Comparative Analyses of Twin Blastocysts

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Comparative Analyses of Twin Blastocysts

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Declaration

I, Laila Ahmed Noli, declare that this PhD thesis, entitled, **"Comparative Analyses of Twin Blastocysts"** contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. However, it contains material that has been published in *Human Reproduction* (2015; 30:2774-84), *Stem Cells and Development* (2016; 25:1853-62) and *Human Reproduction Update* (2017; 23:156-65). I declare that there is no conflict of interest and that the time-lapse systems were used in this work for non-commercial purposes.

This thesis is my own work, except for the following:

1. The collection of unfertilised eggs, embryos that resulted from abnormal fertilisation (1PN, 3PN) post-ICSI/IVF, the culture of embryos until Day 4 or 5 of development as well as the laser ablation of the ZPs on Day 3 in PGD cycles were performed by Guy's ACU embryology team.

2. Assessment of the quality of the embryo post-thaw and the blastomeres biopsy of the donor embryo explained in Chapter 3 as well as the ICMs isolation and plating in validation method III in Chapter 6 were performed in conjunction with Yaser Dajani, an embryologist at Guy's ACU.

3. The preparation of 4% paraformaldehyde used for validation method II and imaging the stained embryos explained in Chapter 5 were performed by Nora Fogarty from the Division of Stem Cell Biology and Developmental Genetics, National Institute for Medical Research, Mill Hill.

4. All the experiments performed in validation method IV, which is explained in Chapter 7, were performed by Dr. Antonio Capalbo from GENERA, the Centre for Reproductive Medicine, Clinica Valle Giulia, Rome.

The details regarding any experimental work that was outsourced are provided in the thesis.

Laila Ahmed Noli

Abstract

Background It is possible to replicate the natural process of twinning by means of artificially spitting early-stage embryos in the laboratory. This method has resulted in applications relevant to agricultural and sporting animals. Embryo splitting, or *in vitro* twinning, has been successfully conducted in various livestock species. Human embryo splitting has been previously reported. The results were, however, inconsistent.

Hypothesis The quality of the human embryos generated by twinning *in vitro* is comparable to the quality of the embryos created by fertilisation.

Experimental Approach A total of 176 twin embryos created by splitting of 88 human embryos from either early (2 – 5 blastomeres, n = 43) or late (6 – 10 blastomeres, n = 45) cleavage stages were analysed in terms of morphokinetics and developmental competence. Data was compared to the morphometrics of embryos created by fertilisation and leading to pregnancy and live birth following single blastocyst transfer (n = 42). Furthermore, comparative analyses of the expression patterns of early lineage-specific markers (n=21 pairs) of twin blastocysts and non-manipulated Day 5 - 6 blastocysts using immunocytochemistry and human embryonic stem cells (hESCs) derivation was attempted (n = 5 pairs). Finally, comparative analyses of micor RNA (miRNA) profiles in spent blastocyst medium (SBM) of human twin embryos created by blastomere biopsy (n = 7 pairs) and SBM of blastocysts that led to a healthy pregnancy and live birth following embryo transfer (n = 7) were also conducted.

Results Morphokinetic data indicated that human preimplantation development is subject to strict temporal control according to a set 'developmental clock'. The size of twin embryos generated in the study was directly proportional to the starting cell number of the embryos used in their genesis. Furthermore, the first commitment decision in terms of cell fate was delayed, with the inner cell mass (ICM) becoming distinguishable later in the study group than in the normal control blastocysts. The ICM, if present at all, was small in size and of low quality. Furthermore, most cells in the twin embryos concurrently expressed both ICM and trophectoderm (TE) markers. Finally, the nature of miRNA secretion in SBM consistently varied between the twin and control embryos.

Conclusion Taken together the data suggested that human twin embryos created *in vitro* by embryo splitting are unsuitable not only for clinical use but also for research purposes.

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Publications

1. Cvoro, A., Devito, L., Milton, F. A., Noli, L., Zhang, A., Filippi, C., Sakai, K., Suh, J. H., H. Sieglaff, D., Dhawan, A., Sakai, T., Ilic, D. and Webb, P. (2015). A thyroid hormone receptor/KLF9 axis in human hepatocytes and pluripotent stem cells. *Stem Cells* **33**: 416-428.
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Abbreviations

9+	more than nine cells
ACU	assisted conception unit
ART	assisted reproductive technologies
ASRM	American Society for Reproductive Medicine
B	blastocyst
BM	blastocyst medium
C	cavitation
CM	cleavage medium
Ct	cycle threshold
DAPI	4',6-diamidino-2-phenylindole
EB	expanded blastocyst
eDET	elective double embryo transfer
EGA	embryonic genome activation
EnSCs	endometrial stromal cells
EPI	epiblast
ESCs	embryonic stem cells
eSET	elective single embryo transfer
hESCs	human embryonic stem cells
HFEA	Human Fertilisation and Embryology Authority
HFFs	human foreskin fibroblasts
HLA	human leukocyte antigen
HSA	human serum albumin
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
iPSCs	induced pluripotent stem cells
IVF	*in vitro* fertilisation
LED	light-emitting diode
M	morula
mESCs	mouse embryonic stem cells
miRNA	micro RNA
MZ	monozygotic
NGS	next-generation sequencing
NT	nuclear transfer

PBS	phosphate-buffered saline
PE	primitive endoderm
PGD	pre-implantation genetic diagnosis
RNA-Seq	RNA sequencing
SBM	spent blastocyst medium
SNPs	single nucleotide polymorphisms
SPS	serum protein substitute
STR	short tandem repeats
TE	trophectoderm
TLS	Time-Lapse System
UNESCO	The United Nations Educational, Scientific and Cultural Organization
UNG	uracil-N glycosylase
ZGA	zygotic genome activation
ZP	zona pellucida

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Chapter 1

Introduction

1.1 The importance of embryology

The field of embryology is central to the significant body of research currently taking place in human development and is at the forefront of scientific and medical advancements. For instance, this research offers the potential to eradicate genetic disease, with recent advances indicating that direct genome editing is now possible in pluripotent and other stem cells and in embryos during early stages of human development. Furthermore, techniques to eliminate mitochondrial disease are already being applied to human-assisted reproductive technologies (ARTs) (Amato *et al.*, 2014). Embryology therefore provides significant scope for achieving major advances in treatments for human diseases and for increasing our understanding of the events that occur during the key stages of early development. These studies answer fundamental questions related to human and non-human animal biology.

Embryology also provides us with information related to the development of safe ART. The use of ART has been steadily increasing in recent years, and it now accounts for between 1 and 3% of annual births in the developed world (Santos *et al.*, 2010). Therefore, it is of substantial importance to explore embryology to understand the impact of these interventions, especially on subsequent generations. For instance, the potential negative impact of ovarian stimulation on oogenesis, embryo quality, endometrial receptivity and perinatal outcomes has been investigated (Santos *et al.*, 2010), but the underlying mechanisms remain poorly understood, and further research is required. The EpiHealth Consortium, an EU-funded research collaboration, is investigating whether *in vitro* fertilisation (IVF) conceived offspring experience greater health problems later in life, and this type of ongoing research is essential to mitigate potential future risk. Furthermore, enhancing our understanding of early embryonic development is important for clinical applications involved in fertility issues, including the underlying biological causes of miscarriage. Human development is also a major focus of preventive medicine and efforts to improve maternal-foetal health. Extensive epidemiologic

evidence has indicated that the risk of disease in adulthood is associated with adverse environmental conditions early in development (Heijmans *et al.*, 2009), with a growing amount of evidence indicating that epigenetic modifications are associated with a variety of environmental conditions and stressors that mediate their effects by altering the uterine environment. Thus, very early mammalian development is a crucial period for establishing epigenetic markers that persist throughout life (Heijmans *et al.*, 2008) and that are associated with predisposition to disease. This phenomenon is the subject of rapidly advancing scientific research. These insights into fundamental biological processes help us to understand the key mechanisms that are involved in development and explain the increase in the focus on taking a life-course approach to preventing disease (Gluckman *et al.*, 2007).

Embryology is also central to the study of human embryonic stem cells (hESCs), which is an area of research that juxtaposes two fundamental but potentially opposing moral principles: the desire to alleviate suffering and the duty to respect and value of human life, the latter being a central ethical question in all such areas of research, thus ensuring the field attracts ongoing public scrutiny.

These extensive and varied research approaches advance our understanding of human diseases, including their origins and mechanisms, and aid in the development of potential preventive measures and treatments. However, the limited availability of human embryos for scientific and medical research is an ongoing challenge in the field of embryology. Conventional sources of embryos for research purposes include non-viable embryos that would otherwise be discarded following IVF and viable embryos that are left over following IVF and are deemed unnecessary.

Currently, approximately two-thirds of all elective single embryo transfers (eSET) carried out in women seeking fertility treatment are performed via blastocyst transfer, and this proportion does not appear to be declining (Human Fertilisation and Embryology Authority, 2013).

The single embryo transfer policy was first introduced to reduce the likelihood of multiple births in IVF-conceived pregnancies, since this represents the biggest single risk for both mothers and babies. Previous research indicated that transferring two embryos was more likely to result in a live birth than transferring

a single embryo, but the risk of obstetric complications, such as pre-term birth, is increased in these cases (Thurin *et al.*, 2004). Observational studies have demonstrated that eSET and elective double embryo transfer (eDET) result in the same pregnancy rates since younger patients with a better prognosis receive SET, while older women with a poorer prognosis receive DET (Bergh, 2005; Gerris, 2005). Thus, the Human Fertilisation and Embryology Authority's (HFEA), the body responsible for the independent regulation of the licensing and monitoring of fertility clinics and human embryo-based research, introduced a policy for clinics to transfer single embryos to women under the age of 40 (Human Fertilisation and Embryology Authority, 2015), an intervention that has proven successful.

This shift in the paradigm of fertility treatments has also resulted in the majority of embryos donated by couples being at the blastocyst stage of development. The shift towards blastocyst transfer has arisen as a result of a policy that was recommended and implemented by the HFEA's Multiple Birth Policy in 2009 (Human Fertilisation and Embryology Authority, 2013) (see paragraph 2.2).

The HFEA reported that the proportion of blastocyst transfers increased from a pre-policy rate of 12.8% in 2008 to rates as high as 46.8% in 2012 (Human Fertilisation and Embryology Authority, 2013) (Table 1 in paragraph 2.6).

The latest figures from the HFEA show that these types of IVF made up 54% of such procedures in April 2014, with the rate of frozen embryo transfer at the blastocyst stage of development being almost 80% (Figure 1.1). As a result of this policy, a dramatic reduction was achieved in multiple birth pregnancies, as shown in Figure 1.2.

As previously mentioned, the availability of human embryos is a fundamental requirement for the extensive research that is currently taking place in the field of human development. This includes studies involving the latest and much-publicised clustered regularly interspaced short-palindromic repeat (CRISPR) gene editing technique, for instance, and its multiple potential applications. The cas9-endonuclease based method for sequence-specific genome modification is guided by DNA-RNA base pairing to engineer point mutations, deletions and insertions into animal genomes, including humans (Hendriks *et al.*, 2015). This technique allows a high degree of specificity and has therefore generated significant interest from the

research community in terms of its future potential for removing or replacing disease-causing genes in pre-implantation embryos. It is currently being used as a method to further explore the mechanisms that are involved in early development (Callaway, 2016). Thus, it is critical to the advancement of our scientific understanding of this area that human embryos are available for research involving this technique. Since the policy to transfer embryos at the blastocyst stage of development was introduced, there has been a reduction in the number of cleavage-stage embryos that are available for research purposes. Therefore, splitting embryos at the cleavage stage offers the potential to increase the number of viable embryos, and it may therefore be a suitable means of addressing the current shortage in the availability of embryos that are appropriate for these lines of research. This type of approach could alleviate the reliance on obtaining donor embryos via conventional means. This type of study is, therefore, important because it is aimed at investigating potential solutions to the predicted increase in the shortage of human embryos as a result of changes in fertility interventions over time and to pre-empt future potential issues in this vital area of research. This study has the potential to increase the supply of human embryos, and it will therefore provide vital contributions to this field.

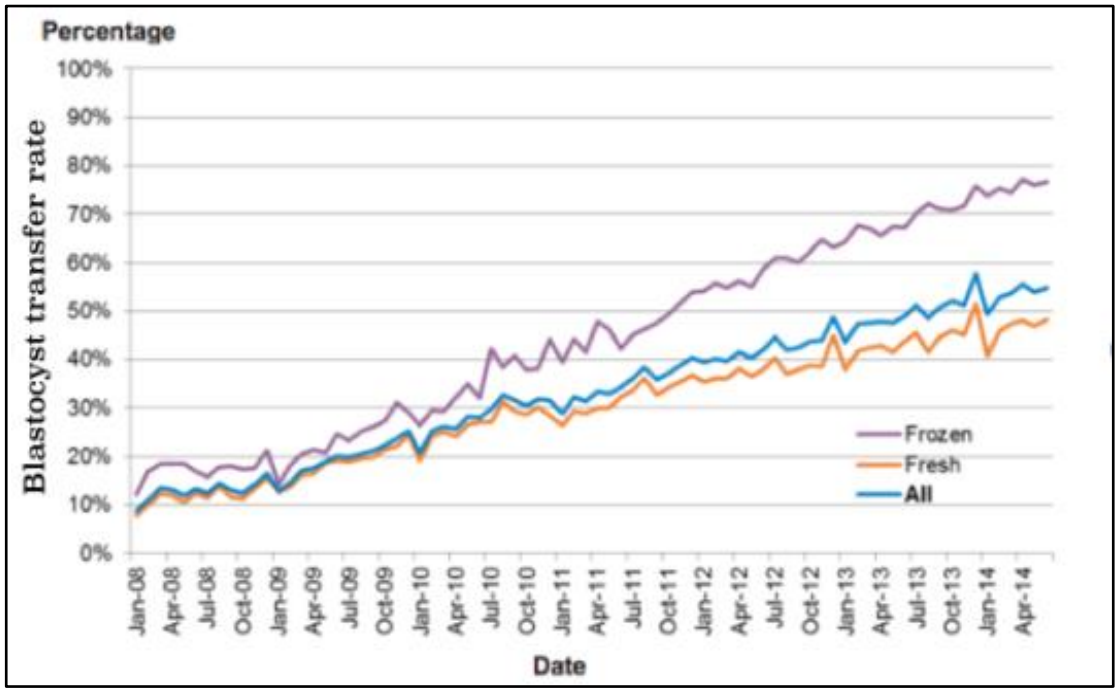


Figure 1.1: The percentage of embryos that were transferred at the blastocyst stage between 2008–2014. The figure shows that there is a trend towards blastocyst transfer over the last five years. The blue line represents overall blastocyst transfer, the purple line represents frozen embryos that were transferred at the blastocyst stage, and the orange line represents freshly transferred embryos. This graph was reproduced from Improving outcomes for fertility patients: multiple births 2015, HFEA (Human Fertilisation and Embryology Authority, 2015).

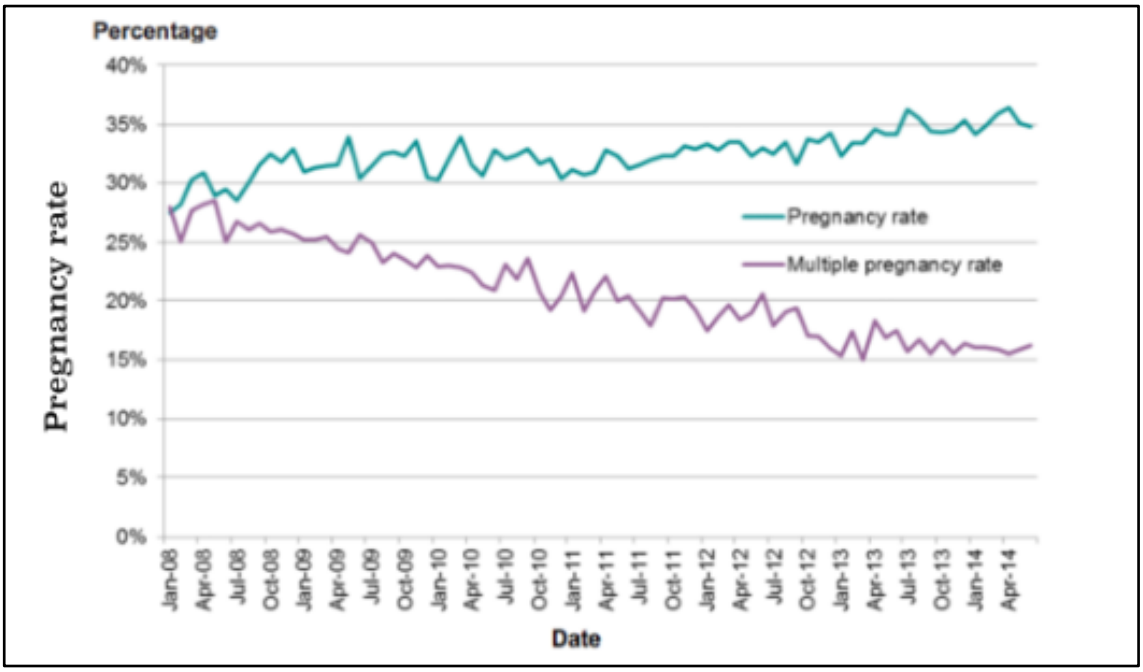


Figure 1.2: A graph showing the pregnancy rate (per embryo transfer) and the multiple pregnancy rate (per pregnancy) for fresh and frozen transfers that were performed between 2008 and mid-2014. The graph shows that the pregnancy rate has been maintained since 2008 and that it has, in fact, begun to increase, despite the wider use of single embryo transfer. In 2008, the overall multiple pregnancy rate was 27%, and it had fallen to 16% in the first half of 2014. This graph was reproduced from Improving outcomes for fertility patients: multiple births 2015, HFEA (Human Fertilisation and Embryology Authority, 2015).

1.2 Embryo splitting

1.2.1 Method of embryo splitting

Using a range of different techniques, embryo splitting or *in vitro* twinning has been performed *in vitro* in several animal species. Early in the twentieth century, experiments in fish showed that lowering the incubation temperature or reducing the oxygen concentration decreased the rate of development and thereby increased the incidence of monozygotic (MZ) twins (Stockard, 1921). Similarly, a large number of more recent studies have demonstrated that delayed fertilisation in rabbits and hypoxia in cultured mouse embryos also led to MZ twinning (Hall, 2003; Aston *et al.*, 2008). It has been suggested that, in these cases, twining may have been induced by disruptions in communication between blastomeres at various stages of development. The theoretical bases for these studies have allowed the continuous progression of techniques used to split embryos, and the latest improvements in microscopy and micromanipulation technologies allow the mechanical induction of MZ twinning to be achieved via blastomere biopsy or blastocyst bisection.

1.2.1.1 Blastomere biopsy

This technique involves removing one or more blastomeres from different cleavage stage embryos and inserting them into a previously prepared empty zona pellucida (ZP) for further development, as shown in Figures 1.3. To achieve this, the donor embryo is first treated with acidified Tyrode's solution, which produces an opening or hole in the ZP. Blastomeres are then removed via a biopsy pipette or aspirating pipette that is inserted through the ZP hole. The free blastomere is subsequently transferred to a ZP that was previously emptied by removing its cellular content. However, the major limitation of this technique is that the extracted blastomeres display reduced developmental capacity, which results in arrested embryos. In horses, embryo splitting using the blastomere biopsy technique has resulted in successful live births, with healthy monozygotic foals that developed to term (Allen and Pashen, 1984) from 2- and 8-cell stage embryos. The success rate of these procedures has been favourable, as has the developmental efficiency of producing twin blastocysts from 2- and 4-cell stage split murine embryos (Illmensee *et al.*,

2005). However, blastomere separation in non-human primates such as rhesus monkeys has not resulted in twin births or comparable success rates (Mitalipov *et al.*, 2002), and the theoretical basis for this difference is not yet known. The results of studies on assisted embryo hatching have suggested that differences in the sizes of the artificial opening in the ZP may cause various developmental abnormalities, such as premature hatching or artificial twinning (Petersen *et al.*, 2005).

An alternative method for making an opening in the ZP is the use of lasers. Laser-assisted biopsies result in reduced handling of the embryo and faster biopsies, which increase the likelihood of the embryos achieving their developmental capacity (Han *et al.*, 2003). Interestingly, laser-assisted biopsy is the technique that is used to perform pre-implantation genetic diagnoses (PGD), with recent studies demonstrating that using biopsies at the cleavage stage to perform PGD significantly reduced the reproductive capacity of human embryos, whereas blastocyst biopsy did not (Scott *et al.*, 2013).

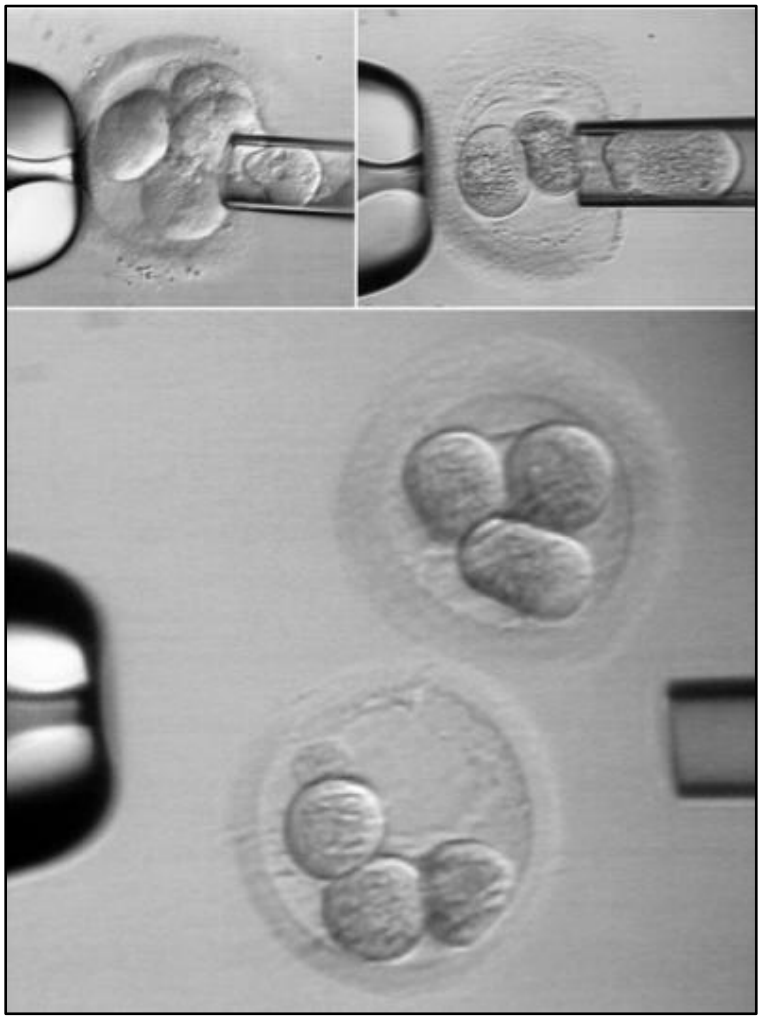


Figure 1.3: The process of twinning using a cleavage stage embryo. The images show the sequential process that is performed during the biopsy of 3 blastomeres from a 6-cell donor embryo (Twin A) and their placement into an empty ZP (Twin B) that was previously prepared. Images were reproduced from (Illmensee *et al.*, 2011).

1.2.1.2 Blastocyst bisection

Blastocyst bisection is another procedure that has not previously been reported in human embryos. Using this technique, a surgical microblade is attached to a micromanipulator and used to bisect the blastocyst into two embryos, with an even distribution of inner cell mass (ICM) and trophectoderm (TE) between the resultant demi-embryos, as shown in Figure 1.4. The MZ twin embryos are then immediately cultured *in vitro* using a culture medium that encourages further development. Although this procedure has not been attempted in humans, blastocyst bisection has been attempted and shown to be effective in a number of mammalian species, including cattle (Ozil *et al.*, 1982; Ozil, 1983; Seike *et al.*, 1989b), mice (Nagashima *et al.*, 1984; Wang *et al.*, 1990), rabbit (Yang and Foote, 1987), sheep (Széll *et al.*, 1994) and pigs (Nagashima *et al.*, 1989; Reichelt and

Niemann, 1994). Commercial exploration and investment in embryo splitting technologies is based on the aforementioned increased likelihood of pregnancy and the efficiency of producing offspring because of the increase in the number of viable embryos transplanted. Hence, success in this sphere has so far resulted from this specific methodology, which involves splitting morulae or blastocysts to produce monozygotic offspring. In non-human animal models, the embryos that are used to perform these procedures are obtained either by flushing the uterus of the mated animal following natural conception or by applying assisted reproductive technology via IVF (Ozil *et al.*, 1982; Ozil, 1983; Voelkel *et al.*, 1985).

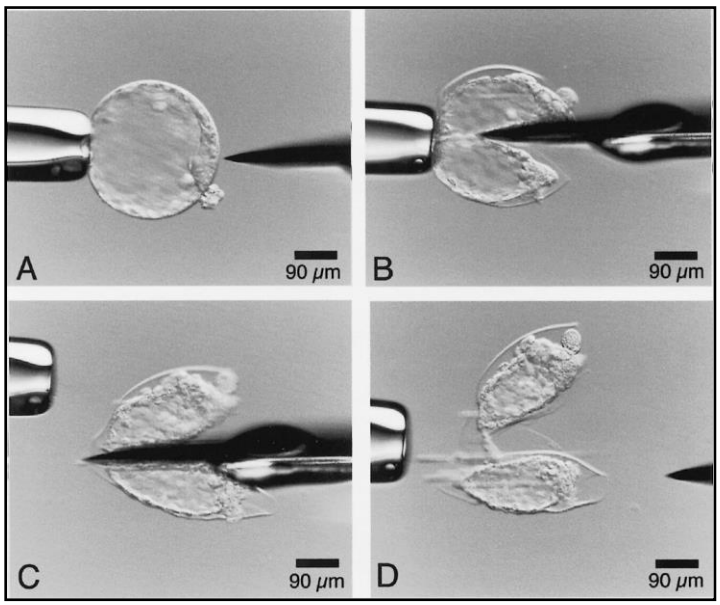


Figure 1.4: The bisection of an embryo at the blastocyst stage. (A) The blastocyst is immobilized using a holding pipette that is held at a position that is diametrically opposite to the ICM. (B) A surgical microblade is used to dissect the ICM into two halves. (C) Bisection is completed and the bisected embryos is released from the holding pipette. (D) A monozygotic split embryo is formed by embryo splitting using the bisection procedure. Images were reproduced from (Mitalipov *et al.*, 2002).

1.2.2 Benefits of embryo splitting

1.2.2.1 Mammals

Embryo splitting provides the opportunity to obtain genetically identical embryos, a feature that is necessary for comparative research. Furthermore, MZ offspring are a valuable phenomenon when considering the creation of progeny in mammals. For example, in the livestock industry, MZ animals are an important method of increasing the number of offspring from genetically superior or valuable parent animals. In veterinary medicine and breeding, embryo splitting has been extensively used to maintain high quality and healthy livestock to supply human

nutritional requirements, such as meat and dairy produce, and consumer demand (Yang *et al.*, 2007). In addition, the use of genetically identical animals can reduce the number of test animals that are needed for comparative research projects (Biggers, 1986; Yang and Anderson, 1992).

To enhance offspring efficiency and to create MZ offspring, a commonly studied method is animal cloning by nuclear transfer (NT). Whereas NT allows adult animals to be cloned from somatic cells, embryo splitting replicates a natural process that results in the formation of MZ twins. Much of the ethical debate surrounding embryo research is based on the future potential of cloning adult humans, and this distinction is therefore important. Cloning of adult animals by nuclear transfer has become relatively common and is used most notably to clone champion polo ponies for competitive sports. Until the cloning of Dolly the sheep in 1997 (Stewart, 1997; Wilmut *et al.*, 1997) (the first mammal to be cloned), many thought human cloning would be impossible. By using embryo splitting technologies in mammals, the number of embryos required for intrauterine transfer is increased, and this has an important effect on increasing the chance of pregnancy. Unlike cloning by nuclear transfer, the number of clones that can be produced from one animal is limited, however, by the degree to which embryo splitting can be efficiently achieved.

1.2.2.2 Humans

Embryo splitting may be employed by researchers to validate new media or laboratory techniques. For example, one of two genetically identical twin embryos can be used as a control, whilst the other may be cultured under test or novel conditions. Employing genetically identical embryos avoids the potential to misinterpret data that result from innate differences between samples that contain variations in their individual genetic makeup. Furthermore, this type of method would require a smaller sample size of test embryos to obtain statistically meaningful data. The quality of the embryos that are generated using these methods remains controversial, however, despite its suitability as a method for increasing embryo numbers (Noli *et al.*, 2015b).

Human embryo splitting has also shown obvious potential in applications related to ARTs. This technique is particularly valuable for couples experiencing infertility

who have a low chance of achieving success through routine IVF, especially in patients with advanced maternal age. Using these methods, high quality embryos can be selected and duplicated to increase the chance of success, and additional high quality embryos would also be available for repeat cycles of IVF when endometrial receptivity is implicated in conception failure. Although the use of this practice is currently restricted in the UK for ethical reasons, it is permitted in the US, essentially because it reproduces a phenomenon that occurs naturally during normal, *in vivo* development in MZ twins (Shikai, 2003).

Although ART is a future potential application for human embryo splitting, the legality and safety of using such a procedure for reproductive purposes has not yet been established. Therefore, the immediate primary goal of this study is the creation of embryos for research purposes, as previously outlined. However, this should, in turn, contribute to the development of the necessary techniques and increase our understanding of these processes, which will ultimately result in achieving the necessary ethical and knowledge base for this work to progress.

Furthermore, in addition to enhancing the potential chances of conception in future human ARTs and in replicating high quality stock in animals, splitting two-cell stage embryos into component blastomeres and following the development of the resulting twin embryos into adulthood could prove hugely informative. It may clarify how human twins that are nearly or completely genetically identical can have such differing phenotypes (Cheung *et al.*, 2008; Katayama *et al.*, 2010). The production of MZ twins is also of particular interest when evaluating the effect of environmentally influenced epigenetic changes, such as altered DNA methylation, on phenotype (Fraga *et al.*, 2005; Whitelaw and Whitelaw, 2006; Haque *et al.*, 2009; Kaminsky *et al.*, 2009). Twins produced from the earliest blastomeres would be the most useful for examining epigenetic differences at this stage, which are likely minimal, and transferring the embryos between different dams could be used to test the effect of maternal diet on the epigenome of the developing embryo (Katayama *et al.*, 2010).

Embryo splitting is also a means to examine early development, the role of individual blastomeres in commitment to particular cell lineages, and how development potential is affected by factors such as the distribution of cytoplasm and informational macromolecules, the plane of cleavage, and gene transcription.

For instance, studies of murine half embryos have shown that cell-cell interactions play a significant role in cell-fate specification in mammalian blastomeres and post-implantation development. During normal development, each blastomere gradually activates an all ICM or TE gene expression pattern with each cell cycle (Lorthongpanich *et al.*, 2012). As cleavage progresses and positional information begins to influence gene expression, some genes are down-regulated, while others are upregulated, depending on the position of the blastomere and its cell-cell interactions. This process continues during subsequent cleavage divisions, resulting in an increasingly specific pattern of gene expression. In this way, using half embryos to investigate the entire process of embryonic development, to define the regulatory capacities of half embryo-derived blastomeres, and to identify the factors on which they are dependent would help us to determine the optimal stage of development at which to perform embryo splitting.

Because of the ethical and legal concerns surrounding embryo splitting, blastocysts resulting from this procedure have not been transferred into a human uterus to investigate their capacity for implantation. It therefore remains unclear whether twin blastocysts produced by embryo splitting are equivalent to non-manipulated embryos in a biological sense. Studies carried out in this field have so far not investigated the characteristics and viability of split human embryos at a genetic or epigenetic level, although studies have been performed to confirm their monozygocity (Illmensee *et al.*, 2011).

1.2.3 History of embryo splitting

Research into embryo splitting in animals dates back to the late 1800s, with early studies by Hans Dreisch on sea urchin embryos providing proof of concept evidence that individual blastomeres from 2- and 4-cell embryos could develop into larvae (Driesch, 1894). Subsequent studies on salamanders by Hans Spemann demonstrated that individual blastomeres of 2-cell stage embryos possess the potential to develop into full organisms. Hans Spemann also performed the first nuclear transfer experiments in 1914, 83 years before Dolly the sheep was created (Spemann, 1921).

1.2.3.1 Embryo splitting in mice

The first reported case of embryo splitting in mammals was reported in mice (Tarkowski, 1959b) in which blastomeres derived from early pre-implantation embryos were examined to determine their developmental potential. Tarkowski discovered that single blastomeres derived from 2-cell embryos undergo normal full-term development, resulting in adult mice (Tarkowski, 1959a; Tarkowski, 1959b). Further studies demonstrated that in some cases, the blastomeres that were isolated from 2-cell stage embryos resulted in genetically identical twin animals (Mullen *et al.*, 1970; Tsunoda and McLaren, 1983; Togashi *et al.*, 1987; Wang *et al.*, 1997; Sotomaru *et al.*, 1998; Tarkowski *et al.*, 2005). Numerous studies have demonstrated that a single blastomere from a 2-cell stage mouse embryo possesses totipotency, whereas a single blastomere from a 4-cell stage embryo is not totipotent (Tarkowski, 1959b; Tarkowski, 1959a; Mullen *et al.*, 1970; Tsunoda and McLaren, 1983; Togashi *et al.*, 1987; Papaioannou *et al.*, 1989; Wang *et al.*, 1997; Sotomaru *et al.*, 1998). Blastomeres derived from embryos at the 4-cell stage formed embryos that could implant, but they often failed to form egg cylinders (Rossant, 1976). However, when blastomeres were isolated from an 8-cell stage mouse embryo, only small trophoblasts formed (Edwards and Beard, 1997). It was suggested that the inability of 4-cell stage or 8-cell stage mouse blastomeres to develop normally might result from the reduced number of cells that are present in the resulting embryos (blastocysts) at the beginning of cavitation. It has been shown that because these blastocysts contained low total cell numbers, they had either a very small ICM or lacked an ICM altogether and that in general; they contained only giant trophoblast cells (Tarkowski and Wroblewska, 1967; Rossant, 1976).

1.2.3.2 Embryo splitting in farm animals

Pregnancies resulting from MZ twins that were generated *in vitro* by embryo splitting or twinning have been show to result in the live birth of healthy animals. Successful twinning has been reported in sheep (Willadsen, 1979), cattle (Seike *et al.*, 1989a; Seike *et al.*, 1991; Johnson *et al.*, 1995) goats (Tsunoda *et al.*, 1985), pigs (Reichelt and Niemann, 1994) and horses (Allen and Pashen, 1984). The most common outcome of producing MZ offspring is twins or singletons, but triplets and quadruplets have also been reported in cattle following the transfer of quartered

embryos (Willadsen and Polge, 1981; Johnson *et al.*, 1995). An increase in the production of cattle from the transfer of bisected embryos that were produced using these approaches has been reported (Leibo and Rall, 1987). In addition to fresh transfer, the use of frozen-thawed demi-embryos has also been attempted (Seike *et al.*, 1991). Remarkably, in the case of cattle, the normal pregnancy rate from whole embryo transfer is ~70%. The equivalent rate for a demi-embryo is ~50-55%, and this method therefore provides a 30-40% increase in the chance of conception (Wood and Trounson, 2000). In addition, no developmental or physiological defects have been reported in the offspring resulting from these split embryos, which develop into healthy animals.

1.2.3.3 Embryo splitting in non-human primates

During the evolution of embryo splitting methods for studying genetically identical offspring in humans, embryo splitting has been investigated in Rhesus macaques. Rhesus macaques are a non-human primate model that is highly related to human beings in evolutionary, genetic and physiological terms. Therefore, they can be used to gain crucial information for human-related research (VandeBerg and Williams-Blangero, 1996). Specifically, the successful development of methods for producing MZ twins in monkeys could lead to significant advancements in the scientific understanding of human disease, monozygotic twinning and the effects of the maternal environment on the epigenetic profile of a developing embryo. In addition, these studies could also lead to the development of better animal models for vaccine trials and tissue transplantation studies (Schramm and Paprocki, 2004b). However, current strategies aimed at producing MZ twins in rhesus monkeys have met with only limited success (Schramm and Paprocki, 2004b). Blastomere separation studies performed in Rhesus monkeys gave rise to blastocysts with significantly different total cell numbers within a given demi-embryo pair (Mitalipov *et al.*, 2002). This may have resulted from the asymmetric distribution of cytoplasm between the blastomeres during separation or difference in the polarity of cells within the embryo. Twenty-two pairs of demi-embryos were created using blastomere separation and then transferred, resulting in a pregnancy rate of 33% (seven out of twenty-two). Among these pregnancies, two twin pregnancies (9%) were initiated, but neither of the twin pairs developed to term (Mitalipov *et al.*, 2002). In one study, a total of 368 embryos were created by

splitting 107 rhesus embryos at the 8-cell stage to produce sets of identical quadruplets, each consisting of two blastomeres, that resulted in four pregnancies. The first non-human primate to be cloned, Tetra, was a healthy female that was born as a result of the transfer of 13 embryos that were produced from splitting eight-cell stage embryos (Chan *et al.*, 2000). Preliminary experiments showed a reduction in the developmental potential of the blastocysts when blastomere separation was performed at later cleavage stages (between the 8- and 16-cell stage) (Chan *et al.*, 2000). However, other studies have shown that blastomere separation at the 2- or 4- cell stage can lead to the formation of demi-embryos that develop into blastocysts that are comparable to non-manipulated control embryos (Mitalipov *et al.*, 2002). The ratio of ICM to TE and the ratio of ICM to total cells in these split blastocysts were similar to the ratios in non-manipulated control blastocysts. However, the total number of cells in the split blastocysts was almost 50% lower than the number in the controls (Mitalipov *et al.*, 2002), similar to results recorded in other species (Willadsen, 1981; Willadsen *et al.*, 1981; Willadsen and Polge, 1981; Willadsen, 1989). In the case of the demi-embryos that were developed using blastocyst bisection methods, a pregnancy rate of 33% (four out of twelve) was achieved. However, no twin pregnancies were established, and all of the pregnancies were singletons (Mitalipov *et al.*, 2002). While blastocyst bisection led to the formation of higher numbers of demi-embryos, the number of clinical pregnancies per oocyte was higher for embryos produced by blastomere separation (Mitalipov *et al.*, 2002). However, in spite of the fact that pregnancies have been established using both methods of embryo splitting in rhesus monkeys, they both resulted in only singleton offspring whether they were implanted in different or the same recipients (Chan *et al.*, 2000; Mitalipov *et al.*, 2002).

1.2.3.4 Embryo splitting in humans

The first human embryo splitting procedure was reported by a team of researchers including Robert Stillman and Jerry Hall from George Washington University in Washington, D.C., in October 1993 in a prize-winning paper titled “Experimental Cloning of Human Polyploid Embryos Using an Artificial Zona Pellucida”, which was presented at a joint meeting of the American Fertility Society and the Canadian Fertility and Andrology Society (Hall *et al.*, 1993). Researchers used polyspermic embryos that would not have survived and would therefore have been

routinely discarded. They separated blastomeres from seventeen 2- to 8-cell embryos, covered them with an artificial ZP and cultured them for up to 32 cell divisions. Although the researchers claimed that their results paved the way for enhanced infertility treatment using this method, it was later found that the study did not possess the valid Institutional Review Board approvals, and the authors were reprimanded and instructed to destroy their data (Fackelmann, 1994; Macklin, 1995). In the wake of protests from the scientific community and media, the American Society for Reproductive Medicine's (ASRM's) Ethics Committee formulated a statement concerning embryo splitting and its use in infertility treatment, which was subsequently accepted by the Board of Directors in December 1995 (The Ethics Committee of the American Society for Reproductive Medicine, 2004)

The literature that is currently available on the subject suggests that the use of these types of embryo splitting techniques may result in the formation of viable and morphologically adequate blastocysts in humans (Van de Velde *et al.*, 2008; Illmensee *et al.*, 2010). However, there have been few comprehensive qualitative analyses of embryos that were created using splitting techniques. In addition, the results of reported studies have been somewhat contradictory. For example, (Van de Velde *et al.*, 2008) reported that blastomeres derived from 4-cell embryos possessed sufficient plasticity to form blastocysts, whereas the results published by another research group stated that blastomeres from 8-cell embryos led to the development of higher quality blastocysts than blastomeres derived from embryos at earlier stages (Illmensee *et al.*, 2010).

In the study published by (Van de Velde *et al.*, 2008), split embryos were evaluated in terms of their size, biological behaviour, morphology and immunocytochemistry. Blastocysts that were derived from individually cultured blastomeres resulted in embryos that were smaller than regular human embryos that were cultured *in vitro* (between Days 3 and 5, they were 4 times smaller than the controls). It was also shown that in spite of their smaller size, the blastocysts underwent compaction on Day 4 and cavitation on Day 5, similar to the control human embryos. However, on Day 6, the majority of these split embryos were able to form complete blastocysts that possessed a distinct ICM and TE, and the yield of cells per embryo was very low. The presence of ICM cells in the split embryos was

confirmed using immunocytochemistry and confocal microscopy analyses to study of the expression of the transcription factor; Nanog homeobox (NANOG). NANOG is a marker that is specifically expressed in ICM cells in expanded human blastocysts, and it is therefore an indicator of pluripotency/stemness within these cells (Niakan and Eggan, 2013). Trophoblast cells in the split embryos were clearly observed using the inverted microscope. In one embryo, all four blastomeres developed into viable blastocysts, each with a cohesive TE and a tightly packed ICM, with some cells expressing NANOG, as shown in Table 1.1. Although the sample size was small, Van de Velde successfully demonstrated that cells isolated from a 4-cell stage human embryo could individually develop into mini-blastocysts with a delineated ICM and TE cells (Van de Velde *et al.*, 2008).

