subsequently, a fertilizing adult (Keefer, Pant et al. 2007).

ESCs can be derived by placing the inner cell mass ICM of the blastocysts into *in vitro* culture (Evans and Kaufman 1981). However, there are a number of aspects that make it difficult to know whether the ICM cells are homogenously pluripotent and whether ESCs are formed by identical pluripotent ICM or other kind of cells that exist within the blastocyst (Oback 2008).

Most attempts to culture bovine ES like cells have been to isolate them from the ICM of day seven blastocysts. In cattle these have been generated by in vitro production (IVP) system, which is a process to get immature oocytes that are grown *in vitro* through reliable procedures of maturation, fertilization and culture to a stage that they are able to be transferred or frozen (Galli, Duchi et

al. 2003). The source of embryonic cell lines is believed to reside within the central ICM where pluripotent epiblast tissue is located (Nichols, Silva et al. 2009). The self renewal characteristic of ESCs depends on the activation of certain transcriptional regulatory networks, such as transcription factor STAT3, which is activated by cytokines to activate a variety of downstream genes (Ying, Wray et al. 2008). In the mouse, ESCs are maintained in culture by the cytokine; leukaemia inhibitory factor (LIF) to activate STAT3 and an inhibitor of differentiation: bone morphogenetic protein (BMP). However, ES cell differentiation occurs by the stimulation of the extracellular single regulated kinases 1 and 2 (ERK 1/2) pathway which is stimulated by fibroblast growth factor 4 (FGF4) (Ying, Wray et al. 2008). Results concluded by (Ying, Wray et al. 2008) found that self renewal was preserved by the blockage of the phosphor-ERK pathway by the use of a more chemically defined medium using the inhibitors (SU5402 & PD184352) (2i). The reason for that was because the effects of undefined growth factors and hormones are unknown. (Nichols, Silva et al. 2009) found that blockage of (Erk) and glycogen synthase kinase- 3 signalling from the 8 cell stage suppresses development of the hypoblast. Moreover, Nanog the epiblast specific marker and an important pluripotency gene was expressed and pluripotency was confirmed by contribution to chimaeras with germline transmission.

The use of 2i for efficient derivation of ESCs provoked the hypothesis that immortalising the pluripotent cells from developing embryos does not depend upon adaptation and selection in culture, but on the blocking of inductive differentiation pathways (Nichols, Silva et al. 2009). Therefore, I presume that cells with ESCs properties exist in the pre-implantation stage of development.

3i /2i media have been used in late stages to assist embryos development. In the present study I used 2i media instead of 3i media, as it was proven that they have similar effects (Nichols, Silva et al. 2009). Day 2, 3, 5 and 7 embryos were used, and blastomeres were disassociated and single and groups of blastomeres were loaded in a 96 well plate in order to produce short term cell lines that express pluripotency markers and have normal karyotyping.

To achieve the goal in the present study, literature review papers are covered in order to have a sufficient understanding of aspects underlying pluripotency in bovine derived ES-like cells. Papers related to *in vitro* production, bovine embryogenesis and gene expression, ESCs in human, mouse and bovine, deriving ESCs in defined conditions and deriving ESCs from single blastomere are presented. In addition a number of markers associated with pluripotency and early development are provided.

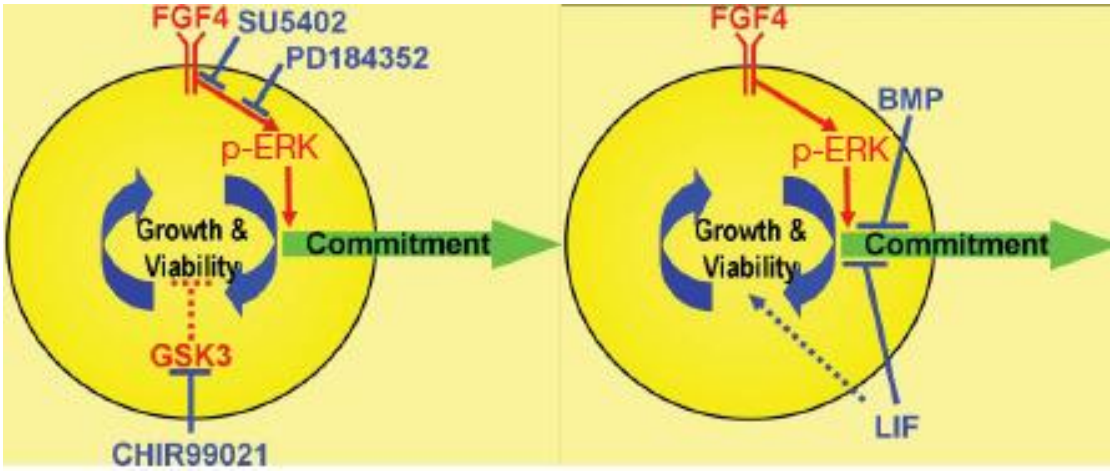


Figure (1.1): The pluripotency state when p-ERK signalling is inhibited upstream by the three inhibitors (CHIR 99021, PD184352 and SU5402) or downstream by LIF and BMP (Ying, Wray et al. 2008).

1.2 Stem cells

Stem cells have two properties, the capacity for unlimited self renewal and pluripotency. Theoretically, these cells can provide a supply of cells for transplantation. Totipotent stem cells have potentially greater utility; they are able to develop into all tissues and organs in the embryo and the extra embryonic tissues that constitute the placenta. Tissue specific stem cells, also known as adult stem cells, are available throughout the body, such as in the bone marrow, liver, skin and brain. These cells work as a lifetime maintenance system to repair damage that may occur during life (Wagers, A et al. 2002). However, these cells can be difficult to obtain and isolate, and when isolated have poor growth in the laboratory making them less useful for tissue

engineering (Vogel 2001). In these cases, pluripotent cells may be beneficial because they provide a more appropriate cell source. Because of their plasticity and potentially unlimited capacity for self renewal, ES therapies have been proposed for regenerative medicine and tissue replacement after injury or disease. In 1964, researchers isolated a single type of cell from a teratocarcinoma, a tumour now known to be derived from a germ cell (Kleinsmith and Pierce 1964). These cells isolated from the teratocarcinoma grew and replicated in cell culture as a stem cell and are now known as embryonic carcinoma cells (ECCs). ECCs can work as a model for early mouse development (Illmensee and Mintz 1976). However, ECCs are cancer derived and usually have an abnormal karyotype, therefore, they are not suitable for clinical applications (Rippon and Bishop 2004).

1.3 Pluripotent stem cells

Pluripotent stem cells can be derived from embryonic, foetal and adult sources. Embryonic carcinoma cells (ECCs) were the first type of pluripotent cells to be identified, firstly from mouse (Kleinsmith and Pierce 1964) and then from human (Andrews 1998). ECCs are pluripotent cells derived from germ cell tumours. Studies on these pluripotent stem cells have led to the derivation of other pluripotent stem cells including embryonic stem cells from blastocyst (ESCs), from in vitro primordial germ cells (PGCs), from adult germ cells, germ line stem cells (GSCs), and from unfertilized eggs, parthenogenetic, (pES). Additionally, the knowledge of the genetic markers diagnostic for ESCs has helped to induce pluripotent stem cells from differentiated adult cells, which are now known as induced pluripotent stem cells (iPSCs). All these different pluripotent cell lines have the general principle of pluripotent stem cells including unlimited proliferation and self renewal, and the ability to give rise to all the cells in the body from the three embryonic germ layers. The production of all cell types can be accomplished by a differentiation system to obtain an embryoid body (EB) or pluripotent cell injection into adult mice (teratoma) or by chimera formation (Daley, Lensch et al. 2009).

1.3.1 Embryonic germ cells (EGCs)

Primordial germ cells (PGCs) are the progenitor cells of the germ cell lineage, which produce gametes in the adult (Bendsen, Byskov et al. 2003). hPGCs are isolated from the foetal gonad about 5 to 10 weeks following gestation, to obtain hEGCs (Swelstad and Kerr 2010). During this time PGCs proliferate in order to undergo sexual differentiation into either an ovary or testis (Labosky and Hogan 2008). PGCs are considered to be unipotent because they are restricted to becoming germ cells and they do not exhibit self renewal (Dolci, Pesce et al. 1993). hEGCs can be derived using the transformed mouse embryonic fibroblast line, Sanadoz Thioganine and Ouabain-resistant mouse fibroblast (STO). Recently, some attempts were made to derive human and mouse EGCs using mouse embryonic feeder cells (MEF) (Dolci, Williams et al. 1991). The derivation of EGCs requires a number of factors, including Forskoline to increase derivation efficiency (Swelstad and Kerr 2010), LIF to maintain human and mouse EGCs pluripotency, stem cell factor for mouse PGCs proliferation and survival (Labosky, Barlow et al. 1994), and fibroblast growth factor (FGF) also for proliferation and survival. Normally, EGC colonies can be obtained within 2 to 3 weeks, with a low efficiency rate, between 10%- 20% (Swelstad and Kerr 2010). In comparison to other pluripotent stem cells, EGCs are considered to be difficult to derive and maintain which hampers the progress in research in this type of cells (Swelstad and Kerr 2010).

1.3.2 Germ-line stem cells (GSCs)

Stevens, 1962 derived teratocarcinomas from primordial germ cells (PGCs). Pluripotent stem cells can be obtained from male germ cells (Guan, Nayernia et al. 2006), which are known as germ line stem cells (GSCs). They were first isolated from mouse spermatogonia (Matsui, Zsebo et al. 1992), and then they were derived from male testicular GSCs biopsies in 2009 (Guan, Nayernia et

al. 2006). Spermatogonia stem cells (SSCs) are present since birth and they develop from PGCs by undergoing the differentiation process which leads to sperm development. GSCs from adult mice can contribute to teratoma and chimaera formation (Labosky, Barlow et al. 1994). Additionally, GSCs are similar to ESCs morphologically, and hGSCs are able to form teratoma (Conrad, Renninger et al. 2008), but a chimaera test cannot be performed using human cells. The derivation of GSCs depends on cell selection from testicular biopsies or spermatogonia and the utilization of cell culture substrates like laminin and collagen (Swelstad and Kerr 2010). GSCs have some characteristics of PGCs, such as genome wide demethylation, erasure of genomic imprints and reactivation of X chromosomes (Labosky, Barlow et al. 1994). The long history of studying SSCs contributes to the success in deriving GSCs (Dym, He et al. 2008). GSCs can provide an adult source for pluripotent stem cells which is easier to use for clinical applications. However, they can only be used in male patients (Swelstad and Kerr 2010).

1.3.3 Parthenogenetic stem cells (pESCs):

Parthenogenesis can be defined as the development of a diploid embryo from a female gamete without the contribution from a male (Swelstad and Kerr 2010). Parthenogenesis occurs in some invertebrate and vertebrate species naturally, but it is rare in mammals. This process can be induced in vitro using ethanol, ionomycin or cycloheximide, which induce Ca^{+2} oscillations. Additionally, it can be induced using physical stimulation, including mechanical stimulation, cold temperature treatment and electrical shock (Rougier and Werb 2001). Also, cytoskeletal inhibitors (6-dimethylaminopurine) are needed to keep the second polar body to create a diploid cell like zygote (Swelstad and Kerr 2010). The lack of paternally derived genes leads to the inability of parthenogenetic embryos to develop past an early post implantation stage (Surani, Barton et al. 1984). But, Wu and colleagues were able to use the appropriate imprinting genes which help to produce offspring (Wu, Kumagai et al. 2006). In addition, the first human specific pESC lines were produced in 2008, in which they

expressed pluripotency markers, and were able to differentiate to cells from the three germ lines and induce teratoma formation (Revazova, Turovets et al. 2008).

Recent research demonstrates that pESCs are able to be derived at rates approaching 10%-16% (De Sousa, Gardner et al. 2009). Haploid parthenogenetic embryos can also be created by eliminating the cytoskeletal inhibitor step to extract the second polar body after oocyte activation. By this process, obtaining heterozygous pESCs can be avoided (Revazova, Turovets et al. 2008).

The culture conditions and the derivation system resemble those of ESCs. The system of ICM isolation and cell culture are similar to ESCs. To culture pESCs they need either human or mouse fibroblast feeder, and growth media including human serum or serum replacement. LIF is not required to derive pESCs (Lin, OuYang et al. 2007). The positive factor associated with those cells is that they can be produced successfully from fresh oocytes, which make these cells beneficial for women able to produce oocytes (Swelstad and Kerr 2010). However, recently pESCs were obtained using cryopreserved oocytes, suggesting that this could be promising in the treatment of elderly women and women diagnosed with cancer (Fengying, Zhenfu et al. 2009).

1.3.4 Induced pluripotent stem cells (iPSCs)

The production of iPSCs involve the use of somatic cells and and the introduction of certain gene expression for reprogramming, to enable these cells to be pluripotent or embryonic (Swelstad and Kerr 2010). Lineage reprogramming can occur naturally in lower vertebrates and in mammals as well, by dedifferentiation, in which a differentiated cell reverts to an early stage of development, and transdifferentiation in which a differentiated cell turns into to another type. The study of natural transdifferentiation may help reveal the molecular machinery that underlies reprogramming. That can be used to improve and develop better reprogramming mechanisms (Swelstad and Kerr 2010). The first iPSCs were produced in 2006 by Yamanaka. Since then, iPSCs

were able to be derived in mouse, human, rat and monkey cells, using transcription factors. The original method used viral integration to facilitate the appropriate gene expression (Swelstad and Kerr 2010). However, this technique cannot be suitable for clinical applications, because of the use of the viral fusion technique. Recently, iPSCs have been produced using transduction with proteins, or by the use of nonintegrating vectors that express reprogramming factors. To reprogram somatic cells, numbers of pluripotency genes are used including NANOG, OCT4, and SOX2 and additionally some oncogenic factors like KLF4 and c-MYC (Amabile and Meissner 2009). The studies on iPSCs demonstrated the requirement of SOX2 and OCT4 for somatic cell reprogramming, and the use of KLF4 and c-MYC for better efficiency (Swelstad and Kerr 2010). The major problem associated with iPSCs is the possibility that these cells will generate a tumour later on. That is because of the integration of oncogenic factors into the host genome. To avoid this issue, transfection is used by the utilization of chemical inhibitors to reprogram proteins (Stadtfeld, Nagaya et al. 2008). Chemical inhibitors are used for DNA methylation, histone methylation and acetylation to improve reprogramming efficiency (Stadtfeld, Nagaya et al. 2008). However, chemical reagents are not enough for full programming and they can introduce some modifications that result in deregulation of genes (Palii, Van Emburgh et al. 2008). It takes approximately 1 to 4 weeks to generate iPSC-like colonies after transfection. Then these colonies are selected for clonal propagation depending on their morphology. Another 4 weeks are needed for pluripotent gene expression, and then the process of selection is started depending on the expression of cell surface markers including TRA-1-60, TRA-1-81 and SSEA4 (Brockes and Kumar 2002). The low success rate of transformation is another issue. Transformation can provide between 0.001% to 0.1% efficiency rates. However, the addition of other molecules can improve the efficiency of transformation to about 3% in human iPSCs and 10% in mouse iPSCs (Stadtfeld, Nagaya et al. 2008). Additionally, the use of cells from an earlier developmental tissue can improve the efficiency of transformation by

approximately 8 fold. Although the generation of iPSCs brings a greater hope for therapeutic uses and avoids the use of embryonic material, still there is an urge to find better pluripotency regulation and cellular reprogramming mechanisms (Swelstad and Kerr 2010).

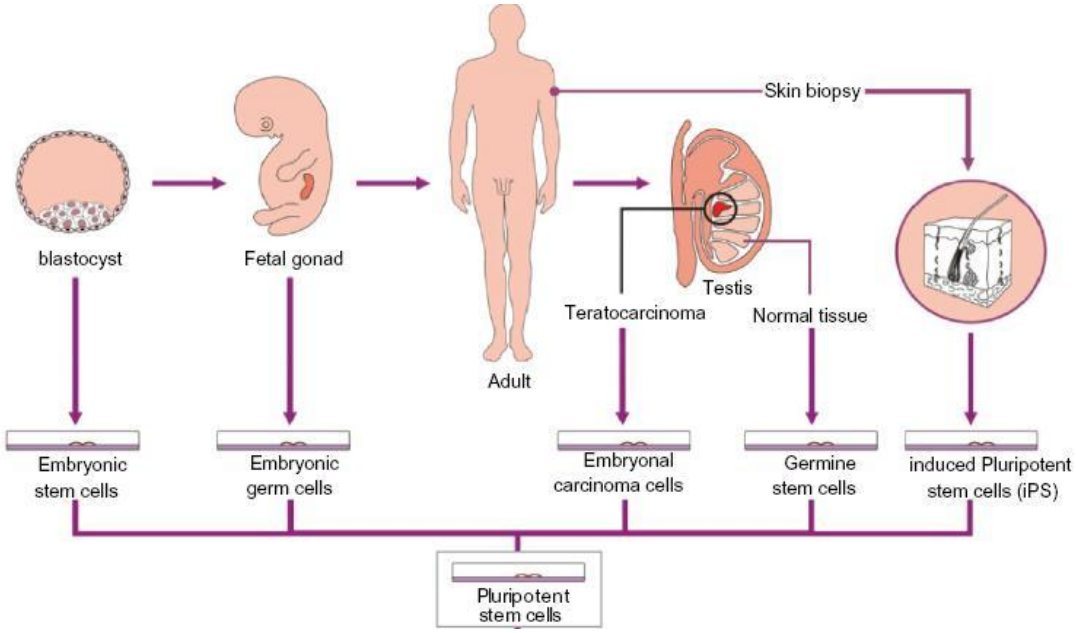


Figure (1.2): Different types of human pluripotent stem cells and their derivation systems including embryonic stem cells derived from blastocyst, embryonic germ cells generated from primordial germ cells, embryonal carcinoma cells isolated from adult teratocarcinoma, germ line stem cells derived from spermatogonia and induced pluripotent stem cells produced by reprogramming unipotent cells (Swelstad and Kerr 2010).

1.3.5 Embryonic stem cells (ESCs)

Embryonic stem ESCs are pluripotent cells that are capable of differentiating into cells of all three germ layers lineages in vitro and in vivo (Martin 1981). These cells can divide symmetrically and self renew, therefore, they can provide an unlimited source of any type of cells. Totipotency is defined as the capacity of a single cell to produce fertile offspring. This totipotency is lost when a cell becomes more specialised, and becomes pluripotent, unipotent, and finally a specialised cell. The totipotent cell is able to divide on its own and produce tissues for foetal development and implantation. Mouse embryonic stem ESCs were first described about 20 years ago. They were isolated from the inner cell mass ICM of the developing blastocyst and grew in vitro (Evans and Kaufman 1981; Martin 1981). ESCs can contribute to all cell lineages, including the germ line (Nagy, Gocza et al. 1990). In vitro, ESCs can proliferate in the undifferentiated state indefinitely, unless they are induced by appropriate signals. In this case, they will differentiate to all mature somatic phenotypes. ESCs can provide a model system for studies of early embryonic development and cellular differentiation (Rippon and Bishop 2004). Also, they can be used in cell therapies to provide a source of cells or tissues in the laboratory. This new era in therapy can solve many problems associated with diseases, organ damage, and the body's repair. ESCs are isolated from the inner cell mass ICM of a day 5 to 7 blastocyst. The quality and the quantity of the ICM are important to obtain ESCs (Lerou, Yabuuchi et al. 2008). ESCs lines are mostly obtained from IVF embryos, for human ESCs this is an issue because of the poor quality of these embryos. Daley et al. have reported a better method used to obtain ESCs from embryos considered to be not optimal for implantation by using hypoxic conditions (Daley, Peters et al. 2008; Lerou, Yabuuchi et al. 2008). Yamanaka had a similar finding with iPSCs production under hypoxic conditions (Lerou, Yabuuchi et al. 2008). In addition, in order to eliminate more stress from these poor embryos, manipulation techniques can be avoided such as immunosurgery. The derivation of hESCs using animal

materials, limits the use of these cells in cell therapies. Therefore, using animal free conditions, serum free and feeder free medium is advised.

Pluripotent stem cells	**Source**
Embryonic stem cells	blastocysts, early stages of development
Epiblast stem cells	Epiblast
Induced pluripotent stem cells	Unipotent cells
Embryonal carcinoma cells	teratocarcinoma
Parthengenic stem cells	chemically activated unfertilised oocyte
Germline stem cells	spermatogonia stem cells from fetal or adult males
Embryonic germ cells	primordial germ cells (mouse only)

Table (1.1): Different types of pluripotent stem cells.

1.4 MOUSE EMBRYONIC STEM CELLS (mESCs)

Early mouse embryos have the ability to form teratocarcinomas if they are transferred to extra-uterine sites like kidney (Stevens 1970). This ability of mouse embryos points to the fact that they have a group of cells that can develop into pluripotent stem cells. mESCs were first derived from the ICM of mouse blastocysts using fibroblast feeder layers and serum (Evans and Kuafman, 1981, Martin, 1981). These mESCs are karyotypically normal cells and can contribute to cells of all three germ lines. This mechanism enables the modification of the mouse germ lines (Bradley, Evans et al. 1984). In mice, the ability to derive ESCs is largely affected by mouse strain type (Ledermann and Bürki 1991). Additionally, mESCs can be derived from early cleavage stage embryos and from a single blastomere from two to eight cell stage embryos (Chung, Klimanskaya et al. 2006). To obtain mESC lines, the culture system

needs to be supplemented with two protein kinase inhibitors. The first one: is PD0325901 that blocks mitogen- activated protein kinase (ERK/MAPK) signal pathway to block differentiation (Chung, Klimanskaya et al. 2006); the second one is CHIR 99021, which acts through bFGF to up regulate the glycogen synthase kinase (GSK) (MEK/ERK) pathway to promote proliferation and self renewal (Kang, Kim et al. 2005). (Hanna, Markoulaki et al. 2009), derived LIF dependent mESC (NOD strain) by using two systems: up regulation of C-MYC and KLF4; or supplementing the medium with small compounds that activate the C-MYC and KLF4 pathways. The first component is CHIR (has similar action to C-MYC), which inhibits the GSK pathway. The second is kenpaullone (KP), which is a selective inhibitor of GSK and cycline (Hanna, Markoulaki et al. 2009), which substitutes for KLF4 actions. By these systems, LIF dependent mESC were obtained and chimaeric mice were acquired. LIF is not enough to stop differentiation of mESCs in serum free medium, bone morphogenetic protein (BMP), a member of the TGFβ family, is also required (Ying, Nichols et al. 2003). BMP acts through the S mad pathway to induce the expression of the inhibitor of differentiation (Yu and Thomson 2008). Epiblast ESCs (EpiSCs) are different to mESCs, in that they are isolated from day 5.5 to 6.5 post implantation mouse embryos. However, they resemble hESCs in terms of culture requirements, gene expression and pluripotency tests.

1.5 Human Embryonic stem cells (hESCs)

Human ESCs are derived in a similar way to that of mouse ESCs. Initially, human ICM was cultured in media supplemented with LIF and serum, which is suitable for mESCs derivation and culture, but not for hESCs, and resulted in differentiation (Yu and Thomson 2008). In 1995, ESCs were derived from rhesus monkey and marmoset (Thomson, Kalishman et al. 1996). The research on those cells has enabled the culture conditions of IVF embryos to be improved (Gardner, Schoolcraft et al. 1998), which led to the derivation of hESCs (Thomson, Itskovitz-Eldor et al. 1998). hESCs have been derived from morula, blastocyst (Stojkovic, Lako et al. 2004), single blastomeres (Irina,

Young et al. 2006) and parthenogenetic embryos (Lin, OuYang et al. 2007). However, it has not been determined yet if these different pluripotent cell lines have equivalent developmental potential (Thomson, Itskovitz-Eldor et al. 1998). To culture hESCs, mitotically inactivated fibroblast feeder layers and serum medium were used in both mouse and human ESCs. However, LIF is only used for mouse, because hESCs do not express the LIF pathway, LIFR, gp130 and JAK1 and 2 (Brandenberger, Wei et al. 2004), and STAT3 is activated at a low level (Dahéron, Opitz et al. 2004). The BMP pathway components are all present in hESCs, but the addition of BMPs to the culture medium will introduce differentiation (Xu, Chen et al. 2002). For self renewal, FGF and TGFβ/ Activin / Nodal are critical for hESCs, like mouse epiblast ESCs (Brons, Smithers et al. 2007). bFGF has a positive regulation effect on the expression of TGFβ in the feeder layer and hESCs, which enhances hESCs self renewal (Greber, Lehrach et al. 2007). Activin and TGFβ promote undifferentiated proliferation of hESCs in lower concentrations of FGFs (Beattie, Lopez et al. 2005).

1.6 BOVINE EMBRYONIC LIKE STEM CELLS (bES-like cells)

Research in bovine ES-like cells have established since 1996 (Mitalipova, Beyhan et al. 2001), to create models for human genetic diseases, and cell transplantation therapies. In addition, bES-like cells can provide useful tools to improve animal traits, products, and for disease resistance, which can be obtained by somatic cell nuclear transfer (SCNT) or by chimaera formation. However, to this day it is still unknown if these cells can be produced *in vitro* (Roach, Wang et al. 2006). The poor results may be due to some issues with the starting materials such as, embryos produced from an *in vitro* system and the treatments for bES-like cells maintenance *in vitro*. ESCs were first established from *in vivo* day 3.5 mouse embryos in 1981 (Evans and Kaufman 1981). ESC lines were also derived from human (Thomson, Itskovitz-Eldor et al. 1998). In the 1990s, there were attempts to derive ESC from porcine ICM, but there was no proof that these cells were pluripotent (Notarianni, E et al. 1990). The

success in obtaining ESC from primates encouraged workers to obtain similar lines from other species, including sheep (Notarianni, E et al. 1990). Unfortunately, successful ESC lines were only obtained from another rodent species, the rat (Buehr, Meek et al. 2008). The efforts attempted in another species did not result in true ESC lines, because these cells were not able to proliferate and, or differentiate to all cell types from the three germ layers (mesoderm, endoderm, and ectoderm) (Wobus and Boheler 2005). ESC from domesticated ungulates such as cow could be a beneficial tool for agriculture, and for regenerative medicine. Large species like cow can be better models for human diseases than the rodents, because organ size, physiology and anatomy are more similar to humans, which can be introduced into recipients for transplantation issues. In addition, the ability to knock in and knock out genes can create good models in order to study gene functions. For agricultural benefits, production genes can be modified to bring more commercial value. However, it has been difficult to derive bESCs lines up to now, and there has not been any bESCs lines reported (Keefer, Pant et al. 2007). In cattle, most attempts to isolate ESC were from blastocysts (Stice, Strelchenko et al. 1996) and from 12-14 embryos with an embryonic disc(Gjorret and Maddox-Hyttel 2004). Additionally, some attempts used early cleavage embryos starting from the two cell stage to the morulae stage to derive ESC. Most of these attempts failed (Mitalipova, Beyhan et al. 2001) except for one from a 16 cell stage embryo, that survived for 9 months (Stice, Strelchenko et al. 1996), and another one from a 2 cell stage embryo that survived for 3 years (Mitalipova, Beyhan et al. 2001). In terms of differentiation, those cell lines were able to differentiate as they formed embryoid bodies, but they could not form teratomas. Although these cell lines were able to survive for a period of time and had a restricted ability to form teratomas, they were not considered to be true ESC. The poor results obtained can be related to the stage of derivation and/or the culture medium. There are a number of factors that contribute to the difficulty to sustain bES-like cells in culture for a long time. These factors can be related to features of both intrinsic and extrinsic embryonic development.

1.6.1 Intrinsic factors

1.6.1.1 Developmental competence

True ES cell lines were only obtained from two strains of mouse; strains 129 and CS7BL16. EpiSC had been derived from only two other species, human and monkey (Thomson, Itskovitz-Eldor et al. 1998). (Telugu, Ezashi et al.) believed that mESC are laboratory artefacts that were selected by chance and by improvements in the culture conditions of the early stages of outgrowth. However, genetic background information enabled the understanding of the development requirements of mouse and therefore, improves the culture system for mESC. Mouse can be a model that can be used to understand pluripotency networks and the factors that contribute to it. This understanding can be applied to other species, and may contribute to the improvement in the culture requirements.

1.6.1.2 Differences in developmental timing

Early embryonic development up to epiblast formation is not similar in different species, especially between mouse and cow. The time of differentiation is different, the first differentiation to occur in mouse takes place by day 3.5, and an additional layer of cells is formed called extra embryonic endoderm. All of these three layers are formed by day 5 in mouse, but this is not the case in other species. In human and cow, blastocysts formation takes place several days after mouse. Mouse form the epiblast structure by the proliferation of the ICM cells by day 6, and gastrulation is initiated on day 6.5. However, distinguishing between the ICM and the epiblast is difficult in cows. In cows, the ICM is smaller than the TE, but ICM cells proliferate to form epiblast by day 7-8 (Hall, Christensen et al. 2009) and then persist for a long time. Additionally , a thin layer of TE cells overlaying the epiblast (Rauber's layer) degenerates by day 12 in cow, exposing the epiblast to the uterine lumen contents (Maddox-Hyttel, Alexopoulos et al. 2003). The loss of the Rauber's layer coincides with rapid growth of the embryo. In the mouse, attachment to

the uterus occurs by day 4.5, while in cattle this period is extended to14 days, and full attachment occurs by day 21. Lastly, the development in cattle is not like mouse and human. There is a longer time for trophoblast expansion, whereas the embryo expansion is delayed. Furthermore, because the epiblast is not enclosed within the trophoblast and exposed to the uterus fluid, the nutritional requirements can differ in bovine, rodent and human. That is why it is not easy to select the appropriate stage at which to derive bES-like cells, and develop culture requirements.

1.6.1.3 Lack of pluripotency markers:

Early embryology and molecular factors that accompany each decision in cattle are not fully known. Molecular markers and surface markers in human and rodent are more specific to the species, that is why they may not be completely accurate for detecting bES-like cells. NANOG is an important pluripotency marker that has been detected in mouse and human ESC (Mitsui, Tokuzawa et al. 2003). NANOG is also detected in bovine blastocysts, ES like cells and in both ICM and TE (Muñoz, Rodríguez et al. 2008). SOX2 transcripts are specific for epiblast (Prasanna Kumar personal communication), but there are a number of papers suggesting that it is also expressed in the trophoblast (Magnani and Cabot 2008). Additionally, bovine OCT4 expression is not like mouse, because it is expressed in both the ICM and TE, and continues to be expressed in the TE for a longer time in cattle (He, Pant et al. 2006) until day 12 which then is only expressed in the epiblast (Gjorret and Maddox-Hyttel 2004). Therefore, OCT4 is considered to be a pluripotency marker for ICM and epiblast. Surface markers, stage specific embryonic antigens (SSEA1, SSEA3, SSEA4), and keratin sulphate antigens (TRA-1-60, TRA-1-80) transcripts are localised in both the bovine ICM and TE. Therefore, bES-like cells lack specific pluripotency markers (Keefer, Pant et al. 2007). It is important to check a wide list of markers to define bES-like cells, which may be not consistent with human and mouse ESC markers.

