
CELL ALLOCATION PATTERNS DURING MAMMALIAN PRE-IMPLANTATION DEVELOPMENT

by

Lessly Pilar Sepulveda Rincon BS, MMedSci

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**Division of Child Health, Obstetrics and Gynaecology
School of Medicine
United Kingdom**



**The University of
Nottingham**

DECLARATION

I hereby declare that this thesis is my own work and that it has not been submitted anywhere for any other degree or award. The work presented herein is my own work and where other sources of information have been used, they have been duly acknowledged.

Lessly Pilar Sepulveda-Rincon

ABSTRACT

The first cell differentiation in the mammalian embryo occurs during pre-implantation development. At blastocyst stage two cell lineages can be distinguished: the inner cell mass (ICM), situated at the embryonic pole and the trophectoderm (TE) at the abembryonic pole. In murine embryos, it has been suggested that the first cleavage plane might be related with the embryonic-abembryonic (Em-Ab) axis at the blastocyst stage. So the daughter cells of the two-cell embryo might be already predisposed to a specific cell lineage further on development. The objective of the present thesis is to investigate the effects of assisted reproductive technologies (ART) factors affecting cell allocation patterns during pre-implantation development.

This was addressed by observing cell allocation patterns during mammalian pre-implantation development on embryos produced using different ARTs. Using live-cell tracing, it has been concluded that cell allocation patterns during pre-implantation embryo development are potentially conserved among mammals, or at least among mouse and bovine embryos. Pre-determined (orthogonal and deviant patterns), as well as stochastic development (random pattern), have been identified in mouse and bovine embryos. The incidence of these cell allocation patterns was not affected by maternal age, oocyte production, oocyte fertilisation/activation method, cleavage-stage biopsy or species.

In addition, differences on epigenetic profiles, coping mechanisms after cell removal and further organ development were present among patterned embryos. Future work is advised to understand the basis of the mechanism(s) driving or driven by cell allocation patterns; particularly its relation with organ development.

TABLE OF CONTENTS

DECLARATION	i
ABSTRACT	ii
TABLE OF CONTENTS	iv
ACKNOWLEDGEMENTS.....	vii
LIST OF PUBLICATIONS.....	viii
LIST OF FIGURES.....	ix
LIST OF TABLES.....	xi
LIST OF ABBREVIATIONS	xii
1. CHAPTER ONE: LITERATURE REVIEW.....	1
1.1. Assisted Reproduction Technologies.....	1
1.1.1. Superovulation protocols	4
1.1.2. Insemination or fertilisation methods	6
1.2. Embryo assessment for embryo transfer	7
1.2.1. Non-invasive methods	9
1.2.2. Invasive methods	16
1.3. Morphological and molecular events during mammalian pre-implantation development.....	21
1.3.1. First Cell Lineage specification in mice	24
1.1.2. Epigenetics and development	40
1.4. Rationale and hypothesis	42
1.5. Aims and Objectives of the study	43
2. CHAPTER TWO: GENERAL MATERIALS AND METHODS.....	46
2.1. Protocols using mouse tissue	46
2.1.1. Embryo production.....	46
2.1.2. Embryo collection and embryo culture	49
2.2. Protocols using bovine tissue	50
2.2.1. Oocyte collection and oocyte maturation	50
2.2.2. <i>In-vitro</i> fertilisation and embryo culture	50
2.3. Non-invasive cell tracing method	51
2.4. Blastocyst assessment	53
2.4.1. Pre-patterning.....	53
2.4.2. Total cell count	54
2.5. Statistical analysis.....	54
3. CHAPTER THREE: EFFECT ARTs ON CELL ALLOCATION PATTERNS DURING PRE-IMPLANTATION DEVELOPMENT.....	56
3.1. Introduction	56
3.2. Objectives	57
3.3. Methods.....	58
3.3.1. Embryo production.....	58

3.3.2.	Live cell tracing	58
3.3.3.	Time-lapse culture for cell tracing observation.....	58
1.1.3.	Gender determination	59
3.3.4.	Statistical analysis	60
3.4.	Results.....	61
3.4.1.	Cell tracing in mouse embryos	61
3.4.1.1.	<i>Non-invasive cell tracing method</i>	61
3.4.2.	Cell tracing in bovine embryos	72
3.5.	Discussion	76
3.6.	Conclusions	82
4.	CHAPTER FOUR: EFFECTS OF EMBRYO CELL REMOVAL ON CELL ALLOCATION PATTERNS DURING PRE-IMPLANTATION DEVELOPMENT	83
4.1.	Introduction	83
4.2.	Objectives	86
4.3.	Methods.....	86
4.3.1.	Embryo biopsy	87
4.3.2.	Cell tracing on bisected two-cell mouse embryos.....	89
4.3.3.	Blastocyst assessment	90
4.3.4.	Statistical analysis	90
4.4.	Results.....	91
4.4.1.	Cell lineage allocation patterns in biopsied mouse embryos	91
4.4.2.	Cell allocation patterns in biopsied bovine embryos	97
4.4.3.	Twinning	100
4.5.	Discussion	105
4.6.	Conclusion.....	112
5.	CHAPTER FIVE: EPIGENETICS AND CELL ALLOCATION PATTERNS IN PARTHENOGENETIC MOUSE EMBRYOS.....	114
5.1.	Introduction	114
5.2.	Objectives	116
5.3.	Methods.....	116
5.3.1.	Embryo production by fertilisation.....	116
5.3.2.	Oocyte activation by parthenogenesis	117
5.3.3.	Immunocytochemistry	118
5.3.4.	Histone modifications image analysis.....	119
5.3.5.	Statistical analysis	120
5.4.	Results.....	121
5.4.1.	Cell allocation patterns in fertilised and parthenogenetic mouse embryos	121
5.4.2.	Histone modifications in mouse blastocysts	123
5.5.	Discussion	130

5.6. Conclusion.....	135
6. CHAPTER SIX: EFFECT OF CELL ALLOCATION PATTERNS IN OFFSPRING DEVELOPMENT	137
6.1. Introduction	137
6.2. Objectives	138
6.3. Methodology	139
6.3.1. Embryo culture and manipulation	139
6.3.2. Pseudo-pregnant mouse surrogates	139
6.3.3. Non-surgical embryo transfer.....	139
6.3.4. Offspring assessment.....	141
6.3.5. Statistical analysis	142
6.4. Results.....	143
6.4.1. Pre-implantation embryo development.....	143
6.4.2. Embryo transfer and live birth results	145
6.4.3. Organ morphometry.....	148
6.5. Discussion	150
6.6. Conclusions	160
7. CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSIONS	161
7.1. General Conclusions	174
REFERENCES.....	161
APPENDICES.....	202
I. Mouse IVF media preparation	202
a) Sperm pre-incubation medium.....	202
b) High calcium HTF medium	202
II. Time-lapse embryo imaging	203
III. Embryo twinning in mammalian species	206
IV. Thawing instructions for one-cell mouse embryos from Embryotech	207
V. Activation media for mouse parthenotes.....	208
a) CBZ medium.....	208
VII. Non-surgical embryo transfer in mice	209

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LIST OF PUBLICATIONS

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REVIEWS

Sepulveda-Rincon, L.P., del Llano-Solanas, E., Serrano-Revuelta, E., Ruddick, L., Maalouf, W.E. & Beaujean, N. 2016. Early epigenetic reprogramming in fertilized, cloned and parthenote embryos. *Theriogenology*, 89 (1), 91-98. This work was presented by Dr Nathalie Beaujean at the International Congress of Animal Reproduction Annual Meeting 2016, Tours, France. 26-30 June, 2016.

INTERNATIONAL CONFERENCES

Sepulveda-Rincon, L.P., Islam, N., Campbell, B.K., Beaujean, N. & Maalouf, W.E. 2016. O-268 Cell allocation patterning between the two-cell to the blastocyst stage might be associated with organ development in mouse offspring. *Human Reproduction*, 31 (suppl 1), i116-i116. This work was presented as oral presentation at the European Society of Human Reproduction and Embryology Annual Meeting 2016, Helsinki, Finland. 3-6 July, 2016. Abstract O-268.

Sepulveda-Rincon, L.P., Dubé, D., Adenot, P., Laffont, L., Ruffini, S., Maalouf, W.E., Duranthon, V. & Beaujean, N. 2014. 78 noninvasive cell lineage tracing in bovine embryos from two-cell stage up to blastocyst stage. *Reproduction, fertility, and development*, 27, 132-132. This work was presented by poster at the International Embryo Transfer Society Annual Meeting 2015, Versailles, France. 10-13 January, 2014. Abstract 78.

Sepulveda-Rincon, L.P., Campbell, B.K., Beaujean, N. & Maalouf WE. 2014. Effect of 8-cell Stage Biopsy on Cell Lineage Allocation in Mammalian Embryos. *Fertility and Sterility*; 102, E309-E309. This work was presented at the American Society of Reproductive Medicine Annual Meeting 2014, Honolulu, Oahu, Hawaii. 18-22 October, 2014. Abstract P-520.

LIST OF FIGURES

Figure 1.1 Invasive and non-invasive pre-implantation embryo assessments.	8
Figure 1.2 Pre-implantation development in mouse embryos.	23
Figure 1.3 Mammalian embryo at blastocyst stage.	24
Figure 1.4 Principal theories on cell lineage commitment in pre-implantation mammalian embryos.	28
Figure 1.5 Cell distribution at blastocyst stage according to the first cell cleavage plane in pre-implantation mouse embryos.	32
Figure 2.1 Lipophilic dye injection in two-cell mouse and bovine embryos.	52
Figure 2.2 Cell distribution at blastocyst stage according with first cell cleavage plane in pre-implantation mouse embryos.	53
Figure 2.3 Blastocyst nuclear staining with DAPI.	54
Figure 3.1 Non-invasive cell tracing in two-cell mouse embryos.	62
Figure 3.2 Second cell cleavage on injected two-cell mouse embryos.	62
Figure 3.3 Cell allocation of blastomeres of the two-cell mouse embryo at blastocyst stage.	63
Figure 3.4 Cell allocation patterns on 3D in mouse embryos.	63
Figure 3.5 Cell allocation patterns on mouse blastocysts after CM-Dil injection at the two-cell stage.	65
Figure 3.6 Total cell count in mouse blastocysts from superovulated and non-superovulated females.	67
Figure 3.7 Gender determination on mouse embryos by immunostaining.	68
Figure 3.8 Non-invasive cell tracing in bovine embryos.	73
Figure 3.9 Start of the cell intermingling on bovine embryos.	73
Figure 3.10 Cell allocation patterns in bovine embryos.	75
Figure 3.11 Cell allocation pattern incidence among bovine embryos.	75
Figure 3.12 Total cell counts in bovine blastocysts.	76
Figure 4.1 Bovine embryo cleavage stage biopsy.	88
Figure 4.2 Twinning technique in mouse embryos.	89
Figure 4.3 Live tracing on bisected mouse embryos.	89
Figure 4.4 Cell allocation patterns in biopsied mouse blastocysts.	93
Figure 4.5 Cell allocation patterns across control and biopsied mouse embryos.	93
Figure 4.6 Blastocyst rates by SART system in biopsied and non-biopsied mouse embryos.	97
Figure 4.7 Incidence of different cell allocation patterns in biopsied and non-biopsied bovine embryos.	99
Figure 4.8 Total cell counts in bovine blastocysts.	99
Figure 4.9 Example of uneven size of blastocysts from two-cell bisected mouse embryos. ...	101
Figure 4.10 Couplet of blastocysts from bisected two-cell embryo following the random pattern.	102

Figure 4.11 Pre-implantation development of twin mouse embryos bisected at two-cell stage.	104
Figure 4.12 Four cell configuration on twin embryos.	105
Figure 5.1 Fertilised and parthenogenetic activated oocytes.	118
Figure 5.2 Incidence of cell allocation patterns in fertilised and parthenogenetic mouse embryos.	123
Figure 5.3 Histone 3 lysine 9 acetylation in mouse blastocysts.	124
Figure 5.4 Relative H3K9ac levels in fertilised mouse embryos.	124
Figure 5.5 Histone 3 lysine 9 acetylation in two-cell mouse parthenotes.	126
Figure 5.6 Relative H3K9ac levels in two-cell stage mouse parthenotes.	126
Figure 5.7 Immunodetection of H3R2me2 levels in four-cell mouse parthenotes.	127
Figure 5.8 Relative H3R2me2 levels in four-cell mouse parthenotes.	127
Figure 5.9 Relative H3K9ac levels on mouse blastocysts from parthenogenetic activated oocytes.	129
Figure 5.10 Total cell count (TCC) in blastocysts from parthenogenetic activated mouse oocytes.	130
Figure 6.1 Non-surgical embryo transfer procedure using NSET device.	141
Figure 6.2 Mouse embryo cell allocation patterns at blastocyst stage.	144
Figure 6.3 Cell allocation pattern incidence at blastocyst stage of thawed one-cell mouse embryos.	144
Figure 6.4 Blastocysts before non-surgical embryo transfer.	145
Figure 6.5 Blastocyst expansion grades before embryo transfer.	145
Figure 6.6 Live offspring analysis.	146
Figure 6.7 Boxplots comparing the relative organ weights from 2 week old mice.	149
Figure 7.1 Differences between mammalian embryos following different cell allocation patterns.	164
Figure 7.2 Diagram of possible mechanisms controlling cell differentiation.	169
Figure 7.3. Time-lapse of mouse embryo following the orthogonal cell allocation pattern... ..	203
Figure 7.4. Time-lapse of mouse embryo following the deviant cell allocation pattern.	204
Figure 7.5. Time-lapse of mouse embryo following the random cell allocation pattern.	205

LIST OF TABLES

Table 1.1 Morphological assessment in pre-implantation embryos.....	11
Table 1.2 Candidates for Pre-implantation Genetic Diagnosis/Screening.	19
Table 1.3 Available embryo genetic testing techniques.	19
Table 1.4 Pre-implantation embryo biopsies for Pre-implantation Genetic Diagnosis/Screening (PGD/S).....	19
Table 1.5 Methodologies used for cell lineage tracing studies in mouse embryos.	28
Table 1.6 Proponents of the pre-patterning theory on mouse embryos.....	33
Table 1.7 Opponents of the pre-patterning theory on mouse embryos.	36
Table 2.1 Mouse strains and media used during this study.....	47
Table 3.1 Cell allocation pattern incidence across embryos from superovulated and non-superovulated (control) mouse females.	65
Table 3.2 Sex ratio in embryos from superovulated and non-superovulated mouse females.	68
Table 3.3 Sex ratios among cell allocation pattern in mouse blastocysts from superovulated and non-superovulated mouse females.	68
Table 3.4 Morphokinetic parameters during pre-implantation development between embryos from superovulated and non-superovulated mouse females.	70
Table 3.5 Morphokinetic parameters during pre-implantation between embryos following different cell allocation patterns.....	70
Table 3.6 Cell allocation patterns incidence across embryos from young and aged superovulated mouse females.....	71
Table 3.7 Cell allocation pattern incidence across embryos from superovulated mouse females and after natural or <i>in-vitro</i> fertilisation.....	72
Table 3.8 Cell allocation pattern incidence among mouse and bovine embryos.	77
Table 4.1 Biopsied cell staining status and cell allocation patterns in mouse embryos.	92
Table 4.2 Morphokinetic analysis between patterns from the three-cell stage up to the eight-cell stage.	95
Table 4.3 Embryo morphokinetics after blastomere removal at the eight-cell stage.	95
Table 4.4 Total cell count in mouse blastocysts.....	96
Table 4.5 Blastocyst grading by blastocoel expansion among control and biopsied mouse embryos.	97
Table 4.6 Morphokinetics among bisected two-cell embryos.	103
Table 5.1 Total cell count (TCC) in blastocysts from parthenogenetic activated mouse oocytes.	130
Table 6.1 Embryo transfer and live birth data.	147
Table 6.2 Mice total body weight.	148

LIST OF ABBREVIATIONS

Abreviation	Description
Ab	Abembryonic
ART	Assisted Reproduction Techniques
AV	Animal-Vegetal
BWS	Beckwith Wiedemann Syndrome
cc2	Duration time from the 2-cell stage to the 3-cell stage
CCs	Cumulus Cells
CDX2	Caudal-type homeobox-2
CGH	Comparative Genomic Hybridisation
COC	Cumulus cells-oocyte complex
COS	Controlled Ovarian Stimulation
DNA	Deoxyribonucleic acid
DOR	Diminished Ovarian Reserve
dpc	Days post coitus
E3	Embryonic day 3
E5	Embryonic day 5
E-Ab	Embryonic-Abembryonic axis
EGA	Embryo Genome Activation
Em	Embryonic
EPI	Epiblast
ESHRE	European Society of Human Reproduction and Embryology
ET	Embryo transfer
F/P	Foetal/Placental
FISH	Flourescent <i>in-situ</i> hybridisation
FSH	Follicle-Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
H	Histone
H3K9ac	Histone 3 Lysine 9 acetylation
hCG	Human chorionic gonadotropin
HDACis	Histone deacetylase inhibitors
HLA	Human Leukocyte Antigen
ICM	Inner cell mass
ICSI	Intracytoplasmic Sperm Injection
IP	Intra-peritoneal
IU	International units
IUI	Intra-uterine Insemination
IVC	<i>In-Vitro</i> Culture
IVF	<i>In-Vitro</i> Fertilisation
K	Lysine
Klf5	Kruppel-like zinc-finger transcription factor
LH	Luteinising Hormone
mRNA	Messenguer Ribonucleic Acid

NSET	Non-surgical embryo transfer
OHSS	Ovarian hyper-stimulation syndrome
PB	Polar Body
PCOS	Polycystic Ovaries Syndrome
PCR	Polimerase Chain Reaction
PE	Primitive Endoderm
PGD	Pre-implantation Genetic Diagnosis
PGS	Pre-implantation Genetic Screening
PN	Pronuclear
POA	Premature Ovarian Aging
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
s2	Duration time from the 3-cell stago to the 4-cell stage
SCNT	Somatic cell nuclear transfer
SEP	Sperm entry point
SET	Single Embryo Transfer
SI	Standar Incubation
SNP	Single Nucleotide Polymorphism
TE	Trophectoderm
TLM	Time Lapse Monitoring
TSA	Trichostatin A
UK	United Kingdom
XEN-like	Primitive Endoderm cells-like
YAP	Yes-associated protein
ZP	Zona pellucida

1. CHAPTER ONE: LITERATURE REVIEW

1.1. Assisted Reproduction Technologies

Great expectations in the battle against human infertility were raised after the success of the first *in-vitro* fertilisation (IVF) treatment performed by Robert Edwards and Patrick Steptoe in 1978. Among mammals, humans are highly inefficient in reproduction. In a normal menstrual cycle there are only around 30% of probabilities that a woman will become pregnant (Zhang et al., 2013b). Also, it has been estimated that around 70 million couples worldwide present some case of infertility; representing the 15% of the couples at reproductive age (Zhao et al., 2011).

Infertility has been defined as the failure to conceive after one or two years of regular unprotected sexual intercourse (HFEA, 2013). The causes of infertility vary, but they are usually related to peritoneal or uterine disorders (10%), tubal damage (20%), ovulatory disorders (25%), idiopathic (25%), factors affecting male fertility (30%) and in the 40% of the cases it is a combination of female and male infertility factors (National Institute for Health and Care Excellence, 2013). Over the past years, infertility has increased due to different factors affecting gamete quality as eating, smoking and exercise habits as well as postponement of motherhood (Feuer and Rinaudo, 2012). Consequently, there has been a growing interest in assisted reproduction techniques (ARTs). These encompass superovulation protocols, intra-uterine insemination (IUI), IVF, intracytoplasmic sperm injection (ICSI),

pre-implantation genetic diagnosis/screening (PGD/S) and gametes/embryo cryopreservation.

Currently, more than 6.5 million babies have been born by IVF treatments in the world (Brown, 2016). In some European countries, babies born after ARTs represent up to 4% of the total births (Nyboe Andersen et al., 2009). Nonetheless, ARTs success rates still remain low. Probabilities of delivering a baby after an IVF cycle are around the 33% in women younger than 35 years old (Botros et al., 2008) and no more than 23.1% when they are older (HFEA, 2013). Several factors have been related with pregnancy outcomes after ARTs as gametes/embryos precedence and state (autologous or donation and fresh or thawed), female stimulation treatment, patient age, number of previous treatments and day of embryo transfer on day 3 or day 5 to mention the most common treatments (Meseguer et al., 2012). In order to maximize pregnancy rates by ARTs, multiple embryo transfer was initially proposed. However, this approach has been extensively debated due to high multiple pregnancy rates and its entailed complications. These complications include high indices of baby and mother morbidity and mortality, as well as new born complications such as low birth weight, cerebral palsy and pre-term delivery (Feuer and Rinaudo, 2012, Seli et al., 2010).

Significant progress in understanding *in-vitro* embryo production and culture (Kilani et al., 2009), has led to the promotion of single embryo transfer (SET) policy by European authorities (Guerif et al., 2010, Sifer et al., 2011). With SET

policy increasing, the embryo selection method is crucial to rise pregnancy and “take home baby” rates across fertility clinics. Embryo selection method needs to ensure the selection of the embryo of higher quality and with better chances of implantation (Assou et al., 2010). Currently, embryo selection methods across fertility clinics, are based on embryo morphology. However, several studies report that good embryo morphology does not totally correlate with embryo implantation or correct genetic content (Assou et al., 2010, Marhuenda-Egea et al., 2011, Yang et al., 2012, Cruz et al., 2011, Kroener et al., 2012). Therefore, the lack of a reliable embryo selection method for embryo transfer seems to be one of the most important factors of implantation failure among advanced maternal age, severe male infertility, genetic disorders or chromosomal abnormalities (Wong et al., 2013, Harper and Harton, 2010, Seli et al., 2010). Different approaches at metabolomic, proteomic, transcriptomic or genomic levels have been developed to assess embryo quality (Brison et al., 2007).

The application of ARTs varies according to the specific diagnosis of the patients. In general, a fertility treatment starts with a proper evaluation and diagnosis of both partners (female and male); followed by an ovulation/superoovulation protocol, sperm sample preparation, fertilisation protocol (IUI, IVF or ICSI), embryo culture and further embryo transfer. These protocols will be discussed briefly below according to the Cochrane latest review (Myers et al., 2008). It is worth noting that in case of severe fertility diagnosis, female and male gametes can be obtained from donors.

1.1.1. Superovulation protocols

Different ovulation protocols are suggested depending on the patient diagnosis and proposed treatment. For female patients presenting anovulatory cycles and when IUI is recommended; oestrogen inhibitors, insulin sensitizers and gonadotrophins can be used. The most used treatment is based on the administration of oestrogen inhibitors as clomiphene citrate; which acts as oestrogen antagonist and agonist. Clomiphene citrate reduces the production of endogenous oestrogens and therefore promotes the release of the follicle-stimulating hormone (FSH), triggering the follicular growth and the release of luteinising hormone (LH) needed for ovulation (Meridis and Lavery, 2006). Nevertheless, it has been reported that 80% of women under clomiphene citrate treatment get to ovulate and only 40% of those will get pregnant (Meridis and Lavery, 2006).

When IVF or ICSI are recommended, controlled ovarian stimulation (COS) protocols are widely advised in fertility clinics. Superovulation protocols are expected to promote multiple follicular growth and oocyte maturation. These protocols involved the use of gonadotrophins, gonadotrophin releasing hormone (GnRH) agonists or oestrogen inhibitors for superovulation depending on the patients diagnose (Urbancsek et al., 2000). COS protocols aim to increase the number of embryos available and theoretically, the chances to deliver a baby.

It has been suggested that pregnancy rates differ among gonadotrophins protocols. Higher doses of gonadotrophins increase the risk of ovarian hyperstimulation syndrome (OHSS) and the additional use of GnRH might also increase the multiple pregnancy rates. Therefore, strict follow up of the treatment is required. Suppression of the release of FSH and LH from the pituitary can be achieved by the use of GnRH agonists, then the follicle growth is controlled by exogenous FSH administration and finally oocyte maturation is promoted by human chorionic gonadotrophin (hCG) administration; which acts as LH inducing ovulation (Elder, 2010). The desirable time for hCG administration is when the biggest follicle rises around 17 mm of diameter (Coughlan et al., 2011). Oocyte retrieval is usually guided by a trans-vaginal ultrasound probe after 24-36 hours of the LH surge that can be measured on the urine (Urbancsek et al., 2000). Nevertheless, complications of the procedure such as anaesthetic complications, bleeding, injuries to abdominal organs (specially the bowel) and possible infections have been reported (Weinerman and Grifo, 2012). Similarly, mouse studies regarding the effects of superovulation protocols on oocyte and embryo quality have suggested significant differences on deoxyribonucleic acid (DNA) methylation and gene expression patterns when compared with oocytes and embryos produced on natural ovulatory cycles (reviewed in (el Hajj and Haaf, 2013)). Hence, further research of superovulation effects on embryo development is desirable.

1.1.2. Insemination or fertilisation methods

IUI is considered *in-vivo* oocyte fertilisation; while IVF and ICSI require oocyte fertilisation outside the body (Orvieto et al., 2000). For IUI the sperm insemination must be performed on a time frame of two days before ovulation as the lifespan of the oocyte is 12 to 24 hours post-ovulation. Also, the spermatozoa have the ability to fertilise the oocyte during the first 40 up to 80 hours on the feminine track (Priehl et al., 2000). For IVF or ICSI, sperm insemination is usually performed 4 to 6 hours after oocyte retrieval. ICSI treatment is recommended in case of recurrent fertilisation failure and severe male infertility factor (Coughlan et al., 2011). The first baby after ICSI treatment was born in 1992 (Palermo et al., 1992). It has been reported that ICSI is a more effective fertilisation method when compared with IVF (Hwang et al., 2005). However, additional oocyte manipulation is needed when performing ICSI. The oocytes are exposed to hyaluronidase for cumulus cells removal and then the zona pellucida (ZP) and the oolema are breached with a needle containing one single spermatozoon that will fertilise the oocyte (Woodward, 2007). These manipulations might increase the risk of embryo contamination, damage of oocyte spindle or worst of oocyte destruction (Orvieto et al., 2000, Fleming and King, 2003). Nevertheless, in the United Kingdom (UK) around half (52.9%) of the embryos produced by ARTs are produced by ICSI (HFEA, 2013).

Finally, embryo transfer is currently performed on day 3 (cleavage stage) or on day 5-6 (blastocyst stage) depending on the patient medical history and the clinic facilities. In case of surplus embryos, these can be cryopreserved for future transfer, donated to other couples or destroyed. Within the UK, around 20% of the embryo transfers use cryopreserved/thawed embryos (HFEA, 2013).

1.2. Embryo assessment for embryo transfer

The available embryo assessments can be seen on Figure 1.1. Embryo assessment aims to identify the embryo(s) with higher quality and higher implantation potential. Embryo assessments can be classified into invasive and non-invasive methods. Non-invasive methods include: morphological criteria regarding the appearance of pronuclei, nucleoli and blastomeres, as well as fragmentation rates, blastomere number, embryo symmetry; -omics assessment as the measurement of different molecules on the culture media, oxygen consumption rates and embryonic transcriptome, proteome and metabolome; and time-lapse monitoring (TLM). On the other hand, PGD/S represent an invasive embryo assessment method. All embryo assessments are stage developmental dependent.

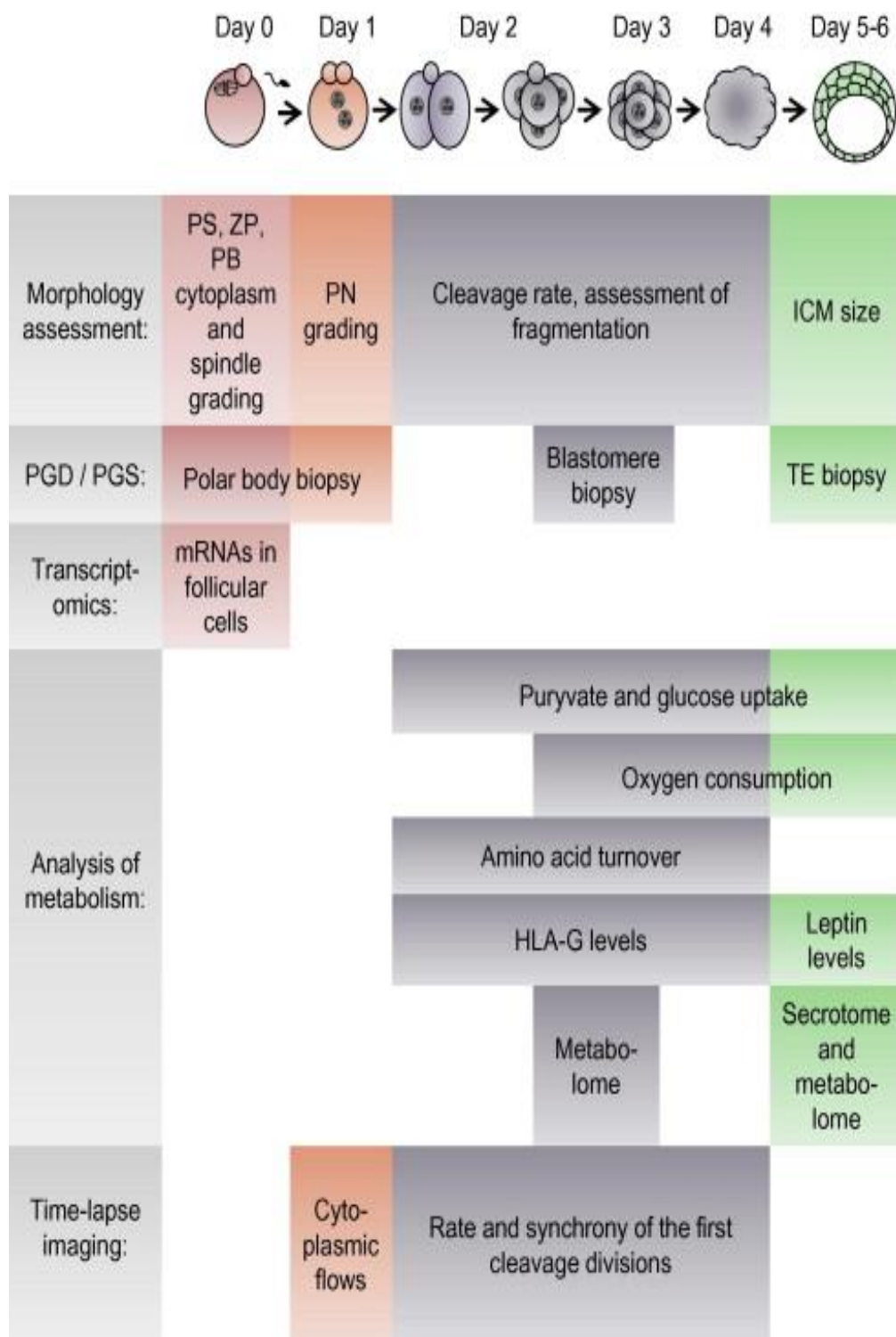


Figure 1.1 Invasive and non-invasive pre-implantation embryo assessments. Different embryo assessment is performed depending on embryo developmental stage, morphology and molecular content. The application of these assessment methods depends on the fertility clinic and the technology available. Non-invasive methods include morphology assessment, transcriptomics, analysis of metabolism and time-lapse imaging; while invasive methods include Pre-implantation Genetic Diagnosis/Screening (PGD/S). Extracted from (Ajduk and Zernicka-Goetz, 2013).

1.2.1. Non-invasive methods

1.2.1.1. Morphological assessment

The most used methods for embryo selection are based on embryo morphology, as it is a simple assessment and it does not required sophisticated equipment. As mentioned before, embryo morphology assessment varies according to the developmental stage of the embryo (Table 1.1). Morphological observation starts after oocyte pick-up. First, the assessment aims for oocytes with several layers and expanded cumulus cells. In case of ICSI treatment, cumulus cells are removed and a good quality oocyte must present smooth membrane with one polar body. Around 16 to 18 hours post insemination (either IVF or ICSI), the two pronuclei (female and male) must be lined central with same number of nucleoli and two polar bodies must be observed. The embryo should have even blastomeres' size through the first 5 cell cycles and present only one nucleus per blastomere with less than 25% of fragmentation to be considered as a good quality embryo. After the fifth cell cycle, the embryo cavity (blastocoel) will appear and grading will be according to the blastocoel expansion and the morphology of the outer cells or trophectoderm (TE) and the inner cell mass (ICM).

Currently, embryo transfer is performed on embryonic day 3 (E3) or E5-6 depending on the patient and the clinic protocol. The main advantage of transferring embryos on E3 is that there are more available embryos at cleavage than blastocysts on E5-6. It has been suggested that embryos arising

the blastocyst stage present better chances for further development as extended embryo culture might act as embryo self-selection process (Reh et al., 2010). Nonetheless, the latest Cochrane report states that there is no quality evidence supporting increased cumulative pregnancy and live birth rates after blastocyst embryo transfer when compared with cleavage stage embryo transfer (Glujovsky et al., 2016). At present, the golden embryo selection method for blastocyst transfer is the Gardner and Schoolcraft embryo grading system where ICM quality is preferred over TE quality based on the fact that the ICM will give rise to the future offspring and the TE to the external accessories. Nonetheless, a recent publication has questioned this preference. Based on a retrospective analysis, it was determined that TE grade might be a better embryo implantation predictor than ICM grade; so that an embryo graded 3BA (see Table 1.1) has higher implantation probabilities than an embryo graded 4AB (Ahlström et al., 2011). Results might be related with embryo implantation potential as TE is directly involved in this process.

Morphological grading is still a subjective assessment and lately, the correlation between good embryo morphology and embryo quality has been questioned. It has been suggested that “pretty” embryos do not always implant or give rise to healthy babies (Nel-Themaat and Nagy, 2011). Thus, given the constraints of embryo morphology assessment, research has been carried out comparing not competent and competent embryos in relation with their transcriptomic, proteomic and metabolic profiles.

Table 1.1 Morphological assessment in pre-implantation embryos. Quality assessment based on morphology from oocyte pick-up to embryo transfer. Adapted from (Ahlström et al., 2011, Bar-Yoseph et al., 2011, Herrler and Beier, 2000).

Embryonic day	Morphological aspect observed	Criteria and grading
Day 0	Cumulus cells-oocyte complex (COC)	1. Immature oocyte at prophase I: no visible polar body and dense and compact cumulus cells surrounding the oocyte. 2. Nearly mature oocyte at metaphase I: neither polar body nor germinal vesicle present, expanded cumulus cells (three to five times oocyte diameter). 3. Mature/ovulatory oocyte at metaphase II: cumulus cells very expanded. Polar body difficult to identify. Smooth ooplasm. 4. Premature oocyte: ooplasm a little bit dark and no even smoothness. First polar body still present (fragmented or intact). 5. Atretic oocyte, nonviable: Nucleus and polar body degenerated, no cumulus cells observed with the ooplasm jagged and dark.
Day 0-1	Membrane, pronuclei, nucleoli and polar bodies	Membrane: smooth or jagged Pronuclei: equal size, central, apposed, other Nucleoli: equal numbers and sized, ≥ 3 , other Polar bodies: $<90^\circ$ apart, $<45^\circ$ apart, other
Day 2	Blastomeres and blastomere nucleus	1n, $\geq 75\%$ of blastomeres with one nucleus p1n, $<75\%$ of blastomeres with one nucleus NV, no visible nuclei MN, >1 nuclei in one blastomere Blastomeres size: even or uneven
Day 3	Blastomeres	1. Six to eight-cell embryo, equally sized, spherical blastomeres and $<10\%$ fragmentation 2. Six to eight-cell embryo, uneven or irregular shaped blastomeres and 10-25% fragmentation 3. Four to five-cell embryo with $<10\%$ fragmentation or six to eight-cell embryo with 25-50% fragmentation 4. Non-viable embryos with lysed, contracted or dark blastomeres or with $>50\%$ fragmentation
Day 5	Blastocoel expansion	1. Early blastocyst with blastocoel $<50\%$ 2. Early blastocyst with blastocoel 50-80% 3. Full blastocyst 4. Expanded blastocyst 5. Beginning of hatching 6. Hatched blastocyst
	ICM	A. Many cells and tightly packed B. Few cells and loosely grouped C. Very few cells
	TE cells	A. Many cells B. Few cells C. Very few cells

1.1.1.1. Time-lapse Monitoring

Time-lapse monitoring allows the recording of embryo development during the *in-vitro* culture. This emerging technology has allowed morphokinetic embryo assessment, which is gathering momentum in fertility clinics. TLM technology comprises an incubation system coupled with a microscope that

allows picturing the embryos every couple of minutes through all *in-vitro* culture until embryo transfer. Even though embryos on TLM are exposed to light-emitting diode lighting, this exposure is less than 50 seconds per day and no significant differences have been found when compared against embryos cultured on standard incubation (Cruz et al., 2011). Currently, there are several TLM systems available as the EmbryoScope, the well of the well and the poly (dimethylsiloxane) four well dish.

The main advantages of TLM is that it allows a non-invasive assessment without disturbance of culture conditions (Wong et al., 2013). Also, it permits the observation of cell divisions as well as pronuclei, nucleus, fragmentation and cell movements (Meseguer et al., 2012). Conventional embryo culture only allows embryo assessment at one specific time of development (Sakkas et al., 2004). Thus, TLM features let to a better embryo assessment as morphology changes a lot through pre-implantation development. For example, pronuclear (PN) morphology changes from 16 to 20 h post ICSI, therefore traditional embryo culture and assessment might be susceptible to misdiagnosis (Montag et al., 2011).

Furthermore, different conclusions have been raised from TLM in human embryos. Blastocysts presenting good morphology have been related to synchronised cell division (Cruz et al., 2012). Similarly, it has been suggested that synchronised appearance of pronuclei in both blastomeres during the second cell cycle could be related with pregnancy rates (Lemmen et al., 2008).

Also, unfragmented embryos present better chances of being euploid embryos (Chavez et al., 2012). Moreover, it has been suggested that assessment of the morphology and timing of the first cleavage is an important parameter to consider on day 3 embryo transfers (Hesters et al., 2008). Hesters and colleagues have reported better implantation rates in double embryo transfers when both embryos are early cleaved embryos with even blastomeres and less than 20% fragmentation.

Coupled with the above, two main research groups have published consistent data regarding embryo cleavage timing and blastocyst rates; Wong group (Wong et al., 2010) and Cruz group (Cruz et al., 2012). Wong and colleagues used thawed one-cell human embryos produced by IVF while Cruz and colleagues worked with human embryos after ICSI treatment. Even though the embryo production technique differs between studies, both groups have concluded that the duration of the two- to three-cell stage (cc2), the three- to four-cell stage (s2) and mainly the time of arrival to the five-cell stage are important parameters related with the potential to reach blastocyst stage. Cruz group also established the relation between embryo kinetics and morphology with embryo implantation rates. Still, it is worth noting that all studies mentioned are not randomised; thus, results might be biased and inconclusive. The number of embryos transferred in Cruz's study were not constant and Wong's study used donated oocytes without taking into consideration the ovarian stimulation protocol and maternal age.

In concordance with previous studies, Meseguer and colleagues performed a retrospective study from ten IVF clinics and reported 20% improvement on clinical pregnancy rates using TLM versus standard incubation (Meseguer et al., 2012). The same research group developed a grading system to predict embryo implantation. This system is based on timing of five-cell stage and the intervals between three- to four-cell and two- to four-cell stage (Meseguer et al., 2011). In this developed system, the embryos presenting higher implantation rates were not those cleaving faster but those in the middle quartile. However, other clinics have failed to improve pregnancy rates using this system. Therefore, it is possible that embryo morphokinetics might be affected by other factors and embryo selection assessment must be adapted to each clinic.

1.2.1.2. -Omics assessment

Metabolomics embryo assessment has been developed as an alternative to morphological assessment. Gene analysis of cumulus cells (CCs) has detected possible biomarkers for predicting embryo implantation as genes NF1, PCK1 and BCL2L11 (Assou et al., 2010) and also CDC42 and PGK1 (Hamel et al., 2010). Still, variations on CCs gene expression can be present from patient to patient in response to different hormonal treatments, as well as other factors (Wathlet et al., 2011). Thus, repeatability and reproducibility might be difficult to achieve in gene profile assessment.

As well as gene expression profile assessment, other technologies have been developed and have presented higher challenges. Ribonucleic acid (RNA) analysis, which is highly expensive and technically demanding, present the risk of rapid RNA degradation and sample contamination (Seli et al., 2010). On the other hand, an estimation of the embryo proteome is around 1 million of proteins. Some of these proteins are secreted by the embryo and can be detected in the culture medium (Katz-Jaffe and Gardner, 2008). Proteomics assessment is not absolutely considered a quantitative approach as results might vary from patient to patient (Brison et al., 2007).

At present, metabolomics approaches are under intensive development. The metabolome analysis presents the main advantage of being the last product of gene expression, representing a link between environmental conditions and the physiology of an organism (Botros et al., 2008). Metabolic pathways within embryo development have been deeply studied and this knowledge suggest a link to embryo quality. The major assessed pathways during embryo pre-implantation development are pyruvate, glucose, oxygen, lipid and amino acid consumption (Nel-Themaat and Nagy, 2011).

Important knowledge of competent embryo metabolism is now widely known, as the change of pyruvate uptake to glucose as energy source from morula stage (Seli et al., 2010). Also, amino acid profiles before the morula stage are different between competent and non-competent embryos (Houghton¹ et al., 2002). Nevertheless, these results are questionable as

embryo's gender seems to influence the amino acid turnover during development (Picton et al., 2010). Still, in the near future, -omics embryo examination might give more specific clues of the pre-implantation embryo quality.

1.2.2. Invasive methods

Two different genetic embryo assessments are available: PGD and PGS (Peyvandi et al., 2011). According to the European Society for Human Reproduction and Embryology (ESHRE), PGD should be offered to patients with high risk of transmitting severe genetic disorders or chromosomal abnormalities to their progeny (Seli et al., 2010). Whereas, PGS should be offered to patients presenting repeated embryo implantation failure, miscarriages, advanced maternal age or severe male infertility (Harper and Harton, 2010). So far, about ten thousand babies have been born after a PGD/S cycle (Peyvandi et al., 2011).

Pre-implantation genetic diagnosis/screening (PGD/S) involves genetic counselling to the interested couple, superovulation protocol, *in-vitro* fertilisation (by conventional IVF or ICSI), *in-vitro* embryo culture, embryo biopsy, genetic testing and sometimes embryo cryopreservation/thawing and further embryo transfer. As mentioned before, most of the implantation failures or pregnancy arrests are caused by embryo genetic abnormalities (Simpson, 2012). Hence, genetic diagnosis before embryo transfer is advised. The main objectives of PGD/S are improvement of birth rates in those

patients presenting any kind of genetic disease or disorder, and to reduce spontaneous abortions (Munné et al., 2010).