Table 1.1: Summary of the results of splitting six 4-cell stage human embryos in a study performed by Van de Velde *et al*, 2008. The columns represent the number of blastomeres that remained viable following the biopsy procedure on Day 2 and the number that divided on Day 3, demonstrated evidence of compaction on Day 4, had a cavity on Day 5, and finally formed into full/expanded blastocysts on Day 6. The last column also provides the number of blastocysts with an inner cell mass (ICM)/the number of ICM that expressed NANOG (ND, not done because the sample was lost during fixation). Table was adapted from (Van de Velde *et al.*, 2008).

Embryo	Day 2 Survived	Day 3 2-cell	Day 4 Compaction	Day 5 Cavity formation	Day 6 Full-expanded blastocyst (ICM/TE)
1	4	4	3	3	2(1/ND)
2	4	4	3	3	3(2/2)
3	3	3	3	3	3(1/1)
4*	4	4	4	4	4(4/4)
5	4	3	3	3	3(3/2)
6	4	1	3	3	1(1/0)
Total	23	19	19	19	16 (12/9)

More recently, (Illmensee *et al.*, 2010) showed that the ideal developmental stage for splitting human embryos is the 6-8 cells stage, in terms of both splitting and developmental efficiency. The author claimed that the number of blastocyst-stage embryos that formed significantly exceeded the original number of embryos that were split at this stage. The rationale is that because embryonic genome activation probably occurs around the 4- to 8-cell stage (Braude *et al.*, 1988), a split at this point should not interfere with the dependence of the blastomeres on maternally deposited RNA. The split embryos appeared to hatch earlier, however, possibly because of compromised zona pellucida integrity that resulted from the blastomere biopsy (Illmensee *et al.*, 2010). This effect may enhance the implantation capacity of embryos, especially in patients who may have experienced multiple implantation failures (Primi *et al.*, 2004; Petersen *et al.*, 2005). The data from Illmensee *et al.*

(2010) could be explained by unknown variables in experimental design (e.g., the size of the pipette).

In a second study by the same group, the monozygocity of twin blastocysts was demonstrated (Illmensee *et al.*, 2011). The authors showed that the MZ characteristics of triploid embryos were not altered by embryo splitting, with the resulting twin embryos containing the same allelic short tandem repeats or (STR) sequences. Six selected polymorphic STR markers in the Human leukocyte antigen; (HLA) locus on Chromosome 6 were selected and subjected to nested multiplex PCR analysis using fluorescently labelled primers. Fluorograms from five pairs of twin blastocysts showed that peak positions for the detected STR profiles were identical between twin embryos. This was the first study to demonstrate the monozyogocity of twinned human embryos at the DNA level (Illmensee *et al.*, 2011). Finally, Noli *et al.* (2015b) investigated embryo splitting in humans and the impact of using this methodology on the embryonic developmental clock. The results of that study will be presented as part of this doctoral thesis (Noli *et al.*, 2015b).

1.2.4 Ethical considerations and regulatory framework

Despite the fact that spontaneous MZ twinning is a natural form of cloning, artificially splitting human embryos continues to be a matter of ethical debate. These ethical considerations have given rise to a regulatory framework to restrict research and development in human cloning, which in the UK, includes the particular methodology of embryo splitting. Research utilizing non-human animal embryos lays the foundation not only for commercial exploitation in agriculture and sports involving animals but also for further research into human embryos with the ultimate possibility of applications relevant to human ART.

The process of embryo splitting falls under the generic heading of human cloning, which is an emotive and controversial topic. It is distinct from the process of nuclear transfer, which transfers nuclear content from somatic cells for the purposes of creation of a child or therapeutic application. Nuclear transfer effectively duplicates a fully formed human being, whereas embryo splitting replicates the natural process that forms MZ twins during embryogenesis. Whereas nuclear transfer circumvents normal gametogenesis and fertilisation and prevents

the normal programming of an embryo's genome, embryonic stem cells obtained from a cloned embryo are functionally the same as those that develop during normal development and during IVF (Jaenisch, 2004). In addition, the split embryos are dichorionic and diamniotic, with a separate placenta and amnion, which reduces the risk of common complications in twin pregnancies, such as cord entanglement or twin-twin transfusion.

Furthermore, since MZ twinning is also a natural phenomenon, significant information can be obtained from analysing the behaviour of twins. Finally, the embryo splitting procedure familiarises parents with the possibility of twin pregnancies and their risk, which better prepares them for these events.

Hence, although biological barriers are likely to prevent human reproductive cloning by nuclear transfer in the foreseeable future, it is more likely to be achieved by embryo splitting. The ethical debate regarding embryo splitting is therefore more likely to attract public attention and scrutiny.

Most mammalian reproductive cloning that is performed using nuclear transfer gives rise to offspring that either die during gestation or suffer from large offspring syndrome, which is typified by respiratory and metabolic abnormalities and an enlarged, dysfunctional placenta (Jaenisch, 2004). Clones that do survive usually have a normal phenotype and are physiologically able to produce healthy offspring (French *et al.*, 2006), and no significant behavioural or psychological problems related to monozygotic twining have so far been reported (Rutter and Redshaw, 1991; Kendler *et al.*, 1996). Hence, the ethical debate centres on whether human reproductive cloning by embryo splitting, if possible in the foreseeable future without increasing the risk of abnormalities in the child, is ethically justifiable. There are various issues to consider in this ethical debate, including the right to life of the embryo and the interests of the child, the societal consequences and teleological perspectives (Strong, 2005). For example, one controversial and highly discussed aspect of embryo splitting is whether artificial twinning violates the right of an unborn child to be unique. However, given that embryo splitting replicates a natural process, none of these arguments carry sufficient ethical justification to warrant a total ban on human reproductive cloning using this methodology. It is widely accepted that embryo splitting must not be used for unethical purposes, such as the generation of histocompatible embryos with the

intention of organ transplantation. Therefore, the main consideration, from both a scientific and a clinical perspective, is whether this methodology can be used without an increased risk of abnormalities.

In terms of regulation, the two sides of the Atlantic mirror each other with regard for the acknowledged complexity of the moral arguments that are related to this research. Since Dolly the sheep was born in 1997, the international community has expressed concern about the potential for reproductive cloning in humans, and numerous countries have formulated bans either through laws, decrees or official statements (The United Nations Educational, Scientific and Cultural Organisation Committee, 2004).

In terms of regulation at the international level, the General Conference of UNESCO unanimously acclaimed the Universal Declaration on the Human Genome and Human Rights in 1997. This international instrument was subsequently endorsed by the General Assembly of the United Nations in 1998, which declared that human reproductive cloning is a practice against human dignity (The United Nations Educational, Scientific and Cultural Organisation Committee, 2004).

At the European level, the Additional Protocol to the Convention of the Council of Europe for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine on the Prohibition of Cloning Human Beings was developed in 1998 and took effect in 2001. It states that "*any intervention seeking to create a human being genetically identical to another human being, whether living or dead, is prohibited"* (Council of Europe, 1997).

In terms of the UK and the US regulations, following controversy over original research in the United States, the ASRM published a statement in 1995 concerning embryo splitting stated that *"splitting one embryo into two or more embryos could serve the needs of infertile couples in several ways"* and that they did not recognise a significant ethical objection to the placement of two or more embryos with the same genome in the recipient uterus with the aim of resulting in a single pregnancy, as long as the parents undergoing the fertility treatment were duly apprised of the outcome of this procedure. In the summary of their report, the ASRM stated that "*since embryo splitting has the potential to improve the efficacy*

of IVF treatments for infertility, research to investigate the technique is ethically acceptable' (The Ethics Committee of the American Society for Reproductive Medicine, 2004).

A bill was subsequently passed by the House of Representatives in 2003 that banned reproductive and therapeutic cloning. The bill paved the way for legislation to be passed in different states that outlawed either reproductive cloning or both therapeutic and reproductive cloning. Fifteen states have laws on human cloning. These laws specifically define cloning as an embryo that is achieved via nuclear transfer (National Conference of State Legislatures, 2003) and does not include embryo splitting. Thus, in the United States, legislation on reproductive cloning relates specifically and exclusively to nuclear transfer methodologies. Furthermore, since the 1995 statement by the ASRM, legislation has allowed embryo splitting as an infertility treatment. The UK includes cloning both by embryo splitting and nuclear transfer in the same legislation. There is therefore a difference in laws between these two countries.

This is reflected in the reaction to earlier decisions relating to original embryo splitting research in the United States in which the views of the ASRM have not been supported by international regulatory bodies. For instance, the opinion of the HEFA in the UK was different. The original HFEA Act 1990, which regulates the medical and scientific manipulation of embryos, defined an embryo as a "live human embryo where fertilisation is complete", and therefore the Human Reproductive Cloning Act 2001 was brought into force in 2001 to cover embryos created by reproductive cloning techniques. It prohibits reproductive cloning and states that "a person who places in a woman a human embryo which has been created otherwise than by fertilisation is guilty of an offence and this offence carries up to 10 years and/or an unlimited fine" (The Human Reproductive Cloning Act, 2001) (Chapter 23).

In 2002, a ruling came into force that allowed for clones produced by cell nuclear replacement to be classified as embryos, and reproductive cloning therefore subsequently fell under the HFEA Act (1990). The act and associated Code of Practice that was produced by HFEA allows embryonic research to be conducted for the following purposes:

- to increase knowledge about the causes of congenital disease,
- to increase knowledge about the causes of miscarriages,
- to develop more effective methods of contraception, and
- to develop methods for detecting the presence of genetic or chromosomal abnormalities (Human Fertilisation and Embryology Authority, 1990).

In 2001, further regulations allowed for the creation of embryos for the following purposes:

- to increase knowledge about the development of embryos,
- to increase knowledge about serious disease, and
- to enable any such knowledge to be applied in developing a treatment for a serious disease (Human Fertilisation and Embryology Authority, 2001).

Furthermore, the 6th HFEA Code of Practice (paragraph 8.9 ii) specifies that the embryo splitting procedure must not be used by fertility clinics to produce embryos for treatment purposes (The Human Fertilisation and Embryology Authority, 2003). The HFEA now stipulates that a license must be granted for therapeutic cloning research. The first license was awarded by the HFEA in 2004 to scientists from the University of Newcastle to create human embryonic stem cells via cell nuclear transfer (Human Fertilisation and Embryology Authority, 2004).

Chapter 2

Validation System

The embryo splitting procedure must be tested in two stages if the technique is to achieve broader scientific and clinical use. First, the method of splitting must be optimally achieved using the most appropriate technique, as previously outlined, and second, the quality of the resultant twin blastocysts must be ascertained in terms of their viability and developmental potential. These factors are important for both the use of embryos in scientific research and for future potential applications in ART.

The ideal means of determining the viability and development potential of embryos derived from embryo splitting would be to use split embryos to establish pregnancies *in utero* and evaluate the offspring born from such a pregnancy. However, for obvious ethical and, in the UK, legal reasons, these experiments cannot be performed in human subjects (The Human Fertilisation and Embryology Authority, 2003). Therefore, the most appropriate validation method is a thorough examination of embryonic development and a comparison of the genetic and epigenetic characteristics of split embryos with profiles derived from non-manipulated controls. This approach could be used to determine and study any impairment that is observed in embryos derived from embryo splitting procedures. Accordingly, the developmental potential, pluripotency and reproductive competence of twin embryos were validated in several stages, as follows:

1. A morphokinetic analysis of embryonic development using a time-lapse imaging system.
2. A study of the appearance and localization of lineage-specific transcription factors of both the ICM and the TE using immunohistochemistry.
3. The derivation of hESCs to study the pluripotency of the ICM in split embryos.
4. Molecular analyses of the split embryos:
    a. Transcriptome and
    b. Epigenetics, including DNA methylation and micro RNAs (miRNAs).

2.1 Validation method I: Morphokinetic analysis of embryonic development using a time-lapse imaging system

Since the birth of the world's first IVF baby, Louise Brown, in the 1970s, the field of clinical embryology has witnessed incremental changes in the evolution of related technologies, including the development of improved culture media and advancements in ambient incubation conditions. In addition, the field has also undergone revolutionary changes in available techniques, such as intracytoplasmic sperm injection (ICSI) and embryo biopsy. These developments have increased the number of treatment options that are available to specific patient groups who would previously have remained childless. Over the years, novel technologies have become available that can provide significant information about the dynamics of cleaving human pre-implantation embryos cultured *in vitro*. These technologies have played an important role in the gathering of evidence that has increased the likelihood of pregnancies following successful embryo implantation.

Traditionally, embryos are examined daily to assess their cleavage status and quality to obtain optimal results in terms of pregnancy and implantation rate. Although frequently examining developing embryos using microscopic examination outside the incubator allows the researcher to assess the timing of developmental events, it also subjects the developing embryos to unwanted changes in temperature, gas composition and humidity (Fujiwara *et al.*, 2007; Zhang *et al.*, 2010). Therefore, when using conventional incubators to culture embryos, there is a trade-off between the gathering of meaningful information about the events of embryo development and the undesirable outcomes that are associated with compromising the steady culture conditions that the developing embryos are exposed to.

New technologies include the design of safe, fully sealed incubation systems within which embryos can be cultured undisturbed for several days while simultaneously being subjected to high-frequency time-lapse imaging system (TLS) to gather data. The use of cameras to carry out time-lapse monitoring within the incubation chamber circumvents the limitations of traditional incubators, thereby providing a feasible means for longer inspection times and allowing dynamic parameters to be included in the morphological evaluation. Several published research articles and

reviews (Lemmen *et al.*, 2008; Pribenszky *et al.*, 2010a; Wong *et al.*, 2010; Meseguer *et al.*, 2011; Kirkegaard *et al.*, 2012a; Kovacs, 2014; Rubio *et al.*, 2014; Yang *et al.*, 2014; Kirkegaard *et al.*, 2015) have suggested potential roles for time-lapse monitoring in the selection of embryos that demonstrate clinical competence.

The acquisition of sequential, photographic, time-lapse images of a patient's embryos is rapidly gaining importance as a non-invasive tool for monitoring and selecting embryos. Although the first use of 'time-lapse cinematography' to study fertilisation and developmental kinetics in early human embryos was reported more than 15 years ago, the availability of highly sophisticated, commercially available systems has enabled this technique to be routinely used in present-day IVF clinics. Several publications have used time-lapse imaging to report on markers related to embryonic viability and putative implantation ability and to assess morphokinetic variables during embryonic development (Meseguer *et al.*, 2011; Dal Canto *et al.*, 2012; Aguilar *et al.*, 2014; Basile *et al.*, 2015). Time-lapse photography, when used to analyse embryonic development, is called morphokinetics because it analyses both morphological criteria that are typically used for embryo grading and the kinetics of development in each embryo at certain predefined time points. Morphokinetic parameters that are used by embryologists to evaluate embryos include assessments of the appearance or fading of pronuclei, cell cleavage (including its pattern and rate), and the time from insemination to the start of blastulation. A study by Desai *et al.* (2014) showed that there was variability in early morphokinetic characteristics between high-quality blastocysts and embryos that failed to undergo blastulation. In addition, the group found that there were variations in recorded parameters between high quality blastocysts that were transferred to patients, in terms of whether they implanted or did not (Desai *et al.*, 2014). Another study revealed that aneuploid embryos experienced delayed initiation of blastocyst formation and delayed development to full blastocysts compared to their euploid counterparts (Montag, 2013). Thus, this evidence indicates that the extensive use of time-lapse imaging technologies and the associated increase in the development of a large number of selection models and algorithms are increasing.

Chavez *et al.* (2012) conducted a study that combined non-invasive time-lapse imaging with the karyotypic reconstruction of all blastomeres. Four-cell human

embryos were used to investigate whether blastomere behaviour may be indicative of ploidy status during the first two cleavage divisions. This study concluded that the timing of cell cycle parameters is precise in all euploid embryos until the four-cell stage. Conversely, only 30% of aneuploid embryos displayed such normal timing. They also concluded that cell cycle and fragmentation parameters of individual blastomeres are indicative of ploidy status, that these parameters can be tracked, and that they provide the basis for a clinical diagnostic tool to exclude the transfer of embryos that may be prone to miscarriage. This is quite important, as previous research has demonstrated that aneuploidy in human embryos is more frequent than perhaps expected, with some 50-80% of cleavage-stage human embryos shown to have abnormal chromosome numbers (Vanneste *et al.*, 2009).

2.1.1 History of time-lapse imaging systems

Time-lapse imaging has been used to study fertilisation and the early developmental kinetics of human embryos since the 1990s (Payne *et al.*, 1997). In its initial form, time-lapse cinematography collected images of fertilised oocytes every minute over a time period of 4 hours. This was accomplished by positioning a low-light polychromic video camera inside a temporary incubation chamber that was fabricated from Perspex and placed on an inverted microscope at 200x magnification. These images were magnified into 1064x on a monitor. Since this nascent version in the 1990s, the technology has significantly progressed, and it now allows the continuous, non-invasive monitoring of an oocyte during the entire duration of the culture period, beginning at insemination and lasting through embryo transfer or freezing, while obviating the requirement to remove the embryos from their stable culture environment. In contrast to traditional once-a-day microscopic observations, time-lapse technology now enables the acquisition of several hundred images, the precise identification of the timing of key events during the embryo's development, and the potential to review cell division patterns and to visually detect brief but critical changes. The sequence of continuous images is stitched using software to demonstrate the progressive development of the embryo over a significant period of time into a film that may be only a few minutes long. These videos detail the performance of each embryo and can be archived to create a time-lapse database. The retrospective re-annotation of images and analyses of such data is also possible with time-lapse analysis, keeping the fate of

the embryo and its developmental outcome in mind. Pribenszky and his colleagues were the first to report the occurrence of a live birth following time-lapse imaging of embryos in 2010 (Pribenszky *et al.*, 2010b). Since then, time-lapse imaging of embryos has evolved as an immensely useful tool in both research and clinical use in the field of human fertility. It is, however, associated with extensive laboratory requirements. The most important components of a time-lapse system include a high-resolution microscope, a specialized culture dish that is capable of holding the embryo to minimise its movement, computer software designed to capture, analyse and record images and, finally, a non-disturbed culture system in which the whole setup is placed (Campbell and Fishel, 2015).

2.1.2 Types of time-lapse imaging systems

A number of time-lapse systems are available from commercial vendors for use in clinical IVF facilities. Every system allows variations in terms of its design and modes of operation. The key element in all of these systems is the manual or semi-automated interpretation of key events and the process of recording or annotating particular points in the pre-implantation development of embryos. The time-points at which recording or annotation take place can be defined by either the user or the system. The associated software can then be used to calculate the timing of desired events, such as the time taken by the embryo to proceed from one cleavage division to the next, or to annotate the appearance or disappearance of specific structures in a precise manner. The supplementary data that are acquired from these annotations and calculations can then be used to select embryos for potential use in further experiments, transfer or cryopreservation (Campbell and Fishel, 2015).

These commercially available time-lapse systems are manufactured by various companies, including Embryoscope® (Vitrolife), Primo Vision (Vitrolife) and EEVA (Early Embryonic Viability Assessment, Auxogyn, Inc). Of these, the most widely established technologies, the Primo Vision (Vitrolife) and EmbryoScope (Fertilitech) systems, make use of bright field technology. In comparison, the EEVA system utilizes dark field technology to carry out live time-lapse imaging. The common features of all of these time-lapse systems is a digital inverted microscope that captures images of the embryos at intervals of between 5 and 20 minutes. These images are subsequently processed using a customised image

acquisition programme and displayed on a computer screen. Finally, the images acquired at pre-set intervals are stitched into short movies that can then be rewound and fast forwarded through to facilitate a more thorough analysis.

2.1.2.1 EmbryoScope (FertiliTech, Denmark)

The EmbryoScope, which was developed by Fertilitech in Denmark, is a stand-alone incubator unit that contains a built-in microscope that can accommodate embryos from six patients in specialized slides (Vitrolife) as shown in Figure 2.A. The incubator chamber is non-humidified and consists of an internal circulation system of UV-sterilised HEPA-purified air and an activated carbon filter for volatile organic compounds. The system has a built-in low-intensity camera with red light-emitting diode (LED) (635 nm) illumination that is capable of performing steady and unhindered time-lapse imaging of the resident embryos. Water pans are located in the incubator for humidification and to help eliminate the condensation of water and associated fungal growth on surfaces. The EmbryoScope system can simultaneously monitor and image as many as 72 embryos. The system is capable of imaging the oocytes/embryos every 10-20 minutes, and the monitored embryos can be evaluated through as many as nine equidistant focal planes while being cultured within the incubator. The software accompanying the system includes modelling software that can be used to select embryos and to develop an embryonic development database via a retrospective analysis of development data. Specialised culture dishes (EmbryoSlide, Fertilitech) that can hold up to 12 embryos are used in the EmbryoScope system. A microwell with a central depression radius of 0.2 mm and that can contain 25 µl of media is used to culture each embryo (Figure 2.1 B).

The secure handling and firm grip of the slide is ensured by a vertical tail-fin, and the easy detachment of the lids is facilitated by a small fin. A distinctive identifier for each embryo in the slide is provided in the form of a micronumeral next to the bottom of the well that can be visualised through a dissection microscope. An EmbryoSlide tray can be perfectly transferred into an instrument slide holder for direct heat transfer to the wells containing culture media. The combination of the hydrophobic polymer used to fabricate the slide and the layer of immersion oil prevent the dehydration of the embryos, especially during handling and storage in laboratories with low humidity and in dry incubators. The accompanying software,

EmbryoViewer Workstation, allows the user to annotate, re-examine and draw comparisons between synchronised time-lapse videos of embryos of interest (Figure 2.1 C). A single workstation can be attached to several instruments, and the real-time monitoring of embryos from a remote location is also possible using this system. Secure and reliable data-sharing can be enabled within the facility and amongst clinics using EmbryoScope, as supported by the Zoi server. This facilitates the collection of patient data from several connected incubators and allows this information to be assimilated in a common facility for storage and analysis.

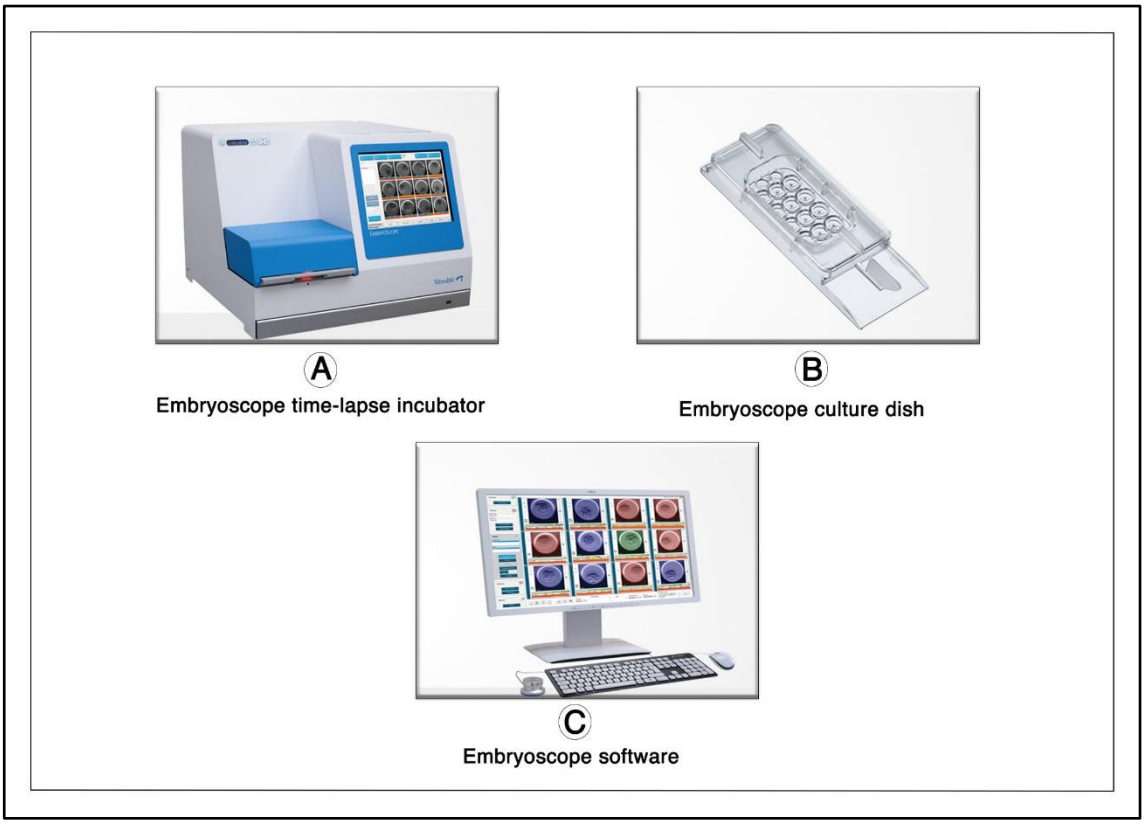


Figure 2.1: The EmbryoScope system. (A) The EmbryoScope time-lapse incubator. (B) The EmbryoSlide culture dish. (C) The EmbryoViewer software. Images were taken from (Vitrolife) website.

2.1.2.2 Primo Vision (Vitrolife)

The Primo Vision is a compacted digital inverted microscope system that is appropriately sized for installation inside different sizes of traditional incubators (Vitrolife). A controlling unit located outside the system is used to control the microscope, construct a patient database, analyse embryonic development and make decisions concerning the embryos. Multi-well dishes (Primo Vision embryo culture dish, Vitrolife) containing 9-16 wells are used to culture the embryos in the Primo Vision system. In these culture dishes, an individual drop of culture medium is used to cover each embryo well. The advantage of the Primo Vision system is that it enables a single embryo to be observed while also maintaining the benefits of group culture. As many as 16 embryos from the same patient can be cultured and monitored using the Primo Vision system. Up to six units can be connected to a controlling unit that is located outside the incubator and connected using a USB. The setup uses a low intensity green LED (550 nm) illumination system and is capable of examining the embryos in 11 focal planes. Each controlling unit is

capable of simultaneously monitoring and evaluating a total of 96 embryos (Figure 2.2).

Figure 2.2: The Primo Vision time-lapse embryo monitoring system. Image was reproduced from (Vitrolife) website.

2.1.2.3 EEVA (Auxogyn)

Similar to the Primo Vision system, the EEVA system (Eeva Test) operates via the placement of a specialised microscope inside an incubator. The EEVA system utilises dark field illumination to highlight cell membranes, and specialised EEVA dishes are used to culture the embryos. The software chooses the embryos with the maximum probability of progressing to the blastocyst phase according to the timing of the initial cleavage events up to the 4-cell stage.

2.1.2.4 Differences between various types of TLS

The main difference between the three time-lapse systems is the way in which they monitor the embryos being cultured. In the EmbryoScope system, the tray holding the slide containing the embryos is subjected to continuous movement to bring each embryo individually into the field of view. Hence, when using a fully loaded tray with 72 embryos, there is an interval of 20 minutes between two photos of any given embryo. The significant length of this interval hampers the ability of the embryologist to detect rapid changes in an accurate manner (e.g., S1: the time to complete a synchronous division, which should take <30-35 min). The continuous movement of parts, the electromagnetic effects, the presence of volatile organic

compounds and the heat released from the lubricants within the system may potentially exert negative influence on the embryos, although no such negative impacts have so far been reported. In addition, this technology enables the system to maximise resolution. In the case of the Primo Vision system, as many as 16 embryos can be continuously and simultaneously monitored without subjecting the embryos to any movement. Therefore, when using the Primo Vision system, it is possible to culture embryos in a totally unperturbed environment. Because all 16 embryos can be monitored concurrently, images can be acquired less frequently, resulting in the embryos being exposed to much lower levels of light and electromagnetic effects than in the EmbryoScope system. However, the resolution that can be achieved when using this system is not as high as that of the EmbryoScope. It should be noted that both the EmbryoScope system and the Primo Vision system expose embryos to significantly lower level of light than they are exposed to under traditional brightfield microscopy (Campbell and Fishel, 2015).

The third system, EEVA uses a different technology, dark-field illumination, to observe the embryos in culture. This method of illumination facilitates more accurate observation of the blastomere membranes, which increases the accuracy of monitoring cell divisions. However, this system provides significantly less data about intracellular morphology and reduces the ability to follow embryonic development beyond Day 2, when the number of cells increases. Because of the automated nature of the system, large fragments of embryos could potentially be falsely identified as blastomeres, thereby reducing the precision of embryo selection. Furthermore, of the three TLS systems, the EEVA, with its dark-field illumination technology, subjects the embryos to the highest light load. On the basis of observations of early markers up to Day 2 of development, the software attached to the EEVA system can indicate which embryos are most likely to develop to blastocyst stage. The use of the EEVA system has also been reported to reduce inter-observer variability and to increase accuracy when identifying the most suitable embryos (Conaghan *et al.*, 2013). Table 2.1 summarizes the features of the three different systems.

Table 2.1: Comparison of currently available time-lapse systems

Time-lapse system	EmbryoScope	Primo Vision	EEVA
Design	Incubator built-in time-lapse system	Microscope placed inside standard incubators	Microscope placed inside standard incubators
Imaging and Illumination	Bright-field Red LED	Bright-field Green LED	Dark-field
Culture dish (es)	EmbryoSlide	9-16 well Primo vision embryo culture dish	EEVA dish
Planes of view	Up to 9	11	1
Number of monitored embryos	72	96	Variable depend on the dish(es) used

2.1.3 Limitations and benefits of time-lapse technology

In spite of the relatively recent development of time-lapse technology, IVF clinics around the world have adopted its use, which often results in the addition of substantial fees ranging from several hundred to several thousand U.S dollars to the cost of fertility treatment to patients. However, reports of time-lapse imaging in the mainstream media have been very positive, based on preliminary journal publications (Campbell *et al.*, 2013; Devlin, 2013; Sample, 2013; Armstrong *et al.*, 2015). The hype surrounding this technology results from the tenuous claim that it can triple the success rate of IVF treatment and increase the rate of live births by 78%, with scant regard for the limitations of the study design described in the publications that are describing the effectiveness of these newly developed technologies (Campbell *et al.*, 2013; Devlin, 2013; Sample, 2013; Armstrong *et al.*, 2015). The manufacturing industry that is behind these time-lapse systems has supported the extensive adoption of these technologies by citing 'improved success rates' and the merits of 'bringing the latest technology to patients' and 'adding value to the treatment cycle' (FertiliTech). There is, however, an absence of robust evaluation.

In addition to embryo monitoring, time-lapse technology can also be used in several other applications in an IVF laboratory. For instance, it can be used to validate traditional static assessment protocols, to compare the effect of impactful variables, such as the culture media or drug regimens, on morphokinetics, to forecast

embryonic viability and outcomes, to examine transient or anomalous morphological observations, and to assure quality control. These are only some of the applications that can benefit from the use of time-lapse systems.

2.1.4 The use of time-lapse systems as validation systems

In terms of splitting embryos, in spite of evidence showing the successful production of healthy offspring in, for instance, farm animals and mice, there is still a burden of proof on the scientific community to demonstrate that this technology is safe in humans and that there are no potential risks to offspring conceived using this method. Time-lapse imaging is one way to obtain such evidence. In relation to the procedural risks associated with embryo splitting, there are similarities with the blastomere biopsy procedures that are involved in PGD, which is used to select healthy embryos for couples who have a well-defined genetic risk factor. Blastomere biopsy is used in these instances to obtain embryonic DNA for analysis, and it has historically been the most frequently employed method for this purpose (Harper and Harton, 2010). Whether it is used for PGD or during embryo splitting, a blastomere biopsy is an invasive procedure, and both techniques result in the disruption of cell adhesion and the breaching of the ZP to isolate one/two blastomere (in PGD) or half the number of blastomeres for transfer into a previously empty ZP (in embryo splitting). Kirkegaard *et al.* (2012b) used a time-lapse system to monitor human embryonic development after using blastomere biopsy for PGD and found that blastomere biopsy resulted in the prolongation of biopsied cell stages and a delay in compaction that was accompanied by altered embryo hatching.

An early study by Hardy *et al.* showed that blastomere biopsies used for PGD resulted in a reduced cell number in both the TE and the ICM in the resulting blastocyst, but no effect on development (Hardy *et al.*, 1990). In relation to splitting, recording the time point at which an embryonic stage is initiated allows a thorough comparison with the donor embryo (Twin A), from which half the blastomeres are removed and then placed into recipient B (Twin B), the ZP of which was previously emptied. The two Twin embryos can then be compared to a normal control that was obtained at the same developmental stage to obtain

valuable information about any developmental differences that might occur between the embryos.

Taken together, these studies explore and evaluate broader applications for blastomere biopsy, which is currently used for PGD, and widen the availability of evidence in the scientific literature regarding morphokinetics. These data led to conclude that this technology would be an optimal tool for analysing the quality of split embryos. Therefore it was chosen as a validation tool for this study.

2.2 Validation method II: Study of the appearance and localization of lineage-specific transcription factors in both ICM and TE using immunocytochemistry

Human embryonic development begins in a relative transcriptional silence in which the oocyte ceases gene expression while undergoing meiotic maturation. The transition from zygote to embryo begins with the intermingling of chromosomes on the metaphase plate of the first mitotic division. This is followed by sequential cleavage divisions and culminates in major embryonic genome activation (EGA) on day 3 during human development (Braude *et al.*, 1988; Dobson *et al.*, 2004). EGA results in the generation of novel transcripts/proteins that were not expressed in the oocyte that allow further development. After day 3, 8-cell embryos undergo a process of compaction that gives rise to a tightly packed cell mass called a morula. The transition from morula to blastocyst is the starting point of morphologically visible cell differentiation and lineage segregation. Genes encoding transcription factors, epigenetic modifiers and chromatin remodelling factors are upregulated at this time. During the early blastocyst stage, the first lineage to form is the TE, an extra-embryonic epithelial monolayer of cells that surrounds the ICM. By the time implantation occurs (late blastocyst stage), a second extra-embryonic layer forms at the ICM surface called the primitive endoderm (PE). The ICM is composed of plenipotent cells, and these cells maintain their pluripotency as they mature into epiblasts (EPIs). The ICM ultimately gives rise to the three germ layers (ectoderm, endoderm and mesoderm) and germ-line cells during gastrulation. The positioning of plenipotent cells at the inner or outer part of the morula for later differentiation has not been well described. The exposure of outer cells to the outer environment is likely to be the cause of the series of molecular events that leads to the activation

of different signalling pathways in these cells than those the inner cells undergo, with the result being that these cells take on different fates.

Our understanding of early mammalian lineage specification and its importance to subsequent developmental events is largely based on the results of murine studies. For instance, such studies have shown us that the TE and PE are important for successful implantation into the maternal uterus, and they provided information about axis specification and nutrient transfer (Boroviak and Nichols, 2014). Thus, founder cells are needed by the foetus, and the differentiation of extra-embryonic tissues is required for implantation, nutrition and patterning, which are themselves necessary for normal development and for the embryo to successfully implant in the maternal uterus (Boroviak and Nichols, 2014). Once the cells have committed to a particular lineage, a state of 'naïve' pluripotency must be maintained to retain the capacity of the cells to develop into the multiple tissue types that are present in a fully formed organism (Nichols and Smith, 2009). In other words, they must not prematurely differentiate so that they can remain susceptible to the positioning and temporal patterning signals that are required for further development (Nichols and Smith, 2009). Thus, the concept of a 'developmental clock' (Noli *et al.*, 2015b) to govern the appropriate timing of differentiation is important during implantation and development to term and can therefore also be used as a marker of the quality of split embryos compared to normal control embryos. Hence, although mouse embryos are able to compensate for disruptions in cell numbers and structures, with split embryos leading to successful live births, disturbances to the formation of a normal TE lineage can lead to implantation failure (Chawengsaksophak *et al.*, 2004; Strumpf *et al.*, 2005).

In mice, the three rounds of cleavage that follow zygote formation produce an 8-celled embryo made of plenipotent blastomeres that retain the capacity to become all embryonic and extraembryonic lineages (Tarkowski and Wroblewska, 1967; Kelly, 1977; Suwinska *et al.*, 2008). After this stage, the first major differentiation occurs. This occurs during compaction, where the area of cell-cell contact is increased through an E-cadherin-dependent process, resulting in the formation of the morula (Johnson and Ziomek, 1981; Pratt *et al.*, 1982; Larue *et al.*, 1994; Fierro-Gonzalez *et al.*, 2013). At the 16-cell stage, the morula has a layered structure, with the outer layer forming the TE during subsequent development

stages (Tarkowski and Wroblewska, 1967) and the inner cells forming the ICM, and with the appropriate polarity that is required for correct lineage specification during development (Plusa *et al.*, 2005; Alarcon, 2010). The formation of the blastocyst through cavitation (Smith and McLaren, 1977) includes the transition of the inner cells from totipotency to plenipotency and pluripotency, whilst the outer cells commit to a TE lineage, resulting in the loss of their potential to develop into other lineages. Blastocyst formation occurs around the 30-cell stage, a stage during which the embryonic-abembryonic axis is also formed (Smith and McLaren, 1977). Subsequently, some ICM cells begin to differentiate into the PE (the equivalent of the hydroblast in other species) (Saiz and Plusa, 2013). This stage is therefore critical, since correct TE commitment is vital for implantation, and these cells also form the placenta, whereas the PE forms the yolk sac and is central to axis signalling during gastrulation (Gardner and Johnson, 1972; Gardner *et al.*, 1973; Copp, 1979; Gardner and Rossant, 1979; Gardner, 1983). Each stage of development is controlled by a complex array of transcription factors, including factors required for lineage segregation into TE, PE and EPI. Some of the key regulatory pathways involved in these processes are particularly well defined in mouse models.

In terms of lineage segregation, in the mouse, the first segregation arises from the reciprocal inhibition of the homeodomain proteins POU class 5 homeobox 1 (OCT4/POU5FI) and Caudal type homeobox 2 (CDX2) in the ICM and TE (Niwa *et al.*, 2005; Ralston and Rossant, 2005; Strumpf *et al.*, 2005; De Paepe *et al.*, 2014), and the second arises from interactions between NANOG and GATA binding protein 6 (GATA6) that result in the formation of the PE and EPI (Chazaud *et al.*, 2006). Some of the major regulatory pathways that are responsible for lineage segregation have been determined, including Hippo signalling (Nishioka *et al.*, 2008; Nishioka *et al.*, 2009), and various models have been proposed to explain these processes. The stochastic model proposes that the lineage segregation rely on variations in the levels of NANOG, POU5FI and CDX2 (master proteins) between blastomeres and that the subsequent sorting of cells or changes in their positioning are dependent on overall differences in gene expression (Dietrich and Hiiragi, 2007). These mechanisms are associated with the first differentiation during the compaction stage, in which blastomeres move considerably during each cleavage stage (Kurotaki *et al.*, 2007). Similar processes are involved in the second

differentiation event, which is caused by a "salt and pepper" or random pattern of gene expression of EPI- and PE-related transcription factors (e.g., NANOG and GATA6) in the ICM. Cells subsequently segregate into different cell lineages (Chazaud *et al.*, 2006). A different model explains lineage segregation in terms of cell position, where cells on the outside of the embryo develop into the TE, while inner cells develop into the ICM (Tarkowski and Wroblewska, 1967). This model, which is appropriately named the "inside-outside model", proposes that differences in the surrounding milieu of each cell group and associated differences in cell-cell contacts influences the commitment of cells to specific fates. A third model, the cell polarity model, associates lineage segregation with polarity. This model suggests that cellular polarisation is the result of differences in the levels of expression of transcription factors, with blastomeres at the 8-cell stage experiencing increased cell-cell contacts during compaction and exhibiting polarisation along the apical-basal axis (Johnson and McConnell, 2004). Polarisation during the subsequent two divisions (from 8 to 16 cells and from 16 to 32 cells) is therefore affected by the orientation of the cleavage plane in the blastomeres, with symmetric divisions giving rise to outer cells that are polarized and asymmetric divisions producing apolar inner cells and polar outer cells. Polar cells subsequently develop into the TE, and apolar cells develop into the ICM before ultimately differentiating into the PE and EPI. It seems likely that a hybrid of polarity and positional models could explain lineage segregation, with gene expression patterns that are associated with specific lineages being influenced by both the position of the cell and cell-cell contacts (Lorthongpanich *et al.*, 2012). A final model has suggested that the sequential waves of cell divisions direct lineage segregation, with the first phase leading to the EPI and the second to the PE (Bruce and Zernicka-Goetz, 2010).