1.6.2 Extrinsic factors:

1.6.2.1 Mixing cell types:

bES-like cells are usually derived from isolated ICM from day 7 or 8 blastocysts using the mechanical or immunosurgery systems, and from earlier stages of development including 2,4,8,and16 cells. However, it is possible that the isolated ICM cells had some degree of contamination with other cells like TE cells. (Talbot, Powell et al. 1995) presented that the contamination with TE can be sufficiently high to exceed the number of ICM cells.

1.6.2.2 Culture conditions:

The use of a mouse and, or human feeder layers to derive bES-like cells may be inappropriate. The use of heterologous culture cells like STO and mouse embryonic fibroblast (MEF) is still questionable (Brevini, Gandolfi et al. 2007). These heterologous factors might not be effective in supporting bES-like cells, and still more research is needed to find out better culture systems for the growth of bES-like cells. hESCs need basic fibroblast growth factor (bFGF) and a feeder layer for proliferation and do not require leukaemia inhibitor factor (LIF), while mESC depend on LIF (Dahéron, Opitz et al. 2004). (Vejlsted, Avery et al. 2005) reported that human LIF is essential for bES-like cell derivation. Human LIF has been reported to have negative effects on bES like cells (Vejlsted, Avery et al. 2005). Although, signals to maintain pluripotency in mouse and human cells are clear, it remains elusive in bovine. So, the combination of ICM derived ESC and the epiblast derived ESC medium additives, 2i in bovine, may up regulate the LIF/STAT3 signalling pathway and promote the outgrowth of bES-like cells.

1.7 Deriving ESCs in defined conditions

The unique feature of ESCs is their ability to differentiate *in vivo* and *in vitro* into many cell types. Therefore, they provide a useful tool to study development and can be used in clinical therapies. Banks of hESC lines with

different human leukocyte antigen (HLA) were established to provide hESCs consistent for clinical therapy. Additionally, nuclear transfer NT may be able to generate homologous ESCs in the future and provide cells for regenerative medicine (Dvash, Mayshar et al. 2004). Tissue culture technology was established in 1950s (Ludwig, Levenstein et al. 2006). Mitotically inactivated fibroblasts were first used to support HeLa cells, the oldest and the most commonly used human cell line derived from cervical cancer (Ludwig, Levenstein et al. 2006). Later on ESCs and mouse carcinoma were cultured on feeder layers. It was lucky that fibroblast feeder layers support both human and mouse ESCs. hESCs lines are usually derived in a medium with animal products, which may cause a number of problems as (Lu, Hou et al. 2006) mentioned. Firstly, ESC lines may contain immunogenic or/ and toxic proteins that may cause immune responses and lead to rejection of the transplant tissue and organs. Animal products express Neu5Gc, which is a non human immunogenic sialic acid on cells for human transplantation. Secondly, the isolation of hESCs from the feeder layer is time and labour consuming. Finally, undefined conditions may cause complications in developmental studies. Therefore, it is important to use a defined culture system to derive hESCs without the use of animal products. The derivation of hESCs need a number of requirements such as, bFGF for self renewal, a feeder layer and condition medium or cytokines such as TGF or Wnt3a, matrix and FCS or serum replacement. (Lu, Hou et al. 2006) developed a mixture that had chemically synthesized recombinant and human derived factors to support hESCs growth, which they named hESC Cocktail. In addition, other groups have reported the use of defined culture conditions including (Ludwig, Levenstein et al. 2006).

1.8 The extra cellular matrix (ECM)

The extra cellular matrix (ECM) is the structure that gives multicellular organisms their strength and keeps cells in their correct positions. Organisms are formed from many types of tissues; these tissues are connected to each

other by the ECM at special places called cell-cell junctions (Alberts, Johnson et al. 2002). The natural extra cellular molecules ECM are important to support tissue structure, function and organs in vivo and in vitro to direct cell fate and behaviour (Lutolf and Hubbell 2005). In addition, they have a number of roles in regulating cellular functions such as proliferation, growth, migration, morphogenesis, differentiation, gene expression and survival (Lutolf and Hubbell 2005). The ECM is composed of proteins that are secreted from neighbouring cells to form an organized network. The ECM concentrate on connective tissues and the amount of ECM varies greatly between different organs. The variation in the components of the ECM leads to different types of matrix molecules, each with different functions. ECM from fibrous proteins including collagen, fibronectin and laminin, has mainly adhesion functions (Alberts, Johnson et al. 2002). Each type of matrix is not appropriate to support all cell types (Hughes, Postovit et al. 2010). ECM can be extracted from living cells to be used for more sensitive types of cells (Hughes, Postovit et al. 2010). In addition, advanced tissue engineering enables the design of artificial materials which resemble the natural environment. To obtain a clinically acceptable source of ESCs, they need to be derived in fully defined conditions. To derive ESC in defined conditions, surface coating is needed to act as an attachment material for ESC. Surface coatings consist of a combination or individual ECM proteins, such as laminin-11, collagenIV, gelatin, Marigel and fibronectin, which are not fully defined and homospecific (Domogatskaya, Rodin et al. 2008). ECM components are important in regulating ESC self renewal and in maintaining pluripotency. ECM is regulated by the integrin family of cell surface adhesion receptors (Hynes 2002). Integrin induced by cell-ECM interactions are important for pluripotency maintenance and viability (Almeida, Huovila et al. 1995). Integrin-ECM interactions act through integrin-liked kinase (ILK) or focal adhesion kinase (FAK) signalling pathways, and additionally through the activation of PI3K/Akt and MAPK pathways (Ramirez and Rifkin 2003).

Members of the integrin family are expressed in patterns depending on their function during embryogenesis (Hayashi, Furue et al. 2007). For instance, integrin β1 is essential for ICM proliferation (Hayashi, Furue et al. 2007).

1.8.1 COLLAGEN

The collagens are the most common matrix in all multi-cellular animals. They are secreted by the connective tissue and other cell types. Collagens are the major protein component of bone and skin, and they form about 25% of the proteins in animals. The collagen molecule is long with a triple helical structure, in which three α chains are wound around each other. There are 25 different types of collagen α chain, which make about more than 10,000 types of collagen only 20 have been identified so far including type I, II, V and XI. Collagen I is important for bone and skin structure. For example, types IX and XII create the surface of collagen fibers whereas type VI and VII are network forming collagen. Collagen type I and IV have been reported to maintain pluripotency in mouse ESCs (Alberts, Johnson et al. 2002).

1.8.2 FIBRONECTIN

Fibronectin is a large glycoprotein which is found in most if not all vertebrates. It helps to organise the matrix and regulate the attachment of cells to it. The main type of fibronectin is type III. Fibronectin that exists in soluble forms helps in blood clotting and phagocytosis, while the fibrillar form assemble on the cell surface and are deposited in the ECM. There are some proteins that prevent fibronectin assembly in inappropriate places such as, ultroglobin. Fibronectin is very important in early development. That was proved by gene inactivation experiments in mice (Alberts, Johnson et al. 2002). These mice were unable to form fibronectin and therefore died during embryogenesis because they were not able to form proper blood vessels. That can happen because of the lack of interaction between cells and the surrounding ECM, which usually have fibronectin (Alberts, Johnson et al. 2002).

1.8.3 LAMININ:

Laminins are components of basement membranes, which are the first substrate to attach to the early stages of embryonic development, starting from two to four cells in murine embryos (Hayashi, Furue et al. 2007). Mice that lack laminin, die early during embryogenesis because they are unable to form basal lamina. Laminin 511 is one type of the laminins that attach to the ICM of the blastocysts and promote adhesion and proliferation (Rodin, Domogatskaya et al. 2010). Therefore, laminins are useful molecules in culturing ESC in vitro. Laminin-1 is a large protein that composed of very large polypeptide chains (α, β, and γ). These chains can arrange differently to form distinct types of laminin. About 15 different combinations of these macromolecules have been found (Rodin, Domogatskaya et al. 2010). Each type of the laminins has different locations and functions, some presented in early embryos, or epithelial cells, or in tumour cells (Domogatskaya, Rodin et al. 2008). Laminin-511 is common in basement membranes in embryonic stages such as ECM between cells in the ICM of the blastocysts (Domogatskaya, Rodin et al. 2008), and in adult tissues as well (Hayashi, Furue et al. 2007). Laminins are important for cell adhesion, proliferation, migration and apoptosis resistance, which make them an appropriate candidate for ESC culture (Domogatskaya, Rodin et al. 2008). The linkage between these different types of laminins and other macromolecules makes it difficult to isolate them (Domogatskaya, Rodin et al. 2008). However, there are a number of laminins that can be extracted including, laminin 111, laminin 211, laminin 322, laminin 411. (Domogatskaya, Rodin et al. 2008) found that laminin 511 is capable of facilitating mESC self renewal for more than 150 days, even in the absence of differentiation inhibitors.

1.8.4 MATRIGEL

Matrigel is a combination of factors and not a fully defined mixture of extra cellular matrix proteins that were extracted from the Englbreth-Holm-Swarn mouse tumours (Hughes, Postovit et al. 2010). It consists of laminin, collagen IV, fibronectin and a number of growth factors such as FGF2, EGF,EGF,

PDGF, NGF and TGF-β (Hughes, Postovit et al. 2010). One of the important roles of matrigel is its use for growing ESC; because of its ability in mimicking the cell-ECM interactions in vivo (Hughes, Postovit et al. 2010). However, (Mieszawska and Kaplan 2010) reported that it can limit ESC development. It is used in the culture of pluripotent stem cells, but it can be use to induce differentiation, increase tumour growth, and support duct formation. However, it has variable components that differ from patch to patch, which can affect reproducibility of the culture system and the safety standard of pluripotent stem cells that is used in cell therapy (Hughes, Postovit et al. 2010). Matrigel can be replaced with more purified components including IV collagen, fibronectin, laminin and vitronectin in combination or alone. In addition some substrates have been reported to support hESCs pluripotency and the binding with integrins including vitrovictin collagen and laminin-511(Nagaoka, Si-Tayeb et al. 2010).

1.9 Deriving bovine embryonic stem like cells from a single blastomere

Using stem cells in human for cell therapy and to repair damaged tissues is real. However, the use of stem cells is a problematic topic involving ethical and political issues. There is a division among the public and politicians, whether it is ethical to use ICM to create hESC lines because it involves destroying embryos. However, the use of one or two blastomeres from a pre-implantation embryo to produce stem cell lines may solve this problem as it does not involve embryo destruction. Additionally, isolated blastomeres from mammalian embryos have other significant applications. Isolated blastomeres can be used for genotype analysis for superior animals, for sex determination, and screening for genetic disorders as for pre-implantation genetic diagnosis (PGD) (Chung, Klimanskaya et al. 2006; Simpson 2006). PGD is a common technique used to detect genetic diseases. The technique involves the removal of a single blastomere from an 8 cell stage embryo through a hole in the zona pellucida. The single balstomere is then analysed to detect genetic abnormalities. If there was no abnormality, the 7 blastomere embryo is then transferred to the mother

uterus to produce the foetus. In addition, PGD can be used to decrease miscarriage, increase implantation rates and increase live birth in some situations. Normally, the embryo is transferred back to the uterus within 24-48 hr., but the time can be extended to the blastocyst stage. The removal of a single blastomere will not affect the implantation and development of the embryo, because only one blastomere is removed from the embryo. However, it is beneficial to culture the isolated blastomere with its parent embryo up to the blastocyst stage to enhance blastomere division and the derivation of ESC lines. However, this may affect the implantation procedure because of the long culture *in vitro*, which is not similar to the normal environment. The milieu of the human reproductive system has positive effects on promoting the expression of the correct genes and the imprinting pattern. The pluripotentcy of single blastomeres can be determined by the transfer into the uterus. Offspring have been produced from 2 cell stage mice embryos (Geens, Mateizel et al. 2009). Four cell stage bovine embryos are the only example able to be developed into live offspring (Johnson, Loskutoff et al. 1995). (Moore 2006) found that about 33% of the blastomeres derived from 4 to 6 cell porcine embryos continue their development normally *in vivo*. However, it is still unknown when blastomeres lose their totipotentcy during pre-implantation development. (Lorthongpanich, Yang et al. 2008) found that blastomeres from early stage embryos do not have equal competence for development. In addition, single blastomeres are not equally competent to continue development and to express ICM markers (Lorthongpanich, Yang et al. 2008). In addition, lower rates of development have been observed *in vitro*. ESC lines are usually derived from blastocyst stage embryos, which involve the destruction of human embryos. However, use of a single blastomere was the method that allowed stem cell line derivation and preserved the embryo to continue its implantation and development. This technique was done firstly in mice by (Chung, Klimanskaya et al. 2006), and then in the human by (Irina, Young et al. 2006). Three different cell fates were observed when using this system to obtain ESC lines; 1- cells resemble a trophoblast cells, 2- cells similar to ESCs, but they

start to differentiate and 3- cells similar to ESCs and contribute to proliferation and express ESCs markers. These normal ESCs were able to differentiate into cells from the three germ layers, and form teratomas. Further study is needed to find out whether these cell lines are different from blastocyst derived ESC lines. However, the downside of this system are: 1- possibility of causing damage to the embryo during cell removal, 2- the embryo may not respond to the procedure, 3- the use of surplus IVF embryos, which usually have genetic defects and 4- the low success rate compared to the blastocyst derived ESC lines (Chung, Klimanskaya et al. 2006; Irina, Young et al. 2006; Simpson 2006). Ethical and political complications in the use of human embryos may not apply for cattle embryos. However, the use of this system in bovine may be beneficial because it has been a challenge to obtain the naive state of ESCs in bovine. This can be attributed to a number of factors such as, the long period before implantation, the lack of sufficient information about an optimal culture system and the lack of specific bovine pluripotency markers. Thus, it is useful to investigate the pluripotency of blastomeres derived from early bovine embryos. In addition, a large number of bES-like cell lines can be obtained from a few embryos. Furthermore, this system allows genetic diagnosis of the resulting cell lines before obtaining them.

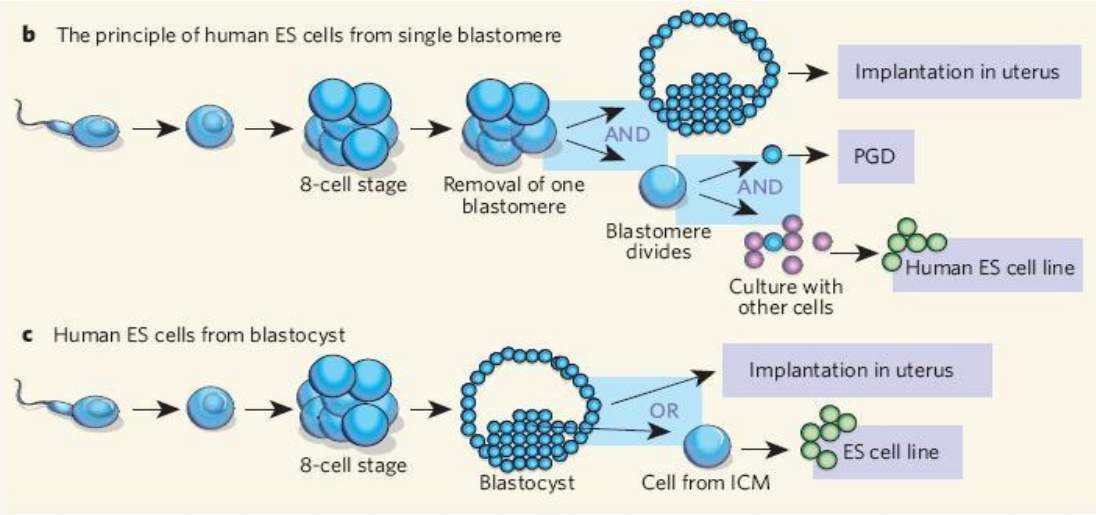


Figure (1.3): Derivation method of ESC lines. (b): from single blastomere, (c): from blastocyst (Simpson 2006).

1.10 Markers detected in bovine outgrowths:

1.10.1 NANOG

LIF maintains self renewal of mouse ESCs by the activation of Stat3, but it is not sufficient to maintain pluripotency alone. Thus, this pathway is not essential for pluripotency. Many genes have been found to maintain pluripotency, but none of them is independent of LIF/Stat3 function. OCT4 is a Pou family transcription factor that is expressed in pre-implantation embryos, ESCs, epiblast and germ cells (Scholer, Dressler et al. 1990). Suppression of OCT4 induces differentiation (Hitoshi, Jun-ichi et al. 2000). OCT4 over expression causes differentiation, which resembles the one caused by Stat3 inactivation, suggesting interaction between the two transcription factors. SOX2 also functions with OCT4 (Guo, Costa et al. 2002), which indicates a cross link with Stat3. The LIF/Stat3 pathway is an independent factor that determines pluripotency in both the ICM and ESCs. Nanog is a transcription factor that works independently of the LIF/Stat3 pathway. It contains a homedomain protein of ESC self renewal and is also expressed in early embryonic stages. Nanog was named after the land of the ever young (Tir nan og). Nanog cDNA has 2184 nucleotides with a single open reading frame encoding a polypeptide of 305 amino acids. It has a long un-translated region with a repetitive element. Nanog null cells differentiate into endoderm. It has regulation effects in maintaining ESCs pluripotency. It works independently from the LIF/Stat3 pathway to prevent differentiation into endoderm cells. In pre-implantation embryos, Nanog expression is low and OCT4 is the transcription factor that determines cell fate. At the blastocyst stage Nanog is expressed in the ICM to cause the cells to remain pluripotent. However, it is not sufficient to maintain pluripotency without LIF/STAT3 signals.

1.10.2 SOX2

In mouse and human ESCs, OCT4, SOX2 and NANOG are involved in pluripotency maintenance and have a common target site in the regulatory regions of a number of genes. SOX2 repression in mESCs induces trophectoderm differentiation (Masui, Nakatake et al. 2007), but over expression of SOX2 causes non specific lineage differentiation, neuronal differentiation and cell death (Kopp, Ormsbee et al. 2008). SOX2- null mice enable the maintenance of the ICM and trophoblast development (Avilion, Nicolis et al. 2003), suggesting that SOX2 is important for both, ESCs maintenance and trophoblast development. Additionally, reduction in SOX2 expression in hESCs causes differentiation of ESCs with an increase in trophetoderm markers (Fong, Hohenstein et al. 2008). Also, suppression or over expression of SOX2 in hESCs causes trophectodermal differentiation, which indicates the important role of SOX2 in the maintenance of trophoblast development (Adachi, Suemori et al. 2010).

1.10.3 OCT4

OCT3/4 is a transcription factor that is encoded by the POU5F1 gene from the Pou-homeodomain family. It has an essential role of regulating pluripotency during early development (Morrison and Brickman 2006). OCT3/4 is expressed in embryo including epiblast and primordial germ cells, ESCs and EGCs (Pesce and Schöler 2001). OCT4 is down regulated during gastrulation and acts as an ESCs maintenance factor (Boiani and Schassler 2005) and it's down regulation cause the loss of the pluripotent state (Nichols, Zevnik et al. 1998). In addition, OCT4 can be used in reprogramming somatic cells (Bortvin, Eggan et al. 2003). OCT4 regulates the pluripotency state by the activation and the repression of hundreds of target genes (Boyer, Lee et al. 2005). (Abu-Remaileh, Gerson et al. 2010) found that the fate decisions are regulated by the interaction between OCT4 and the Wnt/β-catenin signalling pathway (Abu-Remaileh, Gerson et al. 2010).

1.10.4 FGF4

FGFs consist of a large family of proteins. Those that interact with a family of receptors are designated (FGFG-1 to 4). The FGF4 gene was discovered firstly in human testicular germ cell tumours. It starts expression in early embryonic stages up to the pre-implementation blastocysts (Rappolee, Basilico et al. 1994). FGF4 has an important role in embryogenesis as knock out FGF4 mice embryos die after implantation (Feldman, Poueymirou et al. 1995). FGF4 is expressed only in the primitive streak, and then in the tail bud (Kosaka, Sakamoto et al. 2009). Its expression is restricted in the ESCs and embryonic carcinoma (Kosaka, Sakamoto et al. 2009), and can be activated through SOX2/ OCT3-4 complex, which is important for development and pluripotency. Actually, OCT3-4 is an important regulator for FGF-4 expression. FGF4 activates the Erk 1/2 signalling cascade in mouse ES (Kunath, Saba-El-Leil et al. 2007), while its inhibition does not affect ESCs proliferation (Kosaka, Sakamoto et al. 2009), but restricts ESCs ability to differentiate. The blockage of FGF-4-ERK signalling by the inhibitors (SU5402) and (PD184352), maintains ESCs self renewal (Ying, Wray et al. 2008). In addition, in pre-implantation embryos, FGF4 inhibits trophectoderm differentiation in the placenta (Kosaka, Sakamoto et al. 2009). Therefore, FGF4 maintains the pluripotency of ESCs and enhances self renewal of trophoblast stem cells (TSCs).

1.10.5 CDX2

CDX2 is a cell fate determining gene that plays an important role in trophectoderm specification (Nishioka, Yamamoto et al. 2008). CDX2 down regulates the expression of NANOG and OCT4 (Strumpf, Mao et al. 2005). At the blastocyst stage, CDX2 expression becomes restricted in the trophectoderm (TE) (Strumpf, Mao et al. 2005). It has been reported that CDX2 may be involved in allocation of blastomeres either at the ICM or TE, because CDX2 expression is hetergenous in blastomeres at the 8- cell stage (Ralston and Rossant 2008). In addition, the level of CDX2 expression can affect cell

allocation as it can be detected in TE while not in the ICM. Thus, CDX2 early expression at the 8- cell stage embryo stage can influence polarity and therefore cell position. At the same time, the positioning of cells influences CDX2 expression, later, in the 32- cell stage embryo (Jedrusik, Parfitt et al. 2008).

1.10.6 SALL4

Sall4 is an important transcription factor that maintains mESCs in the undifferentiated state through the binding and activation of the POU5f1 promoter (Hamatani, Carter et al. 2004). Reduction of Sall4 induces ESCs differentiation to the trophectoderm cells (Jinqiu, Wai-Leong et al. 2006). Similar findings have been reported *in vivo*, as Sall4 reduction in pre-implantation embryos causes a reduction in OCT4 expression and an increase CDX2 expression, the trophectoderm marker, in ICM cells. That suggested the importance of cooperation between OCT4 and Sall4 in early development. Also, Sall4 has an important role in regulating OCT4 activity in order to maintain ESCs in an undifferentiated state.

1.10.7 KLF4

KLFs are zinc finger containing transcription factors with different functions. Krupple-like factor 4 (KLF4) is highly expressed in epithelial tissues such as the gut and skin (Shields, Christy et al. 1996). Additionally, its expression has been detected in other tissues including testis, lung, lymphocytes and endothelial cells (Shields, Christy et al. 1996). KLF4 has many biological functions including cell cycle control, transcriptional regulation, apoptosis, differentiation and cell fate determination (Yusuf, Kharas et al. 2008). Knock out KLF4 mice were born with normal phenotype and histology (Katz, Perreault et al. 2002), but they die soon after birth, because of many complications including an inability to feed and the rapid loss of body fluid. KLF4 is a specific gene for goblet cell formation (Katz, Perreault et al. 2002). Thus, KLF4 is an essential factor in regulating many biologically functions. In addition, over expression of KLF4 inhibits cell proliferation in culture (Shields,

Christy et al. 1996). KLF4 involves in regulating ESCs self renewal (Li, McClintick et al. 2005), as KLF4 over expression has a great effects on ESCs self renewal. In addition, (Kim, Chu et al. 2008) reported that KLF4 has a crucial role in somatic cell reprogramming and ESCs self renewal maintenance (maintain immortality). KLF4 has been used in combination with other genes including OCT4, SOX2 and c-Myc in reprogramming somatic cells, as it cooperates with c-Myc to establish the immortal state of iPSCs.

1.10.7 DPPA3

Dppa3 is a gene expressed especially in oocytes, pre-implantation embryos and pluripotent cells (Sato, Kimura et al. 2002). Stella expression is activated during germ cell specification (Sato, Kimura et al. 2002). After the formation of the zygote, Dppa3 is expressed in the pro-nuclei until the blastocyst stage (Saitou, Barton et al. 2002). Then, Dppa3expression is down regulated until the formation of primordial germ cells (Saitou, Barton et al. 2002). (Payer, Saitou et al. 2003) found that the maternal inheritance of Dppa3 is important for embryonic development. Repression of Dppa3 from oocytes affects development and causes complications in blastocyst formation, implantation and poor development. In addition, Dppa3 transcription factor can bind to DNA and RNA in vitro (Payer, Saitou et al. 2003), suggesting that it might be involved in linking chromatin with RNA. Lack of Stella can affect other genes that are involved in development. The interaction between Dppa3 and NANOG can be associated with pluripotency, teratocarcinomas and germ cell tumours.

1.11 In vitro production of embryos (IVP)

The role of reproduction is fundamental to all life. Animal breeding, reproduction, selection and wild life conservation allow the maintenance and dissemination of species and breeds (Galli and Lazzari 2008). For example farm animal reproduction needs genetic selection to improve traits in the next generation (Galli and Lazzari 2008). Some of the technologies used are

genomics, proteomics and marker-assisted selection, which have become a necessity to improve traits. The improvement of assisted reproduction techniques in farm animals has increased our knowledge of the molecular mechanisms involved in reproductive systems. This has also improved our understanding of the roles played by male and female gametes and aspects of their fusion, the development of embryos, and lately the manipulation of early development embryos (Galli and Lazzari 2008). Therefore, reproductive technologies support an understanding of the biology related to reproduction. The technology that has had the greatest impact on animal breeding is in vitro production of embryos (IVP) (Galli and Lazzari 2008). IVP allows embryos to be obtained from infertile donors (Gordon 2003). IVP developed after the realisation that immature oocytes after in vitro maturation (IVM) can be used to produce viable embryos with high efficiency (Staigmiller and Moor 1984). The focus was on the female germline to overcome the physiological limitation to make better use of female gametes (oocytes) instead of wasting them by atresia (Galli and Lazzari 2008). By this technique, the outcome of female gametes has greatly amplified. The first in vitro fertilised embryo was obtained in rabbit (Chang 1959). The mouse was the next mammalian species to be fertilised in vitro (Whittingham 1968). The first IVF calf derived from in vitro matured oocytes was in 1982 (Gordon 2003). Oocyte maturation and fertilisation techniques were developed by Lu and colleagues in 1987, and in1988 the first calves were born from a totally IVP system. Oocytes play the most crucial role in breeding (Staigmiller and Moor 1984). The production of a large proportion of good quality oocytes using in vitro techniques opened a new door in embryo production. The (IVP) of embryos began with work on a number of approaches, such as, the control of meioses, in vitro fertilization (IVF), and embryo culture in vivo and in vitro. IVP first applied rodents in order to isolate and study the biology of intact follicles, and then grow them in culture (Gordon 2003). In livestock this was a challenge because of the large size and connective tissue of the ovary. In 1992, Lazzari developed a protocol to isolate a large number of primordial oocytes from new born piglets' ovaries, by

enzymatic digestion and centrifugal elution. This technique was improved by Miyano and Manabe 2007 by obtaining oocytes from secondary follicles. However, culture requirements to support the development of ovary follicles were complex. It was obvious that oocytes were present in secondary follicles of farm animals. More work was done to find the appropriate media for maturation in vitro. Successful results were obtained in bovine, in pig and in horse. IVM was better understood after applying IVF. Factors that affect the development of oocytes were identified such as, follicle size (Galli and Moor 1991) and the interaction between oocytes and follicular cells (Staigmiller and Moor 1984). The retrieval of oocytes from live bovine donors, ovum pick up, (OPU) was a major step forward in developing an effective IVP system (Gordon 2003). In addition, IVF results were poor until heparin was used for sperm capacitation (Parrish, Susko-Parrish et al. 1986). Also, separation of X and Y sperm was obtained by flow cytometry to get embryos of the desired sex (Gordon 2003). However, there are number of concerns in producing embryos by IVP. For example, the low number of blastocysts obtained from every procedure, large offspring syndrome (LOS) (Young, Sinclair et al. 1998), and their lower ability to withstand freezing and thawing. Also, in vitro produced embryos have different molecular and cellular markers than those produced naturally (Gordon 2003).

Still, IVP is a popular system, in 2005, as IETS reported 260,000 IVP embryos were transferred in farm animal because of its high yield of low cost embryos for transfer. These embryos were used for diagnosis, somatic cell and embryo cloning and for the production of transgenic cows, as well as for basic research on the mechanisms of embryogenesis (Gordon 2003). The IVP system is applied in most reproductive technology labs. The production of large numbers of embryos enables workers in the field to use them for research, which has a significant effect on the amount of knowledge related to oocyte maturation, fertilisation and the development of early embryos. Because of this knowledge in bovine and the similarities between the bovine and human early

development, the bovine model may replace the mouse model for early mammalian development (Wrenzycki, Herrmann et al. 2001).

1.12 In vitro embryo production in cattle

The cattle industry has a number of difficulties such as, cattle diseases and increased demand worldwide. These difficulties have reduced the outcome of cattle breeding, and have required the introduction of new techniques in order to produce embryos more efficiently and more cheaply. There are two major systems to produce cattle embryos, (MOET) for multiple ovulation embryo transfer and IVP for in vitro embryo production. The second system is used to produce embryonic stem cells, and will be focused on here. In this system, cattle embryos can be produced by using immature oocytes collected from the ovaries of donors of various age and physiological status. Following that, reliable procedures maintain these oocytes in vitro using a number of culture protocols to allow them to grow up to the stage suitable for transfer, freezing and culture.

IVP comprises three different biological steps: in vitro maturation IVM, in vitro fertilization IVF, and in vitro culture IVC.