Embryo genetic testing is possible due to the great plasticity of the mammalian embryo. Mammalian embryos can overcome different manipulations and develop to term (Oron and Ivanova, 2012, Tabansky et al., 2010). These manipulations include *in-vitro* culture, cell rearrangement, cell addition, cell removal or blastomere injection. David Gardner and Robert Edwards are the pioneers on embryo biopsy, in 1968 they sexed rabbit embryos before transfer by TE biopsy (Theodosiou and Johnson, 2011). Still, it was not until 1990 that PGD was applied to human embryos by Alan Handyside and co-workers. Embryos from two couples carrying X-linked diseases were biopsied and sexed by polymerase chain reaction (PCR). Then, only female embryos were transferred, producing healthy babies (Harper, 2001).

The candidates for PGD/S are listed in Table 1.2. PGD/S candidates include patients with history of chromosomal disorders, specific gene disorders, mitochondrial diseases and recurrent pregnancy failure (Liebaers et al., 2000). There are five main technologies available for embryo genetic testing: amplification based on PCR, fluorescent in-situ hybridisation (FISH), comparative genomic hybridisation (CGH) array, single nucleotide polymorphism (SNP) array, quantitative SNP array analysis and karyomapping (Hens et al., 2013). The use of these techniques varies according to the

genetic assessment needed (Table 1.3). The mentioned techniques are out of the scope of this study and have been reviewed elsewhere (Seli et al., 2010, Dahdouh et al., 2015).

An embryo biopsy is needed to obtain the biological embryo material. Different biopsy techniques can be used: first and second polar body biopsy (before oocyte fertilisation and at one-cell stage respectively), blastomere biopsy at cleavage stage and trophectoderm biopsy at blastocyst stage (Brison et al., 2007). Previous ZP breaching is needed before any cell removal. This could be achieved by three different methods: using laser, using Tyrode's solution or using fine needles (Sermon, 2002). At present, ZP breaching using laser is the most used method (De Rycke et al., 2015).

Furthermore, the removal of the cells (polar bodies (PBs), blastomeres or TE cells) is performed largely by the aspiration method; which involves the suction of the needed cells by an aspiration pipette. Other removal methods have been described such as extrusion by displacement for blastomeres or needle dissection for TE cells (De Vos and Van Steirteghem, 2001). The advantages and disadvantages of the different biopsy types can be seen in Table 1.4. In general, there are several risks that can be associated with PGD on mammalian embryos. Embryo damage can be caused by technical problems during the biopsy (Harper and Thornhill, 2002). Also, misdiagnosis due to pitfalls of the diagnosis technique might be present (Brezina et al., 2012).

Table 1.2 Candidates for Pre-implantation Genetic Diagnosis/Screening. Patients presenting the following characteristics are highly advised to follow an IVF/ICSI cycle coupled with pre-implantation genetic diagnosis/screening before embryo transfer, according to each specific case. Adapted from (Brezina et al., 2012).

Candidates for PGD/S
Autosomal recessive diseases in which both parents are known genetic carriers, such as cystic fibrosis or sickle cell disease
Autosomal dominant diseases in one or both parents, such as Huntington's disease
X linked diseases (such as haemophilia)
Any parent who harbours certain balanced chromosomal translocations or inversions
Not appropriate for: medical conditions in parents in whom a definitive genetic cause has not been identified, testing for non-medical phenotypic traits (such as eye or hair colour)
Diagnosis is controversial for: sex selection for the purposes of family balancing, HLA ¹ matching for the purposes of creating a tissue donor for an existing diseased sibling
¹ Human leukocyte antigen

Table 1.3 Available embryo genetic testing techniques. Techniques available depending on single gene defects, human leukocyte antigen (HLA) compatibility and chromosomal anomalies. Adapted from (Hens et al., 2013).

	Single gene defects	HLA typing	Chromosome screening		Reciprocal and Robertsonian imbalance
			Aneuploidy	Deletions/duplication	
Amplification based PCR	X	X	X	X	X
FISH¹			X	X	X
CGH² array			X	X	X
SNP³ array	X	X	X	X	X
Karyomapping and quantitative SNP array analysis	X	X	X	X	X

¹Fluorescence *in situ* Hybridisation

²Comparative Genome Hybridisation

³Single Nucleotide Polymorphism

Table 1.4 Pre-implantation embryo biopsies for Pre-implantation Genetic Diagnosis/Screening (PGD/S). Adapted from (Brison et al., 2007, Dahdouh et al., 2015). Advantages and disadvantages of the available cell biopsies of pre-implantation mammalian embryos.

Day post- fertilisation / Embryo biopsy	Disadvantages	Advantages
Day 0 / Polar bodies	ZP breaching cannot be chemical Only maternal inherited material is available for analysis High number of zygotes must be tested Sequential biopsy recommended	2 cells for analysis Internal embryonic material undisturbed No need of embryo cryopreservation before transfer on Day 3 or 5 No legal or ethical concerns
Day 2-3 / Blastomere	Concerns in long terms effects of blastomere removal on embryo development or lower implantation rates Blastomere mosaicism	Up to 2 cells for analysis Blastomere removal does not interfere with blastocyst formation No need of embryo cryopreservation before transfer on Day 5 Suitable for all diagnosis
Day 5-6 / TE cells	Information of TE cells related to ICM still questionable Blastocysts culture Need of embryo cryopreservation before transfer	ICM is not perturbed More than 2 cells for analysis Small number of embryos tested Suitable for all diagnosis

ZP: Zona Pellucida, TE: Trophectoderm, ICM: Inner Cell Mass

Polar body biopsy is recommended for analysis of maternal inherited material and chromosome analysis. Nevertheless, paternal inherited material is out of the scope of this analysis (Zhuang and Zhang, 2003). It is argued that polar body biopsy is a non-invasive procedure, as the PBs do not present any contribution on the pre-implantation embryo development. Still, two biopsies are performed at different times, increasing the embryo manipulation time outside the incubator. On the other hand, blastomere biopsy at cleavage stage is recommended as paternal and maternal defects can be assessed. However, mosaicism at cleavage stage is higher than blastocyst stage (Handyside, 2011). Thus, TE biopsy has been suggested with the great advantage of providing more than one cell for the genetic analysis. Yet, the number of embryos tested is reduced as only around 50% of the *in-vitro* fertilised embryos arrive to blastocyst stage (Basille et al., 2009).

According to the PGD Consortium data, the most used biopsy technique across European clinics is aspiration of one or two blastomeres at cleavage stage (Harper et al., 2012, De Rycke et al., 2015). Cleavage stage biopsy is performed at six- to eight-cell stage including the removal of one or two blastomeres (Kirkegaard et al., 2012). Different retrospective studies have suggested that embryos after single cell removal at cleavage stage have better chances to implant than those with double cell removal (Haapaniemi Kouru et al., 2012). Latest studies reported that there is no difference on morphological events between biopsied embryos at six- to eight-cell stage and non-biopsied embryos (Kirkegaard et al., 2012). Furthermore, human

embryos from abnormally fertilised eggs were subjected to cleavage stage biopsy by laser ZP drilling without impairing blastocyst formation (>70%) even after freezing/thawing embryos presented good morphology (Wang et al., 2008). Hence, it is suggested that cleavage stage biopsy does not have detrimental effects during pre-implantation development.

Moreover, in addition to the predisposition of genetic diseases, embryo aneuploidy has been studied. Embryo aneuploidy has been related with advance maternal age (Kroener et al., 2012, Fragouli et al., 2013). Older women on ART cycles are reported to be the target group for PGS (Gleicher and Barad, 2012). Yet, a meta-analysis of randomised controlled trials suggested that this target group is specifically affected when performing PGS and birth rates are decreased (Mastenbroek et al., 2011). These results might be strongly related to oocyte quality reduction because of advanced maternal age and not only because the PGS procedure. As embryo quality and further implantation seem affected by several factors, a greater knowledge on pre-implantation embryo development is desirable.

1.3. Morphological and molecular events during mammalian pre-implantation development

Mammalian embryo development is characterised for two developmental stages: pre-implantation and post-implantation, being the first a unique feature of placental mammals (Bergsmedh et al., 2011). Embryo development starts with the fusion of a specialised haploid female and male gametes

(oocyte and sperm), followed by the activation of maternal metabolism to form a diploid zygote (Gilbert, 2010b, Yamagata and Ueda, 2013). Pre-implantation embryo development comprises from the formation of the zygote until the blastocyst stage (Guo et al., 2010) (Figure 1.2). After fertilisation, the embryo undergoes to a series of mitotic divisions, being the first division the longest (Marikawa and Alarcón, 2009). During the first cell cleavage rounds, the embryo is regulated by the maternal genomic information and afterwards the embryo depletes this maternal material and starts transcribing its own RNA (Rodriguez-Osorio et al., 2011). This transition is called embryo genome activation (EGA).

Afterwards, around the third-fourth round of divisions, cell compaction begins and cavitation starts just after the fifth round of mitosis. Cavitation initiates and the liquid cavity continues to expand until the blastocyst is formed (Oron and Ivanova, 2012, Kurotaki et al., 2007, Fujimori, 2010). It has been observed that cavitation can start at different locations within the embryo, forming more than one cavity until they get together and form a single cavity (Motosugi et al., 2005). Cavitation is maintained by an osmotic gradient between the environment surrounding the embryo and the embryo cavity, which promotes water influx (Marikawa and Alarcon, 2012).

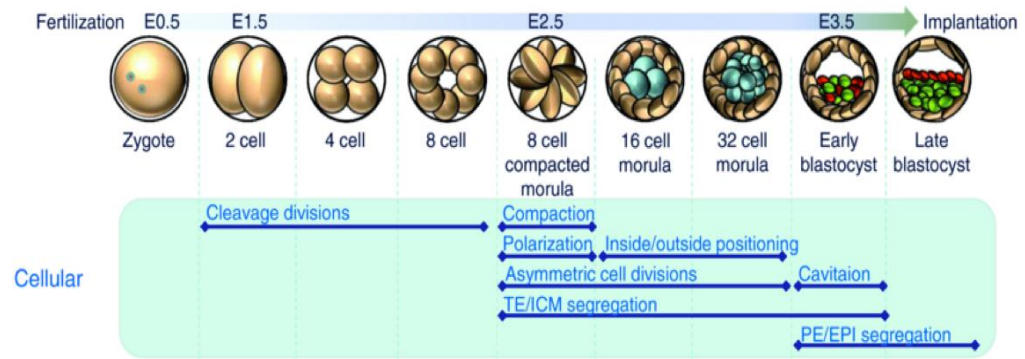


Figure 1.2 Pre-implantation development in mouse embryos. Cleavage timings on embryonic days (E) in mouse embryos during the first five to six cell cycles. After the third cleavage division the embryo starts compaction and polarisation prior cavity formation at the fifth cell cycle. Figure adapted from (Oron and Ivanova, 2012).

The zygote is considered to be totipotent as from it, all the cells will be formed (Saiz and Plusa, 2013). Nonetheless, during pre-implantation development, the totipotency of embryo cells will be lost and these cells will first specialise into two different cell lines: TE and the ICM (Morris et al., 2010). This two cell lines are distinguishable at blastocyst stage when the embryo can be divided by the Embryonic-Abembryonic (Em-Ab) axis (Figure 1.3). The Em axis comprises the ICM and the polar TE; while the Ab axis contains the mural TE (Fujimori et al., 2009). Whereas the TE cells will go further specialisation to the different placenta layers, the ICM presents a pluripotency state being able to form all cell types of the body after implantation (Rossant, 2011, Johnson, 2011). Cell lineage specification plays a crucial role on embryo development and different factors leading this process will be discussed below.

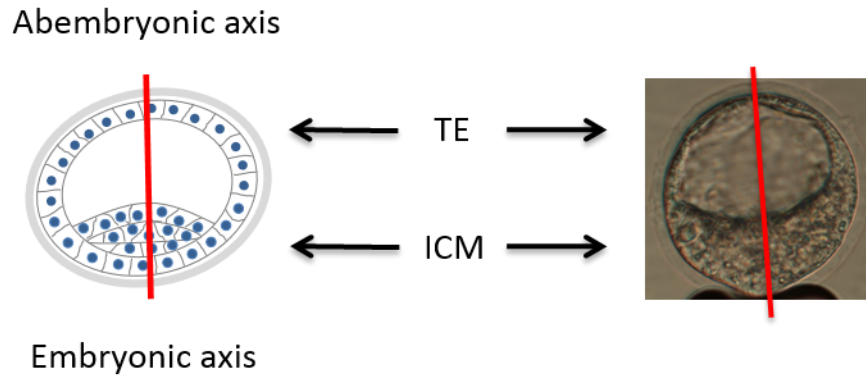


Figure 1.3 Mammalian embryo at blastocyst stage. The red line corresponds to Embryonic-Abembryonic axis. Embryonic axis comprises the inner cell mass (ICM) and mural trophoblast while the abembryonic axis the polar trophoblast (TE).

1.3.1. First Cell Lineage specification in mice

Across evolution, it is usually believed that some developmental mechanisms are conserved as any changes might compromise further developmental events. Nevertheless, early embryonic development is very diverse through all the species (Slack, 2013). Cell lineage specification mechanisms are well established in non-vertebrate species. In nematodes, sea urchins and ascidians the yolk amount within the egg is important not only affecting cell cleavage patterns, but it also affects the cytoplasmic determinants inherited from one cell to its daughters (Wolpert et al., 2011a). Furthermore, early development in vertebrates has been mainly studied in zebrafish, frog and chicken embryos due to their accessibility and easy visualisation/manipulation (Arnold and Robertson, 2009). In species like frogs or tunicates, factors within the egg, sperm entry point and morphogenic features in early embryos are related with further cell fate (Hiiragi and Solter, 2006). Hence, it is believed that cells are pre-patterned to be a specific cell lineage since early embryo stages (Herr et al., 2008). Nonetheless, birds and mammals seem to have

different developmental routes as this cell patterning has not been proven (Wolpert et al., 2011b). To date, the first crucial factor(s) and the starting point of cell lineage specification are still unknown (Fujimori et al., 2009).

The preferred model for mammalian pre-implantation development has been so far the mouse. This model has allowed a wide analysis of its genome, regulatory networks on development and easy embryo manipulation among other factors (Xenopoulos et al., 2012, Guo et al., 2010, McDole et al., 2011, Hiiragi and Solter, 2006, Niakan et al., 2012, Yamanaka and Ralston, 2010, Gilbert, 2010a, Feuer and Rinaudo, 2012). Latest investigations have been trying to elucidate what is driving cell lineage specification in pre-implantation mammalian embryos and when the fate of each cell is established. Cutting-edge publications have suggested that the great regulative nature on mammalian embryos might be biasing cell specification process in mammalian embryos (Zernicka-Goetz, 2006, Morris, 2011). In frog embryos this plasticity ends at the eight-cell stage, in chick embryos it ends when the formation of the primitive streak starts and in mouse embryos plasticity ends even after primitive streak stage (Wolpert et al., 2011b). Manipulations within this plasticity window might result in normal offspring. Therefore, the great flexibility of these embryos against different external manipulations as rearrangement, addition or removal of blastomeres and *in-vitro* culture might hide some lineage specification patterns in mammalian embryos (Bischoff et al., 2008, Cockburn and Rossant, 2010, Johnson, 2009). Yet, it is not known if

cell commitment in mammalian embryos is just a series of stochastic events or there is a hidden pattern.

Until recent years it was believed that during the first rounds of cell cleavage, the cells in the mammalian embryo were identical and had the same potential to become ICM or TE (Zernicka-Goetz, 2011). Extensive research has been performed aiming to elucidate this matter and a hot debate is actually going around this particular subject. Different aspects of pre-implantation embryo development as sperm entry point (Piotrowska and Zernicka-Goetz, 2001, Piotrowska-Nitsche et al., 2009, Motosugi et al., 2005), ZP shape (Gardner, 2007, Plusa et al., 2005, Katayama et al., 2010), nucleoli movements, first cell cleavage, cell cleavage patterns and morphokinetics have been assessed without finding any clear relation between the afore mentioned aspects and cell lineage commitment. Different methodologies have been applied across the literature (Table 1.5) and it has been difficult to compare due to their great variations. The literature reports that the above techniques do not impair normal embryo development. Nonetheless, the negative effects of different embryo manipulations are not exempted (Chroscicka et al., 2004).

Different models have been proposed to explain when cell fate is established and what is driving cell fate in pre-implantation mammalian embryos (Figure 1.4). The first theory on cell differentiation was suggested by Tarkowsky and Wroblewska (Tarkowski and Wróblewska, 1967). The proposed model is known as the inside-outside model (Figure 1.4b). This model refers to cell

position within the sixteen-cell embryo, where the outer cells are committed to become TE and inside cells to ICM (reviewed in (Zernicka-Goetz, 2005)). However, a few years later another model was proposed, the cell polarity model (Johnson and Ziomek, 1981) (Figure 1.4c). This model states that instead of cell position driving cell fate, it is cell polarity what is driving it (Yamanaka et al., 2006). Asymmetric blastomere divisions at the eight-cell stage will give rise to one polar and one apolar cell, whereas symmetric cleavage will give rise to two polar cells. Furthermore, at the sixteen-cell stage polar cells are biased to become TE and apolar cells will form the ICM.

A recent study using disrupted mouse embryos suggested that cell position is not sufficient for driving cells into TE and the authors lean towards the cell polarity model (Kondratiuk et al., 2012). Kondratiuk and colleagues reached to this conclusion as Cdx2 transcription factor is crucial for TE differentiation and in turn, it is closely linked to cell polarisation and not to cell positions. Nevertheless, is still unknown what is triggering cell polarisation at the eight-cell stage and cell contact is not the only mechanism involved (Tabansky et al., 2010).

Table 1.5 Methodologies used for cell lineage tracing studies in mouse embryos. Different methodologies reported on the literature for the study of cell allocation on mammalian embryos using the mouse as animal model.

Method	Reference
Fluorescein isothiocyanate (FITC)	(Johnson and Ziomek, 1981)
Horseradish peroxidase (HR)	(Balakier and Pedersen, 1982)
Lipophilic tracer Dil dissolved in corn oil saturated solution	(Gardner, 1997)
Embedded embryos on alginate gel and/or oil drop injection on the ZP	(Gardner, 2001)
Blastomere labelling at two-cell stage with lipophilic tracers (Dil, DiO or DiI)	(Piotrowska et al., 2001)
Fluorescent latex microspheres injected on the ZP	(Gardner, 2002)
Blastomere injection at two-cell stage with Cre recombinase, CMV Cre or pCAG-CRE	(Fujimori et al., 2003)
Blastomere injection at two-cell stage with fluorochrome-Conjugated dextran tracer	(Chroscicka et al., 2004)
Use of males expressing EGFP-H2B for mating	(Piotrowska-Nitsche and Zernicka-Goetz, 2005)
Cell lineage tracing by photo conversion	(Kurotaki et al., 2007)
Two-photon microscopy	(McDole et al., 2011)
Rainbow transgenic mouse line	(Tabansky et al., 2013)

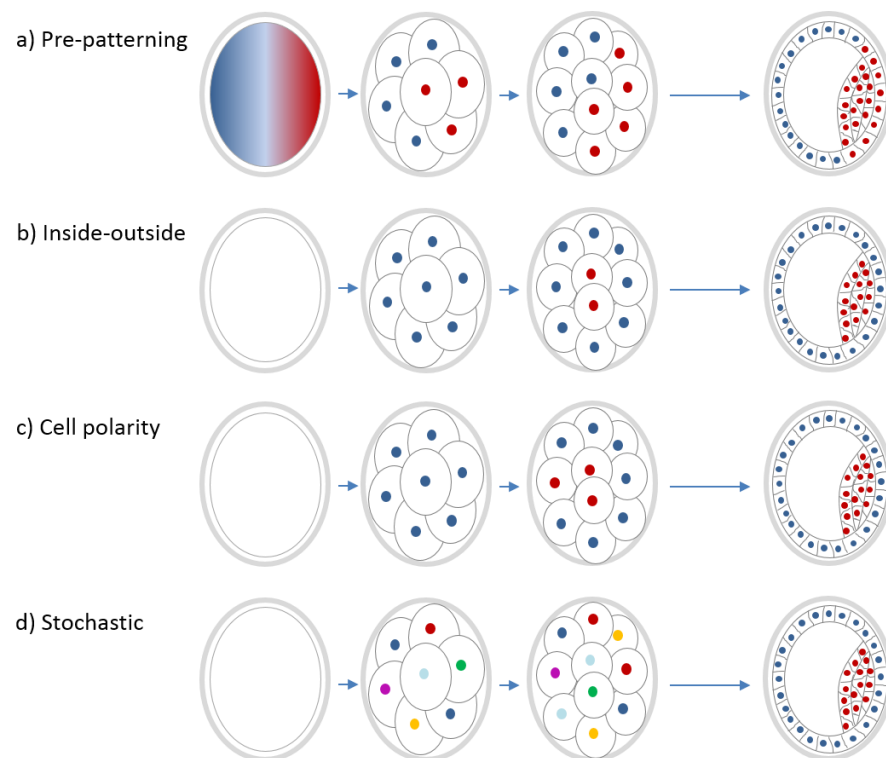


Figure 1.4 Principal theories on cell lineage commitment in pre-implantation mammalian embryos. a) Pre-patterning theory suggesting that cell fate is established since very early embryo developmental stages as one-cell stage and therefore one blastomere of the two-cell embryo will give rise to the inner cell mass (ICM) and the other to the trophectoderm (TE). b) Inside-outside model claiming that at sixteen-cell stage outer cells will give rise to the TE and inside cells to the ICM. c) Cell polarity model states that at eight cell stage symmetric cleavages will become the TE while asymmetric cleavages will give rise to the ICM. d) Stochastic patterning describes that cell lineage specification is a random process and all cells might become either TE or ICM. Figure adapted from (Wennekamp and Hiiragi, 2012).

Another proposed theory is called the Pre-patterning or biased theory, which refers to the different potential of the twin blastomeres at the two-cell stage embryo to become ICM and polar TE or mural TE (Figure 1.4a). Whereas some studies propose that the Em-Ab axis is established since syngamy (Piotrowska et al., 2001, Piotrowska and Zernicka-Goetz, 2002, Fujimori et al., 2003, Piotrowska-Nitsche et al., 2005, Torres-Padilla et al., 2007, Gardner, 2007, Bischoff et al., 2008, Katayama and Roberts, 2010, Liu et al., 2012), others suggest that cell fate is a random event and it is not determined before the eight-cell stage ((Motosugi et al., 2005, Waksmundzka et al., 2006, Kurotaki et al., 2007, Alarcón and Marikawa, 2003, Alarcón and Marikawa, 2008, González et al., 2011, Wennekamp et al., 2013)(Figure 1.4d). Nonetheless, the literature shows discrepancies in both sides of the debate.

The pre-patterning theory comes from other vertebrate species as mentioned before. The first attempts to study the pre-patterning in mammalian embryos were done with horseradish peroxidase labelling by Balakier and Pedersen (1982). Furthermore, the use of lipophilic tracers for studying the cell lineage allocation comes from some experiments on sea urchin embryos. McCain and McClay (1994) introduced this technique on embryos dissolving DiI and DiO dyes in alcohol and afterwards in soybean oil before injecting one of the two-cell blastomeres of urchin embryos without affecting embryo development (McCain and McClay, 1994). This technique was then applied on

mouse embryos by Gardner (1997). Gardner's main focus was to observe the second polar body movements during pre-implantation development. The second polar body remained attached to the embryo (by an intercellular bridge) allowing a specific marker of the animal-vegetal pole and this was orthogonally oriented to the Em-Ab axis at blastocyst stage (Gardner, 1997). This study has been recently debated as the second polar body does not present relationship with the first cleavage on mouse embryos (Zheng et al., 2012). The main studies agreeing on pre-patterning in mouse embryos can be seen in Table 1.6, while studies against this theory can be observed in Table 1.7.

The first study regarding the cell lineage commitment in early mammalian development using lipophilic tracers at two-cell stage was performed by Zernicka's group (Piotrowska et al., 2001). Two main observations were made in this study. First, the authors observed 2 different clusters of cells at blastocyst stage; each one from the descendants of one of the two-cell embryo. Second, they observed that usually the daughter cells of the twin blastomere cleaving first, gave rise to the embryonic part of the blastocyst and the boundary line between clusters was orthogonal to the Em-Ab axis (Figure 1.5a). Thus, these observations agree with the idea that blastomeres at the two-cell stage might be pre-patterned to have a specific allocation in the mammalian embryo. The described cell pre-patterning was observed in the majority of the embryos (80%), but it was not the only pattern observed. Zernicka and colleagues stated that yet the boundary between the daughter

cells can be draw clearly; in some cases some cells can pass this boundary line. Consequently, the blastocyst's classification in this study was related to the number of cells trespassing the boundary line.

Additional studies performed by the same research group suggested that this cell pre-patterning varies across embryos produced by different methods as natural fertilised versus parthenogenetic embryos. Natural fertilised embryos presented a higher orthogonal pre-patterning incidence (> 70%) compared with their parthenogenetic counterpart (30%) (Piotrowska and Zernicka-Goetz, 2002). Later on, another research group applying different technology (pCAG-CRE injections), was able to observe the pre-pattern previously described by Zernicka's group (Fujimori et al., 2003). However, the incidence of the pre-patterned embryos differed. Around 41% of the embryos presented a tendency of one twin blastomere descendants to be part either of the Em or the Ab part (Figure 1.5a), 13% presented two different cell clusters (each cell cluster from descendants of one twin blastomere) and the boundary line between cell clusters was overlapping the Em-Ab axis (Figure 1.5b). Furthermore, in 43% presented a mixture of descendants of both twin blastomeres was observed (Figure 1.5c). Even though, Fujimori and colleagues agreed with the embryo pre-patterning, they did not observe that the first blastomere cleaving to the three-cell stage (leading blastomere) only formed part of the ICM. Other research groups have arrived to the same conclusion (Katayama and Roberts, 2010, González et al., 2011, Waksmundzka et al., 2006).

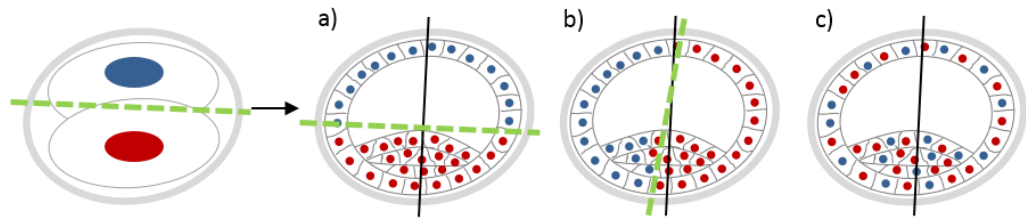


Figure 1.5 Cell distribution at blastocyst stage according to the first cell cleavage plane in pre-implantation mouse embryos. a) Orthogonal pattern where first cleavage plane (green dotted line) is orthogonal ($\pm 30^\circ$) to the Embryonic-Abembryonic (Em-Ab) axis (black line) at blastocyst stage. b) Deviant pattern where first cell cleavage plane is aligned ($\pm 30^\circ$) to the Em-Ab axis. c) Random pattern where the first cell cleavage plane does not predict cell allocation at blastocyst stage and descendants from each twin blastomere are randomly distributed.

Furthermore, a few months after Fujimori's publication, Alarcon and Marikawa (2003) questioned the importance of the pre-patterning in mouse embryos. They also saw two main cell clusters at blastocyst stage. Nevertheless, only 25% of the embryos presented the boundary line orthogonal to the Em-Ab axis, named orthogonal pattern; while the 75% the boundary line was overlapping the Em-Ab axis (Alarcón and Marikawa, 2003), named deviant pattern. The pre-patterning theory was rejected, as post-implantation embryo development between the two pre-patterned blastocysts using two different mouse strains was not compromised. Equally, no significant differences regarding live birth rates between the pre-patterned embryos were observed. Similarly, Liu and co-workers did not observed any difference regarding live birth rates in natural fertilised embryos. However, they only got offspring within the orthogonal group in clones from somatic cell nuclear transfer (SCNT) embryos (Liu et al., 2012).

Table 1.6 Proponents of the pre-patterning theory on mouse embryos.

Reference	Mouse strain and superovulation procedure	Blastomere labelling method at two-cell stage	Assessment at blastocyst stage	Main outcomes	Weak points
(Gardner, 1997)	Closed-bred PO mice on a natural breeding and natural mating	Dil or HRP	Focused on the second polar body movements and blastocyst symmetry	The second PB remains attached to one blastomere and its descendants. Blastocysts are bilaterally rather than radially symmetrical where one blastomere will give rise to one side of the Em-Ab axis.	Does not report incidence of cell distribution
(Piotrowska et al., 2001)	F1 (C57BL/6xCBA) females superovulated with 7.5 international units (IU) PMSG/7.5 IU hCG mated with same genotype males. Embryos collected at two-cell stage.	Dil, DiD or DiO	Classification according to cell distribution and cells crossing the borderline between cell clusters.	80% of the embryos the borderline between cell clusters is orthogonal to the Em-Ab axis while the 20% is parallel. The first blastomere cleaving to the three-cell stage usually gives rise to the ICM.	Embryo classification might be subjective
(Piotrowska and Zernicka-Goetz, 2002)	F1 (C57BL/6 x CBA) females superovulated with 7.5 IU PMSG/7.5 IU hCG. Parthenogenetic activation at 16-17h post hCG and natural fertilised zygotes were obtained at 1 cell stage	Dil, DiD or DiO	Classification according to cell distribution and cells crossing the borderline between cell clusters.	70% of natural fertilised embryos presented the borderline between cell clusters orthogonally to the Em-Ab axis, while natural fertilised embryos with the fertilisation cone removed only presented 50% and the parthenotes only 30%. The results suggested that fertilisation methods might affect embryo development and also that what is happening after sperm entries the oocyte is important.	Classification might be subjective
Fujimori et al. 2003)	BDF1 females superovulated with 5 IU PMS/5 IU hCG mated with homozygous GAG-CAT-Z transgenic males	CMV Cre, pCAG-CRE or Cre recombinase	Blastomere's descendants distribution at blastocyst stage	Three different types of cell distribution at blastocyst stage, where the 41.5% the borderline of cell cluster was orthogonal to the Em-Ab axis, 13.2% was parallel and 42.8% was intermingled.	Methodology of embryo transfers is missing
(Plusa et al., 2005)	CAG: H2B=EGFP females superovulated with 7.5 IU PMSG/7.5 IU hCG mated with males with the same genotype. Zygotes collected at 1-cell stage	Dil and DiD *Embryos constraint in sodium alginate	Blastomere's descendants distribution at blastocyst stage	85% of embryos presented orthogonal pattern, 15% deviant and no intermingling of cells was observed. It was suggested that is more about the first cleavage plane rather than the portion of animal or vegetal pole inherited	Only embryos with PB at one of the long axis were selected for the study

Table 1.6 Continuation

Reference	Mouse strain and superovulation procedure	Blastomere labelling method at two-cell stage	Assessment at blastocyst stage	Main outcomes	Weak points
(Piotrowska-Nitsche et al., 2005)	F1 (C57BL/6 x CBA) females superovulated with 7.5 IU PMSG/7.5 IU hCG and mated with transgenic males H2B-GFP. Embryos collected at two-cell stage.	Dil	Blastomere's descendants cell distribution at four-cell stage	*Chimera construction separately with meridional or equatorial blastomeres at four cell stage and embryos transfer at blastocyst stage. Chimeras with E- blastomeres in random four-cell embryos presented normal post-implantation development.	Pups numbers were not specified
(Piotrowska-Nitsche and Zernicka-Goetz, 2005)	F1 (C57BL/6Xcba) females with males expressing EGFP-H2B or F1 males	Dil *Further Dil injection at one of the four blastomeres	Blastomere's descendants cell distribution at blastocyst stage	73% of embryos presented orthogonal pattern, 27% deviant and there was no cell intermingling reported. Embryos in which animal and vegetal parts are separated in both two-cell blastomeres have significantly reduced viability.	
(Torres-Padilla et al., 2007)	Natural breeding F1 crosses (C57BL/6 x CBA/H)	Green fluorescent beads or rhodamine dextran or DsRed alone or combined with CARM1	Blastomere's descendants cell distribution at blastocyst stage	When second cleavage is similar to the first one (EE or MM) then the embryo presents a random pattern, when they differ (EM) the embryo presents an orthogonal and when is ME the first blastomere cleaving to the three-cell stage contributes mainly to the embryonic pole. In ME embryos the progeny from M blastomere presented higher H3R26 methylation levels at four-cell stage and corresponds usually to the formation of embryonic pole. In this ME embryos the vegetal blastomere always presented lower levels of H3R26me. Injection of CARM1 at two-cell stage drives that blastomere to embryonic part	Does not mention embryo collection stage and the main focus of the study is on ME embryos
(Gardner, 2007)	PO closed-bred albino strain females with natural breeding or superovulated. Mated with normal or vasectomised males. Embryo collection at two-cell stage	Small drops of soya oil injected into the ZP or Dil *ZP removal in some groups	Blastomere's descendants cell distribution at blastocyst stage	Embryo rotations at morula stage happen very seldom. First cleavage plane is mostly orthogonal to the EM-Ab axis at blastocyst stage related with the lesser diameter of the ZP and the bilaterally of the mouse blastocyst	Does not mention the proportion of orthogonal and non-orthogonal blastocysts

Table 1.6 Continuation

Reference	Mouse strain and superovulation procedure	Blastomere labelling method at two-cell stage	Assessment at blastocyst stage	Main outcomes	Weak points
(Bischoff et al., 2008)	F1 (C57BL/6Xcba) females with males expressing EGFP-H2B Natural breeding	EGFP-H2B and DIC	Blastomere's descendants cell distribution at blastocyst stage	61% of the embryos presented orthogonal pattern, 12% deviant and 27% cell mixture. There are different cleavage patterns for an embryo to form the blastocysts. Nonetheless, the orthogonal pattern seems to prevalence, this pattern might give some clues of the importance of the distribution of component of the AV axis. Nevertheless, deviant and mixed patterns might undergo to different process that might then hide some early developmental patterns.	
(Katayama and Roberts 2010)	CF-1 or NiH-Swiss females natural breeding and superovulated with 5 IU PMSG and 10 IU hCG, mated with CF-1 males. Embryos collected at two-cell stage.	CM-Dil	Blastomere's descendants cell distribution at blastocyst stage	Embryos from natural breeding females presented around 33% orthogonal patterned embryos and the rest deviant; while 75% of the embryos from superovulated females presented orthogonal pattern. Leading and lagging blastomeres can contribute to either Em or Ab poles.	
(Liu et al., 2012)	F1 (C57B1/6 x DBA/2) females (8 weeks) superovulated with 10 IU PMSG/ 10 IU hCG mated with same genotype males and for SCNT R1 embryonic stem cells were used as donor cells.	Dil	Blastomere's descendants cell distribution at blastocyst stage	42% of natural fertilised embryos presented orthogonal pattern compared with 49% of SCNT embryos. Higher expression of Sox2 gene in orthogonal group and higher implantation rates in orthogonal SCNT blastocysts but not in normal fertilised (compared with their counterparts deviant embryos)	Very low numbers of implanted embryos after transfer
(Tabansky et al., 2013)	Rainbow transgenic mice produced by pronuclear injection into BDF2 embryos crossed to C57/Bl6 collected at two-cell stage. Females were superovulated with 5 IU PMSG/ 5 IU hCG.	Exposure to 4-hydroxytamoxifen (4-OH) at four cell stage for rainbow activation	Blastomere's descendants cell distribution at blastocyst stage	Many embryos presented bias in their four-cell stage blastomere development; nonetheless they presented no specific commitment to a certain cell lineage (ICM or TE). Only in 30% of the embryos presented significant bias to this contribution	

Table 1.7 Opponents of the pre-patterning theory on mouse embryos.

Reference	Mouse strain and superovulation procedure	Blastomere labelling method at two-cell stage	Assessment at blastocyst stage	Main outcomes	Weak points
(Alarcón and Marikawa, 2003)	B6D2F1 (C57BL/6 x DBA/2) females and CD-1 were superovulated with 10 IU eCG/10 IU hCG and mated with males of the same genotype. Embryos obtained at two-cell stage	DiI	Blastomere's descendants cell distribution at blastocyst stage and further embryo transfer to foster mothers	Both strains presented similar incidence in patterns, where around 25% were orthogonal and 75% deviant. Similar implantation rates and normal fetuses rates. Cell distribution at blastocyst stage is not affecting implantation rates.	Only two embryo patterns reported, orthogonal and deviant
(Chroscicka et al., 2004)	F1 (CBA/H x C57Bl10 or C57Bl10 x CBA/H) females superovulated by 10IU PMSG/10 IU hCG. Embryos obtained at two-cell stage	Dext-Rho in one blastomere and Dext-Fluo in the other	Blastomere's descendants cell distribution at blastocyst stage	Any specific pattern in the distribution of blastomere's descendants cells from the two-cell embryos. The intermingling of cells started during the fourth or fifth cell cleavage round (at 8-16 cell stage), increasing further in next divisions.	No report about pre-patterned embryos
(Motosugi et al., 2005)	(C57BL/6 x C3H) F1 females superovulated with 5 IU hCG (no mention of PMSG) mated with (C57BL/6 x DBA/2) F1 males. Embryo collection at 2 cell stage.	DiI, DiD or DiA	Blastomere's descendants cell distribution at blastocyst stage	34% of the embryos presented orthogonal pattern, 35% deviant and 31% cell intermingling With respect of the SEP there any developmental differences when transferring just one blastomere with the sperm entry point or the twin. Both twin blastomeres undetermined in cell fate. No polarity until blastocyst stage. ZP exerting spatial constraint determining Em-Ab axis	
(Waksmundzka et al., 2006)	F1 (CBA/H X C57Bl10) or F1 (C57Bl10 X CBA/H) females (2-4 months old) superovulated with 10IU eCG/10IU hCG	Fluorochrome-conjugated dextran	TCC and cell distribution between blastomere descendants	Both blastomere give rise to the embryonic and abembryonic parts within the blastocyst	

Table 1.7 Continuation

Reference	Mouse strain and superovulation procedure	Blastomere labelling method at two-cell stage	Assessment at blastocyst stage	Main outcomes	Weak points
(Kurotaki et al., 2007)	C57Bl/6 females mated with homozygous R26H2BEGFP mice	Photo conversion	Blastomere's descendants cell distribution at blastocyst stage	71% embryos presented orthogonal pattern and 29% deviant Extrinsic factors as ZP shape have an effect on blastocyst formation and cell lineage allocation and not intrinsic factors as cell cleavages within the early embryo	Only embryos with the second PB on one of the blastomeres instead of between them were taking into account
(Alarcón and Marikawa, 2008)	F1 (C57BL/6 c CBA), F1 (C57BL/6 x DBA/2) and CD1 female mice superovulated with PMSG and hCG and mated with F1 (C57BL/6 x DBA/2) or CD-1 males. Embryo collection at two-cell stage.	Dil *Alginate solution on the culture medium	ZP measurements and blastomere's descendants cell distribution at blastocyst stage	60% embryos on the alginate group presented the orthogonal pattern while the rest the deviant one. Results different from the control group with only 19% embryos presenting orthogonal pattern and the majority deviant one. Mechanical constraint model as suggested that orthogonal patterned embryos are promoted due to less movements within the pre-implantation embryo development	Only ME embryos were taking into account for this study
(González et al., 2011)	F1 (129/Sv x C57Bl/6) females superovulated with 5 IU PMSG/5 IU hCG. Embryos collected at two-cell stage.	Dil	ICM and TE contribution from lagging and leading blastomere	Fate of lagging blastomere not determined as it can form ICM, TE or both. 4 blastomeres at four-cell stage can produce ESC line. Leading blastomere in three-cell embryos equatorial tends to be TE while meridional does not have any predilection between ICM and TE.	Does not mention cell distribution according cell clusters

Later on, the pre-patterning theory was rejected once again by Chroscicka and colleagues as they did not observed any pre-patterning in mouse embryos, claiming that daughter cells from blastomeres of the two-cell embryo start intermingling at the eight-cell stage onwards (Chroscicka et al., 2004). It has been argued that the aforementioned discrepancies between studies might be because the use of different parameters as: mouse strain, fertilisation methods, superovulation protocols, culture conditions and cell lineage tracing and assessment methods. Nevertheless, the controversy around the subject has continued and the possible parameters causing the pre-patterning (or otherwise the stochastic distribution of cells within the pre-implantation mammalian embryo) have increased; making the story of cell lineage commitment very complex. The aforementioned parameters will be discussed promptly.

Motosugui and colleagues observed the same incidence between pre-patterned and non-prepatterned embryos. They decided to go further and investigate about the sperm entry point (SEP) on embryo development. The experiment consisted on the production of two different chimeras, one containing two blastomeres with the SEP (from two different embryos) and the other containing two blastomeres without the SEP. There was no significant difference between chimeras in blastocyst formation neither in live births rates (Motosugi et al., 2005). Hence, the results suggested that SEP do not exert a developmental bias on mouse embryos. Furthermore, Motosugui also noticed that Em-Ab axis could be predicted by ZP spatial constraint. Other

authors have observed some biasing by the ZP on cell allocation at blastocyst stage. Whereas Gardner claimed that the lesser diameter of the ZP is related with the first cleavage plane and further Em-Ab axis (Gardner, 2007), Alarcon and Kurotaki suggested that this pre-patterning is due to mechanical constrain by the ZP; reducing cell movements within the embryo (Alarcón and Marikawa, 2008, Kurotaki et al., 2007). Plusa also observed the above as the majority of the studied embryos with the second PB at one side of the long axis (and therefore ellipsoidal ZP shape) were pre-patterned (85%) (Plusa et al., 2005). This mechanical constrain model has been indirectly supported by other research group that observed a higher incidence in the random cell allocation on embryos cultured without ZP (Katayama and Roberts, 2010). Then again, Plusa agrees with Gardner and both suggested that first cleavage plane rather than the proportion of animal or vegetal portion within the twin blastomeres is driving cell position at blastocyst stage.

In order to elucidate if the inherited portion of the animal or vegetal pole exerts some bias in embryo development, Piotrowska and colleagues constructed different chimeras using tetrahedral embryos previously injected with Dil at two-cell stage (Piotrowska-Nitsche et al., 2005). The chimeras were made with blastomeres inheriting most of the vegetal pole. Even though they were able to form blastocysts, they obtained significant lower implantation rates when compared with chimeras made from blastomeres inheriting most of the animal pole. In addition to this, similar experiments performed by other research group arrived to the same conclusions (Morris et al., 2012).

Nevertheless, further investigations on this subject suggested that embryos in which the first cleavage is aligned to the animal-vegetal (AV) axis present inferior viability than those in which the first cleavage is orthogonal to this axis (Piotrowska-Nitsche and Zernicka-Goetz, 2005). New technologies as the Rainbow cell tracing method have been adapted in mice for the pre-patterning study. Results shown that only the 30% of the embryos presented the orthogonal pattern (Tabansky et al., 2013). Authors agreed that pre-patterning in mouse embryos is possible but is not happening in all embryos.