In human embryos, most maternally derived RNA breaks down during the 2- and 4-cell stage, with the remaining transcripts gradually disappearing over time (Dobson *et al.*, 2004; Vassena *et al.*, 2011). There are then three waves of transcription during the 2-, 4-, and 8-cell cleavage stages (Braude *et al.*, 1988; Vassena *et al.*, 2011). These may affect the balance between totipotency and differentiation, and several studies have analysed the temporal and spatial localization of transcription factors that are associated with lineage segregation during preimplantation development (Cauffman *et al.*, 2005; Cauffman *et al.*, 2009; Niakan and Eggan, 2013). Detecting the transcription factors that are associated

with the TE lineage (e.g., CDX2) is only possible from the blastocyst expansion stage onwards in the outer layer of cells (Niakan and Eggan, 2013) (Niakan and Eggan, 2013), and little is known, in general, about commitment to the TE lineage in humans. The role of Yes associated protein 1 (YAP1) in human embryonic development is also poorly understood (Kuijk *et al.*, 2015), although it is known to be activated when fibroblasts are reprogrammed into induced pluripotent stem cells (Lian *et al.*, 2010), indicating that it could be involved in the development of pluripotency. In terms of the ICM, the expression of the markers NANOG, POU51 and SRY-related HMG-box (Sox)-containing protein (SOX2) has been described in hESCs (Hyslop *et al.*, 2005), with studies demonstrating that they bind to their own promoters, thereby providing a regulatory mechanism for maintaining pluripotency and self-renewal (Boyer *et al.*, 2005). In developmental terms, NANOG is observed only at the full/expanding blastocyst stage, in the nuclei of some ICM cells (Hyslop *et al.*, 2005; Cauffman *et al.*, 2009; Niakan and Eggan, 2013). POU5FI is found in the inner and outer cells during compaction and at the blastocyst stage in both the ICM and TE (Cauffman *et al.*, 2005; Niakan and Eggan, 2013), whereas SOX2 is expressed from the 8-cell stage but is only found in the inner cells at compaction or in the ICM at the full blastocyst stage. By the second differentiation, GATA6, GATA binding protein 4 (GATA4) and SRY-box 17 (SOX17) are detectable in progenitor PE cells in human expanded blastocysts (Kuijk *et al.*, 2012; Roode *et al.*, 2012), a stage at which NANOG expression is elevated and GATA6 expression decreases in some inner cells. After hatching, the segregation of PE and EPI cells is indicated by differences in the expression of GATA6 and NANOG (Kuijk *et al.*, 2012; Roode *et al.*, 2012). SOX17 is first detected in early blastocysts (Niakan and Eggan, 2013). At the expanded blastocyst stage, there is a high level of SOX17 in the nuclei of all ICM cells, whereas in hatched blastocysts SOX17 expression is restricted to the putative PE within the ICM (Niakan and Eggan, 2013; De Paepe *et al.*, 2014).

A major difference between mouse and humans is in the timing of key developmental stages, starting with EGA initiation, which occurs at the 8-cell stage in humans (Braude *et al.*, 1988), and between the 1- and 2-cell stage in mice (Aoki *et al.*, 1997). Blastocyst formation also occurs later in humans (4-5 days post-fertilisation or dpf) (Hertig *et al.*, 1959; Steptoe *et al.*, 1971) than in mice (3-3.5 dpf). Furthermore, human embryos undergo an additional cell division prior to

implantation at 6-8 dpf. Consequently, although the transcription factors associated with lineage segregation are largely the same in humans and mice, the localization and timing of their expression are different (Kimber *et al.*, 2008; Cauffman *et al.*, 2009; Bernardo *et al.*, 2011; Roode *et al.*, 2012; Niakan and Eggan, 2013; Blakeley *et al.*, 2015). For instance, the response to the activation or inhibition of fibroblast growth factor (FGF) signalling, which is thought to be central to EPI/PE lineage specification in mice, is different in humans (Kuijk *et al.*, 2012; Roode *et al.*, 2012). Another significant difference is in the localization of the TE-associated transcription factor, CDX2, which is involved in the lineage specification of the TE in mice (Strumpf *et al.*, 2005) and humans (Bernardo *et al.*, 2011; Niakan and Eggan, 2013; Blakeley *et al.*, 2015) but is not detected at the human morula stage or in early blastocysts when the TE is visible. The timing of the expression of the EPI transcription factors NANOG and SOX2 also differs between mice and humans, with SOX2 transcripts detected at the 4-cell stage in mice (Kimber *et al.*, 2008) and at the compacted morula stage and in the ICM of early blastocyst stage embryos in humans (Cauffman *et al.*, 2009). NANOG has been shown to co-localise with Cyclin E1 (CCNE1) in the ICM of expanding human blastocysts, with NANOG expressed in only a sub-set of ICM cells at the mid-blastocyst stage (Roode *et al.*, 2012) and CCNE1 expressed throughout early development from the 4-cell stage and playing an important role in hESC derivation (Krivega *et al.*, 2015). By the end of the preimplantation stage of development, the localization of key lineage-specific transcription factors is similar in mice and humans, although the length of this stage differs between the two species. Thus, the appropriate timing for an analysis of these markers is during the preimplantation stage.

Ethical concerns and the paucity and inconsistency in the quality of embryos produced *in vitro* make direct observations of human pre-implantation development a challenging task. Several factors contribute to the completion of a successful pregnancy after IVF. In addition to the health of the mother, the developmental potential of the embryo is a key factor, and it, in turn, depends on the successful formation of all three embryonic lineages. A reduction in number of cells in any of the first three cell lineages in the developing mammalian blastocysts has a profound effect on further development. It has been reported in many such cases that embryos in this condition are unable to progress beyond the

implantation stage (Feldman *et al.*, 1995; Nichols *et al.*, 1998; Mitsui *et al.*, 2003; Chawengsaksophak *et al.*, 2004; Ralston and Rossant, 2008; Kang *et al.*, 2013; Schrode *et al.*, 2014; Wicklow *et al.*, 2014). A significant challenge in ART is the identification of human embryos with the most promising developmental potential (Filho *et al.*, 2010). There is currently no consensus regarding the most accurate methods for assessing embryo quality. However, in theory, all assessment methods rely on regular evaluations of embryo morphology, which are carried out in the IVF laboratory using either automatic (time-lapse imaging) or semi-automatic analyses of microscope images (Filho *et al.*, 2010; Montag *et al.*, 2011). However, the generation of a morphologically normal blastocyst does not definitively imply that correct lineage formation has occurred. Mutant mouse embryos with incorrectly specified early cell lineages cannot be visually distinguished from wild-type littermates until they reach the blastocyst stage; however, they are unable to develop beyond the peri-implantation stage (Nichols *et al.*, 1998; Mitsui *et al.*, 2003; Chazaud *et al.*, 2006; Nishioka *et al.*, 2008; Kang *et al.*, 2013; Schrode *et al.*, 2014; Wicklow *et al.*, 2014).

Thus, if we understand the timing of key events in both humans and mice and extrapolate on the data available from lineage segregation studies in mice to assess the impact of disrupting implantation and subsequent developmental events, then performing an immunocytochemical analysis of the appearance and localisation of lineage-specific transcription factors during specific stages of development should provide reliable information related to subsequent human developmental potential. This approach is necessary due to the obvious presence of strong ethical and medical concerns that are related to testing for correct lineage specification in human embryos that are meant for implantation, and there has been considerable difficulty in determining the relationship between failed lineage segregation and failed pregnancies following embryo transfer.

Furthermore, this type of analysis contributes to our ability to increase the rate of successful human pregnancies by developing optimal conditions for culturing embryos in non-human animals to promote correct lineage specification and, consequently, high quality embryos. Many published articles have reported that *in vitro* culture conditions have a significant influence on lineage allocation and lineage-specific gene expression in preimplantation embryos in mammals. For

instance, rabbit embryos cultured *in vitro* demonstrate increased transcription levels of *NANOG* and *SOX2* (Henderson *et al.*, 2014), and *POU5F1/OCT4* (Saenz-de-Juano *et al.*, 2013) than *in vivo*-derived embryos. The medium used to culture the embryos can be renewed to avoid this effect (Saenz-de-Juano *et al.*, 2013). In a recent report, a study using *in vitro*-derived equine embryos indicated that the composition of the culture medium, and specifically the glucose concentration of the medium, can influence the allocation of cells to particular lineages in pre-implantation embryos (Choi *et al.*, 2015). To the best of our knowledge, the impact of various culture paradigms on lineage formation has not yet been thoroughly evaluated in human embryo culture systems. This is partly the result of the fact that lineage specification data in humans remains very scarce, and that no 'gold standard' for studying lineage development in pre-implantation human embryos has therefore been decided upon.

In 2009, it was reported that as many as over 40% of deliveries following IVF in the USA involved twin or multiple births. In standard IVF cycles, the usual practice is to transfer multiple embryos to increase the probability of pregnancy. However, multiple pregnancies carry an additional risk of pre-eclampsia, maternal haemorrhaging, pre-term labour, uterine rupture and the need for operative delivery (Crosignani and Rubin, 2000). Currently, complications associated with IVF are circumvented by transferring a smaller number of embryos (preferably a single embryo) into the uterus during each IVF cycle (Filho *et al.*, 2010; Center for Disease Control And Prevention (CDC), 2012; US Department of Health and Human Services (HHS), 2014). As a result, a system is needed that would allow viability and lineage formation to be reliably assessed in the embryos that are to be transferred. Several research groups have addressed some aspects of this process. However, more studies need to be performed to establish what precisely constitutes a healthy human embryo. Because ethical concerns restrict experimentation with human embryos, early human embryology can be best understood only by extrapolating data obtained from studies of development in non-human mammalian species. However, relying on data from mouse models alone is insufficient, because several features of pre-implantation development and lineage formation are significantly different between mice and humans. Finding an appropriate mammalian model for studying human embryonic development is therefore an unsolved challenge in the field of developmental biology.

A thorough analysis of the temporal and spatial patterns of protein localisation during human pre-implantation developmental stages has been reasonably well established (Niakan and Eggan, 2013). A complete analysis of the molecular mechanisms that are involved in the process of lineage restriction in twin blastocysts that are formed from embryo splitting would help us to understand whether these embryos are developmentally normal. Given that extensive studies in mice have demonstrated that restricted development, including implantation failure, can occur when lineage segregation is disrupted, and given the difficulty of replicating similar studies in human models, an immunocytochemical analysis of the expression of key developmental regulators was determined to be an optimal validation method for this study.

2.3 Validation method III: Derivation of human embryonic stem cells

The presence and correct functionality of embryonic stem cells (ESCs) in developing embryos are vital prerequisites for normal early development to occur, since their capacity for pluripotency is required for blastocysts to develop into the many different tissue types that are present in a fully formed organism. ESCs therefore provide a potential experimental model that could be used as a marker of normal embryogenesis.

ESCs have different pluripotent states: naïve and primed (Nichols and Smith, 2009; Van der Jeught *et al.*, 2015). In mice, mESCs exist in an uncommitted naïve state, whereas hESCs exist in a more advanced state (Van der Jeught *et al.*, 2015). Naïve ESCs are thought to exhibit greater pluripotency compared to that of primed ESCs due to their capacity to generate chimeras when reintroduced into the pre-implantation blastocyst (Bradley *et al.*, 1984; Gu *et al.*, 2012). Female naïve stem cells maintain both X chromosomes active (XaXa) and undergo reduction in global DNA methylation (Rossant, 2008; Bao *et al.*, 2009; Hayashi and Surani, 2009; Han *et al.*, 2010; Nichols and Smith, 2011; Van der Jeught *et al.*, 2015). Naıve ESCs are also less prone to primordial germ cell (PGC) differentiation in vitro (Van der Jeught *et al.*, 2015). Conversely, primed mEpiSCs are very inefficient in generating chimeras, as female ESCs have already been through X chromosome inactivation (XiXa), and they exhibit an increase in global DNA methylation. The capacity for

naïve pluripotent stem cells to self-renew is dependent on leukaemia inhibitory factor (LIF) and bone morphogenetic protein (BMP), whereas the self-renewal capacity of primed pluripotent stem cells results from basic fibroblast growth factor (bFGF) and Activin (Rossant, 2008).

Characteristics of naïve pluripotency include the transcription of Oct4 (also known as Pou5f1) driven by its distal enhancer, retention of a pre-inactivation X chromosome state, and a reduction in global DNA methylation as well as a lowered H3K27me3 repressive chromatin mark on developmental regulatory gene promoters (Gafni *et al.*, 2013). Epigenetic properties associated with naïve pluripotency include the use of the proximal enhancer element to maintain OCT4 expression, the increased likelihood of inactivation of the X chromosome in the majority of female human ES cells, a higher level of DNA methylation, including significant levels of H3K27me3, and bivalent domain acquisition on lineage regulatory genes (Gafni *et al.*, 2013). In mice, the embryonic stem cells in the naïve pluripotent state cause a more robust development potential compared to primed epiblast cells. Attempts to isolate and derive hESC lines in a similar naïve pluripotent state have been successful only recently (Ware *et al.*, 2014; Duggal *et al.*, 2015; Pastor *et al.*, 2016; Xu *et al.*, 2016; Zimmerlin *et al.*, 2016). However, more work remains to gain a clear insight into the molecular mechanisms governing the naïve pluripotency of hESC lines as well as to define the derivation strategy, and more importantly, maintenance conditions.

The first report to describe the derivation of ESCs from the ICM of blastocyst-stage mouse embryos was published by Evans and Kaufman in 1981 (Evans and Kaufman, 1981). The evolution of this research as well as successful derivation of ESCs from non-human primates led to the derivation of the first hESC line that was obtained from the ICM of pre-implantation blastocyst-stage human embryos in 1998 (Thomson *et al.*, 1998). The early adoption of this technique involved using ICM cells to derive hESC lines (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000), with the best rate of derivation being from Day 6 blastocysts (Chen *et al.*, 2009). Single blastomeres obtained from 4- and 8-cell stage embryos have also been used to generate hESC lines, indicating that these early blastomeres possess pluripotency (Klimanskaya *et al.*, 2006; 2007; Feki *et al.*, 2008; Geens *et al.*, 2009; Ilic *et al.*, 2009). hESCs derived from either the ICM or blastomeres possess similar

transcriptional profiles, implying that a similar precursor cell in the embryo gives rise to the hESC line during *in vitro* culture and derivation (Giritharan *et al.*, 2011; Galan *et al.*, 2013).

hESCs, like mouse ESCs (mESCs), display a high nucleus to cytoplasm ratio and tend to grow as small colonies. However, hESC colonies have a morphology that is distinct from that of mESCs. ESCs possess two essential and fundamental features. First, they have an unlimited capacity for self-renewal, and they undergo a number of symmetric cell divisions to maintain their undifferentiated state and plenipotency. The self-renewal process in ESCs is controlled by certain intrinsic factors, such as the transcription factors Pou5f1 and Nanog, and other external influences, such as extrinsic growth factors like Basic Fibroblast Growth Factor; bFGF. Second, ESCs have demonstrated pluripotent potential, which indicates their ability to differentiate into the three main embryonic germ layers: endoderm, ectoderm and mesoderm. Therefore, hESCs can potentially give rise to all the cell types found in the body. The evidence for hESC pluripotency has been gathered from *in vitro* assays aimed at studying the differentiation of hESCs during the formation of embryoid bodies, which contain derivatives of all three germ layers, via a mechanism that is, to a certain degree, reflective of the process of embryonic development. *In vivo*, the pluripotent nature of hESCs is demonstrated by the formation of teratomas when ESCs were injected into severe combined-immunodeficient (SCID) mice and the formation of chimaeras when ESCs were injected into pre-implantation embryos (in experiments involving rodent ESCs).

Because hESCs do not appear in this form during native embryonic development *in vivo*, they can be regarded as artefacts of culture. Upon *in vivo* transplantation, pluripotent hESC cells form a teratoma rather than a foetus. This is because the *in vitro* derivation of hESC lines results in the loss of the spatio-temporal cues that are necessary for normal *in vivo* development, suggesting that their ability to contribute to the formation of extraembryonic tissue may not have been retained. Notwithstanding these data, hESCs recapitulate several *in vivo* early developmental processes and are therefore a valuable tool for studying early mammalian development.

Pluripotent hESCs can be used in two significant applications. First, they possess remarkable potential for deployment in cell-based therapies. Second, they can also

act as a suitable model for studies aimed at increasing our understanding of early developmental processes. Apart from embryo-derived ESCs, somatic cells have also been used to generate pluripotent stem cells using two techniques. The first, somatic cell nuclear transfer, involves replacing the nucleus of the oocyte with the nucleus of an adult cell that was derived from a donor. Cell division can then be stimulated in the fused cell, resulting in the formation of an embryo from which ESCs can be derived (Tachibana *et al.*, 2013). More significantly, somatic cells can be reprogrammed and induced to become undifferentiated, pluripotent cells known as induced pluripotent stem cells (iPSCs). For example, the retrovirus-mediated introduction of *POU5F1, SOX2*, Kruppel-like factor 4 (*KLF4*) and v-myc myelocytomatosis viral oncogene homologue (*C-MYC)* into human dermal fibroblasts (Takahashi *et al.*, 2007), or *POU5F1, SOX2, NANOG* and lin-28 homologue (*LIN28*) into human foetal fibroblasts (Yu *et al.*, 2007) led to the formation of iPS cells.

hESC pluripotency is maintained *in vitro* by important transcription factors that play key roles in embryonic development. Some of these are the homeodomain proteins POU5F1, NANOG, and SOX2. *SOX2* and *POU5F1* bind the *NANOG* promoter, thereby contributing to its regulation, to some degree (Rodda *et al.*, 2005). *NANOG, SOX2 and POU5F1* are believed to regulate as many as 350 genes in hESCs by binding to their promoter regions (Boyer *et al.*, 2005). *POU5F1* can be detected throughout murine and human oocyte development and preimplantation development (Rosner *et al.*, 1990; Hansis *et al.*, 2001; Cauffman *et al.*, 2005; Kimber *et al.*, 2008). Furthermore, it is extremely important for retaining pluripotency in hESCs (Nichols *et al.*, 1998; Niwa *et al.*, 2000; Bodnar *et al.*, 2004). The downregulation of *NANOG* results in the subsequent down-regulation of *POU5F1* and the loss of hESC cell-surface antigens (Hyslop *et al.*, 2005). During human development, *SOX2* transcripts are expressed starting during the four-cell stage (Kimber *et al.*, 2008), whereas in mice, *Sox2* transcription starts at the advanced morula stage (Avilion *et al.*, 2003). Intriguingly, a maternal component of *Sox2* has been implicated in decisions that are made during early cell fate patterning events in mouse development (Avilion *et al.*, 2003). *NANOG* transcripts have been detected in pronuclear human embryos after the eight-cell stage (Kimber *et al.*, 2008). *NANOG* appears at the later stages during morula development in mice (Dietrich and Hiiragi, 2007) and its expression has been

observed in both human and mouse ESCs.

Several extrinsic influences are involved in regulating the expression of these transcription factors and the cues that lead to either self-renewal or differentiation. These external signals can be provided by the underlying feeder cells, foetal calf serum in the culture medium or as growth factors via exogenous supplementation. Some of these factors include bFGF, insulin-like growth factor, heparin sulphate proteoglycans (Koivisto *et al.*, 2004; Bendall *et al.*, 2007; Levenstein *et al.*, 2008), Notch (Chiba, 2006; Zhang *et al.*, 2007), activin A (Beattie *et al.*, 2005), and Wnt proteins (Villa-Diaz *et al.*, 2009). In addition, it has been reported that epigenetic mechanisms are crucial to maintaining the pluripotent nature of hESCs and that their differentiation is regulated by these mechanisms (Surani, 2001). Epigenetic reprogramming occurs in two crucial stages during embryonic development: gametogenesis and the pre-implantation embryonic stage (Reik *et al.*, 2001; Reik, 2007; Reik and Kelsey, 2014). hESCs possess a unique epigenetic signature that has been convincingly linked to the global permissivity of gene expression and the pluripotency of hESCs (Atkinson and Armstrong, 2008).

Because the derivation of hESCs and associated analyses have provide a valuable information about normal embryonic development at the molecular level, it was judged that they would, in combination with the immunocytochemical analysis of lineage-associated transcription factors, provide a robust method for evaluating embryo quality. Since hESCs provide a potential experimental model that could be used to assess pluripotency in embryos and an analysis of transcription factors that are associated with lineage segregation would collectively provide a method for evaluating whether correct cell fate decisions have been made in the embryo, these analytical tools were used as complimentary markers of the developmental potential of the embryos. Hence, for this study, analysis of hESCS was chosen in combination with immunocytochemistry as suitable methods for validation.

2.4 Validation method IV (a): Transcriptomics

In contrast to the genome, which is similar in all of an organism's cells (apart from specific cell lineages, such as beta-lymphocytes or mutated cells), the transcriptome is extremely dynamic. More precisely, the transcriptome represents the total RNA content of the cells, including mRNA, miRNA, piRNA, tRNA, rRNA and non-coding

RNAs, and it provides a snapshot of the transcriptional and regulatory profile of a cell at a specific time.

Because the transcriptome represents the output of transcripts in a cell, it can be used to describe the intracellular conditions that underlie different physiological and developmental processes. Therefore, transcriptomic studies at a molecular level have been performed in an attempt to determine the fundamental transcriptional structure of genes by gaining descriptions of their 5' and 3' ends and the post-transcriptional modifications they are subjected to. From a spatial and temporal perspective, transcriptomic assays are used with the aim of creating libraries of transcripts at crucial time points throughout development and under both healthy and disease conditions (Beane *et al.*, 2011; Qian *et al.*, 2013).

Because transcriptomic studies have shown great potential, several methods have been developed or used to perform research in this field. In the past, microarrays were widely used to study the transcriptome. Microarrays are two-dimensional arrays of single, short, fluorescently labelled DNA strands (probes) that are anchored on a solid substrate. When a cDNA sample is loaded on a microarray chip, the complementary strands hybridise, and a signal can be detected (Allison *et al.*, 2006). The analysis of the resulting signal provides information about the expression of genes of interest. Microarrays are an inexpensive and high-throughput technology that has been very well defined and discussed in the scientific literature. However, even though microarrays have the potential to simultaneously analyse a high volume of genes, the greatest disadvantage of the method lies in the fact that the probes have to be predefined (Marioni *et al.*, 2008). Microarrays are therefore most suitable for measuring the expression levels of genes that are already well studied. Additional disadvantages of the method include high noise in the readings, which can result from unspecific binding (Okoniewski and Miller, 2006; Royce *et al.*, 2007), and difficulty in comparing the expression levels of different transcripts in the same sample (Marioni *et al.*, 2008).

To meet the need for a more sophisticated method to decipher the transcriptome, RNA sequencing; (RNA-Seq) was developed based on next-generation sequencing (NGS) technologies (Metzker, 2010). RNA-Seq can accommodate, to a greater extent, the complicated needs of transcriptomics research. First, it allows the highly accurate measurement of expression levels and the ability to make

comparisons between samples and between genes within the same sample (Mortazavi *et al.*, 2008; Nagalakshmi *et al.*, 2008). In addition, it supports the current need for gaining information at a genetic level because it can be used to detect alternate splicing events (Morin *et al.*, 2008; Pan *et al.*, 2008) and alternate transcription initiation sites (Nagalakshmi *et al.*, 2010). The latter of these two entities developed during evolution to increase the complexity of the genome.

Furthermore, RNA-Seq can be used to study allele-specific expression (Degner *et al.*, 2009), gene fusion events (Edgren *et al.*, 2011), exon/intron boundaries and 5' and 3' ends (Wang *et al.*, 2009b). Finally, RNA-Seq can be used to detect novel or rare transcripts (Guttman *et al.*, 2010; Cabili *et al.*, 2011) and sequence variations, such as single nucleotide polymorphisms (SNPs) (Cloonan *et al.*, 2008; Morin *et al.*, 2008), unlike microarrays, which can only be used to detect genomic variations in previously well-characterised genes.

In mammals, during the pre-implantation stages of development, there is a step-wise decay of maternally stored RNAs that occurs simultaneously with the dramatic induction of new transcripts from the embryonic genome via a mechanism known as zygotic (or embryonic) genome activation (ZGA or EGA) (Schultz, 2002; Schier, 2007; Walser and Lipshitz, 2011). Determining gene expression profiles during human pre-implantation embryonic development has been hindered by the limited availability of blastomere samples and the quality of available quantitation platforms (Dobson *et al.*, 2004; Zhang *et al.*, 2009; Xie *et al.*, 2010; Vassena *et al.*, 2011). However, with the dramatic improvements that have occurred in RNA-sequencing technologies, such as single-cell RNA-Seq (Tang *et al.*, 2010; Islam *et al.*, 2011; Hashimshony *et al.*, 2012; Ramskold *et al.*, 2012), there is now an unparalleled opportunity to study the regulation of genes during early human development at a high resolution. These novel developments in the field of single-cell RNA seq-based approaches to transcriptomics have provided researchers with more extensive means to increase our understanding of the transcriptional programmes that control human embryogenesis (Xue *et al.*, 2013; Yan *et al.*, 2013; Piras *et al.*, 2014).

Although previously conducted studies have analysed differences between the transcriptomes of mouse and human pre-implantation embryos (Xue *et al.*, 2013; Piras *et al.*, 2014), studies aimed at increasing our understanding of the

mechanisms that underlie lineage specification have been limited. Other studies have made use of microarrays to analyse whole embryos, but the inherent heterogeneity among cells makes it complicated to identify gene expression profiles that are specific to particular cell types (Zhang *et al.*, 2009; Xie *et al.*, 2010; Madissoon *et al.*, 2014). In a recently published study, Blakeley *et al.* (2015) report the significant differences between early human and mouse development and gained insights into the process of human embryogenesis and its relationship with stem cells using a single RNA-seq approach.

Thus, given the aforementioned requirement to establish the safety of embryo splitting, a highly sensitive and reproducible method, such as RNA-seq could potentially be used to examine the effects of different procedures on the transcriptomic profile of the resulting twin embryos and to compare the study data to a profiles derived from non-manipulated human embryos. However, because this type of analysis of mRNA does not allow the researcher to verify the expression of the associated protein, which can only be confirmed using immunocytochemistry, this method was not ultimately chosen as a validation tool for this study.

2.5 Validation method IV (b1): Epigenetics, DNA methylation

Human embryogenesis includes the processes, such as cell division and differentiation that take place during the early phases of development. In biological terms, these processes involve the growth of the embryo from a newly fertilised egg, or zygote, to an adult human being. Fertilisation is said to occur at the point at which a sperm cell penetrates an oocyte, and in mammals, the resulting process of early embryonic development is highly complex. Fertilisation is followed by a sequence of precisely controlled events that result in the formation of a multicellular organism that consists of different types of cells. Although every cell in the human body contains an identical genome, there are many different types of cells with various physiologies and properties. Each cell type develops from a totipotent cell derived from the embryo and progresses towards an increasingly specialised pathway. Totipotent cells are able to develop into a new organism (integrated body plan) and produce offspring. These cells ultimately differentiate into multiple cell types, whilst retaining the same underlying DNA sequence; that is the same as the zygote from which it originally derived (with the exception of

mammalian B and T cells). Different cells carry out diverse functions due to the tissue-specific patterns of gene expression that are put in place during the course of development. Once cell-fate is ascertained through epigenetic mechanisms, it is rigorously maintained in all subsequent divisions. Therefore, it is logical to conclude that development, by definition, is an epigenetic process. In differentiated cells, specific gene expression programmes are controlled by a more flexible system, which can dynamically switch on and off the genes that are responsible for homeostasis or react to environmental stimuli (Reik, 2007).

A potential mechanism through which the early environment of the developing embryo can induce an alteration in phenotype is by altering the epigenetic regulation of gene expression. For example, changes in the diet of the mother have been reported to cause long term changes in gene expression (Bertram *et al.*, 2001; Bertram and Hanson, 2001; Armitage *et al.*, 2004; Armitage *et al.*, 2005; Bogdarina *et al.*, 2007), with a complex inter-relationship between maternal nutrient intake and embryonic development being mediated by the placenta. The word 'epigenetic' was first introduced by the researcher Conard Waddington in 1942 to describe the phenomenon by which phenotypic modifications were induced by environmental influences. Waddington's research into developmental plasticity led him to conclude that a single genotype could result in the expression of multiple phenotypes that vary according to the effect of varying environmental influences during development. For instance, he described the phenomenon of polyphenism in insects. He reported in his work that exposing wild type Drosophila melanogaster pupae to heat shock led to the development of altered vein patterns in their wings (Waddington, 1952; 1959).

The term epigenetics refers to a series of mechanisms by which gene expression is altered in a stable manner without changing the underlying DNA sequence. Access to promoter sequences and other 5' regulatory sequences by the transcriptional machinery is tightly regulated by the architecture of chromatin (combination of proteins and DNA). This architecture is orchestrated in a precise and timely manner by interplay between various molecules that interact either directly or indirectly with chromatin to modify it. These modifications control the transcription of DNA to RNA while maintaining the underlying DNA sequence, and they are therefore said to be "epigenetic", a word derived from the Greek epi

(above) and genetikos (origin) (Dupont *et al.*, 2009). Several types of epigenetic modifications are known to affect expression levels, including changes in nucleosome positioning and conformation, histone modifications, acetylation, ubiquitination, DNA methylation (which is the most well-studied), and non-coding RNAs including miRNA.

DNA methylation at the cytosine residues of CpG dinucleotides is an epigenetic modification that plays an important role in gene expression (Jin *et al.*, 2011; Petrussa *et al.*, 2014), with most eukaryotic genomes being modified by this mechanism. It is a stable and heritable covalent addition that is propagated through DNA replication and cell division (Wigler *et al.*, 1981; Stein *et al.*, 1982; Klose and Bird, 2006). DNA methylation is believed to function by hindering the transcription factors from recognizing their target response elements. It is involved in numerous processes, such as X chromosome inactivation, genomic imprinting, embryonic development, tumour suppression and chromosome stability and, in general, with the formation of heterochromatin (Prokhortchouk and Defossez, 2008). Nucleosome positioning may also be affected by modified cytosine residues, leading to the assembly of specialized nucleosome structures that aid in the repression of transcription (Kass *et al.*, 1997).

In recent years, high-throughput sequencing techniques have been used to study genome-wide DNA methylation patterns in early stage human embryos (Guo *et al.*, 2014; Smith *et al.*, 2014). In these studies, samples of eggs, sperm, zygotes, and cleavage and blastocyst stage embryos have been analysed. These data were corroborated by both research groups, who showed that the DNA in eggs was moderately methylated, whereas the DNA in sperm was highly methylated, similar to what had been previously observed in the methylation profiles of mouse sperm and eggs (Smallwood *et al.*, 2011; Kobayashi *et al.*, 2012; Smith *et al.*, 2012). However, it was observed that 2-cell stage embryos and zygotes had lost a significant fraction of this methylation. Specifically, Guo *et al.* (2014) observed that the male, sperm-derived genome underwent a significant amount of demethylation, whereas, the maternal egg-derived genome, in comparison, underwent a more modest amount of demethylation. In blastocyst stage, low levels of methylation were observed. This finding was consistent for all blastocyst cells, including the pluripotent cells of the inner cell mass. A previously published study (Lee *et al.*,

2014) suggested that in order for embryonic cells to become pluripotent, the epigenetic memory of the cells must be erased, providing a possible explanation for global demethylation. In contrast, it was also reported by both groups that when the cells developed tissue-specific identities post-implantation, DNA methylation levels sharply increased to values typical of differentiated cells. Following its almost complete elimination, the epigenetic memory framework was therefore subsequently restored (Guo *et al.*, 2014; Smith *et al.*, 2014), Figure 2.3.

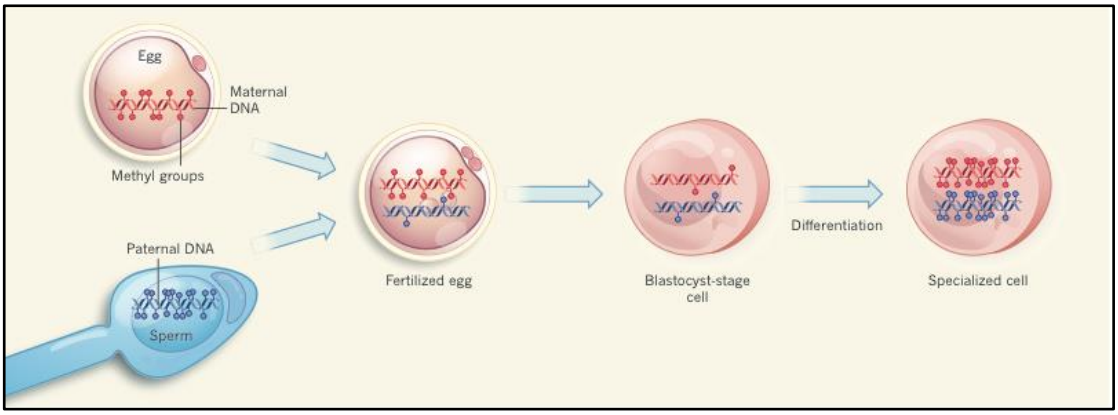


Figure 2.3: Tracking the state of DNA methylation. Guo *et al.* and Smith *et al.* analysed DNA methylation during early human embryogenesis. The DNA of human sperm is richly methylated. Conversely, oocyte methylation levels are lower (sperm and egg not drawn to scale). However, following the fertilisation of the oocyte, methylation is almost entirely erased — with a greater degree of the reprogramming dedicated to alterations of the paternal-derived genome methylation than its maternal counterpart. As embryonic development begins, maternal genome-derived demethylation continues to occur in cells until the blastocyst stage. In subsequent stages, DNA becomes re-methylated in differentiating cells. Thus, the control of gene transcription is inherited by daughter cells in newly specialised cells. Image taken from (Reik and Kelsey, 2014).

Since cloning mammals using NT generally results in gestational or neonatal failure, with a small percentage of the manipulated embryos resulting in live births, determining the safety of any cloning technique is of key significance. Although cloned embryos that were derived from donor cells, such as embryonic stem cells, that require little to no early developmental genes to be reprogrammed are capable of substantially better developmental progress beyond implantation than NT clones that are derived from somatic cells, there must still be a robust evaluation of any proposed human model before any such methodology can be considered for research or clinical applications. This is true regardless of the fact that studies have demonstrated that survival to birth and beyond is possible in cloned animals, even with substantial transcriptional dysregulation. This is consistent with mammalian development being rather tolerant to epigenetic abnormalities, with lethality resulting only when a threshold of faulty gene reprogramming encompassing multiple loci is passed (Rideout *et al.*, 2001).

Furthermore, research involving pigs has demonstrated that aberrant epigenetic patterns are often observed in the genome of cloned offspring. However, even in successful live births that avoid postnatal death, the disruption of methylation in imprinted genes is still observed, which could explain their adult pathologies and reduced lifespan (Shen *et al.*, 2012) .

Hence, the possibility that embryo splitting results in an unequal cell distribution and associated epigenetic discordance in the generated twin embryos has been proposed as a potential limitation of the technique in applications involving human reproductive technologies. However, it has been found that cell distributions are not an impediment to normal development. There is precedent for the unequal distribution of cells in the twin embryos, resulting in genetic and phenotypic differences among healthy monozygotic twins (Alikani *et al.*, 2003). As a result, it has been suggested that epigenetic changes caused by differences in DNA methylation during embryonic and foetal development may be a factor that underlies the discordance observed in monozygotic twins (Singh *et al.*, 2002).

In fact, studies have shown that MZ twins exhibit a wide range of within-pair differences at birth but show discordant levels that are generally lower than those observed in dizygotic DZ pairs. Analyses of comparative data describing DNA methylation between groups of MZ and DZ twins provide the opportunity to explore the likely relative proportions of genetic and shared and non-shared environmental factors that contribute to variations in phenotypes. (Gordon *et al.*, 2012), proposed that the largest contributors to variation include the combined effects of gestation in a non-shared intrauterine environment and stochastic factors. Theirs was the first study to analyse DNA methylation on a genome scale in twins at birth, and their results further highlight the importance of the intrauterine environment in shaping the neonatal epigenome. Subsequent studies have demonstrated that discordance in twins with asthma was associated with differential DNA methylation patterns (Murphy *et al.*, 2015), while in cancer-discordant MZ twins, specific genes that were associated with cancer, rather than global methylation patterns, were different between the twins (Roos *et al.*, 2016).

Phenotypic variations among adult monozygotic twins can result from different locus-specific 5-methylcytosin DNA and histone acetylation (Fraga *et al.*, 2005). In addition, genetic variations between monozygotic twins may also result from

chromosomal mosaicism or point mutations in nuclear and mitochondrial genes. These revelations have considerably modified our simplistic opinion that monozygotic twins represent perfect genetic clones (Shur, 2009).

Furthermore, DNA methylation is generally understood to have a 'silencing' effect on transcription. This function of DNA methylation was originally proposed in the 1970s. With the advent of enhanced genome-scale mapping of methylation, DNA methylation has now been observed at various genomic locations. The relevant locations at which methylation has been observed to occur include transcriptional start sites with or without CpG islands, gene bodies, regulatory elements and repeat sequences (Jones, 2012). A growing amount of evidence suggests that the function of DNA methylation seems to change according to the prevalent circumstances and that the role DNA methylation plays in transcriptional control is more nuanced than was once thought. Therefore, exploring the function of DNA methylation in different contexts would be important when analysing the relevant data (Jones, 2012), as well as the results of any analysis as part of a validation system in this PhD study .

Other studies have published methylation data that was acquired from human preimplantation embryonic cells and embryonic stem cells. The genome-scale DNA methylation maps developed from these cell sources demonstrate that the global hypomethylation that has been observed at this stage of development is not retained in subsequent stages (Smith *et al.*, 2014).

A further study investigated the expression patterns of four known DNA methyltransferases (DNMTs) in human oocytes and IVF-conceived embryos and compared them to the expression patterns observed in controls (healthy, high quality, fresh embryos/abnormally developing embryos/both embryo groups following cryopreservation) using immunocytochemistry (Petrussa *et al.*, 2014). DNMTs are the enzymes that are responsible for the functional control of methylation patterns in terms of both establishing and maintaining methylation, and they are important for epigenetic control mechanisms during normal embryogenesis. DNMT1o was found to be the most important factor for maintaining DNA methylation during early development, while DNMT3b was the most important for remethylating global DNA in preimplantation blastocysts. These results differed from those described in prior mouse studies. Therefore,

researchers have concluded that there are species-specific differences in mammalian methylation enzymes. Further results have demonstrated that DNMT alters expression patterns in embryos that underwent cryopreservation and abnormally developing embryos (Petrussa *et al.*, 2014). The consequences of these effects during long-term development are not known, and they therefore require further research.

In terms of validation methods for evaluating the effects of embryo splitting, ESCs derived from the ICM of a developing blastocyst can be grown indefinitely on a tissue culture dish while retaining their ability to differentiate into all cell types and used a cell source for methylation analysis. This approach requires a clearly defined ICM, though the derivation of hESCs that can be grown in culture does provide the opportunity to study epigenetic changes during the early stages of human development (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000). Thus, researchers have endeavoured to understand how, for example, chromatin regulates the pluripotency of these cells. A number of recent studies have investigated chromatin structure in human and mouse embryonic stem cells using ChIP assays in combination with quantitative PCR, microarrays and high-throughput sequencing (Azuara *et al.*, 2006; Roh *et al.*, 2006; Barski *et al.*, 2007; Mikkelsen *et al.*, 2007; Pan *et al.*, 2007; Zhao *et al.*, 2007).

However, methodological limitations, in terms of the potential difficulty of isolating samples of ICM, caused us to hesitate before including this validation method in this study. In addition, because the unequal distribution of cells in the healthy MZ twin embryos results in genetic and phenotypic differences between healthy MZ twins and the potential for variation among twin groups, it is not possible to determine the reliability of using data derived from such a validation method as an absolute indicator of quality. Furthermore, the low availability of human embryos for research purposes, together with a consideration of the evolving theories regarding epigenetic dynamism during early development and a more nuanced understanding of the effects of DNA methylation on transcription led to finally excluding methylation analysis as a validation tool in this study.

2.6 Validation method IV (b2): Epigenetics, micro RNA (miRNA) analysis

miRNAs are small, single-stranded RNA molecules that are approximately 22 nucleotides in length. miRNAs are evolutionarily conserved sequences of non-coding RNA that have important post-transcriptional functions in many significant cellular processes, such as developmental timing, metabolism, signalling pathways, apoptosis, brain development, myogenesis and cardiogenesis. They have also been implicated in the development of human pathologies, such as genetic disorders, cancer and viral diseases (Kloosterman and Plasterk, 2006). Approximately 1048 miRNAs have so far been discovered in the human genome (Griffiths-Jones, 2004; miRBase, 2014). Unlike the previously held view that miRNAs operate exclusively negative regulators of gene expression via sequence-dependent 3' untranslated region (UTR) binding to mRNAs, recent research has shown that miRNA regulation involves more complex mechanisms. It has been shown that miRNAs are involved in both repressing and activating their mRNA targets by binding to the UTR and coding promoter regions of target protein-coding messenger RNAs (Breving and Esquela-Kerscher, 2010).