1.13 Grading embryos

In vitro produced mammalian embryos have differences in their characteristics in comparison to in vivo embryos. These characteristics include, lower cell numbers, an altered inner cell mass ICM: trophectoderm (TE) ratio, irregular size of blastomeres and cytoplasmic fragmentation, which lead to the reduction in developmental competence (Kruip and den Daas 1997). Cytoplasmic fragmentation is common in vivo and in vitro (Pereda, Cheviakoff et al. 1989; Alkani 1999). However, the degree of fragmentation can vary from a few fragments to extensive fragmentation. Apoptosis can happen naturally in *in vivo*-produced mammalian blastocysts (Byrne, Southgate et al. 1999), which

eliminates damaged or abnormal embryos (Hardy 1999). In addition, apoptosis is a major cause of embryonic arrest, in vitro, in bad culture conditions, such as an excess of embryo: media ratio, heat shock, excess of oxygen free radical concentrations, and exposure to a high concentration of spermatozoa during IVF (Tremoleda, Stout et al. 2003). The increasing use of blastocyst culture in an IVP system has encouraged the analysis of blastocyst parameters, and their effects on embryos death (Tremoleda, Stout et al. 2003). Parameters such as cell number and apoptosis rate can be indicators of the health and developmental potential of IVP produced embryos (Hardy 1999). But, to select good embryos the most common parameters are: the degree of fragmentation and the developmental stage. Extensive fragmentation can be associated with reduced blastocyst formation, and problems with implantation , despite that good quality embryos with no fragmentation can still be arrested (Hardy, Stark et al. 2003).

Total	Grading
15	(13) 1c,(4) 2c
11	EB2, B2, B2, B2, TM3, 8c, TM2, 8c, XB2, XB1, B2
11	XB2, B3, XB2, B2, B2, B2, 8c, 4c, XB2, XB2, B1
11	XB2, TM2, TM2, XB3, B2, B1, XB2, 8c, B2, B1, XB3
11	XB1, B2, HB2, B2, XB2, TM2, TM2, TM2, B2, XB1, B2
12	(10) 1c, (2)2c
10	EB2, XB2, XB1, B1, B2, XB1, TM2, B2, TM2, 8c
11	EB1, EB2, XB1, XB2, TM2, TM1, B2, B1, XB2, B1, B3
11	B1, B2, 8c, TM2, XB2, XB2, XB1, B2, TM1, B2, TM2
10	B2, B2, B2, XB3, XB2, XB1, TM2, B3, TM2, TM1
14	(11) 1c, (3) 2c
10	XB2, EB2, dg, B3, B2, XB1, EB2, XB2, XB1, XB2
11	XB1, XB2, B3, B2, B1, TM2, EB2, 6c, B1, B2,B3
10	B1, B2, B1, XB2, B2, TM2, EB2, EB2, 4c, B3
10	XB2, B2, B3, TM2, B1, B2, 8c, EB2, XB2, B2

Table (1.2): Example of grading day 7 bovine embryos. c, cell, TM, tight morula, EB, early blastocyst, B, blastocyst, B1, blastocyst grade 1, B2, blastocyst grade 2, B3, blastocyst grade 3, XB, expanded blastocyst, HB, hatched blastocyst.

Chapter Two

Materials and Methods

2.1 In vitro production of bovine embryos

2.1.1 Aspiration of ovaries

An important factor in the maintenance of oocytes is temperature. It was important to avoid temperature shock and therefore the temperature must be monitored during the collection procedure. Ovaries were collected from the reproductive tract using scissors from slaughtered cows, and then placed in a warm saline solution in thermos flasks at the abattoir and brought to the aspiration lab within about 2-4 hours. Before aspiration was started the ovaries were washed three to four times in sterile 0.9% saline (30◦C) in another wide mouthed thermos flask to remove blood. Fifteen ml conical tubes were then filled with 2ml warm aspiration medium. 18 gauge needles were placed into the tubes and the blunt needle was then pushed into the second hole on the aspiration bung. The blunt needles were connected to a negative pressure (-45 mmHg) from the aspiration machine. Follicles between 2-8 mm in diameter were aspirated. Tubes were filled up to three quarters, and then they were changed. From each ovary about 5 oocytes were expected. Full tubes were taken to the embryology lab to be analysed.

Figure (2.1): Aspiration machine and tube warmer machine. Aspiration device was set on -40 to -45 mmHg and tube warmer was sat on 28˚C.

2.1.2 In vitro maturation (IVM)

IVM plates were prepared in the morning (two hours before IVM) in a laminar flow hood. Six cm Petri dishes were used and 12 drops of 40 µl of maturation medium supplemented with cysteamine (Sigma) were placed using a dropper pipette. Drops were then overlaid with enough mineral oil to prevent evaporation. Plates were labelled with initials, date, and IVM, and placed in a 5% CO_2 incubator at 38.5. Ovaries were then aspirated as described previously. About 3ml of aspiration medium was added to a 9 cm plate, and the sediment, which was the oocytes aspirated and other cells, was added using a sterile glass Pasteur pipette with a syringe. The plate was then placed in a grid plate underneath a warm microscope stage. Cumulus-oocyte complexes (COCs) were searched for, and (COCs) that were light in colour with an even cytoplasm and surrounded by cumulus cells were selected, using a low volume pipettor and sterile pipette tips. Oocytes without cumulus cells or with a large number of cumulus cells layers were left behind, because they were either immature or too mature and this can lead to poor blastocyst development later. (COCs) were washed twice in 35mm Petri dish containing Hl99+ 10% FCS, and then washed in 35mm Petri dish containing Bl99+ 10% FCS. Ten (COCs) were then transferred to 40µl drops of IVM media, until all (COCs) were transferred. The time and the number of (COCs) in each plate were written on the Petri dish, before being placed into the 5% CO_2 incubator for 22-24 hours at 38.5˚C.

2.1.3 In vitro fertilisation (IVF):

After 22-24 hours of oocyte maturation, they were then fertilised by the use of washed, frozen and thawed semen of a bull of known in vitro fertility (Dustin). IVF media is a synthetic oviduct fluid SOF medium, without glucose, with further addition of 10µg/ml heparin, 1µM pyruvate, and 1µM penicillamine (Sigma) and 2µM hypotaurine (Sigma) (IVF+ PPHH). The number of

spermatozoa that was needed was calculated after sperm counts using a haemocytometer. The required amount of IVF medium was added to the semen solution.

2.1.3.1 Plate preparation

Plates were prepared in a laminar flow hood. Chemicals were added to increase zygote development. 1μM Pyruvate was added to increase the developmental potential of the embryos, the 1μM penicillamine/ 2μM hypotaurine solution was added to maintain sperm motility, and heparin (1μl/ml) was added to capacitate the sperm and increase the chances of fertilization. Twelve drops of 30μl and two 40μl drops for washing were placed in a 6 cm plates. Plates and IVF+ PPHH were placed in the 5%CO_2 incubator at 38.5˚C for at least 2 hours to equilibrate. Also HSOF was placed into the incubator at 38.5˚C with the cap tightened to warm up.

2.1.3.2 Sperm preparation

Stock solutions and the percoll were warmed to room temperature before making the percoll solution to prevent precipitation. Sperm preparation was carried out in the laminar flow hood. Percoll gradients were prepared by pipetting 1ml of 45% percoll into a 15ml conical tube and carefully under laying it with 1 ml of 90% percoll solution. Warm water (~35˚C) was prepared in a beaker to thaw the semen straw. Straws were taken from the liquid nitrogen container and placed in the water beaker for 30 sec (one straw can fertilise 150 oocytes). One semen straw is 0.25 ml, and contained approximately 1×10^8 spermatozoa/ml. Straws were dried and wiped with 70% ethanol to prevent contamination. The straw contents were placed into the tube by cutting the edges of the straw with sterile scissors. The tube with the percoll gradient/ sperm was centrifuged (Biofuge primo 75005181 centrifuge) at 700 g

(2200rpm) for at least 20 min. Sperm were recovered immediately when the centrifuge came to rest to prevent motile sperm from swimming up the gradient. Sperm were removed from the bottom of the tube using a sterile glass pipette with a syringe. The sperm pellet was then re-suspended in 1 ml of HSOF medium, and centrifuged again (Biofuge primo 75005181 centrifuge) at 200g for 5 min. During this time 190µl of water was pipetted into a glass tube, and the Haemocytometer was prepared. The supernatant was then removed and the sperm pellet re-suspended in 200µl of equilibrated IVF+PPHH medium. A 10µl aliquot of the solution was added to the 190µl of water to make a 1:20 dilution. Using a pasture pipette and syringe the remaining volume of the solution was measured, and then placed in the CO_2 incubator again at 38.5˚C to complete the sperm calculation.

2.1.3.3 Sperm calculation

The haemocytometer consisted of two grids; each one had 25 large squares with triple lines and 400 smaller squares, and a thick cover glass for an accurate count. The haemocytometer was prepared by placing a cover slip over the support shoulders. Seven to10µl of the 1 in 20 sperm dilution was placed in the both edges of the cover slip, by touching the edge and the cover slip and allowed the sample to flow under the cover slip. The sperm were counted in the 25large squares under the microscope on (x20). Only spermatozoa whose head was within the triple lines were counted. Both grids were counted and then the mean of the two was used to calculate the final concentration of 1 million sperm/ ml, using this formula:

(Volume measured (A) × Average number of sperm count (B)) ÷ 25 =Total volume (C).

The total volume (C) – Volume measured (A) = Volume of IVF+ PPHH medium to add (D) to sperm dilution.

When dealing with sperm, the dilution medium was added gently and slowly so the sperm to prevent dilution shock. Also, the diluted sperm were kept in the CO_2 incubator during the calculation period to keep the pH and the temperature at the optimal level. After the calculation was done and the medium was added, 10µl of diluted sperm was added to each drop containing oocytes. IVF drops were checked to ensure sperm motility. IVF plates were placed in the CO2 incubator for 18-24 hours at 38.5˚C.

2.1.3.4 Oocyte preparation

During sperm centrifugation, oocytes were prepared, by removing them from IVM drops using a large volume pipettor and transferring them to a 35mm Petri dish containing HSOF. Cumulus cells were removed by pipetting the complexes up and down many times until most the cumulus cells were removed. Oocytes were then transferred to another HSOF dish, then to an IVF+PPHH plate. Five oocytes in 10µl of IVF+PPHH were placed in IVF drops, and then plates were placed again in the 5% CO_2 incubator at 38.5˚C.

2.1.4 In vitro culture (IVC)

18-24 hours after fertilisation of oocytes, the cumulus was removed and presumptive embryos were placed in culture in a modular incubator chamber for 5 days. The day of fertilisation was day 0, and the first day in culture was day 1. Medium was changed on day 5 and plates were placed in a modular incubator chamber for 2 days. Embryo development was scored on day 7. For example if IVM was done on Monday, IVC would be done on Wednesday, changeover would be on Sunday, and embryos development would be on

Tuesday. Plates for IVC were prepared in the laminar flow hood. Five drops of 20µl early SOF and 2 of 40µl of washing drops were placed in 35mm plate. Drops were covered with mineral oil. Plates were placed in a modular incubator chamber, and gas was turned on (5%CO_2, 7%O_2 and 88%N_2) for 5 min. Modular incubator clumps were closed to keep the gas in, and it was placed in a dry incubator at 38.5˚C for two hours for equilibration of the plates.

The modular incubator chamber was prepared by placing a small amount of sterile water in the 60 mm plate (only the bottom side of the plate) to humidify the chamber. A 35mm plate of BI99+ FCS was placed in the chamber as an indicator (it should remain salmon pink colour) to ensure that the chamber was gassed. The black o-ring was greased with lubricant, the lid of the modular incubator was placed, and clamps were closed. Embryos were pulled using a pipette and cumulus was removed in the washing drops of IVF by pipetting up and down several times. Embryos then were transferred to a 35mm plate of warm HSOF to be washed; this step was repeated for two or three times. Using a mouth pipette, embryos were placed in the first washing drop of ESOF then to the second. 10-15 embryos were placed in each culture drop. Each plate was marked with the number of embryos, and placed in the chamber. After processing all of the plates, they were placed in the chamber to be gassed for 5 minutes (5%CO_2, 7%O_2 and 88%N_2). The closed chamber was then placed in the dry incubator for 5 days at 38.5˚C. On day 5 embryos were removed from ESOF medium by mouth pipette to late synthetic oviduct medium (LSOF) plates that were prepared with the same ESOF plates. Plates were returned to the chamber and gassed (5%CO_2, 7%O_2 and 88%N_2). Chamber was placed in the dry incubator at 38.5˚C for more two days. On day 7 embryos were graded.

2.2 Grading embryos

Day 7embryos were graded depending on the degree of fragmentation and the stage of development, using the criteria described.

2.3 Single blastomere culture in different substrates

2.3.1 Coating plates

96 well plates were used (Nunclon, Lot no. 105851). The number of wells that were used for every substrate was determined depending on the experiment. Substrates varied in some experiments depending on their availability and the plan of the experiment. Coating the 96-well plates or other types of plates was done in the laminar flow. On the lid, the date of the experiment, my initials, the media used in the experiment including additives and the substrates used with lines to illustrate their place in the plate, were written. Fifty µl of each substrate was added to every well in the 96-well plate. With the lid on, plates were put in the CO_2 incubator at 38.5 C for 30 minutes to dry. Excess coating was tipped out, and 100µl PBS (Sigma) was added to every well for washing. Plates were left to dry for another 30 min with the lid off in the laminar flow. Then, 100 µl of ESOF was added to every coated well. In case of using 2i or 3i media, supplements were added to the ESOF media including CHIR 99021(Biovision), PD0325901 (Stemgent) and PD173074. Plates were gassed using triple gas in the incubator chamber, and left over night. It is important not to coat all the wells in the 96-well plates, as the loading of blastomeres takes time, which may affect the pH of the media.

2.4 Immunosurgery

THSOF-BSA (20µl 50 X Essential amino acid/ml + 10µl Non- essential amino acid) was placed in the 38.5˚C CO_2 incubator to warm up before use it in the procedure. Anti bovine serum developed in rabbit (RαB) (Sigma) and Guinea pig compliment serum (GP) (Sigma) working solutions were prepared by the addition of 150µl THSOF-BSA to a 50µl RαB aliquot and centrifuging for 5

min at 2000 rpm before the use. GP was prepared by the same way. 40µl droplets for washing and 20µl for incubation were made in 6cm for both RαB and GB. Drops were covered with mineral oil and plates were placed on the warm stage. Zona pellucid was removed from the selected blastocysts by placing them in pronase drops. Blastocysts were washed in THSOF-BSA to stop pronase activity, this step was repeated three times. 5 blastocysts were placed at a time in the washing drop of RαB then to other washing drops to remove THSOF-BSA. Then they were placed in 20µl drop for 45 min in the CO_2 incubator at 38˚C. During this time antibodies were bind to the TE cells. After 45 min blastocysts were removed from RαB drops and washed in THSOF-BSA plates. Again 5 blastocysts were placed in the washing drop of GP then in the incubation drop. Blastocysts were left for 45 min in the CO_2 incubator at 38˚C. During this time finely drawn Pasteur pipette were made. After incubation, blastocysts with lysed TE cells were placed in THSOF-BSA and by the pipette TE cells were dislodge from the ICM cells. ICMs were then placed in the pre-coated wells and placed in the chamber to be gassed (5%CO_2, 7%O_2 and 88%N_2).

2.5 Dissociation method

Before starting, the required stage of embryonic development was selected with the emphasis placed on the quality of embryos selected. The zona pellucida was removed from the embryos by placing 30 embryos in 50µl drops of 0.5% pronase in HSOF –BSA+Ca+Mg 1mg/ml of PVA (sigma P811 lot 016k1523). When the zona pellucida began to lose shape, embryos were transferred to HSOF in 35mm plate to be washed twice.

Nine ×30 µl HSOF drops were placed in 60mm Petri dish, covered with mineral oil and left on the warm stage to warm up. Zona free embryos were placed in these drops to be dissociated using a pooled mouth pipette. The size of mouth pipette is important; it needs to be slightly larger than embryos to ease the dissociation procedure. The embryos were dissociated by lifting them up and down in the mouth pipette. After embryos were dissociated, the

microscope was placed in the lamina flow hood, and the transfer was done in the hood to avoid contamination. A single blastomere, a group of blastomeres, a zona free whole embryo, or a zona intact whole embryo was placed in the wells following the experimental plan. Ninety-six well plates were placed in the incubator chamber to be gassed, and left for 5 days.

On day 5 the plates were graded, and wells with outgrowth were marked. On day 7, 100 µl of N2B27 (invitrogen) + 20% FCS was added to each well with an outgrowth. All procedures related to 96-well plates were done in the hood to avoid contamination.

2.6 Blastomere culture using dimples

In the previous blastomere culture experiments there was a poor outcome associated with a low number of blastomeres. The goal of these set of experiments was to obtain embryonic stem cells from a single blastomere.

The separation of blastomeres of embryos at the eight cell stage was applied at day two of culture. The zone pellucida was removed by the exposure of the embryos to 0.5% pronase in HSOF –BSA+Ca+Mg 1mg/ml of PVA (sigma P811 lot 016k1523). About 30 embryos were loaded into 50 µl drops of pronase, and left for 1-2 min or until the zone pellucida was removed. Embryos were then transferred to 35mm HSOF plate to be washed. Then, each embryo was placed in 30 µl drops of dissociation media (HSOF-Ca+3mg/ml BSA+0.02% EDTA+ 5µg/ml CB) and gentle pipetting with a pooled "tapered" pasteur pipette was performed. Then, blastomeres were placed in HSOF drops to be washed. Then, dissociated blastomeres were transferred to plates with dimples using a very fine pooled pasture pipette (slightly larger than a blastomere size). The number of blastomeres in each dimple depends on the experimental plan. Plates were placed in the modular chamber under a gas phase of (CO_2 5%, O_2 7%, and N_2 88%) with humidity at 38.5C° in the dry incubator to be gassed, and then left in the dry incubator at 38.5˚C. These

procedures were conducted on a warm stage (36-38˚C) and at room temperature (24-26˚C).

2.6.1 Preparing dimples

Conical wells or dimples were prepared on the bottom of a 60 mm culture dish. One, 3, 6 and 12 conical wells were created in groups by pressing the bottom of the dish using a sterile (rod). Each group of micro wells was covered with 20µl drop of 0.1% gelatin, then left to dry, with the lid on. Embryonic stem cell culture medium then was covered with mineral oil. Plates were left to equilibrate for 2 hr under a gas phase of (CO_2 5%, O_2 7%, and N_2 88%) with humidity at 38.5C° in the modular chamber in the incubator, before blastomeres were placed.

2.6.2 Single blastomere culture in different substrates using tissue culture plates

60 mm tissue culture plates (Falcon, U.S.A) were used for the experiment. 20×5µl drops of every substrate (collagen, gelatin, poly-lysine, fibronectin) were placed in each plate. Drops were left to dry in the laminar flow. Access of substrates was removed using a mouth pipette, Five µl ESOF drops were placed in place of the substrates drops. Drops were covered with mineral oil, and left in the modular chamber to equilibrate. The same dissociation method was followed. In case of using 32 cell stage embryos, dissociation media (HSOF-Ca+3mg/ml BSA+0.02% EDTA+ 5µg/ml CB) was used to dissociate embryos.

2.7 Counting cells in bovine outgrowth derived from dissociated blastomeres

Cell culture media (DMEM/F12+ 10% FCS) and trypsin (0.05% / 1mm EDTA) were placed in the incubator to be warmed. Wells with outgrowths were marked and ES media was aspirated off the outgrowths. Outgrowths were then washed with an appropriate volume of warm PBS. Enough trypsin (0.25%/ 1mm EDTA) to cover the outgrowths was added to each well. The plate was left in the incubator for 6-10 min. Then, cells were pipetted up and down using a plugged Pasteur pipette to break the outgrowths. The plate was returned into the incubator for another 6 min, and then outgrowths and the enzyme were pipetted to get single cells. Then each outgrowth was transferred to a 15ml conical tube and the enzyme activity was blocked by the addition of warm culture media (DMEM/F12+10% FCS). Tubes were spun for 3min at 1000 rpm. Supernatant was aspirated off and the pellet was resuspended in an appropriate amount of ES culture media. Ten µl of the solution was loaded in the haemocytometer for counting. Then the solution was transferred into 4 well tissue culture plates to reattach. In the haemocytometer there was 5 large squares, only shiny cells were counted in those. The total number of cells in each outgrowth was calculated using the following equation:

(no. of cells counted ÷ no. of squares) x volume (ml) x 10,000

2.8 Passage bovine outgrowth derived from dissociated blastomeres

2.8.1 Mechanical passaging

When the outgrowth filled the well it was passaged and transferred to a larger well to let the outgrowth extend its proliferation. Before the passage, the new plate was prepared with coating substrate and the media as mentioned before and left in the CO_2 incubator at 38.5˚C to equilibrate. To passage the outgrowth, sterile needles were used to cut the outgrowth into parts, and then

these pieces were transferred using a pipette to the new well with the fresh media. After passaging all the outgrowths, the new plate was placed in the modular chamber to be gassed with the triple gas, and then left in the dry incubator at 38.5˚C.

2.8.2 Enzymatic passaging

To dissociate bovine outgrowths, trypsin (0.25%) (Gibco), Accutase (Gibco), dispase (0.5 mg/ml) Gibco and type IV collagenase (Gibco) were used. The enzyme was added to the outgrowth and then left in the CO_2 incubator for 5 min. The outgrowth was lifted up and easy to be transferred to gelatin 0.1% pre-coated plate. The plate was placed in the modular chamber to be gassed with the triple gas.

2.9 KARYOTYPING:

Nocodozole (0.1µg/ml) was added the night before karyotyping (1:2000). This is recommended for counting, as it shortens the chromosomes and gives a more accurate count. DMEM was in the CO_2 incubator for 10-15 min to be warm prior the use. The medium was aspirated, and then trypsin was added to the wells with outgrowths and left in the incubator for 1-2 min. Trypsin activity was blocked with DMEM media. Outgrowths with the media were spun for 3 min at 1000 rpm. The supernatant was aspirated, and about 5ml of 0.56% KCl was added to the sediment with the little media and then, incubated for 30 min in the water bath at 37˚C. Five ml of KCl from the freezer was added slowly to the side of the tube. The tube was spun for 3 min at 1000 rpm, and the supernatant was aspirated. Five ml of KCl from the freezer was added slowly without pipetting. The tube was incubated in the fridge for 30 min. The last two steps were repeated twice. Then about 100µl of ice cold KCl was added, and the solution was pipetted up and down until it was cloudy. The solution was returned to the fridge until Pasteur pipettes were made, and slides prepared. Using the Pasteur pipette, 3 drops of the cell solutions were made in each slide

by holding the Pasteur pipette vertically on the slide. Slides were left to dry, and then labelled. Slides were stained using 5% Giemsa stain for 20 min, and then washed using tap water.

2.9.1 Slide preparation

Clean microscope slides with a frosted end were used. Slides were wiped with 70% ethanol, and then they were placed into a vertical slide holder and put into a -20 freezer for 2-3 min.

2.9.2 Slide staining

Gurr buffer was prepared by dissolving 1 Gurr buffer tablet in 100 ml of MQ H_2O. Giemsa stain was prepared by adding 2.5 ml of giemsa stock solution to 47.5 ml of Gurr buffer (PH6.8). Slides can be stained at the same day of counting chromosomes if spreads are dry. Gloves must be worn when putting slides into the staining solution. Slides were placed into Giemsa stain for 20-25 min, and then they were washed carefully and gently with tap water. Slides were left to dry vertically, and then they were ready for examination under the microscope.

2.10 Nucleic Acid Manipulation

2.10.1 Total RNA isolation

Gloves were worn at all times in the molecular lab, and work space and pipettes were wiped with ethanol before starting. All steps were carried out on ice. Each sample was given a code number to distinguish it from others. Samples were kept in Trizol (Invitrogen, NZ) in -20 freezer. One µl of ms2RNA (200 ng/ µl) (Invitrogen, NZ), a carrier, and 5µl alpha globin (1pg/µl)

(Sigma) was added to make the RNA visual and sticks to the side on the tube to prevent the loss of RNA from the sample, and then they were mixed by pipetting. An appropriate amount of ice cold chloroform CHCl3 was added (1:5 ratio), and then spun at 7000 rpm (Biofuge fresco 75005521centrifuge) at 4˚C for 10 min. In empty tubes 4µl of liner poly acrylamide (5 µg/ ml) was added, which help to sterile the precipitate, while waiting for the centrifuge to stop. The top layer (aqueous phase) was transferred carefully to the tube with linear polyacrylamide, and then an equal volume of isopropanol was added, which binds to the RNA and makes it precipitate. Tubes were incubated for 10 min at room temperature, and then spun for 30 min at 13000 rpm (Biofuge fresco 75005521centrifuge) at 4˚C orientated tubes to make it easier to locate the pellet. Supernatants were decanted by aspiration carefully, and then 70% ethanol was added for washing. Tubes were spun at 13000 rpm (Biofuge fresco 75005521centrifuge) for 10 min (orientated tubes), and then supernatants were decanted again. The pellets were allowed to dry at 95˚C for 2 min, and then they were re-suspended in ~ 8 µl of DEPC water, and then centrifuged (Eppendorf Minispin plus 5453). Tubes were incubated at 65˚C for 2 min in the PCR machine, and then kept at 4˚C for 10 min to redissolve before putting on ice for 30 min.

2.10.2 Dnase treatment

One µl of 10X Dnase buffer (1ml) (Invitrogen, NZ) and 2 µl of Dnase I (1U/µl) were added, and then spun down (Biofuge fresco 75005521centrifuge). Tubes were incubated for 60 min at 37˚C in the PCR machine. EDTA (25 mM, Invitrogen, NZ) was added to stop the Dnase reaction, and then tubes were incubated at 65˚C for 10 min. Ten µl of Sodium Acetate NaOAc and 100% ethanol were added, and then tubes were left in -80 freezer for an hour. Then after freezing, samples were centrifuged at 13000 rpm (Biofuge fresco 75005521centrifuge) for 30 min at 4˚C, and then aspirated to remove the supernatants. Ethanol was used to wash the pellet, and then the tubes were

centrifuged for 10 min at 13000 rpm (Biofuge fresco 75005521centrifuge). Ethanol was aspirated and then the samples were allowed to dry. The samples were re-suspended in 11 µl of DEPC water and then vortexed, and then re-dissolved by incubating foe 2 min at 90˚C. Samples were left on ice for 2 min.

2.10.3 cDNA synthesis

One µl of both Random hexamers (Invitrogen, NZ) and dNTP mix (10mM, Invetrogen, NZ) were added on ice, and then incubated for 5 min at 65˚C. Samples were left on ice for 1 min, and then spun down in the mini centrifuge (Eppendrof Mini plus 5453). Master Mix was added to every RNA sample (2 µl 10X First strand buffer, 4 µl MgCl2 (25mM), 1 µl RNase OUT, and 1 µl Superscript III, not added to the RT control, Invitrogen, NZ). Then, samples were vortexed and then 10µl of master mix was added to 13 µl of RNA/ buffer, and then incubated for 10 min at 25˚C, then at 85˚C for 5 min. Samples then were placed in a -20 freezer to chill. One µl of RNase H (Invitrogen,NZ) was added, and then samples were incubated at 37˚C for 20 min. The RT control is mRNA that has not been transcribed to cDNA, which helps to find out any DNA contamination and should not be amplified in the PCR.

2.10.4 RT- PCR

Before starting the PCR, samples were vortexed and centrifuged shortly (Eppendrof minispin plus 5453) and all pipettes and work area were wiped with ethanol, before use. For each PCR run a positive control (group of blastocysts) and H_2O (no cDNA sample just DEPC water) were included in the analysis. Master mix was prepared by adding (20.8µl of DEPC, 2.5 µl of 10X buffer + MgCl2, 0.5 µl of dNTPs (10 mM), 1.0 µl of F+R primer and finally 0.2 µl of Taq, all from Invitrogen, NZ). Not, the volumes are per sample, if 10 samples were to be run, the volume was multiply by 10. Twenty-five µl was added to 1

µl of the cDNA sample to be run, and then samples with master mix were placed in the PCR machine Light Cycler®, by putting the capillaries in the wells. Capillaries must be locked and pushed down, before locking the lid. The soft ware was prepared prior to carrying out the run, with the appropriate annealing temperature. PCR should be run as quickly as possible. The machine was run at 95˚C for 5 min for polymerase activation, followed by 35 cycles for denaturation at 95˚C for 30 sec, annealing at the appropriate temperature for 30 sec, then elongation at 72˚C for 30 sec in each cycle, and then at 72˚C for 5 min. Capillaries were frozen at -4˚C following the run in order to load the samples later into to the gel.

2.10.5 Agarose gel electrophoresis

The gel was prepared using 1% agarose (Invitrogen, NZ) with TAE buffer (2M Tris, 250 mM Glacial Acetic Acid, 50mM EDTA+ 1L H_2O). Super safe gel stain (10000X in DMSO) (Invitrogen, NZ) was added to the gel before loading the warm gel into the clean appropriate size box to contain the gel. Clean tape was placed in both sides of the box to keep the gel inside the box. The comb was placed at one end of the gel insert to place the PCR samples later. The gel was poured into the gel box and left to set for 30 min. Bromo-methylene dye solution was added to the samples to make them sink. The gel tray was placed into the machine, with the care that the comb ends at the negative electrode. The reservoir was filled to just under the maximum mark and covered the gel with 0.5% TAE buffer. The comb was removed carefully, and then each sample (15µl) was loaded in order along the wells following the template of the samples. Between each load, the tip of the pipette was washed in the TAE buffer to avoid contamination between samples. At the end 5µl of the marker was loaded, 1KB plus DNA ladder (Invitrogen, NZ) to check the fragment size later. The lid was placed on the reservoir and the machine was turned on. For small gels the sitting was placed on low 80-90 volts and for larger gels it was

130 volts. The machine was turned off when the blue line was at the end of the gel, and then the gel was lifted out the machine in a paper towel.

2.10.6 Reading the gel

The gel was placed inside a gel-reader, and the computer was turned on using the (Quality One) programme, File: gel.doc. The visible light was turned on and the image was focused to insure that the wells can be seen clearly. Visible light was turned off, and then the UV was turned on. By using the live focus button and adjusting the intensity of light, the image can be visualised. Image was printed and saved for analysis.