1.1.2. Epigenetics and development

Recently, the study of epigenetics during pre-implantation development has increased. Epigenetics studies are focused on heritable changes that can be seen on the phenotype, but do not represent any change on DNA sequences (Kouzarides, 2007). Reprogramming of the gametes and preimplantation embryos is crucial for its development into a new organism (Léandri et al., 2009, Hales et al., 2011). Genome epigenetic modifications include histone modifications (such as methylation, acetylation, sumoylation, ubiquitination, phosphorylation, etc.), DNA methylation and remodelling of the chromatin (Shi and Wu, 2009, Wang et al., 2011). Therefore, histone modifications, by causing chromatin structure alteration, will affect the expression or silencing of genes facilitating or restricting access of controlling gene expression transcription factors (Parfitt and Zernicka-Goetz, 2010). Nonetheless, it is not totally understood how epigenetic remodelling mechanisms and gene

expression are related during embryogenesis (Sepulveda-Rincon et al., 2016b).

Two crucial epigenetic marks in regulation of gene expression are acetylation of lysine tails on histone 3 (H3) and histone 4 (H4) (Jafarpour et al., 2011, Paul and Knott, 2013). H3 acetylation initiates at lysine (K) 14, then 23, after 18 and finally at lysine 9 (Wu et al., 2011). Thus, H3K9 being the last H3 to be modified, its acetylation and methylation are considered important epigenetic modifications, since they have been related to active and inactive chromatin configurations respectively (Santos et al., 2003). H3K9 acetylation may prevent the heterochromatin downstream assembly by maintenance of genome regions transcriptionally competent (Sawai et al., 2012). The possible mechanisms of this is that the affinity for DNA is reduced because the acetylation on the histone tails neutralises the charge of the histone tails. Hence, the conformation of the nucleosome is changed to an open configuration which is accessible for transcriptional factors (Wang et al., 2011).

Overall, the pre-patterning study not only focuses on further cell allocation, but also attempts to clarify if there is any molecular difference between twin blastomeres or blastocysts showing different pre-patterns and if they have different potentials in further developmental stages. Studies regarding the expression of *Carm1*, which is an enzyme mediating histone modifications transferring methyl groups to residues of arginine and seems to be over

expressed on the ICM in mouse blastocysts (Torres-Padilla et al., 2007). It suggests that injecting this enzyme to one blastomere at two-cell stage can bias the fate of that blastomere into ICM (Parfitt and Zernicka-Goetz, 2010). Thus, it has been suggested that epigenetic modifications can be important factors driving cell fate on mammalian embryos (Hemberger and Dean, 2007). Still, the contribution of histone modifications on cell lineage commitment during development is far from being understood (Paul and Knott, 2013).

1.4. Rationale and hypothesis

Currently, one of the main concerns about ARTs is related to the safety of these techniques, regarding pregnancy complications and long-term outcomes related to genetic and birth defects (Grafodatskaya et al., 2013, Feuer and Rinaudo, 2012, Kondapalli and Perales-Puchalt, 2013). A number of studies have reported that imprinting disorders like Angelman and Beckwith-Wiedemann syndromes (BWS) and different cancer types (Hargreave et al., 2013) are more common in ART babies compared with babies conceived spontaneously (reviewed in (Pinborg et al., 2013)). Recent meta-analysis suggested that there is a 1.32 increased risk for ART babies in having a birth defect when compared with non-ART babies (Hansen et al., 2013). However, other long-term ART consequences have not been investigated yet. In the past 30 years of the development of ARTs, the main focus has been to increase live birth rates with little emphasis on the possible effects that ARTs might have on short and long-term embryo development.

The hypothesis in the present study is that cell allocation patterns in mammalian embryos are affected by different embryo manipulation techniques. In case that this hypothesis is accepted, the factors or circumstances causing these cell allocation patterns in mammalian embryos should be thoroughly investigated. Possible candidates are different embryo production methods and embryo manipulations. As well, it is important to elucidate the correlation of the different cell allocation patterns observed at blastocyst stage with embryo quality and any significant developing difference between them (if any).

1.5. Aims and Objectives of the study

First cell lineage commitment is a crucial process for embryo survival and proper further development (Cockburn and Rossant, 2010). It has been proposed that first cleavage plane can be related with the Em-Ab axis, thus first cell lineage specification. In Chapter three, the present study aims to elucidate if cells of the mammalian embryos are pre-patterned to occupy a specific location during pre-implantation or their development is a stochastic process. First, cell allocation patterns will be identified in mouse embryos by non-invasive live cell tracing. Furthermore, it will be determined if cell allocation pattern incidence is changed by superovulation, advanced maternal age or *in-vitro* fertilisation. Despite the great progress in the understanding of mouse pre-implantation development, it remains unclear how close it is to human embryo development (Cockburn and Rossant, 2010). Bovine and

humans are monoparous species, and embryos are closer in terms of crucial embryo developmental events (as EGA). In addition, a close correlation on offspring defects as the large offspring syndrome in cattle and the BWS in humans has been reported (Chen et al., 2013b). It is worth noting that humans and cattle are outbred species not as inbred mice used on science. Thus, cell allocation patterns will be explored in other mammalian species that resembles more to the human embryo, the bovine.

Moreover, PGD/S is an invasive technique taking momentum in fertility clinics worldwide. Only in the UK, the number of IVF cycles involving PGD has increased 13.5% from 2009 to 2010 (Yu et al., 2009). Thus, in Chapter four a special interest will be made on embryo biopsy and its effects on cell allocation patterns during pre-implantation development. This will be with the aim to assess how this invasive procedure might be shifting cell allocation patterns, possibly promoting cell rearrangements within the pre-implantation embryo. Likewise, the capacity of the blastomeres of the two-cell mouse embryos to develop into blastocysts will be explored as a possible alternative to increase embryo quantity for embryo transfer or for cell therapy.

On the other hand, different epigenetic changes are responsible for gene expression or silencing. Hence, in Chapter five, changes on epigenetic marks will be explored among embryos following the different cell allocation patterns. Similarly, symmetry of epigenetic marks will be investigated in blastomeres of the two- and the four-cell stage embryos. The above with the

aim to investigate further the possible mechanisms causing different cell allocation patterns during pre-implantation development.

Finally, in Chapter six, the aim will be to determine if cell allocation patterns are related with further embryo development. Implantation rates and organ morphometry after birth will be analysed after embryo transfer of embryos following different cell allocation patterns.

2. CHAPTER TWO: GENERAL MATERIALS AND METHODS

All experiments were performed according to the Animals Scientific Procedures Act 1986 and the European Convention on Animal Experimentation, under the project licence 40/3480 issued by the Home Office, held by the Professor Bruce K Campbell and the project licence 78-95 issued by the French veterinary regulatory service, held by Dr Nathalie Beaujean. Mouse experiments in Chapters 3-6 were performed at the University of Nottingham (Nottingham, UK); while mouse experiments in Chapter 5 and bovine experiments were performed at the French National Institute for Agricultural Research (INRA, Jouy-en-Josas, France).

2.1. Protocols using mouse tissue

Mice were housed in the Biomedical Sciences Unit (BSU) at the School of Medicine of the University of Nottingham or at the INRA animal facility following a 12 hours light/12 hours dark cycle. Male mice were housed individually and female mice were housed in groups of 3 or 4.

2.1.1. Embryo production

2.1.1.1. Natural ovulation and mating

For natural ovulation, promotion of the Whitten effect was achieved by placing dirty bedding from a male's cage into the female's cage two days before placing the females with the males. Then, vaginal plugs were checked

daily and in case of presence, females were separated for embryo collection. Non-plugged females were sacrificed after 5 days of being in the male's cage and were not used for the study.

2.1.1.2. Superovulation protocol

Five to seven weeks old females (Charles River, Margate, UK) were superovulated with one peritoneal injection of 100 μ l containing 5 international units (IU) of pregnant mare serum gonadotrophin (PMSG, Intervet, Buckinghamshire, UK) followed by one peritoneal injection of 100 μ l containing 5 IU of human chorionic gonadotrophin (hCG, Intervet, Buckinghamshire, UK) after 48 hours. The different mouse strains used to produce this thesis are specified on Table 2.1.

Table 2.1 Mouse strains and media used during this study.

Chapter	Mouse strain	Origin	Embryo culture medium	Embryo handling medium
Chapter three	CD-1	Charles River, UK	KSOM	EmbryoMax
Chapter four	B6CBA-F1	Charles River, UK	M16	M2
Chapter five	B6CBA-F1	INRA, FR and Charles River, UK	M16	M2
Chapter six	B6C3-F1 x B6D2-F1	Embryotech, USA	KSOM	EmbryoMax

2.1.1.3. In-vitro fertilisation

The IVF mouse protocol was followed according to the European Mouse Mutant Archive (EMMA) (Bin Ali et al., 2014). Briefly, sperm pre-incubation dishes were prepared placing one drop 90 μ l of sperm pre-incubation medium (see Appendix Ia) under mineral oil (Merck Millipore) in a 35 mm Petri Dish

(Falcon 351008) and equilibrated at 37°C and 5% CO₂ for 10-20 min prior use. After, one CD-1 male (9-24 weeks old) was sacrificed by cervical dislocation (only one male was used per repetition). The cauda epididymis were dissected and all adipose and vascular tissue was removed under a dissecting microscope and light from above. Then, the epididymis were placed into the oil next to the sperm pre-incubation medium drop and the apex of the cauda epididymis was nicked with a small tweezers and a small sperm ball was dragged into the medium drop. The sperm was allowed to disperse in the medium for 60 min at 37°C and 5% CO₂ without disturbance.

Fertilisation dishes were prepared 10-20min before oocyte collection and placed at 37°C and 5% CO₂. HTF medium (see Appendix Ib) was modified with the addition of 30.7 mg of L-Glutathione (Sigma, G4251) to 1 ml of HTF medium. Further, 10 µl of this solution were added to 4 ml of HTF medium and passed through a 0.22 µm filter. One drop of 200 µl of the modified HTF medium was placed in a 35 mm petri dish. One dish was prepared for every three superovulated females. CD-1 females (5-7 weeks old) were previously superovulated following the protocol on section 2.1.2.2 and were sacrificed by cervical dislocation 13-15 h post-hCG. Then, oviducts of three females were dissected and placed into the oil in the fertilisation dish. After, swollen ampullas were identified and were teared releasing the cumulus-oocyte-complexes (COCs). COCs were dragged to the fertilisation drop.

Furthermore, 3-5 μ l of the sperm in the drop edges was removed using a wedge-shaped 10 μ l pipette tip (Axygen, T-400 R) and was added to each fertilisation drop. Then, fertilisation dishes were placed at 37°C and 5% CO₂ for 3-4 hours. After incubation, the presumptive embryos were washed three times in HTF medium, previously pre-equilibrated overnight under mineral oil. After, presumptive embryos were cultured additionally in HTF medium for 5 hours. Finally, embryos were placed in KSOM medium for further culture up to blastocyst stage.

2.1.2. Embryo collection and embryo culture

For one-cell embryo collection, females were checked at 16-20 h post-hCG for visible vaginal copulation plug and were sacrificed at 22-24 h post-hCG. Mice were sacrificed by cervical dislocation, then they were dissected and the uterine horns were identified. Later, oviducts were then cut and placed in embryo handling medium (Table 2.1), previously pre-equilibrated at 37°C. Then, ampullas were punched by a needle and one-cell embryos were released. Embryos were placed in embryo handling medium with hyaluronidase (1 mg/ml) for a few seconds for cumulus cell removal. After several washes on embryo handling medium under mineral oil, embryos were cultured in embryo culture medium (Table 2.1) under mineral oil at 37°C with a gaseous atmosphere of 5% CO₂. The embryos were cultured either in a normal incubator or in the EmbryoScope™ (Unisense Fertilitech, Denmark).

2.2. Protocols using bovine tissue

2.2.1. Oocyte collection and oocyte maturation

Ovaries from the local slaughterhouse were collected and placed in phosphate-buffered saline (PBS) at 30°C for around 2 hours during their transportation to the laboratory. Then, immature oocytes from antral follicles of 2 to 8 mm in diameter were aspirated with a 19-gauge needle under constant vacuum. COCs with three or more compact layers of cumulus cells were selected and rinsed in HEPES-buffered TCM 199. Then, COCs were cultured in maturation medium 25 nM bicarbonate-buffered TCM 199 supplemented with 10% foetal calf serum (FCS) (Gibco, Grand Island, NY, USA), 10 µg/ml of follicle stimulating hormone (FSH) (NIDDK, Stimufol, Sart-Tilman, Belgium), 10 µg/ml of luteinising hormone (LH) (NIDDK, Beckers) and 1 µg/ml of oestradiol 17β (E2) at 39°C in a humidified environment of 5% CO₂ for 22 hours. After, the COCs were incubated in HEPES-buffered TCM 199 supplemented with 0.5% (w/v) hyaluronidase for 5 minutes. Then, the COCs were gently pipetted in order to remove some of the cumulus cells until only 2 or 3 layers of granulosa cells were left.

2.2.2. *In-vitro* fertilisation and embryo culture

Sperm frozen sample was previously thawed at 30°C during 1 minute and motile sperm were selected by the swim-up technique as previously described (mAdamiak et al., 2005). Furthermore, sperm capacitation was performed

with heparin. Then, as previously described (Fouladi Nashta et al., 1998), disaggregated COCs were washed once in oocyte medium containing NaCl, 6.8 g/L; KCl, 230 mg/L; NaHCO₃, 160 mg/L; Na₂HPO₄, 47 mg/L; HEPES, 4.8 g/L; kanamycin monosulfate, 75 mg/L; pyruvic acid, 11 mg/L; BSA, 6 g/L; 60% syrup lactic acid, 1.86 ml/L; MgCl₂ 6H₂O, 100 mg/L; CaCl₂ 2H₂O, 840 mg/L; pH 7.4, osmolarity 282 mOsmol/kg H₂O. After, the COCs were transferred into 500 µl of fertilisation medium in a four-well dish (40-50 oocytes per well) containing sperm (1.0×10^6 sperm/ml) and cultured for 20 h at 39°C in a humidified environment of 5% CO₂, 5% O₂ and 90% N₂. After 20 hours (day 1), all embryos were washed twice in HEPES-buffered modified synthetic oviductal fluid (H-SOF) medium and transferred into mSOFaa media supplemented with 3 mg/ml of bovine serum albumin (BSA) for 24 hours (day 2). On day 2 bovine embryos were cultured in mSOFaa media supplemented with 10% FCS at 39°C in a humidified incubator with a gaseous atmosphere of 5% CO₂, 5% O₂ and 90% N₂ from day 2 until assessment.

2.3. Non-invasive cell tracing method

As previously described in Alarcón and Marikawa (2003), CM-Dil (Chloromethyl Dil, Molecular probes, C7001) was dissolved in virgin olive oil at 60°C at a final concentration of 2 mg/ml. When cooled down and prior to labelling, the injection pipette was backfilled with the dye. Mouse and bovine embryos at two-cell stage (46-48 hours post-hCG and 30-32 h post-fertilisation respectively) were placed in embryo handling medium at

37°C during the micromanipulation. Injections on Chapters 3, 4 and 6 were performed on a Leica DMI3000 B inverted microscope using Eppendorf TransferMan NK 2 micromanipulators with a coupled Eppendorf FemtoJet microinjector. Injections on Chapters 4 and 5 were performed on a Nikon Diaphot-TMD inverted microscope using Narishige micromanipulators with a coupled Eppendorf 5242 microinjector. The micropipette was pushed through the zona pellucida and pressed against one of the blastomere membrane, and then a microdrop was deposited (Figure 2.1). After the injection, labelled embryos were cultured in 25 µl drops of embryo culture medium under mineral oil under culture conditions. It is worth noting that <5% of bovine embryos cleaved to the two-cell stage before 24 h post-IVF and were excluded from the study as at 30 h post-IVF these embryos were already at the three- or four-cell stage. Similarly, embryos that did not reach the two-cell stage at 32 h post-fertilisation were not included in the study.

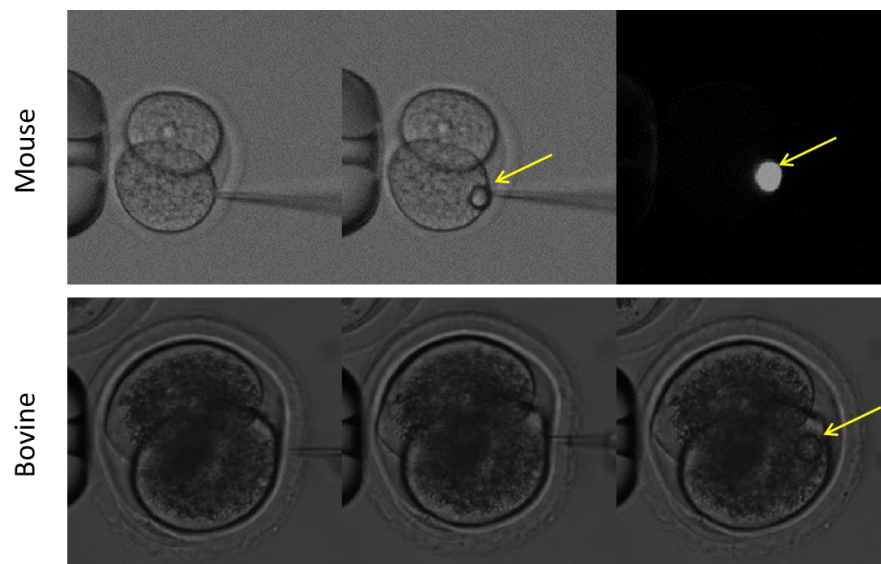


Figure 2.1 Lipophilic dye injection in two-cell mouse and bovine embryos. Yellow arrows point the injected oil drop containing the lipophilic tracer CM-Dil for mouse embryos (≈ 5 pl) and Dil for bovine embryos (≈ 8 pl).

2.4. Blastocyst assessment

2.4.1. Pre-patterning

Blastocysts were placed in 5 μ l M2 or Hepes medium drops on a 60 mm petri dish and on a Leica DMI3000 B inverted microscope, coupled with red fluorescent filters corresponding to maximum excitation/emission wavelengths of 553 nm and 570 nm respectively. For better visualisation, embryos were rotated in order to place the blastocoel cavity floor and the boundary line between the fluorescent and non-fluorescent cells in the same focal plane, as previously described (Alarcon and Marikawa, 2005). Then, embryos were classified into three categories: orthogonal (**Figure 2.2a**), if two defined clusters of cells were observed (stained and not stained) and if the Em-Ab axis was orthogonal $\pm 45^\circ$ to the boundary line between stained and non-stained cells; deviant (**Figure 2.2b**), if two defined clusters of cells were observed (stained and not stained) and if it was parallel $\pm 30^\circ$ to the Em-Ab axis and random (**Figure 2.2c**), if stained cells were intermingled with non-stained cells.

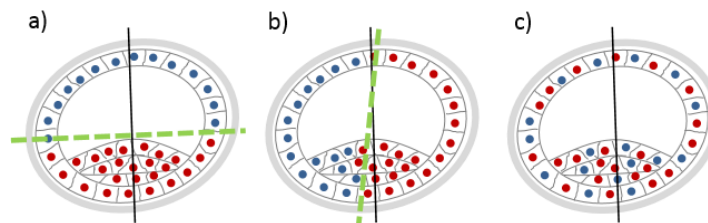


Figure 2.2 Cell distribution at blastocyst stage according with first cell cleavage plane in pre-implantation mouse embryos. a) Orthogonal pattern where first cleavage plane (blue dotted line) is orthogonal to the Embryonic-Abembryonic (Em-Ab) axis (black line). b) Deviant pattern where first cleavage plane is aligned ($\pm 30^\circ$) to the Em-Ab axis. c) Random pattern where the first cleavage plane does not predict cell allocation at blastocyst stage and descendants from each twin blastomere are stochastically distributed.

2.4.2. Total cell count

Blastocysts were permeabilised in 0.1% Triton-100X/PBS during two minutes at room temperature (RT) and then rinsed twice in PBS. Furthermore, embryos were mounted on a SuperFrost microscope slide using Vectashield-DAPI as mounting medium and nuclear staining. Total cell count was effectuated on a Nikon Eclipse Ti 90x microscope, along with a Hamamatsu digital camera (C4742-80-12AG) and a fluorescent filter corresponding to the excitation 350 nm and 470 nm of emission for DAPI. The DNA amount of all cells in blastocysts fluoresced blue (Figure 2.3).

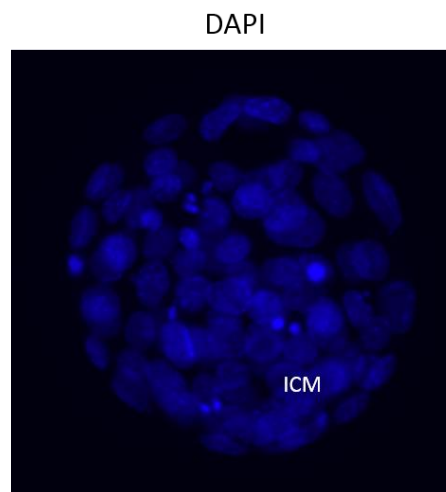


Figure 2.3 Blastocyst nuclear staining with DAPI. Cell nuclear staining can be observed in blue.

2.5. Statistical analysis

Data sets were checked for normality using Shapiro-Wilk test employing IBM SPSS version 23. In general, values are reported as mean $\pm$ SEM. For SEM calculation, the total number of replicates of each experiment was used as

N value when reporting incidence of cell patterns. Total number of embryos (n value) was used to calculate SEM value when reporting TCC in blastocysts and morphokinetics. In order to compare blastocysts rates and morphokinetics between 2 treatments, T-test was used. Analysis of variance ANOVA test was performed for single or two-factor when comparing more than two variables. A p-value less than 0.05 was considered as statistically significant. In case a $p < 0.05$ was obtained after ANOVA test, post-hoc Bonferroni test was used to investigate further differences between groups.

3. CHAPTER THREE: EFFECT ARTS ON CELL ALLOCATION PATTERNS DURING PRE-IMPLANTATION DEVELOPMENT

3.1. Introduction

Mechanisms behind the first lineage specification during early embryo development in mammals are not fully understood. It is still unknown the exact time when this specification begins and if this process presents some grade of reversibility (Artus and Chazaud, 2014). As mentioned in Chapter 1, it is still debatable if the first lineage specification is established at early embryo stages. Whereas some authors propose that Em-Ab axis is related with the second cell cleavage (Bischoff et al., 2008, Fujimori et al., 2003, Gardner, 2007, Katayama and Roberts, 2010, Liu et al., 2012, Piotrowska et al., 2001, Piotrowska and Zernicka-Goetz, 2002, Torres-Padilla et al., 2007), others suggest that lineage specification cues start after the four-cell stage or even until morula stage (Guo et al., 2010, Kurotaki et al., 2007, Motosugi et al., 2005, Waksmundzka et al., 2006, Wennekamp et al., 2013, Alarcón and Marikawa, 2003, Alarcón and Marikawa, 2008).

It is believed that one of the greatest determinants for a successful pregnancy is oocyte competency (Zhang and Smith, 2015). Ovarian pool and oocyte quality seem diminished with age, especially after 36 years in women (Braude, 2013). According to the literature superovulation protocols, different oocyte fertilisation methods or prolonged *in-vitro* culture might affect further embryo development. In mouse, it has been suggested that superovulation

protocols in addition to *in-vitro* culture might alter imprinting gene expression; therefore, superovulation might be affecting/altering oocyte and further embryo development (Fortier et al., 2008, Market-Velker et al., 2010).

3.2. Objectives

How cells, within the mammalian embryo, commit to the two first cell lineages (ICM and TE) is a crucial step during embryo development. Thus, the aim of this chapter is to identify different cell allocation patterns in mouse embryos during pre-implantation development. Furthermore, as part of looking into the effects of ART factors affecting short and long term development, the research progresses to: comparison of cell allocation patterns in embryos produced from PMSG/hCG gonadotrophin stimulated versus non-stimulated mice. Equally, cell allocation patterns in embryos produced from aged female mice or by oocyte *in-vitro* fertilisation will be compared with embryos produced naturally.

In order to further elaborate and understand the origin and consequences of embryo patterning on the pre-implantation embryo development, additional secondary outcomes have been recorded wherever possible. Secondary outcomes includes blastocyst rate of *in-vitro* development, morphology grades, morphokinetic development and gender differences between embryos following different cell allocation patterns. Additionally, cell allocation patterns will be identified in bovine embryos in order to determine if its incidence is similar than in mouse embryos.

3.3. Methods

3.3.1. Embryo production

For this chapter two models were used: mouse and bovine. Mouse embryos were produced by four different route: first embryos were obtained after natural mating non-superovulated CD-1 females (See Section 2.1.1.1); second, embryos were obtained after natural mating of superovulated CD-1 females (See Section 2 1.1.2); third, embryos were produced after *in-vitro* fertilisation of oocytes from superovulated CD-1 females (See Sections 2.1.1.2-3); finally, embryos were obtained after natural mating of superovulated 30-35 weeks old CD-1 females (see Section 2.1.1.2). Bovine embryos were produced as stated on Section 2.2.

3.3.2. Live cell tracing

For single cell labelling the protocol on Section 2.3 was followed. For double staining on two-cell embryos, Dil injection was performed first as the deposited oil drop helped recognised the injected blastomere. Then, the non-injected blastomere was injected in the cytoplasm with a solution of 150 nM Ras-eGFP mRNA. (Moriyoshi et al., 1996).

3.3.3. Time-lapse culture for cell tracing observation

In order to establish if the cell staining was inherited only to daughter cells from the injected blastomeres, time-lapse observations of single and double stained mouse/bovine embryos were performed on a Zeiss LSM700 confocal

microscope (MIMA2 platform at INRA, Jouy-en-Josas, France). The inverted microscope AxioObserver Z1 was coupled with an incubation system. A maximum of 10 mouse or 6 bovine embryos were cultured in 20 μ l drops of embryo culture medium under mineral oil in glassed bottom dish (Ibidi, Germany). Bright field and fluorescence images within different focal planes with 7 μ m intervals every 2 h using Zeiss Zen software. To improve signal it was used a 2.5 airy unit pinhole size (scaling Z = 7.2 μ m) and 0.2% laser power (laser at 488 nm of 10mW, laser at 555 nm of 1 mW)

1.1.3. Gender determination

Embryos were fixed with 4% paraformaldehyde (PFA) in PBS at room temperature for 20 min or overnight at 4°C and rinsed a couple of times in PBS. Then, embryos were permeabilised in 1% Triton X-100 (Sigma-Aldrich) during 30 min at RT. Embryos were rinsed three times in PBS and incubated for 1 hour in BSA/PBS 2% to saturate non-specific sites. Later, embryos were incubated in 10 μ l drops of the diluted 1:200 primary antibody H2AK119Ub (Cell Signalling Technology, Ma., USA) under mineral oil overnight at 4°C. Then, embryos were rinsed three times in PBS and incubated in 10 μ l drops of the diluted 1:200 Anti-Rabbit IgG secondary antibody (Alexa Fluor 488-conjugated) under mineral oil at RT. From this point and on the embryos were kept away from light. Finally, embryos were rinsed once in PBS and transferred in minimum volume to a drop of mounting medium Vectashield + DAPI nuclear staining (Vector Laboratories, USA, see Section

2.4.2) on a SuperFrost microscope slide (ThermoScientific) and dragged continuously to the bottom of the slide. Once the embryos remain at the bottom, they were transferred to a different drop of the mounting medium and a glass coverslip was placed on the slide. Gender determination was performed on a Nikon Eclipse Ti 90x microscope, along with a Hamamatsu digital camera (C4742-80-12AG) and a fluorescent filter corresponding to the excitation 488 nm of emission for fluorescein isothiocyanate (FITC). Paternal X chromosome inactivation occurs in female embryos around blastocyst stage. Histone 2A ubiquitin (H2AUb) can be found in the paternal inactive X chromosome (Xi), consequently, a Xi patch will be seen in cells of female embryos.

3.3.4. Statistical analysis

Data sets were checked for normality using Shapiro-Wilk test employing IBM SPSS version 23. Values are reported as mean $\pm$ SEM. For SEM calculation, the total number of replicates of the experiment was used as N value when reporting incidence of cell patterns. Total number of embryos (n value) was used to calculate SEM value when reporting TCC in blastocysts and morphokinetics. In order to compare blastocysts rates and morphokinetics between 2 treatments, T-test was used. Analysis of variance ANOVA test was performed for single or two-factor when comparing the pattern incidence between treatments and blastocysts total cell count and when comparing morphokinetics between patterns. Chi-square test was used to determine any

skew on embryo gender between treatments. A p-value less than 0.05 was considered as statistically significant.

3.4. Results

3.4.1. Cell tracing in mouse embryos

3.4.1.1. Non-invasive cell tracing method

First, embryos were observed by time-lapse in order to determine if the staining was only visible on the injected blastomere and its descendants. As shown in Figure 3.1, after a couple of minutes of the CM-Dil injection, the lipophilic dye spreads on the injected blastomere. Furthermore, it was investigated if the CM-Dil injection had an adverse effect on cell cleavages. In Figure 3.2 it can be observed that the injected blastomere is able to cleave first or after the non-injected one at similar rates. From a total cohort of 170 embryos within 3 replicates; in $49 \pm 3.7\%$ of the embryos the injected blastomere-cleaved first, in $41 \pm 2.8\%$ cleaved second and in $10 \pm 1.8\%$ injected and non-injected blastomeres cleaved synchronously. It is worth noting that $<5\%$ of the injected blastomeres presented cell death after injection. Nevertheless, these embryos were able to reach blastocyst stage.

In order to better observe the distribution of descendant cells of the blastomeres of the two-cell mouse embryo, each blastomere was injected with a different dye colour, dextran-FITC (green) or Dil (red). Three cell allocation patterns were identified at blastocyst stage (Figure 3.3). Blastocysts

were classified as “orthogonal” (Figure 3.3a) if the two cell clusters were visible (green and red) and if the borderline of these two was orthogonal $\pm 45^\circ$ (perpendicular) to the Em-Ab axis. Embryos presenting two defined cell clusters but where the borderline was parallel $\pm 30^\circ$, were classified as “deviant” (Figure 3.3b). Finally, if green and red labelled cells were intermingled and not defined cell clusters were observed, embryos were classified as “random” (Figure 3.3c). Tri-dimensional observation of the cell allocation patterns previously described can be observed in Figure 3.4. Time-lapse images during pre-implantation development of embryos following the orthogonal, deviant and random patterns can be observed in (Appendix II).

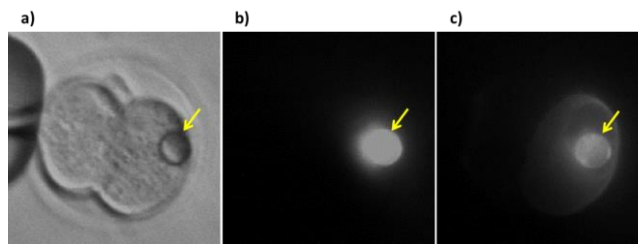


Figure 3.1 Non-invasive cell tracing in two-cell mouse embryos. The yellow arrow points to the injected oil drop containing CM-Dil. a) Two-cell mouse embryo on bright field, b) CM-Dil staining immediately after injection, c) CM Dil staining 10 min after the injection.

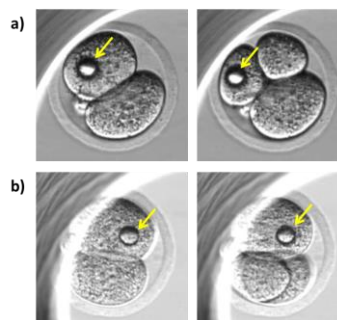


Figure 3.2 Second cell cleavage on injected two-cell mouse embryos. a) Two-cell embryo injected with CM-Dil tracer on one blastomere (left picture), where the injected blastomere cleaves first than its counterpart the non-injected blastomere (right picture). b) The injected blastomere (left picture) cleaves after the non-injected one (right picture).

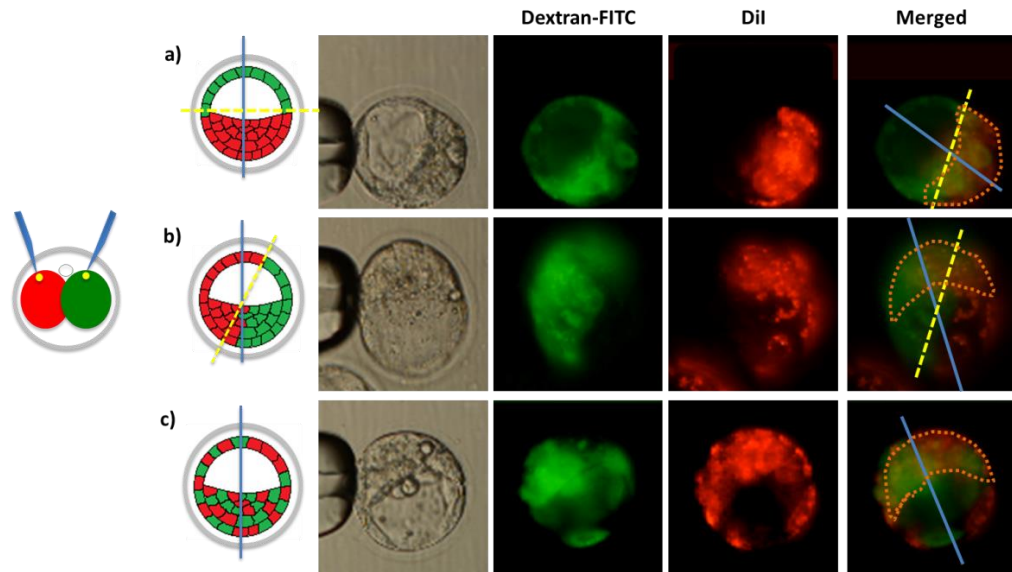


Figure 3.3 Cell allocation of blastomeres of the two-cell mouse embryo at blastocyst stage. Two-cell mouse were injected with Dextran-FITC on one blastomere and with Dil on the other one. At blastocyst stage the descendants of each blastomere were tracked and embryos were classified into: a) Orthogonal, b) Deviant and c) Random embryo. Blue line denotes the embryonic-abembryonic axis, dotted orange line shows the inner cell mass and dashed line denotes borderline between stain and not stained cells.

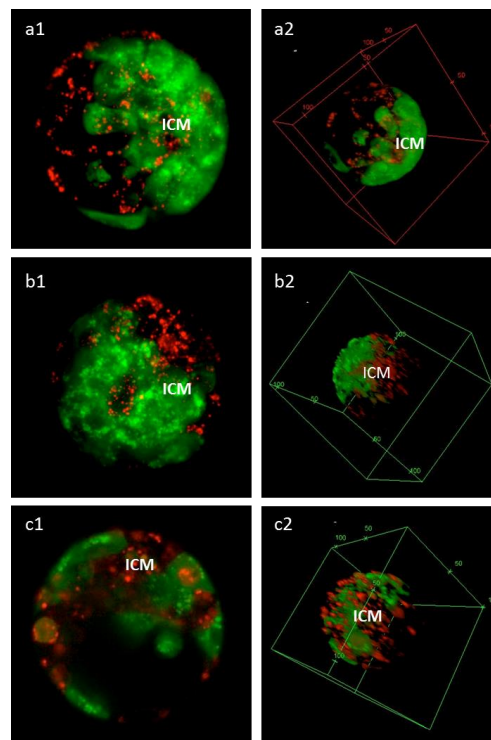


Figure 3.4 Cell allocation patterns on 3D in mouse embryos. Two-cell mouse embryos were injected on one blastomere with Dextran-FITC and on the other with Dil for cell tracing. **a1** shows an orthogonal embryo and its 3D reconstruction on **a2**. **b1** shows a deviant embryo and its 3D reconstruction on **b2**. **c1** shows a random embryo and its 3D reconstruction on **c2**.

3.4.1.2. PMSG/hCG superovulation protocol in mice

does not influence embryo cell allocation patterns

With the aim of seeing the effect of superovulation protocol (PMSG/hCG), widely used in fertility clinics, mouse embryos were obtained from superovulated females and non-superovulated females after natural fertilisation. In order to trace the descendent cells from the two-cell embryos, one blastomere membrane was injected with a lipophilic tracer. After injection embryos were placed back in culture. Statistical T-test showed no significant differences ($p = 0.126$) on blastocyst rates between embryos from superovulated and control females during 5 replicates of the experiment, ($n = 270$ embryos, $67.9 \pm 4\%$ and $n = 324$ embryos, $78.0 \pm 4\%$ respectively). A total of 258 blastocysts for the superovulated group and 240 for the control group were successfully classified according to their cell allocation patterns into the three previously observed patterns in double labelled embryos: orthogonal, deviant and random embryos (Figure 3.5).

Incidence of the different patterned embryos can be seen in Table 3.1, where for control and superovulated groups, the random pattern was significantly predominant ($p < 0.001$ by one-way ANOVA test, $57.6 \pm 3.7\%$ and $48.6 \pm 2.8\%$ respectively) over the orthogonal and deviant ones. Within the control group, the incidences of the orthogonal and deviant patterns are comparable ($p > 0.05$ by post-hoc Bonferroni test, $19.5 \pm 2.9\%$ and $22.7 \pm 3.9\%$ respectively). However, significant differences were found on the incidences

of orthogonal and deviant patterns within the superovulated group ($p < 0.018$ by post-hoc Bonferroni test, $19.9 \pm 2.8\%$ and $31.3 \pm 2.3\%$ respectively).

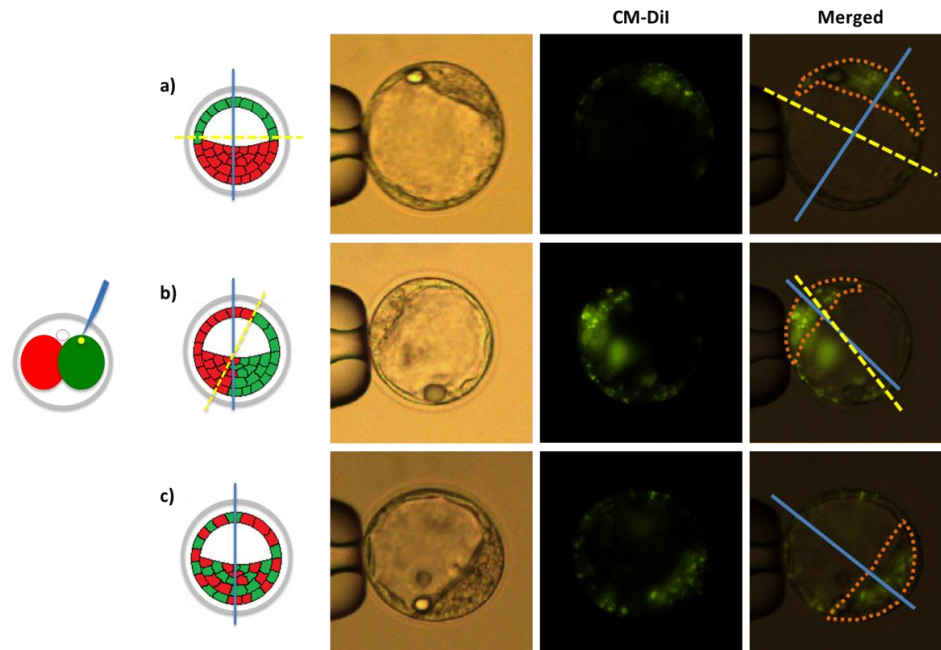


Figure 3.5 Cell allocation patterns on mouse blastocysts after CM-Dil injection at the two-cell stage. Mouse embryos were injected in one blastomere at the two-cell stage with the lipophilic tracer CM-Dil and cultured up to the blastocyst stage. **a)** Orthogonal embryo, **b)** Deviant embryo and **c)** Random embryo. Blue line denotes the embryonic-abembryonic axis, dotted orange line shows the inner cell mass and dashed line denotes borderline between stain and not stained cells.

Table 3.1 Cell allocation pattern incidence across embryos from superovulated and non-superovulated (control) mouse females. Differences on cell allocation pattern incidence between embryos produced after natural cycle ($n = 240$) or superovulation ($n = 258$) treatment during 5 replicates of the experiment. Values are expressed on percentages $\pm$ SEM values, $N=5$ replicates.

Group	Orthogonal	Deviant	Random
Control	$19.5 \pm 2.9^a\%$	$22.7 \pm 3.9^{a,b}\%$	$57.6 \pm 3.7^c\%$
Superovulated	$19.9 \pm 2.8^a\%$	$31.3 \pm 2.3^{b\%}$	$48.6 \pm 2.8^c\%$
P-value	0.947	0.095	0.083

Different subscripts denote significant differences ($p < 0.05$) by One-way ANOVA test and post-hoc Bonferroni test.

Even though a tendency of an increase of the incidence of the deviant pattern after superovulation protocol and a decrease of the random pattern were observed, no significant differences were found ($p > 0.05$ by two-way ANOVA)

when comparing the variation on the incidence on each pattern across the control and the superovulated groups. Therefore, the superovulation protocol used in the present study might have a minimal or null effect on cell allocation patterns during pre-implantation embryo development.

A total of 277 blastocysts were used for nuclear staining with DAPI, from those, 137 were from the control group and 140 from the superovulated group. No significant differences were found between blastocysts from the control and the superovulated group ($p = 0.357$) regarding the total cell counts. Similarly, TCC were comparable between patterns within the control ($p = 0.120$) and the superovulated groups ($p = 0.183$, Figure 3.6). A tendency of having a higher number of cells can be observed within the embryos following the deviant pattern (75.4 ± 2.3 and 73.2 ± 3.4 cells for the control and superovulated groups respectively) when compared with embryos following the orthogonal (70.2 ± 3.9 and 61.0 ± 4.4 cells for control and superovulated groups respectively) and random patterns (66.4 ± 1.6 and 68.7 ± 1.7 cells for control and superovulated groups respectively).

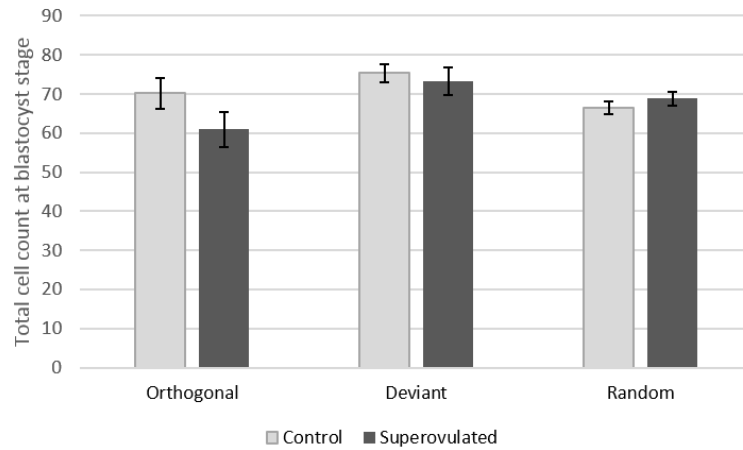


Figure 3.6 Total cell count in mouse blastocysts from superovulated and non-superovulated females. Total cell count was performed by nuclear staining with DAPI. No significant differences ($p > 0.05$) were found between groups by univariate ANOVA test.

3.4.1.2.1. Sex ratio

In order to determine if sex ratio was affected by superovulation protocols or related to cell allocation patterns on mouse embryos, embryo gender was determined at blastocyst stage by immunostaining (Figure 3.7). No significant difference (χ^2 with one degree of freedom = 3.245, $p = 0.072$, Table 3.2) was found between the sex ratio of superovulated and non-superovulated groups. Additionally, no differences were found on sex ratio among cell allocation patterns ($p > 0.05$, Table 3.3). Nevertheless, a tendency of more males within the non-superovulated group and less males in the superovulated group can be observed (Table 3.2 and Table 3.3).