As previously mentioned, the miRNA-mediated regulation of protein-coding mRNAs involves the miRNA binding to the 3' UTR of a target mRNA. The target region is often present in the mature mRNA, but it does not code for any amino acids (Bartel, 2004; Lim *et al.*, 2005). Depending on the level of base-pairing, the miRNA and the 3' UTR of the mRNA may recognise and interact with each other. The typical length of a miRNA is 21 nucleotides. However, all 21 nucleotides in the miRNA sequence do not need to complement the target mRNA for miRNA regulation to succeed. The nucleotides from position 2 to position 8 are the key nucleotides. If this short section contains enough bases that are complementary to the target mRNA for the two RNA strands to bind to each other, the resulting binding of the miRNA prevents the translation of the mRNA. Furthermore, if the two sequences on the miRNA and the target mRNA are perfectly matched, binding between them can trigger the destruction of the mRNA by enzymes that are attached to the miRNA molecule (Bartel, 2009). The amount of protein expression that results from an mRNA can be altered by miRNAs at two stages: both when translation is initiated and after translation has been initiated (Breving and

Esquela-Kerscher, 2010). Thus, miRNAs regulate mRNA functions both post-transcriptionally and post-translationally. Single miRNAs can target several mRNAs on the basis of base-pair complementarity alone. However, a significant proportion of these putative interactions may be dependent on cell type and context (Bartel, 2009) and on the binding of additional cofactors (Jacobsen *et al.*, 2010).

2.6.1 The discovery of miRNAs

The non-parasitic nematode *Caenorhabditis elegans* has been established as a model organism because the developmental fates of its cells are well known and the timing and order of its developmental stages are tightly regulated. One of the primary regulators of these developmental stages is a protein lin-14. Expression of the lin-14 gene is highly upregulated during very early embryonic stages, but as the worm moves from larval stage 1 to larval stage 2, LIN-14 expression is downregulated. When the *Lin-14* gene is mutated, the developmental stages of the nematode occur at the wrong times. If the expression of the LIN-14 protein extends beyond the early development stages, the worm repeats the early stages of development. Similarly, if LIN-14 expression is reduced prematurely, the organism prematurely progresses into later larval stages. In either case, when LIN-14 expression is not correctly regulated, the organism undergoes abnormal development and the normal adult structures of the resulting *C. elegans* fail to develop. Fundamental insight into the mechanisms affected by LIN-14 expression was gained by two groups who were working independently in 1993. These groups found that the key event the controlled LIN-14 expression was the binding of a small ncRNA molecule to the LIN-14 mRNA. As a consequence of this finding, the first miRNA was discovered, and its role in the regulation of *C. elegans* development was recognised (Lee *et al.*, 1993; Wightman *et al.*, 1993; Carey, 2012) (Figure 2.4).

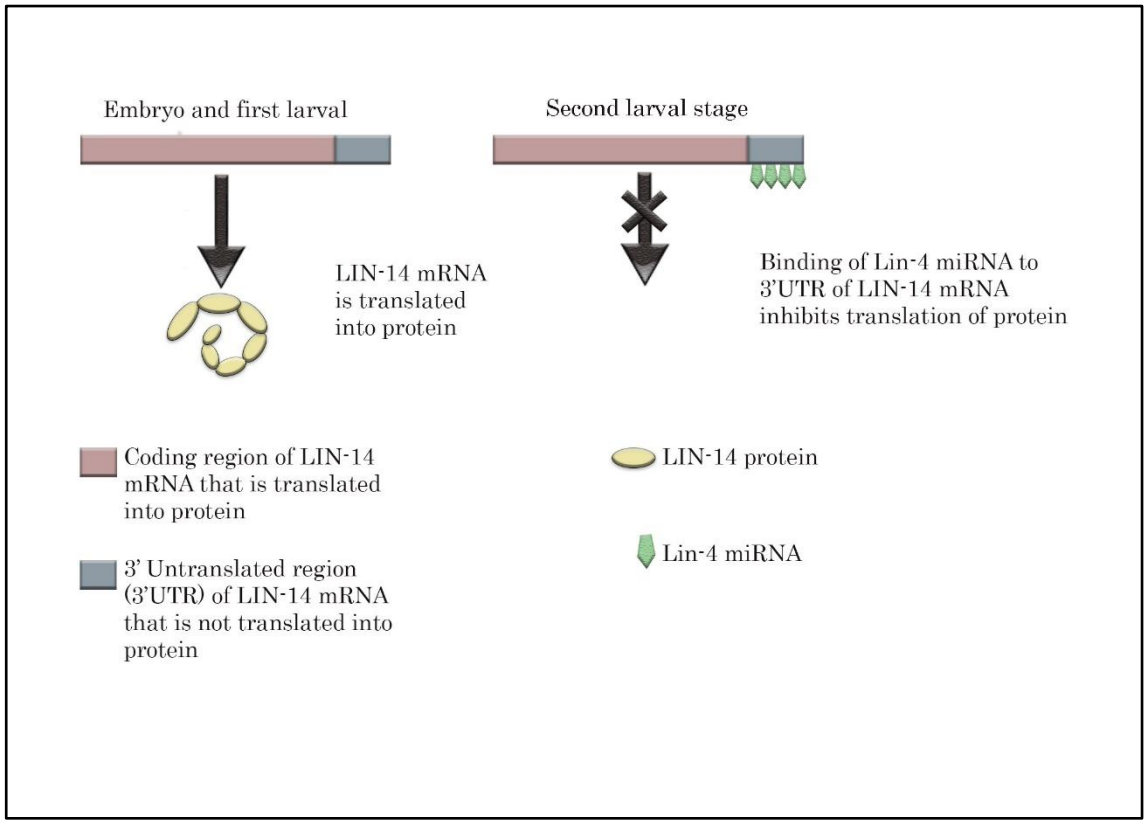


Figure 2.4: Schematic illustrating how altering the expression of the *Lin-4* miRNA at specific developmental stages in *C. elegans* can lead to significant alterations in the expression of the *LIN-14* gene. Modified from (Carey, 2012).

In humans, *LET-7* was the first miRNA to be identified. The *let-7* miRNA was found to be involved in the regulation of developmental timing (Rougvie, 2001). Upon further examination, it was found to be highly conserved across diverse species (Pasquinelli *et al.*, 2000). In addition, *let-7* is also highly conserved among microbes, plants and animals, indicating that it is involved in a significant regulatory pathway (Grosshans and Slack, 2002). In the human genome, almost 60% of all genes are subject to regulation by the 1500 miRNAs that have so far been entered into the human microRNA database (Friedman *et al.*, 2009; miRBase, 2014).

The full extent and significance of miRNA-dependent gene regulation was demonstrated following the discovery of the let-7 miRNA and its conservation between nematodes (e.g., *C. elegans*) and humans (Pasquinelli *et al.*, 2000; Reinhart *et al.*, 2000). The mechanism used by both lin-4 and let-7 to regulate protein expression was mediated by the binding of the miRNA to the 3′ UTR of a target protein-coding mRNA (e.g., lin-14 and lin-41) and the suppression of its translation during important transitory stages in nematode development

(Wightman *et al.*, 1993; Reinhart *et al.*, 2000). Therefore, the discovery of these miRNAs shed light on an entirely new mechanism of genetic regulation: post-transcriptional gene silencing. In this mechanism, the production of a target protein-coding mRNA is allowed, but the translation of the target mRNA into protein is prevented by the miRNA.

2.6.2 Biogenesis, transcription and processing of miRNAs

miRNAs are formed by the sequential cleavage of double stranded RNAs (dsRNAs,; termed primary miRNAs or pri-miRNAs) by a specialised ribonuclease III enzyme (RNase III) that is coupled with specific dsRNA-binding proteins (Kim *et al.*, 2009; Axtell *et al.*, 2011) (Figure 2.5). miRNA biogenesis is regulated by both transcriptional and post-transcriptional mechanisms (Carthew and Sontheimer, 2009; Siomi and Siomi, 2010). Alternate pathways that produce specific miRNAs involve replacing standard biogenesis steps with RNA processing controls from pre-mRNA splicing or RNA degradation pathways (Yang and Lai, 2011).

miRNAs are transcribed by RNA polymerase II. Long pri-miRNAs are produced from independent genomic transcription units or the introns of protein-coding genes by the enzyme RNA polymerase II (Cai *et al.*, 2004; Lee *et al.*, 2004; Rodriguez *et al.*, 2004; Aboobaker *et al.*, 2005; Baskerville and Bartel, 2005; Xie *et al.*, 2005; Ozsolak *et al.*, 2008; Corcoran *et al.*, 2009). miRNAs generated from introns do not require splicing for their production (Kim and Kim, 2007), and the processing of pri-miRNAs into precursor miRNAs (pre-miRNAs) does not affect the splicing of the host pre-mRNA (Ballarino *et al.*, 2009; Kataoka *et al.*, 2009; Ameres and Zamore, 2013).

Drosha crops pri-miRNAs into shorter pre-miRNAs. In animals, pri-miRNAs are converted into pre-miRNAs by the RNase III enzyme Drosha. Pre-miRNAs are ~60 nucleotide stem–loop structures that are formed when Drosha excises one or more pre-miRNAs from a pri-miRNA (Lee *et al.*, 2003; Denli *et al.*, 2004; Gregory *et al.*, 2004; Landthaler *et al.*, 2004). Therefore, a single pri-miRNA can produce several pre-miRNAs. Polycistronic miRNAs enable several miRNAs to be co-expressed by a single promoter, and polycistronic miRNAs can therefore bring about the coordinated expression of multiple target factors that belong to a single pathway (Kim *et al.*, 2009). The enzyme Drosha is a component of a larger complex that is

known as the microprocessor. This is a nuclear complex that has been reported to exist in two forms: a ~600 kDa complex of unknown function and a smaller heterodimer that is composed of Drosha and its dsRNA-binding protein (DGCR8 in mammals and Pasha in other animals) (Gregory *et al.*, 2004; Landthaler *et al.*, 2004; Lee *et al.*, 2004; Han *et al.*, 2006). This coupling between the dsRNA-binding protein Pasha and the RNase III enzyme Drosha is a characteristic of small RNA biogenesis that leads to the restriction of the substrates that are processed by the complex, its increased affinity for certain substrates, and the improved accuracy of its targeting of cleavage sites. The distribution of miRNA-producing enzymes within the cell indicates that in animals, pri-miRNAs are cropped in the nucleus, whereas pre-miRNAs are processed in the cytoplasm (Wu *et al.*, 2000; Billy *et al.*, 2001; Lee *et al.*, 2002; Provost *et al.*, 2002; Lee *et al.*, 2003; Zeng and Cullen, 2003). The pre-miRNAs are exported from the nucleus to the cytoplasm through nuclear pores by the nuclear transport receptor exportin 5 (Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Lund *et al.*, 2004; Zeng and Cullen, 2004; Ameres and Zamore, 2013).

Dicer causes miRNA–miRNA duplexes*. In the cytoplasm, a ~22 nucleotide miRNA-miRNA duplex is cleaved from the pre-miRNA by a second RNase III enzyme called Dicer (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001). In flies, Dicer-1 is responsible for cleaving pre-miRNAs, whereas Dicer-2 causes the formation of siRNAs (Han *et al.*, 2004). Similar to Drosha, Dicer-1 acts by identifying specific structures in a target RNA molecule and then cleaving it at a fixed distance from the base of the pre-miRNA stem before cutting off the loop to produce a ~22 nucleotide mature miRNA–miRNA* duplex (Zhang *et al.*, 2002; Zhang *et al.*, 2004). However, it is not always essential for pre-miRNAs to be diced. For example, pre-miR-451, in zebrafish and mice, has a hairpin loop that is too short to be recognized by Dicer. It is therefore assimilated into the RNA-induced silencing complex for subsequent processing into a mature miRNA by Protein Argonaute-2 (AGO2) (Pase *et al.*, 2009; Cheloufi *et al.*, 2010; Yang *et al.*, 2010).

Partner proteins shape Dicer functions. Similar to the Drosha-Pasha complex, which consists of an RNase III enzyme partnered with a dsRNA-binding protein, the Dicer-1 in *Drosophila melanogaster* partners with two isoforms of the dsRNA-binding protein Loquacious (Loqs) that are called Loqs-PA and Loqs-PB.

These proteins are responsible for increasing the affinity of Dicer-1 for pre-miRNAs (Forstemann *et al.*, 2005; Jiang *et al.*, 2005; Saito *et al.*, 2005; Fukunaga *et al.*, 2012), and there are therefore essential for the effective processing of miRNAs in flies. On the contrary, when Dicer-2 partners with the protein R2D2, it prevents Dicer-2 from processing pre-miRNAs, thereby restricting it to processing only long dsRNA substrates (Cenik *et al.*, 2011). Other combinations with different partner proteins also modulate the specificity of individual Dicer proteins for various substrates. This is likely to be the explanation for organisms like worms and mammals having only a single Dicer enzyme that is capable of producing a range of miRNAs and siRNAs (Ameres and Zamore, 2013).

In mammals, the Dicer enzyme partners with two proteins: transactivation-response RNA-binding protein (TRBP) and protein kinase R-activating protein (PACT) (Chendrimada *et al.*, 2005; Haase *et al.*, 2005). The roles of these partner proteins extend beyond defining Dicer substrate specificity. Dicer partner proteins also play a role at the site of Dicer-mediated cleavage within a pre-miRNA (Fukunaga *et al.*, 2012; Lee and Doudna, 2012). For instance, the presence of TRBP alters the site at which mammalian Dicer cleaves a few pre-miRNAs, such as pre-miR-132. However, the same change does not occur when Dicer binds with PACT. In flies, the miRNAs that arise from interactions between Dicer-1 and a small subset of pre-miRNAs display altered lengths and seed sequences when Dicer partners with the protein Loqs -PB.

A seed for target recognition. Once the sequential pathway involved in the processing of miRNA precursors is complete, a constituent single strand of the miRNA duplex directs the AGO proteins to complementary mRNA sequences, resulting in the down-regulation of the expression of the target mRNA (Kawamata and Tomari, 2010; Czech and Hannon, 2011). The key determining factor that influences binding between the AGO protein and its target mRNA is a 6–8 nucleotide-long sequence domain at the 5′ end of the miRNA. AGO interacts with this region to create the 'seed' (Bartel, 2009). Nucleotide sequences that contain enough base pairs that are complementary to the seed sequence (‘seed matches’) are sufficient to cause a small but noticeable decrease in the expression of the target mRNA. Although seed matches can occur in any region of an mRNA, a decrease in the expression of the mRNA is most likely to occur when the seed

match is located in the 3′ UTR of the target mRNA (Grimson *et al.*, 2007; Forman *et al.*, 2008; Gu *et al.*, 2009; Forman and Coller, 2010). Because of the short length of the region that is required to produce the seed, greater than half of all protein-coding genes in mammals are subject to regulation by miRNAs, and a large number of other mRNAs are negatively selected to circumvent seed matches with miRNAs that are present in the same cells (Farh *et al.*, 2005; Lewis *et al.*, 2005; Stark *et al.*, 2005; Friedman *et al.*, 2009; Ameres and Zamore, 2013).

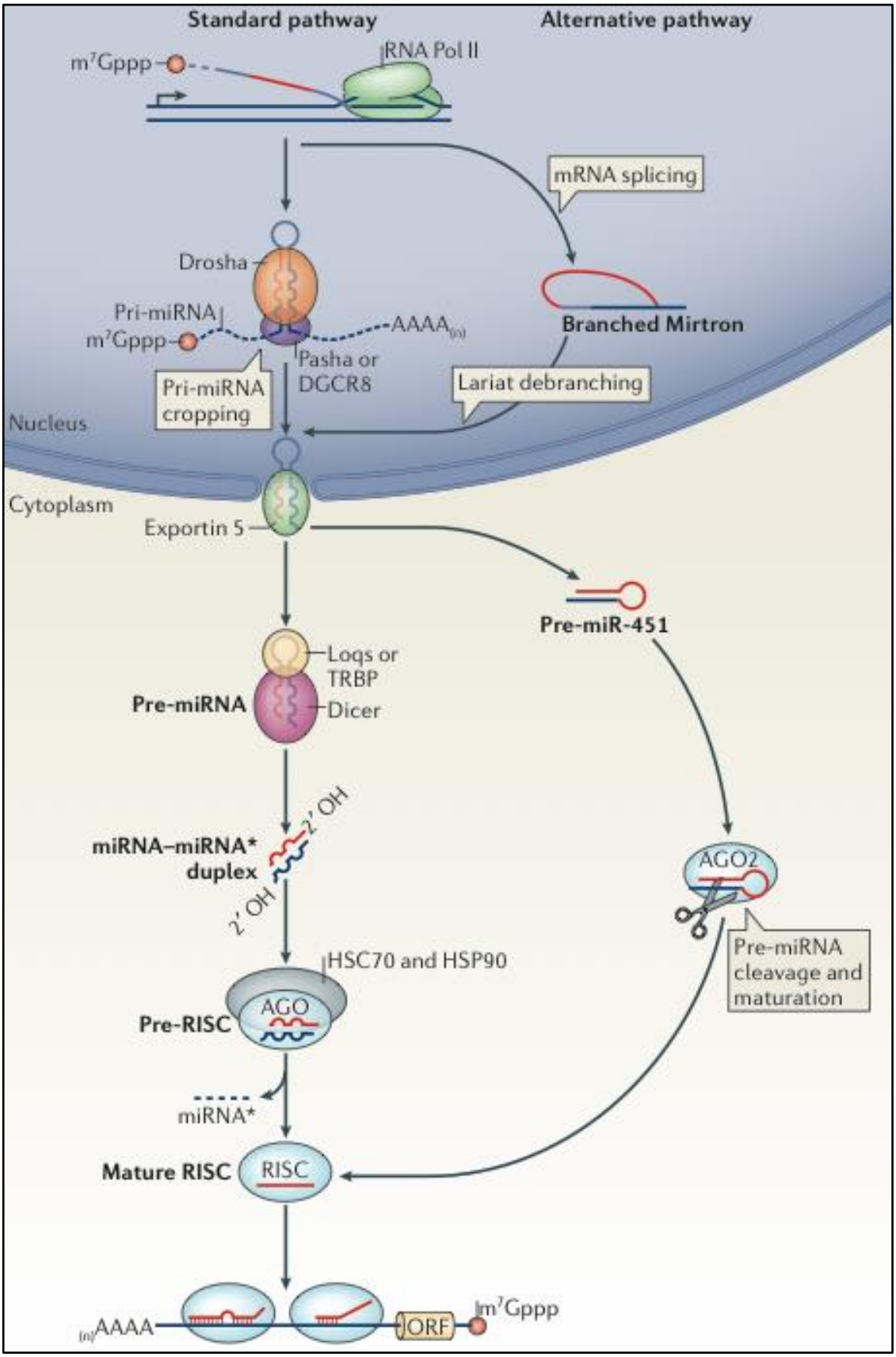


Figure 2.5: Pathways involved in microRNA biogenesis. In the standard microRNA (miRNA) biogenesis pathway, Drosha processes the primary miRNA (pri-miRNA) transcript in the nucleus and subsequently partners with the enzyme Dicer in the cytoplasm. The pri-miRNA, which begins with a 7-methylguanosine cap (m^7Gppp) and ends with a 3′poly (A) tail is transcribed by RNA polymerase II

(Pol II). The endonuclease Drosha and its double-stranded RNA (dsRNA)-binding protein partner DGCR8 (in mammals) or Pasha (in flies) are responsible for cleaving a stem-loop structure from the pri-miRNA in the nucleus. Exportin 5 is responsible for the export of the resulting precursor miRNA (pre-miRNA) from the nucleus. Subsequently, the pre-miRNA is further cleaved by the endonuclease Dicer and its dsRNA-binding partner TRBP (transactivation-response RNA-binding protein in mammals) or Loquacious (Loqs in flies) to cause the liberation of a miRNA-miRNA* duplex. This duplex is loaded as a dsDNA onto an Argonaute (AGO) protein with the help of the HSC70-HSP90 chaperone machinery. Further steps in the maturation process of the miRNA result in the miRNA being expelled *, which leads to the formation of a mature RNA-induced silencing complex (RISC). Individual steps during miRNA precursor processing may be replaced by alternative pathways, as shown on the right side of the figure. Nucleases from alternative cellular pathways, such as the general RNA degradation machinery or pre-mRNA splicing factors, can replace pri-miRNA cropping activity. In these cases, the pri-miRNA is formed from a branched mirtron structure that undergoes lariat debranching. Specifically, in the case of pre-miR-451, the pre-miRNA eludes Dicer processing after it is exported from the nucleus, and it is instead loaded directly onto the AGO2 protein, which triggers its maturation into a single-stranded miRNA. (2′ OH, 2′ hydroxyl group; HSP, heat shock protein; ORF, open reading frame). The image was reproduced from (Ameres and Zamore, 2013).

2.6.3 Secreted miRNAs

MicroRNAs are secreted in membrane-bound exosomes and microvesicles. They are bound to stabilizing proteins and are found in all body fluids, including blood, urine, saliva, tears, breast milk, semen, amniotic fluid, cerebrospinal fluid, peritoneal fluid, and pleural fluid, and in culture media collected from different cell lines (Wang *et al.*, 2010a; Wang *et al.*, 2010b; Weber *et al.*, 2010). In addition, miRNAs secreted by donor cells can be internalised into recipient cells, where they may subsequently perform regulatory functions. In blood serum samples, miRNAs were found to be highly stable and able to withstand extreme environmental conditions such as freezing and thawing (Chen *et al.*, 2008). This highlights their potential usefulness as effective biomarkers. Therefore, there is significant interest in identifying miRNAs in bodily fluids that can be used as non-invasive biomarkers for the early detection of diseases. For example, the miRNAs miR-122, miR-499 and miR-141 have been implicated in the occurrence of myocardial infarction, prostate cancer and liver injury caused by drugs, respectively (Chen *et al.*, 2008; Mitchell *et al.*, 2008; Wang *et al.*, 2009a; Adachi *et al.*, 2010; Wang *et al.*, 2010a; Weber *et al.*, 2010).

2.6.4 MiRNAs in human pre-implantation embryos

A large number of miRNAs are also expressed by human blastocyts (Rosenbluth *et al.*, 2013). These miRNAs are secreted and have been found in embryonic culture media. Specific miRNAs detected in cell media have been linked to the ploidy status of the embryo and its reproductive competence and used to predict the

outcome of *in vitro* fertilisation. Several such miRNAs have been identified (Mineno *et al.*, 2006; McCallie *et al.*, 2010; Kropp *et al.*, 2014; Rosenbluth *et al.*, 2014; Capalbo *et al.*, 2016). While further research is necessary, a comprehensive profile of the miRNAs that are secreted by human pre-implantation embryos into spent culture media is currently underway (Thouas *et al.*, 2015).

A very recent prospective cohort study by Capablo *et al.* (2016) reported a full characterization of a population of miRNAs that were secreted by human blastocysts into spent culture media. These miRNAs were found to be responsible for transmitting information from the developing blastocyst to neighbouring endometrial stromal cells (EnSCs) and to thereby have an impact on the success of the implantation. The significance of this study lies in its suggestion that miRNAs from spent culture media can be used as non-invasive, easy to collect biomarkers for embryo selection during IVF cycles. In their subsequent work, they developed a quantitative qPCR-based protocol for miRNA purification and analysis. hESCs were cultured at different concentrations, and spent blastocyst medium (SBM) were used to validate their protocol both with and without biological variability. The authors compiled miRNA profiles of TE cells and their culture media and compared them among these specimens. Further, the miRNA profiles of SBM samples were collected from embryos at various stages of pre-implantation development, such as the cleavage, morula, and blastocyst stages, to identify when the process of miRNA secretion begins during the pre-implantation window. Finally, the miRNA profiles of SBMs that were collected from implanted embryos were compared to the profiles that were generated from unimplanted euploid blastocysts to evaluate their potential use as biomarkers for embryo selection (Capalbo *et al.*, 2016).

The authors investigated the miRNA profiles of the culture media that were collected from embryos in the cleavage, morula, and blastocyst stages. The SBMs were prospectively collected from euploid implanted (n = 25) and unimplanted blastocysts (n = 28), and these samples were compared to each other. The comparison of TE and SBM samples revealed that 96.6% (57 of 59; 95 CI, 88.3–99.6) of the miRNAs that were found in the SBM were of TE origin and were expressed by TE cells. The miRNA profiles of the culture media that were collected from cleavage and morula stage embryos showed an expression pattern similar to

that of the negative controls, suggesting that miRNA profiles of spent culture media may be effective only when evaluating blastocysts. The miRNA analysis of the SBM obtained from euploid implanted and unimplanted blastocysts demonstrated that two miRNAs, miR-20a and miR-30c, were present at higher concentrations in the implanted blastocysts. These two miRNAs were found *in silico* to play roles in 23 implantation-related pathways (Capalbo *et al.*, 2016).

Previous research has suggested that splitting human embryos might result in the formation of morphologically adequate, viable blastocysts (Van de Velde *et al.*, 2008; Illmensee *et al.*, 2010). However, qualitative analyses of such embryos have been relatively limited. Because the SBM is considered a waste material, and because miRNA analyses of these materials can be hugely informative, the potential importance of analysing the miRNA profile of SBMs has been well-established by our collaborator, Dr. Capalbo. This validation method was therefore used to examine whether the functional and reproductive ability of embryos that were generated by embryo splitting was associated with their miRNA secretions. Further to determine the effect of embryo splitting on the miRNA secretion profile of the Twin embryos. The miRNA profiles of SBMs that were sourced from twin blastocysts were compared to the profiles of blastocysts that were generated by *in vitro* fertilisation. All of the control blastocysts resulted in live births upon single embryo transfer.

2.7 Aims and objectives

2.7.1 Aim

Because there has been a shift towards the cryopreservation of blastocysts in clinical practice, the number of cleavage-stage embryos that are available for research purposes has been reduced. This shortage and the associated potential future restrictions on human development research were the main reasons for investigating the safety and efficacy of the embryo splitting technology. This study was performed to determine whether twin embryos that were generated by splitting a donor embryo are suitable for use as a research tool. The quality of the human embryos that were generated by twinning *in vitro* will be compared to the quality of embryos that were created by fertilisation. These data will also indicate the future potential usefulness of this application in the research field as well as in human ARTs.

2.7.2 Objectives and validation methods

2.7.2.1 Objectives

1. To assess the developmental, pluripotent and reproductive competence of twin embryos that were generated by embryo splitting using blastomere biopsies using four different validation methods.
2. To compare the quality of the twin embryos against the quality of control embryos that were created by fertilisation.

2.7.2.2 Validation methods

Validation method I: A comparative analysis of the morphokinetic parameters of 176 twin embryos that were created by splitting 88 human embryos from either early (2 – 5 cell stage, n = 43) or late (6 – 10 cell stage, n = 45) cleavage stages, as recorded using a time-lapse imaging system. Then compare these data with the morphokinetic data obtained from embryos that were created by fertilisation and that resulted in pregnancy and live birth upon single blastocyst transfer (n = 42).

Validation method II: A comparative analysis of the expression patterns of early lineage-specific transcription factors in twin blastocysts that were derived from split embryos and non-manipulated Day 5 and Day 6 blastocysts using immunocytochemistry.

Validation method III: The derivation of hESCs from twin embryos and a comparison between these hESCs and hESC lines that were previously derived from non-manipulated embryos on Day 5 and Day 6 at the assisted conception unit (ACU) at Guy's Hospital, King's College, London.

Validation method IV: A comparative analysis of the miRNA profiles of SBM taken from twin embryos that were created from blastomere biopsies (n=7 pairs) and control blastocysts that were generated by fertilisation and resulted in live births upon single embryo transfer (n=7).

Chapter 3

Materials and Methods

To generate twin embryos, half of the blastomeres in cleavage-stage embryos were biopsied and transferred into empty ZPs, which were prepared in advance. The embryos the blastomeres were biopsied from were named the "donor" or "Twin A" embryos, and the new embryos with empty ZPs into which the biopsied blastomeres were transferred were named the "recipient" embryos or "Twin B" embryos. The method used to create Twin A and Twin B embryos is explained in this chapter.

The developmental, pluripotency and reproductive competence of the twin embryos that were generated by embryo splitting were assessed using four different validation methods (Figure 3.1):

Validation method I: A comparative analysis of the morphokinetic parameters of the twin embryos was performed by recording the embryos development using a Time-Lapse Imaging System, and the results were compared to morphometric data obtained from control embryos that were created during fertilisation. The detailed methods for this procedure are explained in Chapter 4.

Validation method II: A comparative analysis of the expression patterns of early lineage-specific markers in the twin blastocysts and non-manipulated Day 5-6 blastocysts was performed using immunocytochemistry. The detailed methods for this procedure are explained in Chapter 5.

Validation method III: The derivation of human embryonic stem cell lines from the twin embryos and a comparison of these cell lines to hESC lines that were previously derived from normal, non-manipulated Day 5-6 embryos at the ACU of Guy's Hospital, King's College, London. The detailed methods for this procedure are explained in Chapter 6.

Validation method IV: A comparative analysis of the miRNA profile of the SBM obtained from the twin embryos and control blastocysts that were generated

during fertilisation. The detailed methods for this procedure are explained in Chapter 7.

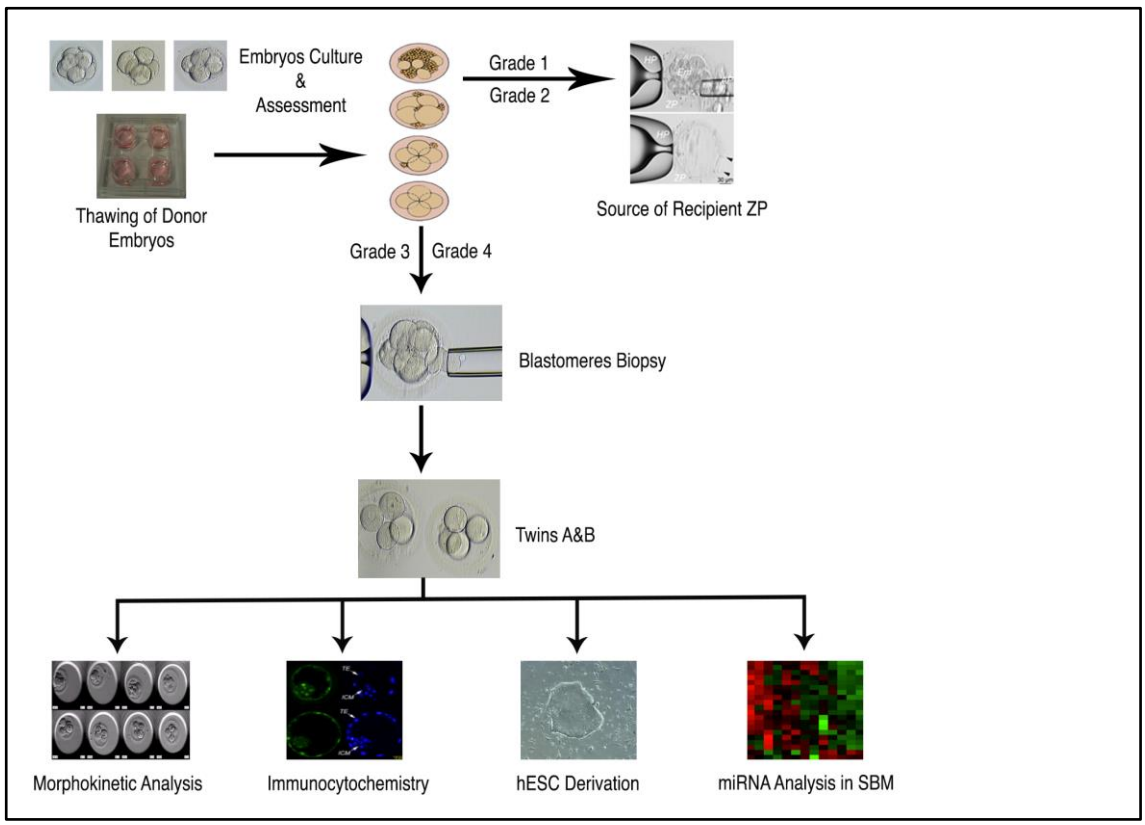


Figure 3.1: Experimental design.

3.1 Study conduct

The research in this project was conducted under a license obtained from the UK Human Fertilisation and Embryology Authority (HFEA Research License Numbers: R0075 and R0133) and with local ethical approval (UK National Health Service Research Ethics Committee Reference: 06/Q0702/90). The parents of all subjects in the study provided informed consent to participate in this experiment, in conformity with the principles outlined by the World Medical Association (WMA) Declaration of Helsinki and the NIH Belmont Report that was created by the National Commission for the Protection of Human Subjects of Biomedical and Behavioural Research. No financial incentives were offered for embryo donation.

3.2 Sources of the embryos used in the project

The generated Twin A and Twin B embryos were compared to different groups of control embryos using four different validation methods. The sources from which the donor, recipient and control embryos were obtained, are defined in this section.

3.2.1 Sources of control embryos

The sources of the control embryo groups were different for each of the four validation methods and are presented in Table 3.1.

Table 3.1: Details of the control groups associated with each of the four validation methods used in this project.

Validation method	Control group
I	Routine morphokinetic recordings were collected from fresh embryos that were undergoing a PGD analysis for clinical purposes and that resulted in a clinical pregnancy upon single embryo transfer. Detailed descriptions of the methods used for embryo collection, fertilisation, culture and use are included in section **4.1.1.**
II	Cryopreserved research embryos at the blastocyst stage were either donated by Guy's ACU patients or imported from different IVF clinics across the UK.
III	No embryos were used as controls. The controls were previously derived hESC lines that were obtained from the ACU at Guy's Hospital, King's College, London.
IV	SBM that was previously collected from cryopreserved blastocysts that resulted in a healthy pregnancy and a live birth following single embryo transfer. The SBM was acquired from GENERA, the Centre for Reproductive Medicine, Clinica Valle Giulia, Rome. For a detailed description of the methods used for embryo collection, fertilisation, culture and use, are included in section 7.2.2 and 7.3

3.2.2 Source of donor embryos

A total of 400 supernumerary donor embryos were obtained according to approved HFEA Research Licences (R0075 and R0133) from Guy's Hospital and various IVF clinics across the UK. The donated embryos were cryopreserved using the slow freezing method at the following stages: 2PN, Day 2 and Day 3.

3.2.3 Source of the empty ZP(s) used as recipient embryos

Unfertilised eggs, embryos resulting from abnormal fertilisation (1PN, 3PN) post-ICSI/IVF that were not suitable for treatment, and lower-quality cleavage-stage embryos that were donated for research (Grade 1 and Grade 2, see section 3.4.3) and that were not suitable for splitting were used as sources for the ZPs used as recipient embryos in this study.

3.3 Preparation of recipient embryos: Twin B

Empty ZPs were prepared on the day of the splitting procedure by removing the cellular content of the source embryos as described in section 3.2.3 and as shown in Figure 3.4. All emptied ZPs were kept in culture dishes containing Quinn's Advantage™ Cleavage Medium (CM) (Sage, Cooper Surgical) supplemented with 10% Quinn's Advantage Serum Protein Substitute (SPS) (Sage, Cooper Surgical) at 37°C in 6% CO_2 and 5% O_2 until further use. The details of the biopsy procedure that was used to remove the cellular contents are explained in section 3.5.2.

3.4 Preparation of donor embryos: Twin A

3.4.1 Thawing of cleavage-stage donor embryos

Embryos were thawed using Quinn's Advantage Thaw Kit (Sage, Cooper Surgical) according to the manufacturer's instructions. Briefly, cryostraws containing frozen embryos were transferred from a liquid nitrogen dewar to an embryology laboratory using a small portable Dilvac Flask (Day-Impex) containing liquid nitrogen. All kit solutions were equilibrated to room temperature (RT) before the experiment was performed. The cryostraws were removed from liquid nitrogen, held in the air for 45 seconds and then immersed in sterile 30ºC water for 30

seconds with the coloured plug end up. The coloured plug was then individually removed from each straw using a metal rod that acted as a plunger. The contents of each straw were expelled onto the upturned lid of a Nunc thaw dish (Thermo Scientific). The embryos were transferred with a minimal amount of cryosolution using a Flexipet holder (Research Instruments Ltd, RI) to a 4-well Nunc dish containing 0.5 ml of 0.5 M Sucrose Thawing Medium supplemented with 12 mg/mL human serum albumin (HSA) using a Flexipet equilibrated with 0.5 M Sucrose Thawing Medium. They were then thoroughly washed and incubated for 10 minutes. The embryos were then transferred into 0.5 ml of 0.2 M Sucrose Thawing Medium containing 12 mg/mL HSA for 10 minutes. Each embryo was transferred from one medium to another using a new pipette each time to minimize carry-over of the cryoprotectant from one solution to the next. The embryos were then transferred to a well containing 0.5 ml Quinn's Advantage Thaw Diluent Solution supplemented with 12 mg/mL HSA using a pipette that was primed with the Diluent Solution. The embryos were then incubated for 5 minutes on a heated stage at 37°C away from light.

3.4.2 Post-thaw culture of cleavage stage donor embryos

Culture dishes were prepared in advance. Eight microdrops of 40 µl CM supplemented with 10% SPS were arranged in a 60 mm Nunc dish and covered completely with 9 ml of mineral oil (Sage, Cooper Surgical). The dishes were labelled and left overnight (> 6 hours) to equilibrate in a humidified atmosphere at 37°C in 6% CO_2 and 5% O_2 in a Heraeus incubator (Thermo Scientific) before use. Day 2 and Day 3 donor embryos were cultured post-thaw in the pre-equilibrated CM for at least 4 hours at 37°C in 6% CO_2 and 5% O_2 in a Heraeus incubator (Thermo Scientific). The 2PN embryos were cultured for a longer period (24 or 48 hours) under the same culture conditions to achieve the desired developmental stage for splitting. All manipulations were conducted under sterile conditions in a Class II laboratory in a compact laminar flow hood built on a heated microscope stage.

3.4.3 Assessment of the quality of donor embryos

The quality of the post-thaw donor cleavage stage (Day2 and Day3) embryos and the post-culture 2PN embryos was assessed using the 1 to 4 grading system described by (Bolton *et al.*, 1989). This morphological assessment system for cleavage-stage embryos is based on the following three parameters: i) the number of blastomeres, ii) the regularity of blastomere sizes and shapes, and iii) the level of fragmentation. The highest-quality embryos were classified as Grade 4, and the lowest quality embryos were classified as Grade 1. The grade criteria are detailed in Figure 3.2 and Figure 3.3.

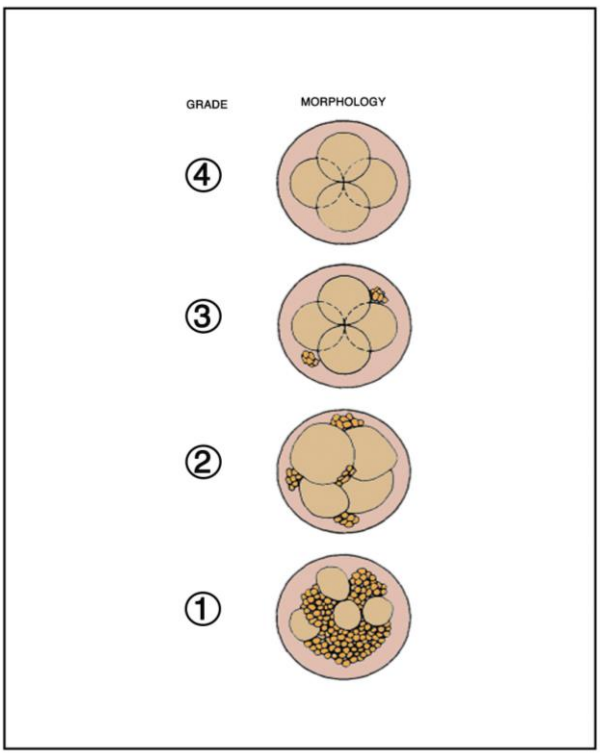


Figure 3.2: Assessment of cleavage-stage donor embryos at the 4-cell stage. Grade 4: regular, spherical blastomeres with no extracellular fragmentation. Grade 3: regular, spherical blastomeres with some extra cellular fragmentation (up to 10%). Grade 2: blastomeres were slightly irregular in size and shape with considerable extracellular fragmentation (up to 50%). Grade 1: barely defined blastomeres with considerable extracellular fragmentation (>50%). This image was modified from (Bolton *et al.*, 1989).

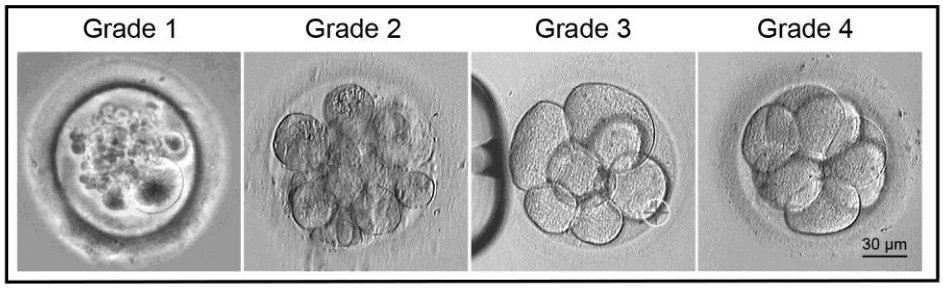


Figure 3.3: Grading of 8-cell cleavage stage embryos based on Bolton *et al.* (1989). Grade 4 indicated the top quality, and Grade 1 indicated the lowest quality. Embryos that were Grade 1 or 2 were used to obtain empty ZPs for recipient embryos (Twin B), and embryos that were Grade 3 or 4 were used as donor embryos (Twin A).

3.5 Splitting method for obtaining twin embryos

The donor embryos in this study were categorized into two groups according to their surviving post-thaw cell numbers. In Group 1, the donor embryos were split at the 2- to 5-cell stage (early cleavage stage), and in Group 2, the donor embryos were split at the 6-10 cell stage (late cleavage stage). Group 1 embryos could be divided into four subgroups that comprised donor embryos with initial cell numbers of 2, 3, 4 or 5. Group 2 contained five subgroups in which the donor embryo cell numbers were 6, 7, 8, 9 or 10.