2.10.7 NANOG (Invitrogen) mRNA analysis

PCR analysis was carried out according to the RT-PCR method. The primers for NANOG were:

Forward primer: CACCCATGCCTGAAGAAAGT

Reverse primer: TGCATTTGCTGGAGACTGAG

Annealing temperature: 56˚C

Fragment size: 438 bp

2.10.8 OCT4 (Invitrogen) mRNA analysis

PCR analysis was carried out according to the RT-PCR method. The primers for OCT4 were:

Forward primer: GGTTCTCTTTGGAAAGGTGTTC

Reverse primer: TGGCGACGGTTGCAAAACCA

Annealing temperature: 56˚C

Fragment size: 333 bp

2.10.9 Sox2 (Invitrogen) mRNA analysis

PCR analysis was carried out according to the RT-PCR method. The primers for Sox2 were:

Forward primer: CTA TGA CCA GCT CGC AGA

Reverse primer: GGA AGA AGA GGT AAC CAC G

Annealing temperature: 58˚C

Fragment size: 152 bp

2.10.10Klf4 (Invitrogen) mRNA analysis

PCR analysis was carried out according to the RT-PCR method. The primers for Klf4 were:

Forward primer: TCCCACCGCTCCATTAC

Reverse primer: ATGAGAACTCTTCGTGTAGG

Annealing temperature: 60˚C

Fragment size: 158bp

2.10.11 Dppa3 (Invitrogen) mRNA analysis

PCR analysis was carried out according to the RT-PCR method. The primers for Dppa3 were:

Forward primer: TCT TAC CCC TCT CCG CCT AT

Reverse primer: TGC AAG TTG CCA CTC AAC TC

Annealing temperature: 55˚C

Fragment size: 296 bp

2.10.12 Sall4 (Invitrogen) mRNA analysis:

PCR analysis was carried out according to the RT-PCR method. The primers for Sall4 were:

Forward primer: CGG GTG CTC CAA TGA ACT AT

Reverse primer: TGT CCT TCA AGA TGA GCA CG

Annealing temperature: 55˚C

Fragment size: 195bp

2.10.13 β-actin (Invitrogen) mRNA analysis

PCR analysis was carried out according to the RT-PCR method. The primers for β-actin were:

Forward primer: GGCATCCTGACCCTCAAGTA

Reverse primer: CACACGGACCTCGTTGTAGA

Annealing temperature: 52˚C

Fragment size: 100bp

2.10.14 Lefty1 (Invitrogen) mRNA analysis

PCR analysis was carried out according to the RT-PCR method. The primers for Lefty were:

Forward primer: CAG GGA CTA TGG AGC TCA GG

Reverse primer: CTG GAT GCT GAC AAT CAT GG

Annealing temperature: 55˚C

Fragment size: 268bp

2.10.15 FGF4 (Invitrogen) mRNA analysis:

PCR analysis was carried out according to the RT-PCR method. The primers for FGF4 were:

Forward primer: GCGACTTCCTCTTCTTCCC

Reverse primer: GCAGATGGAAACCGATGC

Annealing temperature: 55˚C

Fragment size: 151bp

2.10.16 Cdx2 (Invitrogen) mRNA analysis:

PCR analysis was carried out according to the RT-PCR method. The primers for Cdx2 were:

Forward primer: AGACAAATACCGGGTCGTGTACA

Reverse primer: TTTGCTCTGCGGTTCTGAA

Annealing temperature: 60˚C

Fragment size: 162 bp.

Primer	Forward/ Revese	source	Primers sequence	Annealing temperature	Fragment size
Cdx2	F	bovine	AGACAAATACCGGGTCGTGTACA	60°C	162
Cdx2	R	bovine	TTTGCTCTGCGGTTCTGAA		
Dppa3	R	bovine	TCT TAC CCC TCT CCG CCT AT	55°C	158
Dppa3	F	bovine	TGC AAG TTG CCA CTC AAC TC		
Fgf5.2	F	bovine	GCGACTTCCTCTTCTTCCC	55°C	151
Fgf5.2	R	bovine	GCAGATGGAAACCGATGC		
Klf4	F	bovine	TCCCACCGCTCCATTAC	60°C	158
Klf4	R	bovine	ATGAGAACTCTTCGTGTAGG		
Lefty 1	F	bovine	CAG GGA CTA TGG AGC TCA GG	55°C	268
Lefty 1	R	bovine	CTG GAT GCT GAC AAT CAT GG		
Nanog	F	bovine	CACCCATGCCTGAAGAAAGT	56°C	438/295
Nanog	R	bovine	TGCATTTGCTGGAGACTGAG		
Oct 4	F	bovine	GGTTCTCTTTGGAAAGGTGTTC	60°C	333
Oct 4	R	bovine	TGGCGACGGTTGCAAAACCA		
Sall4	F	bovine	CGG GTG CTC CAA TGA ACT AT	55°C	195
Sall4	R	bovine	TGT CCT TCA AGA TGA GCA CG		
Sox2	F	bovine	CTA TGA CCA GCT CGC AGA	60°C	152
Sox2	R	bovine	GGA AGA AGA GGT AAC CAC G		
β-Actin	F	bovine	GGCATCCTGACCCTCAAGTA	52°C	100
β-Actin	R	bovine	CACACGGACCTCGTTGTAGA		

Table (2.1): The primers used in PCR, their reverse and forward primers, annealing temperature and fragment size. Bovine mRNA sequences were obtained from the AgResearch-DNA sequence database and primers were designed by invitrogen.

2.11 Statistical analysis

All values are presented as a mean ± SD, unless indicated otherwise. Statistical significance was accepted at $P < 0.05$ and determined using the two-tailed Fisher-test in 2x2 tables or two-taled t-test with unequal variance).

2.12: Reagents, solutions and media composition

Embryonic stem cell media:

78% DMEM/F12 base media (Global Science and Technology) Cat# BR014G with 20% FCS(Gibco) Cat# 10091-148 and fresh 1% mercaptoethanol (Sigma) and 1% non essential amino acids (Sigma).

B199:

Bicarbonate buffered medium M199 with 1mM glutamine and 0.2mM pyruvate with antibiotics: kanamycin sulphate from streptomyces K1377-25G with stock B: 10ml of sodium bicarbonate S5 761-500G (Sigma) and stock C: .06ml of sodium pyruvate P1562-100G.

IVM medium:

IVM medium containes: B199, FCS, stock FSH, LH. B199 (base medium)+10% FCS (foetal calf serum) (Life Technology) with 10µg/ml ovine follicle-stimulating hormone (FSH) (Ovagen; Immuno- Chemical Products [ICP], Auckland, New Zealand), 1µg/ml ovine lutanizing hormone (LH) (ICP), 1 µg/ml β-estradiol E2758-1G in absolute ethanol E/0650DF/17 (Fisher Scientific) and 10µl/ml cysteamine just prior to use.

HSOF:

Hepes-buffered synthetic oviduct fluid (SOF) with 107.7 mM NaCl, 7.15 mM KCl, 0.3mM KH2PO4, 5mM NaHCO3, 3.32 mM sodium lactate, 0.069 mM kanamycin monosulfate, 20 mM Hepes, 0.33 mM pyruvate, 1.71 mM CaCl2. $2H_2O$, 3mg/ml fatty-acid free bovine albumin (ABIVP; ICP) (Thompson et. al., 1990)

Aspiration media:

H199+925 IU/ml Heparin (CP Pharmaceuticals Ltd., UK) + 20% (w/v) Albumin concentrate (ICP).

IVF SOF medium:

107.7 mM Nacl, 7015 mM KCl, 0.3 mM KH2PO4, 25 mM NAHCO3, 3.32 mM sodium lactate, 1.71 mM CaCl2.$2H_2O$, 0.04 mM kanamycin monosulfate, 8 mg/ml fatty-acid free bovine albumin (ABIVP, ICPbio), supplemented with 0.33 mM sodium pyruvate, 0.001 mM Heparin, 0.2 mM penicillamine and 0.1 mM hypotaurine.

Redigrad™

Composition: Silica sol with cavalently linked saline

Density (g/ml): 1.130 ± 0.005

Osmolarity (mOsm/kg H_2O): max. 30

Viscosity (cP): max. 15 at 20˚C

PH: 9.4 ± 0.5 at 20 ˚C to 25˚C

Endotoxin (EU/ ml): max. 2

GE Healthcare Life Science, Level 1, 8 Tangihua Street, Auckland 1010, New Zealand.

Biphasic AgR SOF (ESOF):

Biphasic AgResearch Synthetic Oviduct Fluid medium (AgR SOF, AgResearch, Hamilton, New Zealand). AgR SOF is modified formulation it contains:107.7 mM NaCl, 316 mM KCl, 100x 90 mM KH_2PO_4, 1.32g/ml 60%wv $C_3H_5O_3Na$, $C_{18}H_{36}N_4O_{11}H_2SO_4$, 25 mM $NAHCO_3$, 33mM $C3H_3O_3Na$, 171mM CaCl2 $2H_20$, 49mM MgCl2 $6H_2O$, 316 mM KCl, 60mM $C_6H_{12}O_6$ and Non Essential Amino Acid 100x, Gluta-Max 1 and (BSA).

Biphasic AgR SOF (LSOF):

LSOF contains: 107.7 mM NaCl, 316 mM KCl, 100x 90 mM KH_2PO_4, 1.32g/ml 60%wv $C_3H_5O_3Na$, $C_{18}H_{36}N_4O_{11}H_2SO_4$, 25 mM $NAHCO_3$, 33mM $C3H_3O_3Na$, 171mM CaCl2 $2H_20$, 49mM MgCl2 $6H_2O$, 60mM $C_6H_{12}O_6$ and Non Essential Amino Acid 100x, Gluta-Max 1, BM Essential Amino Acid and (BSA).

Phosphate-buffer saline (PBS):

1.9 mM/l sodium dihydrogen orthophosphate 1-hydrate, 8.4 mM/l disodium hydrogen orthophosphate 2-hydrate and 150 mM/l sodium chloride.

concentration	**Code**	**Chemicals**	**Chemicals Formula**
171 mM	A	Calccium Chloride 2 hydrate	$Ca\ Cl_2\ 2H_2O$
60 mM	D	D-(+)-Glucose	$C_6\ H_{12}\ O_6$
250 mM	E	Hepesfree acid	$C_8\ H_{17}\ N_2\ O_4\ S$
250 mM	F	Hepes Sodium salt	$C_8\ H_{17}\ N_2\ O_4$ Sna
1MU	G	Heparin Sodium salt 1,000,000	
100 MG	H	Hypotaurine	$C_2\ H_7\ NO_2$ Sna
	I	Kanamycin Monosulphate	$C_{18}\ H_{36}\ N_4\ O_{11}\ H_2SO_4$
49 mM	K	Magnesium Choloride	$Mg\ Cl_2\ 6H_2O$
316 mM	O	Potassium Chloride	KCl
100x 90 mM	P	Potasium Phosphate mono-basic	$KH_2\ PO_4$
33 mM	S	Pyruvic acid	$C_3H_3O_3$ Na
250 mM	Q	Sodium Bicarbonate	Na HCO3
107.7mM	U	Sodium Chloride	Na Cl
1.32g/ml 60%wv	X	DL-Lactate Acid	$C_3\ H_5\ O_3$ Na

Table (2.2): The chemicals used in the different media, their concentration, code and their formula.

Chemical number and company	**code**	**chemicals**
17-5445-01 (Healthcare)	1	Percoll Plus
M-7145 (sigma)	2	Non-Essential Amino Acid 100x
B6766 (sigma)	3	BM Essential Amino Acid 50x
35050 (GIBCO)	4	Gluta- Max 1
H-3149 (Sigma)	5	Heparin 5,000 1μ/ml
30044-333 (GIBCO)	6	Fetal Bovine Serum (FCS)
30036-578 (GIBCO)	7	Albumin Concentrate (BSA)
M6500-25G (Sigma)	8	Cysteamine
P4875-25G (Sigma)	9	Penicillimine
H1384-1G (Sigma)	10	Hypotaurine

Table (2.3): The chemicals used, the company they were obtained from and their code number.

medium	chemicals code
HSOF	UOPXIQEFSA7
IVF	UOPXIQSA7
ESOF	UOPXIQSAKOD247
LSOF	UOPXIQSAKPD2347

Table (2.4): The chemicals used in the different media. Each chemical was given a letter code as described in table (2.2) and (2.3).

DEPC-H_2O:

0.1% (v/v) diethyl procarbonate in mili-Q water. Mixed overnight then autoclaved for 30 min at 121˚C.

50xTAE:

242g Tris base with 571µl Glacial acetic acid and 100µl 0.5M EDTA (pH=8.0), make up to 1 L with DEPC H_2O.

Dissociation media:

HSOF-CA, 3mg/ml BSA, 0.02% EDTA and 5µg/ml cytoclasin B (CB)

Substrates:

Collagen: homemade collagen from rat tail, one part of collagen into five parts of distilled water.

Collagen (2): sigma collagen type I from rat tail (3.85 mg/ml). Product number: C3867

Gelatin: 0.1% gelatin (Sigma).

Poly-L-lysine: Sigma (0.01%). Product noumber: P4707

Fibronictin: human Fibronectin from BD Biosciences (Cat number 354008) 1mg lyophilized.

Laminin: (Invitrogen) Cat. number 23017-015, Lot. number 771124.mouse laminin from BD Biosciences (Cat. number 354232).

substrate	Stock	per 96-plate	time	temperature	post coating	supplier	cat-no
fibronectin	1 mg/ml	100 ul stock + 4900 ul PBS --> 5 ml working	>45 min	RT	remove fluid, air-dry	SIGMA	F1141
collagen I	~25 mg/ml (=2.5%)	100 ul stock + 4900 ul PBS--> 5 ml working	>hours-o/n	RT/37C or 2-8C (o/n)	remove fluid, air-dry	SIGMA	C3867
gelatin	1 mg/ml (0.1%)	5 ml stock/working	>hours-o/n	RT/37C or 2-8C (o/n)	remove fluid, air-dry	SIGMA	
g/c (1:2)	as above	1.7 ml gelatin + 3.4 ml collagen working	>hours-o/n	RT/37C or 2-8C (o/n)	remove fluid, air-dry	as above	as above
poly-L-lysine	0.1 mg/ml (0.01%)	1000 ul stock + 4000 ul PBS--> 5 ml working	>1h	RT	wash PBS, air-dry	SIGMA	P4707
laminin	1 mg/ml	50 µl stock+ 450 µl PBS-->0.5 ml working	>1h	RT	wash PBS, air-dry	Invitrogen	23017-015

Table (2.5): Summary of the substrates used for coating, their concentration, coating method and their supplier.

Chapter Three

Results

3.1 IVF development

3.1.1: Introduction

To obtain bovine outgrowths, first bovine embryos were produced by the standard IVP system. The resulting embryos were graded on day seven based on their stage of development and morphology as described in section 2. Embryos were given a grade 1, 2 or 3, depending on their quality with those graded 1 suggested to be of the best quality. The grading was recorded and development was determined and the cleavage rate at day one was recorded. The table below gives a summary of the total number of embryos used, their cleavage rate and blastocyst number (B) with the different grades. Embryos were cultured for a certain period of time depending on the experimental plan. There were a number of factors that affected the cleavage rate and the grade of embryo obtained, including the quality of the starting materials, the season in which the oocytes were obtained and technical factors. With the blastocysts graded 1 to 3 at day seven the cleavage rate was 25.0% and with blastocysts graded 1 to 2 it was 12.7%. The average cleavage rate in all the experiments, including all embryos whether they remained in culture until day seven or not, was 55.0%.

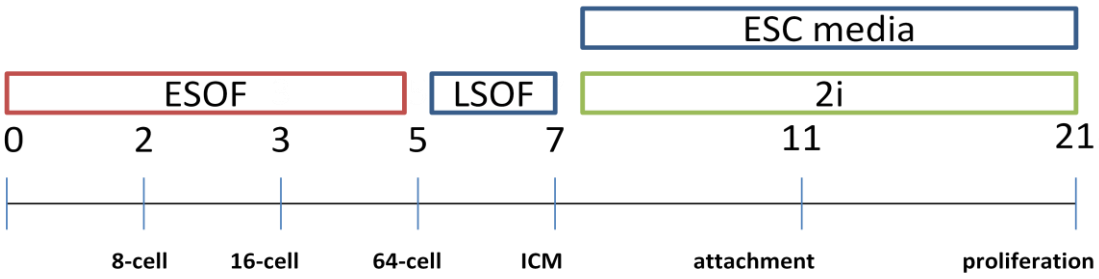


Figure (3.1): Outline of different developmental stage experiments and medium used for IVP embryos. Treatments ESOF: from day one to five, LSOF: from day five to seven, 2i or ESC media: from day 7.

n.	no. ovaries	nIVM	oocyte recovery	nIVF	nIVC	>1-cell	% cleaved (± SD)	TM 1-3	%TM 1-3 (± SD)	B1-3	%B1-3 (± SD)	B1-2	%B1-2 (± SD)	%B1-3/ cleaved	% B1-2/B1-3 (± SD)
52	1714	10517	6.14	10122	9777	5410	55.0% ±20.0 %	-	-	-	-	-	-	-	-
27	676	3735	5.53	3635	3557	1503	42.0% ±20.0 %	139	3.9% ±4.1%	889	25.0 % ±17.3 %	453	12.7 % ±8.7 %	59.0%	51.0% ±26.5 %

Table (3.1): Embryo development in IVP. The cleavage efficiency of the embryos is recorded. The first line represents runs that produced embryos for 8-cell, 16-cell and morula experiments and the second line represents runs used for ICM experiments. The results describe a summary of the average number of ovaries used, the number of oocytes obtained, the total number of embryos, the cleavage rate and the percentage of morulae, the number of blastocysts at each of the different grades. All percentage values were calculated based on the number of each category related to the initial number of embryos used. nIVM is the number of oocytes matured, nIVF is the number of embryos used, nIVC is the number of embryos cultured, TMI-3 is the number of morula obtained, B1-3and B1-2 are the number of blastocysts graded 1-3 and 1-2 respectively.

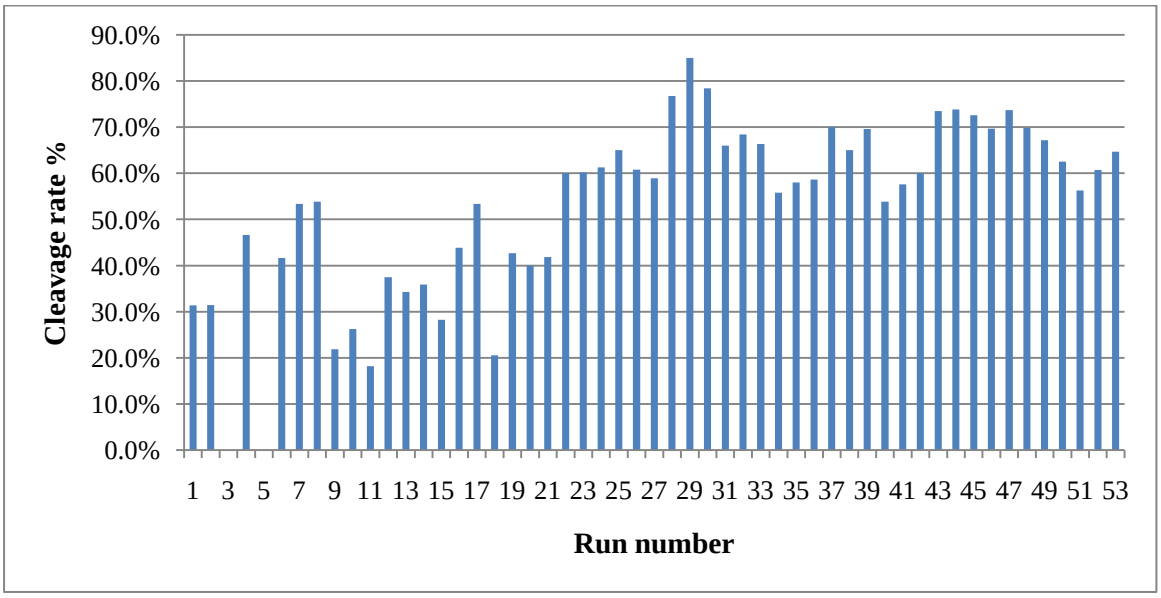


Figure (3.2): The percentage of embryos cleaved. The percentage was calculated based on the number of embryos cleaved at day one relative to the initial number of oocytes obtained. The number of embryos used in each run varied from 70to 410 with a mean value of 200.

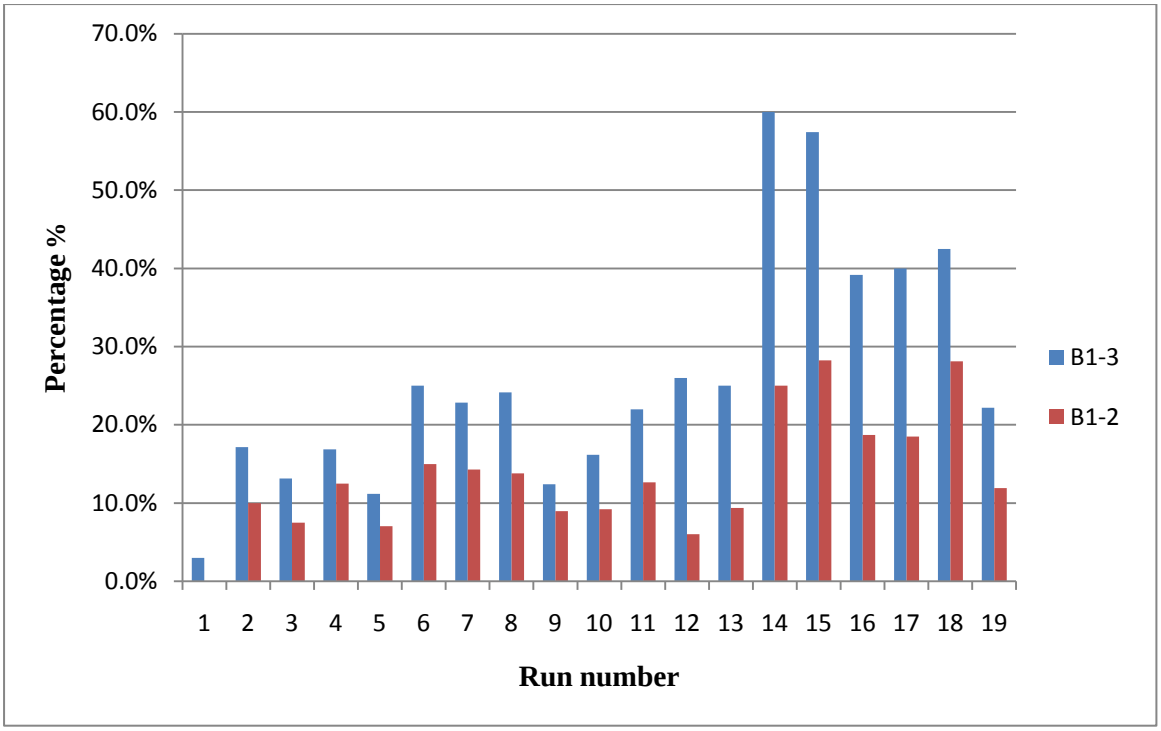


Figure (3.3): The percentage of Grade 1-3 blastocyst formation (blue) and percentage of Grade 1-2 blastocyst formation in the treatments that used day 7 embryos. The percentage was calculated based on the number of blastocysts relative to the initial number of embryos used. The number of embryos used in each run varied from 70 to 320 with a mean value of 176.

3.2 Developmental stage

3.2.1. The effects of the stage of development on attachment

3.2.2 Introduction

Embryos at different stages of development were used to examine the affect of developmental stage on attachment in a number of different substrates. ICM or 64cell, 16cell or 8cell, dissociated embryos were placed in pre-coated wells to analyse the stage of development associated with the highest plating efficiency.

3.2.3 ICM culture

ICM was isolated from day 7 embryos by immunosurgery. Isolated ICMs from grade 1 and 2 embryos were plated on pre-coated 4-well plates with the different substrates; gelatin, collagen, laminin and poly-L-lysine. The purpose of the experiment was to determine the plating efficiency of the ICM relative to that found for other stages of development. Plating efficiency was examined in the different extra cellular matrixes. The results are shown in (table 3.2) ICM cells plated on the different substrates attached to the surface, but with varying efficiency. Overall ICM attachment efficiency was (59%) 19/32.

stage	No.well	culture vessel	Average plating efficiency % ±SD
ICM	2	4-wellplate	58% (13/48) ±32.76%

Table (3.2): ICM plating efficiency in the different substrates. The plating efficiency was calculated based on the number of outgrowths produced relative to the initial number of wells used.

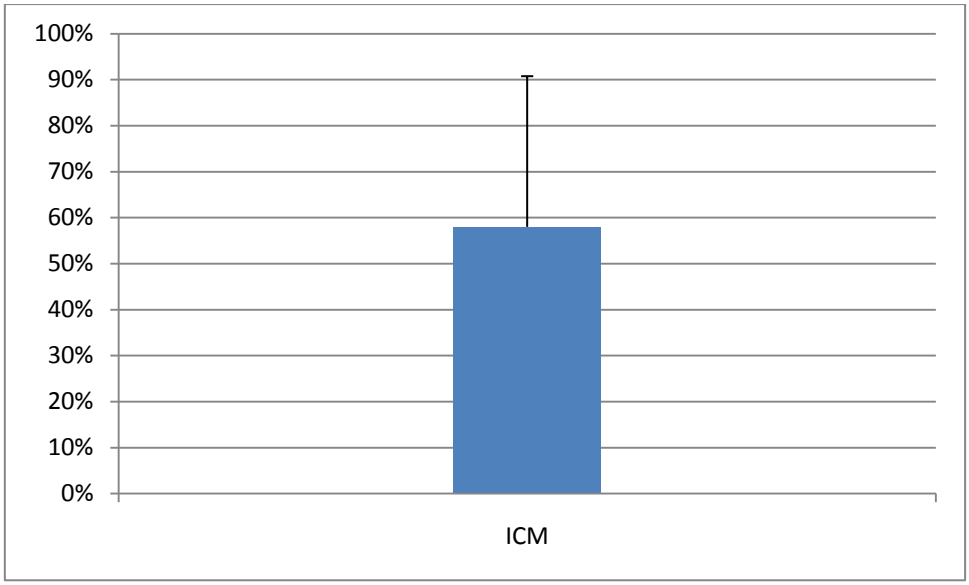


Figure (3.4): The average plating efficiency of ICM on the different substrates; gelatin, collagen, laminin and poly-L-lysine. The plating efficiency was calculated based on the number of outgrowths produced relative to the initial number of wells used.

3.2.4 Morulae culture

Dissociated blastomeres derived from day 5 embryos were placed in pre-coated 4-well plates. Plates were coated with the different substrates; gelatin, collagen, laminin, and poly-L-lysine. Attachment was recorded on day 2 post plating. Also, the medium was changed on day 2 post plating from LSOF to N2B27. Also two inhibitors CHAIR and PD032 were added to the medium at this time to inhibit differentiation, starting from 4-6-2010. The average efficiency of attachment in the different substrates was (32%) 22/68.

stage	culture vessel	plating efficiency ±SD
day 5	4 well plate	31.00% (22/68) ±32.46%

Table (3.3): The average plating efficiency of dissociated morulae (day5) in the different substrates; gelatin, collagen, laminin and poly-L-lysine. The average was calculated based on the average of plating efficiency of each substrate, and the plating efficiency was calculated based on the number of outgrowths relative to the initial number of wells used for each substrate.

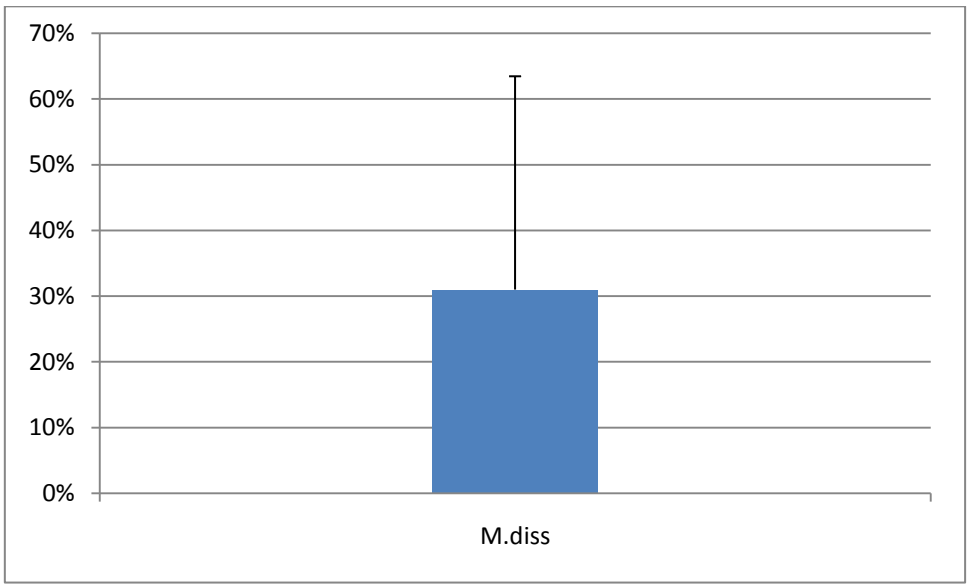


Figure (3.5): The average plating efficiency of dissociated morulae in the different substrates; gelatin, collagen, laminin and poly-L-lysine. The average was calculated based on the average of plating efficiency of each substrate.

3.2.5 16-cell culture

Dissociated blastomeres derived from day 3 embryos were placed on 96-well plates, as described in section (2). Dissociation media was used for blastomere separation to reduce pipetting the embryos. Either one or twenty blastomeres were placed in each well along with wells containing zona free blastomeres from non dissociated embryos. The medium was changed on day 6 post plating from ESOF to N2B27. Attachment was recorded on day 11 post plating and outgrowth production was recorded on day 20.The average of 16-cell attachment was (21%) 61/288. There was a significant different between (ZF not dis.) and 1 and 20 blastomeres plating efficiency ($p < 0.05$).

blastomeres/well	**culture vessels**	**%plating efficiency ± SD**
1b	96 well plate flat bottom	7% (6/72) ±9.19%
20b	96 well plate flat bottom	11% (10/72) ±14.85%
ZF not dis (16)	96 well plate flat bottom	54% (41/72) ±11.31*

Table (3.4): The average plating efficiency results of 1blastomere, 20 blastomeres and zona free non dissociated embryos derived from day 3 (16-cell) embryos were plated in 96well flat bottom plates. Blastomeres were plated in pre-coated wells with gelatin and collagen, and on wells without any coating. Plating efficiency was calculated based on the number of attachments relative to the initial number of wells used for each treatment. * indicates significant results.