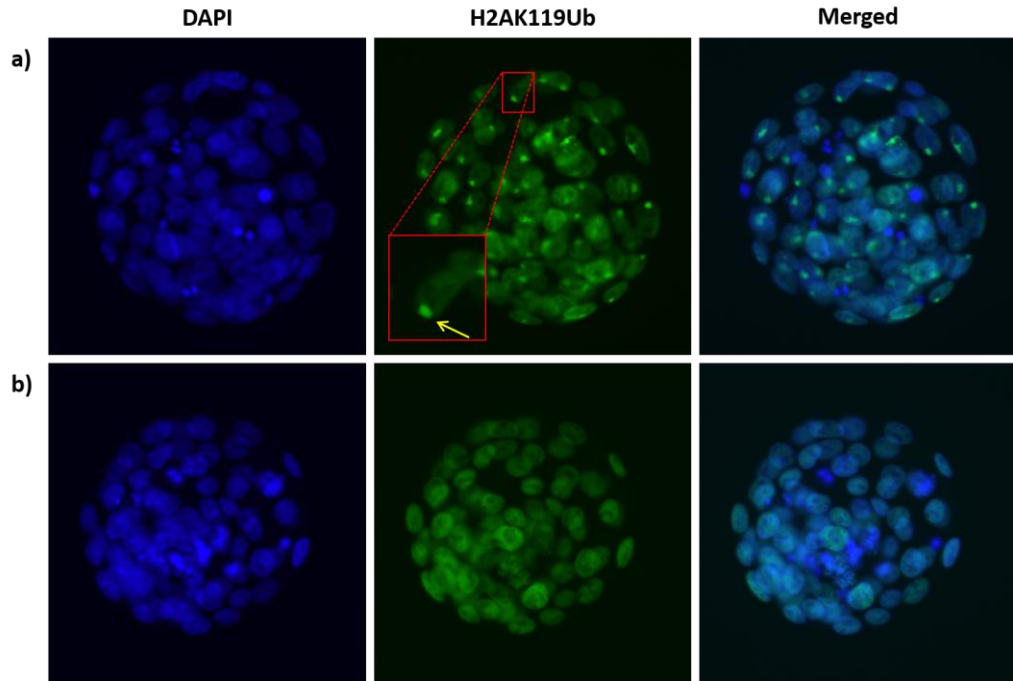


Figure 3.7 Gender determination on mouse embryos by immunostaining. Mouse blastocysts were stained with DAPI for nuclear staining and immunostained with the polyclonal antibody targeting the histone H2A with the ubiquitinated lysine 119 (H2AK119Ub) for detection of the paternal inactive X chromosome (Xi). **a)** Female embryo presenting altered H2AK119Ub staining patterns representing Xi patches (yellow arrow). **b)** Male embryo with unaltered H2AK119Ub staining patterns indicating the absence of Xi.

Table 3.2 Sex ratio in embryos from superovulated and non-superovulated mouse females. No significant difference was found between treatment groups ($p = 0.072$ by Chi-square test).

Gender	Non-superovulated	Superovulated
Female	58	71
Male	81	64
Ratio M/F	1.39	0.90
n	139	135

Table 3.3 Sex ratios among cell allocation pattern in mouse blastocysts from superovulated and non-superovulated mouse females. No significant differences were found between patterns on the non-superovulated ($p = 0.468$) nor on the superovulated ($p = 0.577$) groups by Chi-square test.

Group	Pattern	Orthogonal	Deviant	Random
Non-superovulated	Female	13	22	23
	Male	23	23	35
	Sex ratio	1.76	1.04	1.52
Superovulated	Female	11	21	39
	Male	10	14	40
	Sex ratio	0.90	0.66	1.02
χ^2		0.230	0.323	0.259

3.4.1.2.2. Morphokinetics

With the objective to determine if PMSG/hCG superovulation protocol was affecting the embryo morphokinetics, embryos were cultured in the EmbryoScope for time-lapse recording. Oocyte fertilisation timings between embryos from superovulated and non-superovulated females cannot be determined with precision. Thus, in order to have the same reference point between embryos from the control group (n = 33 embryos, from those 13 were females and 20 males) and the superovulated group (n = 71 embryos, from those 35 were females and 36 males), different intervals were annotated: from two-cell stage to three-cell stage (t2-t3), from two-cell stage to four-cell stage (t2-t4), from three-cell stage to four-cell stage (t3-t4), from four-cell stage to five-cell stage (t4-t5), from five-cell stage to eight-cell stage (t5-t8), from eight-cell stage to nine-cell stage (t8-t9), from eight-cell stage to morula stage (t8-tM) and from morula stage to start of cavitation (tM-cav) (Table 3.4). Significant differences between control and superovulated groups were found only for the intervals t2-t3 ($p < 0.001$) and t2-t4 ($p < 0.001$ by T-test). Furthermore, differences between female and male embryo morphokinetics were observed only in the t2-t3 interval ($p = 0.012$), where female embryos cleaved faster when compared with males (16.98 vs 18.89 h respectively). On the other hand, no significant differences were found between embryos following the orthogonal, deviant and random patterns for any mentioned interval (Table 3.5).

Table 3.4 Morphokinetic parameters during pre-implantation development between embryos from superovulated and non-superovulated mouse females. Intervals are expressed in hours. Differences between groups were calculated by T-test and P-values < 0.05 were considered significantly different.

Interval	Control (hrs $\pm$ SEM)	Superovulated (hrs $\pm$ SEM)	P-value
t2-t3	15.42 $\pm$ 1.06	20.75 $\pm$ 0.18	< 0.001
t2-t4	16.31 $\pm$ 1.09	22.00 $\pm$ 0.25	< 0.001
t3-t4	1.33 $\pm$ 0.15	1.24 $\pm$ 0.10	0.718
t4-t5	12.33 $\pm$ 0.35	12.40 $\pm$ 0.25	0.868
t5-t8	3.06 $\pm$ 0.30	3.18 $\pm$ 0.19	0.662
t8-t9	6.32 $\pm$ 0.54	5.14 $\pm$ 0.30	0.051
t8-tM	14.89 $\pm$ 0.62	14.65 $\pm$ 0.48	0.691
tM-Cav	14.64 $\pm$ 1.48	13.92 $\pm$ 0.74	0.328

t2: two-cell stage, t3: three-cell stage, t4: four-cell stage, t5: five-cell stage, t8: eight-cell stage, t9: nine-cell stage, tM: morula stage and Cav: start of cavitation.

Table 3.5 Morphokinetic parameters during pre-implantation between embryos following different cell allocation patterns. Intervals are expressed in hours. Differences between groups were calculated by one-way ANOVA test and P-values < 0.05 were considered significantly different.

Interval		Orthogonal (hrs $\pm$ SEM)	Deviant (hrs $\pm$ SEM)	Random (hrs $\pm$ SEM)	P-value
t2-t3	Control	9.75 $\pm$ 0.00	14.60 $\pm$ 1.93	16.11 $\pm$ 1.33	0.506
	Superovulated	20.59 $\pm$ 0.16	20.78 $\pm$ 0.34	20.78 $\pm$ 0.27	0.938
t2-t4	Control	12.3 $\pm$ 5.05	14.87 $\pm$ 1.95	17.63 $\pm$ 1.32	0.277
	Superovulated	21.73 $\pm$ 0.22	22.17 $\pm$ 0.36	21.98 $\pm$ 0.38	0.875
t3-t4	Control	1.16 $\pm$ 0.08	1.30 $\pm$ 0.38	1.36 $\pm$ 0.19	0.934
	Superovulated	1.14 $\pm$ 0.21	1.50 $\pm$ 0.19	1.14 $\pm$ 0.86	0.270
t4-t5	Control	11.64 $\pm$ 1.03	12.36 $\pm$ 0.41	12.46 $\pm$ 0.54	0.767
	Superovulated	13.47 $\pm$ 0.68	12.04 $\pm$ 0.47	12.35 $\pm$ 0.33	0.240
t5-t8	Control	3.62 $\pm$ 1.18	2.80 $\pm$ 0.60	3.06 $\pm$ 0.36	0.739
	Superovulated	3.07 $\pm$ 0.52	3.43 $\pm$ 0.39	3.07 $\pm$ 0.24	0.697
t8-t9	Control	6.19 $\pm$ 1.56	7.03 $\pm$ 1.44	6.06 $\pm$ 0.62	0.764
	Superovulated	3.25 $\pm$ 0.89	5.56 $\pm$ 0.57	5.28 $\pm$ 4.51	0.086
t8-tM	Control	14.07 $\pm$ 2.03	15.89 $\pm$ 1.54	14.65 $\pm$ 3.15	0.637
	Superovulated	13.95 $\pm$ 1.83	14.48 $\pm$ 0.91	14.89 $\pm$ 0.60	0.816
tM-Cav	Control	13.54 $\pm$ 5.03	10.78 $\pm$ 1.73	16.60 $\pm$ 2.01	0.234
	Superovulated	13.67 $\pm$ 2.09	12.41 $\pm$ 1.24	14.87 $\pm$ 1.02	0.324

3.4.1.3. Cell allocation patterns are not affected by mouse female age up to 35 weeks

As females undergoing ART are close to the end of their reproductive span, aged CD-1 females (30-35 weeks old) were superovulated and placed with a male partner for natural fertilisation. In order to determine if female age was affecting embryo cell allocation patterns during pre-implantation. However,

< 8% of the superovulated females successfully mated with their partners during 3 repetitions of the experiment. The obtained embryos presented similar blastocysts rates ($p = 0.918$ by one-way ANOVA, $n = 36$ embryos, $69 \pm 7.7\%$) compared with those from younger females (6-9 weeks old, $n = 270$ embryos, $67.9 \pm 4\%$). The results suggest that there is no difference on cell allocation patterns between embryos produced from young females and older females ($p > 0.05$, Table 3.6). Once again, the difference on the incidence of the patterns is significant ($p < 0.001$). Still, the incidence of the orthogonal and deviant groups was comparable in the older group; which was not the case for the younger group.

Table 3.6 Cell allocation patterns incidence across embryos from young and aged superovulated mouse females. Significant differences between patterns were found ($p < 0.001$) by One-way ANOVA test.

Group	Orthogonal	Deviant	Random
Young	$19.9 \pm 2.8\%^a$	$31.3 \pm 2.3\%^b$	$48.6 \pm 2.8\%^c$
Aged	$16.8 \pm 9.1\%^{a,d}$	$27.9 \pm 10.5\%^{b,d}$	$55.1 \pm 1.3\%^c$
P-value	0.652	0.607	0.338

Different subscripts denote significant differences ($p < 0.05$) by post-hoc Bonferroni test.

3.4.1.4. In-vitro fertilisation does not affect cell allocation patterns

In order to determine if there is any influence of the fertilisation method on cell allocation patterns in mouse embryos, mouse oocytes were produced after PMSG/hCG superovulation protocol and *in-vitro* fertilisation. Fertilisation rates of $68.4 \pm 7\%$ and blastocysts rates of $60.4 \pm 7\%$ (from fertilised embryos) were obtained during 3 replicates of the experiment. A total of 165 blastocysts were obtained after IVF. *In-vitro* fertilised blastocysts presented similar incidence of the different cell allocation patterns (Table 3.7). Once

again, incidence of the random pattern was significantly higher ($p < 0.001$ by One-way ANOVA) than the incidence of deviant and orthogonal patterns. Even though an increase of the random pattern across the IVF embryos was observed, differences were not significant when compared with their counterpart, naturally fertilised embryos. Thus, these results suggest that IVF does not affect cell allocation patterns.

Table 3.7 Cell allocation pattern incidence across embryos from superovulated mouse females and after natural or *in-vitro* fertilisation. Significant differences were found between the incidence of different cell allocation patterns among IVF and superovulated groups ($p < 0.001$ by one-way ANOVA).

Group	Orthogonal	Deviant	Random
Superovulated	$19.9 \pm 2.8\%^a$	$31.3 \pm 2.3\%^b$	$48.6 \pm 2.8\%^c$
IVF	$17.1 \pm 2.7\%^a$	$27.1 \pm 4.7\%^b$	$55.6 \pm 5.8\%^c$
P value	0.581	0.413	0.175

Different subscripts denote significant differences ($p < 0.05$) by post hoc Bonferroni test. Reported p values were obtained by T-test when comparing cell allocation pattern incidence between superovulated and IVF groups.

3.4.2. Cell tracing in bovine embryos

Bovine embryos have a higher lipid content than mouse embryos. As Dil dye is a lipophilic tracer, first it was determined if the tracer stayed on the descendants of the injected blastomere. The Dil staining was only passed to the descendants of the injected blastomere during further cell cleavages (Figure 3.8), as it was previously observed on mouse embryos. After the third cell cycle, a cell separation of sister blastomeres was observed in some embryos (Figure 3.9b). Presumably these embryos are starting to develop the random cell allocation pattern; and it is from this point and on that cell intermingling occurs. Embryos where sister cells remain together (Figure 3.9a), will then follow the orthogonal or deviant patterns. Further

mathematical modelling to predict if sister cells will or will not remain together is advised. Potential parameters that might be used for this correlation are: ZP shape, ZP diameter, blastomere size, cell division orientation (symmetric or asymmetric), cell division rate and order.

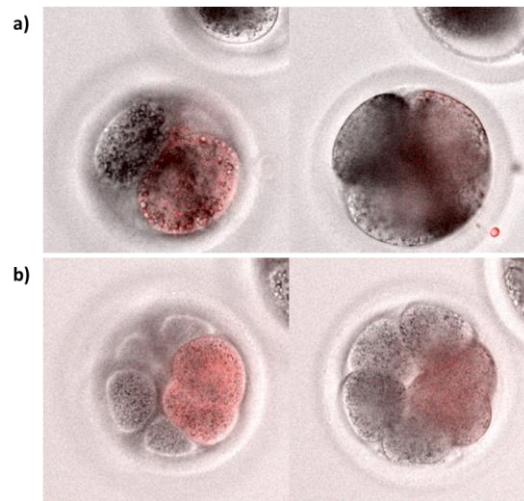


Figure 3.8 Non-invasive cell tracing in bovine embryos. One blastomere of the two-cell bovine embryo was injected with Dil dye. Dil staining is present only on the descendant cells of the injected blastomere. Left pictures represent the top focal plane of the embryo; while the right one the bottom. **a)** Four-cell bovine embryo with two blastomeres stained. **b)** Eight-cell bovine embryo with only four blastomeres stained.

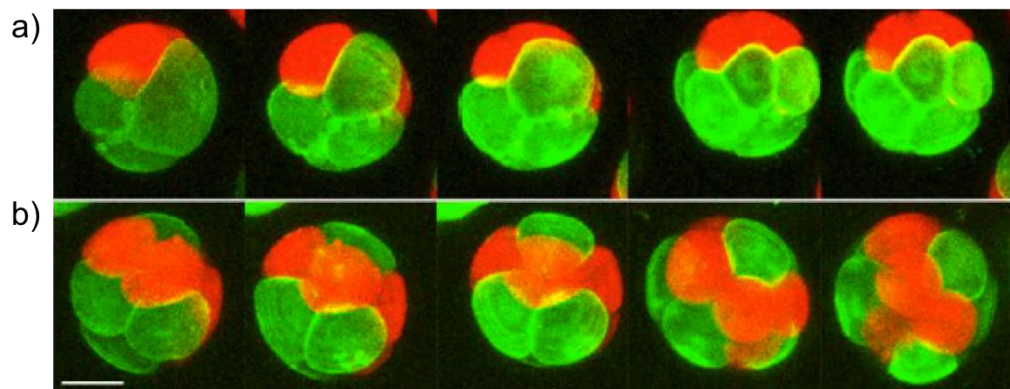


Figure 3.9 Start of the cell intermingling on bovine embryos. One blastomere of the two-cell stage bovine embryo was injected with Dil and the other with Ras-GFP to trace descendant cells from different blastomeres **a)** Eight-cell embryo where sister cells of each of the injected blastomeres remain together. **b)** Eight- to ten-cell stage embryo where sister cells of each of the injected blastomeres start to intermingle with the sister cells of the other injected blastomere, thus intermingling of cells starts. Scale bar = 50 μ m. Figure extracted from (Sepulveda-Rincon et al., 2016a).

As in mouse embryos, three-cell allocation patterns were successfully identified at blastocyst stage ($n = 346$ embryos over 40 replicates, Figure 3.10). Similarly to mouse embryos, the random pattern was significantly predominant when compared with the orthogonal and deviant ones ($62.9 \pm 2.6\%$, $n = 210$ embryos, $p < 0.001$ by One-way ANOVA, Figure 3.11). Bonferroni multiple comparisons test did not show differences on the incidence between orthogonal and deviant embryos ($p > 0.05$, $14.8 \pm 2.3\%$, $n = 56$ embryos and $22.1 \pm 2.5\%$, $n = 80$ embryos respectively).

Total cell counts at blastocyst stage differed among embryos with different cell allocation patterns ($p < 0.001$) (Figure 3.12). Embryos following the deviant pattern presented significantly higher TCC at blastocyst stage (140.2 ± 8.6 cells, $n = 23$ embryos) when compared with orthogonal (91.2 ± 11.1 cells, $n = 15$ embryos, $p < 0.001$) and random (108.4 ± 4.7 cells, $n = 45$ embryos, $p = 0.004$) embryos. No statistical difference was found between orthogonal and random embryos TCC ($p = 0.365$).

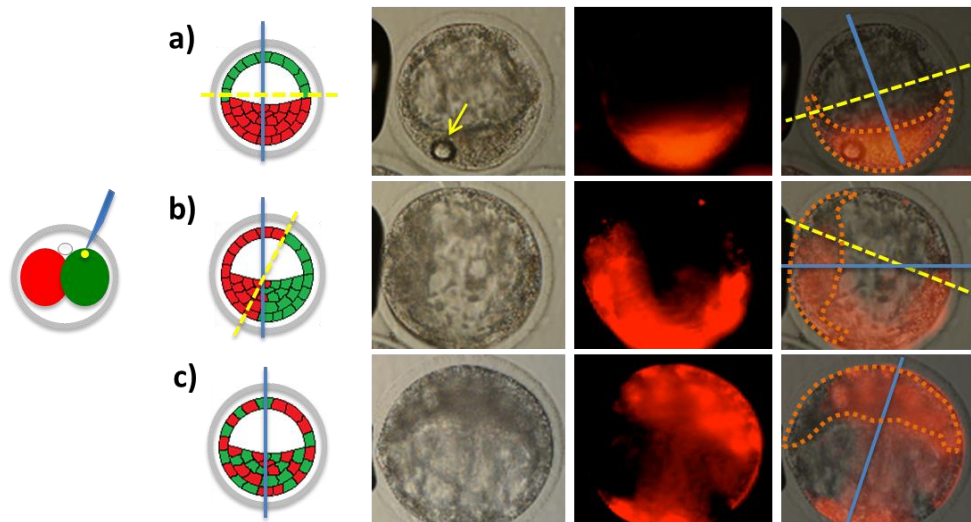


Figure 3.10 Cell allocation patterns in bovine embryos. Bovine embryos were injected with Dil (yellow arrow) on one blastomere of the two-cell embryos. At blastocyst stage, first cell cleavage was correlated with the embryonic-abembryonic axis (blue line). Dotted orange line denote ICM and dashed yellow line denotes the borderline between stained and not stained cells. Blastocyst were classified according their cell allocation pattern into: **a)** Orthogonal, **b)** Deviant or **c)** Random.

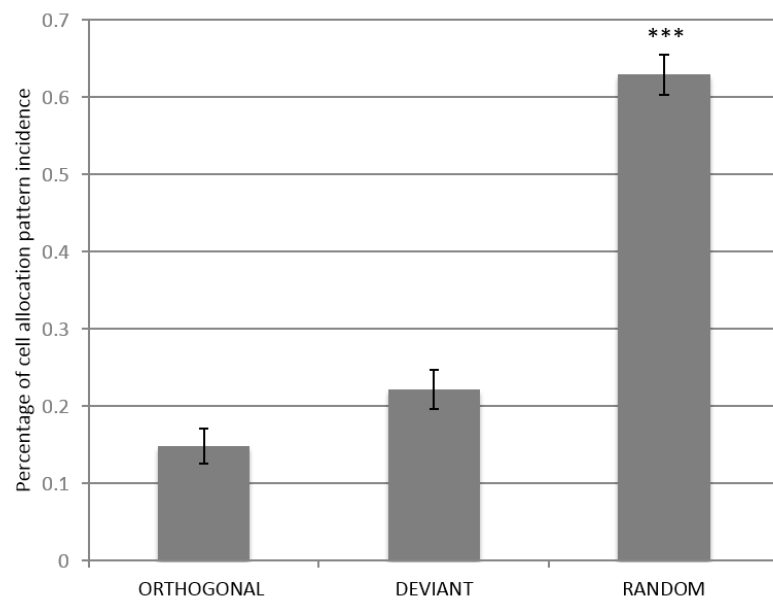


Figure 3.11 Cell allocation pattern incidence among bovine embryos. *** Denotes statistical difference ($p < 0.001$) by One-way ANOVA test.

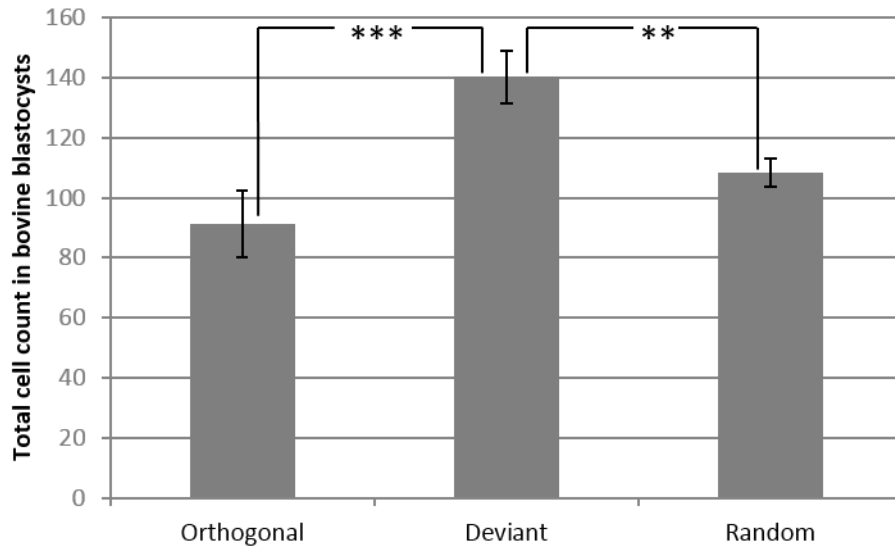


Figure 3.12 Total cell counts in bovine blastocysts. Significant differences regarding TCC at blastocyst stage were found among embryo patterns ($p < 0.001$ by One-way ANOVA). *** Denotes $p < 0.001$ and ** denotes $p = 0.004$ by post-hoc Bonferroni test.

3.5. Discussion

Live cell lineage tracing in mouse and bovine embryos seem to be a safe procedure that does not delay further cell cleavages. The injected blastomere has the same chances of cleaving first after injection than its non-injected counterpart. As previously discussed in Chapter 1, the pre-patterning theory is not completely accepted in mammalian embryos. In the present chapter, the presence of pre-patterned (orthogonal and deviant) and non-patterned (random) embryos was observed in mouse and bovine embryos. It is interesting that the incidence of these patterns was similar among embryos despite the oocyte/embryo production method (Table 3.8). Thus, the presented results suggest a conserved similarity between two different mammalian species.

Table 3.8 Cell allocation pattern incidence among mouse and bovine embryos.

Group	Orthogonal	Deviant	Random
Control CD-1 (n=240)	19.5 ± 2.9%	22.7 ± 3.9%	57.6 ± 3.7%
Superovulated CD-1 (n=258)	19.9 ± 2.8%	31.3 ± 2.3%	48.6 ± 2.8%
Aged CD-1 (n=36)	16.8 ± 9.1%	27.9 ± 10.5%	55.1 ± 1.3%
IVF CD-1 (n=165)	17.1 ± 2.7%	27.1 ± 4.7%	55.6 ± 5.8%
Bovine (n=346)	14.8 ± 2.3%	22.2 ± 2.6%	62.9 ± 2.6%

n represents total number of blastocysts

These results differ from those reported previously in the literature. Authors that agree with the pre-patterning theory have reported that the orthogonal pattern is predominantly present (>60%) among mouse embryos (Piotrowska-Nitsche and Zernicka-Goetz, 2005, Piotrowska and Zernicka-Goetz, 2002, Piotrowska et al., 2001, Katayama and Roberts, 2010); on the contrary to the present study, where orthogonal embryos represent only less than 20% of all embryos. In agreement with these results, in ovine embryos it has been very recently reported that random pattern is the most predominant pattern (Hosseini et al., 2016).

On the other hand, the present results show a tendency of the bovine embryos to have a lower incidence of embryos following the orthogonal pattern when compared with mouse embryos (Table 3.8). One of the theories explaining why embryos follow the orthogonal pattern refers to the ZP exerting constraint on embryo cells; hence, preventing cell movements within the embryo (Alarcón and Marikawa, 2008, Kurotaki et al., 2007). The constraint theory might explain in part the presented results, as the ZP in bovine embryos is different from the one of mouse embryos. Bovine ZP contains 5 different glycoproteins (Topper et al., 1997) while mouse ZP

contains only 3 (Wassarman, 2008). Crosslinking between glycoproteins ZP3 and ZP2 in mouse embryos might confer a more rigid structure to its ZP (Green, 1997).

Additionally, it was observed that orthogonal and deviant embryos do not present separation of sister cells throughout pre-implantation development. Whereas, cell intermingling in random embryos was observed from the fourth cell cycle onwards (Sepulveda-Rincon et al., 2016a). These results are in agreement with previous observations of intense cell movements in bovine and mouse embryos (Holm et al., 1998, Chroscicka et al., 2004). The nature of these increased cell movements within the random embryos is still unknown.

It has been reported that embryos from superovulated females seem to arrive to blastocyst stage at a slower pace than embryos from non-stimulated females (Ertzeid and Storeng, 2001). In the present study there was no difference on morphokinetic time points from the four-cell stage up to the blastocyst stage between embryos from superovulated females and non-superovulated ones. However, embryos from superovulated females cleaved at a slower pace during the two- and four-cell stage when compared with embryos from non-superovulated females. Chromosomal abnormalities, DNA damage or lack of sufficient energy might be responsible for prolonged cell cycles (Ajduk and Zernicka-Goetz, 2013). Furthermore, studies in mice have reported that superovulation affects genomic imprinting (loss of DNA methylation) during pre-implantation development in a dose-dependent

manner (Market-Velker et al., 2010). Therefore, if there is a correlation between patterns, imprinting and morphokinetics; it might be the case that the effect of superovulation was not observed. The superovulation protocol used on the present thesis was only 5 IU of PMSG and hCG, in which the hormone doses are lower than those previously reported (Market-Velker et al., 2010, Ertzeid and Storeng, 2001). Also, superovulation treatments have been related with diminished oocyte quality (Tarin et al., 2014) and in the present study such effect was not observed, thus suggesting that cell allocation patterns might not be related with oocyte quality per se.

Furthermore, advanced maternal age has been associated with poor oocyte quality (Tarin et al., 2014). In addition, it has been suggested that aneuploidy might be related with the interkinetochore distances in mouse oocytes and this distance tends to be bigger in oocytes from older females (Merriman et al., 2012). However, aneuploidy rates on oocytes from 3 and 9 months old mouse females seem to be the same. This might be related with the obtained results where no difference was found between the embryos produced from young and older females in terms of cell allocation patterns. It is latent that the group of females used in the present study (30-35 weeks old) were not old enough to see any detrimental age effect. On the other hand, higher numbers of blastocysts on the aged group might reduce the great variability on the cell allocation pattern incidence and make the analysis more robust.

In the present study, sex ratio was not affected among cell allocation patterns in mouse embryos. Similarly, it has been reported that sex ratio is not affected or biased by ART in mice (Byrne et al., 2006), bovine (Avery et al., 1992) or humans (Richter et al., 2006). However, some discrepancies across literature can be found where some authors suggest that there is a gender bias depending of the semen source at least in bovine (Totey et al., 1996). Also in humans, it has been reported a gender bias towards male embryos after IVF (Hentemann et al., 2009) and while some studies claim that there is a gender skew towards male embryos after ICSI (Dumoulin et al., 2005, Maalouf et al., 2014); others have reported the opposite (Hentemann et al., 2009). Similarly, it has been suggested that fast developing embryos tend to be male embryos in human (Alfarawati et al., 2011, Ménézo et al., 1999), mouse (Valdivia et al., 1993), bovine (Avery et al., 1992, Xenopoulos et al., 2012) and ovine (Bernardi and Delouis, 1996) embryos. Nevertheless, some studies have not found differences on embryo morphokinetics between female and male embryos (Holm et al., 1998, Totey et al., 1996). It is worth noting that time-lapse embryo culture technology has become recently available, therefore previous studies used static morphologic assessment, which might be less accurate.

Overall, correlation between human embryo morphokinetics and implantation potential, blastocyst development and aneuploidy prediction remains questionable and remains to be seen (Kirkegaard et al., 2015). Even in humans with a large data set, it has been difficult to determine if there is a direct correlation (if any) between morphokinetics and chromosomal status. An

extensive review can be found elsewhere (Swain, 2013). Mouse morphokinetics have been correlated with metabolomics and post-implantation embryo survival (Lee et al., 2015). Arrival time to five-cell stage and cavitation was related with blastocyst cell numbers but not with further post-implantation development. Similar results have been reported suggesting that embryos developing at slower pace present lower total cell numbers at blastocyst stage (Market Velker et al., 2012). The lack of significant differences in morphokinetics analyses within the different patterned embryos might indicate that within each type of embryo there are slow and fast embryos. Nevertheless, there is an observed tendency for deviant embryos to present higher total cell counts at blastocyst stage and for orthogonal embryos to present lower total cell counts. A possible explanation is that deviant embryos might present an accelerated cell division pattern once the blastocyst stage has been reached; as morphokinetics analyses did not show any difference on cell cleavages or reaching to blastocyst stage. Moreover, ICM/Total cell count ratio in bovine embryos seem to be comparable among different embryos (data not shown, (Sepulveda-Rincon et al., 2016a)) and within the normal parameters (Su et al., 2014). Hence, cell allocation patterns might be related with blastocyst size but not with ICM/TCC ratio.

3.6. Conclusions

There is a comparable incidence of the three identified cell allocation patterns on mammalian species, mouse and bovine (Table 3.8); suggesting that cell behaviour during pre-implantation embryo development might be a conserved mechanism among mammals. This mechanism might conserved some vestiges of developmental mechanisms from other studied lower species (Bruce and Zernicka-Goetz, 2010) as first cleavage plane of orthogonal and deviant embryos seem to be related with the Em-Ab axis at blastocyst stage. Nevertheless, stochastic development is predominant in mouse and bovine embryos as first cleavage plane is not related to the Em-Ab axis on the majority of the embryos (random embryos). Overall the results presented in this Chapter provide little evidence to support the hypothesis that ARTs such as superovulation and *in-vitro* oocyte fertilisation have an effect on cell allocation patterns in embryos from either monovulatory or polyovular species. Additionally, cell allocation patterns were related with TCC at blastocyst stage at least in bovine embryos. Thus, these patterns might mask different mechanisms involved in further cell cleavages during embryo development. More invasive ARTs such as embryo biopsy might shift these cell allocation patterns and further investigation will be useful.

4. CHAPTER FOUR: EFFECTS OF EMBRYO CELL REMOVAL ON CELL ALLOCATION PATTERNS DURING PRE-IMPLANTATION DEVELOPMENT

4.1. Introduction

The mammalian embryo seems to have a plastic development at least during fertilisation and the first three cell cleavages, as it can overcome different manipulations and develop to term (Oron and Ivanova, 2012, Tabansky et al., 2010). ARTs have been applied for more than three decades and success rates still remain low; the probabilities of delivering a baby after an IVF/ICSI cycle are around 33% in women younger than 35 years old (Botros et al., 2008) and no more than 23.1% when they are older (HFEA, 2013). Several factors have been related with pregnancy outcomes after ARTs as gametes/embryos precedence and state (autologous or donation and fresh or thawed), female stimulation treatment, patient age, number of previous treatments and day of embryo transfer (D3 or D5) to mention the most common ones (Meseguer et al., 2012). Yet, embryo implantation failure remains the main cause of the low success rates on ARTs (Hesters et al., 2008). Most of embryo implantation failures or pregnancy arrests are caused by embryo genetic abnormalities (Simpson, 2012); therefore genetic diagnosis before embryo transfer is advised. Embryo pre-implantation genetic diagnosis/screening (PGD/S) is increasingly offered in fertility clinics worldwide with blastomere biopsy being the most common technique for obtaining the biological material (Harper et al., 2012). So far, more than ten thousand babies have been born after a

PGD/S cycle (Peyvandi et al., 2011). Therefore, further research on the effect of embryo biopsy on embryo development must be done.

On the other hand, the need for poor respondent patients to have more embryos for transfer or patients looking for compatible human leukocyte antigen (HLA) typing for further stem cell therapy, has led to the suggestion of applying twinning or embryo splitting. This is a procedure used in farm animal species to increase production of high merit animal into an assisted reproductive technique (Willadsen, 1979), however due to ethical reservations and lack of scientific data, no clinical births from in vitro splitting of normal fertilised human embryos have been reported in the literature to our knowledge. In a number of mammalian species, monozygotic twinning has been reported naturally. However, the exact time when this phenomenon happens is still unknown but it is believed to occur after blastocyst formation and even after embryo implantation (reviewed in (Arias et al., 2013)). In humans, monozygotic twinning is presented in approximately 0.4% of all natural births and around 3% after ART births; in both cases it seems to decrease by advanced maternal age (Franasiak et al., 2015, Knopman et al., 2014). It is interesting that monozygotic twins conceived naturally or by ART present the same distribution. Around 80% of naturally conceived monozygotic twins are dichorionic (Kristiansen et al., 2015, Knopman et al., 2014); which means that the embryo splitting occurred before the morula stage or this can be the result of a fusion of membranes instead of a twinning

process (Herranz, 2015). The other 20% of the monozygotic twins are monochorionic, where the embryo splitting occurred during days 5-8 of development (diamniotic) or later on (monoamniotic). Nevertheless, reports on the literature have been inconclusive regarding the stage of development that embryo cells change from being totipotent to pluripotent or the mechanism(s) allowing twinning. Embryo loss is present more often in monochorionic pregnancies than dichorionic (Kristiansen et al., 2015). Thus it makes sense that embryo splitting at earlier stages might increase the survival chances of both twins.

Previous studies reported that even though sister blastomeres might be proficient compensating cell loss; molecular expression patterns do not seem to be equal between them (Lorthongpanich et al., 2008). Therefore, the homogeneity between embryo blastomeres is questionable. Nonetheless, embryo bisection or splitting has been practiced successfully in mammalian species (ovine, bovine, caprine, porcine, equine, rabbit, rat, mouse, non-human primates and human) at different pre-implantation stages, producing blastocysts (Willadsen, 1979, Willadsen, 1981, Malik et al., 2013, Dang-Nguyen et al., 2011, Dang-Nguyen et al., 2014, Krzyminska et al., 1990, Tang et al., 2012, Illmensee et al., 2005, Katayama et al., 2010, Wang et al., 1997, Illmensee et al., 2010a, Illmensee et al., 2010b, Noli et al., 2015) and healthy mouse and porcine offspring (Reichelt and Niemann, 1994, Nijs et al., 1988) (see Appendix III).

4.2. Objectives

It was hypothesised that due to the disruption of the blastomere adhesion at the eight-cell stage caused by the handling medium during embryo biopsy, cell allocation patterns might be shifted towards the random pattern as cell removal could promote cell rearrangements within the embryo. Hence, the purpose of this chapter is to gain further insights into the plasticity of the mammalian embryos and how the removal of a single cell during pre-implantation embryo development is managed in terms of cell allocation patterns and morphokinetics. The main animal model used was the mouse due to its ease of manipulation.

Furthermore, the bovine model was considered in order to mimic the procedure performed in human embryos where the embryo cell removal is performed on the clinical set up during the EGA time, around the eight-cell stage. Secondly, this chapter aims to explore the totipotency of both blastomeres of the two-cell mouse embryo and their capacity to reach blastocyst stage. The above is done with the purpose of exploring the possibility that twin blastomeres have similar developmental fates.

4.3. Methods

For this chapter two different models were used: the mouse and bovine. B6CBA-F1 mice were used to produce mouse embryos after superovulation protocol and natural mating as previously describe in Section 2.1.2 for

cleavage stage biopsy experiments. For bovine embryo production please refer to Section 2.2. Two-cell embryos were injected on one blastomere for non-invasive cell tracing (See Section 2.3) and returned to culture until embryo biopsy.

For the second part of this Chapter, one-cell frozen B6C3-F1 x B6D2 F1 mouse embryos (Embryotech Laboratories, Inc., Massachusetts, USA) were thawed according to supplier's instructions (Appendix IV). Additionally, mouse embryos were produced as described in Section 2.1.2.

4.3.1. Embryo biopsy

For embryo biopsy at cleavage stage, mouse embryos were assessed at 68-72h post-hCG and six- to eight-cell embryos were indistinctly allocated in control and biopsy groups. In the bovine model, embryos were assessed at 44-46 h post-IVF and six- to twelve-cell embryos were indistinctly allocated in control and biopsy groups. The selected embryos were transferred to a pre-warmed 60 mm ICSI dish (BD Falcon) containing 10 μ l drops of G-PGD™ medium (Vitrolife, Sweden) under mineral oil. The embryo biopsy process can be observed in Figure 4.1. Briefly, embryo biopsy was performed using a 420x XYclone laser objective (Hamilton Thorne Biosciences, MA, USA) mounted on a Leica DMI3000 B inverted microscope. The holding pipette used had a length of 60 mm, an internal diameter of 9-17 μ m, and a bevel of 30° (Swemed, Vitrolife, Sweden). Zona opening was performed with a 300 μ s

pulse non-contact laser (XYCLONE, Hamilton Thorne Biosciences, Beverly, MA) at 100% power. Then, a single blastomere correspondent to the fourth cell cycle (eight-cell stage) was removed regardless its staining status (Figure 4.1). The resulting biopsied embryos were further cultured under normal culture conditions.

For mouse embryo twinning embryos were assessed at 42-46 h post-hCG and two-cell stage embryos were selected (Figure 4.2). Then the above conditions for embryo biopsy were followed and one of the two blastomeres was removed. After embryo twinning embryos were cultured in couplets of blastomeres (zona pellucida (ZP) and zona pellucida free (ZPF) blastomeres) from the same embryo until blastocyst stage. Hence, a single two-cell embryo will give rise to two demi-embryos.

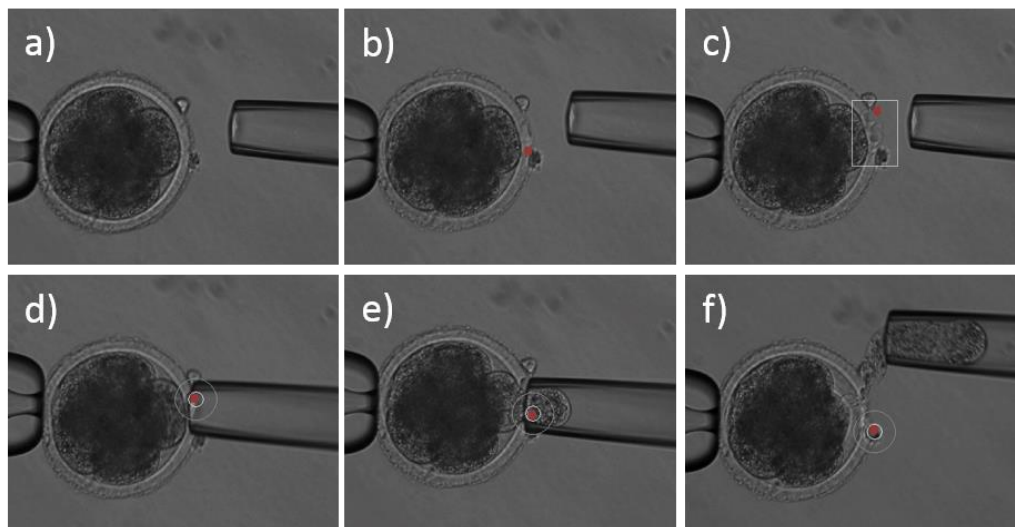


Figure 4.1 Bovine embryo cleavage stage biopsy. Sequence taken during bovine embryo biopsy where a) shows a bovine embryo at the eight- to ten-cell stage; b) red dot represents the laser allocation; c) zona pellucida breaching section (white square) through laser pulses and d) to f) show how a single blastomere is removed by aspiration.

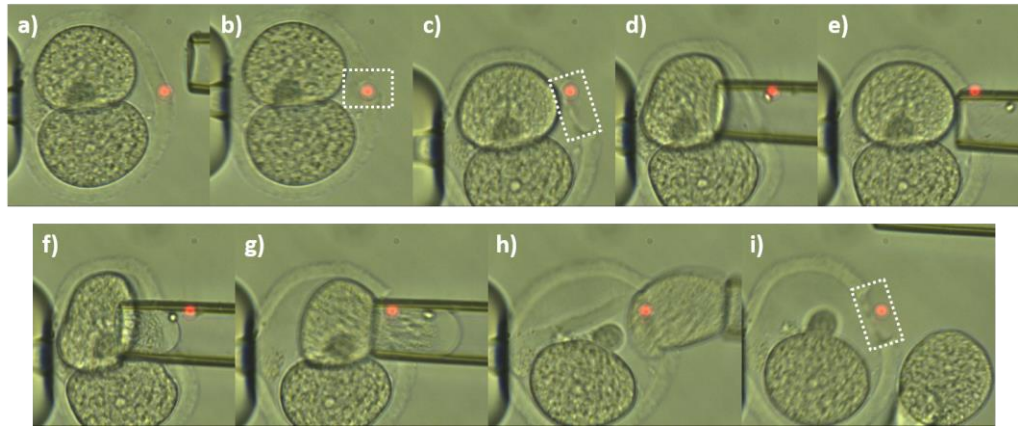


Figure 4.2 Twinning technique in mouse embryos. Sequence of mouse blastomere separation at the two-cell stage. Red dot represents the laser target and with dotted box represents the zona pellucida opening. a-c) Zona pellucida opening with laser, d-i) blastomere extraction.

4.3.2. Cell tracing on bisected two-cell mouse embryos

During the following cell cleavage after the twinning procedure, the lipophilic tracer CM-Dil was deposited on the membrane of one blastomere of the bisected embryos without ZP (Figure 4.3a) and with ZP (Figure 4.3b).