3.5.1 Biopsy medium preparation

To loosen cell-cell adhesions and facilitate the biopsy, 50 x 9 mm ICSI dishes (Falcon) were prepared for each biopsy by adding 2 x 20 µl droplets of Quinn's Advantage Ca^{2+}/Mg^{2+}-Free Medium with HEPES (Sage, Cooper Surgical) supplemented with 10% SPS and then overlaying the medium with 4.5 ml of mineral oil. Immediately before conducting the biopsy, the dishes were warmed to 37°C for 30 minutes.

Before each blastomere biopsy procedure, one donor embryo (the future Twin A) and one emptied ZP (the future Twin B) were transferred into the same 20 µl microdrop of biopsy medium and incubated for 3 minutes on a warm microscope stage at 37°C. The label for each embryo was written on the underside of the ICSI dish.

3.5.2 Blastomere biopsy of the donor embryos

The biopsy procedure was performed on the heated stage of a Nikon IX-70 microscope that was equipped with micromanipulation tools (RI) in a 20% O_2 atmosphere. Prior to the blastomere biopsy, both the holding and the aspirating pipette (RI) (the latter is also known as the blastomere biopsy pipette) were primed with a small amount of biopsy medium. Under the lowest power objective (4x), the fine focus joystick was used to lower the holding pipette and bring the cleavage-stage donor embryo into focus. Gentle sucking and blowing was applied to the holding pipette to move the embryo into the correct position before securing the desired blastomere for biopsy at the 5 o'clock position. The blastomere biopsy pipette (with a diameter of 35 µm) was gently lowered into the drop next to the embryo, close to the blastomere to be biopsied. To prepare an opening for the blastomere biopsy pipette to penetrate, the ZP was targeted with a laser target cursor, and a 30-50-µm hole was drilled using a laser pulse length of 750 µsec that was generated by a Saturn 3™ laser system (RI) and RI Viewer Imaging Software (RI). This particular size of a hole in the ZP is required to avoid bulging of the transferred blastomeres during micromanipulation and embryo development or premature embryo hatching. Typically, the early cleavage-stage donor embryos (group 1 with 2, 3, 4 or 5 cells) with larger blastomeres were given an opening diameter in the ZP that was 40-50 µm, whereas the later-stage or embryos (group 2 with 6, 7, 8 9 or 10 cells) with smaller blastomere sizes were given a hole in the ZP with a diameter of 35-40 µm. Because one laser shot at 750 µs will generate a hole that is 12.5 µm, three to five overlapping laser shots were applied to achieve the required hole size in the ZP. The blastomere biopsy pipette was pushed through the hole to ensure that it was the required size and completely free of ZP remnants. Blastomeres were carefully aspirated one by one using the biopsy pipette that was inserted through the opening in the ZP. Once the blastomere(s) were removed and expelled into the drop, the biopsy pipette was removed to prevent back suction. At

the end of the biopsy, the donor embryo (Twin A) was released from the holding pipette by turning the holding air syringe clockwise (positive pressure). Clinically unsuitable oocytes and embryos and lower-grade embryos were emptied using the same technique that was used for the blastomere recipients, as shown in Figure 3.4. The previously emptied ZP was secured by the holding pipette, and the biopsied blastomere(s) were carefully inserted one by one into the emptied ZP(s) to create a recipient embryo (Twin B), as shown in Figure 3.5.

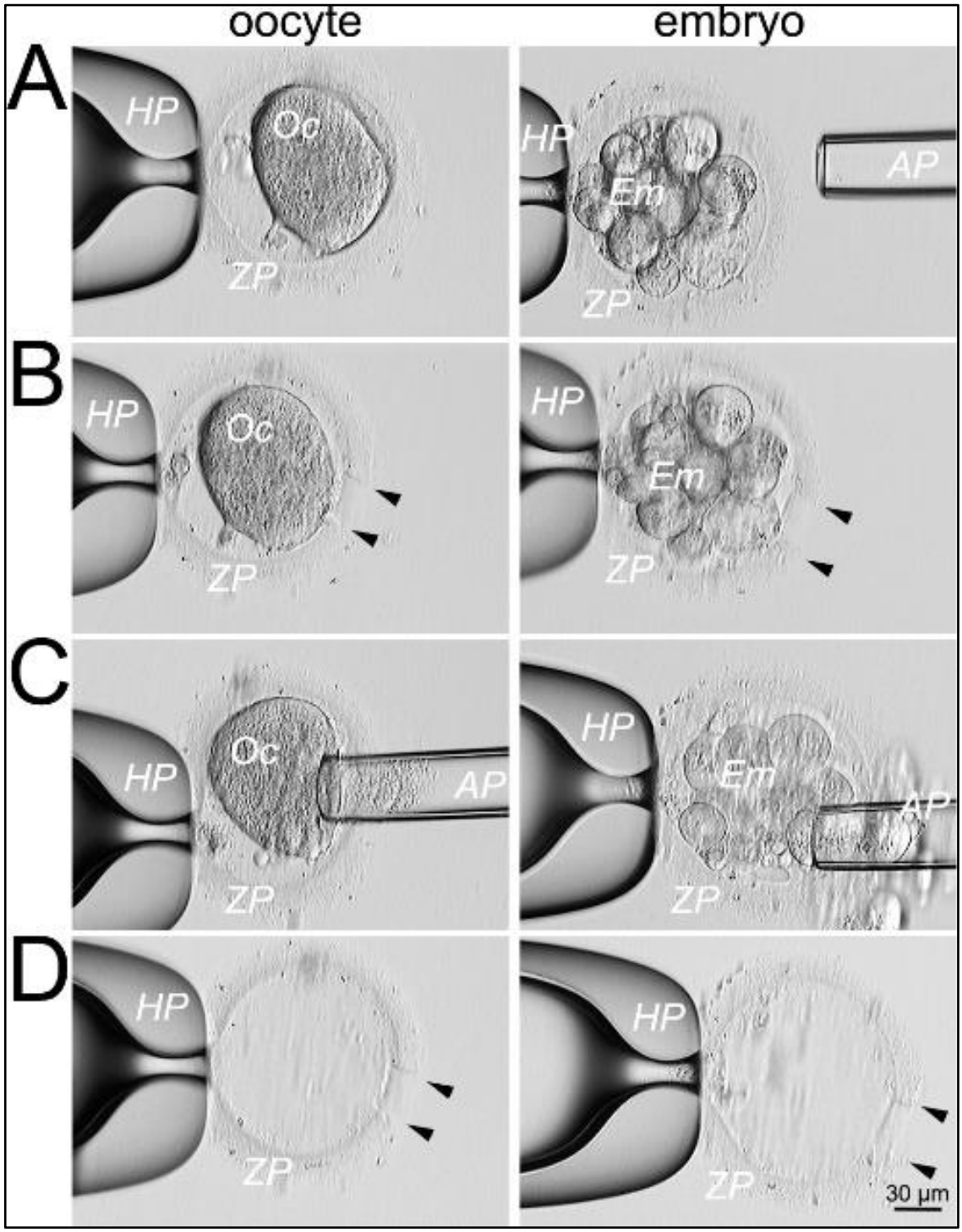


Figure 3.4: Preparation of blastomeres recipient zona pellucidae. (A) The blastomere recipient ZP was prepared from unfertilised oocytes (left) or from embryos that resulted from an anomalous fertilisation (right) and were therefore unsuitable for clinical use. (B) An opening was created in the ZP using a laser. (C and D) The cellular material within the ZP was aspirated until the ZP was completely empty to produce a vacant ZP shell, which was then used as a recipient for biopsied blastomeres. (AP, aspirating pipette; Em, embryo; HP, holding pipette; Oc, oocyte; and ZP, zona pellucida. The images were reproduced from Noli *et al* (2015b).

In cases where a donor embryo contained an even number of blastomeres (2, 4, 6, 8 or 10 cells), half of the blastomeres were transferred into a previously empty ZP to create a recipient embryo, as shown in Figure 3.5. In cases where a donor embryo contained an odd number of blastomeres (3, 5, 7 or 9 cells), the blastomeres usually varied in size. The larger blastomeres were randomized between Twin A versus Twin B. A similar blastomere biopsy strategy was used for 5-, 7- and 9-cell donor embryos.

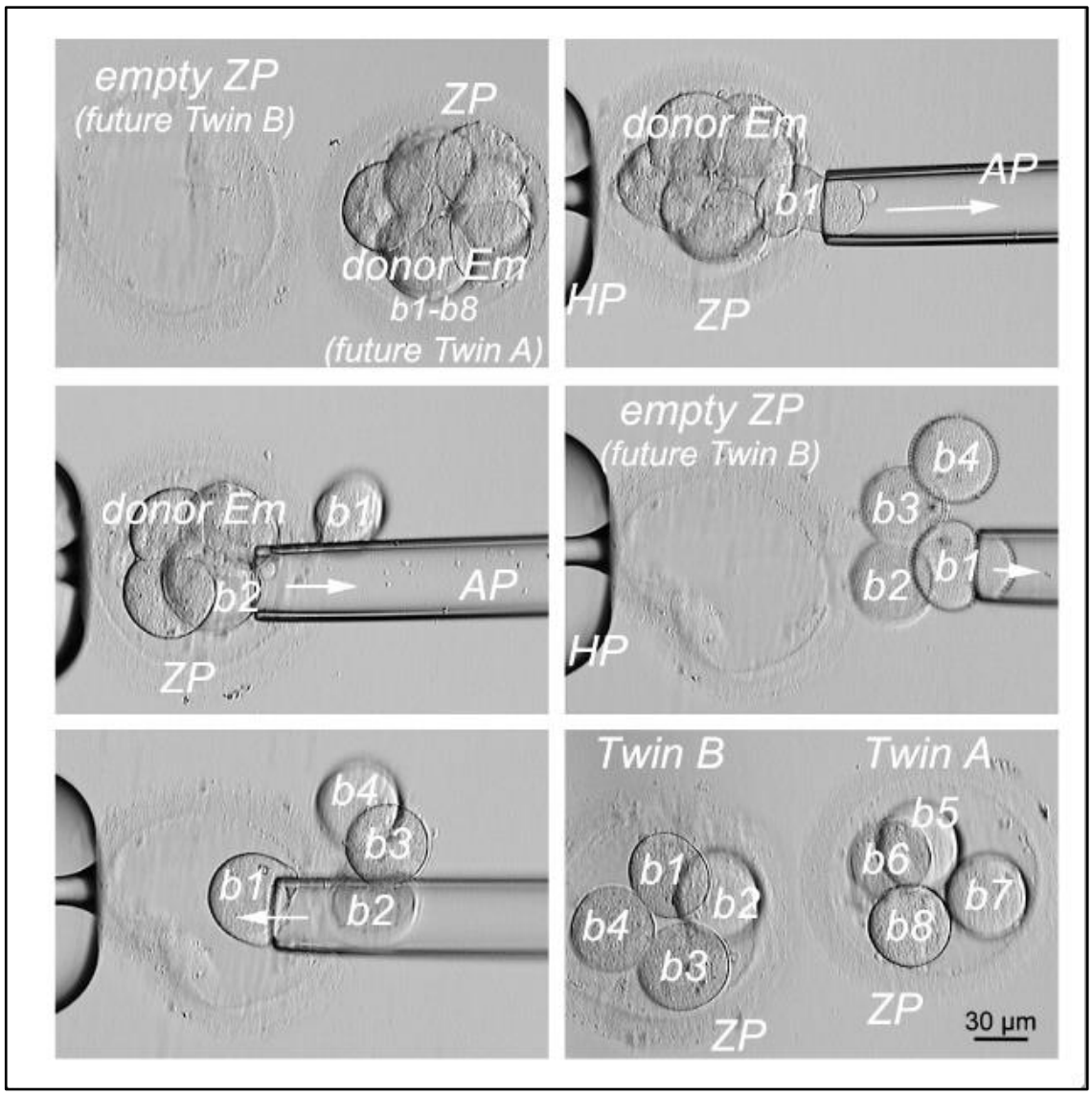


Figure 3.5: Process of cleavage-stage embryo twinning. Images showing the sequential process of a biopsy of 4 blastomeres (b1-b4) from an 8-cell donor embryo (Twin A) and their placement into a previously donated and prepared empty ZP (Twin B). The direction of the arrows indicates the movement of the blastomere and whether it was aspirated or expelled through the pipette. (AP, aspirating pipette; b, blastomere; Em, embryo; HP, holding pipette; ZP, zona pellucida). Images were reproduced from Noli *et al* (2015b).

3.5.3 Post-biopsy *in vitro* time-lapse culture of Twin A and B embryos

After blastomere transfer, the donor (Twin A) and recipient (Twin B) embryos were cultured *in vitro* in separate wells containing 25 µl of pre-equilibrated Quinn's Advantage Blastocyst Medium (BM) supplemented with 10% SPS and covered with 1.2 ml of mineral oil in previously prepared EmbryoSlide® culture dishes (Vitrolife). The EmbryoSlide® culture dishes containing BM were prepared a minimum of 6 hours in advance and equilibrated to 37°C in 6% CO_2 and 5% O_2. To distinguish the donor embryos from the recipient embryos, the donor embryos were placed in the EmbryoSlide® culture wells with odd numbers (1, 3, 5, 7, 9 and 11), and the even-numbered wells (2, 4, 6, 8 and 12) were used to culture the recipient embryos. During the splitting procedure used to create Twin A and Twin B, the EmbryoSlide® was initially placed in a Heraeus incubator at 37°C in 6% CO_2 and 5% O_2. Once the procedure was completed, the EmbryoSlide® containing 12 twin embryos (6 Twin A and 6 Twin B) was placed into an Embryoscope™ tri-gas incubator with a time-lapse image recording system (Unisense Fertilitech) and the embryos were incubated at 37°C in 6% CO_2 and 5% O_2. On average, approximately 1 hour was required to split 6 donor embryos, with an average of 10 minutes required per donor embryo.

Chapter 4

Validation Method I: Morphokinetic Analysis of Embryo Development using a Time-lapse Imaging System

4.1 Study hypotheses

The first method used to validate the quality and developmental competence of the twin embryos was an analysis of the morphokinetic parameters of the twins using a time-lapse imaging system. The morphokinetic data obtained from the twin embryos were compared to the morphometrics of the control embryos that were created by fertilisation and that resulted in live births after single blastocyst transfer. The study hypotheses were the following:

1. There are no differences in the percentage of embryos that reach each stage of development (9+; more than nine cells, M; morula, C; cavitation, B; blastocsyst, EB; expanded blastocyst) between Twin and control embryos.
2. There are no differences in the developmental dynamics of embryos that reach the EBs stage between Twin and control embryos or between the A and B Twin embryos.
3. There are no differences in average embryonic diameter between Twin and control embryos or between the A and B Twin embryos.
4. There are no differences in the percentage of embryos with ICM grades B and C between Group 1 and Group 2 embryos.

4.2 Control embryos

A standard short protocol was used to induce ovulation and control the timing of oocyte retrieval. All patients used a GnRH antagonist regimen during which gonadotropin injections were performed at a dose of 150-300 IU/day after an ultrasound scan on day 2 or 3 of the cycle to confirm the quiescence of the ovaries and the thinness of the endometrium (≤5 mm) and to determine AFC. The GnRH antagonist cetrorelix (Cetrotide; Merck-Serono) was administered at a dose of 0.25 mg daily when the lead follicle had reached a diameter of 14 mm. Both the gonadotropin and cetrorelix injections were continued until the administration of hCG was initiated. Transvaginal oocyte retrieval was performed 36 h later. ICSI was performed when the spermatozoa were of poor quality. Otherwise, standard *in* IVF procedures were performed. Following oocyte and sperm collection, whether with or without ICSI, the fresh embryos were transferred into a previously prepared EmbryoSlide® culture dish containing pre-equilibrated single step medium (Sage, Cooper surgical) and covered with 1.2 ml of mineral oil. The control embryos were exposed to the same culture conditions as the twin embryos: 37°C in 6% CO_2 and 5% O_2 in an Embryoscope. On Day 3, the embryos underwent laser-assisted zona ablation and were then returned to incubate in the Embryoscope. The herniated TE cells were biopsied on Day 5 or Day 6. Next, the blastocysts were vitrified using Cryotop/Kitazato reagent, and after PGD analysis was performed on the biopsied TE cells, the genetically suitable embryos were thawed and transferred. There was no actual physical use of the embryos. The purpose was solely to obtain morphokinetic data from normal embryos that were used for single embryo transfer and that subsequently resulted in a clinical pregnancy. These data were used as the control group.

4.3 Time-lapse monitoring

No morphokinetic movies were acquired of the donor embryos prior to splitting because the donor embryos were thawed and then cultured in a standard Heraeus incubator to the desired stage for splitting, as described in sections 3.4.1 and 3.4.2. Once the blastomere biopsy was completed, the resulting donor (Twin A) and recipient (Twin B) embryos were cultured in the EmbryoSlide® and placed in the Embryoscope under the same conditions, and time-lapse image recordings were

initiated to study embryo morphokinetics. The recordings used to determine the control embryo morphokinetics were made following a fertilisation check at the 2PN stage. All images were automatically recorded in seven focal planes every 20 minutes at 15 µm intervals (1280 × 1024 pixels per image, which was equivalent to 3 pixels per µm, in monochrome at 8-bit, and ≤ 0.032 s per image using a single red LED). The time-lapse movies of embryo development were all manually annotated by the same observer using EmbryoViewer® software version 5.3.202.8202. An example of a time-lapse image series of a single embryo with its accompanying annotation is shown in Figure 4.1.

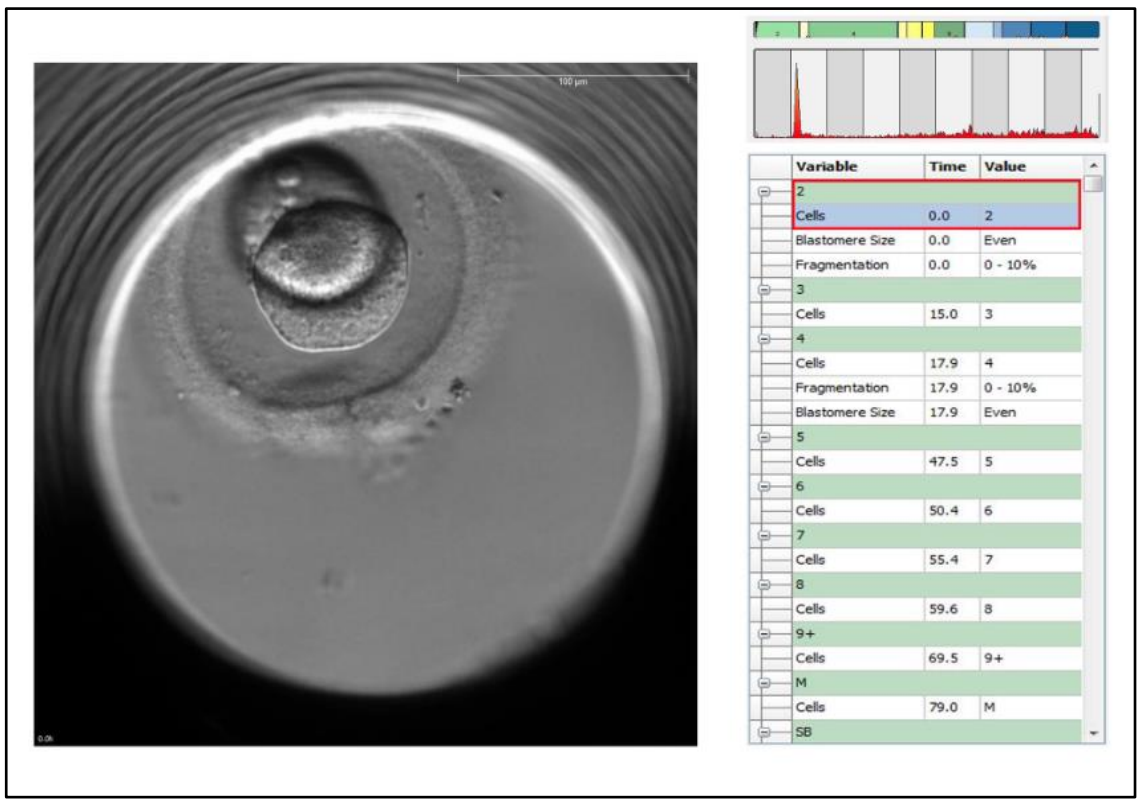


Figure 4.1: The intuitive embryo evaluation interface (Annotation tool). A manual annotation was made for each cell division event (for both the Twins and Controls). This was displayed as a table, which provided an easy overview of the observations that were made for each developmental stage. The annotation data shown here represent embryo #29, which was a Twin B embryo (E29B). This Twin originated from a donor embryo that was split at the 4-cell stage into two embryos, each with two cells, and t2 was taken to indicate time zero (selected in red).

4.3.1 Definition of morphokinetic parameters

4.3.1.1 Early morphokinetic parameters

The donor embryos used for splitting experiments were previously slow-frozen at different developmental stages (2PN, Day 2 or Day 3). Therefore, the following very

early morphokinetic parameters could not be accurately assessed: the timing of second polar body extrusion (tPB2), the timing of pronuclear appearance (tPNa), the timing of PN fading (tPNf), and the time at which the cell divided into 2 cells (t2) (t2 was defined in twin embryos after the donor embryo with two cells split into twin embryos each with containing one cell). Because these very early kinetic parameters could not be defined for embryos that had already split, these parameters were disregarded in the control embryos. The reference point for the timing of the split embryo was the first parameter that was recorded during embryonic development and was usually calculated as zero. For example, splitting an embryo at the 4-cell stage resulted in two twin embryos, each with two cells, and t2 was defined as zero hours for both embryos, as shown in Figure 4.1. The next frame corresponds to the next developmental stage (3 cells), which was defined as t3. The time points t4, t5, t6, t7 and t8 were defined as the first frame in which the corresponding number of cells was reached (e.g., t4 indicated 4 cells, t5 indicated 5 cells, and so on). The times were annotated at the time point at which cytokinesis was complete and a clear cell junction could be seen to have formed. Blastomere size was recorded as "even" at the 2- and 4-cell stages when all cells were of equal size (when there was a less than 50% difference in size). Blastomere size was recorded as uneven at the 2- and 4-cell stages when at least one blastomere was >50% larger or smaller than the other blastomeres.

4.3.1.2 Late morphokinetic parameters

The time t9+ (indicating more than nine cells) was defined as the time point at which the first sign of compaction was observed (when the cell membrane was no longer visible). For split embryos, this point was annotated before the 6-cell stage because many twin embryos displayed developmental behaviour that resembled the stage of their original parental donor. In the control embryos, t9+ was only recorded after the embryo completed the 6-cell stage.

The morula stage was reached when desmosomes and gap junctions were observed to have formed and the blastomeres were therefore bound tightly together. The cells become nearly indistinguishable at this stage, and the embryo resembles a spherical ball. This time point was defined as tM. The time point at which the blastocoel cavity began to emerge from the cavitation was annotated as tC. Once the blastocoel cavity was 50% of the size of the embryo, tB (blastocyst) was

annotated. Because an opening was made in the ZPs of both the split and the control embryos, tEB (expanded blastocyst) was defined as the time point at which the TE cells protruded through the opening of the ZP and the embryo began to hatch. Examples of the early and late morphokinetic parameters that were annotated are shown in Figure 4.2.

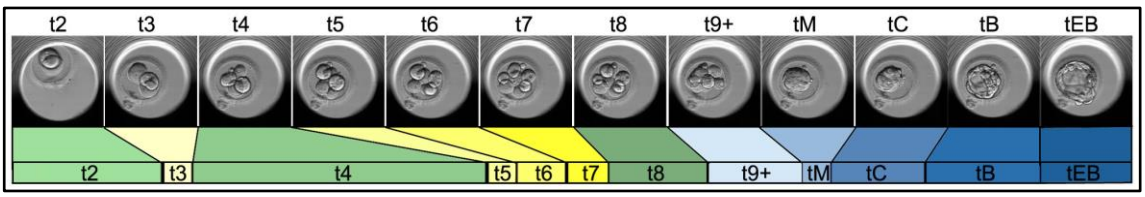


Figure 4.2: The early and late morphokinetic parameters that were annotated for all embryos, when possible. The time at which two cells were visible equalled time zero for the represented embryo, which resulted from a 4-cell-stage donor embryo that was split into two embryos. The early morphokinetic time points t3, 4, 5, 6, 7 and 8 were annotated when the corresponding cell numbers were observed. Late morphokinetic markers included the following: t9+: the time at which more than nine cells were observed; tM: the time at which a full morula was observed; tC: the time at which cavitation started; tB: the time at which the blastocyst had formed; and tEB: the time at which the blastocyst expanded (the start of hatching).

4.4 Assessment of blastocyst size and quality

The sizes of the blastocysts were evaluated in the donor, recipient and control embryos in the image frame in which the time point tB was recorded. The distance tool in the EmbryoViewer software was used to measure the vertical Y diameter (µm) and the horizontal X diameter (µm), as shown in Figure 4.3. The mean of these measurements was used as the calculated mean diameter for each embryo. Blastocyst quality was assessed using the image frame in which the time point tEB was recorded. As shown in Figure 4.4, the status of blastocyst expansion, the quality of the ICM and TE cells was evaluated based on the grading system suggested by Stephenson *et al.* (2007), this classification system is shown in Figure 4.5 (Stephenson *et al.*, 2007).

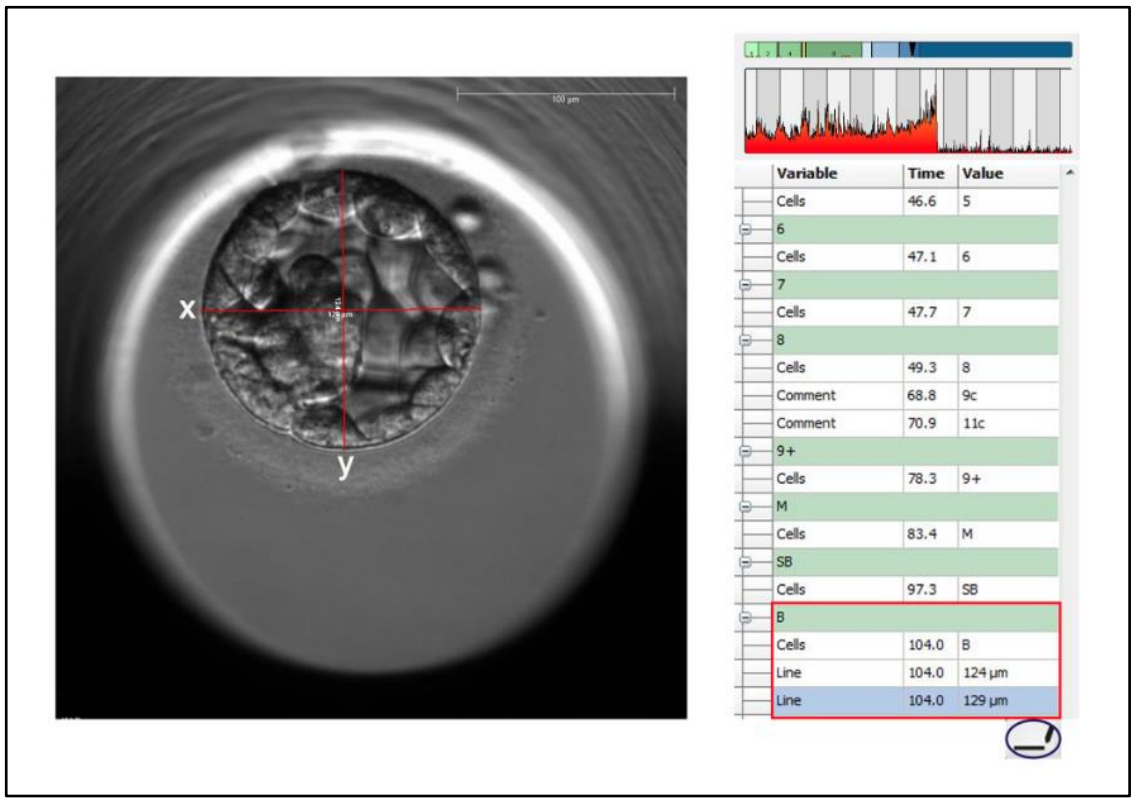


Figure 4.3: An example of the process used to measure the blastocyst diameter using the distance tool in the EmbryoViewer® software (selected in navy). The Y diameter (µm) and the X diameter (µm) were measured for Control embryo 1 (C1). The average diameter was calculated and exported into an Excel sheet for further analysis, as was described in section 4.4.

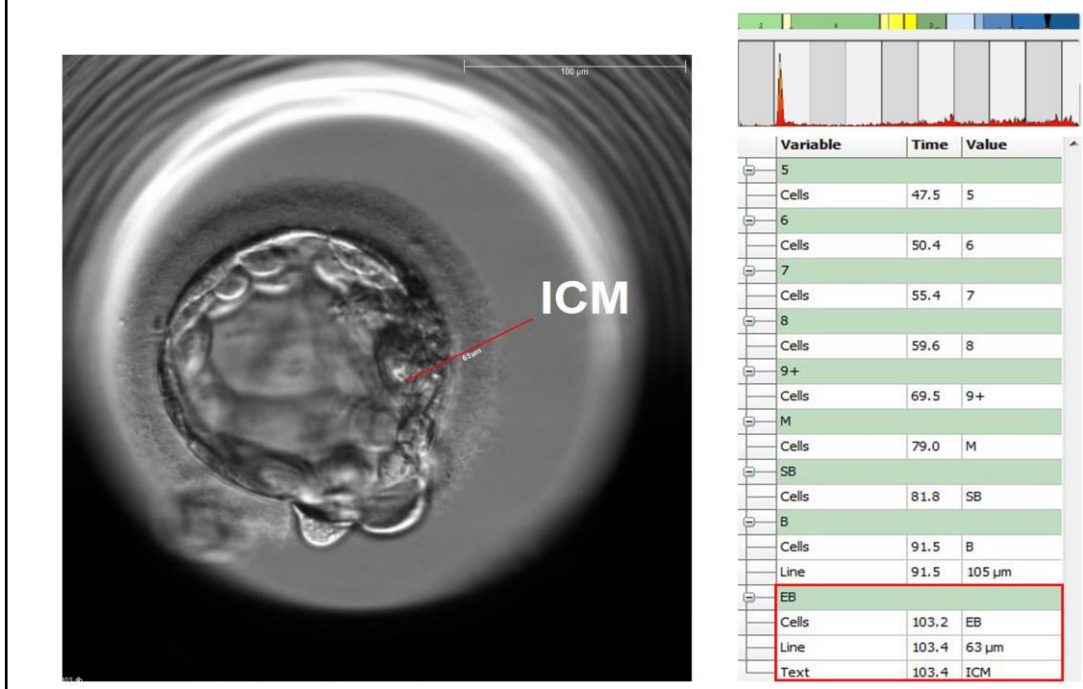


Figure 4.4: The ICM, TE and blastocyst assessments of embryo #29 twin B (E29B). This blastocyst was graded as 5Bβ based on the grading system that was defined by (Stephenson *et al.*, 2007).

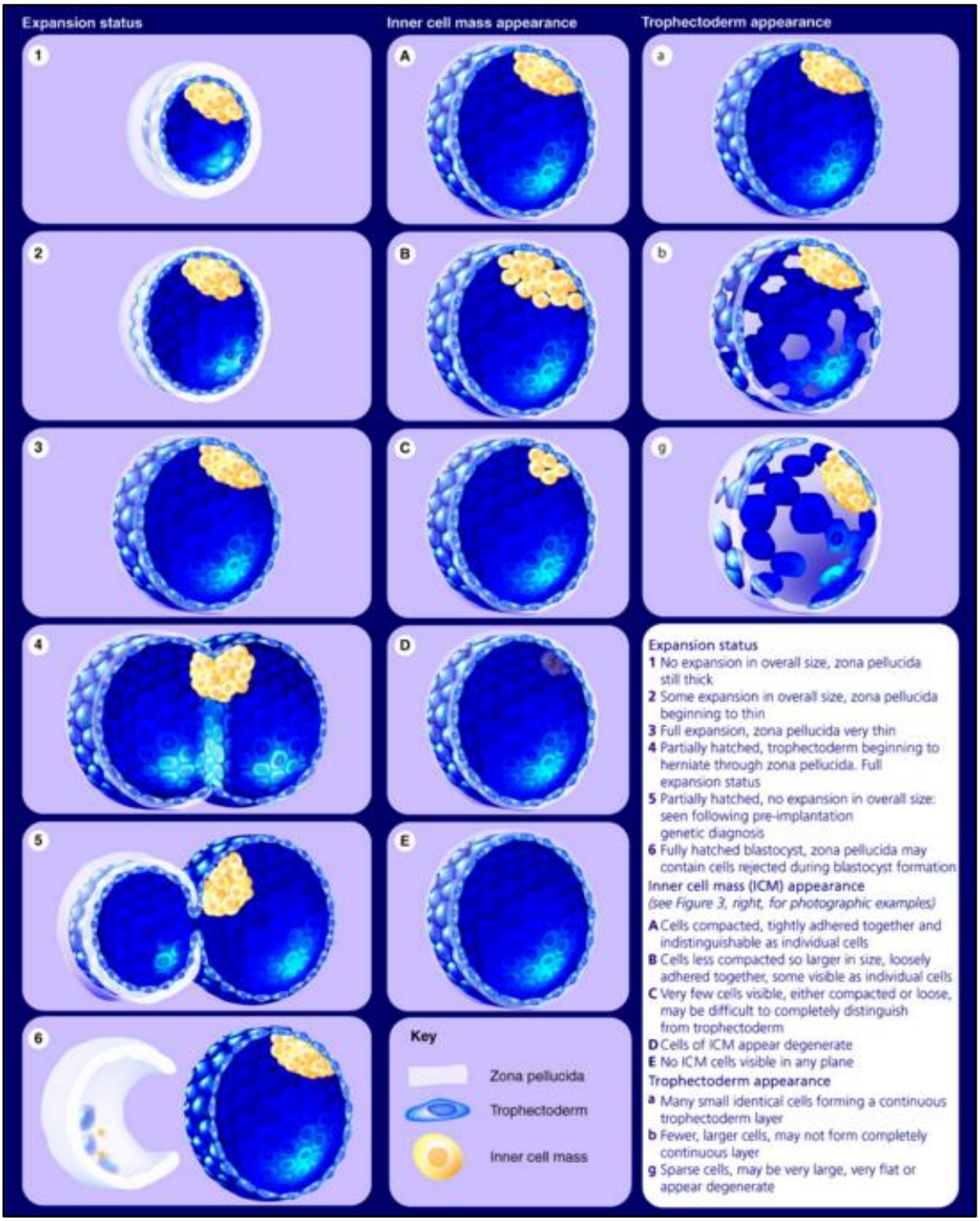


Figure 4.5: Figure showing the classification system that was used to grade the blastocysts that were generated from Twins A and B and the control embryos, as suggested by Stephenson *et al.*, 2007. A schematic depiction of stages 1-6 of Expansion. (A-E) Assessment of ICM quality (α- γ) and the appearance of TE. The images were reproduced from (Stephenson *et al.*, 2007).

4.5 Data collection, exporting and further coding

Annotation data were exported into an Excel document. Further data coding was manually performed, and all data were organised and presented in three different tables (Group 1, Group 2 and Control), as shown in the Results (Chapter 8). A sample data set is shown in Table 4.1, and each column and example is identified in Table 4.2. Time points refer to the first time (in hours) at which the given number of cells or developmental stage was observed and at which the parameter was annotated. When an objective evaluation of late kinetic parameters or specific developmental events was not possible as a results of a lack of or unfocused images, the presence of oil drops or other technical problems such as a failure to record, the respective data point was treated as missing data, and the corresponding embryo was excluded from further analysis.

Table 4.1: A representative sample of an Excel sheet. This sheet represents the data for Subgroup 6 of Group 2. The Donor embryo cell # (6 cells in this case), and the Twin embryo cell # (3 cells) were noted for Twin A (the donor) and Twin B (the recipient), respectively. Late stage morphokinetic parameters: the time period from stage t9 through stage tEB. Bc grading and Bc size are noted in the table.

DONOR EMBRYO cell #	TWIN EMBRYO cell #	Embryo ID	t9+	tM	tC	tB	tEB	Grading			Diameter(μm)		
								Expansion status	ICM	TE	Y	X	average
6.1	3	E8A	31.63	34.46	44.05								
	3	E8B	18.07	31.60	35.13	50.60					62	84	73
6.2	3	E6A	16.68	19.87	29.07	39.50	48.06	5	C	Y	114	116	115
	3	E6B	21.06	27.41	29.74	44.17	58.27	5	0	Y	98	95	96.5
6.3	3	E13A											
	3	E13B	30.59	N/A	50.56	67.90					98	96	97
6.4	3	E14A											
	3	E14B											
6.5	3	E22A											
	3	E22B	41.18	48.03	49.36	64.77					95	91	93
6.6	3	E19A	32.23	47.20	50.87	57.70					91	97	94
	3	E19B	29.70	38.40	44.19								
6.7	3	E41A	43.33	45.60	54.73	71.68					99	101	100
	3	E41B	36.00	52.75	66.96								
6.8	3	E61A	37.82	41.32	49.49								
	3	E61B	41.82	43.32	54.16	65.00					95	83	89
6.9	3	E73A	29.76	34.26	45.13	56.08	63.75	5	0	Y	110	120	115
	3	E73B	26.09	30.93	40.27	50.03	60.42	4	C	Y	113	119	116

Table 4.2: Identification of each column of raw data shown for Group 1, Group 2 and the control group (the columns DONOR EMBRYO cell #, TWIN EMBRYO cell # and Embryo ID were not available for the control embryos) and an example for each column.

DONOR EMBRYO cell #	The first number refers to the number of cells (blastomeres) in the donor embryo (subgroups). The second number refers to the donor ID. E.g., 6.1: six cells in donor 1
TWIN EMBRYO cell #	The number refers to the number of cells (blastomeres) in Twin A and Twin B after the donor embryo was split (the number of blastomeres that was used to create Twin A and Twin B). E.g., 3: Three cells were kept in Twin A and were transferred into empty ZP to form Twin B after the donor embryo (6.1) was split.
Embryo ID	The number refers to the Twin A and Twin B IDs, which are usually the same. The embryo that served as the donor embryo was called Twin A, and the second embryo, which developed in the recipient ZP after the blastomere biopsy procedure, was called Twin B. E.g., E8A: Twin A 8 and E8B: Twin B 8
t+9,tM,tC,tB and tEB	The numbers refer to the values of the late stage morphokinetic parameters, which include stages t9, tM, tC, tB and tEB and are expressed in hours.
Grading	The blastocyst quality assessment was performed using the image frame in which the time point tEB was annotated. ICM and TE grading and expansion status were noted based on the grading system suggested by Stephenson *et al.* (2007). The number refers to the expansion status. The letters (A-E) refer to the ICM quality, and (α- γ) refer to TE appearance. In the case of ICM absence, 0 was used. E.g., in E6A, the blastocyst was graded 5C γ. 5: the embryo was partially hatched, and the TE cells had begun to herniate through the ZP, with no overall change in size. C: The ICM quality reflects the presence of very few visible loose cells, which are difficult to distinguish from TE cells. γ: There were few TE cells and they were very large and flat.
Diameter (μm)	The blastocyst size was determined using the image frame in which the time point tB was annotated. The numbers refer to the Y and X diameters (μm) and the calculated average diameter (μm). The Y and X diameters were measured using the distance tool in the EmbryoViewer software, and the average diameter was calculated. E.g., in E8B, the Y diameter was 62 μm, the X diameter was 84 μm, and the average diameter was 73 μm.

4.6 Analytical methods

In this study, the statistical analysis was focused on late kinetic parameters because early kinetic parameters were not consistently available for all twin embryos as a result of their developmental behaviour post-splitting. The actual recorded time was used to calculate the duration (in hours) between each time point during embryo development, and these durations were used for further analysis.

4.6.1 Description of statistical methods

1. A survival analysis based on calculations of the proportions of embryos that reached each of the following stages of development: 9; M, C, B, and EB based on a group comparison and a comparison of twin cell #, as shown in Table 4.3.
2. An analysis of kinetics or developmental dynamics included only the embryos that reached EB. The analyses were based on comparisons of groups and twin cell # (Table 4.3) and were performed using two different methods: i) calculating the duration of critical developmental events, including compaction (9+ - M), the initiation of cavitation (M–C), blastocyst formation (C – B) and blastocyst expansion (B – EB) in embryos that reached the EB stage and ii) calculating the duration between the 9+ stage and the following stages: M, C, B and EB.
3. An analysis of blastocyst size was performed using the previously calculated average diameter of embryos (μm) (see section 4.4) and was based on group and twin cell # comparisons (Table 4.4).
4. An analysis of ICM quality that was based on calculating the percentage of embryos with an ICM grade of B or C (see section 4.4) in group 1 and group 2 (Table 4.5).