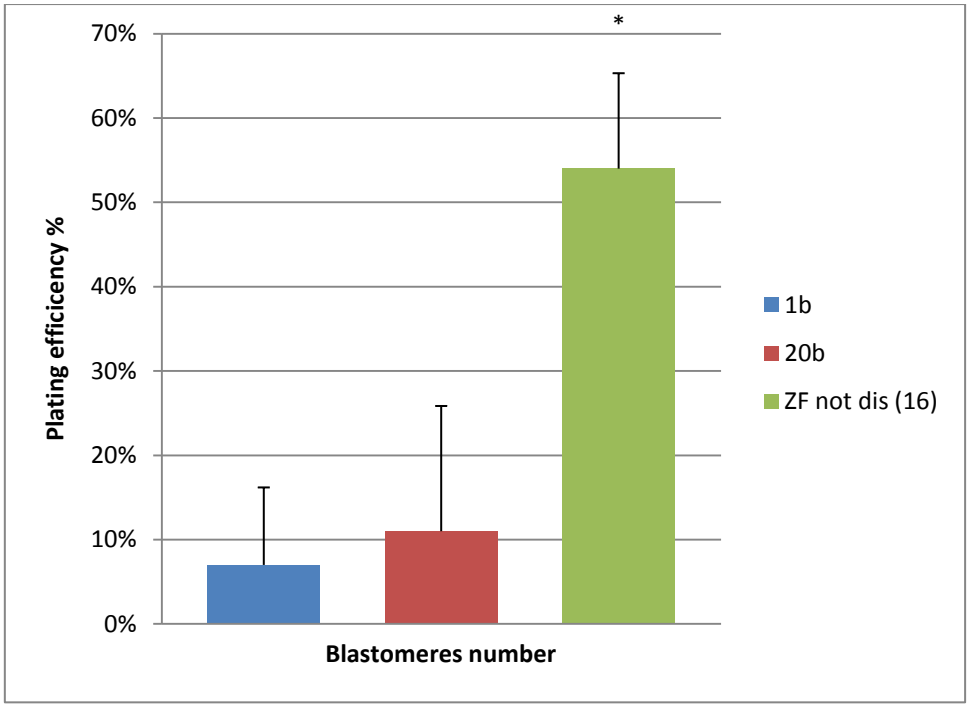


Figure (3.6): The average plating efficiency of 1blastomere, 20 blastomeres and zona free non dissociated embryos derived from 16-cell embryos. Plating efficiency was calculated based on the number of attachments relative to the initial number of wells used for each treatment. * indicates significant results.

3.2.6 8-cell culture

3.2.6.1 Introduction

Dissociated blastomeres from 8 cell stage embryos plated in pre-coated 96 well plates, displayed fluid accumulation during the first 1-2 days. Dissociated plated blastomeres increased in size during this period. Adhesion to the pre-coated culture plate was noticed from day4 in culture. On day 7, complete adhesion was recorded and the medium was changed. However, in some cases blastomeres did not attach to the surface, but were free floating in the medium. Further development or cleavage was associated with the blastomeres that did not adhere to the surface. In some cases, floating blastomeres formed blastocysts or vesicles.

3.2.6.2 Plating efficiency of 8-cell dissociated blastomeres

Blastomeres derived from day 2 dissociated embryos were plated in pre-coated 96-well plates. Different substrates were used to examine blastomere attachment including; gelatin, collagen, laminin, fibrinectin and poly-L-lysine. For each substrate different numbers of blastomeres were placed in the plate, starting from 1 single blastomere to about 20. In this set of experiments, two types of 96-well plates were used; plates with flat bottom wells and plates with conical bottom wells. The results shown in Figure 3.6 suggest that plating efficiencies increased with an increase in the number of blastomeres added to each well over the range of between 1 and 20 blastomeres per well. Overall the attachment efficiency in all the different treatments was (28%) 250/899. A Fisher test was carried out on this data which revealed that 20 blastomeres attachment was significant to the other blastomere number.

Stage	**blastomeres/well**	**%plating efficiency ± SD**
8-c (D2 pm)	1b	4% (12/288) ±6.52%
8-c (D2 pm)	2b	0% (0/48) ±0%
8-c (D2 pm)	4b	8% (4/48) ±22.19%
8-c (D2 pm)	8b	11% (11/96) ±25.52%
8-c (D2 pm)	16b	38% (9/24) ±41.08%
8-c (D2 pm)	20	65% (194/299) ±39.19% *
8-c (D2 pm)	ZF not dis	38% (18/48) ±36.98%

Table (3.5): The average plating efficiency of different blastomere number derived from 8-cell embryos, in 96-well plates. The plating efficiency was calculated based on the number of attachments to the initial number of wells in each treatment.

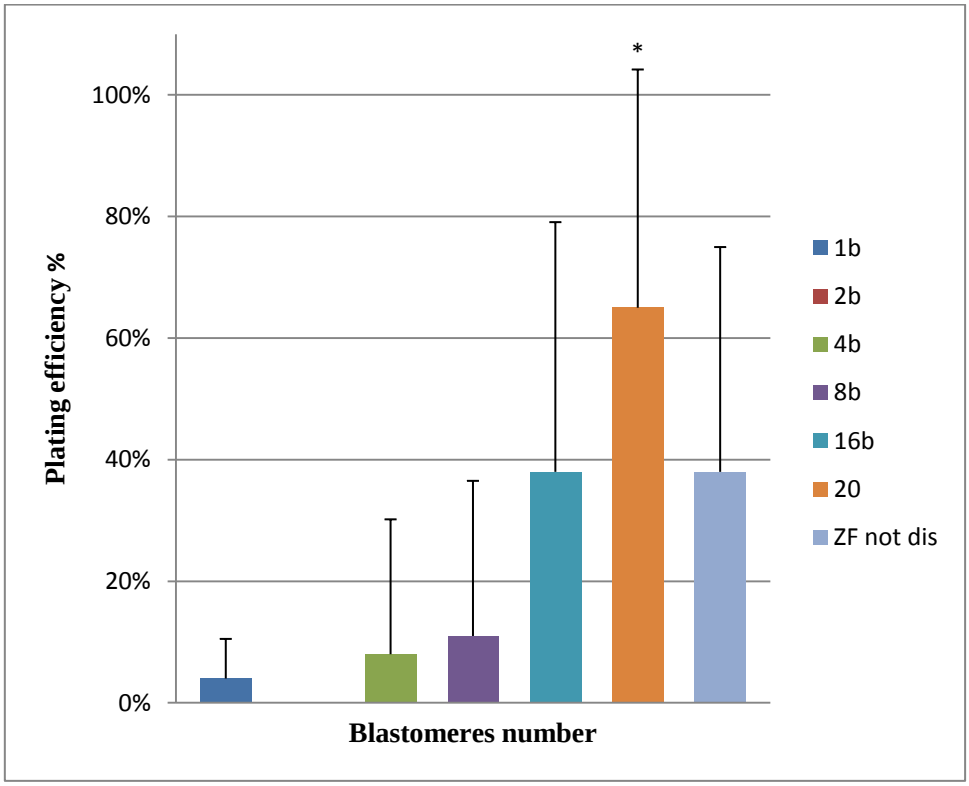


Figure (3.7): The average plating efficiency of different number of blastomeres (1blastomer, 2blastomeres, 4 blastomeres, 8 blastomeres, 16 blastomeres, 20 blastomeres, zona free not dissociated embryos and zona intact embryos) derived from dissociated day2 embryos. Plating efficiency was calculated based on the number of attachment to the initial number of wells used. * indicates significant results.

3.2.7: The effects of the stage of development on plating efficiency:

The results above suggest that the developmental stage has an effect on plating efficiency**.** ICM were associated with the best plating efficiency (58%) 19/32. Dissociated morula was the next with (32%) 22/68. Then, were 8-cell embryos with (28%) 250/899. 16-cell embryos were associated with the lowest plating efficiency with (21%) 61/288. This result is depicted in Figure (3.7). A statistical analysis carried out on this data (Fisher test), which revealed that data for ICM plating efficiency was significant to the other developmental stages ($P < 0.05$).

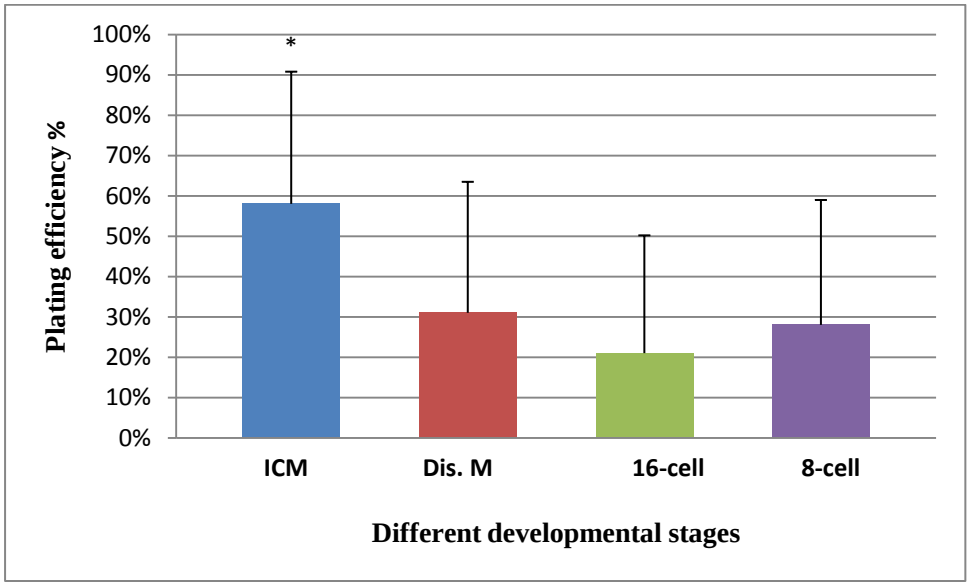


Figure (3.8): The average plating efficiency of embryos from different stages of development; ICM, dissociated morulae, 16-cell and 8-cell. The plating efficiency was calculated based on the number of wells in which ICM or blastomeres attached relative to the initial number of wells used.

3.3 The correlation between the number of blastomeres plated and attachment

3.3.1 Introduction

Blastomeres from 8-cell and 16-cell embryos were plated as described in section 2. The number of blastomeres plated varied as described below. The purpose of plating different numbers of blastomeres was to investigate if there is a correlation between the number of blastomeres plated and the plating efficiency.

3.3.2 16-cell embryo results

As depicted in table (3.4) above, plating zona free non dissociated 16-cells embryos had the highest plating efficiency (54%) 41/72whereas When 1 blastomere and 20 blastomeres were added the plating efficiencies were (7%) 6/72 and (11%) 10/72 respectively. This result is shown in Figure (3.8). A fisher test statistical analysis was carried out on this data, which revealed that plating efficiency of ZF non dis. was significantly different to 1 and 20 blastomeres ($P < 0.05$).

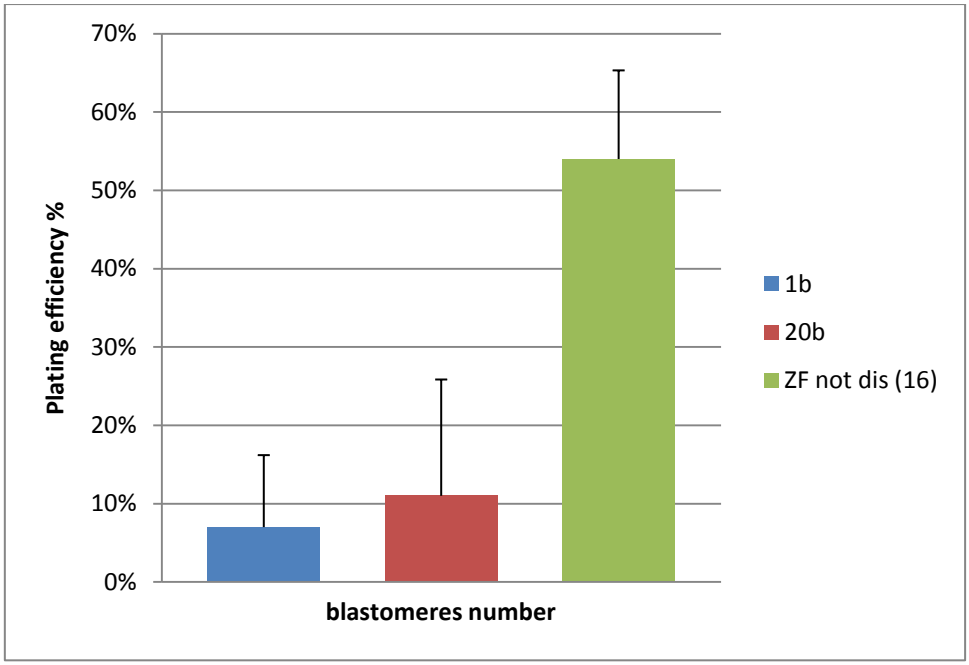


Figure (3.9): The plating efficiency of different numbers of blastomeres derived from day3 embryos. 1b; 1 blastomere, 20 b; 20 blastomeres, ZF; zona free not dissociated. The plating efficiency was calculated based on the number of wells in which embryos attached relative to the number of wells used. * indicates significant results.

3.3.3 8-cell embryo results

As depicted in Figure (3.9) the larger the number of blastomeres plated the better were the attachment results obtained, regardless of the substrate used. The addition of 1 blastomere per well resulted in 4% attachment 12/288. However, the addition of 2 blastomeres per well did not result in attachment of any embryos in experiments using 96 well plates (0%) 0/48. But, the addition of 4,8,16 and 20 blastomeres resulted in attachment and outgrowth, to variable extents. The loading of 4 blastomeres per well resulted in (8%) attachment 4/48 and the loading of 8 blastomeres per well resulted in (11.5%) 11/96. The loading of 16 and 20 blastomeres per well resulted in the highest levels of attachment with (38%) 9/24 and (65%) 194/299 respectively The use of zona free non dissociated 8-cell embryos and embryos resulted in attachment values of (38%) 18/48. A Fisher test was carried out on this data, which revealed that

20 blastomeres attachment efficiency was significantly higher than the other blastomere numbers used in the experiment ($P < 0.05$).

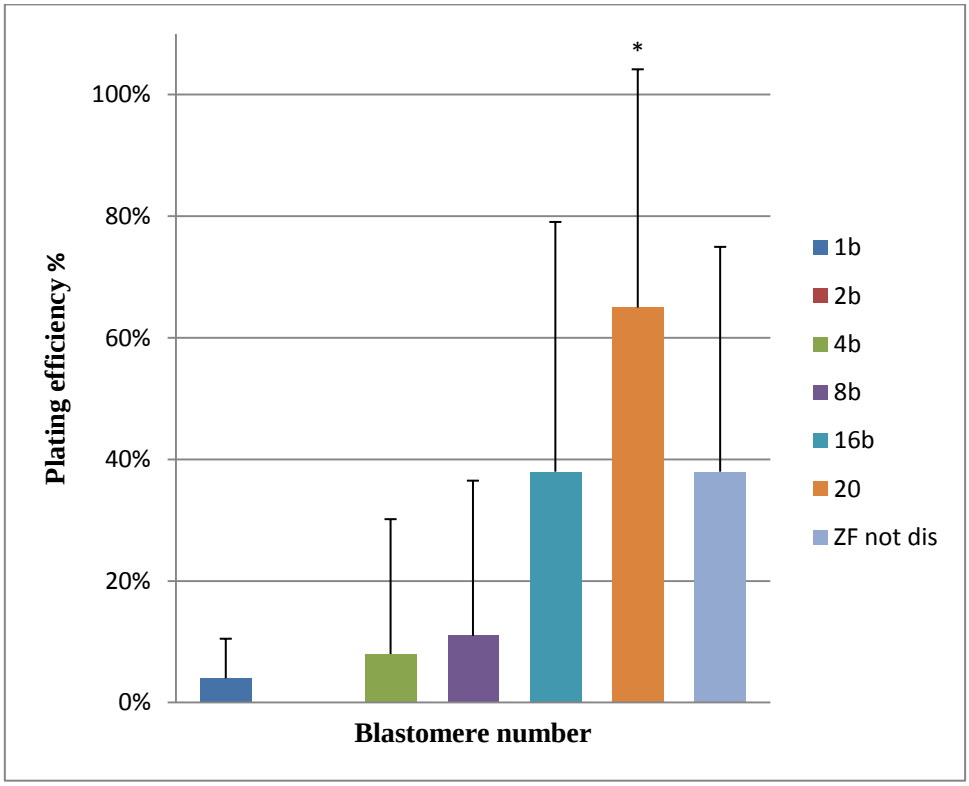


Figure (3.10): The plating efficiency using different numbers of blastomeres (1blastomere, 2blastomeres, 4 blastomeres, 8 blastomeres, 16 blastomeres, 20 blastomeres, zona free non dissociated embryos) derived from 8-cell embryos. Plating efficiency was calculated based on the number of wells in which embryos attached relative to the number of wells used. * indicates significant results.

3.4 Attachment in the different substrates

3.4.1 Introduction

In all the different treatments, plates were coated with different selected substrates as described in section (2). ICM, dissociated morula, 16-cell embryos and 8-cell embryos were analysed and selected. In addition, good quality embryos with an appropriate stage of development were further selected for better results. For ICM experiments, immunosurgery was done to isolate the ICM from the TE, as described in section 2. For the other experiments, blastomeres were dissociated and loaded into the plates as described in section (2).The overall objective of these experiments was to determine the best substrate for attachment. These experiments were repeated a number of times, in which five different substrates were used including gelatin, laminin, collagen, poly-L-lysine and a mixture of gelatin and collagen. The attachment of bovine blastomeres in the various substrates was significantly different as shown in the results below.

3.4.2 ICM attachment in the different substrates

Four different substrates were used for ICM attachment including; collagen, gelatin, laminin and poly-L-lysine. 4-well plates were coated as described in section 2. ICM was isolated by immunosurgery. As depicted in table (3.6) below gelatin and collagen had similar attachment efficiencies (75%) 6/8. Laminin had a lower efficiency of attachment with (50%) 4/8 and poly-L-lysine was the lowest with (38%) 3/8.

stage	no./well	substarte	culture vessel	no. Plated	Average of plating efficiency ± SD
ICM	8	laminin	4-well plate	4	50% ± 40.82%
ICM	8	collagen	4-wll plate	6	75% ± 28.87%
ICM	8	gelatin	4-wll plate	6	75% ± 28.87%
ICM	8	poly-L-lysine	4-wll plate	3	37.50% ± 25%

Table (3.6): The average ICM plating efficiency in the different substrates; collagen, gelatin, laminin and poly-L-lysine. The plating efficiency was calculated based on the number of wells in which embryos attached relative to the initial number of wells used.

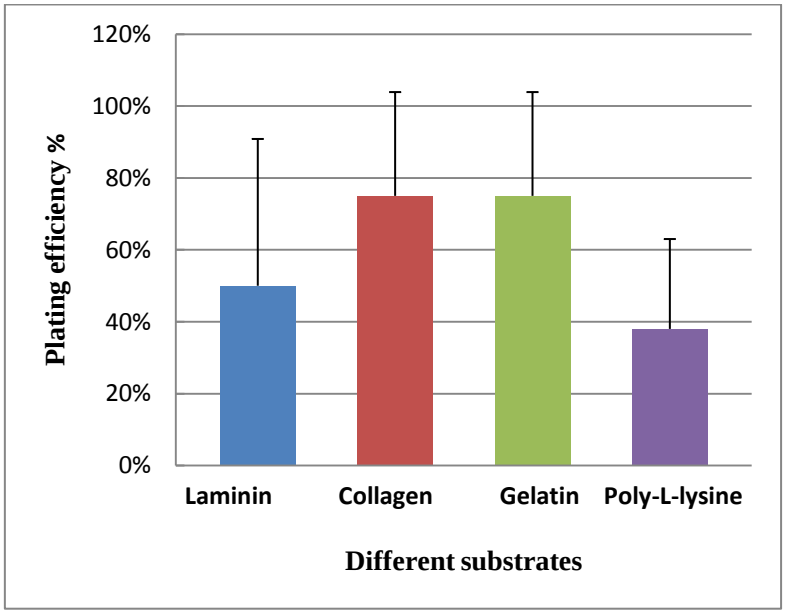


Figure (3.11): The average plating efficiency of ICM in the different substrates; collagen, gelatin, laminin and poly-L-lysine. The average plating efficiency was calculated based on the average number of wells in which embryos attached to the initial number of wells used.

3.4.3 Attachment of dissociated morula in the different substrates

Dissociated morulae were plated on the different substrates; collagen, gelatin, laminin and poly-L-lysine in order to find out if there a substrate that promote attachment. As depicted in figure 3.11 below, gelatin had the best attachment results (50%) 8/16, and then was collagen (38%) 6/16. Laminin and poly-L-lysine had lower plating efficiency with (31%) 5/16 and (18%) 3/16 respectively.

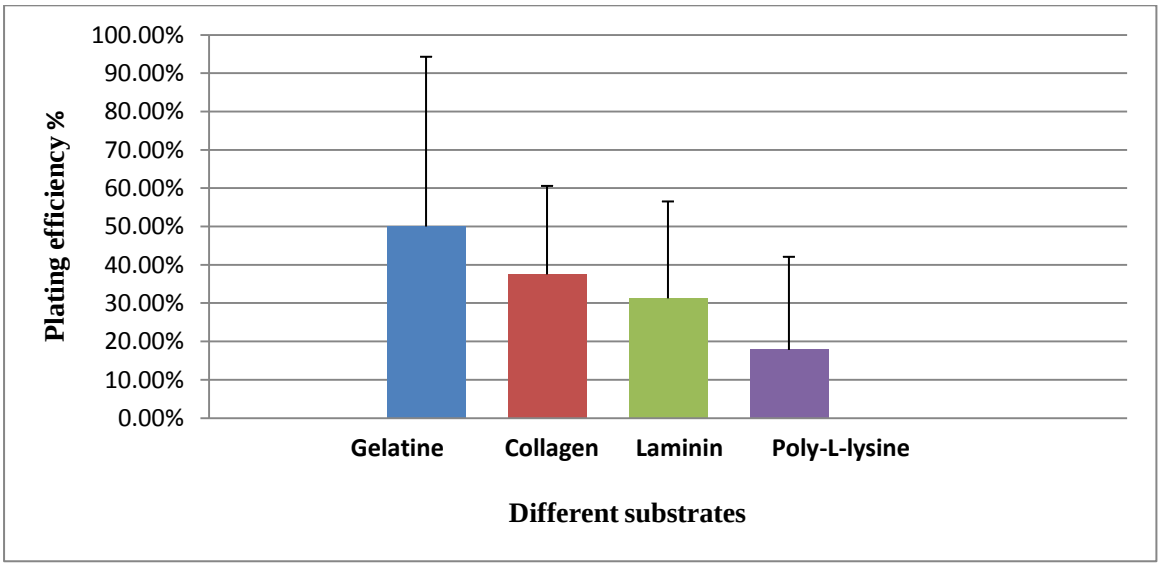


Figure (3.12): The average of plating efficiency of dissociated morulae in the different substrates; collagen, gelatin, laminin and poly-L-lysine; no: no coating was used. The average plating efficiency was calculated based on the average number of wells in which embryos attached to the initial number of wells used.

3.4.4 Attachment of 16-cell in the different substrates

As depicted in figure 3.12 below, 16-cell treatments had slightly similar attachment in collagen (28%) 29/96 and gelatin with (25%) 26/96. However, without any coating (6%) 6/96 embryos were still able to attach.

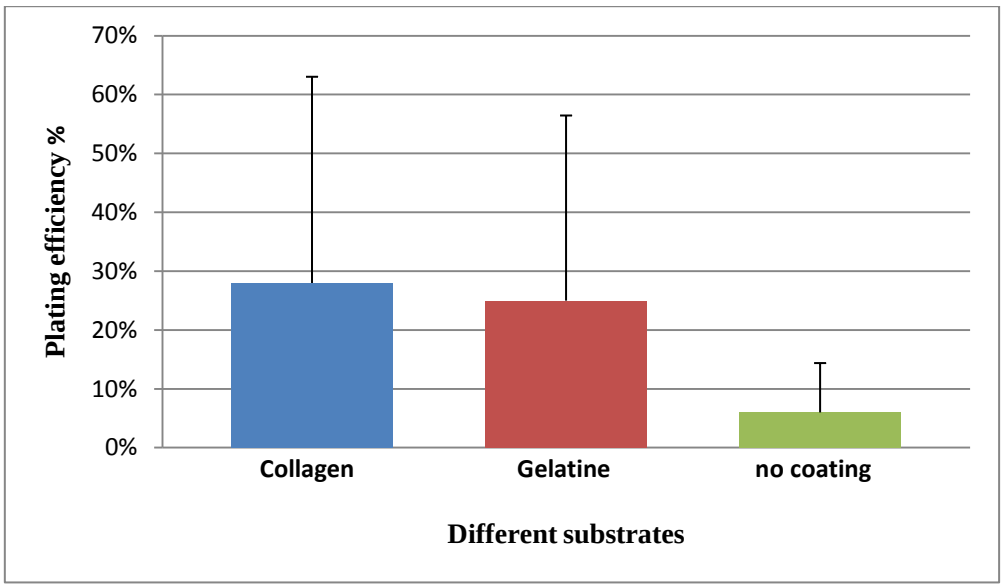


Figure (3.13): The average of plating efficiency of 16-cell different treatments in collagen, gelatin and no coating. The average plating efficiency was calculated based on the average number of wells in which embryos attached to the initial number of wells used.

3.4.5 8-cell attachment in the different substrates

The different treatments of 8-cell were plated on the different substrates; collagen, gelatin, gelatin/collagen, laminin and poly-L-lysine. The purpose of the experiments was to examine the plating efficiency in the different substrates and to find out the substrates that promote attachment. Attachment was scored on day 7 from plating and outgrowth production was scored on day 11 or 12. Media was changed every 3 to 4 days in sterile conditions. Experiment results as shown in figure 3.13 indicated that overall gelatin was the best substrate to promote attachment and outgrowth production

in all the different blastomeres number 144/298 (58%), then laminin 21/76 (28%), collagen 31/183 (17%), and poly-L-lysine was 18/131 (14%). The worst attachment was associated with fibronectin and gelatin/collagen with 4% 2/48 and 3% 2/72 respectively. These results were obtained based on the total number of blastomeres attached from the initial number of wells used. A Fisher test was carried out on this data, which revealed that the attachment efficiency was significantly higher in gelatin than the other substrates ($P < 0.05$).

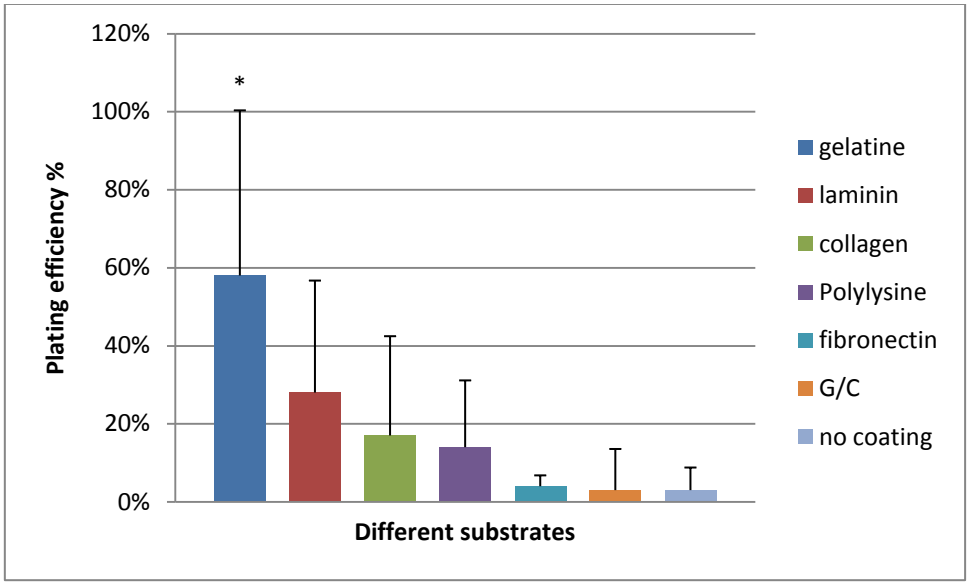


Figure (3.14): The average of plating efficiency of 8-cell in the different substrates; collagen, fibronectin, gelatin, gelatin/collagen, laminin and poly-L-lysine. The average plating efficiency was calculated based on the average number of wells in which embryos attached to the initial number of wells used. * indicates significant results.

3.5 The effects of the different substrates on cell proliferation

The objective of this experiment was to determine if the various substrates affected cell proliferation. Outgrowths after 20 to 21 days in culture were passaged enzymatically and the sells produced were counted as previously described in section (2). The number of cells counted on the different substrates was variable. Outgrowths from gelatin coated wells had more cells than the other substrates. The average cell number on gelatin was 8,290, while 2500 were obtained on collagen, 4080 were obtained on poly-L-lysine and 4640 were obtained on laminin. A two tale t-test was carried out on this data, which revealed that gelatin significantly better for cell proliferation than the other substrates ($P < 0.05$).

stage	**embryos/ well**	**no. cells plated (estimate)**	**Substrate**	**days post-plating**	**Average number of cells ±SD**
8-cell	3	24	gelatin	20	8.29E+03 ±2289 *
8-cell	3	24	laminin	20	4.64E+03 ±1574
8-cell	3	24	poly-L-lysin	20	4.08E+03 ±2515
8-cell	3	24	collagen	20	2.50E+03 ±500

Table (3.7): The average cell number in outgrowths derived from the different substrates; gelatin, collagen, poly-L-lysine and laminin. The number of cells was calculated based on the total number of cells counted from each outgrowth using the haemocytometer. * indicates significant results.