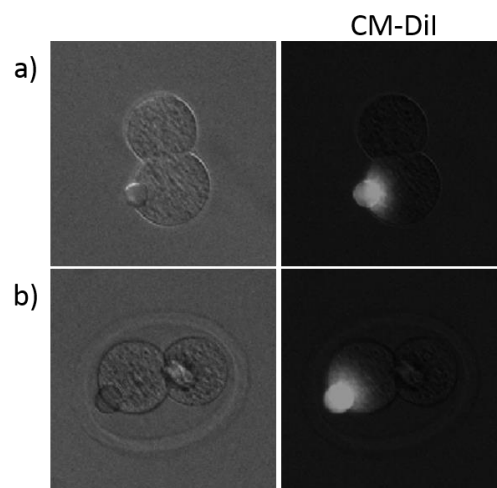


Figure 4.3 Live tracing on bisected mouse embryos. One blastomere of the two-cell mouse embryo was removed and cultured without its zona pellucida (a) and at the two-cell stage was injected with Dil lipophilic tracer. The remaining blastomere within the zona pellucida was also injected at the two-cell stage (b).

4.3.3. Blastocyst assessment

Blastocysts were grade by the society of assisted reproductive technology (SART) system. SART system classifies the embryos into three different categories: good, fair or poor. Embryos were classified as good if their ICM and TE presented high quality with a distinct structure of large number of cells. If embryos presented a distinct structure but with a moderate number cells in their ICM and TE, embryos were classified as fair. Whereas, embryos with no distinguishable ICM or TE structure with few cells were classified as poor. In addition, total cell counts on blastocysts were performed by nuclear staining according to Section 2.4.2.

4.3.4. Statistical analysis

Data sets were checked for normality using Shapiro-Wilk test employing IBM SPSS version 23. Pearson Chi-Square test was used to determine predisposition of staining status of the biopsied cell on cell allocation pattern classification. A p-value less than 0.05 was considered as statistically significant. Values are reported as mean $\pm$ SEM. For SEM calculation, the total number of replicates of the experiment was used as N value when reporting incidence of cell allocation patterns. Univariate two-way ANOVA test was used to determine if cell allocation pattern incidence was changed by cleavage stage biopsy procedure. Furthermore, One-way ANOVA test was

used to determine if morphokinetic parameters were similar between control and biopsied groups and among embryo patterns.

Total number of embryos (n value) was used to calculate SEM value when reporting TCC in blastocysts and morphokinetics. Furthermore, two-way ANOVA test was used to determine any differences on TCC between control and biopsied groups and among patterns. Chi-square test was used then to determine any differences on blastocyst quality and blastocyst expansion among control and biopsied groups. Finally, differences on TCC between demi-embryos was analysed by T-test.

4.4. Results

4.4.1. Cell lineage allocation patterns in biopsied mouse embryos

In order to determine if there was not a predisposition to biopsy a stained or not stained cell, staining status of the biopsied blastomere was noted. Over 4 repetitions of the experiment, a total of 145 blastocysts were analysed after blastomere removal, from those, 73 embryos had the biopsied cell without staining and 71 with staining. Our results pointed out that there was no predisposition of biopsying a stain or not stained cell. Also, the staining status was not affecting the cell allocation pattern distribution (Table 4.1); thus the staining status of the biopsied cell did not bias the cell allocation pattern

distribution ($p = 0.149$ by Pearson Chi-Square). Hence, the staining status of the biopsied blastomere was not noted on the following experiments.

Table 4.1 Biopsied cell staining status and cell allocation patterns in mouse embryos. A single blastomere was biopsied at cleavage stage in mouse embryos. Staining status of biopsied cell did not affect embryo classification by cell allocation patterns at blastocyst stage ($p = 0.149$ by Pearson Chi-Square test).

Pattern	Biopsied cell not stained	Biopsied cell stained
Orthogonal	14	13
Deviant	13	22
Random	47	35

Over 9 repetitions of the experiment, 287 blastocysts on the control group and 214 blastocysts on the biopsied group were successfully classified according to their cell allocation pattern (Figure 4.4, Figure 4.5). Orthogonal embryos represented $21 \pm 5.4\%$ and $19 \pm 3.6\%$ of the blastocysts within the control and the biopsied group respectively. The deviant group represented $21 \pm 3.9\%$ and $23 \pm 3.6\%$ and the random group $58 \pm 4.8\%$ and $59 \pm 4.6\%$ for the control and biopsied group respectively. Univariate two-way ANOVA showed no significant differences between treatment control and biopsied groups ($p = 0.973$). However, there is a significant difference on the incidence of the cell allocation patterns ($p < 0.001$). Post Hoc Bonferroni test revealed that this difference is mainly attributed to the difference between the random group compared with the orthogonal ($p < 0.001$) and deviant ($p < 0.001$).

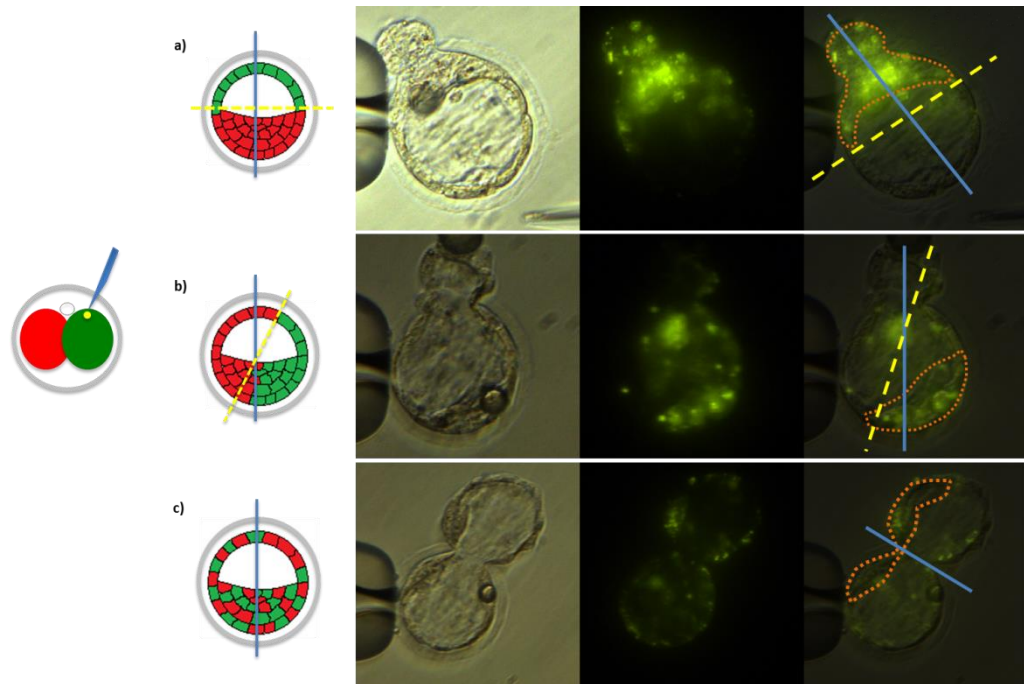


Figure 4.4 Cell allocation patterns in biopsied mouse blastocysts. Two-cell mouse were injected with CM-Dil on one blastomere. At the eight-cell stage a single blastomere was indistinctly removed and embryos were left in culture until blastocyst stage. Three different cell allocation patterns can be observed at blastocyst stage. Blue line denotes the embryonic-abembryonic axis, dotted orange line shows the inner cell mass and dashed line denotes borderline between stain and not stained cells. **a)** Orthogonal embryo, **b)** Deviant embryo and **c)** Random embryo.

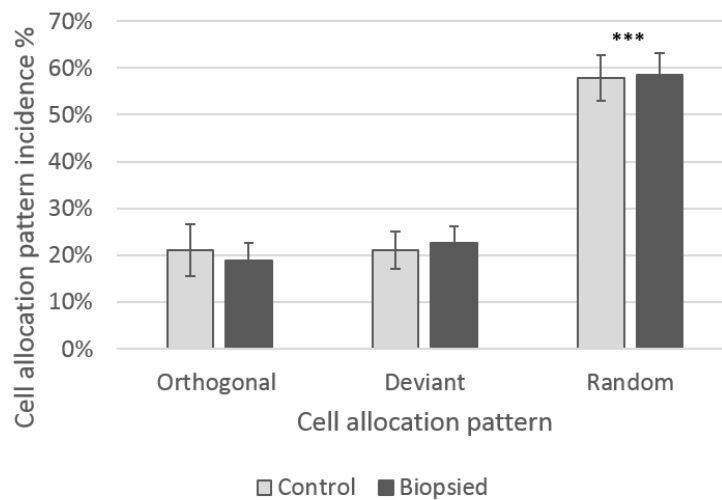


Figure 4.5 Cell allocation patterns across control and biopsied mouse embryos. No significant difference ($p > 0.05$ by one-way ANOVA) on cell allocation pattern incidence was found between control and biopsied groups. Significant differences on cell allocation pattern incidence were found in control and biopsied groups ($p < 0.001$ by one-way ANOVA).

4.4.1.1. *Morphokinetics*

Morphokinetic analysis did not show any significant difference (Table 4.2, $p > 0.05$) on the cleavage timing from the three-cell stage up to the eight-cell stage, before embryo biopsy. After blastomere removal, there was no difference on cleavage timings for t9, tM, tSB or tEB between control and biopsied groups ($p > 0.05$ by T-test). Next, it was determine if the embryo biopsy was having an effect on the duration of embryo cleavages (Table 4.3). When comparing the time from the eight-cell stage to the 9-cell stage, it did not show significant differences between patterns within the control group ($p > 0.05$), however there was a significant difference on the biopsied group ($p = 0.012$). Bonferroni test suggested that this significance can be attributed to the difference between the orthogonal and deviant pattern ($p = 0.011$); where orthogonal pattern was taking longer to cleave from the eight- to nine-cell stage than the deviant one.

Additionally, this difference in cell cleavage timings between groups has remained in further morphokinetic events. The timing between compaction and cavitation was significantly slower on the biopsied group ($p = 0.004$) when compared against control group. Once again the orthogonal pattern seems to take longer to start cavitation in comparison with the deviant ($p = 0.003$) and random ($p = 0.012$) patterns.

Table 4.2 Morphokinetic analysis between patterns from the three-cell stage up to the eight-cell stage. Morphokinetic points are expressed in hours post-hCG injection.

	Orthogonal (n=42)	Deviant (n=50)	Random (n=126)	Kruskal-Wallis test
t3	54.47±0.46	54.39±0.32	54.38±0.20	0.96
t4	55.49±0.53	53.33±0.36	55.37±0.26	0.87
t5	67.48±0.71	66.77±0.40	67.23±0.33	0.70
t6	68.75±0.93	67.58±0.42	67.67±0.38	0.39
t7	68.68±0.85	67.13±0.55	68.66±0.42	0.92
t8	68.47±0.99	69.07±0.49	69.68±0.43	0.90

t3: three-cell stage, t4: four-cell stage, t5: five-cell stage, t6: six-cell stage, t7: seven-cell stage and t8: eight-cell stage.

Table 4.3 Embryo morphokinetics after blastomere removal at the eight-cell stage. Morphokinetic parameters are expressed in hours.

Group	t8 to t9		Compaction to Cavitation	
	Control Mean ± SEM (N)	Biopsied Mean ± SEM (n)	Control Mean ± SEM (n)	Biopsied Mean ± SEM (n)
Orthogonal	9.45±0.49 (16) ^a	10.54±0.47 (26) ^{a,b}	22.59±0.97 (20) ^a	24.03±0.84 (30) ^{a,b}
Deviant	8.95±0.90 (19) ^a	8.66±0.47 (33) ^a	21.72±0.50 (20) ^a	22.01±0.48 (30) ^{a,c}
Random	9.03±0.42 (56) ^a	9.70±0.25 (80) ^{a,c}	22.28±0.64 (57) ^a	22.56±0.45 (73) ^{a,c}

*Different superscripts denote statistical significance ($p < 0.05$ by one-way ANOVA and post-hoc Bonferroni test). t8 to t9: period from eight- to nine-cell stage.

4.4.1.2. Total cell count and blastocyst grades

In order to address whether the biopsy procedure has an effect on the size of the embryo at blastocysts stage, total cell counts of blastocysts were obtained (Table 4.4). Embryos from the orthogonal group presented a TCC of 45.8 ± 0.2 cells and 41.2 ± 0.5 cells within the control and biopsied group respectively. Deviant embryos presented a TCC of 50.4 ± 0.6 cells and 42.0 ± 0.4 cells for control and biopsied group; while random embryos presented a TCC of 47.9 ± 0.2 cells and 43.5 ± 0.2 cells. Univariate test showed no significant difference on the TCC among the three patterns ($p = 0.490$). Still, there was a significant difference on the TCC between the control and the non-biopsied groups ($p = 0.002$). Post-Hoc Bonferroni test suggested that

this difference is mainly attributed to the effect of biopsy on the TCC of the deviant group ($p = 0.028$).

Blastocysts were graded according to the SART system (Section 4.3.3, Figure 4.6). Chi-square test showed no significant difference between control and biopsied groups ($p = 0.089$), neither between cell allocation patterned groups ($p = 0.952$). Majority of embryos ($> 60\%$) among groups and treatments presented good quality, whereas $< 9\%$ presented poor quality.

Due to the fact that SART grading system only includes the ICM and TE grades, embryos were further classified into early, mid, expanded and hatching blastocysts (Table 4.5). Chi-Square test showed no difference between groups ($p = 0.191$). Yet, there was a significant difference on the expansion grades between biopsied and control groups ($p < 0.001$), where the biopsied groups tend to have more hatching blastocysts.

Table 4.4 Total cell count in mouse blastocysts. A single blastomere was removed at eight-cell stage on the biopsied group, whereas the control group was left not biopsied.

Treatment	Orthogonal	Deviant	Random
Control	45.8 $\pm$ 0.2 (n=31)	50.4 $\pm$ 0.6 (n=23)	47.9 $\pm$ 0.2 (n=61)
Biopsied	41.2 $\pm$ 0.5 (n=27)	42.0 $\pm$ 0.4 (n=35)	43.5 $\pm$ 0.2 (n=70)
P value	0.177	0.028	0.082

Univariate ANOVA $p = 0.002$ between treatments. Post hoc Bonferroni $p = 0.028$ for deviant group (control vs biopsied). n represents the total analysed blastocysts.

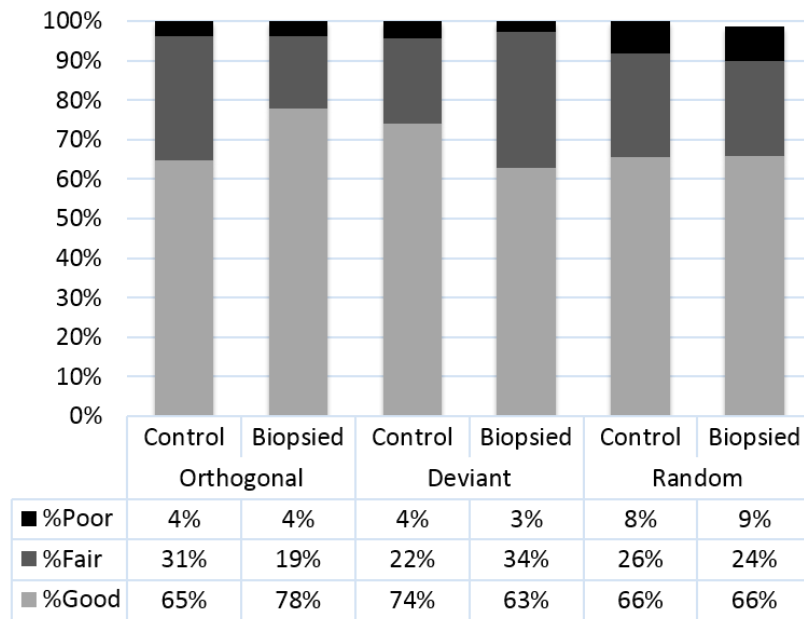


Figure 4.6 Blastocyst rates by SART system in biopsied and non-biopsied mouse embryos. Orthogonal (n = 32 embryos, n = 29 embryos), Deviant (n = 31 embryos, n = 31 embryos) and Random (n = 77 embryos, n = 72 embryos) for control and biopsied groups respectively.

Table 4.5 Blastocyst grading by blastocoel expansion among control and biopsied mouse embryos.

Pattern	Treatment	Early Blastocyst	Mid blastocyst	Expanded blastocyst	Hatching blastocyst
Orthogonal	Control	6%	54%	36%	4%
	Biopsied	31%	13.80%	7%	48.30%
Deviant	Control	9.70%	51.60%	35.50%	3.20%
	Biopsied	32.30%	25.80%	0.00%	41.90%
Random	Control	7.80%	45.50%	42.90%	3.90%
	Biopsied	13.90%	29.20%	2.80%	54.20%

Significant differences were found regarding blastocoel expansion among control and biopsied groups ($p < 0.001$).

4.4.2. Cell allocation patterns in biopsied bovine embryos

A total of 156 bovine embryos at eight-twelve cell stage were biopsied after Dil injection at the two-cell stage during 5 repetitions of the experiment. From those, 107 embryos reached the blastocyst stage ($68 \pm 9.9\%$). The biopsied blastocysts showed the three cell allocation patterns previously described

(Figure 3.10), with the following incidence: orthogonal pattern $21 \pm 3.4\%$, deviant pattern $16 \pm 4.2\%$ and random pattern $63 \pm 1.4\%$ (Figure 4.7). The incidence of the different cell allocation patterns of the biopsied embryos is not significantly different from the non-biopsied embryos ($p > 0.05$). Yet, the difference on the incidence between patterns is significant ($p < 0.001$). A post Hoc Bonferroni test showed that this difference can be attributed mainly to the difference between the incidences of the random pattern compared with the orthogonal ($p < 0.001$) and the deviant ($p < 0.001$) groups.

Overall, a univariate test showed no significant difference ($p = 0.080$) between the total cell count of biopsied and non-biopsied embryos. However, a post Hoc Bonferroni test suggested a tendency of TCC decrease of the biopsied embryos on deviant (95.8 ± 15 cells, $p = 0.018$) and random embryos (90.6 ± 6.6 cells, $p = 0.004$), when compared with their non-biopsied counterparts (136 ± 7.3 cells and 116 ± 5.7 cells respectively) (Figure 4.8). Yet, orthogonal embryos were not affected by the biopsy procedure ($p = 0.065$). Biopsied orthogonal embryos presented a TCC of 116.6 ± 10.6 cells in comparison to their non-biopsied counterpart (91.8 ± 8.0 cells).

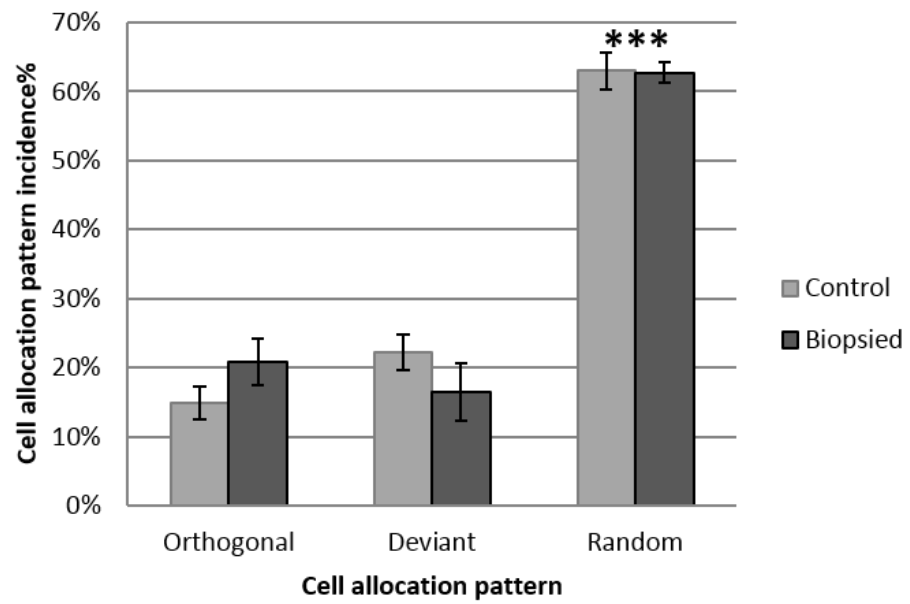


Figure 4.7 Incidence of different cell allocation patterns in biopsied and non-biopsied bovine embryos. No statistical differences were found between the same patterns when comparing the biopsied and non-biopsied groups. *** Denotes $P \leq 0.001$ when comparing the incidence of the random pattern against the orthogonal and deviant ones by two-way ANOVA.

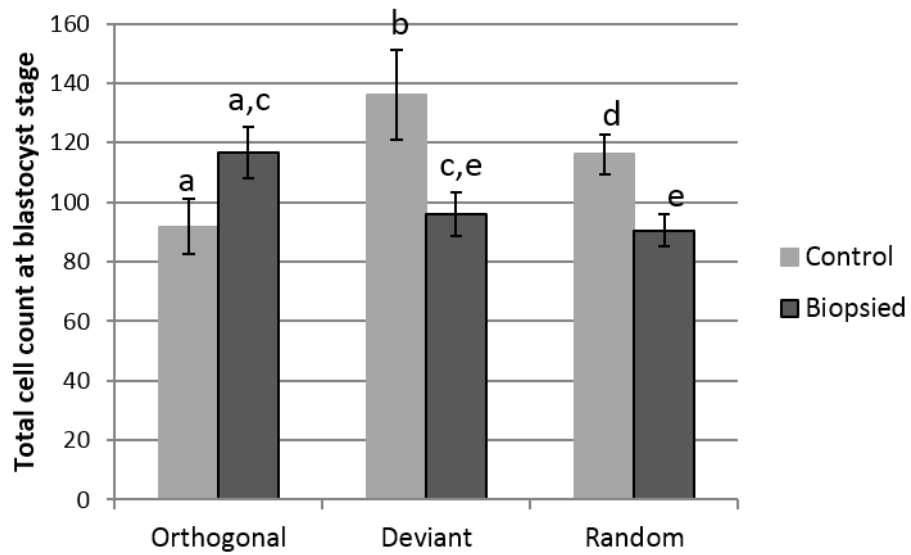


Figure 4.8 Total cell counts in bovine blastocysts. Different letter superscripts indicate significant differences $p < 0.05$.

4.4.3. Twinning

First, in order to reduce the use of animals, one cell frozen embryos were used in this experiment. In a total of three replicates, 282 one cell embryos were thawed and 257 reached the two-cell stage. Embryos were then bisected and indistinctly allocated into control (n = 76 embryos) and injected (n = 181 embryos) groups in order to determine if CM-Dil injection had an effect on the blastomere development. Blastocyst rates on the control group reached 130% (n = 103 embryos) while on the injected group 88% (n = 159 embryos), suggesting a significant effect of CM-Dil injection after twinning. It is worth noting that in 76% (n = 60 embryos) of the control and 58% (n = 106 embryos) of the injected two-cell embryos, at least one of the blastomeres reached the blastocyst stage. On the other hand, only in 52% (43 couplets) and 29% (53 couplets), both blastomeres of the bisected embryos reached the blastocyst stage. Further, from those embryos where only one blastomere reached the blastocyst stage, in the control group 16% (n = 12 embryos) had ZP and 7% (n = 5 embryos) were ZPF; whereas for the injected group it was 19% (n = 34 embryos) and 10% (n = 19 embryos) respectively. No differences were found when comparing TCC of ZP and ZPF blastocysts within the control group ($p = 0.105$, 33.8 ± 1.8 cells and 29.3 ± 1.5 cells for ZP and ZPF blastocysts respectively). However, significant differences were found only on the injected group ($p = 0.011$, by unpaired T-test) when comparing ZP (36.2 ± 3.6 cells) and ZPF (29.0 ± 3.6 cells) blastocysts (Figure 4.9).

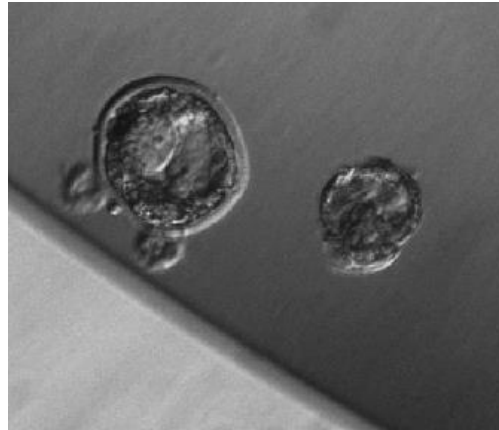


Figure 4.9 Example of uneven size of blastocysts from two-cell bisected mouse embryos. Blastocysts from bisected two-cell embryos. Blastocyst on the right comes from the biopsied blastomere, it was cultured without its zona pellucida. Blastocyst on the left comes from the remaining blastomere after two-cell embryo biopsy, it was cultured with its zona pellucida.

It is worth noting that within the blastocysts couplets of injected group only random pattern was observed in ZP and ZPF blastocysts (Figure 4.10). For the demi-embryos with ZP and without (ZPF), only 5% ($n = 2$ embryos) blastocysts were classified as orthogonal, 9% ($n = 4$ embryos) as deviant, 70% ($n = 30$ embryos) as random and 18% ($n = 8$ embryos) could not be classified. Given the results and to rule out that frozen/thawed embryos were more sensitive to embryo twinning procedure, the experiment was repeated using fresh embryos.

Over three replicates of the experiment using fresh B6CBA-F1 mouse embryos, 185 two-cell embryos were bisected and at the two-cell stage were injected with CM-Dil on one blastomere. The obtained blastocysts rates were of 118% ($n = 218$ embryos), which were similar to the previous results on the control group. From those, 40% (79 blastocyst couplets) both blastomeres reached the blastocyst stage and from the bisected embryos with only one

blastocysts 29% ($n = 48$ embryos) had ZP and 6% ($n = 12$ embryos) did not (ZPF). Similar results were obtained where within the couplets only random pattern was observed. Thus it was hypothesized that CM-Dil injection was exerting some kind of bias on the development of the split embryos. The experiment was replicated once more without CM-Dil injection.

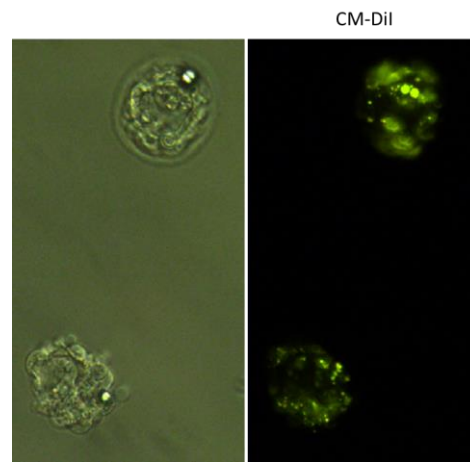


Figure 4.10 Couplet of blastocysts from bisected two-cell embryo following the random pattern. Demi-embryos from two blastomeres of a two-cell mouse embryo. After twinning, zona pellucida and zona pellucida-free embryos were injected with CM-Dil on one blastomere at the two-cell stage. Both blastocysts followed the random cell allocation pattern, in which stained and not stained cells are intermingled (right picture).

Over two repetitions of the experiment, 92 two-cell embryos were bisected. A blastocyst rate of 168% ($n = 155$ embryos) was obtained. From those, 74% (68 couplets) both blastomeres developed into blastocysts and from the rest 12% ($n = 11$ embryos) had ZP and 9% ($n = 8$ embryos) did not (ZPF). Morphokinetics of 38 couplets were analysed in order to determine if there were any differences between twin blastomeres with or without ZP (Table 4.6, Figure 4.11). No statistical differences were found between ZP and ZPF embryos (Table 4.6). Moreover, ZP and ZPF bisected embryos presented diverse four-cell stage configurations (Figure 4.12). In some couplets, ZP and

ZPF embryos presented different four-cell configurations (Figure 4.12a-c), and in other presented similar configurations (Figure 4.12d-f). Furthermore, it was noticed that in some couplets compaction started at different points for ZP and ZPF embryos (Figure 4.12g-h) and in other couplets compaction started at the same time even though ZP and ZPF embryos were at different stages (Figure 4.12i).

Additionally, when looking at the bisected embryos, only one blastomere reached the blastocyst stage; 10 embryos with ZP and 5 embryos ZPF. When ZP embryos developed into blastocysts, 6 out of 10 ZPF did not cleave after the twinning procedure and the other 4 were arrested at t9 without reaching to morula stage. On the other hand when ZPF embryos developed into blastocysts, one ZP embryo was arrested at t6, three embryos at t8 and one embryo at t9.

Table 4.6 Morphokinetics among bisected two-cell embryos. A total of 38 mouse two-cell embryos were bisected in two embryos one with zona pellucida (ZP) and other zona pellucida free (ZPF) and recorded by time-lapse imaging. Consecutive cleavages after twinning were noted as: t4, when bisected embryos cleaved in to two-cell stage; t6, for three-cell stage; t8, for four-cell stage; compaction, at the beginning of compaction; t9, for five-cell stage onwards; morula, for compacted morula before cavitation; cavitation, just the start of cavitation; SB, small blastocyst; B, blastocyst; and EB for expanded blastocyst. No statistical differences were found between demi-embryos by ANOVA. $p < 0.05$ was considered significant.

Period	ZP	ZPF	P-value
t4	55.5 ± 0.5	56.3 ± 0.5	0.302
t6	69.5 ± 0.6	70.4 ± 0.7	0.398
t8	70.4 ± 0.7	71.1 ± 0.8	0.553
Compaction	72.4 ± 0.6	72.0 ± 0.7	0.724
t9	80.9 ± 0.7	81.5 ± 0.8	0.620
Morula	96.2 ± 0.8	94.9 ± 0.8	0.279
Cavitation	96.9 ± 0.8	95.8 ± 0.8	0.348
SB	99.7 ± 0.8	98.5 ± 0.8	0.319
B	105.8 ± 1.1	104.9 ± 1.0	0.573
EB	111.3 ± 0.9	111.64 ± 1.1	0.841

Times are expressed in hours post hCG injection. t4: two-cell stage, t6: three-cell stage, t8: four-cell stage, t9: five-cell stage, SB: small blastocyst, B: blastocyst and EB: expanded

blastocyst

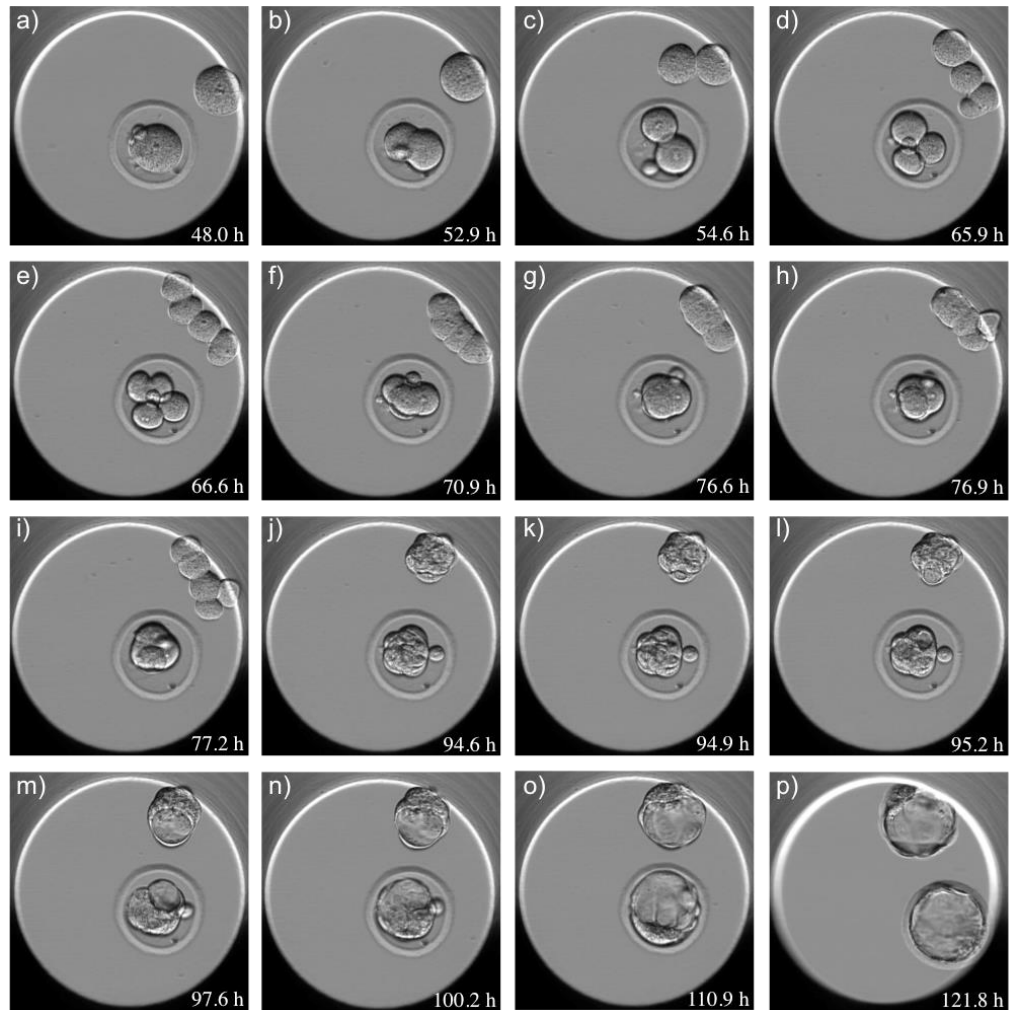


Figure 4.11 Pre-implantation development of twin mouse embryos bisected at two-cell stage. a-p) Twin embryo development from embryo bisection at the two-cell stage up to blastocyst stage. Times refer to hours post hCG injection.

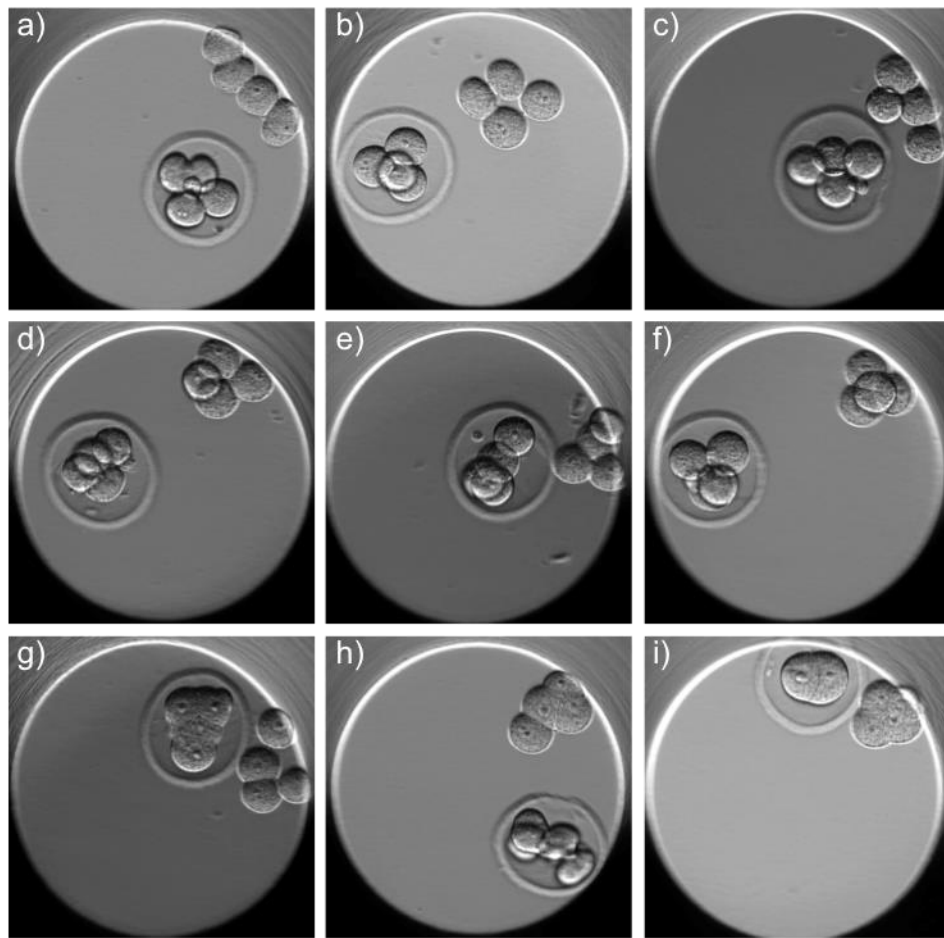


Figure 4.12 Four cell configuration on twin embryos. Two-cell embryos were bisected and cultured on time-lapse imaging. **a-c)** Twin embryos with and without ZP present different configuration at four-cell stage, correspondent to the eight-cell stage. **d-f)** Twin embryos present similar four-cell stage configuration. **g-f)** Twin embryos start compaction at different times. **i)** Early compaction on the ZP twin embryo.

4.5. Discussion

To my knowledge, this is the first study analysing the effects of cell removal during the cleavage stage of embryo development on cell allocation carried out by biopsying a single blastomere around the eight-cell stage for both murine and bovine embryos. This work has been published partially (Sepulveda-Rincon et al., 2016a). Lack of evidence suggest that embryo biopsy

at cleavage stage does not interfere with cell allocation pattern incidence. Thus, this results suggest that even after cells within the embryo readjust after cell removal, cell allocation patterns are not disturbed as they are probably determined at earlier stages.

In this work, two mammalian embryonic models were used not only to confirm the results but also because the bovine model is very similar to human embryos in terms timing of key embryonic events such as embryonic genome activation at the eight- to sixteen-cell stage (Maalouf et al., 2008), compaction after the sixteen-cell stage (Gilbert, 2010a) and similar transcriptomic profile (Jiang et al., 2014). Therefore, cleavage stage embryo biopsy on bovine embryos might bear a closed resemblance to the procedure performed on a clinical set up in humans. Furthermore, it is believed that the first sign of cell differentiation in mammalian embryos occurs during compaction, where cells biased to form the TE, will start to be polarised and its surface will flatten (Roberts et al., 2003, Bakhtari et al., 2014). In the present study, blastomere removal was performed before compaction only in bovine embryos as some mouse embryos were already starting compaction. Thus, it is unlikely that cell allocation patterns, and hence cell lineage fate, are established at compaction. Yet, morphokinetic analysis in mouse embryos suggested that biopsy procedure causes a delay on the following cleavages only for the embryos presenting the orthogonal pattern. A significant delay on hatching time between biopsied and non-biopsied embryos has been

reported in mouse embryos when the biopsy was performed at the four-cell stage (Ugajin et al., 2010). However, these embryos were biopsied at earlier stage than the present study and the adverse effects might be more prominent. In human embryos, a delay on subsequent cell cleavages, compaction and hatching process after cleavage stage biopsy has been observed (Kirkegaard et al., 2012). It would be interesting to explore the morphokinetics in bovine embryos after cell removal, however due to its great lipid content, it is difficult to trace cell cleavages after the six- to eight-cell stage.

In an attempt to link morphokinetics with further embryo development, mouse embryos developing faster have been linked with higher glucose consumption and presented higher foetal development per implantation than slow embryos; nevertheless no differences were found in terms of foetal development (Lee et al., 2015). It would be interesting if these glucose intake profiles can be analysed within the patterned embryos. Moreover, the total cell numbers at blastocyst stage was significantly affected in mouse embryos but not in bovine embryos. In agreement with these results, it has been reported that TCC is not affected by the biopsy procedure at the six- to eight-cell stage in bovine embryos (Tominaga and Hamada, 2004, Park et al., 2001, Kubisch and Johnson, 2007). In the present study, further analysis within each cell allocation pattern groups showed that the deviant group was significantly affected by the biopsy procedure in both species. It might be that

different “types” of embryos are affected differently by embryo biopsy and the way embryo cells arrange during pre-implantation, trigger different compensatory mechanisms for blastomere removal at cleavage stage.

Cell removal during pre-implantation development has been related with placental function alteration, adrenal abnormalities and faster oocyte depletion in mice (Yao et al., 2016, Yu et al., 2013, Zeng et al., 2013, Sato et al., 2014). Nonetheless, the biopsy was done at earlier stages, therefore 25% of the embryo cells were removed instead of the clinical standard of 12.5%. In addition, the *in-vitro* culture factor has not been taken into account in those studies, which in itself has been suggested as a risk for neurodevelopmental disorders (Conti et al., 2013). In humans, it has been reported that the removal of a single blastomere at the eight-cell stage, 12.5% of the embryo, has better outcomes than double cell removal, 25% of the embryo (De Vos et al., 2009). On the other hand, single cell removal at an advanced stage, sixteen- to thirty two-cell stage, in bovine embryos does not have an apparent effect on pre-implantation embryo development and pregnancy rates after embryo transfer (Macháty et al., 1993).

In addition, neurodegenerative disorders have also been related with mouse embryo biopsy in mice (Yu et al., 2009) and in humans it was reported that PGS babies presented higher percentages of complex minor neurological dysfunction at 18 months of age compared to a non-biopsied cohort of embryos (Middelburg et al., 2010, Middelburg et al., 2011). Yet these changes

have not been found on a follow-up study performed by the same group (Beukers et al., 2013) in agreement with other study where no neurological differences were found between babies born after or without PGS (Schendelaar et al., 2013). Likewise, it has been suggested that cell removal during embryo pre-implantation stage might affect somehow the fat metabolism (Holm et al., 2002, Perkel et al., 2015). A reported study in mice, pups born after single blastomere removal at eight-cell stage presented heavier body weights from day 10 after birth (Sampino et al., 2014). With a continually proportion of PGS and PGD procedures, further understanding of the effects of early embryo micromanipulation and follow-up assessment are needed in order to establish the safety of the procedure in short and long term development. Further investigations must be carried out to determine if the procedure of embryo biopsy can cause epigenetic modifications or other changes, as placental function, which could affect the putative offspring.

In order to determine if both blastomeres of the two-cell embryo present the same probability to continue embryo pre-implantation development and hence same developmental potential, blastomeres were separated at the two-cell stage. In theory, if both blastomeres contain the same determinants, both blastomeres should reach the blastocyst stage. However, the present results suggest that this is only true in around 60% of the embryos, when both blastomeres of the two-cell embryo reached the blastocyst stage. In this situation it was noted that both blastocysts presented the random cell

allocation pattern. Therefore, it might be the case that the split twin blastomeres need to be balanced or to promote cell movements during pre-implantation development in order to survive and reach the blastocyst stage.

In the present study it was noticed that twin blastomeres did not present the same cleavage pattern and the four-cell configuration differs. These findings have been previously observed in mouse embryos where only 60% of twin blastomeres followed the same four-cell configuration (Katayama et al., 2010). Nevertheless, when analysing morphokinetics within twin blastomeres, there was not any difference on further cleavages after embryo bisection; suggesting that both blastomeres of the two-cell embryo develop at the same speed at least until the blastocyst stage.

In terms of blastocysts production, the present results suggest that twinning can offer a higher blastocysts production from less quantity of embryos. These results are in agreement with previously reported studies where blastocysts rates go from 50 up to 120% (reviewed in (Arias et al., 2013)). On the other hand, in the present study it was noticed that split blastomeres with ZP were able to develop at a slightly higher rate than ZPF split blastomeres and ZPF two-cell embryos were apparently more sensitive to CM-Dil injection. A decrease, yet not significant, has been previously reported on the development of the split blastomere without ZP, when compared to its twin with ZP (Illmensee et al., 2006). Similarly, it has been reported that mouse

blastocysts from demi-embryos with ZP can develop into living offspring in a much lower extent to those from the control group and no living fetuses were achieved from demi-embryos without ZP (Nijs et al., 1988). Therefore, the present results might also be in agreement with those presented by Nijs and colleagues, suggesting that ZP confers support/protection to the pre-implantation embryo.