Table 4.3: Definition of Analysis Groups-survival and kinetics analyses. The Survival and kinetics analyses were based on group and Twin embryo cell #. Group 1: donor embryos were split at the 2-5-cell stage to generate Twin A and Twin B. Group 1 contained four subgroups corresponding to donor embryos with the following # of cells: 2, 3, 4 and 5. Group 2: donor embryos were split at the 6-10-cell stage to generate Twin A and Twin B. Group 2 contained five subgroups corresponding to donor embryos with the following # of cells: 6, 7, 8, 9 and 10. Overall: Group 1 and Group 2. Twin Embryo Cell #: the number of cells (blastomeres) in each Twin (A and B) after the donor was split. A: Twin A (donor). B: Twin B (recipient). Ctrl: control embryos. *A: comparison of Twin A to different twin embryo cell #s (1-6 blastomeres). B: comparison of Twin B to different twin embryo cell #s (1-4 blastomeres). A vs B: comparison between Twin A and Twin B embryos with equal twin embryo cell #s (1-4 blastomeres).*

Analyses	Group 1		Group 2		Overall		Twin Embryo Cell #		
	A vs B	A or B vs Ctrl	A vs B	A or B vs Ctrl	A vs B	A or B vs Ctrl	*A*	*B*	*A vs B*
1	√	√	√	√	√	√	√	√	√
2	√	√	√	√	√	√	√	√	√

Table 4.4: Definition of Groups for Blastocyst Size Analysis. The blastocyst size analysis based on group and twin cell # comparisons. Group 1: donor embryos were split at the 2-5-cell stage to generate Twin A and Twin B. Group 1 contained four subgroups corresponding to donor embryos with the following # of cells: 2, 3, 4 and 5. Group 2: donor embryos were split at the 6-10-cell stage to generate Twin A and Twin B. Group 2 contained five subgroups corresponding to donor embryos with the following # of cells: 6, 7, 8, 9 and 10. Overall: Group 1 and Group 2. Twin Embryo Cell #: the number of cells (blastomeres) in each of Twins A and B after the donor was split. A: Twin A (donor). B: Twin B (recipient). Ctrl: control embryos. Twin Cell # (1-6): Twin embryos originating from 1, 2, 3, 4, 5 or 6 blastomeres.

Analyses	Group 1		Group 2		Overall	Twin Embryo Cell #
	A vs B	A & B vs Ctrl	A vs B	A & B vs Ctrl	A &B vs Ctrl	Twin Cell #(1-6) vs Ctrl
3	√	√	√	√	√	√

Table 4.5: ICM quality analysis between Group 1 and Group 2. Group 1: donor embryos were split at the 2-5-cell stage to generate Twin A and Twin B. Group 1 contained four subgroups in which the donor embryos contained the following # of cells: 2, 3, 4 or 5. Group 2: donor embryos were split at the 6-10-cell stage to generate Twin A and Twin B. Group 2 contained five subgroups that corresponded to donor embryos containing the following # of cells: 6, 7, 8, 9 and 10. A: Twin A (donor). B: Twin B (recipient).

Analyses	Group 1	Group 2
	A or B vs Group 2	A or B vs Group 1
4	√	√

4.6.2 Statistical analyses

The statistical tests used to compare percentages included the χ^2 test when applicable (expected values ≥ 5). Otherwise, the Fisher's exact test was used with an alpha risk of 5%. To compare durations and mean diameters, Student's T-tests were used, with an alpha risk of 5%. All results were tabulated as shown in Chpater 8 Table 8.1, 8.2 and 8.3. All statistical analyses were conducted using GenoSplice (http://www.genosplice.com/).

Chapter 5

Validation Method II: Immunocytochemical Analysis

5.1 Study hypotheses

The second validation method was performed to analyse the appearance and localization of lineage-specific transcription factors in twin blastocysts derived from split embryos in comparison to the patterns observed in non-manipulated controls in Day 5-6 blastocyst stage embryos. The hypotheses were that a) the twin embryos generated from the splitting procedure were developmentally normal and b) the expression (appearance and localization) of key developmental regulators was not affected in these embryos.

5.2 Control embryos

For the immunostaining experiments, donated blastocyst embryos were acquired with approved consent for use in research. The embryos were thawed as described in section 3.4.1 and cultured in 40-µL drops of BM supplemented with 10% SPS and covered with mineral oil at 37°C in a 6% CO_2 and 5% O_2 environment. Because the blastocysts were in a collapsed state during the thawing procedure and potentially for several hours afterward, after each thaw, a 2-3 hours recovery period was allowed for the blastocoel to re-expand and cell division to resume. Based on the grading system proposed by Stephenson *et al.*, 2007, the good quality embryos with an ICM grading of A or B and a TE of **α or** β (Stephenson *et al.*, 2007) were used. The ages of the Twin and control embryos were matched (Day 5 or Day 6). In the majority of the immunostaining experiments, the Twin and control embryos were processed within the same drops. To gain insight into the expression patterns of lineage-specific markers in Twin and control embryos, the protein expression of the lineage-specific markers CDX2, YAP1, GATA2, NANOG and SOX17 were analysed.

5.3 Immunocytochemical procedure

Immunostaining was performed as described in Niakan and Eggan (2013). Briefly, 4% paraformaldehyde (pH 7.0) was freshly prepared for each experiment and cooled to 4°C by a member of the staff of Francis Crick Institute in London, United Kingdom. The embryos were placed in 500 µl of 4% paraformaldehyde solution in a 4-well non-adherent Nunc plate and then incubated overnight at 4°C on a rotating shaker or for 1 hour on ice. The embryos were subsequently removed from the paraformaldehyde and placed in a solution of Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS; Gibco Life Technologies) containing 0.1% Tween (Sigma). The embryos were washed in 0.1% Tween/PBS three times and then incubated in 0.5% Tween/PBS for 20 minutes on a rotating shaker.

A blocking solution was prepared by adding 10% foetal bovine serum (FBS, HyClone) to 0.1% Tween/PBS. Embryos were incubated in the blocking solution for 1 hour on a rotating shaker. The primary antibody was diluted to 1:500 in blocking solution. The following primary antibodies were used: rabbit polyclonal anti-YAP1 (Cell Signalling Technologies) and anti-GATA2 (Santa Cruz Biotechnology), goat polyclonal anti-NANOG (R&D), mouse monoclonal anti CDX2 (BioGenex) and goat polyclonal anti-SOX17 (R&D). The blocking solution was removed and replaced with the diluted primary antibody, and the embryos were incubated overnight at 4°C on a rotating shaker. The embryos were washed three times in 0.1% Tween/PBS and then incubated in fresh 0.1% Tween/PBS for 20 minutes on a rotating shaker. The secondary antibodies, which included Cy3-, FITC- or Cy5-conjugated donkey anti-rabbit, -mouse or –goat antibodies (Life Technologies), were diluted to 1:300 in 0.1% Tween/PBS. The embryos were then placed in the secondary antibody solution and incubated for 1 hour on a rotating shaker in the dark. The embryos were washed three times with 0.1% Tween/PBS and then incubated for 20 minutes in fresh 0.1% Tween/PBS on a rotating shaker in the dark. Nuclei were visualised using 4',6-diamidino-2-phenylindole (DAPI) in Vectashield mounting medium (Vector Labs). The mounting medium was diluted to 1:6 in 0.1% Tween/PBS. The embryos were then placed in 20 µl of diluted mounting medium. The embryos could be stored at 4°C in this solution for up to one week before imaging. This Vectashield medium enabled the stained embryos to be

stabilized by the viscous nature of the solution. In preparation for imaging, the stained embryos were placed in a 35-mm coverslip-bottomed dish (MatTek).

5.4 Imaging and fluorescence analysis

Imaging was performed in the Light Microscopy Unit at the Francis Crick Institute, London, UK. A Leica SP5 inverted confocal microscope (Leica Microsystems) was used to analyse the fixed and differentially stained blastocysts (controls and Twins). Fluorescent stains for ICM, TE and nuclear integrity were individually analysed and then combined in a Z-stacked image that was generated to enable the quantification of cell data in all focal planes of the blastocysts. ImageJ64 freeware was used with the Cell Counter plugin to quantify the cell numbers because it enables the user to count cells by clicking on individual cells in turn within an image, as shown in Figure 5.1. Every click was recorded by the programme as a coloured dot, and the numbers of dots were totalled and exported to an Excel sheet. These measurements were initially performed in triplicate, but when they were found to be consistent, single readings were recorded. Unmodified images were utilized for cell quantification, and the nucleus was considered to be positive if its staining intensity was visually greater than the intensity of cytoplasmic staining. Co-staining was determined by marking an area in one channel that co-localized with a DAPI. Brightness and contrast were adjusted using Adobe Photoshop CS2 to improve clarity, when necessary.

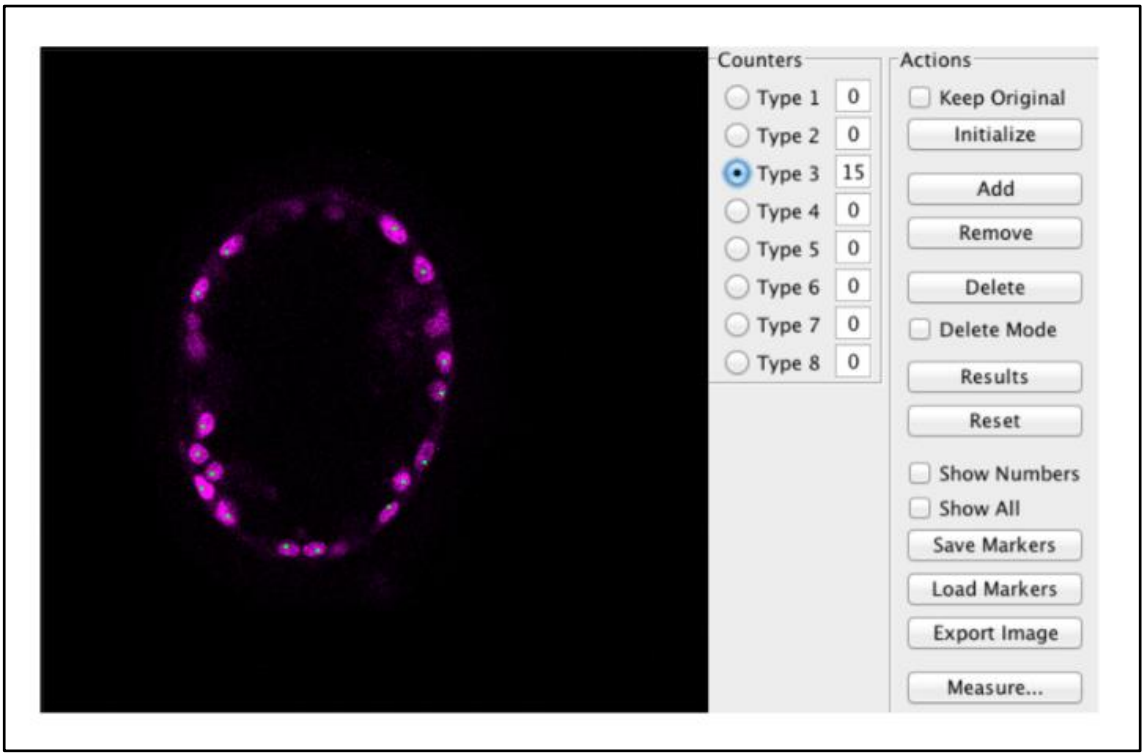


Figure 5.1: Cell quantification was performed using ImageJ64 software with the Cell Counter Plugin. A green dot was used to mark each cell in the embryo in the above image.

Chapter 6

Validation Method III: Derivation of hESC Lines

6.1 Study hypotheses

The capacity of the ICM of the twin blastocysts that were derived from split embryos to develop into a hESC line can be used to confirm their pluripotency. The third validation method used in this study, was to determine the pluripotency competence of the blastocysts that were derived from split embryos by deriving hESC lines from the ICM of the twin blastocysts and comparing them to hESC lines that were previously derived at the ACU at Guy's Hospital, King's College, London. The study hypothesis was that the ICM of the twin embryos that resulted from embryo splitting would be capable of being differentiated into hESCs.

6.2 ICM isolation and plating

The donor embryos used for splitting and the hESCs used in subsequent derivations were donated with informed consent for use in research (under license number R0133) and with local ethical approval (UK National Health Service Research Ethics Committee Reference: 06/Q0702/90). The hESC lines were derived using Day 5 or Day 6 twin blastocysts with a distinct ICM grade of A or B (section 4.4) (n=5 pairs). The derivation was performed according to previously established protocols (IIic *et al.*, 2012; Stephenson *et al.*, 2012). Briefly, a holding pipette was used to secure the blastocyst in the region of the zona pellucida under the ICM. The embryo was cut into two parts using a Saturn 3™ laser system and RI Viewer Imaging Software. The smaller part containing the ICM and adjacent TE cells was transferred into a well in a 4-well dish that contained mitotically inactivated human foreskin fibroblasts (HFFs). Dishes containing HFF feeder cells were prepared by a member of the Embryonic Stem Cell Laboratories in Guy's Assisted Conception Unit.

6.3 Cell culture

The isolated ICM was subsequently plated on mitotically inactivated HFFs and left undisturbed for 48 hours (IIic *et al.*, 2007 and 2010). During the culture period, 50% of the culture medium was replaced every other day. Images of ICM clumps were recorded on a daily basis. All cell cultures were incubated at 37°C in 5% CO_2 in air or in 6% CO_2 and 5% O_2. The cultures were monitored for the appearance of hESC colonies for 15 days. If any hESC-like colonies were visible, they were expanded and cryopreserved at the third passage. Dishes that did not generate hESC colonies were discarded using appropriate protocols.

Chapter 7

Validation Method IV: Analysis of the Secretion of miRNAs into Spent Blastocyst Medium (SBM)

7.1 Study hypothesis

To validate both the functional and the reproductive capacity of twin embryos that were generated using the splitting procedure, a fourth validation method was used in which analysed the miRNA profiles in SBM obtained from twin blastocysts (n=7 pairs) and compared them to the SBM profiles of control blastocysts (n=7) that were generated via fertilisation (non-manipulated) and that resulted in live births upon single embryo transfer. The study design is explained in Figure 7.1. The hypothesis was that there is no difference between the SBM miRNA expression profiles of the Twin and control embryos.

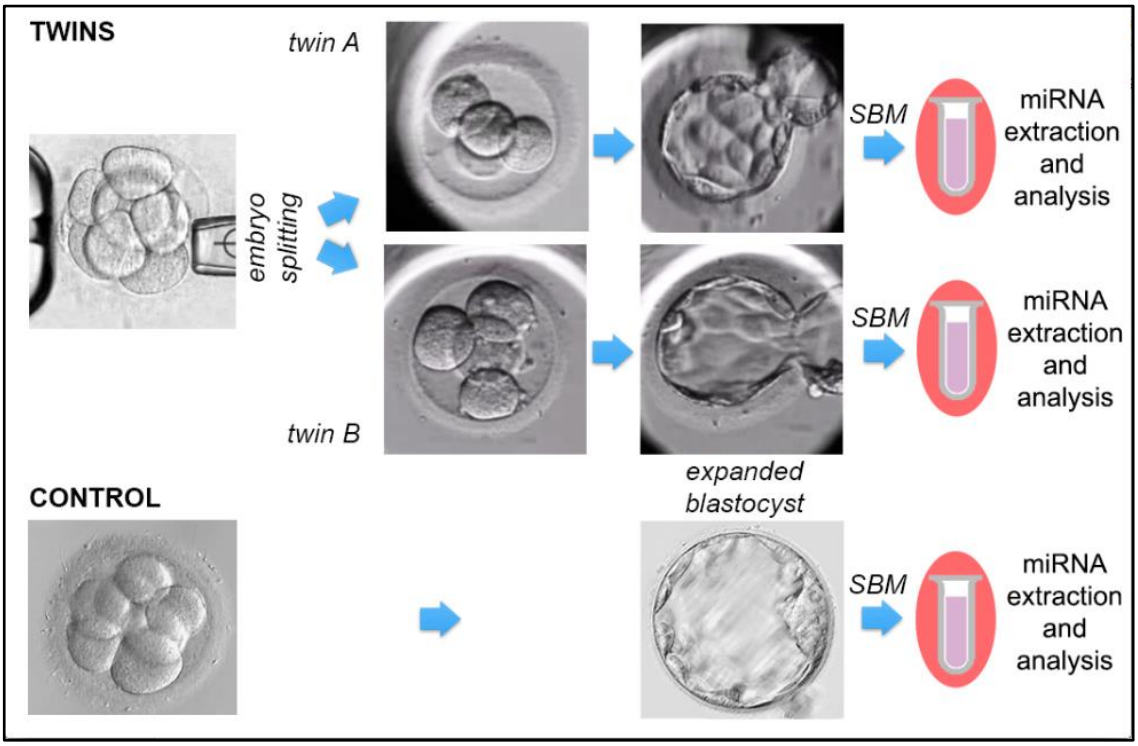


Figure 7.1: Study design. Control embryos were cultured undisturbed until they reached the fully expanded blastocyst stage, and SBM was then collected. All seven blastocysts were diagnosed as euploid and resulted in healthy pregnancies after transfer. Twin embryos were produced after embryo splitting at the cleavage stage. Twin A embryos were cultured up to the blastocyst stage in their own ZP, while Twin B embryos were cultured in host ZPs. SBM was collected at the blastocyst stage. MicroRNAs were extracted and analysed from 7 controls and 7 pairs of twins. The figure was taken from (Noli *et al.*, 2016a).

7.2 Embryo culture

7.2.1 Twin embryo culture

The procedures used to thaw the cleavage-stage donor embryos, to culture the post-thaw embryos, and to perform the quality assessments, the blastomere biopsies of the donor embryos and the post-biopsy *in vitro* cultures were described extensively in sections 3.5.2 and 3.5.3 and in (Noli *et al.*, 2015b). Briefly, 50% of the blastomeres were removed from the donor embryo using aspiration and placed in an emptied ZP. The empty ZP was prepared from a lower grade embryo or oocyte or an embryo that was unsuitable for *in vitro* fertilisation. Both Twin embryos, including the donor (Twin A, n=7) and the recipient (Twins B, n=7), were transferred into an EmbryoSlide® dish containing pre-equilibrated BM supplemented with 10% SPS and covered with 1.2 mineral oil under culture conditions (37°C in 6% CO_2 and 5% O_2) in an Embryoscope™ tri- gas incubator. No morphokinetic analyses were performed for these pairs.

7.2.2 Control embryo culture

SBM samples that were obtained from embryos that resulted in a healthy pregnancy and a live birth following single embryo transfer (n=7) were used as the controls. All experiments performed using control embryos obtained from IVF procedures and the collection of SBM were performed in GENERA, the Centre for Reproductive Medicine, Clinica Valle Giulia, Rome, as described in (Capalbo *et al.*, 2016). Briefly, the embryos were obtained following controlled ovarian stimulation, and fertilised oocytes were cultured sequentially in single 25µl microdrops (Cooper Surgical, Sage) in a humidified environment with 5% O_2 and 6% CO_2 until they reached the blastocyst stage (Days 5–6). On Day 3, cleavage-stage embryos were transferred to a freshly made drop of BM that was supplemented with 5% Quinn's Advantage HSA (Cooper Surgical, Sage). Before the cryopreservation process was performed, blastocyst quality was assessed using the standard guidelines established in (Gardner and Schoolcraft, 1999). The process used for blastocyst vitrification and the warming procedures were performed using a Vitrification and Warming Kit (Kitazato BioPharma) (Kuwayama *et al.*, 2005; Kuwayama, 2007). The entire vitrification and warming procedure was performed under ambient

conditions as previously described in (Rienzi *et al.*, 2010). Once warming was completed, the blastocysts were placed in individually numbered 35 µl microdrops of BM supplemented with 5% HSA and were covered with a film of mineral oil (Sage). The procedures that were previously published in (Capalbo *et al.*, 2015) were used to perform TE biopsies, 24-chromosome aneuploidy screenings, and vitrification. Based on their morphological score, euploid blastocysts were selected for transfer and then warmed and placed in an incubator at 37C in 6% CO_2 and 5% O_2 until transfer.

7.3 SBM collection

When the Twin A, Twin B and control embryos reached the blastocyst stage, the spent culture medium was collected as previously described by (Capalbo *et al.*, 2016; Noli *et al.*, 2016a). Briefly, each Twin blastocyst was gently moved to a new microwell using a Flexipet with care taken to not aspirate any culture medium. The control blastocyst was transferred to either a Nunc for transfer, or to the biopsy dish, or to the vitrification dish.

A new sterile tip that was mounted on a Gilson pipette was used to aspirate the entire SBM (20-25 µl) from each well of the EmbryoSlide® dish/culture dish that contained a Twin embryo/control blastocysts. Care was taken to avoid drawing the mineral oil layer into the pipette. The SBM of the twin embryo/controls was transferred to individually labelled PCR tubes that contained 120 µl of lysis solution. The tubes were then briefly centrifuged and stored at -80°C until they were shipped on dry ice (in the case of SBM collected from twin embryos) to GENERA, the Centre for Reproductive Medicine, Clinica Valle Giulia, Rome, Italy for further analysis.

7.4 Analysis of miRNAs in spent blastocyst media

7.4.1 Isolation, retro-transcription, and pre-amplification of miRNA

miRNA isolation and profiling were performed using previously described methods (Capalbo *et al.*, 2016). Briefly, a magnetic bead-based extraction method (Anti-miRNA Bead Capture purification kit human panel A, Life Technologies) was used to isolate and purify miRNAs from the SBM of the Twin and control embryos. The

entire recoverable volume of the sample was brought to a total volume of 150 µl using ABC buffer. The bead hybridization and miRNA elution steps were performed using a Thermomixer (Eppendorf) according to the manufacturer's instructions. To remove DNA, proteins, contaminants, and residual binding solution while retaining the miRNA that was bound to the beads, a series of washing steps were performed using a DynaMag-2 magnet (Applied Biosystems). The miRNAs were eluted into a final solution volume of 10 µl to keep them concentrated in the minimum possible amount of liquid. The cDNA was generated using the specific Megaplex RT Primer Pool A v3.0 (Applied Biosystems) on a 7900 HT Real-Time PCR System (Applied Biosystems).

To double the final sample volume, a modification of the pre-amplification reaction was used to perform two simultaneous cDNA synthesis reactions. Specifically, two independent reactions were performed in two different tubes to generate PCR products that were pooled at the end of the reaction. Each tube contained 3.5 µl of retro-transcription product, 12.5 µl of TaqMan PreAmp Master Mix (2X), 2.5 µl of Megaplex PreAmp Primers (10x), and 6.5 µl of nuclease-free water. The manufacturer's protocols were used for the PCR reaction, and eight cycles of amplification were performed.

7.4.2 MiRNA analysis using a TaqMan Low-Density Array (TLDA)

To determine the miRNA expression profile of the SBM samples, TLDA miRNA Cards (Panel A) (Applied Biosystems) were used in an Applied Biosystems Viia7 Real-Time PCR System. Fifty microliters of the pre-amplification product was diluted with 400 µl of nuclease-free water. This solution was then mixed with 450 µl of TaqMan Universal Master Mix II containing no Uracil-N glycoslyase (UNG). The mixture was then distributed into the wells of a 384-well plate using centrifugation in a Heraeus Megafuge 40 (Thermo Scientific) with the appropriate TLDA card adapters. The 384-well plate was incubated at 95°C for 10 minutes followed by 40 cycles of amplification at 95°C for 15 seconds and 60°C for 1 minute in a ViiA 7 Real-Time PCR System (Applied Biosystems).

7.4.3 Data processing and statistical analyses

A preliminary manual inspection of all amplification plots was performed. The raw data were subsequently analysed using SDS software (Applied Biosystems). The cycle threshold (Ct) values obtained from the PCR were used as the readouts. All Ct values below 35 were included to capture all potentially meaningful signals from low-input samples for which no comprehensive data for miRNA spectra had been reported. False amplification curves were excluded from the downstream analysis using manual selection. Real-Time StatMiner 5.0 software (Integromics) and SPSS (IBM) were used to process the data. Two blank media control drops were used as the negative controls. These were incubated in the same dishes as the SBM obtained from the embryos, and they were also included in the miRNA analysis as previously described (Capalbo *et al.*, 2016). The data were also compared with the miRNA profiles that were obtained from 5 ICM-free TE samples that were derived from normal blastocysts that were donated for research, as previously described (Capalbo *et al.*, 2016)

7.4.4 Quality control and additional data analysis

Quality control (QC), processing, and further analyses of the data were performed using endogenous control for normalization by GenoSplice Technology. For each miRNA X of each sample S, the samples were normalized according to the formula $2^{-\Delta Ct_{X,S}} = 2^{-(Ct_{X,S} - \text{mean}(Ct_{U6,S}))}$.

7.5 Single assay

Eluted, left-over SBM samples that were obtained from Twin A and Twin B embryos and randomly selected historical controls (euploid implanted blastocysts) were processed for single assay analyses using qPCR. Blanks (molecular biology grade water) and media samples that had never been exposed to embryos were processed in parallel as negative controls. The single assays were tested for U6-snRNA, miR-30c and miR-203. The first of these was chosen to confirm its weak reliability as detected through the miRNome panel, the second was chosen to confirm the differential expression of markers in the comparison between Twin A and Twin B embryos, and the last was chosen to act as a potential normalizer

because it showed the most stable trend among all these samples in the panel analysis (Twin A Ct 26.3±1.1 and 24.1-27.4; Twin B Ct 25.8±1.2 and 23.5-27.3; euploid implanted blastocysts 24.1±1.1 and 22.9-25.5). The minimum variance observed when the median method was performed using the StatMiner software (Integromics) confirmed its appropriateness for this aim. The primers that were used for the retrotranscription reactions in the three assays were pooled together (5 µl each) and diluted with 485 µl of nuclease-free water. The following retrotranscription mixture was used: 6 µl of primer pool, 0.3 µl of 100 mM dNTPs (with dTTP), 3 µl of MultiScribe™ Reverse Transcriptase 50 U/µl, 1.5 µl of 10 × Reverse Transcription Buffer, 0.2 µl of RNase Inhibitor 20 U/µl, 1.5 µl of nuclease-free water (TaqMan® Small RNA Assays protocol, Life Technologies) and 2.5 µl of eluted left-over sample. The following thermal protocol was conducted using a 2720 Thermal Cycler (Life Technologies): 16°C for 30 seconds, 42°C for 30 seconds, 85°C for 5 seconds and 4°C ∞. No preamplification step was used in this protocol, and therefore the threshold Ct level for detection was raised to 37 cycles. The following qPCR reaction mixture was used: 0.5 µl of TaqMan® Small RNA Assay (20 ×), 5 µl of TaqMan® Universal PCR Master Mix II (2 ×) with no UNG, 2 µl of nuclease-free water and 2.5 µl of sample. The qPCR was performed on a ViiA7 machine (Life Technologies) according to the manufacturer's protocol. Each assay was run in triplicate to exclude technical variability resulting from pipetting error. The Ct values used for miR-30c were normalized to the miR-203 values, and the differential expression analysis was performed using the ΔΔCt method (Noli *et al.*, 2016a).

Chapter 8

Results

8.1 Embryos included in the splitting experiment

8.1.1 Donor and recipient embryos

A total of 406 embryos were thawed to use as donor embryos during the embryo splitting experiment. These embryos were sourced from patients at Guy's Hospital and from different IVF clinics around the UK. Of these, 76 were slow frozen at stage 2PN (Day 1). A total of 330 were cleavage stage embryos, with 144 frozen on day 2 and the remaining 186 frozen on day 3. Each embryo was thawed exactly as described in Chapter 3, section 3.4.1, with survival defined as at least 50% of the cells remaining intact following the procedure, or in the case of pronuclear embryos, an intact membrane and normal cytoplasm. Out of the 406 donor embryos, 294 survived post-thawing, and these were distributed across the following stages: 2PN (n=49/76), Day 2 (n=114/144) and Day 3 (n=131/186). After culturing and assessing the quality of the donor embryos, 137 were found to be 'good quality', meaning Grade 4 (n=79) or Grade 3 (n=58). These embryos were grouped based on the number of viable non-degenerated blastomeres that were present as follows. In Group 1, the donor embryos were split using a blastomere biopsy procedure at the 2- to 5-cell stage (early cleavage stage), whereas in Group 2, the donor embryos were split at the 6- to 10-cell stage (late cleavage stage). The remaining 157 embryos that were Grade 1 or Grade 2 did not meet the criteria to become donors and were therefore excluded from the splitting experiments. They were instead used to prepare the empty ZPs that served as the recipients for the blastomere biopsies that resulted in Twin B embryos.

8.1.2 Twin embryos and their further use in different validation assays

A total of 128 out of 137 of the blastomere biopsies were successfully completed, resulting in the formation of 256 Twin embryos: Twin A and Twin B (n=128 pairs). Of the remaining 9 embryos, blastomere lysis occurred during the biopsy

procedure, and the pairs were subsequently excluded from any further analysis. Only 121 pairs out of the 128 pairs of successfully biopsied embryos were used for further analysis, while the remaining 7 pairs were excluded from further analysis because morphokinetic data was unavailable for these pairs. A total of 176 embryos (n=88 pairs) were used for the morphokinetic analysis (validation method I), and 42 embryos (n=21 pairs) were used for the immunostaining procedures (validation method II). Spent culture media was collected for miRNA analysis from 14 embryos (n=7 pairs) (validation method III), and hESC derivation was attempted from 10 embryos (n=5 pairs) (validation method IV), as shown in Figure 8.1.

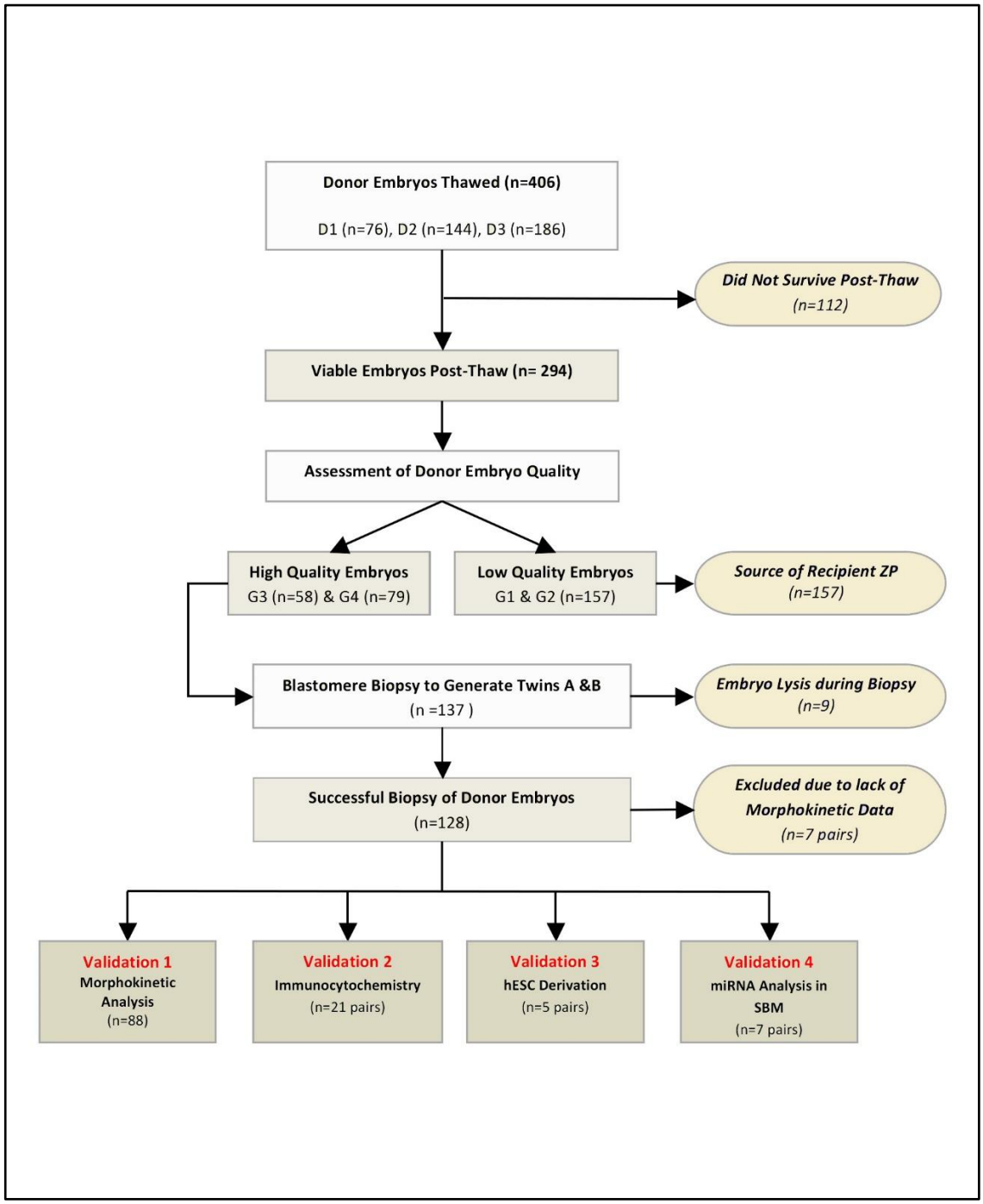


Figure 8.1: Scheme of the donor and recipient embryos that were included in the splitting procedure, showing the successful formation of Twin A and B embryos and their further use in the validation systems that were performed in this study.

8.2 Validation method I: A comparative analysis of morphokinetic parameters

8.2.1 Blastomere donor and blastomere recipient twins have similar developmental potential

A total of 88 cleavage stage embryos were segregated into two different groups: Group 1 (n=43), which contained early cleavage stage embryos consisting of 2-5 blastomeres, and Group 2 (n=45), which contained late cleavage stage embryos consisting of 6-10 blastomeres. Half of the blastomeres in each embryo were biopsied and subsequently inserted into a previously prepared empty ZP (unless an odd number of blastomeres was available in the donor embryo) (Figure 3.5). The empty ZP was produced by removing the cellular material from immature oocytes or embryos that were unsuitable for clinical use, or from Grade 1 and Grade 2 donor embryos (Figure 3.4). The embryos that were used as the donors of the blastomeres were called Twin A in further experiments, while the recipients of the biopsied blastomeres were called Twin B. All the Twin embryos that were produced in this manner were subsequently cultured in an incubator containing a built-in microscope and a camera for the morphokinetic studies. Morphokinetic data from normal embryos was used as the control data set. These normal embryos were subjected to laser ablation of the ZP on Day 3, and they were then cultured up to Day 5-6 and subsequently used for single embryo transfer, which resulted in clinical pregnancy (n=42).

The sequence of events as determined using time-lapse analyses is demonstrated in Figure 8.2 A, and the raw data from the experiments has been compiled in Tables 8.1, 8.2 and 8.3. In the embryo splitting experiments, it was found from general observation that Twins from Group 2 (late cleavage stage) developed into blastocysts that exhibited better quality and higher viability than Twins in Group 1. Of the 86 Twin embryos in Group 1, 26 were EBs (30.23%), whereas in Group 2, the percentage of embryos that were expanding blastocysts was (42.22%), or 38 EBs out of 90 Twin embryos. All data pertaining to the percentage of embryos in Group 1 and Group 2 that progressed to the +9, M, C, B, or EB stage are summarized in Table 8.4

Overall, it appeared that within Group 1, Twin B embryos (23 embryos) developed to a later stage (reached the blastocyst stage, B) than Twin A embryos (13 embryos). However, it must be noted that the only significant difference between Twin A and Twin B embryos was found in blastocyst formation (30.23% vs 53.49%; p=0.049; Figure 8.2 B). Furthermore, the developmental potential of Twin A and Twin B embryos that originated from 1 (n=4), 2 (n=21), 3 (n=11) or 4 (n=24) blastomeres to successfully reach the EB stage was comparable, regardless of their group of origin (Group 1 or Group 2). Comprehensive data about the proportion of embryos that reached each stage are presented Table 8.5. Twin A and Twin B embryos performed similarly (Figure. 8.2C); however, embryos that were created from a higher number of blastomeres (3 and 4) tended to achieve more development than their counterparts that were derived from one or two blastomeres (Figure. 8.2D).

Table 8.1: Data summarizing the developmental potential of Twin A and Twin B embryos from Group 1. The numbers in the rows indicate the hour at which each of the embryos being monitored reached a particular developmental stage (M, morula/fully compacted embryo; C, initiation of cavitation; B, blastocyst; EB, expanding blastocyst). Grading data and blastocyst size was displayed, for Identification of each column of raw data see Table 4.2 in Chapter 4.

DONOR EMBRYO cell #	TWIN EMBRYO cell #	Embryo ID	t9+	tM	tC	tB	tEB	Grading			Diameter (µm)		
								Expansion Status	ICM	TE	Y	X	average
2.1	1	E83A											
	1	E83B											
2.2	1	E30A	60.84	72.46	91.01	98.56	112.83	4	C	Y	90	95	92.5
	1	E30B	61.33	79.33	88.09	92.59	94.47	4	0	Y	92	103	97.5
2.3	1	E31A											
	1	E31B	56.10	64.21	74.96	86.27	109.84	4	0	Y	102	97	99.5
2.4	1	E52A											
	1	E52B											
3.1	1	E82A											
	2	E82B											
3.2	1	E84A											
	1	E84B	57.58	59.42	71.30	84.78					60	72	66
3.3	1	E85A	49.35	57.92	65.92	80.61					95	86	90.5
	2	E85B	48.69	54.76	58.42	67.60	72.31	4	C	Y	108	107	107.5
3.4	2	E43A	60.71										
	1	E43B											
3.5	2	E44A	58.31										
	1	E44B	40.16	50.49	57.76	68.50					60	87	73.5
3.6	2	E49A	27.26	40.88	47.30	52.01	59.26	5	0	Y	106	108	107
	1	E49B	29.54	40.54	44.25	53.10	66.13	4	0	Y	83	95	89
3.7	1	E53A											
	2	E53B											
4.1	2	E86A											
	2	E86B	39.88	45.04									
4.2	2	E87A	47.88	60.28	68.32	76.63	85.31	4	0	Y	97	101	99
	2	E87B	46.05	50.94	54.94	65.61	73.13	5	C	Y	111	116	113.5
4.3	2	E88A	43.89	50.28	62.45	85.48					104	82	93
	2	E88B	39.05	55.62	57.12	69.66	75.64	5	0	Y	105	101	103
4.4	2	E24A											
	2	E24B											
4.5	2	E25A	67.12	73.14	91.09	102.40	112.04	5	C	Y	115	111	113
	2	E25B	49.33	61.02	73.85	83.20	107.65	5	0	Y	102	100	101
4.6	2	E26A	57.53										
	2	E26B											
4.7	2	E32A											
	2	E32B	39.83	45.11	54.37	75.09					104	103	103.5
4.8	2	E33A	42.63	55.12	56.93								
	2	E33B	35.21	40.88	43.34	59.74					101	94	97.5
4.9	2	E34A											
	2	E34B	77.27										
4.10	2	E35A	76.92										
	2	E35B	79.87										
4.11	2	E36A											
	2	E36B											
4.12	2	E27A	62.31	67.71									
	2	E27B	52.11	53.90	69.17	77.08					78	81	79.5
4.13	2	E28A	56.10	64.54	73.50	87.69	108.15	5	0	Y	105	103	104
	2	E28B	52.87	58.92	67.72	94.00	112.26	5	C	Y	93	97	95
4.14	2	E29A	78.57	91.90									
	2	E29B	69.50	78.95	81.78	91.46	103.17	5	B	ß	107	105	106
4.15	2	E37A											
	2	E37B											
4.16	2	E38A	42.10	59.84	62.72	73.10	78.55	5	C	Y	104	109	106.5
	2	E38B	43.48	66.99	72.40	78.56	87.63	5	C	Y	100	100	100
4.17	2	E76A											
	2	E76B	63.91	73.32	76.06	89.40					70	86	78
4.18	2	E77A											
	2	E77B											
4.19	2	E78A											
	2	E78B											
4.20	2	E79A											
	2	E79B											
4.21	2	E80A											
	2	E80B											
4.22	2	E39A	45.41	53.09	57.29	59.63	68.40	5	B	Y	102	104	103
	2	E39B	43.14	48.76	54.18	58.91	66.03	5	0	Y	113	106	109.5
4.23	2	E40A	52.00	61.62	67.31								
	2	E40B											
4.24	2	E42A	33.37	42.28	60.00	70.67					98	111	104.5
	2	E42B	32.51	42.99	48.09	58.93	64.63	5	0	Y	99	109	104
4.25	2	E45A											
	2	E45B	84.82										
4.26	2	E46A	38.22	45.63	59.82	66.95					90	83	86.5
	2	E46B	41.58	51.62	54.17	62.56	68.13	4	0	Y	104	105	104.5
4.27	2	E47A	42.29	54.52	59.63								
	2	E47B	40.35	51.44	52.71	62.00	66.81	4	C	Y	103	110	106.5
4.28	2	E48A	32.17	50.51	64.38								
	2	E48B	38.97	49.08	55.98	65.31	84.83	5	0	Y	103	109	106
4.29	2	E50A	57.01	71.71	76.72								
	2	E50B											
4.30	2	E51A	49.67	59.68	67.69	80.73	85.40	5	0	Y	103	111	107
	2	E51B	47.27	57.68	62.69	73.05	86.40	5	0	Y	103	107	105
5.1	3	E81A	38.99	42.83	47.83	60.40	63.74	5	B	Y	118	113	115.5
	2	E81B	42.66	48.00	50.00	63.91	66.91	4	C	Y	109	111	110
5.2	3	E57A											
	2	E57B											

LEGEND
A
B

# CELLS	# EMBRYOS
2	4
3	7
4	30
5	2
total	43

Table 8.2: Data summarizing the developmental potential of Twin A and Twin B embryos from Group 2. The numbers in the rows indicate the hour at which each of the embryos being monitored reached a particular developmental stage (M, morula/fully compacted embryo; C, initiation of cavitation; B, blastocyst; EB, expanding blastocyst). Grading data and blastocyst size was displayed, for Identification of each column of raw data see Table 4.2 in Chapter 4.