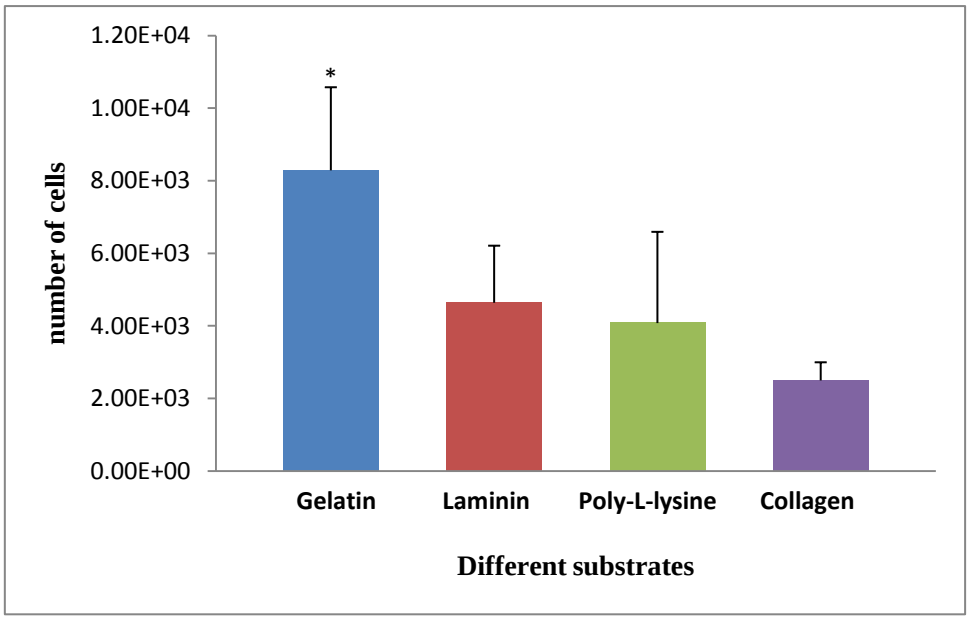


Figure (3.15): The affects of the different substrates on cell proliferation. Outgrowths were in culture for 19-21 days. The results present the average of the total number of cells counted from each outgrowth using the haemocytometer.

3.6 Dissociated 8-cell culture in tissue culture plates

3.6.1 Introduction

Although the previous set of experiments using 96 well plates were successful in terms of outgrowth production, it was difficult to obtain outgrowth from small numbers of blastomeres such as when one or two blastomeres were plated. Therefore, using dimples as micro-wells was an appropriate suggestion to examine the possibility of obtaining outgrowths from 2 blastomeres as a start. The production and coating of the dimples was described in section (2). In these experiments only gelatin, which was the substrate associated with the best outgrowth results, was used.

3.6.2 Dissociated 8-cell culture in tissue culture plates

The results of the experiments, summarised in Table 3.8 below indicated the possibility of obtaining outgrowths from 2 blastomeres, despite the low proportion of outgrowths obtained. Overall, comparison between the two sets of experiments showed that blastomere culture in 96 well plates and in tissue culture plates, produced similar results. Using a larger number of dissociated blastomeres in dimples resulted in better attachment and outgrowth production using twenty blastomeres in one dimple resulted in (79%) 22/28 of outgrowths, which was higher than the percentage obtained in 96 well plates (65%) 194/299 using this number of blastomeres. Using eight dissociated blastomeres in dimples resulted in (36%) 35/96 of outgrowth production, which was also higher than the results obtained from deriving outgrowths in 96 well plates (11.5%) 11/96. Using four dissociated blastomeres in dimples resulted in (10%) 22/216, which was also higher than the results obtained from 96-well plates (8%) 4/48. However, two dissociated blastomeres plated in dimples resulted in (2%) of outgrowth production 9/412, which is low, but still better than the (0%) 0/48 that was obtained from placing 2 blastomeres in 96 well plates. The plating efficiency was obtained based on the total number of wells in which blastomeres attached relative to the initial number of wells used. A Fisher test was carried out on this data, which revealed that 20 blastomeres had significantly better attachment efficiency than the other blastomere numbers used in the experiment ($P < 0.05$).

stage	b./well	Average plating efficiency ±
8-cell	2b	2% (9/412) ±2%
8-cell	4b	10% (22/216) ±3%
8-cell	8b	36% (35/96) ±17%
8-cell	20b	79% (22/28) ±17% *

Table (3.8): The efficiency of attachment of dissociated 8-cell (day 2) embryos plated on gelatin pre coated dimples in tissue culture plates. The percentage was calculated based on the total number of wells in which blastomeres attached from the initial number of wells used. * indicates significant results.

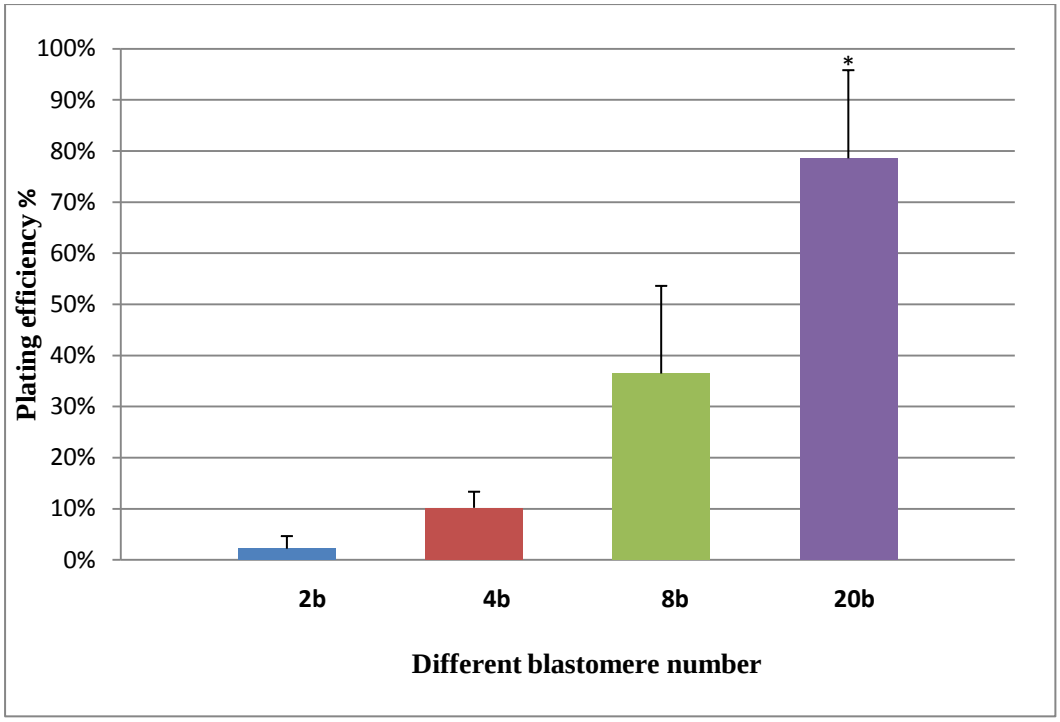
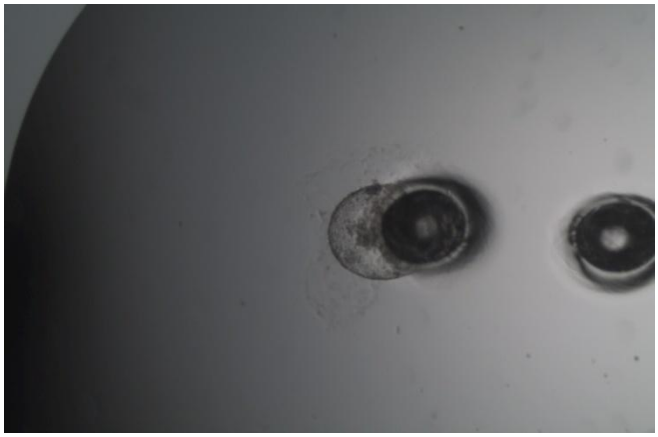
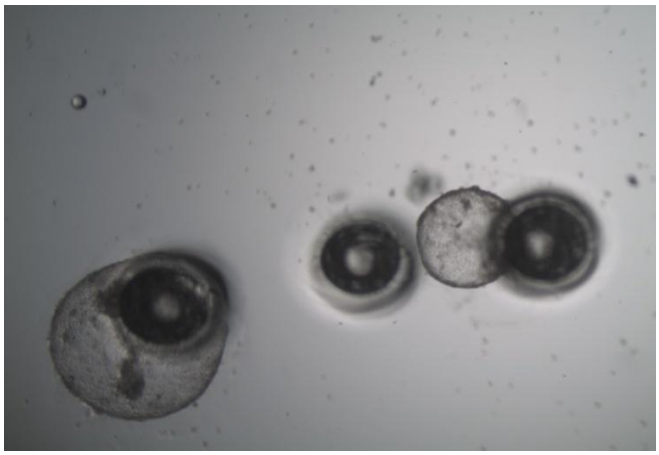


Figure (3.16): The average attachment associated with different blastomere numbers in outgrowths derived from dissociated day 2 blastomeres plated on gelatin coated dimples. The average plating efficiency was calculated based on the average number of wells in which embryos attached to the initial number of wells used.* indicates significant result.

(a)



(b)



(c)

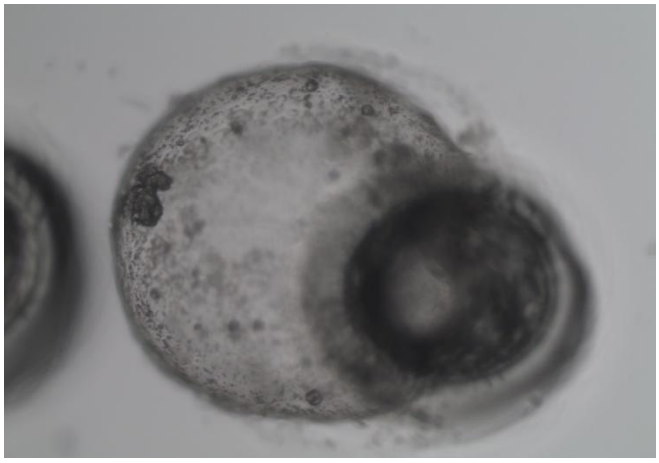


Figure (3.17): Examples of outgrowths plated in dimples coated with gelatin in a tissue culture plate, images were taken on day 21 post plating.

3.7 Passaging bovine outgrowths:

3.7.1 Introduction:

When outgrowths filled the wells containing them, they needed to be passaged. Outgrowths were passaged by transferring them from the initial well to another bigger well or to a number of wells. Outgrowths were first passaged mechanically and then enzymatically. Mechanical passaging involved cutting the outgrowths using needles and enzymatic passaging involved the use of a number of different enzymes to separate the outgrowths from the substrates. The two techniques have different efficiencies and success rates. The enzymatic and mechanical passaging methods were described in section 2.

3.7.2 Mechanical passaging:

Between 86% and 88% of the outgrowths passaged mechanically reattached. Mechanical passaging was performed as described in section (2). The outgrowths derived from dissociated blastomeres were mechanically passaged when the cells filled the wells that contained them. It was necessary to change the media continually in order to maintain the supplements for the outgrowths to be maintained in culture. Mechanical passaging was highly successful in comparison to the enzymatic passaging.

no. passaged	no. Reattached	plating efficiency
15	13	87%
22	19	86%
24	21	88%
14	12	86%

Table (3.9): Re-attachment efficiency of outgrowths passaged mechanically. Outgrowths re-attached on gelatin pre-coated 96 well plates. Plating efficiency was calculated based on the total number of outgrowths re-attached relative to the number of initial outgrowths passaged.

3.7.2 Enzymatic passaging:

As enzymatic passaging is more convenient and results in the better maintenance of a normal karyotype, the outgrowths were processed enzymatically when they filled the wells that contained them. Different enzymes were used to passage outgrowths derived from dissociated blastomeres. The outgrowths were enzymatically processed after at least one treatment of mechanical passaging. The enzymatic passaging method was described in section (2). Nine outgrowths were passaged using Trypsin, 10 using accutase and 9 using dispase. The enzymatic processing was used to lift and detach the outgrowths from the substrate. Although outgrowths were lifted, when they were replated in new gelatin coated wells, they did not attach on most occasions. In a few cases they attached (5/29) (17%), but they developed a different morphology to the undifferentiated outgrowths. Of the five outgrowths that reattached three were treated with dispase, and one was treated with accutase and one was treated with trypsin.

stage	**culture vessel**	**enzyme used**	**Average plating efficiency ±SD**
day 2	96 well plate flat bottom	Trypsin	11% (1/9) ±33.33%
day 2	96 well plate flat bottom	Accutase	10%(1/10) ±31.62%
day 2	96 well plate flat bottom	dispase	33% (3/9) ±50%

Table (3.10). Re-attachment efficiency post enzymatic passaging. Enzymes used for passaging were trypsin, accutase and dispase. Outgrowths were derived from day two dissociated blastomeres plated on pre-coated gelatin dimples. Plating efficiency was calculated based on the number of outgrowths re-attached relative to the initial number of outgrowths passaged.

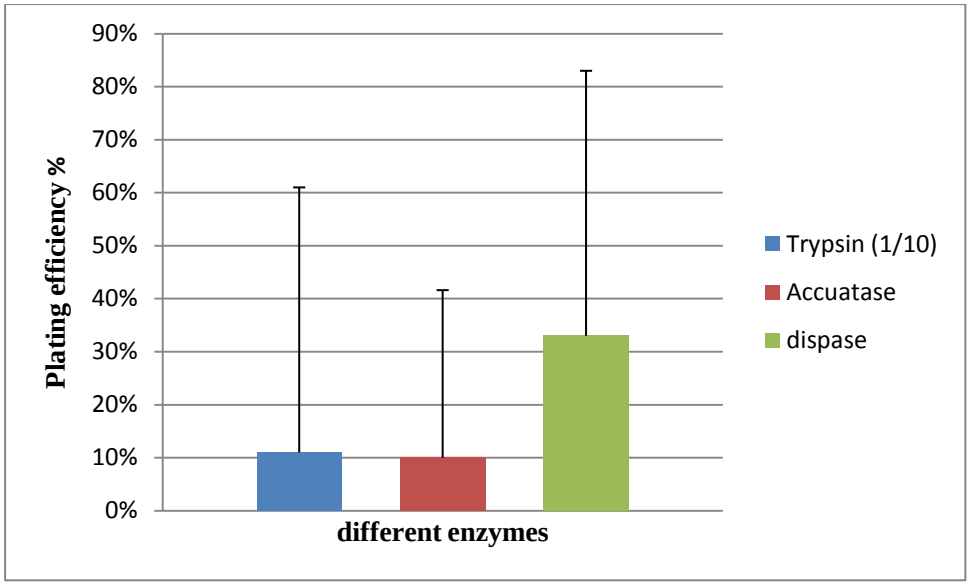
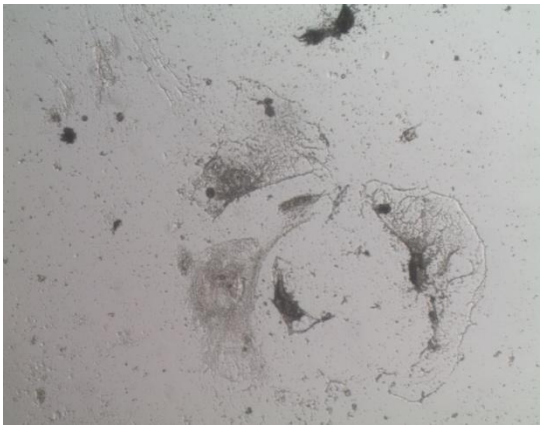
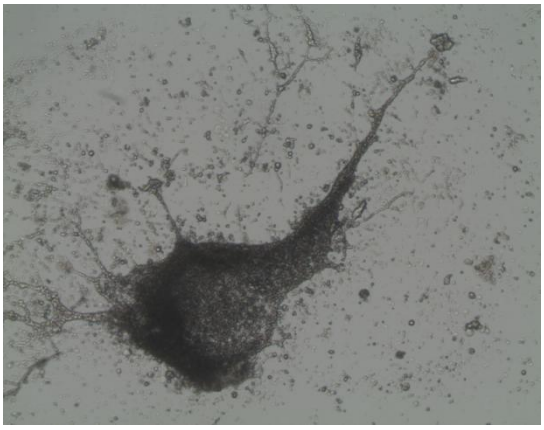


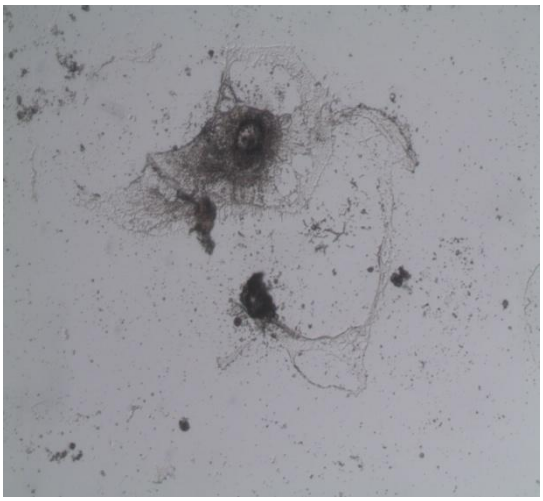
Figure (3.18): Re-attachment efficiency post enzymatic passaging. Enzymes used for passaging were trypsin, accutase and dispase. Outgrowths were derived from day dissociated blastomeres plated on pre-coated gelatin dimples. Plating efficiency was calculated based on the number of wells in which embryos attached to the initial number of wells used.



(a)



(b)



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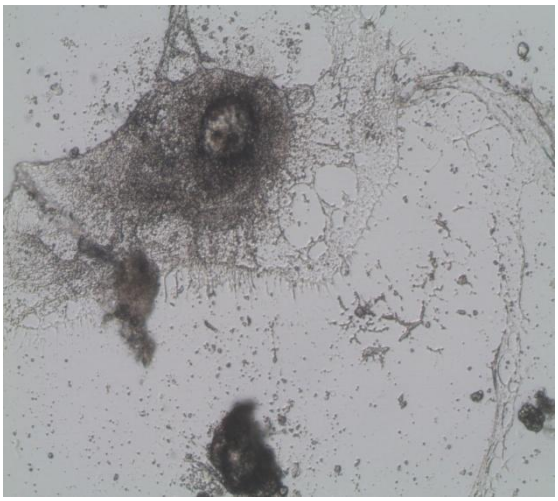


(d)

Figure (3.19): Examples of outgrowths plated on gelatin after enzymatic passaging using trypsin (2.5mg/ml).

3.8 cDNA results

3.8.1 cDNA samples

16 stored outgrowths were used to isolate RNA as described in section (2).Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. mRNA was converted to cDNA in all the samples as described in section (2). cDNA samples were amplified by PCR and the PCR amplicons were resolved using agarose gel electrophoresis as described in section (2). Outgrowths were derived from dissociated day 2 embryos as described in section (2). Those outgrowths were grown in 96 well plates coated with gelatin, gelatin/collagen (2:1) and collagen as described in table (3.12). The number of blastomeres plated in each outgrowth is presented in table (3.12). The production of a PCR amplicon using gene specific primers was used to determine which of the selected genes were expressed in those outgrowths, and if there was a correlation between the substrates used for coating, the number of blastomeres plated and the genes expressed. Bovine mRNA sequences were obtained from AgResearch-bovine database and primers were designed by invitrogen.

primer	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	P	H20
SOX2	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no	no	yes	no
DPPA3	no	no	yes	no	no	yes	yes	yes	no	yes	no	yes	yes	no	no	yes	yes	no
SALL4	yes	no	yes	yes	yes	yes	yes	yes	no	yes	yes	no	yes	yes	ND	yes	yes	no
NANOG	no	no	no	ND	no	no	no	yes	yes	no	no	yes	ND	ND	ND	no	yes	no
OCT4	no	no	no	no	yes	no	yes	yes	no	no	yes	no	yes	no	no	no	yes	no
KLF4	yes	no	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	no
FGF5	no	no	no	no	no	no	no	no	no	no	no	no	no	No	no	no	yes	no
LEFTY	no	ND	yes	no	no	no	ND	yes	no	yes	yes	yes	no	No	ND	no	yes	no
CDX2	no	no	no	yes	no	yes	ND	no	ND	yes	yes	yes	yes	yes	no	yes	yes	no
β-ACTIN	yes	weak	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	weak

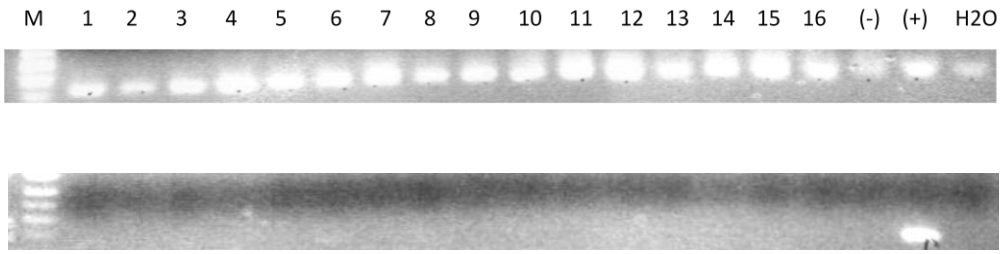
Table (3.11): Summary of results of testing the expression of the 10 genes in 16 samples derived from bovine outgrowths obtained from dissociated day 2 embryos in tissue culture plates, (no): no cDNA amplified ,no expression, (yes): cDNA amplified, positive gene expression (ND): not determined, (P) positive control using genomic DNA sample from foetal liver tissue, H_2O negative control.

sample no.	**substrate**	**blastomere no.**
1	gelatin	24b
2	gelatin	24b
3	gelatin	24b
4	gelatin	24b
5	gelatin	8b
6	collagen	24b
7	gel/col	24b
8	gel/col	16b
9	gelatin	24b
10	gelatin	24b
11	gelatin	24b
12	gelatin	24b
13	gelatin	24b
14	gelatin	24b
15	gelatin	24b
16	gelatin	24b

Table (3.12): The 16 samples used for gene expression analysis, the substrates they were plated on and the initial number of blastomeres used to derive outgrowths.

Representative gels results are shown below:

In figures (3.19, 3.20, 3.21, 3.22, 3.23, 3.24 and 3.25) the gel results testing the expression of the Sox2, Nanog, Oct4, Dppa3, Sall4, Fgf5, Cdx2, Lefty, Klf4 and β-actin genes are shown.



β-Actin (a) and SOX2 (b) gene expression

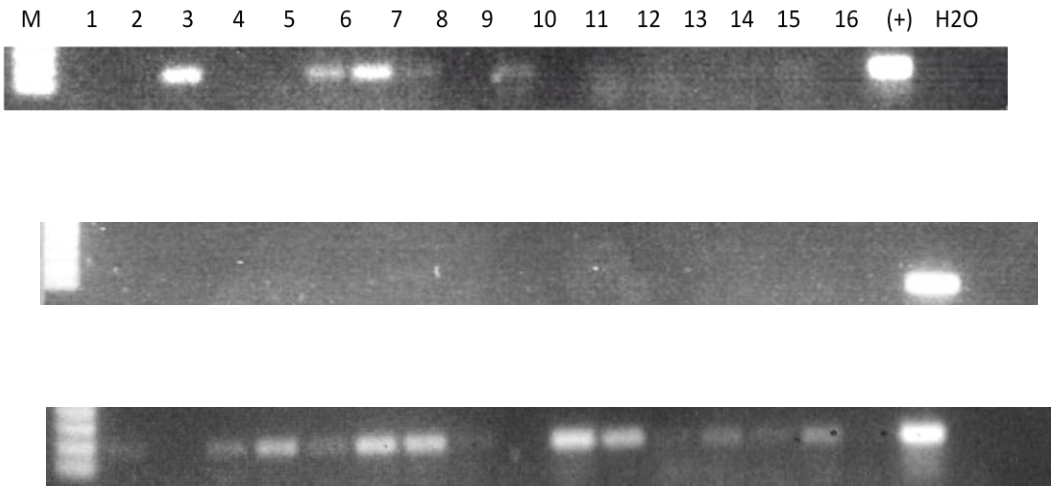
Figure (3.20): Gene expression of β-ACTIN and SOX2 in sixteen bovine outgrowths. Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. The cDNA was then subjected to PCR analysis for gene expression of β-ACTIN and SOX2. Amplified DNA was resolved by agarose gel electrophoresis to detect the expression of the selected genes. The RNA was isolated from outgrowths derived from dissociated day 2 embryos grown on pre-coated plates as described in Table (3.11). The positive control (+) contained genomic DNA from foetal liver tissue and the negative control (H_2O) contained water. A further control labelled (-) contained one blastocyst in error.

β-actin:

The results above indicated that β-actin was expressed in all the samples expect number 2, which had a weak expression. The expression in H_2O was similar to the expression in sample (2) which was negative to all primers (no DNA).

Sox2:

The results above suggest that Sox2 was not expressed in any of the outgrowths detected.



DPPA3 (a), FGF5 (b) and SALL4 (c) gene expression

Figure (3.21): (16) bovine outgrowths gene expression of DPPA3, FGF5 and SALL4. Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. The cDNA was then subjected to PCR analysis for gene expression of DPPA3, FGF5 and SALL4. Amplified DNA was resolved by agarose gel electrophoresis to detect the expression of the selected genes. The RNA was isolated from outgrowths derived from dissociated day 2 embryos grown on pre-coated plates as described in Table (3.11). The positive control (+) contained genomic DNA from foetal liver tissue and the negative control and the water (H_2O).

Dppa3:

The results above indicated that dppa3 was expressed in samples 3, 6, 7, 8, 10, 12, 13, 16.

FGF5:

The results above indicated that all the 16 samples did not express fgf5.

Sall4:

The results above indicated that sall4 was expressed in samples 1, 3, 4, 5, 6, 7, 8, 10, 11, 13, 14, 16.

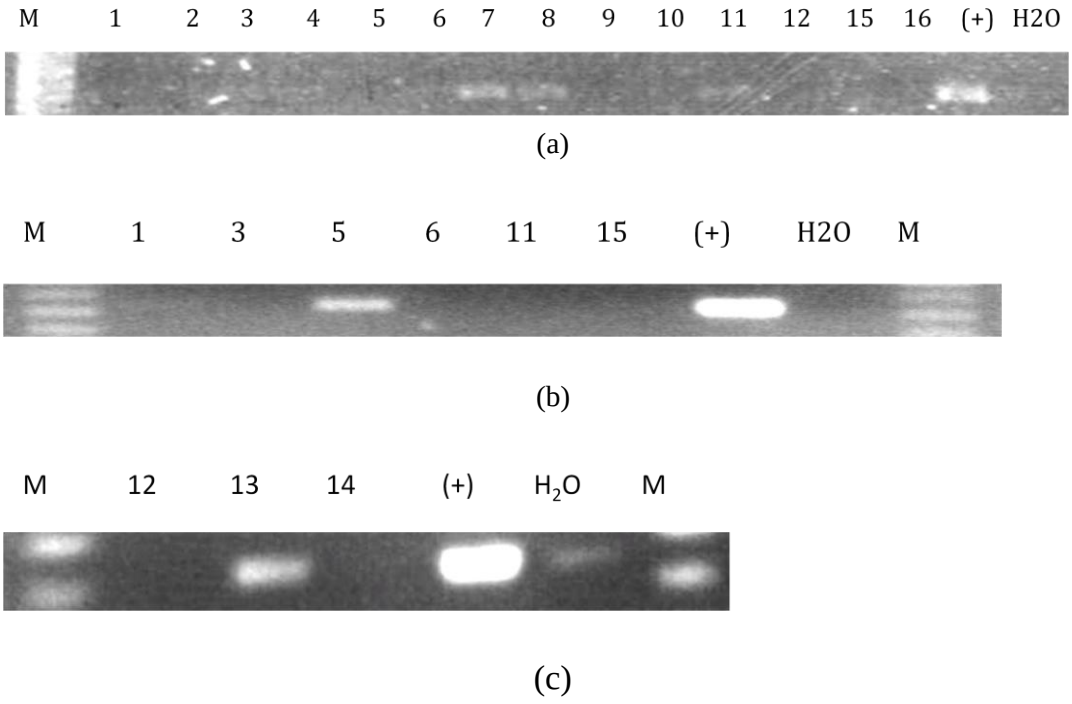
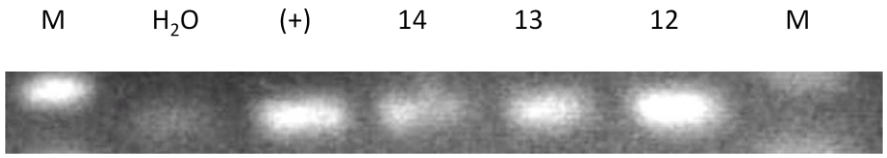


Figure (3.22): (16) cDNA samples produced using primers specific for the Oct4 gene. Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. The cDNA was then subjected to PCR analysis for gene expression of Oct4. Amplified DNA was resolved by agarose gel electrophoresis to detect the expression of the selected genes. The RNA was isolated from outgrowths derived from dissociated day 2 embryos grown on pre-coated plates as described in Table (3.11). The positive control (+) contained genomic DNA from foetal liver tissue and the negative control and the water sample (H_2O).

Oct4:

The results above indicated that oct4 was expressed in the samples 5, 7, 8, 11, 13.

(a)

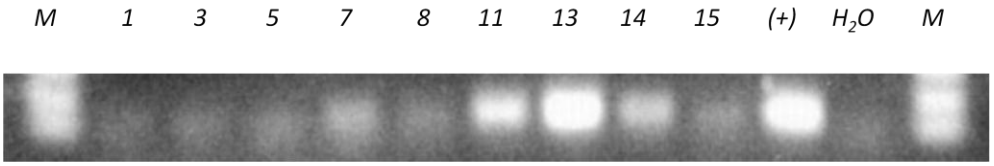


(b)

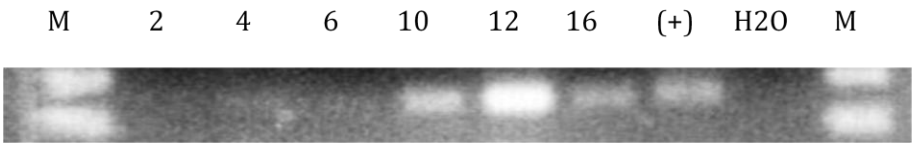
Figure (3.23): (16) cDNA samples produced using primers specific for the Klf4 gene. Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. The cDNA was then subjected to PCR analysis for gene expression of Klf4. Amplified DNA was resolved by agarose gel electrophoresis to detect the expression of the selected genes. The RNA was isolated from outgrowths derived from dissociated day 2 embryos grown on pre-coated plates as described in Table (3.11). The positive control (+) contained genomic DNA from foetal liver tissue and the negative control and the water sample (H_2O).

Klf4:

The results above indicated that klf4 was expressed in all the 16 samples.



(a)

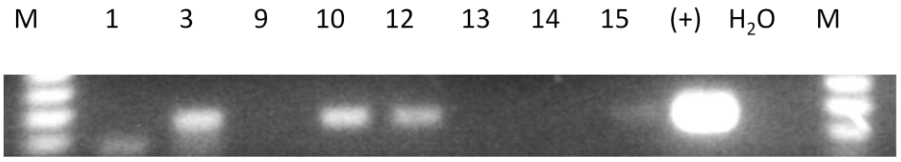


(b)

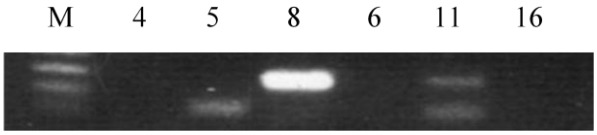
Figure (3.24): (16) cDNA samples produced using primers specific for the Cdx2 gene. Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. The cDNA was then subjected to PCR analysis for gene expression of Cdx2. Amplified DNA was resolved by agarose gel electrophoresis to detect the expression of the selected genes. The RNA was isolated from outgrowths derived from dissociated day 2 embryos grown on pre-coated plates as described in Table (3.11). The positive control (+) contained genomic DNA from foetal liver tissue and the negative control and the water sample (H_2O).