Furthermore, in bovine embryos it was suggested that developmental competence was similar in both blastomeres of the two-cell embryo after twinning (Held et al., 2012). Nevertheless only 12.5% of biopsied embryos developed into two demi-embryos and in 3.1% only one blastomere reach the blastocyst stage. These results denote low developmental rates in bovine embryos after twinning at the two-cell stage.

Overall, twinning can offer a higher blastocysts production from less quantity of embryos. However, the quality or caused changes on these embryos is questionable. It is still not known if twin blastomeres of the two-cell embryo have the same quality and developmental potential. On one side, it has been suggested that there is symmetry between twin blastomeres of the two-cell mouse embryo regarding transcript profiling (Tarkowski et al., 2010). Nevertheless, RNA asymmetry has been shown between sister blastomeres at the two- and four-cell stage (Biase et al., 2014). However, whilst asymmetry on mitochondrial DNA distribution on twin blastomeres has been described in mouse embryos (Kameyama et al., 2010); asymmetries in human embryos

have been reported only in 55% of the analysed embryos (Van Blerkom et al., 2000).

It is important to emphasize that even though twin blastomeres might have totipotent capacity, this does not mean that both blastomeres will present the same tendency towards certain lineage (Biase et al., 2014). In triploid human embryos, the suboptimal splitting stage was determined at six- to eight-cell stage (Illmensee et al., 2009); whereas for mouse embryos the suboptimal splitting stage was determined at two- to four-cell stage (Illmensee et al., 2005). The possibility that totipotency loss is related or triggered around EGA time remains open.

4.6. Conclusion

In conclusion, the findings in this Chapter do not support the hypothesis as there was no evidence suggesting that blastomere removal at the eight-cell stage affects cell allocation patterns during pre-implantation development. Similarly, embryo biopsy around EGA in bovine embryos did not influenced cell allocation patterns. Nevertheless, it is still unknown how cell allocation correlates with coping mechanisms after cell removal. On one side, at least in mouse orthogonal embryos, there is a delay on cell cleavage just after biopsy and also a longer period between embryo compaction and cavitation is observed. In addition, total cell counts at blastocyst stage are reduced after cleavage stage biopsy in deviant embryos. Hence, these results in deviant

embryos suggest a diminution of cell cleavage speed at least after the nine-cell stage. Thus, different embryo cell allocation patterns within pre-implantation development might cover-up different mechanisms which are triggered after *in-vitro* manipulation.

Furthermore, the totipotency state of the blastomeres at the two-cell stage of the mammalian embryo is still debatable. It is clear that twinning is possible in mammalian species; whether twinning is possible in every embryo however remains unclear. Even though the twinning technique can produce a greater quantity of blastocysts, it remains unknown if those blastocysts from demi-embryos have the same developmental potential than normal embryos and if these demi-embryos will develop into a healthy offspring or derived healthy stem cell lines. Further research on epigenetic marks within the twin blastomeres of the two-cell embryo as well as within embryos following the different cell allocation patterns could help to explain the possible different coping mechanisms during pre-implantation development.

5. CHAPTER FIVE: EPIGENETICS AND CELL ALLOCATION PATTERNS IN PARTHENOGENETIC MOUSE EMBRYOS

5.1. Introduction

Asexual reproduction or parthenogenesis, is frequent in some non-mammalian species such as insects, fish, snakes and amphibians, where unfertilised oocytes are activated by parthenogenesis to create a whole new offspring (Cibelli et al., 2006, Paffoni et al., 2008). In mammals, ovulated oocytes progress from metaphase I to metaphase II of the second meiosis and will be arrested there until fertilisation. Unfertilised oocytes in mammalian species can be activated through physical, chemical or electrical treatment (Versieren et al., 2010). Parthenogenetic blastocysts have been successfully obtained in several mammalian species such as mouse, bovine, sheep, goat, pig, rabbit, rat, monkey and human (Cibelli et al., 2006). Nevertheless, parthenotes development is arrested just after forelimb bud stages and there is no documented report of live offspring (without genetic modification) by parthenogenesis in mammals (Paffoni et al., 2008). Parthenotes offer the possibility to study the maternal factors contribution towards development in the absence of paternal contribution (Sepulveda-Rincon et al., 2016b).

After fertilisation, the oocyte undergoes to an important nuclear reorganisation. However, this reorganisation seems delayed in SCNT and parthenogenetic embryos when compared with fertilised embryos (Merico et al., 2007). The use of histone deacetylation inhibitors (HDACis) have been

propose to cause hyperacetylation and therefore, to promote a more relaxed chromatin conformation (Kishigami et al., 2006a). HDACs such as trichostatin A (TSA), have been reported to improved development in SCNT embryos through the increase of acetylation on different histone marks (Maalouf et al., 2009, Rybouchkin et al., 2006). On the other hand, as mention previously in Chapter one, epigenetic marks are involved on cell lineage specification processes in mammals by activating or silencing genes. It has been previously reported that parthenogenetic and SCNT embryos present different epigenetic profiles than their counterpart fertilised embryos (Chen and Yu, 2015). Specifically, differences regarding the H3 methylation among blastomeres of four-cell stage SCNT and fertilised embryos have been reported (Liu et al., 2012). Liu and colleagues showed that a more marked H3 methylation asymmetry among blastomeres was observed in SCNT embryos when compared with fertilised embryos. Furthermore, asymmetries on histone 3 (H3) methylation at arginine 26 (R26) within blastomeres of four-cell mouse embryos have been also observed by other research group (Torres-Padilla et al., 2007). Torres-Padilla and colleagues reported differences on H3R26me levels in blastomeres with meridional or equatorial previous cell divisions. Cells with higher H3R26me levels were predisposed to contribute to the ICM at blastocyst stage.

5.2. Objectives

As mentioned previously, parthenogenetically activated embryos lack of paternal contribution and the epigenetic events during its development differ from their counterpart, normally fertilised embryos. Thus, the principal objective of the present chapter is to observe how the cell allocation patterns during pre-implantation are distributed in mouse parthenotes embryos in comparison with fertilised embryos. Additionally, as the use of histone deacetylase inhibitors (HDACis) (Scriptaid (Wang et al., 2011) or trichostatin A (TSA) (Maalouf et al., 2008)), has been reported to help opening of the chromatin and gene expression in SCNT bovine embryos, the use of TSA on mouse parthenotes will be investigated. Finally as the use of HDACis is supposed to promote histone acetylation, histone 3 lysine 9 acetylation (H3K9ac) mark will be also studied.

5.3. Methods

In this chapter three different groups of embryos will be used: fertilised, for naturally fertilised embryos; parthenotes, for parthenogenetic activated oocytes; and TSA, for parthenogenetic activated oocytes with TSA treatment.

5.3.1. Embryo production by fertilisation

Mouse oocytes naturally fertilised were obtained one cell stage from superovulated B6CBAF1 females naturally mated as previously described on Section 2.1.1.

5.3.2. Oocyte activation by parthenogenesis

Mouse oocytes from superovulated B6CBAF1 female mice were harvested and denuded at 12-14 h post-hCG injection as previously described on Section 2.1.1. Then, MII mouse oocytes were rinsed twice in CZB activation medium (See Appendix V) containing 1 ml of calcium free medium 5 µg/ml of cytochalasin B (Sigma, C6762) and 10 mM strontium chloride hexahydrate (Sigma, 255521). The use of cytochalasin B will prevent second PB extrusion (Figure 5.1), thus producing a diploid parthenote embryo that will no contain paternal chromosomes but two sets of maternal chromosomes. Then, rinsed MII mouse oocytes were indistinctly distributed into 2 groups, control and TSA, and incubated in fresh CZB activation medium with 5 nM trichostatin A (TSA group) or without TSA (control group) at 37°C and 5% CO₂ for 6 h. After activation, mouse parthenotes were rinsed in M16 medium with 5 nM TSA or without and cultured further at 37°C and 5% CO₂ for 4 h. Finally, all embryos were transferred to fresh M16 medium, keeping their identity group.

In order to check if TSA treatment promotes histone modifications during pre-implantation, embryos were taken at two-cell for H3K9ac levels analysis and four-cell stage for H3R2me2 levels analysis. Embryos for cell allocation patterns analysis were left in culture after oocyte activation until Dil injection at the two-cell stage as stated in Section 2.3. After injection, embryos were cultured further until blastocyst stage and were classified into orthogonal, deviant and random according to their cell allocation pattern (Section 2.4.1).

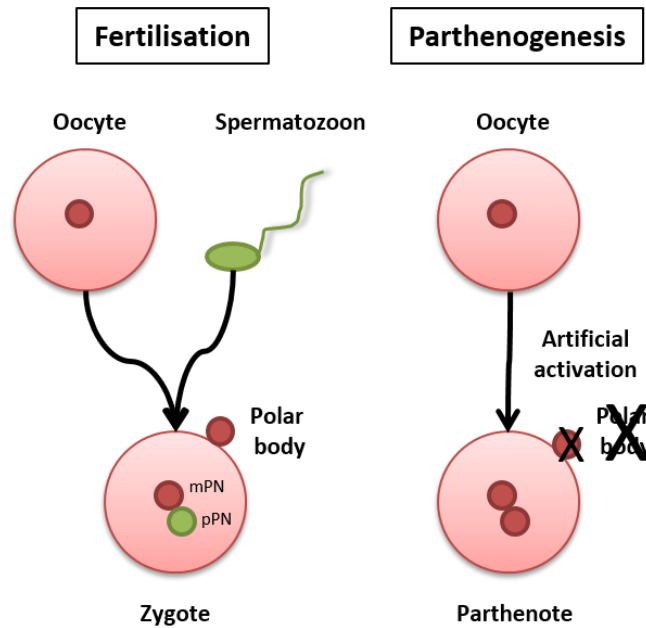


Figure 5.1 Fertilised and parthenogenetic activated oocytes. On the left it can be observed the oocyte fertilisation process by a spermatozoon giving rise a zygote. On the right it can be observed the oocyte artificial activation process giving rise to a diploid parthenote embryo. Figure extracted from (Sepulveda-Rincon et al., 2016b).

5.3.3. Immunocytochemistry

All solutions on this protocol were dissolved on PBS and performed at room temperature unless otherwise stated. Blastocysts were fixed with 4% PFA solution during 20 min and then rinsed in PBS. Embryos were then permeabilised with Triton x-100 1% during 30 min. After permeabilisation embryos were incubated with 2% BSA for 1 h and incubated overnight at 4°C with the primary antibody. The primary antibody used for two-cell embryos and blastocysts was rabbit polyclonal H3K9ac antibody (Active Motive, dilution 1:100 in 2% BSA), whilst for four-cell embryos it was rabbit polyclonal H3R2me2 (Upstate, dilution 1:100 in 2% BSA). Next, embryos were rinsed in PBS and incubated with the secondary antibody (anti-rabbit IgG antibody,

Alexa Fluor 488-conjugated, dilution 1:200 in 2% BSA) for 1 h away from light. From this point onwards light exposure was maintained at a minimum. Afterwards, the embryos were rinsed in PBS and DNA counterstaining was performed with DAPI (dilution 1:200) for 20 min at 37°C. The embryos were then briefly post-fixed with 2% PFA during 10 min and rinsed in PBS before mounting. Finally embryos were mounted with Citifluor (Citifluor Company) on a microscope slide (super frost ultra plus, Thermo Scientific).

5.3.4. Histone modifications image analysis

Mounted embryos were observed using an AxioObserver Z1 inverted microscope equipped with ApoTome slider and a colibri LED illumination system for wide-field fluorescence microscopy (Carl Zeiss, Inc.). Two-cell and four-cell embryos were observed with a 63X oil immersion objective and blastocysts were observed with a 40X oil immersion objective (Plan-Neofluar NA 1.3) and were scanned with a distance between light optical sections of 0.240 μm using AxioVision software version 4.6. Maximum intensity projections of Z-stacks were analysed using Image J software.

For two-cell and four-cell stage embryos, all nuclei were measured. In order to consider not only the ellipsoid volume of the nucleus but its area as well. The following formula was applied: $\pi \cdot 355/113 \cdot \text{radius A} \cdot \text{radius B} \cdot \text{number of stacks}/2$. Then, intensity levels were normalised by dividing the fluorescence intensity of H3K9ac by the total DNA. Further, blastomere with the highest

intensity was classified as first blastomere, followed by the second highest as second blastomere and so on.

For blastocysts, around 20 nuclei per blastocyst were outlined manually on these projections. I tried to keep a consistent selection of different nuclei in the ICM and TE among all blastocysts, however this selection was not random and a possible bias could not be discarded. Mean staining intensities and nuclear areas were then measured for each nucleus. Total fluorescence intensities were calculated by multiplying the mean fluorescence intensities and nuclear areas and then divided by the corresponding exposure time. Fluorescence levels were then normalised dividing the fluorescence intensity of the H3K9ac by the total DNA content.

Finally, the fluorescence levels were normalised against the two-cell embryo, four-cell embryo or blastocysts showing the highest level on each experiment, which was set at 100%. Total cell count was performed as stated on Section 2.4.2.

5.3.5. Statistical analysis

Data sets were checked for normality using Shapiro-Wilk test employing IBM SPSS version 23. Values are reported as mean $\pm$ SEM. For SEM calculation, the total number of replicates of the experiment was used as N value when reporting incidence of cell patterns. Differences on cell allocation pattern incidence between normally fertilised, parthenogenetic and

parthenogenetic/TSA embryos were assessed by two-way ANOVA test and further post-hoc Bonferroni test. Furthermore, differences on H3K9ac levels among parthenotes were identified by one-way ANOVA.

Mann Whitney test was used to compare H3K9ac levels in two-cell parthenotes with and without TSA and also within both blastomeres of the two-cell parthenotes. Moreover, H3R2me2 levels in four-cell embryos parthenotes with and without TSA treatment were analysed by one-way ANOVA test. Similarly, difference on H3R2me2 levels within the four blastomeres of the four-cell parthenotes was studied by one-way ANOVA. Two-way ANOVA was performed to determine any difference between H3K9ac levels at blastocyst stage between parthenote groups.

Finally, total number of embryos (n value) was used to calculate SEM value when reporting TCC in blastocysts. Two-way ANOVA test was performed when comparing blastocysts total cell counts between parthenotes with and without TSA treatment.

5.4. Results

5.4.1. Cell allocation patterns in fertilised and parthenogenetic mouse embryos

Different cell allocation patterns in parthenogenetic mouse embryos among 6 repetitions of the experiment can be observed in (Figure 5.2). There was a significant difference on the pattern incidence within the fertilised ($p = 0.002$,

n = 216 embryos), parthenotes ($p < 0.001$, n = 131 embryos) and TSA ($p < 0.001$, n = 126 embryos) groups. Further analysis revealed that in the fertilised group the random pattern ($49.4 \pm 7.1\%$, n = 112 embryos) was prevalent when compared with the orthogonal ($p = 0.002$, $25.9 \pm 3.9\%$, n = 54 embryos) and deviant ($p = 0.001$, $24.6 \pm 4.4\%$, n = 50 embryos) embryos. No significant differences were observed on the incidence of orthogonal and deviant embryos within the fertilised group ($p = 0.857$). Similarly, embryos on the parthenote group tended to follow the random pattern ($53.6 \pm 5.3\%$, n = 70 embryos) and this tendency was significant when compared with orthogonal ($p < 0.001$, $14.8 \pm 2.7\%$, n = 19 embryos) and deviant ($p = 0.001$, $31.6 \pm 2.2\%$, n = 42 embryos) embryos. Significant differences were found when comparing the incidence of orthogonal against deviant patterned embryos ($p = 0.006$).

Nonetheless, embryos on the TSA group have an inclination towards the random pattern ($45.4 \pm 4.5\%$, n = 58 embryos) when compared with the orthogonal pattern ($p < 0.001$, $17.7 \pm 2.2\%$, n = 22 embryos) but not with deviant pattern ($p = 0.154$, $36.9 \pm 6.3\%$, n = 46 embryos). Likewise, significant differences ($p = 0.002$) were found on the incidence between orthogonal and deviant patterns within the TSA group. However, when the effect on the group on cell allocation patterns was investigated, there were no significant differences for the orthogonal ($p = 0.234$), deviant ($p = 0.181$) or random ($p = 0.381$) patterns among fertilised, parthenotes and TSA groups.

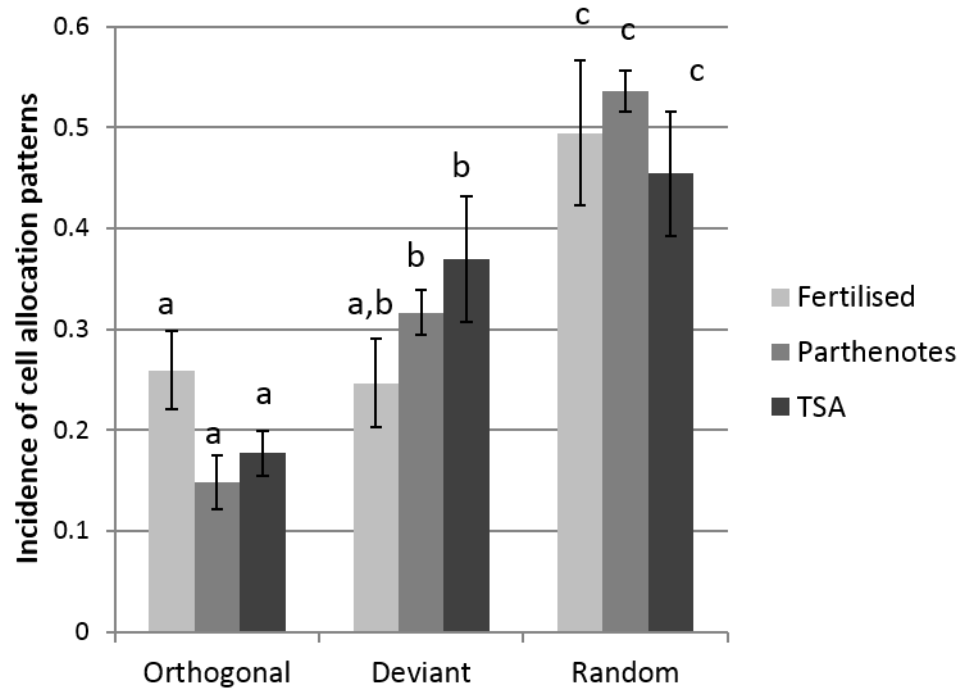


Figure 5.2 Incidence of cell allocation patterns in fertilised and parthenogenetic mouse embryos. Three embryo groups: fertilised, for naturally fertilised oocytes; parthenotes, for parthenogenetic activated oocytes; and trichostatin A TSA for parthenogenetic activated oocytes with TSA treatment. Incidence of embryo patterns were analysed by two-way ANOVA test.

5.4.2. Histone modifications in mouse blastocysts

5.4.2.1. Normal fertilised mouse embryos

Histone 3 lysine 9 acetylation levels were analysed in 92 fertilised blastocysts (Figure 5.3) within 3 repetitions of the experiment showing significant differences among patterns ($p < 0.001$). The orthogonal group ($n = 22$ embryos) presented significantly higher H3K9ac levels when compared with deviant ($p < 0.001$, $n = 41$ embryos) and random ($p < 0.001$, $n = 29$ embryos) groups (Figure 5.4). Nonetheless, blastocysts on deviant and random groups presented similar H3K9ac levels ($p = 0.873$). These results suggest that cell allocation patterns might be affecting some epigenetic marks, thus further

embryo development among the different patterned embryos can be different.

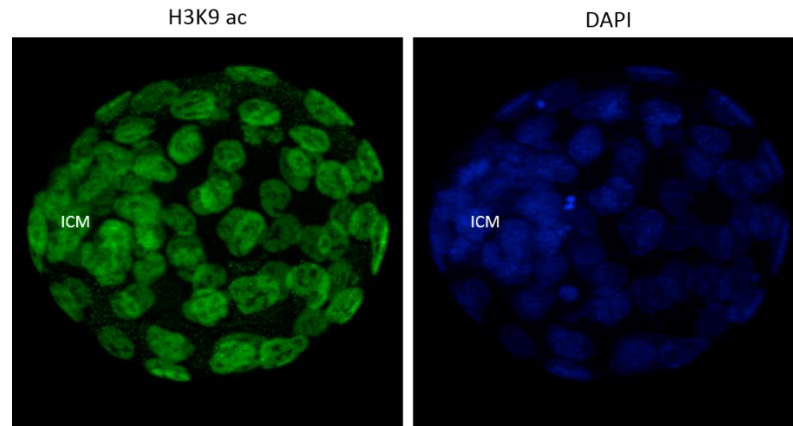


Figure 5.3 Histone 3 lysine 9 acetylation in mouse blastocysts. Immunostaining of H3K9ac marks in mouse blastocysts can be observed in green (left picture). Nuclear staining by DAPI can be observed in blue (right picture).

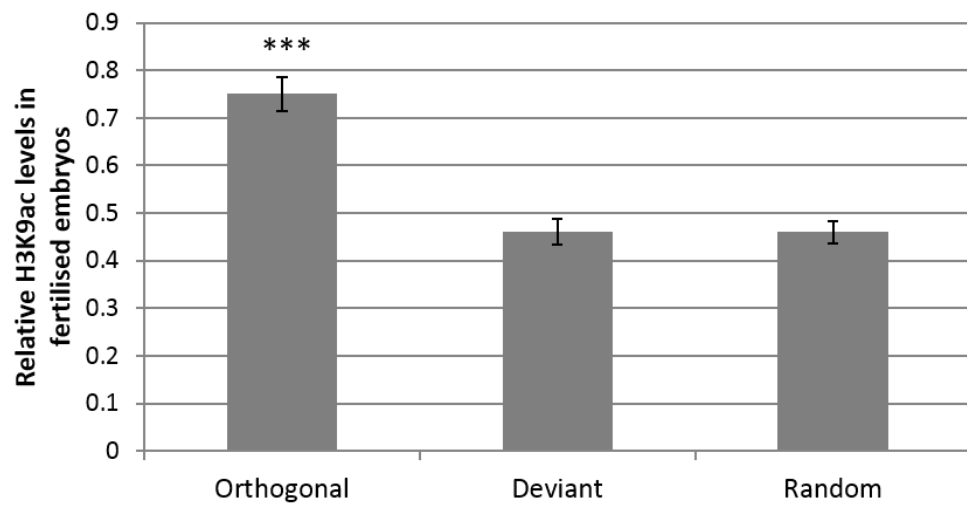


Figure 5.4 Relative H3K9ac levels in fertilised mouse embryos. Blastocysts from fertilised oocytes were analysed for H3K9ac levels by immunocytochemistry. *** Denotes $p < 0.001$ by single-way ANOVA.

5.4.2.2. Mouse parthenotes

A total of 26 two-cell embryos on the control group and 27 on the TSA group were taken in order to see the effects of TSA treatment during oocyte activation on H3K9ac levels (Figure 5.5). When comparing the two different

groups, a significant increase of around 51% on H3K9ac levels ($p = 0.029$ by Man Whitney test) was found among TSA group (Figure 5.6a); showing the promotion of histone acetylation by TSA treatment. Furthermore, when checking for H3K9ac symmetry between twin blastomeres of the two-cell embryos, there was an asymmetric distribution of H3K9ac levels between blastomeres of $25.1 \pm 2.7\%$ for the control group ($n = 12$ embryos) and $19.0 \pm 3.5\%$ for the TSA group ($n = 13$ embryos) (Figure 5.6b). Blastomere asymmetries were significant only within the control group ($p = 0.009$ by Man Whitney test) but not on the TSA group ($p = 0.051$).

In addition, the levels of H3R2me2 were checked on four-cell parthenotes (Figure 5.7) in order to check the effect of TSA treatment on histone methylation and symmetry on blastomeres. Within 3 replicates of the experiment, 85 embryos were analysed (48 embryos on the control and 38 on the TSA group). Overall, there was a significant increase of 13% (Figure 5.8a, $p < 0.001$ by ANOVA test) of H3R2me2 levels between the control and TSA groups. Significant differences were found ($p < 0.001$) between the four blastomeres within the control and TSA groups (Figure 5.8b). Moreover, significant differences were found on the 4th blastomere between treatments ($p = 0.029$).

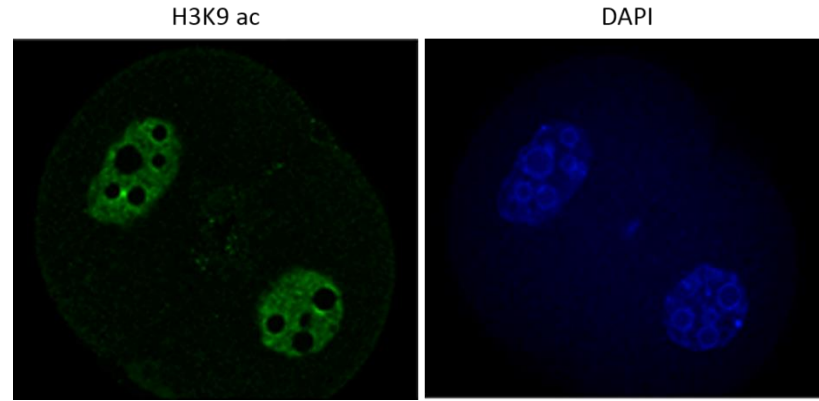


Figure 5.5 Histone 3 lysine 9 acetylation in two-cell mouse parthenotes. Immunostaining of H3K9ac marks in two-cell stage mouse embryos can be seen on green (left picture) and nuclear staining can be seen on blue (right picture).

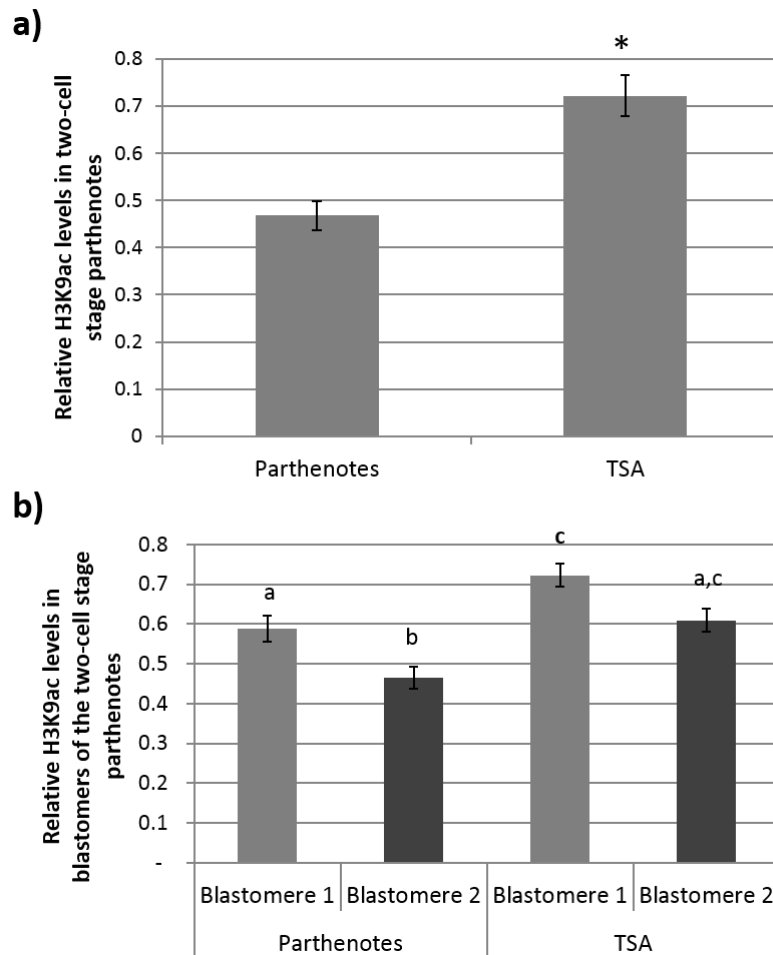


Figure 5.6 Relative H3K9ac levels in two-cell stage mouse parthenotes. H3K9ac levels were detected by immunocytochemistry. **a)** Relative H3K9ac levels in two-cell stage, parthenotes with trichostatin A (TSA) treatment and without TSA treatment. * Denotes $p < 0.05$ by Man-Whitney test. **b)** Relative H3K9ac levels in twin blastomeres of the two-cell parthenotes. Different superscript letters denote statistical significance $p < 0.05$ by two-way ANOVA.

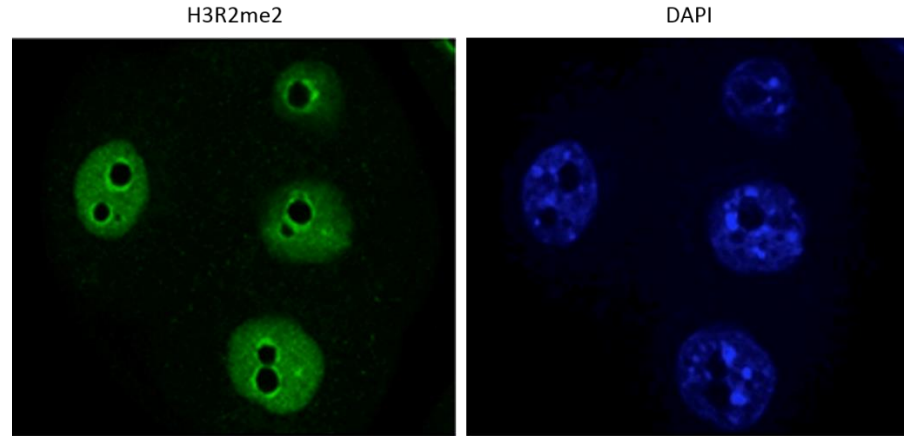


Figure 5.7 Immunodetection of H3R2me2 levels in four-cell mouse parthenotes. Representation of H3R2me2 (green) detection by immunocytochemistry and DNA labelling by DAPI (blue).

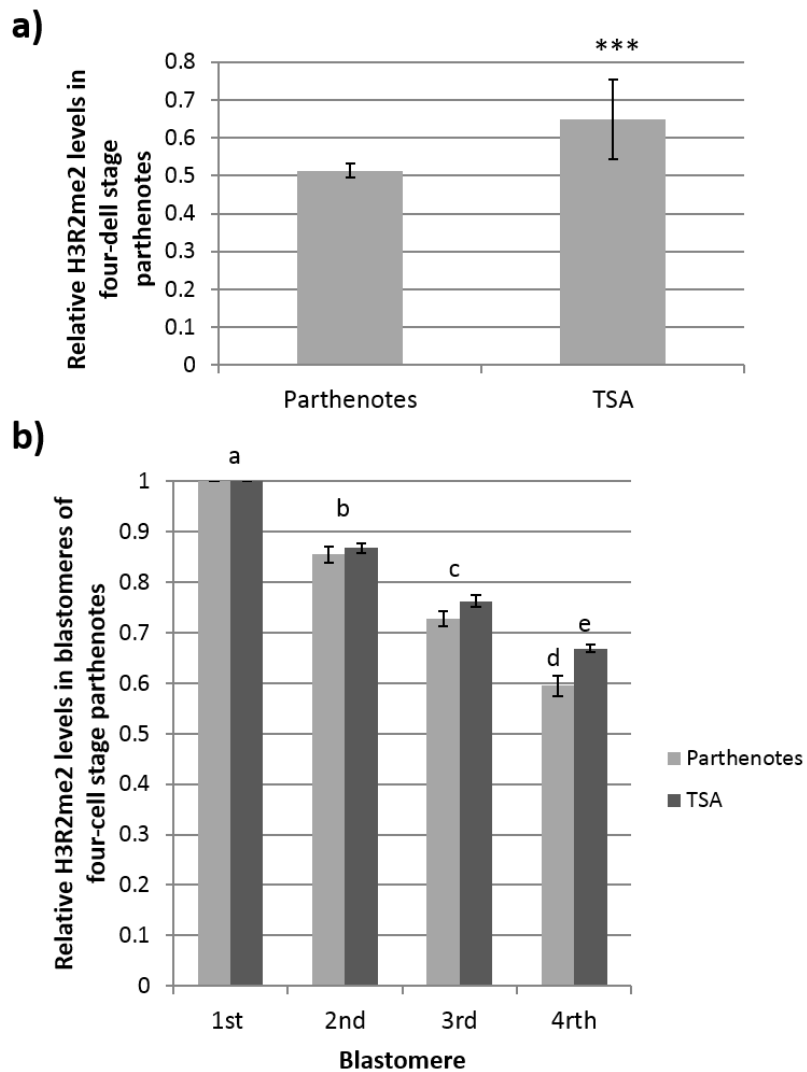


Figure 5.8 Relative H3R2me2 levels in four-cell mouse parthenotes. a) Relative H3R2me2 levels in four-cell parthenotes with and without trichostatin A (TSA) treatment. ***Denotes $p < 0.001$ by Man-Whitney test. b) Relative H3R2me2 levels on four-cell parthenotes among blastomeres. Different superscript letters denote statistical significance $p < 0.05$ by two-way ANOVA.

Among six repetitions of the experiment, blastocyst rates were similar on parthenotes and TSA groups ($82.3 \pm 4.45\%$ and $85.8 \pm 2.1\%$ respectively). Further, H3K9ac levels were measured on 81 blastocysts within 3 repetitions. In general, an increase of 16.5% between in H3K9ac levels after TSA treatment. The orthogonal group showed superior levels ($p < 0.001$) of H3K9ac levels compared with the deviant and random groups. Furthermore, embryos on the TSA group showed different H3K9ac levels from those on the control group (Figure 5.9). Whilst deviant embryos did not show any differences ($p > 0.05$) on H3K9ac levels after TSA treatment, orthogonal embryos showed a significant reduction ($p < 0.05$). In contrast, random embryos presented a significant increase ($p < 0.05$) after TSA treatment.

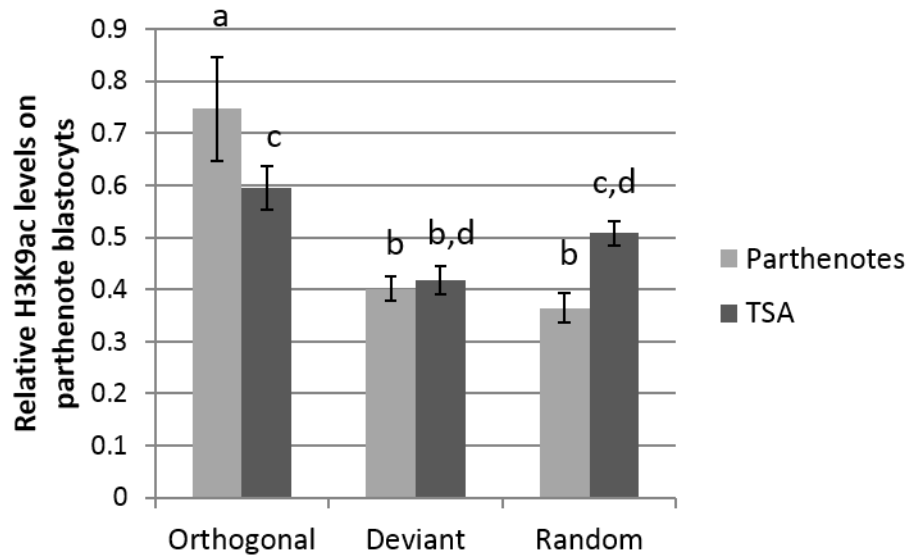


Figure 5.9 Relative H3K9ac levels on mouse blastocysts from parthenogenetic activated oocytes. Different superscript letters denote statistical significance $p < 0.05$ by two-way ANOVA and Bonferroni tests.

Total cell count within parthenote blastocysts ($n = 74$) seems to be affected to certain extent by cell allocation patterns, however significant differences on the parthenote control group ($p = 0.096$) or the TSA group ($p = 0.381$) were not found (Figure 5.10 and Table 5.1). Within the parthenote control group, orthogonal blastocysts presented a significantly lower number of cells when compared with deviant blastocysts ($p = 0.038$), but not when compared with random ones ($p = 0.351$). No difference was found when comparing TCC of deviant and random embryos ($p = 0.120$) on the parthenote control group.

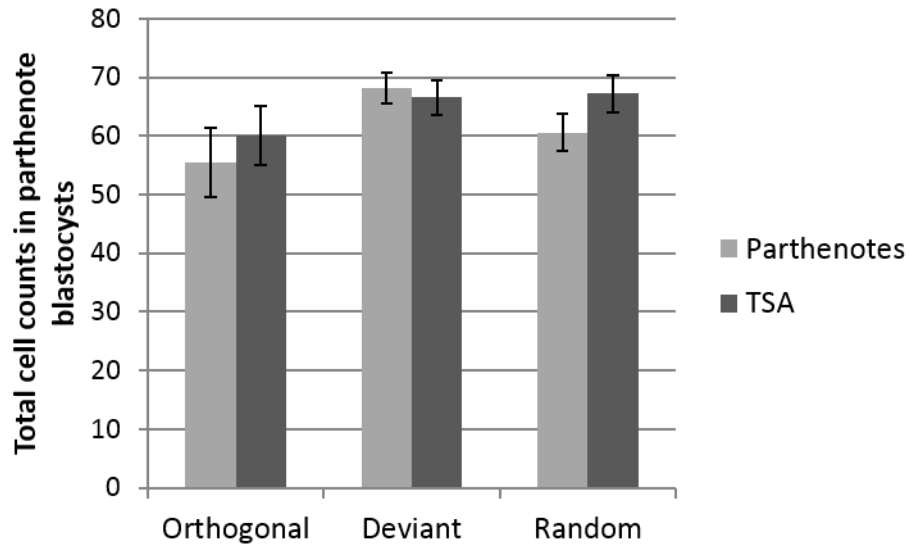


Figure 5.10 Total cell count (TCC) in blastocysts from parthenogenetic activated mouse oocytes. TCC was done by DNA nuclear staining with DAPI using Image J software. No statistical significance ($p > 0.05$) was found between groups and between patterns by two-way ANOVA test.

Table 5.1 Total cell count (TCC) in blastocysts from parthenogenetic activated mouse oocytes. Values are expressed as mean $\pm$ SEM and n as total number of analysed embryos.

TCC	Orthogonal	Deviant	Random
Control	55.4 $\pm$ 5.9, n=7	68.0 $\pm$ 2.6, n=11	60.6 $\pm$ 3.1, n=18
TSA	60.0 $\pm$ 5.0, n=8	66.5 $\pm$ 3.0, n=14	67.2 $\pm$ 3.1, n=15

5.5. Discussion

Based on the presented results, it can be hypothesised that oocyte activation is not responsible of cell allocation patterns during pre-implantation embryo development. These results are in agreement with previously reported data suggesting that paternal contribution does not play a role on the first cleavage and therefore cell allocation at blastocyst stage (Davies and Gardner, 2002).

Nevertheless, the possibility of oocyte fertilisation/activation method having a minimum impact on cell allocation patterns is latent.

There are only three reported studies investigating cell allocation patterns in mammalian parthenogenetic embryos, two of them using pig oocytes (Kim et al., 2012, Park et al., 2009) and the other using mouse oocytes (Piotrowska and Zernicka-Goetz, 2002). Results in this chapter are in partial agreement with those reporting a strong tendency of mouse parthenotes to follow the random pattern (> 50%) (Piotrowska and Zernicka-Goetz, 2002). However, the same group has reported different results on normal fertilised embryos where they tend to present the orthogonal pattern (Piotrowska et al., 2001).

In porcine parthenotes displaying uneven blastomere size, around 50% of the embryos presented an unspecified distribution of descendant cells from the two-cell embryo; besides blastomere size at the two-cell stage (Kim et al., 2012). Furthermore, it was reported that blastomeres of the two-cell porcine parthenotes with high lipid content, tend to occupy the embryonic pole at blastocyst stage 43% of the time (Kim et al., 2012). In addition, lipid rich blastomeres presented a higher expression of *Carm1*. On the other hand, other authors have proposed that in 60% of the cases the lagging blastomere cleaving from the two-cell embryo will give rise mainly to the ICM in porcine parthenotes (Park et al., 2009).

It is interesting that cell allocation patterns can be related with blastocyst total cell count in parthenogenetic embryos. Orthogonal embryos presented

the least TCC numbers whereas deviant embryos presented the larger TCC numbers. Also, it is interesting that TSA treatment might be involved on cell proliferation as the differences on TCC between groups are reduced after TSA treatment.

Remarkably, the presented results show a marked similitude between the H3K9ac levels among cell allocation patterns on natural fertilised and parthenogenetically activated mouse embryos. Orthogonal embryos consistently exhibited higher levels of H3K9ac at blastocyst stage. Hence, suggesting that certain epigenetic routes are affecting cell allocation patterns and it might have an impact later on development.

Furthermore, it is well known that nuclear reprogramming differs between oocytes activated by sperm entry, parthenogenetic activation or by SCNT (Reviewed on (Sepulveda-Rincon et al., 2016b)). Oocytes that are not activated by sperm entry might present a delay on nuclear reprogramming due to its chromatin in a more condensed state (Merico et al., 2007). The use of histone deacetylase inhibitors (HDACis), as Scriptaid or trichostatin A (TSA), has been reported to have beneficial effects on higher blastocysts rates and live births in SCNT mouse embryos (Maalouf et al., 2009, Rybouchkin et al., 2006) and SCNT bovine embryos (Maalouf et al., 2008, Wang et al., 2011). Parthenogenetic embryos seem to have similar problems to SCNT embryos with chromatin reorganization during the first cell cleavages. Thus, the effects

of TSA on cell allocation patterns were investigated in mouse parthenotes in the present chapter.

The effects on TSA were visible at the two- and indirectly at four-cell stage, where a significant increase on H3K9ac levels at the two-cell stage and H3R2me2 levels at four-cell stage were observed. On the other hand, differences on the H3K9ac levels within the twin blastomeres of the two-cell embryo were found on the present study for parthenotes with and without TSA treatment, suggesting that at least at the epigenetic level, early blastomeres present different histone post-translational modification profiles. It is still debatable whether blastomeres of mammalian embryos are homogeneous or heterogeneous during the first cell cycles. Yet, interesting mRNA analysis of two-cell and four-cell mouse embryos suggested that blastomeres presented significant differences on mRNA related with expression factors typically expressed by the ICM and TE (Biase et al., 2014). Thus, suggesting a possible bias on the blastomere's fate even at those early stages.

Unexpectedly, at the four-cell stage asymmetries on histone marks among blastomeres were observed and increased after TSA treatment. It might be the case that asymmetries between blastomeres are carried on from the two-cell stage. Asymmetries on H3 arginine methylation levels in four-cell mouse embryos have been previously reported (Torres-Padilla et al., 2007), suggesting that blastomeres with higher content of H3 arginine methylations

will promote totipotency and will develop into ICM and polar trophectoderm. Also, H4R2me2 has been reported to be asymmetrically distributed on four-cell stage mouse and bovine embryos (Sepulveda-Rincon et al., 2016a). Increase of H4R2me2 levels after TSA treatment could refer to a synergic mechanism between histone acetylation and H4R2me2. Equally, asymmetries between blastomeres have been found at the protein level where relative levels of proteins leptin and STAT3 on four-cell stage mouse and human embryos have been reported to be unequal among four blastomeres (Chen et al., 2009a).