DONOR EMBRYO cell #	TWIN EMBRYO cell #	Embryo ID	t9+	tM	tC	tB	tEB	Grading			Diameter(μm)		
								Expansion status	ICM	TE	Y	X	average
6.1	3	E8A	31.63	34.46	44.05								
	3	E8B	18.07	31.60	35.13	50.60					62	84	73
6.2	3	E6A	16.68	19.87	29.07	39.50	48.06	5	C	Y	114	116	115
	3	E6B	21.06	27.41	29.74	44.17	58.27	5	0	Y	98	95	96.5
6.3	3	E13A											
	3	E13B	30.59	N/A	50.56	67.90					98	96	97
6.4	3	E14A											
	3	E14B											
6.5	3	E22A											
	3	E22B	41.18	48.03	49.36	64.77					95	91	93
6.6	3	E19A	32.23	47.20	50.87	57.70					91	97	94
	3	E19B	29.70	38.40	44.19								
6.7	3	E41A	43.33	45.60	54.73	71.68					99	101	100
	3	E41B	36.00	52.75	66.96								
6.8	3	E61A	37.82	41.32	49.49								
	3	E61B	41.82	43.32	54.16	65.00					95	83	89
6.9	3	E73A	29.76	34.26	45.13	56.08	63.75	5	0	Y	110	120	115
	3	E73B	26.09	30.93	40.27	50.03	60.42	4	C	Y	113	119	116
7.1	3	E3A	24.44	27.63	33.49	41.70	44.96	4	C	Y	108	111	109.5
	4	E3B	20.42	25.11	27.80	40.10	43.46	4	0	Y	98	95	96.5
7.2	3	E12A	8.04	12.21	19.39	30.40	39.68	4	0	Y	100	102	101
	4	E12B	4.00	14.54	18.90	27.30	30.77	5	0	Y	98	110	104
7.3	4	E15A	40.00	46.86	51.02	62.50	70.62	5	0	Y	98	99	98.5
	3	E15B	37.17	44.36	50.53	66.71					88	94	91
7.4	4	E23A	28.10	31.39	34.89	54.90	68.10	5	0	Y	96	86	91
	3	E23B	34.39	37.56	40.35	53.86	66.60	5	0	Y	106	107	106.5
7.5	4	E59A	53.82										
	3	E59B											
7.6	4	E60A	26.14	32.48	39.98	51.99	59.82	4	C	Y	106	118	112
	3	E60B	25.48	32.65	39.65	52.66	61.50	4	0	Y	105	117	111
7.7	4	E55A	22.22										
	3	E55B	19.55	32.56	40.89	50.90					100	82	91
7.8	3	E56A	22.72	25.39	32.23	42.60	60.41	4	0	Y	93	105	99
	4	E56B	19.56	26.72	28.89	49.70					102	94	98
7.9	4	E64A	22.43	37.95	40.79	68.43	77.67	4	0	Y	124	116	120
	3	E64B	24.78	31.29	54.95	76.17	91.85	4	0	Y	130	105	117.5
7.10	4	E65A	26.95	37.96	42.83	50.99	69.43	4	0	Y	90	100	95
	3	E65B	40.63	45.61	59.96	76.50					75	86	80.5
7.11	4	E66A											
	3	E66B	25.12	30.63									
7.12	4	E67A	22.61	29.13	54.30	63.64					102	95	98.5
	3	E67B	26.79	30.46	41.31	50.67					86	94	90
7.13	4	E68A	31.13	40.31	51.67	63.81					112	116	114
	3	E68B	29.80	36.14	59.14								
8.1	4	E1A	17.56	21.24	28.12	34.65	42.02	5	0	Y	99	96	97.5
	4	E1B	17.23	22.08	25.27	35.16	41.86	5	0	Y	97	96	96.5
8.2	4	E2A	14.81	20.08	40.77	47.15	54.99	5	0	Y	94	93	93.5
	4	E2B	17.57	18.74	38.20	55.00					94	100	97
8.3	4	E4A	17.74	35.30	39.05	45.94	54.33	4	C	Y	103	104	103.5
	4	E4B	21.26	27.80	39.05	48.80	51.00	5	C	Y	115	112	113.5
8.4	4	E5A	22.75	24.27	29.78	37.90	49.78	5	C	Y	112	95	103.5
	4	E5B	21.91	28.78	34.10	40.86	49.10	4	B	Y	98	106	102
8.5	4	E7A	8.05	14.39	21.08	26.11					100	104	102
	4	E7B	7.22	N/A	22.59	33.00	43.50	4	0	Y	90	82	86
8.6	4	E9A	24.45	36.13	48.77	58.92					100	102	101
	4	E9B	22.77	26.96	40.38	48.10					101	100	100.5
8.7	4	E10A	18.41	30.14	33.13	37.97	44.74	5	0	Y	95	113	104
	4	E10B	17.08	27.13	31.14	39.50	42.06	4	C	Y	108	117	112.5
8.8	4	E69A	33.20	38.31	66.87								
	4	E69B	31.41	40.34	48.07	66.54					96	84	90
8.9	4	E11A	24.07	26.41	33.75								
	4	E11B	23.07	25.42	29.42								
8.10	4	E18A	23.32	26.65	42.70	49.21					104	104	104
	4	E18B	23.80	26.15	62.04								
8.11	4	E20A											
	4	E20B	13.62	27.16	29.67	51.40	58.88	4	C	Y	103	104	103.5
8.12	4	E21A											
	4	E21B											
8.13	4	E62A	37.58	46.42									
	4	E62B	33.91	46.09									
8.14	4	E63A	34.25	38.42	49.42								
	4	E63B											
8.15	4	E54A	27.38	33.72									
	4	E54B	21.55	34.22	41.06	57.06					100	95	97.5
8.16	4	E70A	14.50	24.41	27.08	36.59	43.84	5	0	Y	109	103	106
	4	E70B	14.33	17.34	25.08	38.42	44.34	5	0	Y	116	103	109.5
8.17	4	E71A	22.42	35.09	41.80	55.57	60.07	5	0	Y	101	112	106.5
	4	E71B	22.25	25.75	36.76	48.37	54.40	5	0	Y	115	106	110.5
8.18	4	E74A											
	4	E74B	27.60	41.10	60.92	72.82	80.82	5	0	Y	90	103	96.5
9.1	5	E16A	15.27	29.49	33.50	43.40	55.90	5	0	Y	104	111	107.5
	4	E16B	25.31	33.16	36.83	47.03					95	94	94.5
9.2	5	E17A	33.00	36.00	43.20	55.40	63.04	4	C	Y	97	106	101.5
	4	E17B	31.50	37.34	40.53	46.87	51.20	4	C	Y	108	105	106.5
9.3	5	E72A	14.34	27.59	34.43	39.76	43.87	4	C	Y	112	111	111.5
	4	E72B	16.18	25.26	33.26	41.14					113	111	112
9.4	5	E75A	31.77	45.64									
	4	E75B	34.77	49.54	56.59	91.33					104	121	112.5
10.1	6	E58A	3.55	21.40	29.40	40.41	59.25	4	C	Y	108	99	103.5
	4	E58B	4.39	16.90	38.58	49.25					98	93	95.5

LEGEND
A
B

# CELLS	# EMBRYOS
6	9
7	13
8	18
9	4
10	1
total	45

Table 8.3: Data summarizing the developmental potential of embryo from control group. The numbers in the rows indicate the hour at which each of the embryos being monitored reached a particular developmental stage (M, morula/fully compacted embryo; C, initiation of cavitation; B, blastocyst; EB, expanding blastocyst). Grading data and blastocyst size was displayed, for Identification of each column of raw data see Table 4.2 in Chapter 4

CONTROL	t9+	tM	tC	tB	tEB	Grading			Diameter (μm)		
						Expansion status	ICM	TE	Y	X	average
C1	78.26	83.38	97.32	104.05	107.67	5	B	β	124	129	126.50
C2	72.60	81.42	94.09	107.21	107.87	5	C	γ	125	133	129.00
C3	84.17	88.83	99.91	106.74	108.24	5	B	β	131	128	129.50
C4	79.25	90.10	95.34	101.50	103.50	5	B	β	125	121	123.00
C5	99.96	105.30	115.53	120.79	123.96	4	B	γ	122	124	123.00
C6	80.26	84.60	92.29	97.80	102.80	4	C	γ	121	123	122.00
C7	69.50	76.87	91.06	97.50	98.19	4	B	β	116	125	120.50
C8	81.29	90.30	97.97	106.65	114.32	5	B	γ	122	127	124.50
C9	96.61	103.94	111.45	114.96	117.66	5	B	β	122	126	124.00
C10	85.30	101.29	106.29	111.29	111.96	5	B	β	123	127	125.00
C11	92.25	99.60	109.11	113.11	117.91	5	A	β	120	118	119.00
C12	85.93	96.60	104.60	119.52	121.19	4	B	γ	120	124	122.00
C13	91.39	98.25	109.26	113.26	115.26	4	B	γ	122	121	121.50
C14	99.33	107.00	114.17	126.94	133.11	4	B	β	120	126	123.00
C15	112.70	121.55	127.22	132.72	139.22	5	B	β	122	122	122.00
C16	66.79	96.14	115.48	122.95	127.29	5	B	β	127	121	124.00
C17	89.22	96.16	101.50	106.17	108.17	4	B	β	124	120	122.00
C18	90.33	94.03	106.38	112.88	115.31	4	B	β	113	115	114.00
C19	101.64	117.62	121.75	133.42	134.09	5	C	γ	118	121	119.50
C20	93.48	100.14	105.14	111.81	114.82	4	C	γ	112	112	112.00
C21	72.96	92.55	98.10	107.39	111.52	5	C	γ	123	127	125.00
C22	87.50	103.81	106.15	112.81	115.48	5	B	β	121	114	117.50
C23	52.82	89.81	94.15	98.48	102.65	5	C	γ	114	115	114.50
C24	85.10	94.98	116.16	117.49	120.17	5	C	γ	122	117	119.50
C25	81.03	103.67	117.18	124.19	126.35	5	B	α	118	116	117.00
C26	81.76	99.57	107.41	111.91	116.15	4	B	β	113	127	120.00
C27	85.01	91.29	110.98	121.68	128.02	4	C	γ	118	116	117.00
C28	99.10	106.11	116.79	123.96	125.96	5	A	β	122	121	121.50
C29	80.01	99.79	106.96	114.80	117.19	5	B	α	119	117	118.00
C30	102.23	109.24	113.24	118.65	120.65	4	B	β	130	126	128.00
C31	53.72	62.23	87.64	91.18	97.34	4	B	β	123	117	120.00
C32	95.56	105.23	113.90	125.74	134.40	5	B	β	125	124	124.50
C33	80.16	92.00	98.00	101.84	107.51	5	B	γ	121	121	121.00
C34	90.74	102.75	114.92	124.97	133.31	4	C	α	142	109	125.50
C35	78.43	89.44	102.24	105.07	108.91	4	B	β	113	117	115.00
C36	76.21	99.89	102.22	112.56	117.77	5	B	γ	112	120	116.00
C37	71.61	89.47	88.10	97.19	99.52	5	B	α	115	126	120.50
C38	66.46	94.07	100.25	112.58	117.60	5	B	γ	130	120	125.00
C39	73.79	90.47	100.14	109.14	111.64	4	B	γ	124	122	123.00
C40	78.63	89.47	94.81	101.99	104.82	4	B	β	117	103	110.00
C41	78.88	97.06	104.41	117.58	119.25	5	C	γ	106	106	106.00
C42	86.57	100.18	108.51	117.17	122.67	5	B	γ	122	130	126.00

Legend
C

Table 8.4: Table summarizing the percentage of embryos from Group 1, Group 2, Group 1 and 2, and the control group that progressed to the +9, M, C, B, or EB stage (*survival by group*).

Stage	Group 1 (%)		Group 2 (%)		Overall (%)		Control (%)
	A n=43	B n=43	A n=45	B n=45	A n=88	B n=88	n=42
9+	55.81	62.79	84.44	91.11	70.45	77.27	100.00
M	46.51	55.81	80.00	91.11	63.64	73.86	100.00
C	41.86	53.49	73.33	86.67	57.95	70.45	100.00
B	30.23	53.49	62.22	75.56	46.59	64.77	100.00
EB	20.93	39.53	46.67	37.78	34.09	38.64	100.00

Table 8.5: Percentage of embryos that progressed to stage 9+, M, C, B, and EB in Twin A embryos that had a different Twin Embryo Cell #, Twin B embryos that had a different Twin Embryo Cell #, and Twin A and Twin B embryos that had the same Twin Embryo Cell # *(survival by Twin cell #).*

Stage	Twin A (%)						Twin B (%)			
	#1 n=8	#2 n=33	#3 n=14	#4 n=28	#5 n=4	#6 n=1	#1 n=8	#2 n=35	#3 n=19	#4 n=26
9+	25.00	63.64	71.43	85.71	100.00	100.00	62.50	62.86	89.47	92.31
M	25.00	51.52	71.43	78.57	100.00	100.00	62.50	54.29	89.47	92.31
C	25.00	45.45	71.43	71.43	75.00	100.00	62.50	51.43	84.21	88.46
B	25.00	30.30	57.14	60.71	75.00	100.00	62.50	51.43	68.42	80.77
EB	12.50	21.21	42.86	42.86	75.00	100.00	37.50	40.00	26.32	46.15

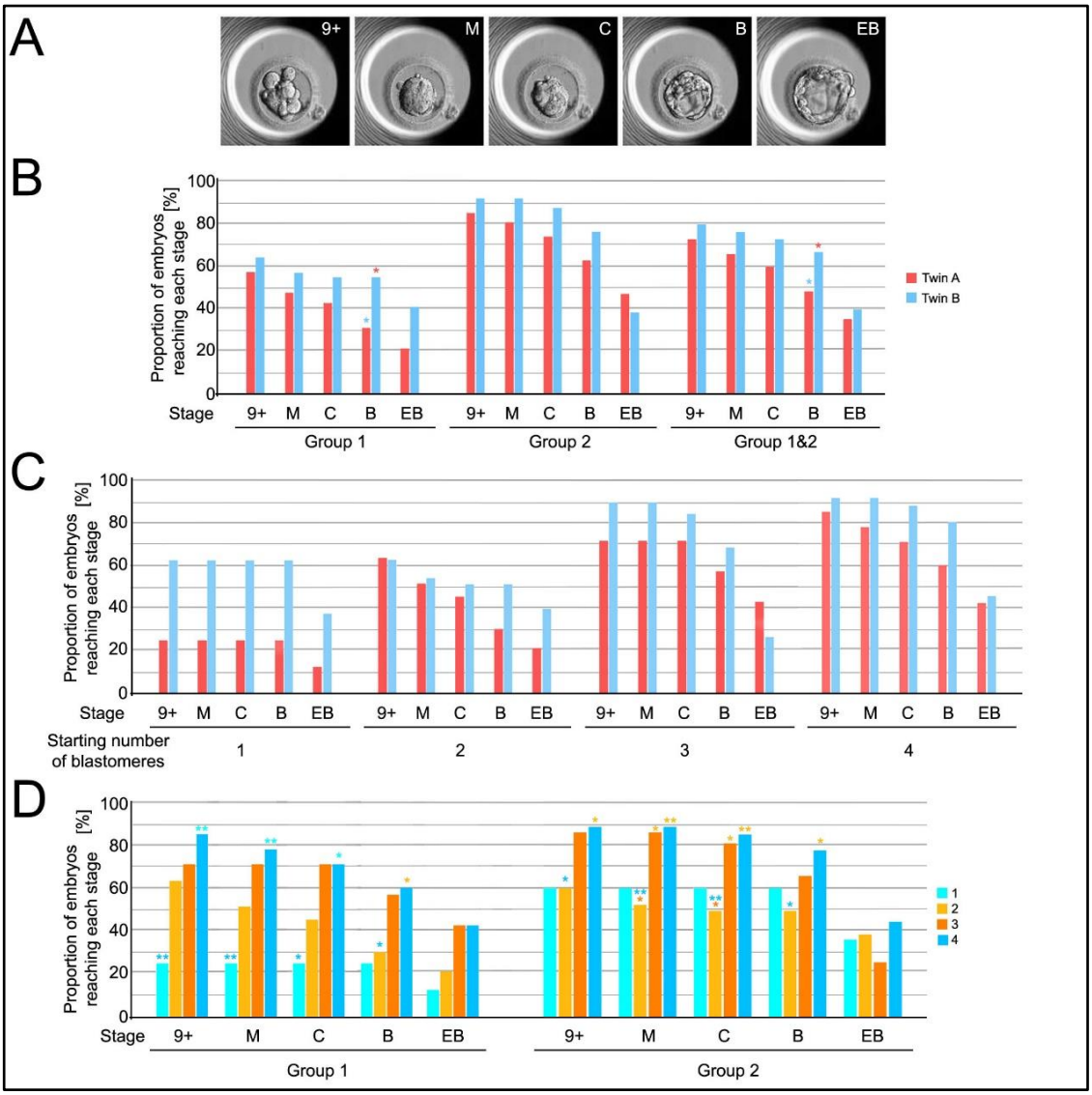


Figure 8.2: Illustration showing that the successful development of Twin embryos into expanding blastocyst was associated with the initial number of blastomeres. (A) Images depicting the developmental stages used for the morphokinetic analyses (9+, more than nine blastomeres; M, morula/fully compacted embryo; C, initiation of cavitation; B, blastocyst; and EB, expanding blastocyst). (B) Both Twin A and Twin B embryos arising from parental embryos in Group 2 tended to display a higher probability of progressing during development than Twins arising from Group 1 parental embryos. In both Group 1 and Group 2, the Twin B embryos demonstrated better developmental progress than Twin A embryos. However, this difference was only found to be statistically significant at the blastocyst stage (B). (C) It was found that Twins that originated from an embryo with a higher number of blastomeres tended to develop further, as demonstrated by the higher percentage of such embryos that reached each progressive stage. Regardless of the initial number of blastomeres, the Twin B embryos showed better developmental progress than Twin A embryos. (D) There was a difference within the group in the proportion of embryos that reached each developmental stage examined in the morphokinetics analyses, and this difference was based on the number of blastomere used to create Twin A and Twin B. However, no overall difference was observed in the proportion of embryos that reached each developmental stage between Group 1 and 2. Images are from (Noli *et al.*, 2015b).

8.2.2 Temporal control of human preimplantation development

The duration of critical landmark events during embryonic development, including compaction (9+ to M), the initiation of cavitation (M–C), blastocyst formation (C – B) and blastocyst expansion (B – EB), were compared in embryos that reached the EB stage (Figure 8.3, Table 8.6). The difference between the control embryos and the Twin embryos in Group 1 was less pronounced than the difference between the control embryos and the Twin embryos in Group 2. However, when pooled together, a significant difference was found across all of the stages, and it was particularly pronounced at the blastocyst formation stage ($p=0.006$ for Twin A embryos and $p\leq0.001$ for Twin B embryos) and the blastocyst expansion stage ($p\leq0.001$ for both Twin A and Twin B embryos) (Figure. 8.3 A). No marked differences were found in developmental dynamics between Twin A and Twin B embryos that originated from 1, 2, 3 or 4 blastomeres, although a significant difference was observed between Twin A and Twin B embryos at the compaction and expansion stages (Figure 8.3 B and C and Table 8.7).

Table 8.6: Average duration of each stage in embryos that reached stage EB (hours) *(kinetics by group).*

Stage	Group 1 (hours)		Group 2 (hours)		Overall (hours)		Control (hours)
	A n=9	B n=17	A n=21	B n=17	A n=30	B n=34	n=42
9+	48.37	46.02	20.93	20.60	29.17	33.31	83.54
M	10.15	10.50	8.16	6.93	8.76	8.77	12.56
C	8.89	5.32	6.57	7.51	7.27	6.39	9.09
B	9.38	10.32	10.70	11.93	10.31	11.12	7.42
EB	9.17	10.54	9.55	7.68	9.43	9.11	3.61

Table 8.7: Average duration of each stage for embryos that reached stage EB (hours) based on Twin cell # *(kinetics by Twin cell #).*

Stage	Twin A (hours)						Twin B (hours)			
	#1 n=1	#2 n=7	#3 n=6	#4 n=12	#5 n=3	#6 n=1	#1 n=3	#2 n=14	#3 n=5	#4 n=12
9+	60.84	47.93	23.44	22.65	20.87	3.55	48.99	45.39	26.36	18.20
M	11.62	10.84	3.59	8.78	10.16	17.84	12.37	10.09	5.60	7.54
C	18.55	8.06	7.49	6.01	6.01	8.00	7.74	4.81	9.03	6.83
B	7.54	9.19	10.59	11.28	9.15	11.01	8.22	10.76	14.39	10.90
EB	14.27	9.27	8.32	9.24	8.08	18.84	12.83	10.05	12.35	5.73

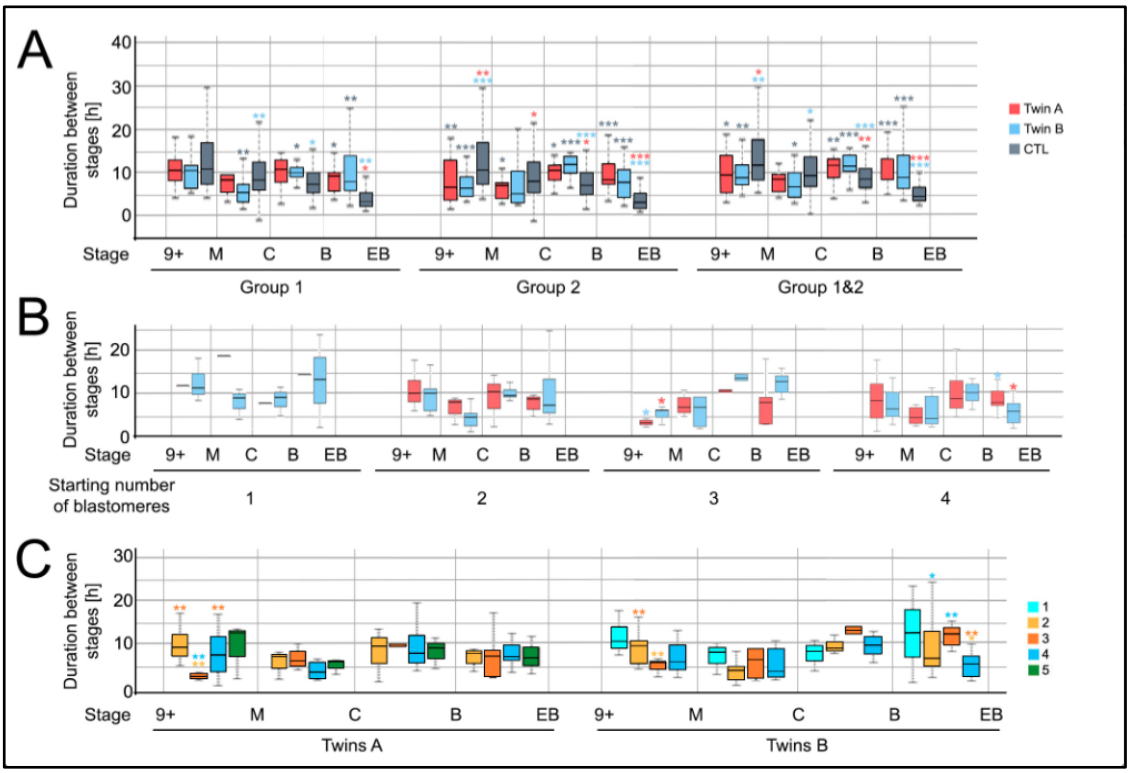


Figure 8.3: Developmental dynamics of Twin embryos. (A) A significant difference was observed in the duration of almost all stages, including compaction (9+ to M), the initiation of cavitation (M–C), blastocyst formation (C–B) and blastocyst expansion (B – EB) when Twins A and B we compared to the control embryos (CTL). More pronounced differences were found between the control embryos and the Twin embryos in Group 2 (B, C). The Twins A and B showed a statistical difference in expansion when they were derived from four blastomeres, and when derived from three blastomeres, a significant difference was recorded in compaction. Because there were an insufficient number of replicates, the duration of each stage could not be statistically compared between Twin A and Twin B embryos that were derived from a single blastomere. Image reproduced from (Noli *et al.*, 2015b).

Overall, when the developmental dynamics of the embryos were analysed using a slightly different approach, it appeared that the differences between the Twin and control embryos was limited to the earlier stages of embryonic development

(Figure. 8.4 and Table 8.8). When the duration from the 9+ stage to the expanded blastocyst stage (9+ to EB) was compared between Twin and control embryos, similar results were observed. However, the durations of the Twins' progress from the 9+ stage to the compaction stage (9+ to M) and from the 9+ stage to cavitation (9+ to C) were significantly shorter than the same periods in the control embryos. These shortened durations were more pronounced in the Twins created from Group 2 embryos, indicating a mechanism of "compensation" for the lower cell numbers in the embryos from Group1 (Figure 8.4 A). No marked differences was observed between Twin A and Twin B embryos except in the time required for the embryos to reach the morula stage, regardless of the number of blastomeres that were used to create the embryos (Figure 8.4 B and C).

Table 8.8: The average duration between stage 9+ and the following stages in embryos that reached the EB stage (hours) *(kinetics by group).*

Stage	Group 1 (hours)		Group 2 (hours)		Overall (hours)		Control (hours)
	A n=9	B n=17	A n=21	B n=17	A n=30	B n=34	n=42
M	10.15	10.50	8.16	6.93	8.76	8.77	12.56
C	19.04	15.82	14.74	14.50	16.03	15.16	21.66
B	28.42	26.13	25.44	26.43	26.33	26.28	29.07
EB	37.59	36.68	34.99	34.11	35.77	35.39	32.69

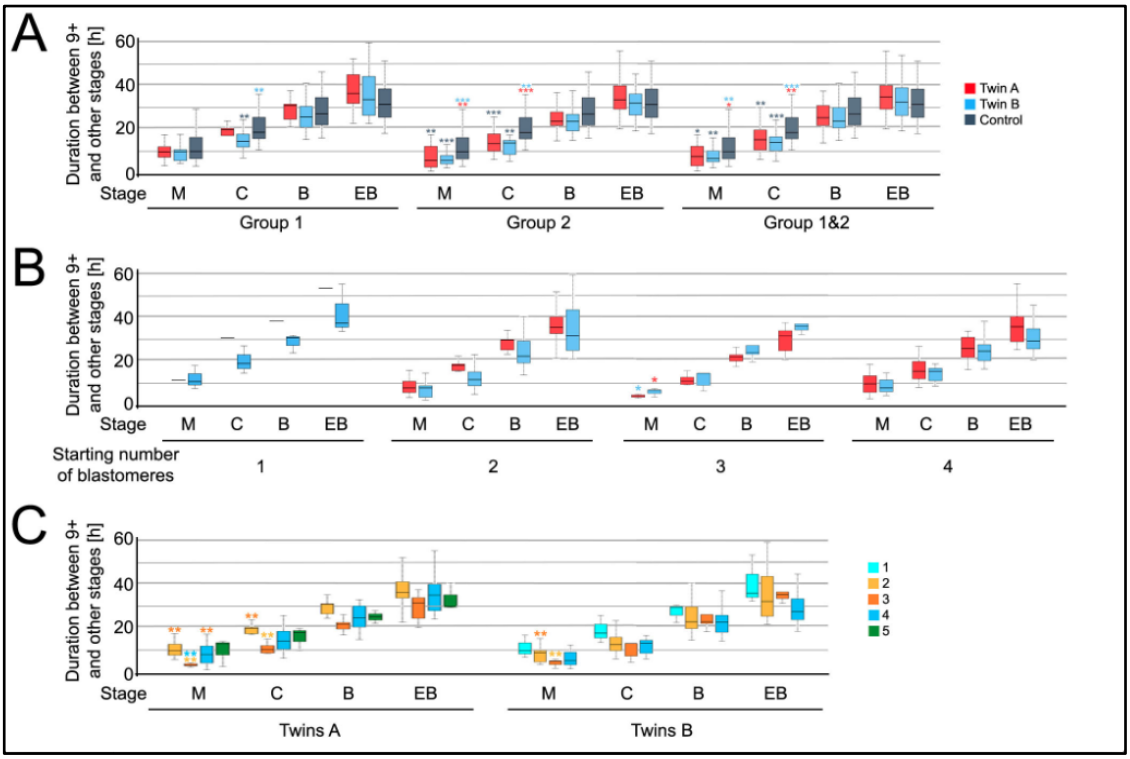
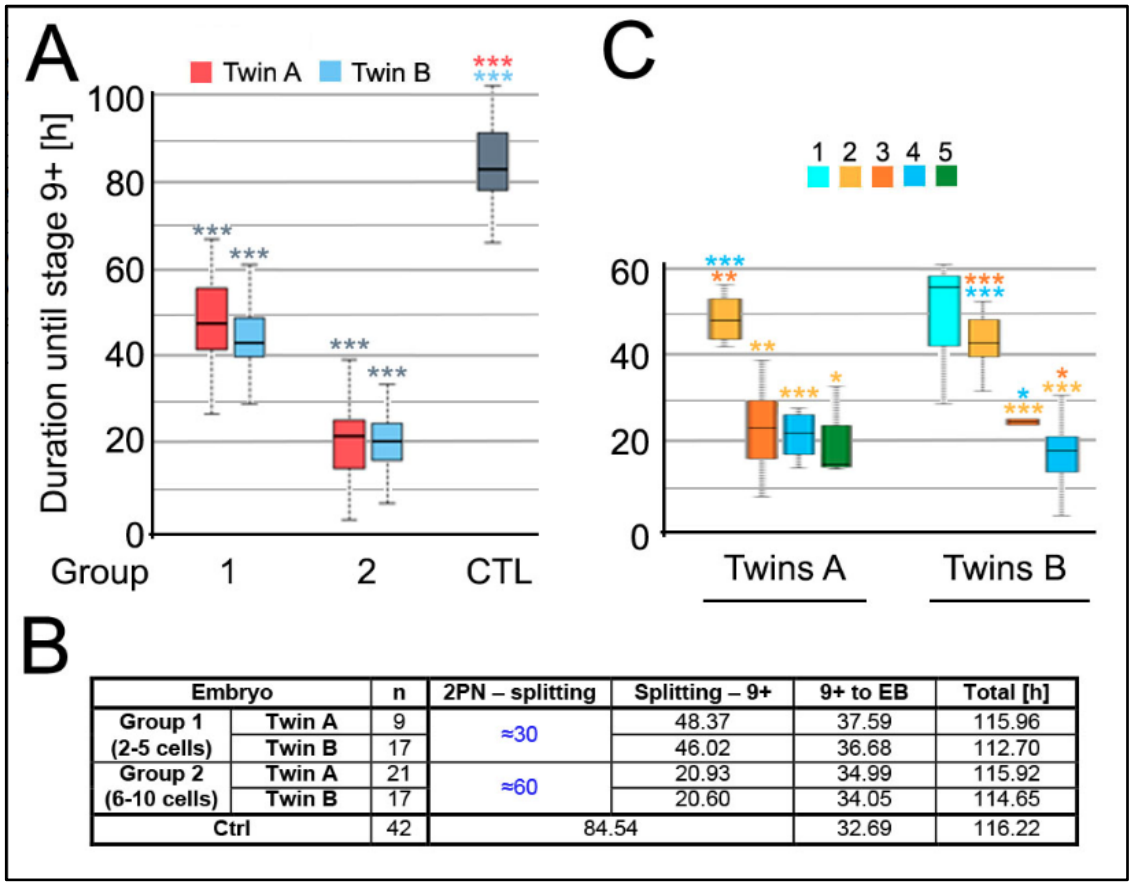


Figure 8.4: Comparison of the durations between the 9+ stage and other stages. (A) Differences between Twin and control embryos were restricted to the earlier stages, including from the 9+ stage to compaction (M) and from the 9+ stage to cavitation. (B, C) No significant differences were observed between Twin A and B embryos except in the time required to reach the morula stage. Raw data from the embryos created from five of the blastomeres were excluded from the statistical analysis as a result of a low number of replicates. Figure 8.4 was reproduced from (Noli *et al.*, 2015b).

All of the embryos, including the Twins (n=64) and the controls (n=42), reached the EB stage within a similar amount of time (average times: 35.82 hours and 32.69 hours, respectively), regardless of the marked differences that were observed in the duration of some stages of development or differences in the number of blastomeres used during their creation. This finding seems to suggest that preimplantation development is governed with strict temporal precision. The average time from embryo creation to the 9+ stage in the control embryos was 83.54 hours (n=42), while in early cleavage stage embryos (Group 1, 2-5 blastomeres), the average time was 48.37 hours for Twin A embryos (n=9) and 46.02 hours for Twin B embryos (n=17). However, the late cleavage stage embryos (Group 2, 6-10 blastomeres) required only half the time to progress to the 9+ stage, averaging 20.93 hours in Twin A embryos (n=21) and 20.60 hours in Twin B embryos (n=17) (Figure 8.5 A). The embryos used in these study as donor embryos were preserved via slow freezing at different IVF centres over several years. The exact number of hours in the culture medium that was required to progress the embryos from the 2 PN stage to 2-5 cells (Group 1) or 6-10 cells (Group 2) was not precisely known. However, from the data in this study, it was estimated, with reasonable confidence, that the embryos in Group 2 generally needed to be cultured twice as long as the embryos in Group 1. If the embryos from Group 1 required approximately 30 hours, then embryos in Group 2 required double the number of hours (~60 hours), then the net time required from the embryos to reach the EB stage from the 2PN stage was similar to the time required in the control embryos (Figure 8.5 B). Most of the Twins that were derived from either one blastomere (n=4) or two blastomeres (n=21) were derived from early cleavage stage embryos and required twice the amount time to reach the 9+ stage (Figure 8.5 C).



B

Embryo		n	2PN – splitting	Splitting – 9+	9+ to EB	Total [h]
Group 1 (2-5 cells)	Twin A	9	≈30	48.37	37.59	115.96
	Twin B	17		46.02	36.68	112.70
Group 2 (6-10 cells)	Twin A	21	≈60	20.93	34.99	115.92
	Twin B	17		20.60	34.05	114.65
Ctrl		42	84.54		32.69	116.22

Figure 8.5: Temporal control of development from the pronuclear (2PN) stage to blastocyst expansion. (A) The mean time required for the embryos to progress from generation to the 9+ stage was the shortest in Group 2. In the control embryos, the mean duration from generation to the 9+ stage was twice as long as the time in Group 1 and four times as long as the time in Group 2. (B) Estimation of the time between the 2PN stage and the blastocyst expansion stage. It is not clear how many hours are required by the embryos to develop in culture from the 2 PN stage to 2-5 cells (Group 1) or 6-10 cells (Group 2). However, it was estimated that embryos in Group 2 may take twice as long as the embryos in Group 1. (C) Twins created from embryos with lower number of blastomeres (one, n=4; two, n=21) were derived from early cleavage stage embryos, and therefore required twice as much time to reach the 9+ stage as the Twins that were derived from embryos with a higher number of blastomeres (3 or more blastomeres). All measurements are shown in hours. n, indicated the number of replicates. Figure 8.5 is reproduced from (Noli *et al.*, 2015b).

8.2.3 The size of the twin blastocysts is proportionate to the number of blastomeres used to create the embryo

As was reported earlier, the Twin blastocysts were smaller in size than the control embryos (Van de Velde *et al.*, 2008). The difference in size between the controls and Twins was always statistically significant, regardless of whether they were Twin A or Twin B embryos or whether they belonged to Group 1 or Group 2 (Figure 8.6 A,B and Table 8.9). If the development of the embryos is temporally strictly controlled, as suggested by the data presented in 8.2.2, the number of cell divisions that the biopsied blastomeres are capable of undergoing before the onset of blastulation and lineage commitment is dictated by their age. As expected, the

diameters of Twin blastocysts varied in proportion to the number of starting blastomeres (Figure 8.6 C). The mean diameter of Twin embryos that were derived from one or two blastomeres was 86.93 µm (n=7) and 101.91 µm (n=28), respectively, whereas the mean diameter in Twins originating from four blastomeres was 102.25 µm (n=38), on average. Finally, Twin embryos arising from five blastomeres measured 106.83 µm (n=3) in diameter. The average diameter of the control embryos was 120.87 µm (n=42) (Table 8.10).

Table 8.9: Average diameter (µm) of blastocysts in different groups *(size by groups).*

	Group 1 n=36	Group 2 n=62	Overall n=98	Group 1		Group 2		Control n=42
				A n=13	B n=23	A n=28	B n=34	
Average Diameter (µm)	99.38	101.53	100.74	101.69	98.07	103.86	99.62	120.87

Table 8.10: Average diameter (µm) of blastocysts derived with different Cell #s in the Twin and control groups *(size by Twin Embryo Cell #)*

	Twin Cell #						Control
	#1 n=7	#2 n=28	#3 n=21	#4 n=38	#5 n=3	#6 n=1	n= 42
Average Diameter (µm)	86.93	101.91	100.05	102.25	106.83	103.50	120.87

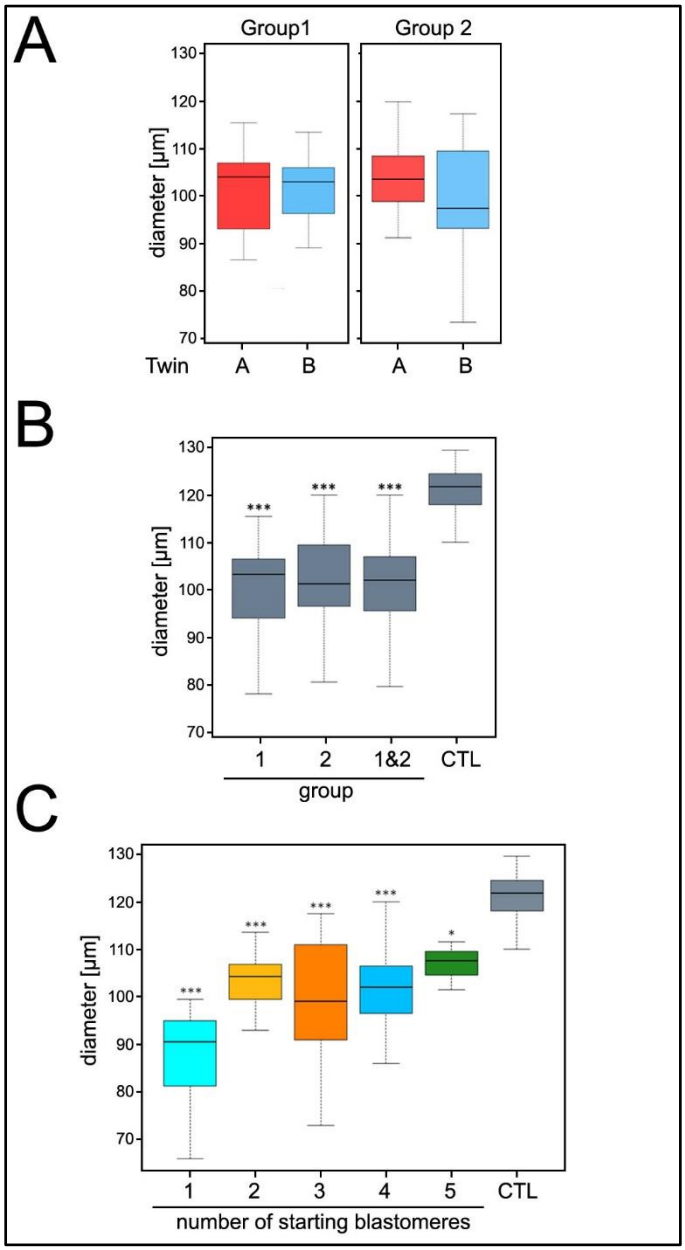


Figure 8.6: Comparison of sizes across the Twin embryos. (A) No significant differences were found in average diameter between Twin A and Twin B embryos in either Group 1 (FC ≈ 1,04 and statistical test *P*-value ≈ 0,32) or Group 2 (FC ≈ 1,04 and statistical test P-value ≈ 0,065). (B) Embryos from both Group 1 and Group 2 had significantly different diameters from the control embryos (FC ≈ 1,20). (C) The diameters of the Twin blastocyst embryos originating from 1 (n=16), 2 (n=68), 3 (n=43), four (n=54) or five (n=4) blastomeres were significantly different from the diameters of the control embryos (1: FC ≈ 1.39 and P-value ≤ 0,001; 2: FC ≈ 1.19 and P-value ≤ 0,001; 3: FC ≈ 1.21 and P-value ≤ 0,001; 4: FC ≈ 1.18 and P-value ≤ 0,001; 5:FC ≈ 1.13 and P-value ≤ 0,05). Twin embryos derived from six blastomeres were not included in the statistical analysis as a result of insufficient replicates (n=1). Statistical analyses were performed using Student's T-tests. Alpha risk: 5% (FC, average fold-change; *** P-value ≤ 0.001; * P-value ≤ 0.5). These images were taken from (Noli *et al.*, 2015b).