Cdx2:

The results above indicated that cdx2 primer was expressed in the samples: 4, 6, 10, 11, 12, 13, 14, 16.



(a)

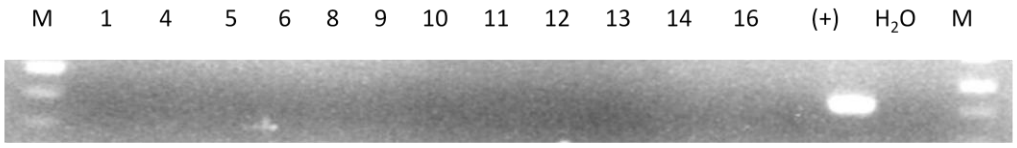


(b)

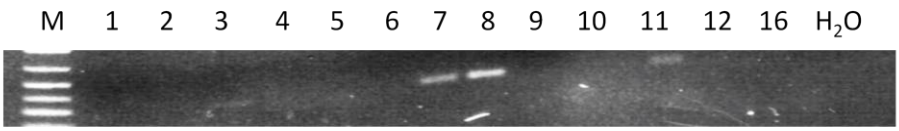
Figure (3.25): (16) cDNA samples produced using primers specific for the LEFTY gene. Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. The cDNA was then subjected to PCR analysis for gene expression of Lefty. Amplified DNA was resolved by agarose gel electrophoresis to detect the expression of the selected genes. The RNA was isolated from outgrowths derived from dissociated day 2 embryos grown on pre-coated plates as described in Table (3.12). The positive control (+) contained genomic DNA from foetal liver tissue and the negative control and the water sample (H_2O).

LEFTY:

The results above indicated that lefty was expressed in the samples: 3, 8, 10, 11, 12.



(a)



(b)

Figure (3.26): (16) cDNA samples produced using primers specific for the NANOG gene. Total RNA was extracted from bovine outgrowths and reverse transcribed into cDNA. The cDNA was then subjected to PCR analysis for gene expression of NANOG. Amplified DNA was resolved by agarose gel electrophoresis to detect the expression of the selected genes. The RNA was isolated from outgrowths derived from dissociated day 2 embryos grown on pre-coated plates as described in Table (3.11). The positive control (+) contained genomic DNA from foetal liver tissue and the negative control and the water sample (H_2O).

NANOG:

The results above indicated that NANOG was expressed in the samples: 8, 9, 12.

3.9 Karyotyping:

Karyotyping of cells in the outgrowths from dissociated blastomeres plated in dimples was carried out after the cells had been mechanically passaged and replated in a (48) well plate. Because of the small size of the wells, 5 wells with outgrowths were pooled to obtain the cells used for karyotyping as described in table (3.13). Karyotyping was done as described in section (2). The karyotyping was processed to determine if those outgrowths had a normal bovine chromosome number (60 chromosomes) and normal appearance. However, the majority of the images taken indicated an abnormal karyotype with varying numbers of chromosomes detected and abnormal chromosome appearance.

Date of plating	stage of embryo	b.no	Medium	Time in culture
3/9/2010	8 cell	8b	standard ES medium	34 days
3/9/2010	8cell	8b	standard ES medium	34 days
3/9/2010	8 cell	4b	standard ES medium	34 days
8/9/2010	8 cell	2b	standard ES medium	30 days
8/9/2010	8 cell	8b	standard ES medium	30 days

Table (3.13): Description of the five wells containing outgrowths that were pooled to produce the cell sample used for karyotyping, and the time they were in culture prior the karyotyping. 8b; outgrowth derived from 8 dissociated blastomeres, 4b; outgrowth derived from 4 dissociated blastomeres, 2b; outgrowth derived from 2 dissociated blastomeres.

Image	chromosomes no.
1	53
2	58
3	53
4	57
5	58
6	26
7	56
9	59
12	60
13	54
20	62
22	16
26	57
31	56
33	59

Table (3.14): The number of chromosomes counted in cells derived from the pooled outgrowths described above. The outgrowths were derived from dissociated day 2 embryos plated on pre-coated dimples in tissue culture plates.

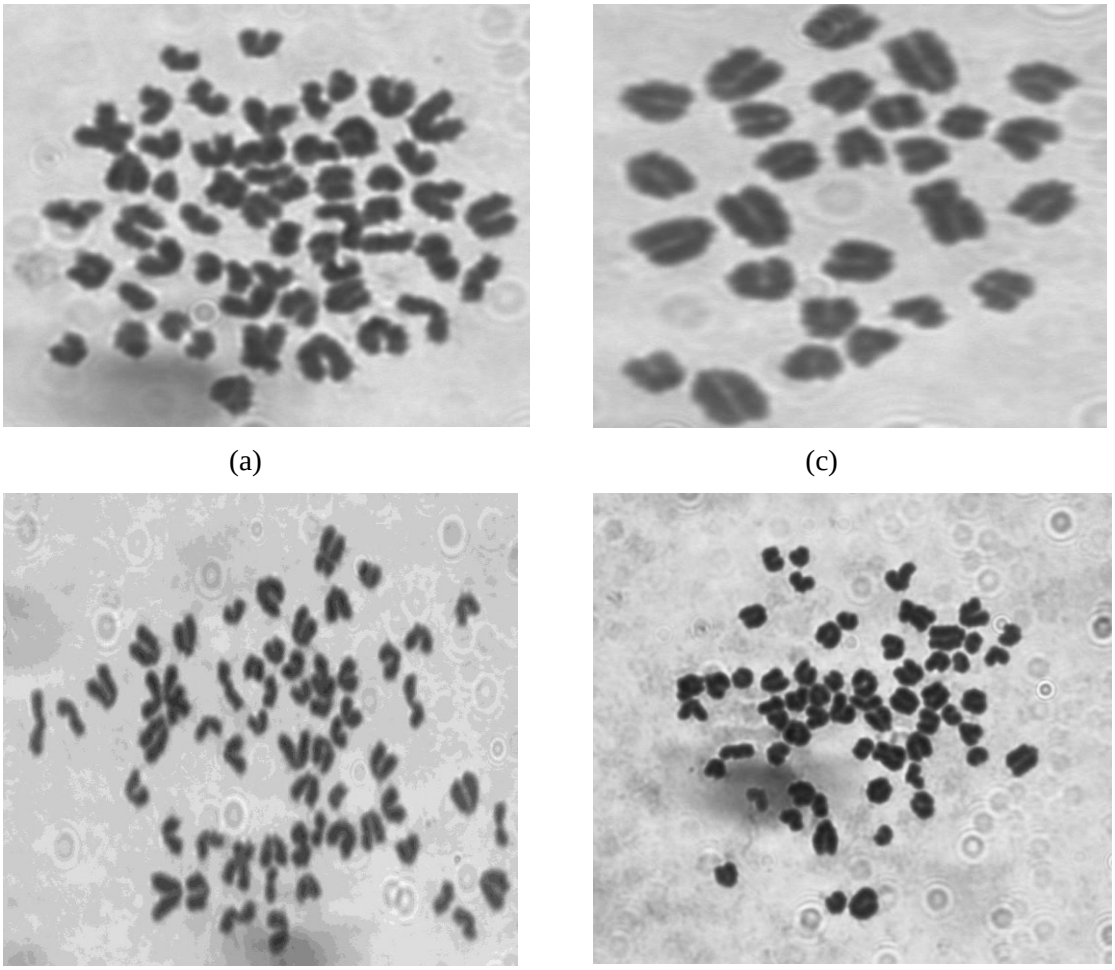


Figure (3.27): Four representative images of metaphase spreads of bovine outgrowths derived from day 2 dissociated blastomeres plated on pre-coated dimples in tissue culture plates. Outgrowths were passaged mechanically and re-plated on gelatin pre-coated 48 well plates. Outgrowths were in culture for 31-34 days prior karyotyping and they were passaged three times. Image (a), (b) and (c) represent abnormal karyotypes of 53, 59 and 26 chromosomes respectively. Image d represents an abnormal karyotype of 60 chromosomes that have unusual sex chromosome morphology.

3.10 The affects of 2i media on outgrowths maintenance

Bovine outgrowths derived from different developmental stages were cultured initially in 2i media. However, outgrowths were not maintained in 2i media, and many died prior the first passage, 22/31 (79%). Therefore, bovine outgrowths were culture in ESC media, in which cells were maintained and proliferated for longer time with medium regular changing. Outgrowths that maintained in culture up to the first passage were more than in 2i media 27/27 (100%). A t-test was carried out on these results which revealed that ESC media was significantly better in bovine outgrowths maintenance than 2i media ($P < 0.05$).

no. outgrowths in 2i media	**no. at passage 1**	**%**	**no. outgrowths in ESC media**	**no. at passage 1**	**%**
31	9	22.03%	27	27	100% *

Table (3.15): comparison between outgrowths cultured in 2i or ESC media. Outgrowths were in culture up to the first passage, and percentage was calculated based on the number of outgrowths at the first passage relative to the initial outgrowths number. * indicates significant results.

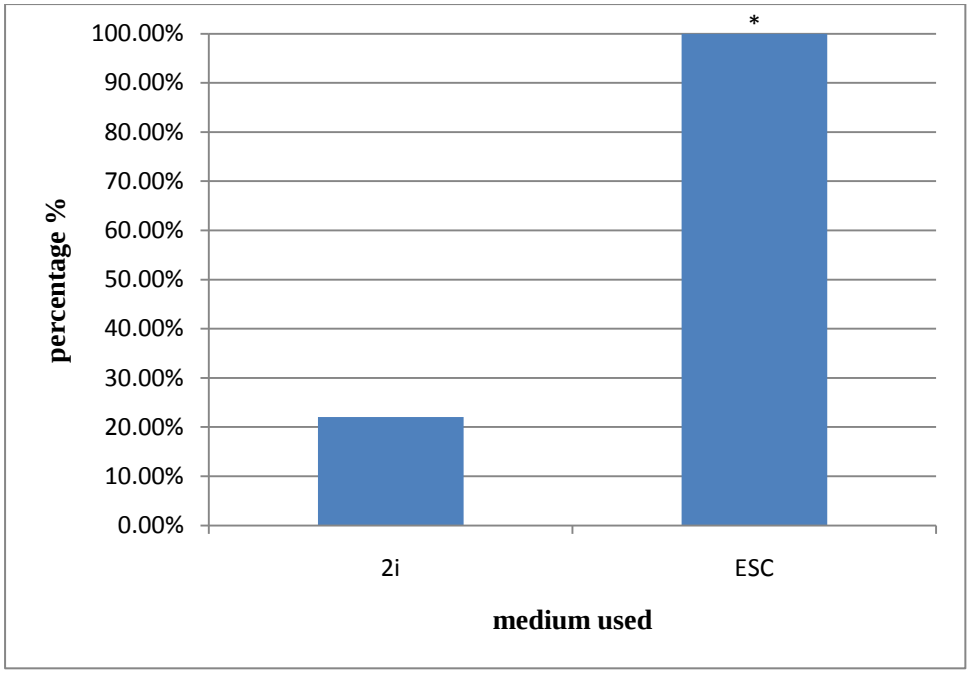


Figure (3.28): The percentage of outgrowths cultured in 2i or ESC media up to the first passage. The percentage was calculated based on the number of number of wells in which embryos attached to the initial number of wells used.

3.11 The affects of 96-well plates (conical bottom) on cell attachment

96-well flat bottom plates were used initially to derive bovine outgrowths. However, poor attachment results were associated with those plates 22/ 108 (20.37%), therefore, they were replaced with the conical bottom ones. The use of conical bottom plates resulted in better attachment 156/182 (85.71%). A Fisher test was carried out on this data, which revealed that 96-well conical bottom plates had significantly better attachment efficiency than the flat bottom ones ($P < 0.05$).

96-well plates type	no. wells	no. outgrowths	plating efficiency ± SD
Flat bottom	108	22	20.37% ± 31.19%
Conical bottom	182	156	85.71% ± 28.27% *

Table (3.16): Plating efficiency using 96-well plates (flat and conical bottom). Data was obtained from 8,16 and 20 blastomeres derived from day 2 and 3 embryos, plated in gelatin. The percentage was calculated based on the number of attachment relative to the initial number of wells used. * indicates that the result was significant.

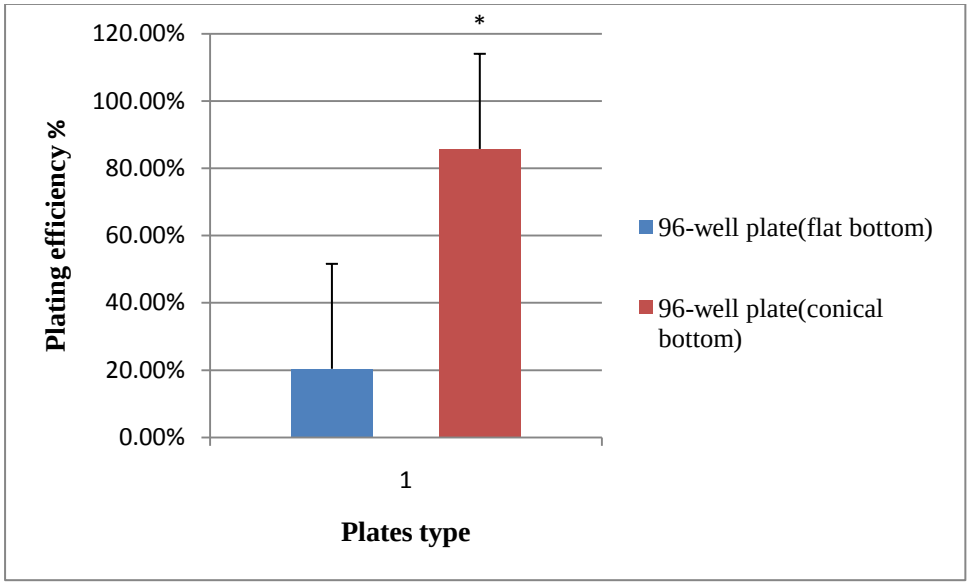


Figure (3.28): Comparison between 96-well flat bottom and conical bottom plates. The average plating efficiency was calculated based on the average number of wells in which embryos attached to the initial number of wells used. * indicates significant results.

Chapter Four

Discussion

4.1 The effects of the stage of development on attachment

In these experiments, embryos at different stages of development were used to examine their attachment efficiency. It is important to investigate the stage associated with the optimal embryonic development and high ESC derivation rate. Based on parameters such as morphological appearance and size of the resulting outgrowth, results of the experiments demonstrated that bovine embryos at different stages of development including; day 2, 3, 5, 7 embryos were capable of forming expanded outgrowths under the appropriate culture conditions. Starting materials were plated into pre-coated wells and cultured in hypoxic conditions using the appropriate medium to support bovine outgrowth.

First, a comparison of the attachment results of the different developmental stages indicates that overall, ICM attachment was significantly higher than the other developmental stages. Day 2, 3, 5 and 7embryos were used, for day 2, 3 and 5 blastomeres were dissociated then plated on the different substrates, whereas ICM cells were isolated by immunosurgery and then plated. ICM experiments were repeated 8 times, however, technical problems and issues associated with the complements led to the recording of only 2 experiments. ICM plating efficiency in all the substrates was (59%) 19/32.

Dissociated morulae experiments were repeated 8 times as well. Attachment was recorded in 22 out of 68 (32%). However, using the same stage of development, bES-like cells have been derived with higher efficiency rates (60-70%) (Strelchenko, Verlinsky et al. 2004).

For pre-compacting stages, day 2 and 3 embryos were examined and different treatments within each stage were applied. In blastomeres derived from 16-cell embryos, four different groups of starting materials were used; 1 blastomere, 20 blastomeres and zona free not dissociated embryos. Overall, attachment efficiency in all these treatments was (21%) 61/288. 8-cell stage experiments were the largest

group in terms of the number of times they were repeated (899). In those experiments successful attachment was recorded in 250 cases and the overall plating efficiency was 28%. This set of experiments had 8 different groups of starting materials to investigate the effects of blastomere number on the plating efficiency within each group.

Concerns have been raised about the ability of earl stage embryos and dissociated blastomeres to form animals. However, researchers have successfully obtained ESCs from early developmental stage embryos. In human, offspring have been produced from dissociated blastomeres derived from 4-cell stage embryos (Geens, Mateizel et al. 2009). In addition, in bovine all four blastomeres derived from 4-cell stage embryos developed into live offspring (Johnson, Loskutoff et al. 1995). HESC lines have been obtained from 2 out of 16 (12.5%) blastomeres derived from 4-cell stage human embryos, which indicated that, at least one out of the 4 cells in the 4-cell stage human embryo is pluripotent. In addition, HESC lines have been derived from single dissociated 8-cell stage human embryos (Klimanskaya, Chung et al. 2007) with a 2% success rate. (Chung, Klimanskaya et al. 2008) have improved the success rate to 20% by the use of a co-culture system with the parent embryos and laminin as an extra cellular matrix to promote adhesion to the surface (Chung, Klimanskaya et al. 2008).

In mouse (Geens, Mateizel et al. 2009) reported that offspring can be obtained from dissociated blastomeres derived from 2-cell stage embryos but they cannot be obtained from embryos at further stages of development (Geens, Mateizel et al. 2009). However, ESC lines have been produced from single dissociated blastomeres derived from 4- and 8- cell stage mouse embryos (Lorthongpanich, Yang et al. 2008). Despite the low success rate of ESCs derived from dissociated blastomeres, this system can be useful in reproduction technologies like PGD without interfering with embryo development (Chung, Klimanskaya et al. 2006). In addition, ESCs derived from pre-compacting embryos can contribute not only

to embryonic tissues, but also, to placental tissues (Mitalipova, Beyhan et al. 2001).

In 16-cell experiments, zona free non-dissociated embryos had a higher attachment rate (21%) than 20 dissociated blastomeres 11%. The lower plating efficiency may be related to the dissociation method that may affect blastomere competence. However, in experiments using 8-cell stage embryos, when 20 blastomeres were plated the highest plating efficiency 65% (194/299) was obtained. The next most efficient used zona free non-dissociated embryos and 16 blastomeres 38% (18/48) and (9/24) respectively. In 8-cell stage experiments, there was a clear positive correlation between the number of blastomeres plated and the attachment efficiency, which has been reported in previous studies (Vajta, Peura et al. 2000).

From these experiments using embryos at different stages of development; day 2, 3, 5 and 7, ICM cells had significantly the highest attachment efficiency. Day 2 and 5 experiments had nearly a similar attachment efficiency and lastly were blastomeres derived from 16-cell stage embryos. This was the overall attachment efficiency in the four groups; ICM, morula, 16- and 8-cells, without taking in account the different treatments in 16- and 8-cells embryos in which different groups with varying number of blastomeres were plated.

ESC lines have been produced from isolated ICM cells (Thomson, Itskovitz-Eldor et al. 1998), morulae (Strelchenko, Verlinsky et al. 2004), single blastomeres of 8-cell stage embryos (Chung, Klimanskaya et al. 2008) and 4-cell stage dissociated blastomeres (Geens, Mateizel et al. 2009). Bovine offspring can be obtained from dissociated 4-cell embryos (Johnson, Loskutoff et al. 1995). In addition, bES-like cells have been derived from day 7-9 blastocysts (Muñoz, Díez et al. 2008), day 12-14 embryos (GjÃ¸rret and Maddox-Hyttel 2004) and from zygotes and early cleavage embryos (Mitalipova, Beyhan et al. 2001). (Muñoz, Díez et al. 2008)

reported that the stage of development did not influence the effectiveness in establishing bES like cells (Muñoz, Díez et al. 2008). However, another study reported that day (8) hatched blastocysts produced more epiblast colonies than day 9 blastocysts (Talbot, Powell et al. 1995). Different views about the best developmental stage make it complicated to determine the appropriate stage to derive bovine colonies. In addition, bovine embryos have a long pre-implantation period compared to mouse and human. Blastocyst forms at day 7 post-fertilization, and then it increases in size especially the endoderm and trophectoderm. At day 12 bovine embryos still express Oct-4, which indicates the presence of pluripotent cells, however, cells derived from day 12 embryos were not self renewable(Gjorret and Maddox-Hyttel 2004). Therefore, it is unclear which stage of development would be better to derive bES-like cells (Gjorret and Maddox-Hyttel 2004).

Human embryos at the 4-cell stage differentially express Oct4 to direct cells either to the ICM or the TE (Deb, Sivaguru et al. 2006). In addition, Cdx2, which is a trophoblast specific gene, is expressed in late 2-cell stage mouse embryos (Hansis, Gifo et al. 2000). In human embryos genome activation occurs between the 4- and 8-cell stages and maternal effects are reduced significantly in the 8-cell stage embryos (Wong, Loewke et al. 2010). (Galan, Montaner et al. 2010) reported that blastomeres fate is not fixed until the 32-cell stage embryos however. (Muñoz, Díez et al. 2008) reported that the outer cells in compacting embryos lose their pluripotency even before the differentiation to ICM or TE. In bovine day 7 embryos, the most favoured stage used to derive bES-like cells, and in which ICM is isolated, it has been reported that ICM cells are not equally pluripotent (Oback 2008).

Second, there was a correlation between the number of blastomeres plated and the attachment efficiency. (Mitalipova, Beyhan et al. 2001) had reported that the efficiency of plating group of blastomeres was higher than blastomeres from single 2-cell embryos (Mitalipova, Beyhan et al. 2001) . In addition, (Tagawa,

Matoba et al. 2008) had better developmental potential from 4 blastomeres derived from 8-cell embryos than from single blastomers derived from 2-cell embryos (Tagawa, Matoba et al. 2008). In my experiments, when 1, 2, 4, 8, 16 and 20 blastomeres derived from 8-cell stage plated, attachment efficiency had a positive correlation with larger blastomere number. When 1 and 2 blastomeres plated, attachment was hardly obtained, however, plating of larger numbers of blastomeres resulted in better attachment efficiency, which supports the previous observations.

4.2 Attachment in the different substrates

The investigation of attachment in the different substrates revealed that the starting materials were able to become attached to all the substrates. However, varying attachment efficiencies were associated with each substrate. One observation in the experiments was that plated blastomeres resulted in either fast attachment or delayed attachment. Fast attachment to the pre-coated surface was associated with gelatin as attachment occured within the first 3-4 days post plating, (before forming blastocysts). The second observation was associated with collagen, gelatin/collagen, laminin and poly-L-lysine in which attachment was delayed to the period post the formation of the blastocyct. Attachment of blastomeres to pre-coated dishes was similar to the results obtained by (Wilton and Trounson 1989), who reported the attachment of mouse single blastomeres to the pre-coated tissue culture plates, which was contrast to the (Saito and Niemann 1991) observation who showed development in blastomeres on pre-coated dishes without attachment to the bottom of the plates. The different observations can be explained by the variation in interaction between dissociated blastomeres and the different extra cellular matrix molecules. Blastomeres surface receptors may have more affinity

for gelatin so they attach to it shortly post plating, whereas they have less affinity for the other substrates so they may attach, but after blastocysts formation.

(Irina, Young et al. 2006) reported that, ESCs derived from dissociated blastomeres developed trophectoderm like cells. To overcome this problem they aimed to recreate the ICM niche to prevent the differentiation to trophectoderm cells by the use of laminin. They found 93% of the obtained colonies were like ICM cells. In addition, they examined a number of substrates and found that laminin is better than fibronectin in promoting ICM-like colonies, because it may have a role in directing blastomeres to differentiate into ICM colonies. In another study, mESCs were undifferentiated when cultured on type I, II collagen and poly-D-lysine, whereas they differentiated when cultured on laminin or fibronectin (Hayashi, Furue et al. 2007). Ying, 2003 has reported that, mESCs proliferated with the preservation of pluripotency when they cultured on gelatin (Ying, Nichols et al. 2003). (Hoffman and Carpenter 2005) used different extra cellular matrixes to examine their attachment efficiency. They reported that laminin, matrigel and fibronectin promote long term growth (Hoffman and Carpenter 2005). A combination of fibronectin and collagen was also reported to promote attachment and proliferation (Lu, Hou et al. 2006).

Fibronectin is the first substrate to appear during bovine embryogenesis, it appears during development at day 7 as it underlies the ICM (Maddox-Hyttel, Alexopoulos et al. 2003). In addition, fibronectin exists in day13 embryos between the hypoblast and trophoblast (Maddox-Hyttel, Alexopoulos et al. 2003). Laminin and collagen IV also exist between the hypoblast and trophoblast (Maddox-Hyttel, Alexopoulos et al. 2003). Related to bovine embryogenesis, fibronectin, laminin and collagen were expected to support bES-like cells derivation and proliferation. In our laboratories laminin has been used for ICM attachment experiments. However, in my experiments, ICM attached better in gelatin and collagen 75%, while ICM plated in laminin had a 50% attachment efficiency. In 16-cell stage

experiments gelatin and collagen had no significant differences in attachment being of the order of 28% and 25% respectively. In 8-cell stage experiments, gelatin was significantly the substrate associated with the highest attachment efficiency and then laminin. This finding was not expected as most of the previous reports recommended different substrates except gelatin.

4.3 The effect of the different substrates on cell proliferation

The size of outgrowths that grew on pre-coated plates with the different substrates varied. The spread of outgrowths in wells was the highest in gelatin as the outgrowths filled the wells within the time outgrowths were in culture (19-20 days). However, there was a concern that cells attached to the other substrates may be more condensed than the ones grown on gelatin. This concern was investigated by counting the number of cells grown on the different substrates. The results of counting confirmed that outgrowths attached to gelatin had significantly ($P < 0.05$) more cells than the other substrates; poly-L-lysine, laminin and collagen.

To start proliferation certain signals are required for DNA replication (Lu, Hou et al. 2006). These signals are transmitted from the extracellular matrix to the nucleus, and proliferation factors act by the binding to receptors on the cell surface (Lu, Hou et al. 2006). Affinity to the extracellular matrix has important affects on attachment and then on proliferation (Lu, Hou et al. 2006). It has been reported that any laminin isoform supports hESCs adhesion and proliferation for a number of passages with the preservation of their pluripotency determined by the high avidity to integrin α6β1 which is expressed by hESCs (Miyazaki, Futaki et al. 2008). Other studies have reported different ECM that support attachment and proliferation (Lu, Hou et al. 2006).

	Growth				
Matrix	(+)	(++)	(+++)	(++++)	(+++++)
Fibronectin			x	X	
Collagen		x		x	
Laminin	x				
Matrigel					X

Table (4.1): A summary of cell proliferation in different coating matrices (Lu, Hou et al. 2006).

4.4 8 –cell culture in tissue culture dishes

It has been reported that outgrowths from 2 cell embryos were obtained when the amount of medium used was reduced from 50µl to 5µl (Mitalipova, Beyhan et al. 2001). In 96 well plates, outgrowths from 1 and 2 blastomeres were difficult to obtain. One possible reason for this was the ratio between the medium volume and the number of cells plated. In our group the standard culture system involves the culture of 10-15 embryos in 20µl drops until day 7 (blastocyst stage). The culture of a group of embryos in the same drop enhances their development because during early stage of development cell to cell communication is important for development and attachment (Nagao, Iijima et al. 2008).

To culture individual or a small number of blastomeres, micro-wells or (well within a well) system is used. The micro-well system is often used to culture mammalian zygotes individually or in small groups to obtain genetically identical animals and to increase the number of offspring from valuable parents (Tagawa, Matoba et al. 2008). Experiments using the micro-well system demonstrated that there is a correlation between the amount of medium and blastomere number. To culture a small number of blastomeres the amount of medium needs to be reduced. Reduction of the amount of medium used in 96-well plates was not possible because this would have led to the evaporation of medium and consequently the

dessication of the blastomeres. Another option was to overlay small volume drops with mineral oil to prevent evaporation, however, this can cause the oil to mix with the medium and lead to negative affects on blastomere development. 96-well plates have the smallest volume of wells, therefore, no other plate option was available. Therefore, depressions in tissue culture plates were the solution in order to obtain wells with a smaller volume for blastomere culture. Groups with different numbers of depressions were made in tissue culture plates, coated and then covered with 20µl medium. In these experiments, outgrowths from 2 dissociated blastomeres were obtained despite the low efficiency and attachment was faster than that found in 96-well plate experiments (4 and 7days) respectively. In addition, as found for experiments using dissociated blastomeres plated into 96-well plates experiments, the number of blastomeres plated into dimples also has a positive correlation with the attachment efficiency, iso that 20 blastomeres had the highest attachment efficiency between the different group numbers plated in depressions. However, it was important to take care to make wells with clean edges to improve the attachment of blastomeres and the spread of outgrowths.

In addition to the micro-well system, to derive blastomere derived ESCs, a co-culture system was used in other studies to enhance the success of the method. Single blastomeres were not able to develop when they were plated into 20-100 µl drops of culture medium (Chung, Klimanskaya et al. 2006). In the co-culture system dissociated blastomeres are cultured with either the parent embryo, ESCs or other dissociated blastomeres (Klimanskaya, Chung et al. 2007). In a number of studies it has been reported that the co-culture system is even essential to derive blastomere derived ESCs (Chung, Klimanskaya et al. 2006), (Irina, Young et al. 2006) and (Klimanskaya, Chung et al. 2007) with the aid of other factors including, the spatial orientation of blastomeres, the feeder layer, ESCs number and media components (Chung, Klimanskaya et al. 2006). However, it was not clear whether the success of this system was attributed to facilitating cell-cell

signals or to substances secreted by ESCs (Chung, Klimanskaya et al. 2006). In addition, co-culture system may lead to the fusion of dissociated blastomeres ESC lines with ESCs or parent embryos used for the co-culture (Chung, Klimanskaya et al. 2006), which may cause contamination of the cell line produced.

However, a co-culture system is not essential to derive bovine outgrowths in dimples (micro-wells). In my experiments attachment was achieved without the use of a co-culture system, which was reported to aid the attachment of small number of blastomeres (Klimanskaya, Chung et al. 2007). Also, (Tagawa, Matoba et al. 2008) has reported that a co-culture system was not essential to derive ESC from single dissociated blastomeres.