It has been reported that fertilised, parthenotes and SCNT embryos present different epigenetic constraints only during the first cleavages but after the eight-cell stages they present similar chromatin organization (Merico et al., 2007). Thus, it can be thought that histone hyperacetylation by TSA treatment might only be seen or only be effective just before the eight-cell stage. The hyperacetylation effect of TSA treatment was not consistent among patterns at blastocyst stage, suggesting that TSA treatment during activation might act immediately and last during the first two cell cleavages; but it might not have a lasting effect. In addition, the present results exhibited no significant improvement on blastocyst yield after TSA treatment. Similar results have been reported in mouse parthenotes where a slight increase on blastocyst rates, yet not significant, was observed after TSA treatment (Kishigami et al., 2006b).

Previously it was hypothesised that cell allocation patterns might be related with different epigenetic profiles. The presented results suggest that even though the incidence of the patterns within the TSA group remained similar when compared with the parthenote control group, there was a slight decrease on the incidence of the random pattern, as well as a slight increase on the orthogonal and deviant patterns. Therefore, as TSA treatment is affecting the embryo epigenetic profiles, these changes might be reflected in the slightly different distribution on the cell allocation patterns.

Epigenetic changes might be just the tip of the iceberg during mammalian embryo development. It has been reported that a decrease of H3K9/14 acetylation levels can be related with transcriptional repression of *Nanog* and *Oct4* when inducing Cdx2 on mouse embryonic stem cells (Carey et al., 2014). Thus, one explanation of the marked difference in H3K9ac levels among patterned embryos might be that deviant and random embryos have already started with trophectoderm differentiation; in contrast with orthogonal embryos, where TSA treatment is allowing these orthogonal embryos to start this differentiation process on time. Further research exploring this idea might help the comprehension of pre-implantation embryo development and how morphokinetic features can be linked with further development.

5.6. Conclusion

In conclusion, cell allocation patterns during embryo pre-implantation development seem related with epigenetic profiles in fertilised and

parthenogenetically activated embryos. Additionally, there is a present asymmetry on histone marks between blastomeres of two- and four-cell stage parthenotes. Even though this asymmetry is observed after TSA treatment, there is a tendency to reduce differences between blastomeres, therefore working towards symmetry. It is important to consider that these epigenetic differences between early blastomeres might be a blastomere cell cycle asynchrony; which it might be triggered by EGA (Roberts et al., 2011, Biase et al., 2014). Also, TSA treatment seems to be reflected, even though not significantly, in cell allocation patterns. Incidence of embryos following the orthogonal was increased after TSA treatment, whereas incidence of embryos following the random pattern decreased. Overall, as the epigenetic mark H3K9ac is affected by cell allocation patterns at blastocyst stage, further research on the effects of this epigenetic difference beyond pre-implantation development must be addressed.

6. CHAPTER SIX: EFFECT OF CELL ALLOCATION PATTERNS IN OFFSPRING DEVELOPMENT

6.1. Introduction

More than 6.5 million babies have been born after the almost 40 years ART have been applied in humans (Brown, 2016), representing up to 4% of born babies in some European countries (Grafodatskaya et al., 2013, Nyboe Andersen et al., 2009). Even though most of these children appear to be healthy, recent systematic literature reviews have reported potential effects of ART on birth defects and offspring health related to immune system, cardiovascular and metabolic problems, epilepsy, cancer, adrenal gland activation and endocrine abnormalities (Hart and Norman, 2013b, Kettner et al., 2015, Hargreave et al., 2013, Hansen et al., 2013, Pinborg et al., 2013). Increased neurological adverse effects after ART encompass cognitive impairment, depression and binge drinking behaviour (Hart and Norman, 2013a). It is still uncertain if these effects are caused by ART *per se* and/or are related with the parental infertility background (Barnhart, 2013).

Animal models have allowed study aspects of the main events occurring during embryo implantation and pregnancy (Cha et al., 2012). However, embryo implantation process changes across mammals. There are two types of embryo attachment: superficial and interstitial (McGowen et al., 2014). Species as bovine, sheep, goat, cow and pig present superficial embryo

attachment, where the trophectoderm invasion into the endometrium is minimal or null (reviewed on (Cha et al., 2012). Non-afrotherian insectivores, bats, rodents and primates share common features during embryo implantation, the interstitial embryo attachment; where the blastocyst is fully embedded within the uterine endometrium (McGowen et al., 2014, Wang and Dey, 2006). Then, trophoblast cells are directly in contact with maternal blood presenting haemochorial placentation (Cha et al., 2012, Wang and Dey, 2006). Thus, due also to its short gestation and easy manipulation and handling, mouse has been the preferred animal model to study embryo development and further development (Melford et al., 2014).

6.2. Objectives

To my knowledge, no previous work has focused on the effects of embryo cell allocation patterns during pre-implantation on offspring organ development. In this chapter, the objective was to assess if there is any difference on embryo implantation and further developmental potential of putative offspring between blastocysts classified by their cell allocation patterns. The end points of the study were pregnancy and live birth rates, offspring total body weight and organ morphometry.

6.3. Methodology

6.3.1. Embryo culture and manipulation

One cell frozen B6C3-F1 x B6D2 F1 mouse embryos (Embryotech Laboratories, Inc., Massachusetts, USA) were thawed according to supplier's instructions (Appendix IV). After thawing, embryos were cultured in KSOM medium under mineral oil at 37°C and 5% CO₂. A lipophilic tracer was injected in one blastomere of the two-cell embryos (See Section 2.3) and left in culture until blastocyst assessment prior embryo transfer.

6.3.2. Pseudo-pregnant mouse surrogates

Six weeks old CD1 male mice were vasectomised by Charles River and complete sterilisation was assessed two weeks after surgery. Nine to eleven weeks old CD1 female mice were whitened two days before placing them with vasectomised 10-11 weeks old CD1 mouse males. Vaginal plugs were daily assessed and females with positive plug were classified as 0.5 days post coitus (dpc) pseudo-pregnant females. Pseudo-pregnant females were housed until they reached 2.5 dpc.

6.3.3. Non-surgical embryo transfer

The protocol suggested on the non-surgical embryo transfer (NSET) device (ParaTechs, KY, USA) was followed (Appendix VII, Figure 6.1). Briefly, blastocysts were classified accordingly to their cell allocation pattern into

orthogonal, deviant or random groups (See Section 2.4.1) and graded accordingly to their grade of blastocoel expansion (Table 1.1). Then, embryos were transferred to 15 μ l drops of KSOM previously equilibrated at 37°C and 5% CO₂ for > 3 h. Then, the NSET device was placed onto a Gilson pipetman P2 and set to 1.8 μ l and 13-18 blastocysts from the same embryo group were loaded. Afterwards, the volume was readjusted to 2.0 μ l and the pipette was left aside carefully. Subsequently, a 2.5 dpc pseudo-pregnant female was placed on the top of a cage with a wire rack, allowing the mouse to grab the cage in such a way the mouse could maintain a vertical position. The smaller speculum was placed into the mouse's vagina and then was replaced for the larger one. Then, the cervix was visualized and the NSET device was inserted into the cervix where embryos were released. NSET device was checked after embryo transfer and any existence of remaining embryos in the pipette were recorded.

Females were caged individually and pregnancy was visually checked on day 14 after embryo transfer. The signs of pregnancy were nice nest building and the presence of bulges on the mouse belly. Pups delivery was checked visually on daily basis from day 16-18 after embryo transfer.

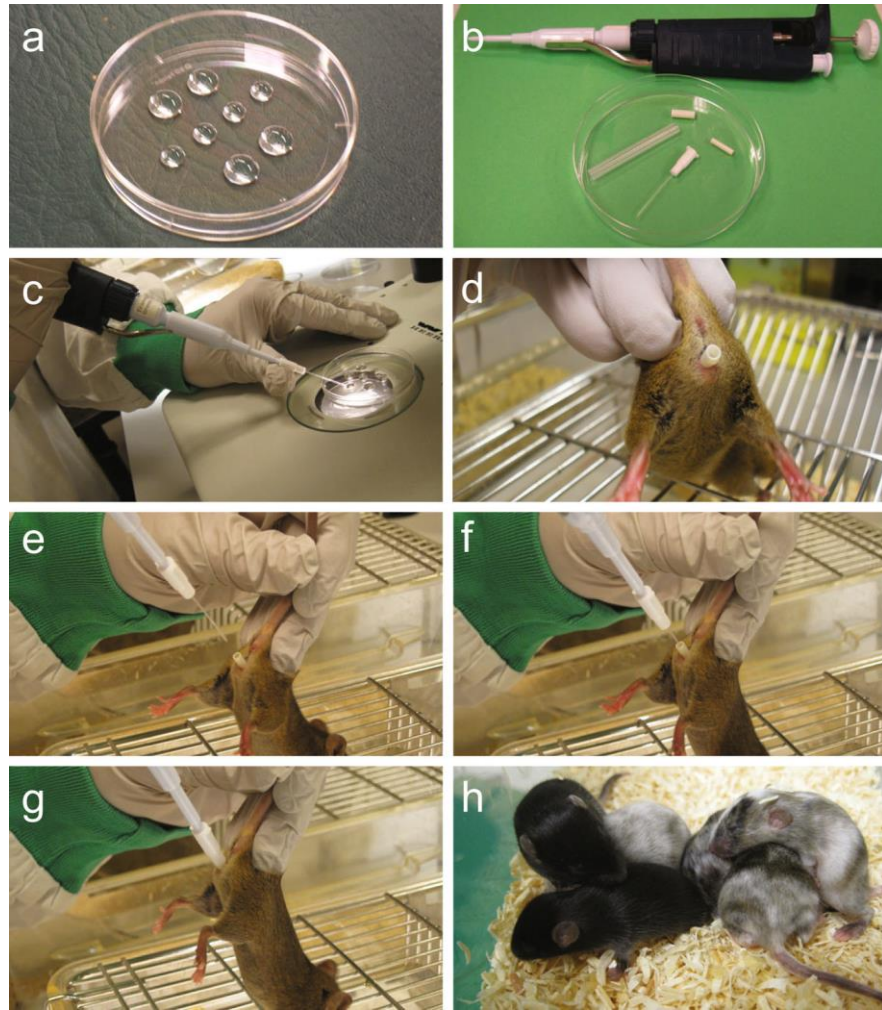


Figure 6.1 Non-surgical embryo transfer procedure using NSET device. a) KSOM drops for embryo rinsing before embryo transfer. b) NSET device with speculums and Gilson pipette P-2 μ l. c) Embryo loading into NSET. d) Speculum insertion into mouse vagina. e-g) Insertion of NSET device into mouse cervix and embryo transfer. h) Live pups after NSET. Figure extracted from (Bin Ali et al., 2014).

6.3.4. Offspring assessment

Live offspring rates and mean live birth rates were calculated as follows:

$$\text{Live offspring rate} = \frac{\text{number of live offspring}}{\text{number of transferred embryos}}$$

$$\text{Mean live birth rate} = \frac{\text{number of live births}}{\text{number of pregnant surrogates}}$$

Pups were weighed and sexed at 1 week after birth. Then, pups were culled 2 weeks after birth by cervical dislocation, weighed and dissected. Different organs (spleen, pancreas, kidneys, liver, lungs and heart) were dissected and weighed in pre-weighed 1.5 centrifuge tubes using a fine balance scale.

$$\text{Relative organ weight} = \frac{\text{weight of organ}}{\text{total body weight}}$$

Foster mothers were also sacrificed by cervical dislocation. Uteri were harvested and implantation sites were noted. Pregnancy loss rates were calculated as follows:

Pregnancy loss rate

$$= \frac{\text{number of implantation sites} - \text{number of live pups}}{\text{number of implantation sites}}$$

6.3.5. Statistical analysis

Data distribution was checked for normality by Shapiro-Wilk test and using SPSS v23 (IBM Software Services, Hamshire, UK). Statistical significance was set at $p < 0.05$ for all analyses. Blastocysts incidence rates within the different cell allocation patterns were compared using ANOVA test. Non parametric test, Kruskal-Wallis, was used to determine any differences among groups for pregnancy, pregnancy loss, mean live birth, live offspring rates as well as litter size comparison. Non-parametric test Kruskal-Wallis was used to determine any differences on relative organ weights among groups; when adequate, Mann Whitney U-test was used to compare groups against each other.

6.4. Results

6.4.1. Pre-implantation embryo development

A total of three repetitions of the experiment were performed, using 568 thawed one-cell embryos in total with $94.4\% \pm 0.6$ cleavage rate. The 536 obtained two-cell embryos were injected with the lipophilic tracer CM-Dil ($94.4\% \pm 0.6$). Overall, a blastocysts rate of $68.7\% \pm 2.3$ ($n = 394$) was achieved. A total of 323 blastocysts (82.1 ± 0.8) were successfully classified into orthogonal, deviant or random accordingly to their cell allocation pattern (Figure 6.2) with an incidence of $27.0\% \pm 4.7$, $28.3\% \pm 5.2$ and $52.3\% \pm 7.2$ respectively (Figure 6.3). Random blastocysts were significantly more predominant ($p = 0.037$ by one way ANOVA test). Expansion grades were similar between orthogonal ($n = 83$), deviant ($n = 85$) and random ($n = 157$) groups (Figure 6.4 and Figure 6.5) ($p = 0.717$ for grade ≤ 3 , $p = 0.917$ for grade 4 and $p = 0.734$ for grade 5 among groups by ANOVA test). The majority of the blastocysts were graded as grade 4; 57.8% for orthogonal, 60% for deviant and 57.3% for random groups. Smaller proportions of blastocysts grade 5 and ≤ 3 were found within the groups; 21.7% and 20.5% for orthogonal, 23.5% and 16.5% for deviant, 24.9% and 17.8% for random groups respectively.

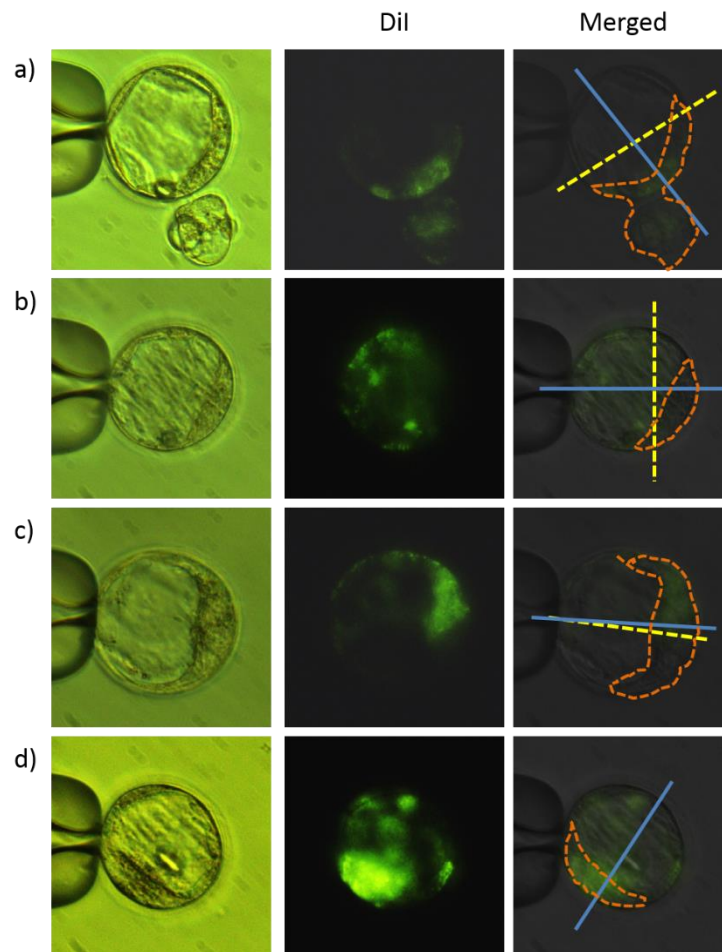


Figure 6.2 Mouse embryo cell allocation patterns at blastocyst stage. a) and b) Orthogonal embryos, c) Deviant embryo and d) Random embryo. Blue line represents Em-Ab axis, dotted yellow line represents the separation of labelled and non-labelled cells cluster and dotted orange line represents the ICM.

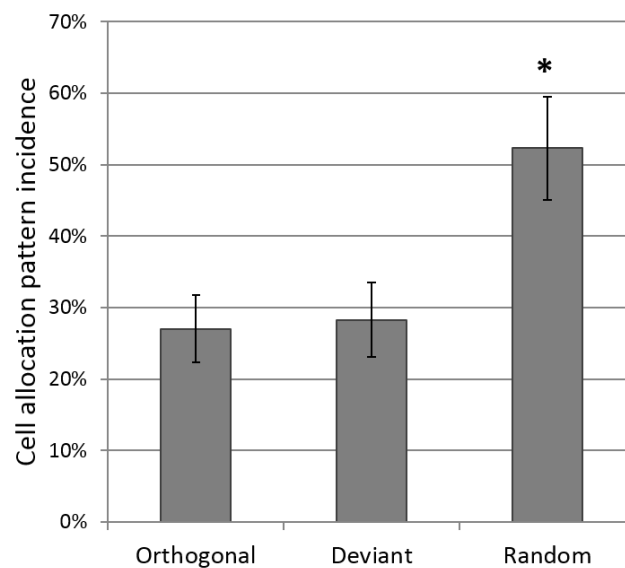


Figure 6.3 Cell allocation pattern incidence at blastocyst stage of thawed one-cell mouse embryos. *Denotes statistical significance by one-way ANOVA ($p < 0.05$).

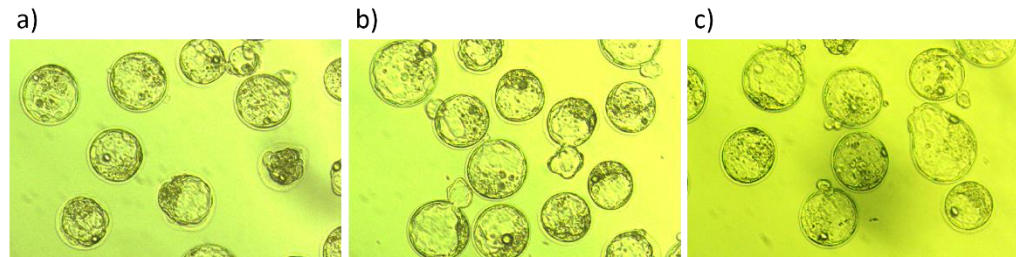


Figure 6.4 Blastocysts before non-surgical embryo transfer. a) Denotes orthogonal embryos, b) Deviant and c) Random.

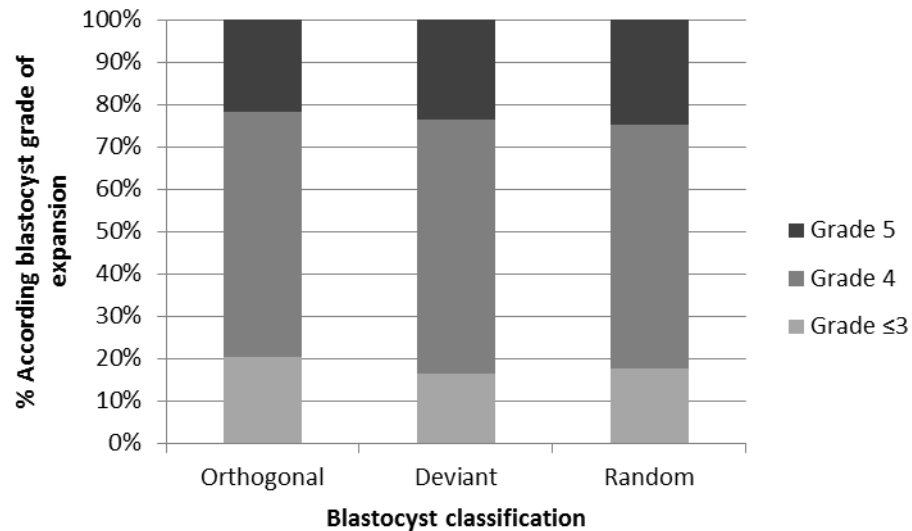


Figure 6.5 Blastocyst expansion grades before embryo transfer. Orthogonal (n=83), deviant (n=85) and random (n=157) blastocysts were classified into grade ≤ 3 if the blastocoel cavity was more than half of the blastocyst or if it was a full blastocyst; into grade 4 if the blastocyst was expanded, blastocoel cavity larger than the embryo and thinning of the zona pellucida was observed; grade 5 if the blastocyst was hatching out of the shell but not completely. No significance was found among groups by Chi Square test ($p > 0.05$).

6.4.2. Embryo transfer and live birth results

Seventeen 2.5 dpc pseudo-pregnant females were used to transfer a total of 275 blastocysts. Due to the higher incidence of embryos classified as random, more foster mothers were used in this group. Nevertheless, the transferred blastocyst number within the three repetitions of the experiment remained constant within the three groups (18, 13 and 16 blastocysts per female respectively). No significant differences were found between groups for

pregnancy rates, implantation rates, pregnancy loss rate, litter size and live offspring rates ($p > 0.05$ by Kruskal-Wallis test, Table 6.1). However, two pregnant females within the deviant group did not deliver live pups. One of them showed clear pregnancy around due date but pups were not seen at any point, while the other foster mother was seen with a dead pup. Cannibalism of the pups was assumed for both foster mothers. Live offspring can be observed on Figure 6.6a, with their foster mother (white fur coat).

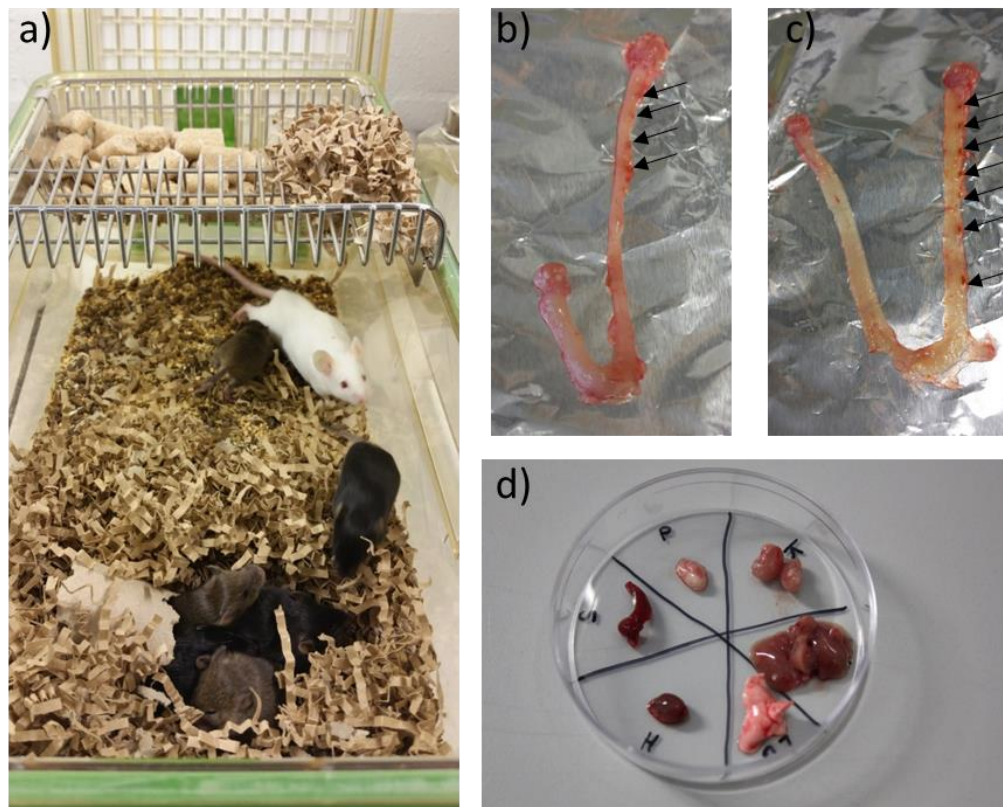


Figure 6.6 Live offspring analysis. **a)** Live offspring can be recognised for the agouti or black fur coat, while the foster mother has a white fur coat. **b)** Retrieved uterine horns of foster mother two weeks after giving birth where implantation sites can be observed (black arrows). **c)** Dissection of uterine horns for better visualisation of implantation sites (black arrows). Implantation sites were only present in one uterine horn. **d)** Offspring organ dissection, where p= pancreas, K= kidneys, L= liver, Lu= lungs, H= heart and S= spleen.

Table 6.1 Embryo transfer and live birth data. Total numbers and different rates (percentages) among groups are not significantly different ($p > 0.05$ by Kruskal-Wallis test).

Embryo group	Orthogonal	Deviant	Random
Transferred blastocysts	76	76	123
Number of foster mothers used	5	5	7
Pregnancy rate	66.6%	83.3%	100.0%
Implantation rate ⁺	42.3%	39.9%	50.7%
Pregnancy loss rate ⁺⁺	49.6%	79.1%	55.9%
Mean live birth rate ⁺⁺⁺	4	2	3.5
Litter size ($n \pm \text{SEM}$)	4 ± 1.5	4 ± 1.0	4.1 ± 0.9
Live pups (litters)	12 (3)	8 (2)	25 (6)
Gender ratio (males/females)	0.71 (5/7)	1.66 (5/3)	1.77 (16/9)
Live offspring rate ⁺⁺⁺⁺	14.7%	11.4%	22.9%

⁺Implantation sites/embryos transferred, ⁺⁺live pups-implantation sites/implantation sites, ⁺⁺⁺live pups/pregnant foster mother, ⁺⁺⁺⁺live pups/transferred embryos.

The median total body weights of offspring from transferred blastocysts can be seen in Table 6.2. Significant difference was found ($p = 0.028$) among groups when 7 days old pups weights were compared. Mann-Whitney U-test revealed that pups from deviant group were significantly lighter (5.60 ± 0.3 g) when compared with pups from random group ($p = 0.015$, 6.55 ± 0.1 g), but not when compared to orthogonal group ($p = 0.230$, 6.28 ± 0.3 g). Fourteen days old pups weights did not vary between groups where orthogonal, deviant and random pups weighted 11.10 ± 0.4 g, 9.77 ± 0.4 g and 10.8 ± 0.2 g respectively. The weight increase between the first and second week of life differed significantly between groups ($p = 0.037$). Furthermore, Mann-Whitney U-test showed that orthogonal group significantly gained more weight (4.81 ± 0.1 g) compared to the deviant group ($p = 0.010$, 4.17 ± 0.1 g) and random group ($p = 0.030$, 4.24 ± 0.1 g). It is worth noting that within the deviant group male pups ($n = 5$) were significantly ($p < 0.05$, Mann-Whitney U-test) heavier than female ($n = 3$) pups during the

first and second week measurements (6.2 ± 0.2 g, 10.4 ± 0.3 g and 4.6 ± 0.1 g, 8.56 ± 0.2 g respectively).

Table 6.2 Mice total body weight. Body weight of pups at 7 and 14 days after birth.

Group	Number of pups	Weight at 7 days post birth (g $\pm$ SEM)	Weight at 14 days post birth (g $\pm$ SEM)	Weight increase (g $\pm$ SEM)
Orthogonal	12	$6.28 \pm 0.3^{a,b}$	11.10 ± 0.4	4.81 ± 0.1^a
Deviant	8	$5.60 \pm 0.3^{*a}$	$9.77 \pm 0.4^*$	4.17 ± 0.1^b
Random	25	6.55 ± 0.1^b	10.80 ± 0.2	$4.24 \pm 0.1^{a,b}$
	P value	0.028	0.105	0.037

*Significance between female and male $p < 0.05$ by Kruskal-Wallis test.

Different subscripts denote $p < 0.05$ within the same column by Kruskal-Wallis test.

6.4.3. Organ morphometry

Relative organ weights from 2 weeks old mice showed no significant differences ($p > 0.05$) among groups for lungs, liver, pancreas and spleen (Figure 6.7a-d respectively). However, a significant difference was found between groups ($p < 0.05$) for heart and kidneys relative weights (Figure 6.7f-g). Pups within the deviant group presented significantly heavier relative heart weight when compared with orthogonal ($p = 0.025$) and random ($p = 0.036$) groups. The pups from the orthogonal group presented the lightest relative kidneys weight when compared with deviant ($p = 0.043$) and random ($p = 0.025$) groups. The Mann-Whitney U-test was applied to determine if gender has an effect on relative organ weights within each group. The results showed no significant difference between genders ($p > 0.05$).

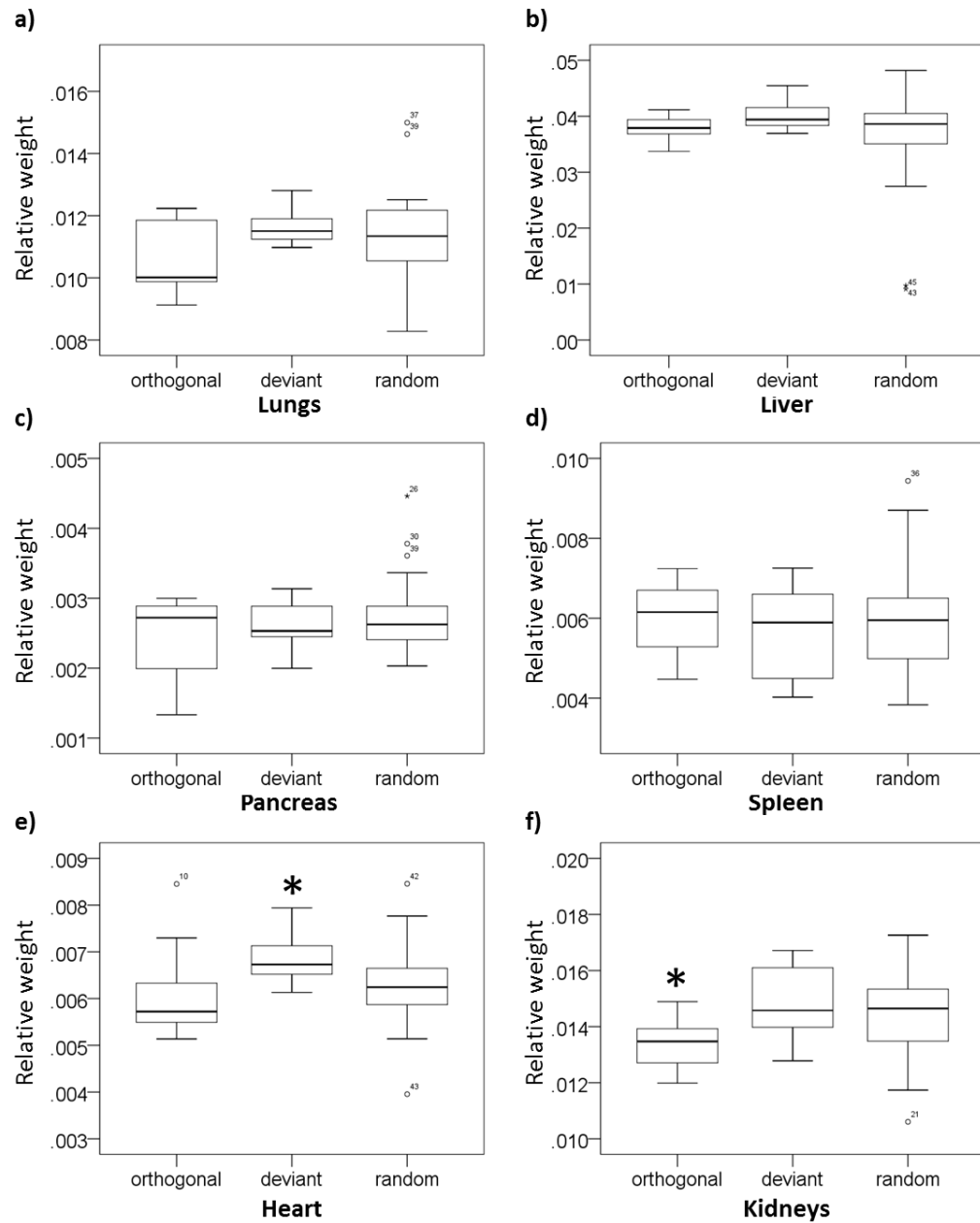


Figure 6.7 Boxplots comparing the relative organ weights from 2 week old mice. a) Lungs, b) Liver, c) Pancreas, d) Spleen, e) Heart and f) Kidneys. Relative organ weights were calculated dividing the organ weight by the total body weight. A total of 45 pups were analysed: orthogonal (n = 12), deviant (n = 8) and random (n = 25). No significant difference was found among groups for lungs, liver, pancreas and spleen. Heart and kidneys relative weights among groups showed difference by Kruskal Wallis-test ($p < 0.05$). *Denotes statistical difference ($p < 0.05$) by Mann-Whitney U-test.

6.5. Discussion

Based on the data from previous chapters 3 and 5, it was hypothesised that embryos classified according their cell allocation patterns during pre-implantation development might have different potential to implant and to develop further because of their differences on total cell count at blastocysts stage and epigenetic profile. Overall, the results of this chapter indicate that blastocysts classified as orthogonal, deviant or random have the same potential to implant and to develop into live offspring. However, the data suggest different effects of cell allocation patterns at blastocyst stage on the first two weeks after birth. This study provides further information about the effects of pre-implantation embryo development on post-natal organ development.

NSET presents the great advantage of animal stress diminution during and after the procedure when compared with the surgical one (Steele et al., 2013). It is a non-invasive procedure, it does not require recovery and it does not present fatality compared with the 3-4% of the surgical procedure (Bin Ali et al., 2014). The first successful non-surgical embryo transfers in mice were reported in 1974 (Marsk and Larsson, 1974). Pregnancy rates in the present study were comparable between groups and in agreement with previously reported studies using NSET device obtaining pregnancy rates higher than 60% (Cui et al., 2014). Even though there was no statistical difference between groups regarding pregnancy rates, it is worth noting that all

surrogate females with transferred random blastocysts became pregnant. A possible theory is that random embryos might produce more hyaluronan promoting cell movements, therefore if this hyaluronan is released to the endometrium, cell migration and implantation might be facilitated (Gardner, 2015).

It is not possible to establish the exact birth day as pups were kept in their nest and most of the times observation through the mice cages was not possible; however the eleven foster females gave birth between days 17 and 18 after ET. Due to the natural behaviour of mice, disturbance during the first days after birth was avoided as it has been reported that might cause cannibalism of the pups due to mother stress (Adams, 2007). In the present study two of the females, both on the deviant group, presumably committed cannibalism. One female was visibly pregnant with several pups on due date but live offspring were never seen, while the other female was seen with one dead pup. Possible explanations are problems at delivery or pups health problems that triggered the death of the pups (Wuensch, 1993). In the present study 13 to 18 blastocysts per female were transferred. It has been reported that embryo transfers higher than 15 blastocysts might result in higher numbers of non-pregnant females or difficulties at giving birth and caesarean sections have been advised (Bin Ali et al., 2014).

Autopsy of foster mothers revealed comparable embryo implantation rates between the three groups, where around 40% of the transferred embryos

implanted. Implantation rates are similar to those reported after surgical embryo transfer in mice (Hemkemeyer et al., 2014). To my knowledge there are only two studies reporting the implantation incidence according to the cell allocation patterns during pre-implantation development. Yet, in Alarcón and Marikawa (2003) and Liu et al. (2012) studies there is only two classifications of embryos, orthogonal and deviant; thus random embryos are assumed to be included within the deviant group. Similar results were obtained in the present study as no significant differences were found between groups regarding implantation rates. However, in SCNT embryos a significant difference between implantation rates of orthogonal and deviant embryos have been reported, where orthogonal embryos presented higher implantation rates (Liu et al., 2012).

Molecular pathways occurring during implantation and cross-talk between embryo and uterus are not clearly understood (Cha et al., 2012, Wang and Dey, 2006) and it is believed that some embryos do not survive embryo transfer procedure due to microenvironment changes and their inability to adapt (Hemkemeyer et al., 2014). Moreover, further research is needed in order to determine at which stage the implanted embryos were lost as pregnancy loss rates were > 50% among groups. In NSET, embryos are deposited only on one of the two uterine horns. The presented results showed that implantation sites were observed indeed only in one uterine horn, therefore it is suggested that embryos do not migrate to the other uterine horn as previously reported (Green et al., 2009). Embryo spacing

occurs in polyovular species (Chen et al., 2009a). In mice embryo spacing can be observed during day 4 (Restall and Bindon, 1971). Possible embryo overcrowding, aberrant embryo spacing or excessive intrauterine fluid might be one of the causes of pregnancy loss (Chen et al., 2011, Lu et al., 2013, Chen et al., 2013a). Better results have been reported when only 6-8 embryos (instead of 13-18) are transferred by NSET (Cui et al., 2014). Also, pregnancy loss might be due to aberrant decidualisation; giving rise to placental defects and therefore affecting foetal growth (Cha et al., 2012). Problems during pregnancy have been documented in mice, where ART embryos (IVC and IVF) presented abnormal placentation (Chen et al., 2015).

A main difference on the embryo transfer procedure performed in mice compared with the one performed in humans is that mouse embryos are at more advanced developmental stage (day 3.5) than the receiving endometrium (day 2.5). Previous studies have demonstrated that these advanced embryos are able to implant in mice (Ueda et al., 2003). A study reports comparable results between 3.5 dpc and 2.5 dpc foster mothers (Bin Ali et al., 2014). In the present study we used 3 surrogate mothers on 3.5 dpc and embryos on day 3.5 were transferred (data not shown), however no signs of pregnancy or live pups were detected. Ten implantation sites were observed in only one of the females.

Litter mean sizes, 4 pups, were akin among groups. Similar results have been reported after NSET (Cui et al., 2014) and surgical embryo transfer (Donjacour

et al., 2014). Litters after embryo transfer tend to be smaller than those after natural conception (Donjacour et al., 2014). These differences might be due to the embryo transfer procedure, superovulation protocol/and IVC. On the present study, live birth rates oscillated between 11-22% as previous reported studies (de Waal et al., 2014). Other studies in mice have obtained higher live offspring rates (> 26% up to 70%) (Marsk and Larsson, 1974, Green et al., 2009, Cui et al., 2014, Donjacour et al., 2014). Even though NSET technique is simple and straight forward, it has been reported that it might take some time in mastering the technique and to obtain similar results of those obtained after surgical embryo transfer (Cui et al., 2014). My limited training performing the NSETs might have contributed to the low live birth rates of the present study. Also, NSET was achieved usually after few attempts of device insertion into the mouse cervix and it has been reported that better results are obtained if NSET is introduced to the cervix on the first try, preventing cervix damage (Bin Ali et al., 2014). The use of anaesthetised foster mothers during embryo transfer, coupled with a mock embryo transfer previous embryo transfer has been suggested in order to avoid difficult embryo transfers (Cui et al., 2014). Overall, absence of significance between groups might be due to the small sample size and further repetitions of the experiment might show if the tendency observed in these results reach significant difference.

Total body weights at 7 days after birth were different among groups, where the pups from the deviant group presented the lowest weight. While some

studies have reported low birth weight after ART when compared with natural conceived babies (Ceelen et al., 2009, Bay et al., 2014), others have reported only a tendency with no statistical difference (Pontesilli et al., 2015). In humans, it has been suggested that low birth weight can be an important risk factor for cardiovascular disease later on in life (Huxley et al., 2007). Low birth weight has been related with foetal growth restriction due to possible malfunctioning of the placenta (Barker and Thornburg, 2013). Increased placental weights have been reported after ART (Koudstaal et al., 2000), which in turn has been related with an increased risk of fatal cardiovascular disease (Risnes et al., 2009). In mice, low birth weight has been linked to metabolic syndromes as type 2 diabetes, obesity and hypertension in adults (Chen et al., 2015). The present study had the purpose of observing any variation between the different classified embryos (orthogonal, deviant and random) after birth, thus having a control group would not be possible as to my knowledge there is no available technology to study embryo cell allocation patterns *in-vivo*. Nevertheless, future investigations should provide a control group in order to have a reference point of normal parameters as even though the present study reports a lower weight on the deviant group, it is still unknown if this weight is above the expected one.

Fourteen days after birth pups among the three groups presented similar total body weight. In humans it has been reported that babies born with low weight “catch-up” within the first months of life (Ceelen et al., 2009, Barker and Thornburg, 2013). On the other hand, in the present study it was

observed that the weight gain of the orthogonal group was significantly higher compared with the deviant group. Rapid weight gain during early age has been correlated with high blood pressure problems later on in childhood (Ceelen et al., 2009).

Organ morphometry assessment revealed significant differences among groups for relative weights of heart and kidneys. It is poorly understood why kidneys, blood vessels and the heart are the most affected by early life events (Thornburg, 2015). Relative heart weights within the deviant group were higher when compared with orthogonal and random groups. It has been suggested that in humans, the increase of heart weight is related to hyperfunction of the myocardium (Vlasov and Volkov, 2004). Literature reports no significant differences when compared heart weights of mouse pups after natural fertilisation or IVF (Donjacour et al., 2014). In Donjacour and colleagues' study there is a shown tendency of higher heart weight within the IVF group and there is a significant difference on the systolic blood pressure and ventricular mass, among other parameters of the cardiovascular phenotype. Therefore, the presented results on deviant pups might reflect further consequences on this cardiovascular phenotype and additional investigations on cardiovascular phenotype might shed some light on how embryo pre-implantation development influences the cardiovascular system. It has been suggested that pre-implantation development has an effect even during foetal life. Alteration of the epigenome during pre-implantation development has been suggested as possible mechanism for the negative

long-term effects of ART offspring (Donjacour et al., 2014). It is believed that early embryonic and late foetal lives are the most susceptible stages for heart development (Thornburg, 2015), therefore epigenetic changes at these stages might have a crucial impact on heart development later on in life. Correspondingly, increase of phospholipid levels in ART foetus in mice have been reported, suggesting that this increase might contribute to cardiovascular or metabolic disorders later in life (Li et al., 2016).

Among the long-term effects of ART, cardiovascular problems are the most concerning health issue as ischaemic heart disease is the main cause of death worldwide; contributing to more than 37% of premature deaths by 2012 (WHO, 2016). In 2013 in England and Wales, ischaemic heart disease was the major cause of death on males and the major second in females among people over 50 (ONS, 2015) and respiratory and cardiovascular disorders were the leading cause of infant deaths in 2012 (ONS, 2014).

An increased symptomatology reflecting cardiovascular problems such as higher blood pressure, higher triglyceride and glucose levels, have been reported on children born after ART when compared with naturally conceived children (Pontesilli et al., 2015). Not only symptomatology has been reported, also congenital heart problems. An extensive review on cardiovascular affections after ART can be seen on Padhee et al. (2015), where 16 studies across the world have reported an increased risk of different congenital heart defects among ART children compared with natural conceived ones. Some of

the reported congenital heart defects are: ventricular defects, atrial septal defect, aortic valve atresia, cardiac neural crest defects, single umbilical artery and arterial trunk. Increased cardiovascular problems have also been reported during foetal life (Reviewed on (Padhee et al., 2015)). The foetal origins hypothesis proposes that different diseases as hypertension, heart stroke, coronary heart disease and type 2 diabetes originate before birth and during infancy (Barker, 2004).

On the other hand, no differences were found between male and female pups for orthogonal and random groups. Similar results have been obtained in *in-vitro* produced mouse embryos (Donjacour et al., 2014). It is worth noting that body weights between female and male pups were significantly different only among the deviant group. Gender differences have been reported for babies born after unassisted or assisted conception (O'Neill et al., 2014). Further investigations on the long-term health between females and males must be addressed as it has been suggested that mouse male offspring might be more at risk of developing glucose intolerance after ART (Donjacour et al., 2014). Differences between genders have been reported as well in humans where girls seem to require higher energy intake than boys (reviewed on (Pontesilli et al., 2015)). It has been advised that steroid hormone anabolic effects might be diverted due to cardiovascular diseases which therefore might affect males or females in different ways (Vlasov and Volkov, 2004). Even though during the present study no differences were found between groups regarding gender skewing, there was a tendency for orthogonal group

to have more females than males and for deviant and random groups to have more males than females. Further investigations are needed in order to determine if gender is related to cell allocation patterns.

This study raises some questions about the cardiovascular system and kidneys development during foetal development and how this might be affected or predisposed since early pre-implantation embryo stages. As mentioned previously, one of the major limitations of this study was the small number of embryo transfers performed. Thus, although the results showed some evidence of effects of pre-implantation cell behaviour on subsequent development, further studies are needed to reassure the presented data. Likewise, this work was carried out in mice and caution should be taken when translating the reported results into humans. Since the main objective of the present chapter was to establish if there is any difference on the developmental potential of blastocysts with different cell allocation patterns, further investigation is required to confirm the reproducibility and consistency of the obtained results. Potential future work could include foetal organ function assessment, placental function, heartbeat and blood pressure monitoring after birth and long-term general health assessment. As well, the number of embryo transfers should be increased to maximise the number of offspring assessed and reach statistical power analysis.

6.6. Conclusions

The presented findings suggest that cell allocation patterns during pre-implantation development can alter further development, which could result in short and long term consequences for foetus or offspring born after embryo transfer. A latent concern has been rising about the effects of ART in further offspring health, especially long-term effects. With the imminent yearly increase of babies born after ART, it is of great importance to be able to identify the group of ART embryos (before embryo transfer) that could be shifting the tendency towards some diseases. A better knowledge on the influence of pre-implantation development in further development is needed not only to improve success rates on ART, but also to ensure the health of the offspring.

7. CHAPTER SEVEN: GENERAL DISCUSSION AND CONCLUSIONS

Based on the literature, there is no consensus about the existence or absence of pre-patterning among mammalian embryos. In this current thesis it was hypothesised that cells within the mammalian embryos were either pre-patterned or stochastically allocated within the embryo through pre-implantation development. As a result, the objective was to investigate cell allocation patterns from the two-cell stage up to the blastocysts stage in mammalian embryos. In addition, the possible perturbation of cell allocation patterns by the most common assisted reproductive technologies was explored as well as the epigenetic profile of the embryos following different patterns. Finally, the correlation between cell allocation patterns and further embryo development was assessed; especially organ morphometry after birth. The main findings of the present work are summarised in Figure 7.1 and as follows:

1. There is a comparable incidence of the three identified cell allocation patterns (orthogonal, deviant and random) on mammalian species, mouse and bovine. These results suggest that cell behaviour during pre-implantation embryo development could be a preserved mechanism among mammals. This mechanism is stochastic but also pre-determined, pointing out that there are some vestiges of developmental mechanisms from lower species (Bruce and Zernicka-Goetz, 2010)(Chapter 3).

2. The effects of superovulation protocol, age of oocyte donor, *in-vivo* fertilisation, *in-vitro* fertilisation or mammalian species do not seem to have a major effect on cell allocation patterns during pre-implantation development. Thus, suggesting that oocyte components might be responsible for cell allocation patterns (Chapter 3).
3. Cell allocation patterns seem to be related with total cell count at the blastocyst stage at least in bovine embryos and possibly also related with gender skewing (Chapter 3).
4. Blastomere removal around the eight-cell stage does not disturb cell allocation patterns during pre-implantation development on mouse and bovine embryos. However, biopsy procedure seems to affect only “pre-patterned” embryos (orthogonal and deviant) in mouse embryos; where orthogonal embryos present a delay on further cell cleavages and deviant embryos present a decrease on total cell count at blastocyst stage (Chapter 4).
5. Totipotency of the twin blastomeres of the two-cell stage mouse embryos remains debatable as not all bisected embryos developed into blastocysts. Where both blastomeres developed into blastocysts, they showed the random cell allocation pattern (Chapter 4).

6. embryos presenting the random pattern are more balanced or homogeneous, thus present the same chances of develop into a healthy offspring.
7. Embryos displaying different cell allocation patterns during pre-implantation show different epigenetic profiles in fertilised and parthenogenetic activated mouse embryos. Embryos following the orthogonal pattern present higher H3K9ac levels at blastocyst stage (Chapter 5).
8. There is an asymmetry on histone epigenetic marks on the blastomeres of the two- and four-cell mouse embryo. This asymmetry is decreased by TSA treatment, resembling more to epigenetic asymmetry shown on normal fertilised embryos (Chapter 5).
9. Cell allocation patterns might hide different mechanisms for further organ development. Two weeks old pups from the deviant group presented bigger hearts and lowest birth weights, indicating there is a latent risk of developing cardiovascular diseases later on in life (Chapter6).

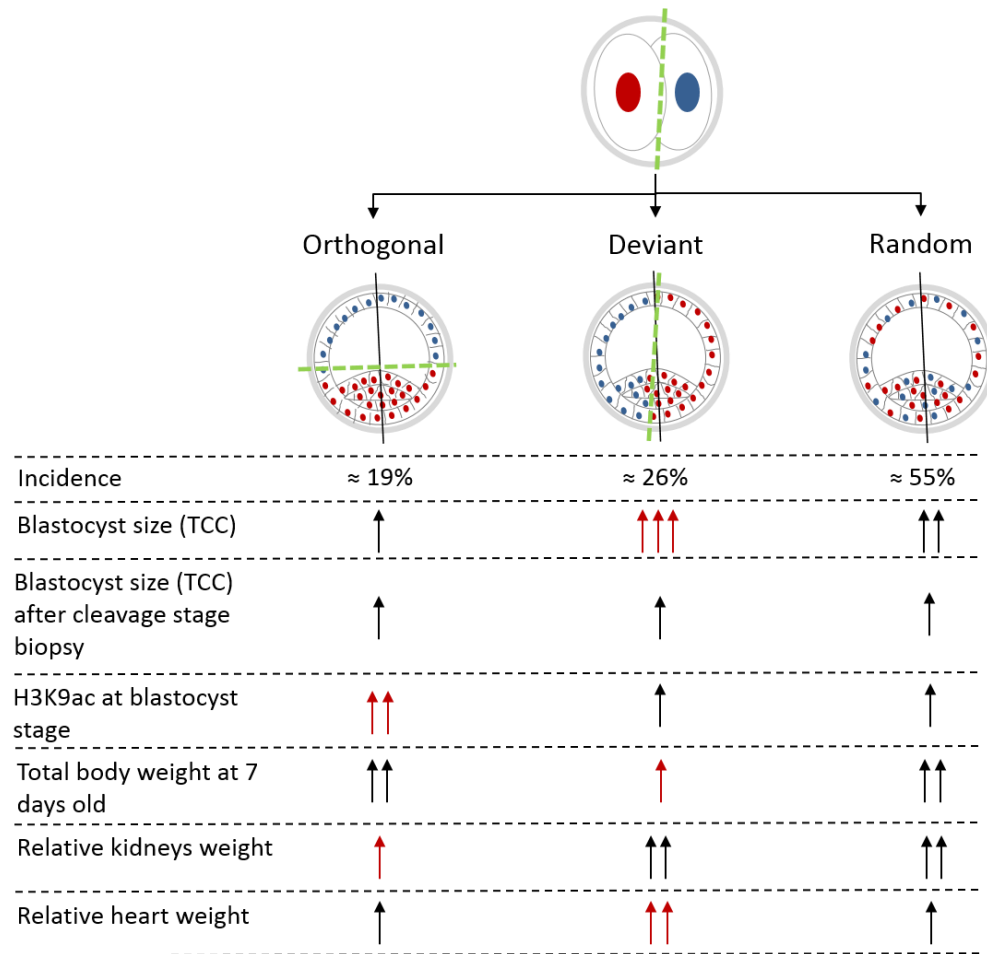


Figure 7.1 Differences between mammalian embryos following different cell allocation patterns. First cleavage plane can be related to the embryonic-abembryonic axis in approximately 45% of the mammalian embryos (orthogonal and deviant embryos); thus descendants of one blastomere of the two-cell embryo will give rise to a specific portion of the embryo at the blastocyst stage. The 55% of the remaining embryos present stochastic distribution of daughter cells of the blastomeres of the two-cell embryo. Arrows indicate quantitative relationship between embryos, where red arrows indicate statistical significant difference. In general, embryos following the deviant pattern present higher numbers of total cell counts at blastocysts stage. However, these numbers are significantly reduced after embryo biopsy at the eight-cell stage. Furthermore, orthogonal blastocysts present higher histone 3 lysine 9 acetylation levels. These epigenetic differences could have further consequences on development as low kidneys relative weight after birth. In mice, deviant embryos are usually lighter up to 7 days after birth and also presented heavier relative heart weights 14 days after birth.

It is difficult to draw a decisive conclusion on the existence of pre-patterning in mammalian embryos; however the present thesis and certain reports indirectly support the idea that it is difficult to affect pre-patterning (if exists) with manipulations at cleavage stage. For example, it has been reported that

injection of siRNA injection targeting SOX2 on bovine zygotes reduces blastocyst rates (Goissis and Cibelli, 2014). In turn, siRNA injection on only one blastomere of the two-cell bovine embryo does not affect ICM composition at blastocyst stage as expected; hence it might be the case that ICM composition is determined during the first cell cleavage (or before fertilisation) and not affected by further manipulation. This can be related to the results obtained throughout the present thesis as on the different experiments performed, the distribution of cell allocation patterns did not change significantly. Evolution might have provided plasticity to mammalian embryos, yet there must be a core mechanism standing to ensure proper development and cell specification; otherwise ultimately chaos might be the pathway into proper development.

It could not be ensured that oocyte components are the only ones driving cell allocation patterns during pre-implantation. It has been reported that the use of superovulation protocols and advanced maternal age have an effect on oocyte quality, therefore oocyte quality should affect embryo development if pre-patterning occurs in mammals. As no change on the incidence of the embryo cell allocation patterns was observed using *in-vitro* oocyte maturation, superovulation protocols or old females; it suggests that cell allocation patterns might not be related to oocyte/embryo quality. It is worth noting that aged female mice used in the present thesis might not be old enough to present diminished fertility. Plus in order to mimic the procedure in humans, further studies using *in-vitro* fertilised oocytes from old females is

recommended instead of natural fertilisation. Similarly, the use of transgenic mouse lines with deficient oocyte quality or mitochondrial diseases, coupled with cell tracing methods might elucidate the role of oocyte on cell allocation patterns during embryo pre-implantation development.

Remarkably, the literature reports that apart from the intrinsic factors within the early embryo blastomeres of ascidians, sea urchin and *C. elegans*, cell interactions seem to have a role on cell fate (Arias et al., 2013). Thus, it seems to be a close communication and shared fate between neighbour cells. Mammalian embryo development might resemble more than it is thought to these mechanisms in lower species. In the present study it was observed that embryos following different cell allocation patterns slightly differ from one another (Figure 7.1). It can be assumed that cell contact is different among embryos, as random embryos seem to have more dynamic cell movements after the third cell cycle when compared with orthogonal and deviant embryos. Study of cell contacts, like cell-cell interactions through catenins, might shed some light in why cells intermingle in only half of the total embryo cohort.

It was interesting that during the present study, deviant embryos seem to be the biggest and orthogonal the smallest blastocysts among groups. Usually bigger embryos are preferred for embryo transfer on a clinical setup. Thus, deviant embryos have been potentially selected over orthogonal and random embryos. Also, deviant and random embryos seem to present a gender skew

favouring the males, opposite to orthogonal embryos which tend to present a skew over females. It has been suggested that fast developing embryos, hence bigger embryos, tend to be male embryos in different mammalian species (Alfarawati et al., 2011, Ménéz et al., 1999, Valdivia et al., 1993, Avery et al., 1992, Xenopoulos et al., 2012, Bernardi and Delouis, 1996).

The present study has shown supportive evidence to suggest that embryos following different cell allocation patterns during pre-implantation might develop differently further on in life. In Chapter 6, embryos following the deviant pattern presented significantly bigger hearts two weeks after birth. As the risk of developing a cardiovascular disease is increased in babies born after ART and with cardiovascular diseases being the most common cause of death worldwide, it is important to investigate the correlation between cell allocation patterns and heart development further. If increased risk of cardiovascular disease development in the deviant group is confirmed, transfer of deviant embryos should be prevented in the future.

The use of animal models to study the consequences of the different ARTs, excluding the background of the parents is a key on the study of the ART effects. Different reported studies in humans have not been conclusive due to the great variety of results confounders as different IVF techniques, different biopsy techniques, parental infertility diagnosis, parental education level, etc. Hence, further experiments in animal models involving ARTs followed by

embryo transfer and long-term follow up should take place in order to ensure the safety of the these procedures.

Different experiments in mouse embryos have proven the great plasticity of the mammalian embryo; where chimeras made from mixing early blastomeres from different embryos or even addition of parthenogenetic embryonic stem cells into embryos complete full embryo development (Chen et al., 2009b, Huang et al., 2008). The possibility that embryos following different cell allocation patterns might have different coping mechanisms (or grade of plasticity) after manipulations or perturbations, indicates that cellular machinery is plastic and adaptable. In the present thesis (Chapter 4), it was noticed that embryos following the deviant pattern presented detrimental effects after embryo manipulation. Deviant embryos presented a significant decrease on total cell count after embryo biopsy. Thus, these results suggest that coping mechanisms of deviant embryos against blastomere removal might be different from those on orthogonal and random embryos. Thus, embryo plasticity does not mean that pre-pattern development does not exist. This might only mean that the mammalian embryo presents a greater grade of plasticity against certain modifications, probably due to the multilineage priming in cells of the early embryo (Arias et al., 2013).

Kojima and colleagues proposed two different clockwork that initiate cell differentiation, one is related with epigenetic regulation and the other one with cell numbers. A harmonic cross-talk communication between cells

throughout embryo development might be driving embryo development (Kojima et al., 2014). In the present study cell allocation patterns were correlated with blastocyst size and histone modifications. Thus, it might be a connection between the mentioned clockworks (Figure 7.2). Cell allocation patterns might not be strictly correlated with embryo quality but with predisposition to certain developmental mechanisms. If epigenetic differences can be seen among patterned blastocysts, it is hypothesized that these differences are present at earlier stages, probably on a minor scale.

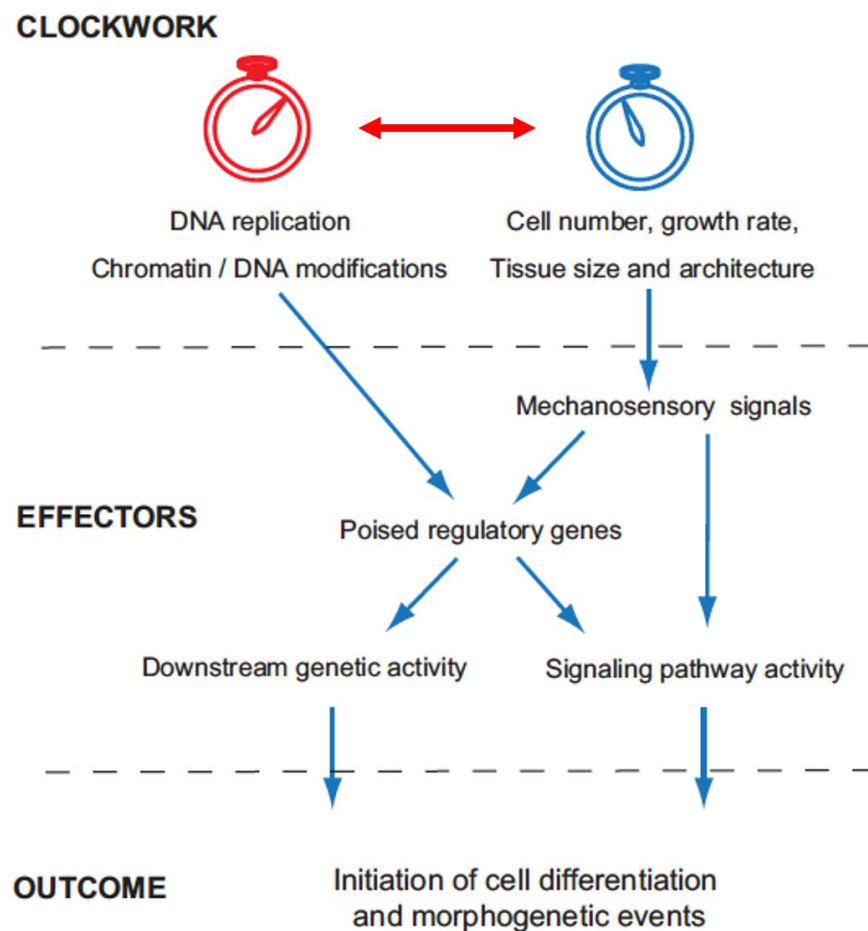


Figure 7.2 Diagram of possible mechanisms controlling cell differentiation. Two related clockworks might be responsible for the initiation of cell differentiation triggering genetic and morphological events. Figure adapted from (Kojima et al., 2014).

Post-translational epigenetic marks as H3K9me3 (histone 3 lysine 9 trimethylated) and H3K27me3 (histone 3 lysine 27 trimethylated) have been related to the first cell lineage commitment in mouse and bovine embryos at late morula stage and early blastocyst stage (Paul and Knott, 2013, Takaoka and Hamada, 2012). It might be possible that post-translational epigenetic marks are involved in cell lineage commitment during the first two embryo cleavages and these histone modifications are carried on. The slightest histone modification asymmetries within blastomeres could be triggering cell lineage specification in mammalian embryos.

As mention in Chapters 4 and 5, different epigenetic profiles among blastomeres within the two- and four-cell embryos sets the question of what mechanisms this epigenetic differences can trigger and how fates might be affected/changed. TSA treatment in parthenotes mouse embryos reduced the epigenetic asymmetry between blastomeres, suggesting that a reduced asymmetry between blastomeres might be better for further embryo development. It is worth noting that, as the same tendency was observed in four-cell stage fertilised (Sepulveda-Rincon et al., 2016a) and parthenogenetic embryos; it might be suggested that fertilisation is not causing this epigenetic asymmetry. Even though during the present study these epigenetic asymmetries (at two- and four-cell stage) were not related with cell allocation patterns during pre-implantation development, significant differences were found on histone modifications between different types of blastocysts.

Orthogonal blastocysts presented the highest levels of H3K9ac in fertilised and parthenogenetically activated mouse embryos.

Different gene expression profiles in TE cells of implanted and non-implanted embryos have been previously reported (Kirkegaard et al., 2015). If HDACs activity must increase during TE specification (Zhang et al., 2013a), then it might be worth measuring histone methylation levels in embryos following different cell allocation patterns in order to determine if TE specification occurs later in *in-vitro* embryos if compared with *in-vivo* embryos. This might be the answer why embryos do not implant. It might be a bad “timing” between embryo/uterine communication/processes. Most of the problems in bovine embryo transfers are related with placentation; maybe in *in-vitro* embryos an increased speed in the TE specification process is detrimental for embryo development. On one side, this thesis suggests that TE specification might be delayed in orthogonal blastocysts while TE proliferation might be accelerated in deviant embryos; therefore triggering a fast and uncontrolled development of the TE tissues. As it has been discussed previously in Chapter 6, this uncontrolled TE development can be translated into bigger placentas; thus, it can represent a higher risk of developing different diseases further on in life. Also, further correlation between cell allocation patterns and reduced foetal/placental weight ratio seems promising as it has been related with increased risk of mortality through cardiovascular disease in humans (Risnes et al., 2009).

Despite several attempts, it has not been possible to strongly correlate embryo morphology with embryo quality and genetic content in human embryos (Kirkegaard et al., 2015). An increasing number of studies are supporting that “ugly” embryos should not be discarded as they have potential in developing into a healthy offspring. Therefore, blastocysts with low quality or from low quality cleavage stage embryos should not be discarded in fertility cycles (Chen and Yu, 2012).

Pre-implantation embryo development is a crucial stage that will have a direct impact on the further embryo survival (Huang et al., 2015). A better understanding on totipotency loss during pre-implantation development would help identify (and control) differentiation pathways which could represent a great advantage for stem cell therapy (Rossant, 2015). It would be interesting to investigate further the potential to obtain stem cell lines from the different classified embryos and see if there is a link between cell allocation patterns and pluripotency.

All things considered, mammalian pre-implantation development might be a combination of extrinsic and intrinsic signals (Dietrich and Hiiragi, 2007) where embryos presenting the random pattern might be the embryos with higher survival odds. Thus, cell displacements and movements during pre-implantation could be the key for proper embryo development. It might be the case that cell heterogeneity in embryos following the random pattern presents the best potential to develop into healthy offspring. Further studies

on cell-to-cell communication are advised, especially after the 3rd cell cycle that it is when cell intermingling occurs on embryos following the random pattern.

Due to ethical issues, cell allocation patterns have not been studied in human embryos. It would be interesting to investigate if the incidence of these patterns is similar in higher primates or human embryos. As it is speculated that oocyte fertilisation/activation does not play a major role on cell allocation patterns, discharged/donated human oocytes might be activated parthenogenetically for this purpose. Specific methodology to ensure embryo viability and good health after implantation is still a priority for scientists and clinicians (Perkel et al., 2015). Caution should be taken when translating the reported results into humans as cell tracing is not available for clinical use; therefore a live-imaging system coupled with optical cell tracing must be developed.

Development of better indicators for embryo quality and more controlled follow-up studies of babies born after ARTs are urgently needed in order to give more informed options to patients (Kamphuis et al., 2014). Meanwhile, whole safety of ARTs should not be assured as extensive studies on procedure safety have not been conducted (LaBonte, 2012). The present study highly recommends the development of quantitative embryo imaging and modelling coupled with characterization of molecular events during embryo pre-implantation development. Consequences of differences in epigenetic

marks might be ambiguous and hard to determine; however it might be the start of different key mechanisms for further development. Thus, further research on different mammalian species on cell asymmetries within the early mammalian embryo could be useful to have a better understanding. Overall, DNA epigenetic changes inherited or caused by *in-vitro* manipulation might not be reversible (Tarin et al., 2014), yet a better understanding of these changes will allow a better approach to counteract them.

7.1. General Conclusions

In conclusion, cell allocation patterns during pre-implantation embryo development are potentially conserved among mammals, or at least among mouse and bovine embryos. As these patterns are not affected by maternal age, oocyte production, oocyte fertilisation/activation method or cleavage-stage biopsy, it is suggested that cell allocation patterns must be established at very early stages, probably before oocyte activation. The great importance of these cell allocation patterns relies on the fact that embryos following different patterns present different epigenetic profiles and also different coping mechanisms after *in-vitro* manipulations like single blastomere removal at cleavage stage. Most importantly, embryos presenting the deviant pattern could be at risk of developing cardiovascular diseases after birth (and possible as well during embryo development). Therefore, future work is required to understand the basis of the mechanism(s) driving or driven by cell allocation patterns in order to being able to identify those embryos at risk prior embryo transfer.

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APPENDICES

I. Mouse IVF media preparation

The supplier for all the reagents was Sigma Aldrich otherwise stated.

a) Sperm pre-incubation medium

Reagent name	Reagent formula	mg/100ml	Cat. number
Sodium chloride	NaCl	697.6	S5886
Potassium chloride	KCl	35.6	P5405
Magnesium sulphate heptahydrate	MgSO ₄ ·7H ₂ O	29.3	M7774
Monopotassium phosphate	KH ₂ PO ₄	16.2	P5655
Sodium bicarbonate	NaHCO ₃	210.6	S5761
Sodium pyruvate	Na-pyruvate	5.5	P4562
Glucose	C ₆ H ₁₂ O ₆	100.0	G6152
Calcium chloride dihydrate	CaCl ₂ ·2H ₂ O	25.1	C7902
Methyl-β-cyclodextrin		98.3	C4555
Penicillin G		7.5	P4687
Streptomycin		5.0	S1277
Polyvinylalcohol		100	P8136

Medium osmolarity should be between 293-320 mOsm/kg with a pH around 7.8 before equilibration at 37°C. The solution should be filtered through a 0.22 µm filter and store in 1 ml aliquots at +4°C for up to three months.

b) High calcium HTF medium

Reagent name	Reagent formula	mg/100ml	Cat. Number
Sodium chloride	NaCl	593.8	S5886
Potassium chloride	KCl	35.0	P5405
Magnesium sulphate heptahydrate	MgSO ₄ ·7H ₂ O	4.9	M7774
Monopotassium phosphate	KH ₂ PO ₄	5.4	P5655
Sodium bicarbonate	NaHCO ₃	210.0	S5761
Glucose	C ₆ H ₁₂ O ₆	50.0	G6152
Sodium lactate	Na-lactate (ml)*	0.34	L7900
Sodium pyruvate	Na-pyruvate	3.7	P4562
Penicillin G		7.5	P4687
Streptomycin		5.0	S1277
Albumin Bovine Serum (BSA), fraction V, fatty acid-free**		400	126575
0.5% Phenol Red (ml)*		0.04	P0290
Calcium chloride***	CaCl ₂ ·2H ₂ O	75.5	C7902

*Indicates volume of reagent. **From Merck supplier. ***There is a tendency for the calcium chloride to precipitate if dissolved in conjunction with the other components of the HTS. Dissolve the calcium chloride in a separate solution and then combined them before being filtered through a 0.22µm filter.

This medium is stored in 10 ml aliquots at +4°C for up to three months.

II. Time-lapse embryo imaging

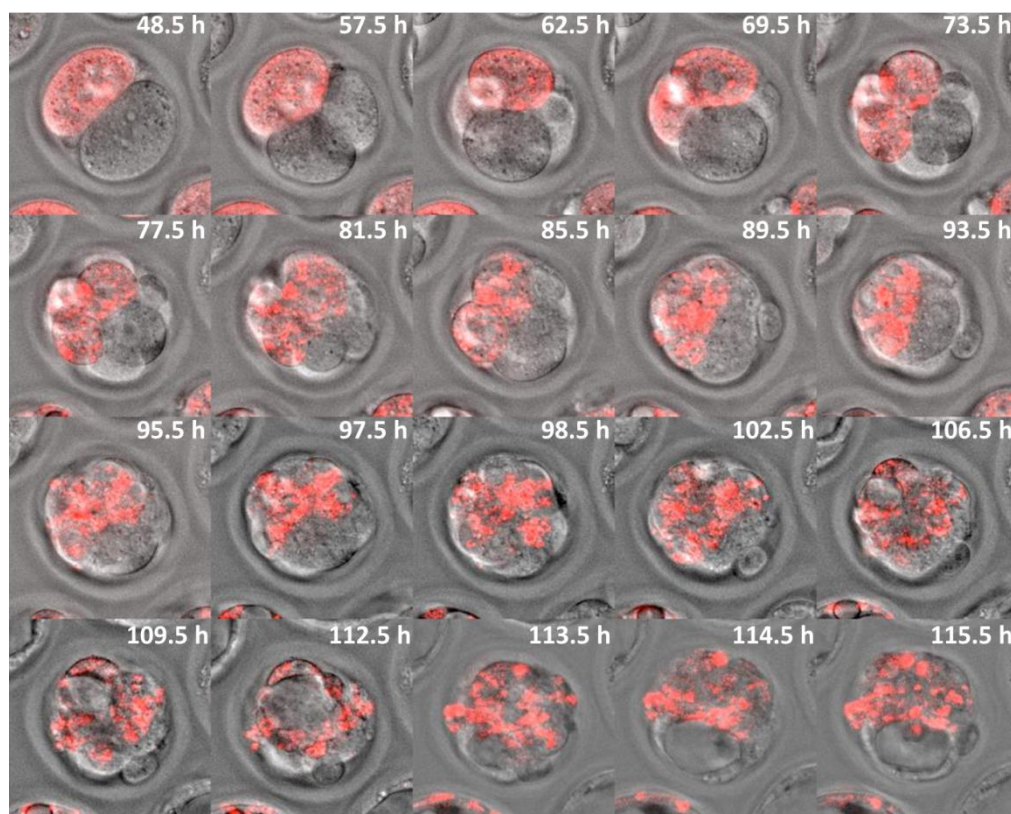


Figure 7.3. Time-lapse of mouse embryo following the orthogonal cell allocation pattern. Mouse embryo was injected on one blastomere with the lipophilic tracer Dil (red) at the two-cell stage. The embryo was observed every 15 min from the two-cell stage up to the blastocyst stage (hours are related to hours post hCG injection). As it can be seen on the picture sequence, daughter cells of the injected blastomere remained together throughout development. At the end of the sequence (115.5 h), the embryo presents the orthogonal pattern.

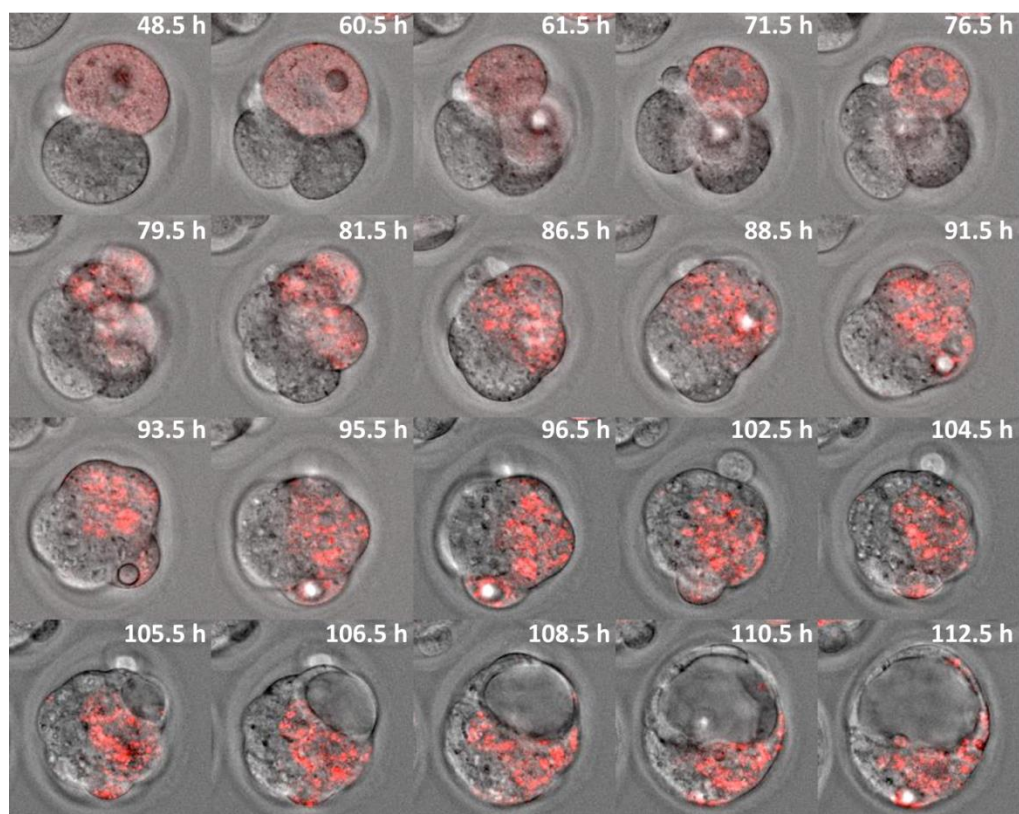


Figure 7.4. Time-lapse of mouse embryo following the deviant cell allocation pattern. Mouse embryo was injected on one blastomere with the lipophilic tracer Dil (red) at the two-cell stage. The embryo was observed every 15 min from the two-cell stage up to the blastocyst stage (hours are related to hours post hCG injection). As it can be seen on the picture sequence, daughter cells of the injected blastomere remained together throughout development. At the end of the sequence (112.5h), the embryo presents the deviant pattern.

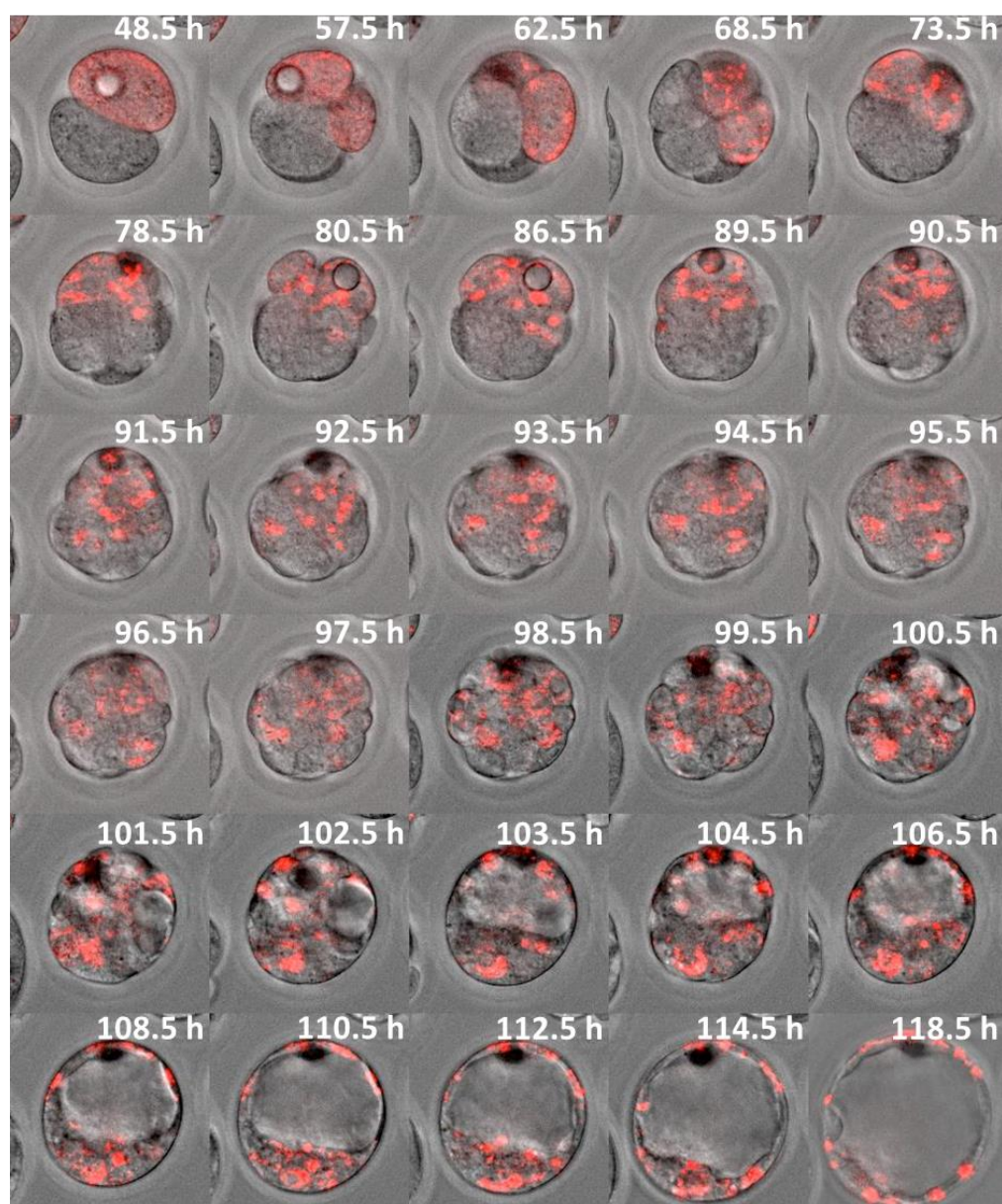


Figure 7.5. Time-lapse of mouse embryo following the random cell allocation pattern. Mouse embryo was injected on one blastomere with the lipophilic tracer Dil (red) at the two-cell stage. The embryo was observed every 15 min from the two-cell stage up to the blastocyst stage (hours are related to hours post hCG injection). As it can be seen from the sequence, daughter cells of one blastomere of the two-cell embryo are intermingled with the daughter cells of the other blastomere of the two-cell embryo. At the end of the sequence (118.5h), the embryo presents the random pattern.

III. Embryo twinning in mammalian species

Species	Embryo stage at bisection	Outcome	Reference
Ovine	Two-cell stage	80% of the couplets developed at similar rates up to blastocyst stage.	(Willadsen, 1979)
	Four- and eight-cell stage	Single blastomeres from four- and eight-cell stage biopsied embryos with ZP donors were transferred into recipients. Live monozygotic lambs were born in both groups.	(Willadsen, 1981)
Bovine	Four-cell stage	Blastomeres of the four-cell stage embryos were cultured with ZP donors and transferred into recipients. Three out of four recipients were pregnant of twin calves.	(Johnson et al., 1995)
Caprine	Two-cell stage	25% developed into blastocyst when one cell was removed	(Malik et al., 2013)
Porcine	Morula and blastocyst stage	66% of the bisected embryos developed in to couplets and only 2.3% resulted in live identical twins	(Reichelt and Niemann, 1994)
	Two-cell stage	Using pronase, ZPF demi embryos presented a 45% blastocyst rate from two-cell embryos. ICM/TE ratios not affected. Expression of transcripts related with cellular-organisation were significantly decreased but not those associated with glucose catabolism	(Dang-Nguyen et al., 2011)
	Two-cell stage	From two-cell stage uneven blastomere embryos. 22% both blastomeres developed into blastocyst and 30% only one. Comparable in TCC, ICM/TE ratios and active mitochondria between small and large blastomeres.	(Dang-Nguyen et al., 2014)
Mouse	Two-cell stage	Live rates lower in demi embryos (30% with ZP and 0% without ZP) compared with 64% of control group	(Nijs et al., 1988)
	Four-cell stage	Splitting into 4 embryos where the blastocyst number were increased but not the total embryos with high quality	(Tang et al., 2012)
	Two-, four-, six- and eight-cell stage	Optimal splitting time at two- to four-cell stage with 74% blastocysts rates compared with 66% for 6-cell stage and 38% for eight-cell stage. They used ZP donors.	(Illmensee et al., 2005)
	Two-cell stage	Different four-cell configuration in twins (>60%). In superovulated group >70% of twins were synchronised and >90% in non-superovulated group	(Katayama et al., 2010)
	Two-cell stage	Using CD-1 and C57CBA-F1 using pronase and mechanical methods for twinning. ~30% of twin pairs developed into blastocyst on CD-1 group and ~90% for agouti F1.	(Wang et al., 1997)
Non-human primates	Two- to five-cell stage and six- to eight-cell stage	Using triploid embryos generated by ICSI with 19% and 66% of efficiency in two- to five- and six- to eight-cell stage embryos	(Chan et al., 2000, Illmensee et al., 2010b)
Humans	Six- to eight-cell stage	Using triploid embryos, same polymorphic STR markers in twin blastocysts. However, no data for half of the embryos	(Illmensee et al., 2010a)
	Two- to five- or six- to ten-cell stage	Best results for six- to eight-cell stage bisected embryos. Only 36% of these twin blastocysts had an obvious ICM graded C.	(Noli et al., 2015)

IV. Thawing instructions for one-cell mouse embryos from Embryotech

The following instructions for one-cell mouse embryo thawing are suggested by Embryotech. First, remove a single straw from storage tank and expose it to room temperature air for 2-3 minutes. Next, place the straw in a 37°C water bath for 1 minute. Remove the straw from water bath and wipe it dry. Furthermore, remove handle and by using a pair of scissors cut the straw between the lower heat seal and the column of media. Then, make a second cut to bisect the cotton plug at the PVA section.

In a petri-dish using the provided stylet, push down on the remaining cotton plug, expelling the contents of the straw into a drop of Hepes buffered medium. Immediately rinse the embryos into 2 additional drops of the Hepes buffered media. Allow embryos to rehydrate for 10 minutes in the final rinse droplet.

V. Activation media for mouse parthenotes

a) CBZ medium

Component	Supplier	Catalogue number	Weight in grams per 200 ml of medium
Potassium phosphate monobasic, KH_2PO_4	Sigma	P5379	0.032
Glucose, $\text{C}_6\text{H}_{12}\text{O}_6$	Sigma	G6152	0.200
Sodium bicarbonate, NaHCO_3	Sigma	S4019	0.422
Sodium pyruvate	Sigma	P4562	0.006
Glutamine	Sigma	G5763	0.0292
Lactate 60%	Sigma	L7900	1.1694 ml*
EDTA disodium salt	BDH	100935V	0.0082
Potassium chloride, KCl	BDH	101985M	0.072
Sodium chloride, NaCl	Fisher	S/3160/30	0.954
Magnesium sulphate heptahydrate	Fisher	M/1050/53	0.058
Water for embryo transfer	Sigma	W1503	Top up to 200ml

*Note that this component needs to be added by volume and not by weight.

CBZ medium was kept at -20°C in 50 ml aliquots until needed. For CZB activation medium preparation, 0.15 g of bovine serum albumin (BSA, Sigma, A3311) and 0.133 g of strontium chloride hexahydrate ($\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, Sigma, 255521) were added to one 50 ml aliquot of CBZ medium. Finally, 5 $\mu\text{g}/\text{ml}$ of cytochalasin B (Sigma, C6762) was added. Osmolarity and pH were adjusted to 280 Osm/l and 7.7 respectively.

VII. Non-surgical embryo transfer in mice

The following instructions are suggested by ParaTechs. Blastocyst stage embryos are recommended for transfer with the NSET device. Embryos may be transferred in KSOM medium or other desired culture media. Pseudo-pregnant female recipient mice should be 2.5 days post-coitum. A demonstration video of the procedure may be viewed at www.paratechs.com/nset. First, 15 μ l drops of culture medium are placed onto a 100 mm petri dish. Furthermore, 12 to 20 blastocysts were loaded into the medium. Then NSET device is then placed onto a P-2 pipette that is set to 1.8 μ l and embryos are loaded on minimum volume. Afterwards, the pipette is re-set to 2.0 μ l.

After, the pseudo-pregnant female is placed on top of a cage with a wire rack and it is allowed to grab the cage bar surface with its forefeet. The midpoint of the mouse tail is then grasped and the tail is angled upward. The smaller speculum is then gently introduced into the mouse's vagina and once in place it is changed for the larger speculum. Once the cervix is visualised with the use of a light source, the NSET device tip is carefully introduced through the cervix. Furthermore, embryos are expelled by pressing completely the pipette plunger. Finally, the NSET device is gently removed without releasing the pipette plunger, followed by the removal of the speculum. The mouse is returned to its cage and no post-procedure monitoring is required.