4.5 Passaging bovine outgrowths

In these experiments bovine outgrowths were not able to be passaged with enzymes. When outgrowths reached the appropriate size they were ready to be passaged to promote proliferation and to increase cell number. In my experiments enzymatic passaging was not successful in most cases. Discrenable outgrowths were obtained by day 11 from plating and wells were filled with cells on day 20-21 from plating, so they needed to be passaged. Cells were passaged first mechanically at least once before the enzymatic passaging. However, when cells were passaged enzymatically they ceased togrow or differentiated. A number of enzymes were used in this study for enzymatic dissociation including; trypsin, accutase, collagenase and dispase to examine their ability to dissociate in bovine outgrowths, however, none of them resulted in attachment and proliferation. In one experiment out of 29 outgrowths only 5 (17%) reattached, but they differentiated (table 3.10). The method of cell passaging is important in order to maintain ESCs. To passage mouse ESCs, trypsin has been used successfully, but the use of trypsin in bovine can cause differentiation and loss of proliferation

(Strelchenko 1996). In addition, (Mitalipova, Beyhan et al. 2001) has reported that bES-like cells were sensitive to trypsin and other enzymes therefore it has been suggested that it is better to dissociate cells mechanically (Mitalipova, Beyhan et al. 2001). However, (Gong, Roach et al. 2010) were able to passage bES-like cells by trypsin and trypLE is expressed without the loss of pluripotency. In another study, accutase which is a combination of a protease and collagenolytic enzymes was reported to be better than trypsin for enzymatic passaging (Nagaoka, Si-Tayeb et al. 2010).

ESCs are diploid primary cells with self renewal ability; they are sensitive to the culture environment and dependent on cell to cell and extra cellular matrix interactions. (Gong, Roach et al. 2010) has reported the importance of using the mechanical method of passaging for the first three times to limit the total dissociation. Cell to cell signals are essential during the early developmental stages to maintain pluripotency. The number of bESCs increased during the three mechanical passages so the cell to cell signals are suggested to have improved. Therefore, it has been suggested that applying enzymatic passaging after a series of mechanical passaging may not affect pluripotency (Gong, Roach et al. 2010).

In addition, (Gong, Roach et al. 2010) have reported the positive effects of using knock out serum replacement (KSR) instead of FCS in bESCs culture media as it may contribute to an improvement in the results of enzymatic passaging. (Kim, Chu et al. 2008) also pointed the negative influence of FCS in mESCs. However, different bovine culture systems have been examined in order to produce bovine ESC lines, but no proliferating cell lines have been obtained (Muñoz, Díez et al. 2008).

Despite the negative effects of enzymatic passaging it is an important method in gene targeting, maintenance and banking of bES-like cells. In addition, enzymatic passaging is important in obtaining single cells in order to derive gene targeted

cell lines. In (Gong, Roach et al. 2010) study of bES-like cells, colonies were passaged enzymatically beyond the third passage using 0.05% trypsin-EDTA, TrypLE expression and liberase 0.15 units/ml. They found positive results with both trypsin and TrypLE expression, however, liberase which is a combination of collagenase and protease, induced differentiation of bES- like cells. The culture medium may be the answer to the difficulties associated with enzymatic passaging. The addition of some factors like rho-associated kinase (ROCK) signalling may reduce apoptosis caused by dissociation (Couture 2010).

However, in my experiments mechanical passaging was highly efficient with 80-85% success rate. Mechanical passage is often used in ESCs system to separate ESC colonies to clumps. Some described the mechanical dissociation as a tedious, inefficient and difficult process with limited reproducibility (Couture 2010). However, others describe it as a reliable method in order to maintain HESC pluripotency (Couture 2010). In my experiments mechanical dissociation was applied once before the enzymatic dissociation, which may be not enough to increase cell number and cell to facilitate cell signals.

4.6 The effects of the culture medium on the development and maintenance of bovine outgrowths:

The affects of the culture medium on the development and maintenance of outgrowths was addressed. Two culture media were used; N2B27 and ESC medium. Both media were capable of maintaining bovine outgrowths in culture. Our culture conditions were capable to establishing and maintaining bovine outgrowths, but only for few passages. Outgrowths were derived at a similar time post plating (day 11) in both media. However, ESC culture medium was superior to N2B27 culture medium especially in outgrowth maintenance. In a number of cases outgrowths cultured in N2B27 medium died within 4-5 weeks post plating

despite the initial attachment and outgrowth production. However, outgrowths cultured in ESC medium were maintained in culture for a longer time. Some of the outgrowths were in culture for 100 days with continuous changing of the medium despite the low proliferating rate. However, the differences between the use of N2B27 medium and ESC medium could not be completely determined as the number of cells in outgrowths cultured in N2B27 was not counted.

It could be that the addition of bovine LIF and the differentiation inhibitors can improve culture medium. Also, the addition of some factors like (ROCK) rho-associated kinase signalling may reduce apoptosis caused by dissociation (Couture 2010). In addition, (Gong, Roach et al. 2010) have reported the positive effects of using knock out serum replacement (KSR) instead of FCS in bESCs culture media. However, in another report, hESCs cultured in medium with serum replacement form differentiated cells in ESCs colonies (Lu, Hou et al. 2006).

4.7 The effects of pronase and dissociation method on blastomere development and attachment

In this study, pronase was used in all experiments to remove zona pellucida, and to act as a dissociation medium in order to separate blastomeres. The use of pronase to remove the zona pellucida has been reported to have some effects on the development of dissociated blastomeres as reported in previous studies (Alfred, Meniono et al. 1983). The zona pellucida gives mechanical protection around blastomeres and it has an important role in enhancing cleavage and development (Alfred, Meniono et al. 1983). However, in these experiments the removal of zona pellucida did not negatively influence blastomere development and attachment (Alfred, Meniono et al. 1983).

In my experiments, embryos were exposed to pronase and the dissociation medium for a few minutes (2-5 min), and dissociated blastomeres were washed in

HSOF medium to remove any toxic substances. There was no clear negative effects associated with the use of pronase and the dissociation medium, which were used to manipulate embryos in all the different treatments, however, variation in attachment and cell number were related to stage of development, initial blastomere number plated and the substrate used. Attachment in some treatments was high such as 20 blastomere attachment on gelatin in conical wells (92%), which eliminated any negative effects of pronase and dissociation method on attachment.

4.8 Gene expression

Genes expressed in association with pluripotency are well established in human and mouse cells. Nanog, Oct-4 and Sox-2 are the major markers associated with pluripotency in human and mouse. In bovine, however, there are no specific markers associated with pluripotency. Nanog, which is a specific marker for ICM in human and mouse, is expressed by the ICM and the TE in bovine blastocysts. In addition, TE cells express OCT4, SOX2 and Nanog, (epiblast markers), and trophoblast specific genes (Cdx2) (Muñoz, Rodríguez et al. 2008). Therefore, these genes may not be associated with pluripotency. Pluripotency can be maintained by the balance of gene expression through the control of pluripotency networks. In the early stages of forming bovine outgrowths, TE cells are still expressing epiblast genes and at the same time trophoblast specific genes are expressed (Muñoz, Rodríguez et al. 2008), which may in part explain the slow differentiation of bovine outgrowths. More work has been suggested to be neseccary in order to identify the specific bovine pluripotency markers (Muñoz, Rodríguez et al. 2008).

To examine gene expression in bovine outgrowths in my experiments, the expression of 10 different genes was investigated including, Nanog, Oct4, Sox2,

Klf4, Dppa3, Sall4, Fgf4, Lefty, Cdx2 and β-Actin. The outgrowths used to isolate cDNA were grown on different substrates, gelatin, collagen and gelatin/collagen mixture to further examination of substrates effects on pluripotency. These genes represent ESCs or naive stem cells genes NANOG, OCT4, SOX2, DPPA3, SALL4 and KLF4, epiSCs genes LEFTY and FGF5, a trophectoderm gene CDX2 and a loading control β-ACTIN. Some of the samples used for gene expression analysis showed expression for ESCs genes, epiblast genes and trophectoderm genes, because of the lack of uniformity. There was thousands of cells in each outgrowth expression of a certain gene did not mean that this gene was expressed in all the cell population because they were heterogeneous. The use of 2i media was to promote uniformity, however, the media did not enhance proliferation and cells maintenance. Therefore, ESC medium was used. More research on 2i medium is needed to find factors that support bES-like cells proliferation and maintenance.

In addition, (Cauffman, De Rycke et al. 2009) reported that markers that define pluripotency in hESCs were unable to identify pluripotency in early developmental stages (pre-implantation embryos). More research is needed to identify gene expression patterns that associated with pluripotency in early stages of development.

4.9 Karyotyping

The karyotype of outgrowths obtained from dissociated blastomeres plated on pre-coated wells was abnormal. Outgrowths were passaged mechanically twice post karyotyping and they remained in culture for 34 days. This period in culture is considered to be short in comparison to other studies. However, it was enough to cause chromosome abnormalities to be observed in all of the metaphase spreads. These chromosome abnormalities suggested that the outgrowths were either initially abnormal or that they had acquired an abnormal karyotype because of the

media or the derivation system. Previous studies have been reported abnormal ESCs (Mitalipova, Beyhan et al. 2001). Abnormalities can be caused by different HESC derivation systems or because of the culture system and can be seen to increase with the time outgrowths spent in culture (Närvä, Autio et al. 2010). A greater time in culture means more passages which may influence karyotyping. Immortal cells become aneuploid and polyploid when they are kept in culture for a long period of time and they develop an unstable karyotyping (Mitalipova, Beyhan et al. 2001). Outgrowths can have an abnormal karyotype because it was generated from embryos with an abnormal karyotype or their abnormal karyotype was acquired in culture (Moore 2006). In contrast, some studies in HESCs reported that chromosomally abnormal embryos can produce normal ESC lines by self correction. In addition, ESCs may have an impaired DNA repair system, so it does not initiate apoptosis in abnormal cells (Närvä, Autio et al. 2010). Also, because ESCs are cultured in hypoxic conditions, their mismatch repair system is down regulated (Närvä, Autio et al. 2010).

4.10 Conclusion

The results of the present study have suggested that bovine outgrowths can be obtained from different developmental stages including dissociated blastomeres derived from day 2 and 3 embryos. The use of different ECMs prevents contamination and promoted attachment and proliferation with variable rates, in which gelatin was the best substrate to promote attachment and proliferation in blastomeres derived from day 2 embryos. Gene expression in obtained outgrowths was did not show a uniform pattern, and markers were expressed in some of the outgrowths related to the lack of homogeneity. In addition, all metaphase spreads of outgrowths at passage three presented abnormal karyotype. 2i media was used to inhibit differentiation, however, it did not promote proliferation. In terms of

tissue culture plates, 96-well conical bottom plates were better than the flat bottom ones in promoting attachment.

4.11 Future research:

The establishment of bES-like cells would be useful to define gene expression during early bovine embryogenesis. However, it is important to find the appropriate culture conditions that would promote bES-like cell proliferation and stabilise their karyotype. The use of 2i media in deriving bES-like cells is useful in order to inhibit differentiation and to introduce uniformity into the cell population, but more research is needed to find out factors that inhibit cell death associated with 2i media and to promote cell proliferation. In addition, in bovine, the genetic background variability is high, therefore more work is needed to decrease this variability and to specify bovine pluripotency markers. In order to maintain outgrowth proliferation in culture, passaging is essential, therefore, improving the enzymatic passaging method is important to work on. Still, the optimal developmental stage with which to derive the naive state is unknown, therefore, further examination of different developmental stages is needed.

Appendix

no. ovaries	nIVM	oocyte recovery	IVF date	sperm	Trtmt	nIVF	nIVC	>1-cell	% cleaved	TM1-3	%TM1-3	B1-3	%B1-3	B1-2	%B1-2	%B1-3/cleaved	% B1-2/B1-3
20	130	6.5	12/11/2009	Dustin	SOP	110	67	21	31.3%	2	3.0%	2	3.0%	0	0.0%	9.5%	0.0%
15	70	4.666667	18/11/2009	Dustin	SOP	70	70	22	31%	0	0.0%	12	17.1%	7	10.0%	54.5%	58.3%
15	100	6.666667	25/11/2009	Dustin	SOP	100	94	0	0%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
10	60	6	2/11/2009	Dustin	SOP	60	60	28	47%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	180	6	26/01/2010	Dustin	B- culture	180	160	0	0%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	220	7.333333	27/01/2010	Dustin	B- culture	215	180	75	42%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	320	10.66667	3/2/2010	Dustin	B- culture	300	300	160	53%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	160	5.333333	4/2/2010	Dustin	B- culture	130	130	70	54%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	170	5.666667	9/2/2010	Dustin	ICMculture	170	160	35	22%	4	2.5%	21	13.1%	12	7.5%	60.0%	57.1%
30	160	5.333333	10/2/2010	Dustin	ICMculture	160	160	42	26%	3	1.9%	27	16.9%	20	12.5%	64.3%	74.1%
30	170	5.666667	16/02/2010	Dustin	ICMculture	170	170	31	18%	3	1.8%	19	11.2%	12	7.1%	61.3%	63.2%
30	150	5	18/02/2010	Dustin	ICMculture	140	120	45	38%	6	5.0%	30	25.0%	18	15.0%	66.7%	60.0%
30	150	5	23/02/2010	Dustin	ICMculture	145	140	48	34%	5	3.6%	32	22.9%	20	14.3%	66.7%	62.5%
30	145	4.833333	24/02/2010	Dustin	ICMculture	145	145	52	36%	6	4.1%	35	24.1%	20	13.8%	67.3%	57.1%
30	140	4.666667	2/3/2010	Dustin	ICMculture	140	145	41	28%	8	5.5%	18	12.4%	13	9.0%	43.9%	72.2%
30	130	4.333333	4/3/2010	Dustin	ICMculture	130	130	57	44%	22	16.9%	21	16.2%	12	9.2%	36.8%	57.1%
30	120	4	10/3/2010	Dustin	ICMculture	110	90	48	53%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	180	6	11/3/2010	Dustin	ICMculture	180	170	35	21%	0%	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
35	170	4.857143	16/3/2010	Dustin	ICMculture	160	150	64	43%	17	11.3%	33	22.0%	19	12.7%	51.6%	57.6%

35	180	5.142857	17/3/2010	Dustin	ICMculture	170	150	60	40%	6	4.0%	39	26.0%	9	6.0%	65.0%	23.1%
30	180	6	18/3/2010	Dustin	ICMculture	180	160	67	42%	9	5.6%	40	25.0%	15	9.4%	59.7%	37.5%
35	220	6.285714	24/3/2010	Dustin	ICMculture	220	220	132	60%	0	0.0%	132	60.0%	55	25.0%	100.0%	41.7%
35	220	6.285714	25/3/2010	Dustin	ICMculture	220	216	130	60%	5	2.3%	124	57.4%	61	28.2%	95.4%	49.2%
36	250	6.944444	8/4/2010	Dustin	ICMculture	240	235	144	61%	18	7.7%	92	39.1%	44	18.7%	63.9%	47.8%
30	210	7	13/4/2010	Dustin	ICMculture	205	200	130	65%	2	1.0%	80	40.0%	37	18.5%	61.5%	46.3%
30	170	5.666667	14/4/2010	Dustin	ICMculture	160	153	93	61%	3	2.0%	65	42.5%	43	28.1%	69.9%	66.2%
60	320	5.333333	20/4/2010	Dustin	ICMculture	310	302	178	59%	20	6.6%	67	22.2%	36	11.9%	37.6%	53.7%
32	250	7.8125	28/4/2010	Dustin	M culture	247	245	188	77%	100	40.8%	0	0.0%	0	0.0%	0.0%	0.0%
30	210	7	29/4/2010	Dustin	M culture	205	202	161	85%	85	42.1%	0	0.0%	0	0.0%	0.0%	0.0%
30	200	6.666667	3/5/2010	Dustin	M culture	200	190	149	78%	80	42.1%	0	0.0%	0	0.0%	0.0%	0.0%
30	160	5.333333	4/5/2010	Dustin	M culture	150	150	99	66%	49	32.7%	0	0.0%	0	0.0%	0.0%	0.0%
30	200	6.666667	12/5/2010	Dustin	M culture	190	190	130	68%	80	42.1%	0	0.0%	0	0.0%	0.0%	0.0%
30	230	7.666667	13/5/2010	Dustin	B- culture	220	220	146	66%	65	29.5%	0	0.0%	0	0.0%	0.0%	0.0%
30	170	5.666667	19/5/2010	Dustin	M culture	170	165	92	56%	67	40.6%	0	0.0%	0	0.0%	0.0%	0.0%
30	260	8.666667	26/5/2010	Dustin	B- culture	250	250	145	58%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	300	10	29/5/2010	Dustin	M culture	290	290	170	59%	84	29.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	260	8.666667	1/6/2010	Dustin	B- culture	255	250	175	70%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	210	7	3/6/2010	Dustin	M culture	200	200	130	65%	50	25.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	150	5	8/6/2010	Dustin	B- culture	130	125	87	70%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
60	410	6.833333	14/6/2010	Dustin	B- culture	400	390	210	54%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%

37	190	5.135135	15/6/2010	Dustin	B- culture	180	165	95	58%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
33	190	5.757576	21/6/2010	Dustin	B- culture	180	175	105	60%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	210	7	22/6/2010	Dustin	B- culture	200	200	147	74%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
32	230	7.1875	28/6/2010	Dustin	B- culture	220	210	155	74%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	190	6.333333	7/7/2010	Dustin	B- culture	180	175	127	73%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
43	300	6.976744	3/8/2010	Dustin	B- culture	285	280	195	70%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
40	310	7.75	9/8/2010	Dustin	B- culture	290	285	210	74%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
37	230	6.216216	10/8/2010	Dustin	B- culture	220	215	150	70%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
34	147	4.323529	17/8/2010	Dustin	B- culture	145	140	94	67%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
40	145	3.625	25/8/2010	Dustin	B- culture	130	128	80	63%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
50	330	6.6	31/8/2010	Dustin	B- culture	320	320	180	56%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
40	150	3.75	1/9/2010	Dustin	B- culture	140	140	85	61%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%
30	160	5.333333	9/6/2010	Dustin	B- culture	155	150	97	65%	0	0.0%	0	0.0%	0	0.0%	0.0%	0.0%

Table (3.1): Summary of the number of ovaries used, number of oocytes obtained, treatments, total number of embryos, cleavage rate and the percentage of morulae and blastocysts produced with the different grades. The percentage calculated based on the number of each category related to the initial number of embryos.

stage	no./well	Substarte	culture vessel	no. Plated	plating efficiency
ICM	2	laminin	4-wellplate	1	50%
ICM	2	laminin	4-wellplate	0	0%
ICM	2	collagen	4-wellplate	1	50%
ICM	2	collagen	4-wellplate	1	50%
ICM	2	gelatin	4-wellplate	2	100%
ICM	2	gelatin	4-wellplate	1	50%
ICM	2	poly-L-lysine	4-wellplate	1	50%
ICM	2	poly-L-lysine	4-wellplate	1	50%
ICM	2	laminin	4-wellplate	1	50%
ICM	2	laminin	4-wellplate	2	100%
ICM	2	collagen	4-wellplate	2	100%
ICM	2	collagen	4-wellplate	2	100%
ICM	2	gelatin	4-wellplate	1	50%
ICM	2	gelatin	4-wellplate	2	100%
ICM	2	poly-L-lysine	4-wellplate	0	0%
ICM	2	poly-L-lysine	4-wellplate	1	50%

Table (3.2): Summary of the results of ICM culture experiments in the different substrates. The plating efficiency was calculated based on the number of outgrowths produced relative to the initial number of wells used.

substrate	no. of wells	no. plated	no. of outgrwoth	pating efficiency
gelatin	2	11	2	100.00%
collagen	2	13	1	50.00%
laminin	2	6	0	0.00%
poly-L-lysine	2	7	1	50.00%
no	1	0	0	0.00%
gelatin	2	10	0	0.00%
collagen	2	8	1	50.00%
laminin	2	4	1	50.00%
poly-L-lysine	2	2	0	0.00%
no	1	0	0	0.00%
gelatin	2	8	1	50.00%
collagen	2	6	1	50.00%
laminin	2	10	0	0.00%
poly-L-lysine	2	2	0	0.00%
no	1	0	0	0.00%
gelatin	2	9	2	100.00%
collagen	2	7	0	0.00%
laminin	2	5	1	50.00%
poly-L-lysine	2	3	0	0.00%
no	1	0	0	0.00%
gelatin	2	11	0	0.00%
collagen	2	8	1	50.00%
laminin	2	5	1	50.00%

poly-L-lysine	2	1	1	50.00%
gelatin	2	9	1	50.00%
collagen	2	6	1	50.00%
laminin	2	8	1	50.00%
poly-L-lysine	2	3	0	0.00%
gelatin	2	7	0	0.00%
collagen	2	9	0	0.00%
laminin	2	7	1	50.00%
poly-L-lysine	2	2	0	0.00%
gelatin	2	7	2	100.00%
collagen	2	6	1	50.00%
laminin	2	0	0	0.00%
poly-L-lysine	2	0	0	0.00%

Table (3.3): Summary of the morulae dissociation experiments, with the substrates used, the number of attached morulae and the number of outgrowths produced. The number of attached blastomeres was estimated, and the plating efficiency was calculated based on the number of outgrowths resulted relative to the initial number of wells used for each substrate.

blastomeres/well	N	substrate	culture vessels	no plated (D11)	%plating efficiency
1	8	-	96 well plate flat bottom	0	0%
1	8	collagen	96 well plate flat bottom	0	0%
1	8	gelatin	96 well plate flat bottom	0	0%
20+	8	-	96 well plate flat bottom	0	0%
20+	8	collagen	96 well plate flat bottom	0	0%
20+	8	gelatin	96 well plate flat bottom	0	0%
ZF not dis (16)	8	-	96 well plate flat bottom	2	25%
ZF not dis (16)	8	gelatin	96 well plate flat bottom	2	25%
ZF not dis (16)	8	collagen	96 well plate flat bottom	7	88%
ZI (16)	8	-	96 well plate flat bottom	0	0%
ZI (16)	8	collagen	96 well plate flat bottom	0	0%
ZI (16)	8	gelatin	96 well plate flat bottom	0	0%
1	8	-	96 well plate flat bottom	0	0%
1	8	collagen	96 well plate flat bottom	1	13%
1	8	gelatin	96 well plate flat bottom	2	25%
1	8	-	96 well plate flat bottom	0	0%
1	8	collagen	96 well plate flat bottom	1	13%
1	8	gelatin	96 well plate flat bottom	2	25%
20+	8	-	96 well plate flat bottom	0	0%
20+	8	collagen	96 well plate flat bottom	2	25%
20+	8	gelatin	96 well plate flat bottom	3	38%
20+	8	-	96 well plate flat bottom	0	0%
20+	8	collagen	96 well plate flat bottom	2	25%

20+	8	gelatin	96 well plate flat bottom	3	37%
ZF not dis (16)	8	-	96 well plate flat bottom	1	13%
ZF not dis (16)	8	collagen	96 well plate flat bottom	7	88%
ZF not dis (16)	8	gelatin	96 well plate flat bottom	7	88%
ZF not dis (16)	8	-	96 well plate flat bottom	1	13%
ZF not dis (16)	8	gelatin	96 well plate flat bottom	7	87%
ZF not dis (16)	8	collagen	96 well plate flat bottom	7	87%
ZI (16)	8	gelatin	96 well plate flat bottom	0	0%
ZI (16)	8	-	96 well plate flat bottom	1	13%
ZI (16)	8	collagen	96 well plate flat bottom	1	13%
ZI (16)	8	gelatin	96 well plate flat bottom	0	0%
ZI (16)	8	collagen	96 well plate flat bottom	1	13%
ZI (16)	8	-	96 well plate flat bottom	1	13%

Table (3.4): Summary of the plating efficiency results from plating day 3 (16-cell) embryos on 96 flat bottom well plates. Different number of dissociated blastomeres was plated, 1blastomere; 20 blastomeres and zona free dissociated embry. Blastomeres were plated on pre-coated wells with gelatin and collagen, and on wells without coating. Plating efficiency was calculated based on the number of attachments related to the initial number of wells used for each treatment.

blastomeres/well	n	Substrate	no plated (D11)	%plating efficiency	no outgrowth (D21)
1	36	-	0	0%	0.00
1	36	collagen	0	0%	0.00
1	36	gelatin/collagen (2:1)	0	0%	0.00
1	36	fibronectin	2	6%	2.00
1	36	poly-L-lysin	5	14%	4.00
1	36	gelatin	2	6%	2.00
8	4	-	0	0%	0.00
8	4	collagen	0	0%	0.00
8	4	fibronectin	0	0%	0.00
8	4	gelatin	0	0%	0.00
8	4	gelatin/collagen (2:1)	0	0%	0.00
8	4	poly-L-lysin	0	0%	0.00
ZF not dis (8)	4	-	0	0%	0.00
ZF not dis (8)	4	fibronectin	0	0%	0.00
ZF not dis (8)	4	gelatin	0	0%	0.00
ZF not dis (8)	4	gelatin/collagen (2:1)	0	0%	0.00
ZF not dis (8)	4	collagen	1	25%	1.00
ZF not dis (8)	4	poly-L-lysin	2	50%	2.00
ZI (8)	4	-	0	0%	0.00
ZI (8)	4	collagen	0	0%	0.00
ZI (8)	4	fibronectin	0	0%	0.00

ZI (8)	4	gelatin	0	0%	0.00
ZI (8)	4	gelatin/collagen (2:1)	0	0%	0.00
ZI (8)	4	poly-L-lysin	0	0%	0.00
1	8	-	0	0%	0.00
1	8	collagen	1	13%	1.00
1	8	gelatin	2	25%	2.00
20+	8	-	0	0%	0.00
20+	8	collagen	2	25%	2.00
20+	8	gelatin	3	38%	3.00
ZI (8)	8	-	1	13%	1.00
ZI (8)	8	collagen	1	13%	1.00
ZI (8)	8	gelatin	0	0%	0.00
ZF not dis (8)	8	collagen	7	88%	7.00
ZF not dis (8)	8	gelatin	7	88%	7.00
ZF not dis (8)	8	-	1	13%	1.00
20+	25	gelatin	24	96%	14.00
20+	24	gelatin	21	88%	0.00
20+	17	gelatin	15	88%	0.00
20+	25	gelatin	22	88%	15.00
20+	20	gelatin	19	95%	13.00
20+	7	poly-L-lysin	1	14%	0.00
20+	7	laminin	2	29%	0.00
20+	7	collagen	5	71%	3.00
20+	7	gelatin	7	100%	4.00
20+	16	collagen	7	44%	2.00

20+	16	poly-L-lysin	10	63%	5.00
20+	16	gelatin	14	88%	9.00
20+	16	laminin	14	88%	6.00
1	4	collagen	0	0%	0.00
1	4	gelatin	0	0%	0.00
1	4	laminin	0	0%	0.00
1	4	poly-L-lysin	0	0%	0.00
2	4	collagen	0	0%	0.00
2	4	gelatin	0	0%	0.00
2	4	laminin	0	0%	0.00
2	4	poly-L-lysin	0	0%	0.00
4	4	collagen	0	0%	0.00
4	4	gelatin	0	0%	0.00
4	4	laminin	0	0%	0.00
4	4	poly-L-lysin	0	0%	0.00
8	4	collagen	0	0%	0.00
8	4	gelatin	0	0%	0.00
8	4	poly-L-lysin	0	0%	0.00
20+	4	laminin	0	0%	0.00
20+	4	poly-L-lysin	0	0%	0.00
20+	4	collagen	1	25%	1.00
8	4	laminin	2	50%	2.00
20+	4	gelatin	4	100%	4.00
1	4	collagen	0	0%	0.00
1	4	gelatin	0	0%	0.00

1	4	laminin	0	0%	0.00
1	4	poly-L-lysin	0	0%	0.00
2	4	collagen	0	0%	0.00
2	4	gelatin	0	0%	0.00
2	4	laminin	0	0%	0.00
2	4	poly-L-lysin	0	0%	0.00
4	4	collagen	0	0%	0.00
4	4	laminin	0	0%	0.00
4	4	poly-L-lysin	0	0%	0.00
8	4	collagen	0	0%	0.00
8	4	laminin	0	0%	0.00
8	4	poly-L-lysin	0	0%	0.00
20+	4	collagen	0	0%	0.00
20+	4	poly-L-lysin	0	0%	0.00
4	4	gelatin	1	25%	1.00
8	4	gelatin	2	50%	2.00
20+	4	gelatin	3	75%	3.00
20+	4	laminin	3	75%	2.00
1	4	collagen	0	0%	0.00
1	4	gelatin	0	0%	0.00
1	4	laminin	0	0%	0.00
1	4	poly-L-lysin	0	0%	0.00
2	4	collagen	0	0%	0.00
2	4	gelatin	0	0%	0.00
2	4	laminin	0	0%	0.00

2	4	poly-L-lysin	0	0%	0.00
4	4	collagen	0	0%	0.00
4	4	laminin	0	0%	0.00
4	4	poly-L-lysin	0	0%	0.00
8	4	collagen	0	0%	0.00
8	4	laminin	0	0%	0.00
8	4	poly-L-lysin	0	0%	0.00
20+	4	laminin	0	0%	0.00
20+	4	poly-L-lysin	0	0%	0.00
8	4	gelatin	2	50%	2.00
4	4	gelatin	3	75%	3.00
20+	4	collagen	3	75%	3.00
20+	4	gelatin	4	100%	3.00
8	4	collagen	0	0%	0.00
8	4	gelatin/collagen (2:1)	0	0%	0.00
16	4	collagen	1	25%	1.00
16	4	gelatin/collagen (2:1)	1	25%	1.00
20+	4	gelatin/collagen (2:1)	1	25%	1.00
20+	4	collagen	2	50%	2.00
8	4	gelatin	4	100%	4.00
16	4	gelatin	4	100%	4.00
20+	4	gelatin	4	100%	4.00
8	4	collagen	0	0%	0.00
8	4	gelatin	1	25%	0.00

8	4	gelatin/collagen (2:1)	0	0%	0.00
16	4	collagen	0	0%	0.00
16	4	gelatin	3	75%	0.00
16	4	gelatin/collagen (2:1)	0	0%	0.00
20+	4	collagen	0	0%	0.00
20+	4	gelatin	3	75%	0.00
20+	4	gelatin/collagen (2:1)	0	0%	0.00

Table (3.5): Summary of the plating efficiency of dissociated 8-cell embryos, in 96-well plates. The plating efficiency was calculated based on the number of attachments related the initial number of wells in each treatment.

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