8.3 Validation methods I and II: ICM quality and comparative analysis of expression patterns of early lineage-specific markers

8.3.1 Poor ICM quality and developmental delays in twin embryos

Although blastomeres from late cleavage embryos (Group 2) resulted in the formation of blastocysts with larger sizes and higher yields, only 36.84% (14 out of 38) of such embryos had a distinguishable ICM. Conversely, in Group 1, 46.15% (12 out of 26) of the blastocysts demonstrated a clear and distinguishable ICM. Of the ICMs observed, only three in Group 1 and one in Group 2 were Grade B. The remainder of the ICMs were Grade C (Stephenson *et al.*, 2007), as shown in Figure 8.7.

During the late morula stage, the first cell fate decision involves spatial segregation, whereby the cells in the outer layers give rise to the TE, the first extraembryonic lineage, while the ICM retains its pluripotency (Johnson and Ziomek, 1981). Following compaction on Day 4 and cavitation on Day 5, by Day 6 the normal human *in vitro* fertilised and cultured embryos had developed into expanded blastocysts with a clearly distinguishable TE and ICM.

To further understand the effect of using the blastomere biopsy technique to perform embryo splitting on lineage specification in pre-implantation embryos in humans, a detailed immunofluorescence analysis was completed. Immunofluorescent staining of Day 5 (n= 3 pairs) and Day 6 (n=3 pairs) blastocysts that were derived from split embryos was performed to determine the presence and localization of lineage-specific transcription factors that are markers for TE (CDX2) and ICM (NANOG). These data were then compared to the control embryos on Day 5 and Day 6 post-fertilisation. Figure 8.8 shows representative pairs from each cohort. Both Twin A and Twin B embryos that were derived from embryo splitting using the blastomere biopsy technique were found to be smaller in size and to display no distinctive ICM. In addition, when Twin A and Twin B embryos were compared to non-manipulated control embryos of the same age, the Twin embryos were found to contain smaller numbers of cells. During the immunofluorescence analysis, on Day 5, not a single cell was detected that was only NANOG-positive

(Figure 8.8). All of the NANOG-positive cells in both Twin A and Twin B embryos also expressed CDX2 in TE cells.

On Day 6, following the expansion of both Twin blastocysts, the ICM was visible in Twin A embryos, but their paired Twin B embryos did not contain a visible ICM. Immunostaining data obtained from Twin A embryos showed that one cell strongly expressed NANOG, while CDX2 expression was downregulated.

Next, markers of TE (CDX2 and GATA2) and ICM (NANOG) were assessed in Day 5 (n=5 pairs) and Day 6 Twin (n=5 pairs) blastocysts. The images shown in Figure 8.9 show representative pairs of embryos obtained from each cohort. On Day 5, there was no discernible ICM in either the Twin A or the Twin B embryo in the pair under consideration. However, a single cell that was positive only for NANOG cell was detected in Twin A (Figure 8.9 and Figure 8.10), indicating that there was potential for ICM formation in this embryo and that it was likely that ICM development was occurring, although it was lagging behind. All of the remaining NANOG-positive cells in both sets of Twins also co-expressed TE markers.

On Day 6, following the expansion of both the Twin blastocysts, the ICM was visible in Twin B but not in Twin A of the pair under consideration. Immunostaining detected cells that were positive only for NANOG in both Twin A and Twin B embryos, supporting the possibility that there was a developmental delay. Twin B embryos were found to be developing two independent ICMs *de novo* (Figure 8.9).

Additionally, cell quantification results for Twin A, B and control embryos are presented in Table 8.11. Interestingly, the total number of cells in Twin A+B combined embryos did not typically exceed that of the single control embryos, and splitting therefore compromised further development.

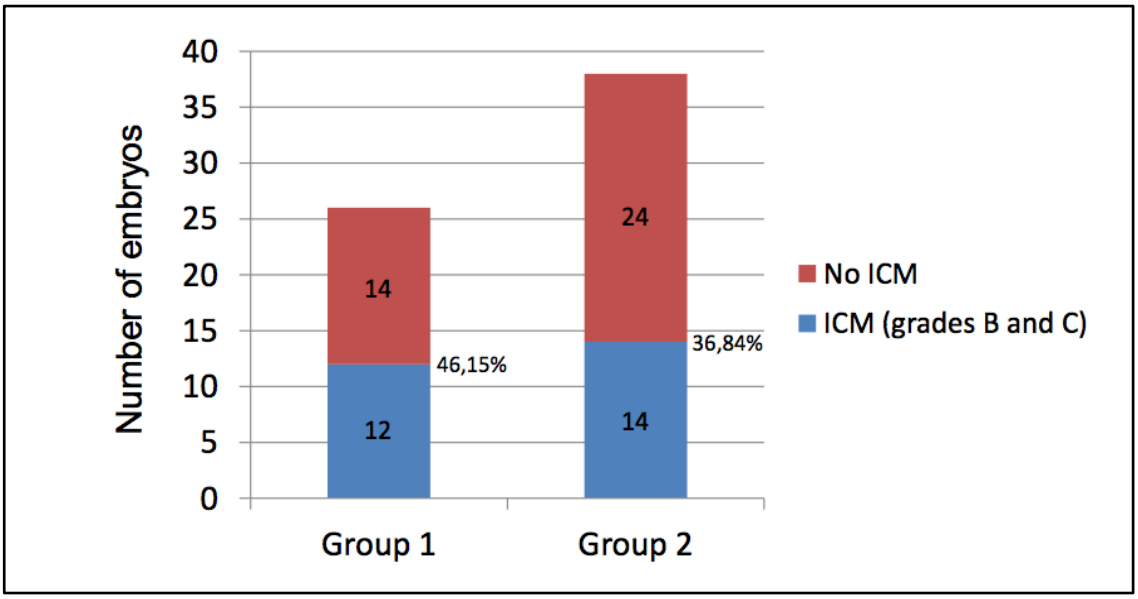


Figure 8.7: ICM grades in Twin embryos. Based on the grading system used in this study (Stephenson *et al.*, 2007), there was no significant difference in the number of embryos with an ICM between the two groups (statistical test p-value = 0,6271), and approximately 41.5% of the embryos had ICM (grade B or C).

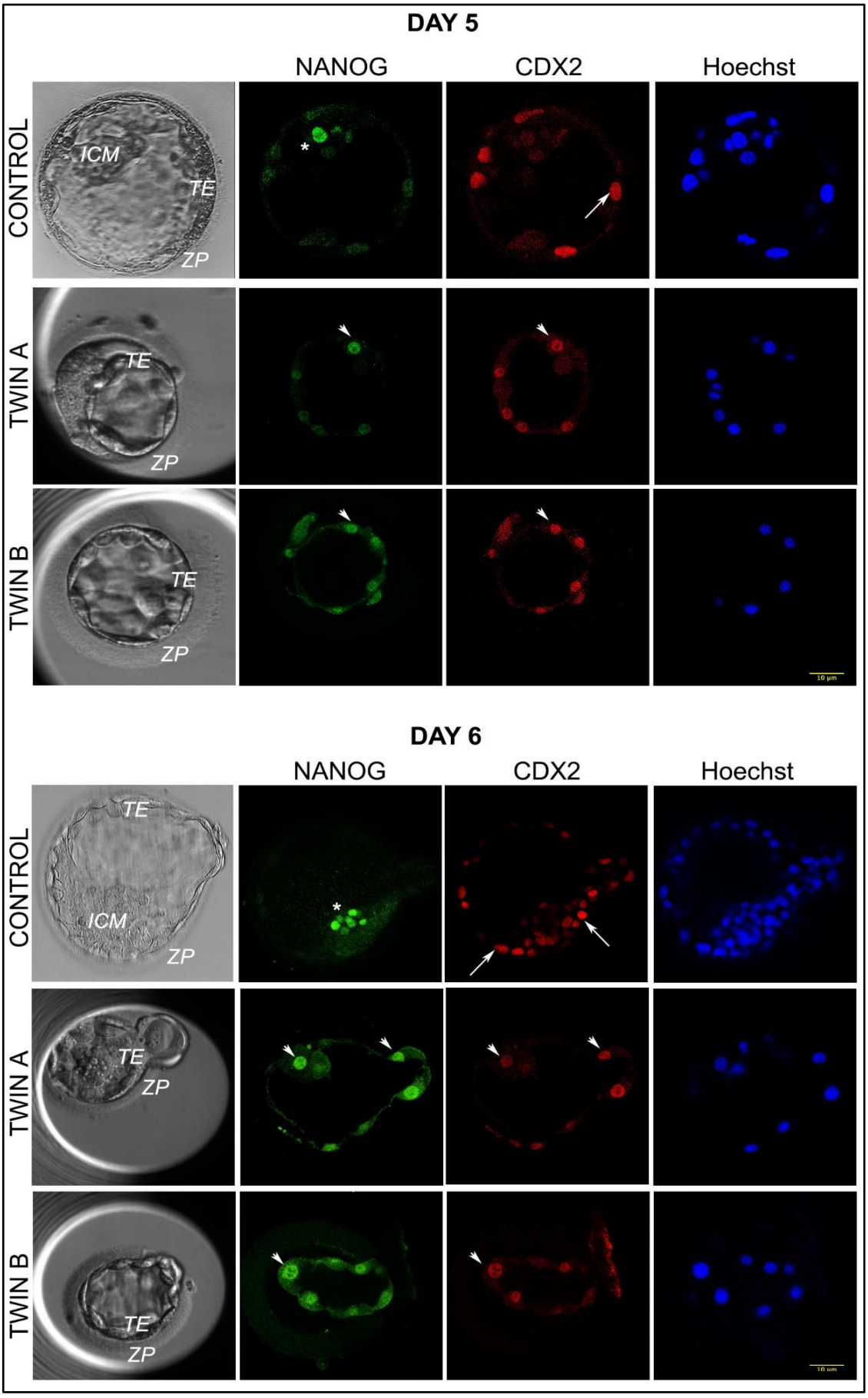

DAY 5
NANOG
CDX2
Hoechst
CONTROL
TWIN A
TWIN B
ICM
TE
ZP
DAY 6
NANOG
CDX2
Hoechst
CONTROL
TWIN A
TWIN B
TE
ICM
ZP

Figure 8.8: Immunostaining for lineage markers (NANOG and CD2) on Day 5 and Day 6 in Twin blastocysts and control non-manipulated blastocysts. Embryo splitting resulted in the formation of Twin embryos that were smaller and that tended to contain no distinctive ICM. When these embryos were compared to non-manipulated control embryos, they were observed to be less cellular, and a majority of the cells in the Twin embryos expressed both ICM and TE markers. No single cell was observed to positive for only NANOG in either of the Twins on Day 5. Both Twins began to expand on Day 6, but the ICM was visible only in Twin A and not in Twin B of the pair under consideration. Immunostaining of Twin A (Day 6) indicated that one cell strongly expressed NANOG and that CDX2 was downregulated (Green: the ICM marker NANOG. Red: the TE marker CDX2. Blue: DNA dye Hoechst 33342). The asterisk indicates the ICM cell that expressed NANOG, and the arrow indicates the TE cell that expressed CDX2 in the control embryo. In Twin A and Twin B, the arrowheads indicate the cells that expressed both NANOG and CDX2. Image reproduced from (Noli *et al.*, 2016a).

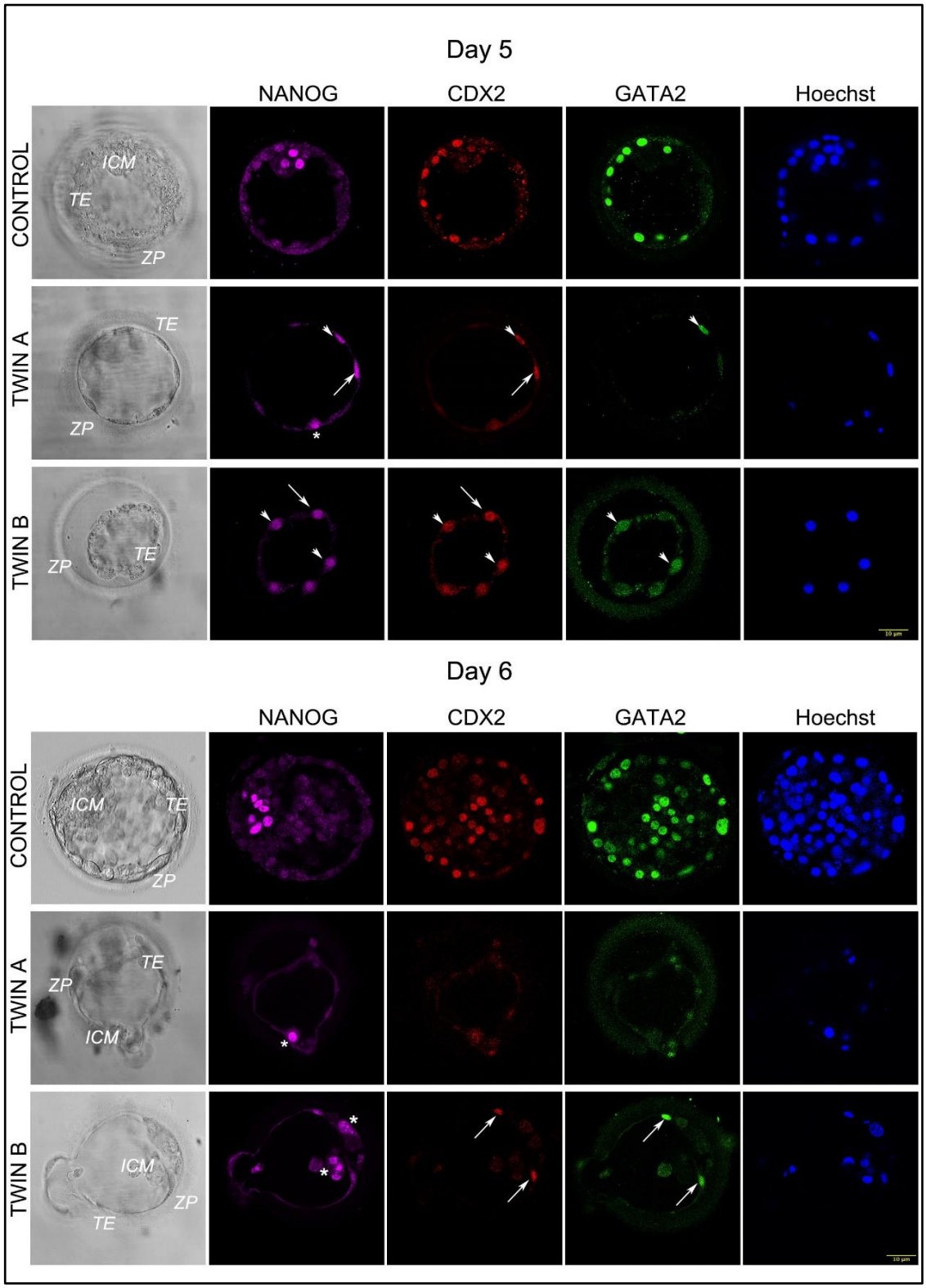


Figure 8.9: Immunostaining for lineage markers (NANOG, CDX2 and GATA2) in control and Twin blastocysts. Day 5 blastocysts derived from split embryos contained cells that co-expressed TE and ICM markers and that showed a badly developed or indistinguishable ICM. In comparison, in the non-manipulated Day 5 control blastocysts, NANOG clearly localized in the ICM, and CDX2 and GATA2 were expressed in the TE. On Day 6, both Twin blastocysts had expanded, and although the ICM was visible only in Twin B, cell that were positive only for NANOG were observed in both Twins. (Magenta: the ICM marker NANOG; Red: the TE marker CDX2; Green: the TE marker GATA2; Blue: DNA dye Hoechst 33342). Arrowheads: cells that express all three markers. Arrows: cells displaying strong expression of both the ICM marker NANOG and the TE marker CDX2. Asterisk: a cell

displaying strong NANOG expression and weak CDX2 expression. The images were taken from (Noli *et al.*, 2015b).

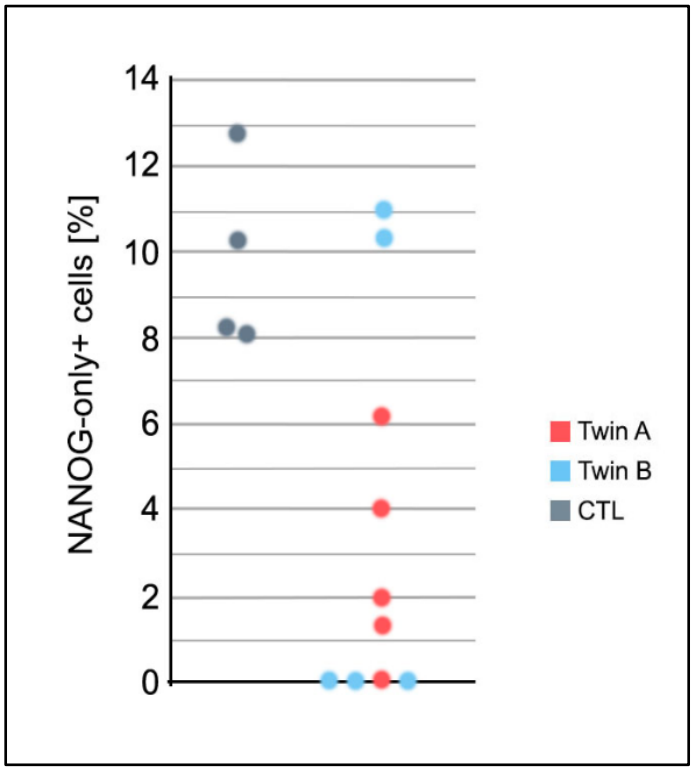


Figure 8.10: Percentage of cells that strongly expressed only NANOG in the control (n=4) and Twin (n=5 for each of Twin A and Twin B) blastocysts. The image was taken from (Noli *et al.*, 2015b).

Table 8.11: Table present the numbers of cells in Twin A+B relative to Control embryos

Embryo ID	Total cells number	Embryo ID	Total cells in A+B
CTL1	173	A1+B1	50+36=86
CTL2	102	A2+B2	38+31=69
CTL3	98	A3+B3	42+38=80
CTL4	125	A4+B4	51+23=74
Mean 124.5		Mean 77.25	

8.4 Validation method III: Derivation of hESCs

The Twin blastocysts could not be cultured beyond Day 6 despite repeated attempts, and the blastocyst media was unable to support the survival of the embryos beyond Day 6/7. Specialized media developed to culture mouse blastocysts beyond the implantation stages (Bedzhov *et al.*, 2014) was also unable to support cultures of human blastocysts, and it is possible that modifications to the protocol would be necessary before it could be adapted for use in human embryonic culture systems (Y. Dajani and D. Ilic, Unpublished observations). The stem cell research group at ACU has significant expertise in deriving hESC lines from intact blastocysts (Ilic *et al.*, 2012; Stephenson *et al.*, 2012; Jacquet *et al.*, 2013) and from single blastomeres (Ilic *et al.*, 2009; Giritharan *et al.*, 2011). However, no hESC lines or initial outgrowth (Figure 8.11) were observed under standard conditions in any of the Twin embryos (n=5 pairs), despite of the presence of clearly identifiable ICM-like structures in the Twin blastocysts. A success rate of 30-50% is usually achieved by the hESC-derivation team at ACU.

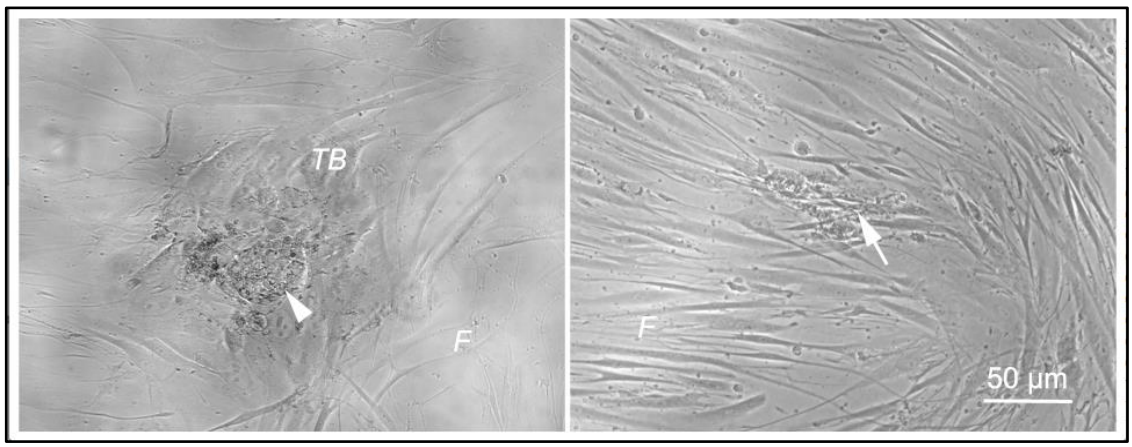


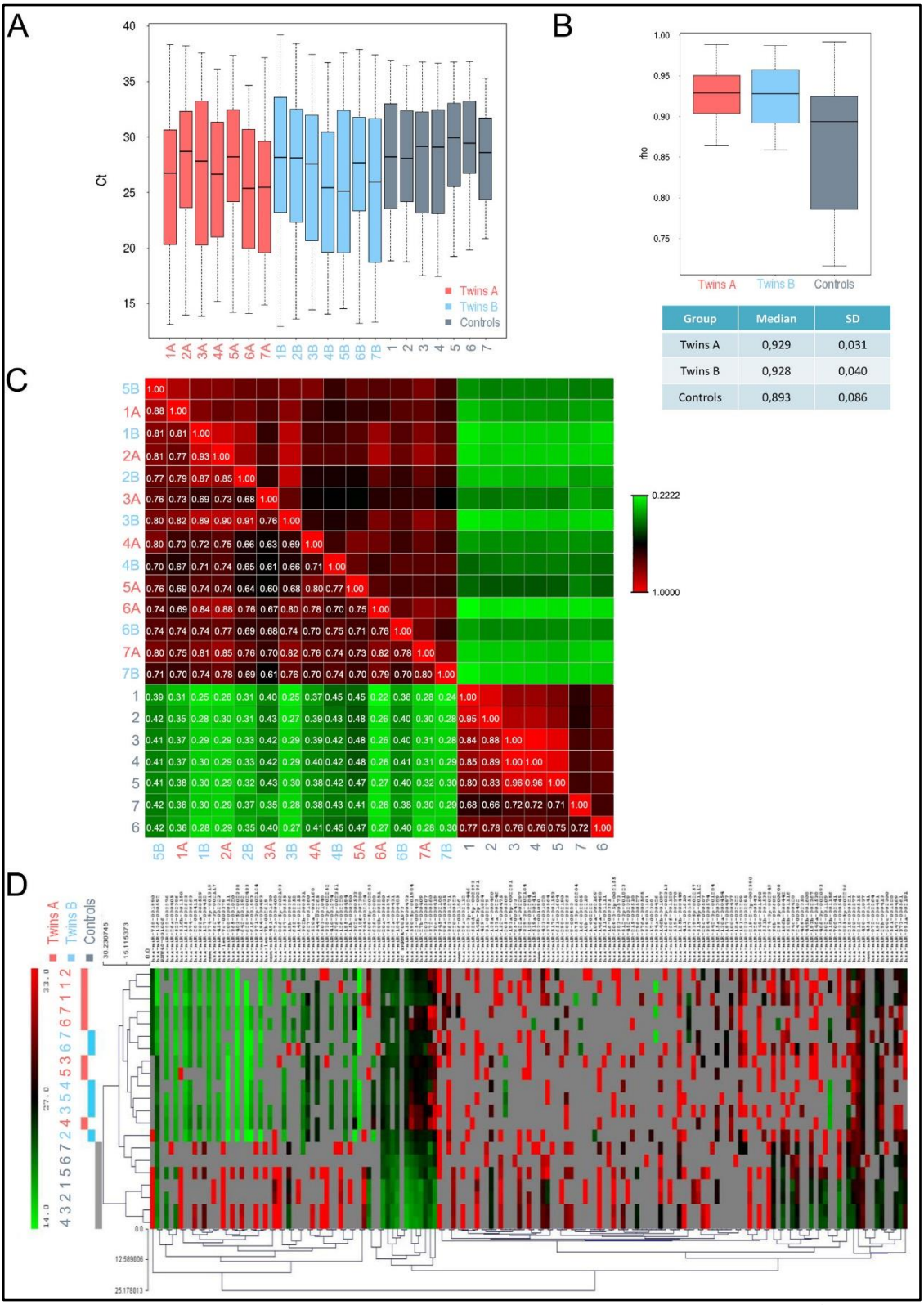
Figure 8.11: Day 7 post-plating of ICM. Left: initial outgrowth of hESCs from ICM (arrowhead); TB, trophoblast cells; and F, feeders. Right: dead cell debris (arrow); and F, feeders

8.5 Validation method IV: Comparative analysis of the miRNA profiles of SBMs

Experiments were performed to determine the functional and reproductive capabilities of the embryos produced using the embryo splitting procedure and the effect of embryo splitting on the miRNA secretion profile of the Twin embryos. A medium throughput analysis was applied, and the profiles of 377 miRNA sequences were studied in SBM obtained from Twin embryos (n=7 pairs) and

control embryos (n=7) that resulted in a live birth following single embryo transfer.

The Ct values and their distributions in Twin A, Twin B and control embryos demonstrated that there was a high median correlation between the samples in each group (Figure 8.12 A). The distribution of Spearman's test *rho* values (Ct values) showed that samples in the Twin A and Twin B groups were similarly correlated, with values of 0.929 ± 0.031 and 0.928 ± 0.040, respectively. However, samples in the control group displayed slightly lower correlations (0.893 ± 0.086) than were observed between the samples in the Twin A and Twin B groups (Figure 8.12 B). A heat map of the Pearson's correlation value matrix of the miRNA raw Ct values that were compiled using values from all the SBM samples analysed (n=21; 7 x Twin A, 7x Twin B, and 7 x Control) clearly distinguished the Twin embryos from the control embryos (Figure 8.12 C). Each square in the matrix describes an exact Pearson's correlation value. A heat map created from an unsupervised hierarchical clustering analysis of miRNA showed that there was no significant clustering according to experimental group. However, a large number of Ct values were missing (Figure 8.12 D). Therefore, to minimize experimental error, only miRNAs with Ct values that were greater than 60% above the set threshold of 35 in each experimental group were analysed (i.e., there were 5/7 valid values in the control SBMs and 9/14 valid values in the Twin SBMs) (Noli *et al.*, 2016a).



Group	Median	SD
Twins A	0,929	0,031
Twins B	0,928	0,040
Controls	0,893	0,086

Figure 8.12: Determination of correlations between replicate samples within each group. (A) Box plot demonstrating the distribution of Ct values for Twin A, Twin B and control embryos. (B) Box plot demonstrating the distribution of Spearman's test *rho* values (Ct values). The values indicate that the median correlations were very high for all sample conditions. The correlation between Twin A (0.929 ± 0.031) and Twin B (0.928 ± 0.040) embryos was similar, whereas a slightly lower correlation was found between the Twin and control samples (0.893 ± 0.086). (C) Comparison of raw Ct data obtained from all analysed samples using Pearson's correlation matrix. The low correlation observed between miRNA profiles in SBM samples that were obtained from *in vitro* cultures revealed a distinct difference between the Twin and control embryos. The numbers indicate the actual correlation value

among all analysed samples. (D) The heat map was constructed using Ct values obtained from an analysis of all of the SBM samples that were collected from Twin A, Twin B and control embryos. The data contained a large number of missing Ct values and did not show any significant clustering by experimental group. Images were reproduced from (Noli *et al.*, 2016a)

8.5.1 Data analysis using common intracellular normalizing markers

The values of intracellular normalization markers, such as the small nuclear RNA U6, tend to display large variation between samples when secreted miRNAs are measured (Xiang *et al.*, 2014). In this study, after the quality control analyses were completed, only one out of three of the endogenous controls could be used for normalization (U6). This was because the endogenous control, the small nucleolar RNA RNU44, did not provide any Ct values and the control RNU48 yielded only 8 Ct values. Although the Ct values of the endogenous control U6 displayed heterogeneity across samples, its Ct values were homogenous within each sample group, and it was therefore selected as the endogenous control (Figure 8.13 A). Because approximately the same Ct values were found for four different U6 reporters in the same sample, U6 was used to compute the $-\Delta Ct$ normalizations for the other miRNAs (Noli *et al.*, 2016a). However, in spite of the good reproducibility of the U6 Ct values across the replicates of samples, because of the U6 Ct in sample 6B, Twins 6A and 6B were excluded from the analysis (Figure 8.13 B and Figure 8.14).

miR-515-5p and miR-490 were present at significantly higher levels, whereas significantly lower concentrations of miR-486-3p, miR-30c and miR-509-3-5p were found in the Twin embryos than in the control embryos ($FC \geq 1.5$, p-value ≤ 0.05) (Figure 8.15). However, as a result of the lack of two additional internal reference genes (RNU44 and RNU 48) and the presence of significant variation in the third reference gene (U6) in the secreted miRNAs, a different kind of normalization, the global normalization strategy, was used to perform the data analysis (Mestdagh *et al.*, 2009).

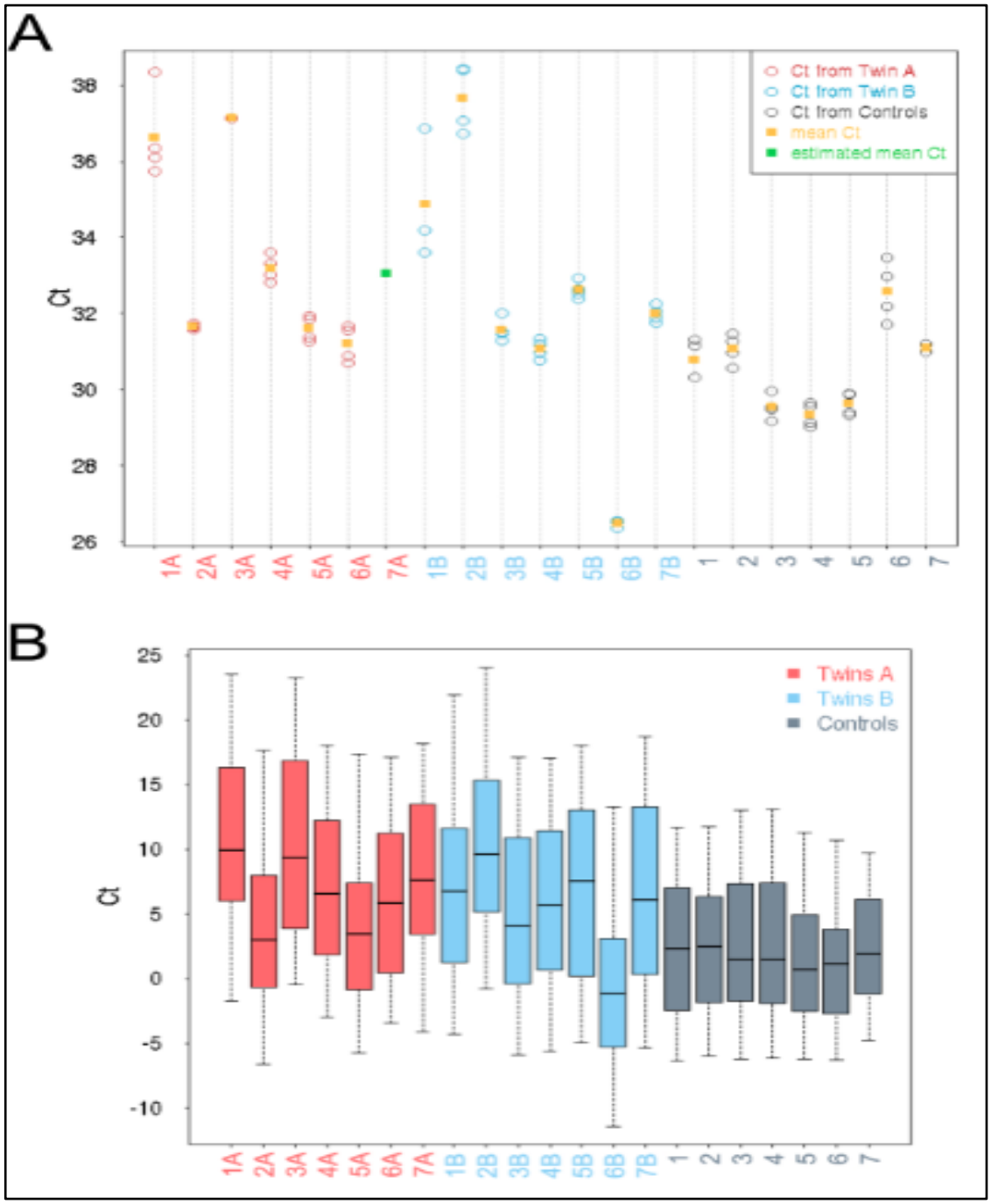


Figure 8.13: The quality of the endogenous control miRNA U6 and the effect of normalization in the endogenous control. (A) Plot showing the distribution of Ct values, demonstrating the heterogeneous distribution of Ct values for U6 between the samples and its homogenous distribution within each sample. Sample 6B displayed a very low Ct value. (B) The distribution of –ΔCt values in samples from the control embryo group was relatively homogenous. More heterogeneity was observed in the samples from both Twin groups, implying that the expression or presence of a miRNA can be different for each sample. Images were reproduced from (Noli *et al.*, 2016a)

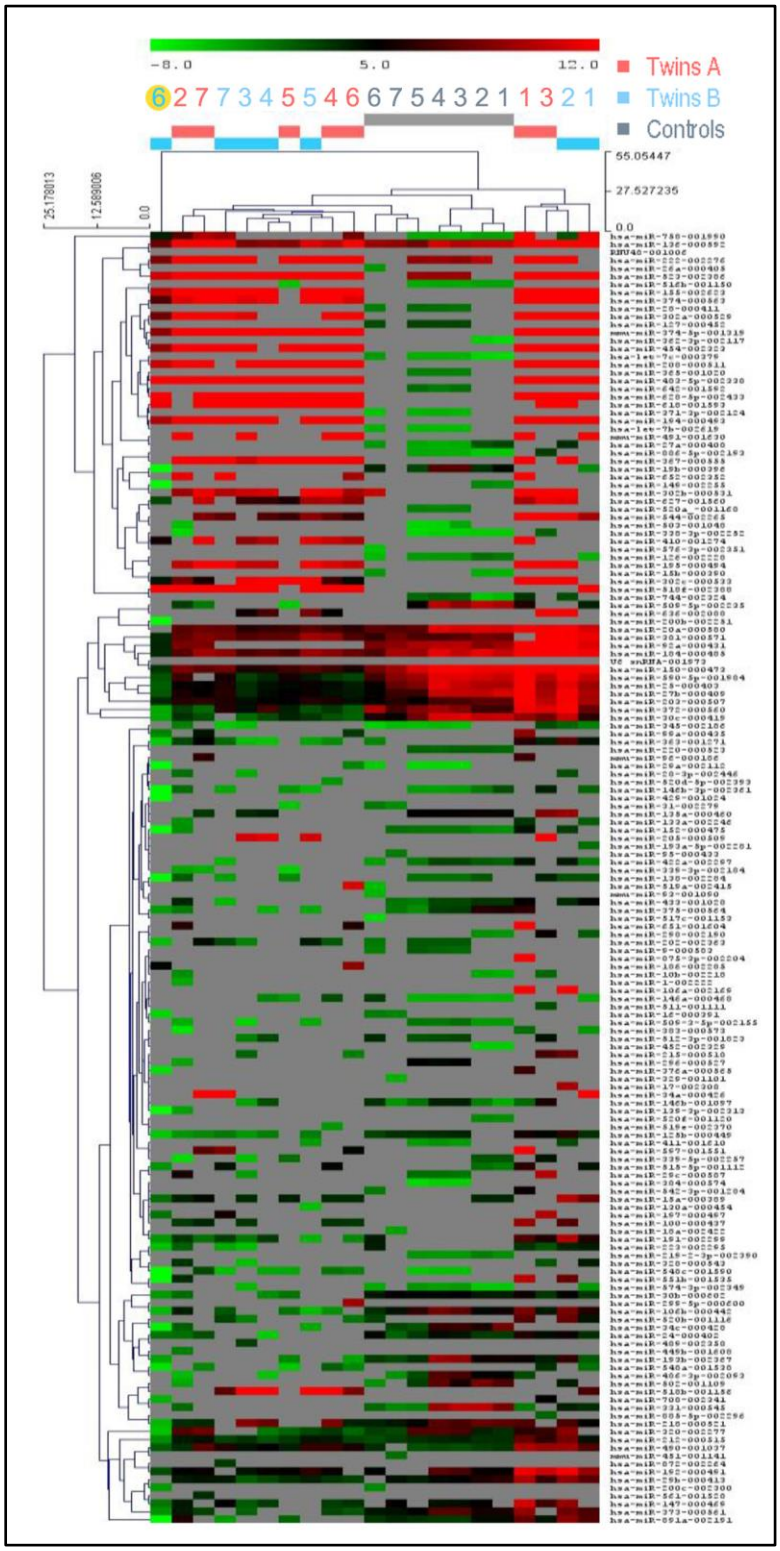


Figure 8.14: Heat map of –ΔCt values from Twin A, Twin B and the control embryos. The heat map demonstrating that there was no significant clustering by experimental group, a large number of missing values, and that the 6B sample (yellow dot) did not cluster with the others. Image reproduced from (Noli *et al.*, 2016a)

A

	Twins A vs. Control		
miRNA	FC	Regulation	p-value
hsa-miR-515-5p-001112	77.27	up	3.10E-01
hsa-miR-490-001037	45.80	up	1.14E-01
hsa-miR-486-3p-002093	30.54	down	4.00E-02
hsa-miR-30c-000419	6.16	down	4.34E-02
hsa-miR-509-3-5p-002155	3.18	down	NA

B

	Twins B vs. Control		
miRNA	FC	Regulation	p-value
hsa-miR-515-5p-001112	25.76	up	4.63E-04
hsa-miR-490-001037	26.50	up	1.72E-01
hsa-miR-486-3p-002093	1286.36	down	NA
hsa-miR-30c-000419	2.87	down	1.88E-01
hsa-miR-509-3-5p-002155	5.12	down	1.16E-02

C

	Twins A and B vs. Control		
miRNA	FC	Regulation	p-value
hsa-miR-515-5p-001112	51.52	up	1.07E-01
hsa-miR-490-001037	36.15	up	2.75E-02
hsa-miR-486-3p-002093	45.27	down	3.86E-02
hsa-miR-30c-000419	4.05	down	6.69E-02
hsa-miR-509-3-5p-002155	4.26	down	5.02E-03

D

	Twins A vs. Twins B		
miRNA	FC	Regulation	p-value
hsa-miR-515-5p-001112	4.52	up	NA
hsa-miR-490-001037	1.73	up	5.72E-01
hsa-miR-486-3p-002093	NA	NA	NA
hsa-miR-30c-000419	1.79	down	7.11E-01
hsa-miR-509-3-5p-002155	NA	NA	NA

Figure 8.15: miRNAs regulated in at least one comparison (FC≥1.5 and p-value ≤0.05) where U6 was used as an endogenous control for normalization. (A) Twins A vs. Control. (B) Twins B vs. Control. (C) Twins A and B vs. Control (D) Twins A vs. Twins B. Green; down regulation and Yellow; up regulation. Figure 8.15 was reproduced from (Noli *et al.*, 2016a).

8.5.2 Data analysis using global geometric means of expression for all detected miRNAs

The global normalization strategy involved three consecutive steps. First, all Ct values over the threshold value of 35 were considered to be noise and were excluded from further analysis. Next, the mathematical average of the Ct values of all detected miRNAs was calculated for each sample. Finally, the calculated average Ct value was subtracted from each individual Ct value for the respective sample (Noli *et al.*, 2016a).This method resulted in the calculation of normalized expression values on the logE scale (with E being the base of the exponential amplification function and 2 being a good estimate). For each individual miRNA, the normalized values were inversely correlated with expression levels. A combination of whole-genome RT-qPCR based miRNA profiling and the use of global mean normalization has been demonstrated to be a highly accurate and reliable strategy for performing high throughput miRNA profiling, and its effectiveness when applied to low input samples as well as SBMs has been validated and reported previously (Capalbo *et al.*, 2016).

8.5.3 The miRNA profile of SBMs was similar among Twin A and Twin B embryos but significantly different from the miRNA profile of control embryos

A comparison of miRNA expression profiles of SBMs obtained from seven pairs Twin embryos to the profiles of SBMs collected from reproductively competent blastocysts was performed. Following quality control procedures and data analysis, it was found that 48 miRNAs were consistently secreted into the culture media by Twin A and Twin B blastocysts, whereas 59 miRNAs were secreted into the SBM by the control embryos. Out of the 48 detected miRNAs, 46 were commonly secreted by both Twin A and Twin B embryos. miR-193b was detected in the SBM of only Twin A embryos, whereas miR-636 was detected only in the SBM of Twin B embryos (Figure 8.16 A). The differences in the miRNA expression profiles in the SBM samples collected from Twin A embryos and the control blastocysts overlapped with the differences observed in the miRNA expression profile of the SBM obtained from Twin B embryos and control blastocysts (Figure 8.16 B) (Noli *et al.*, 2016a).

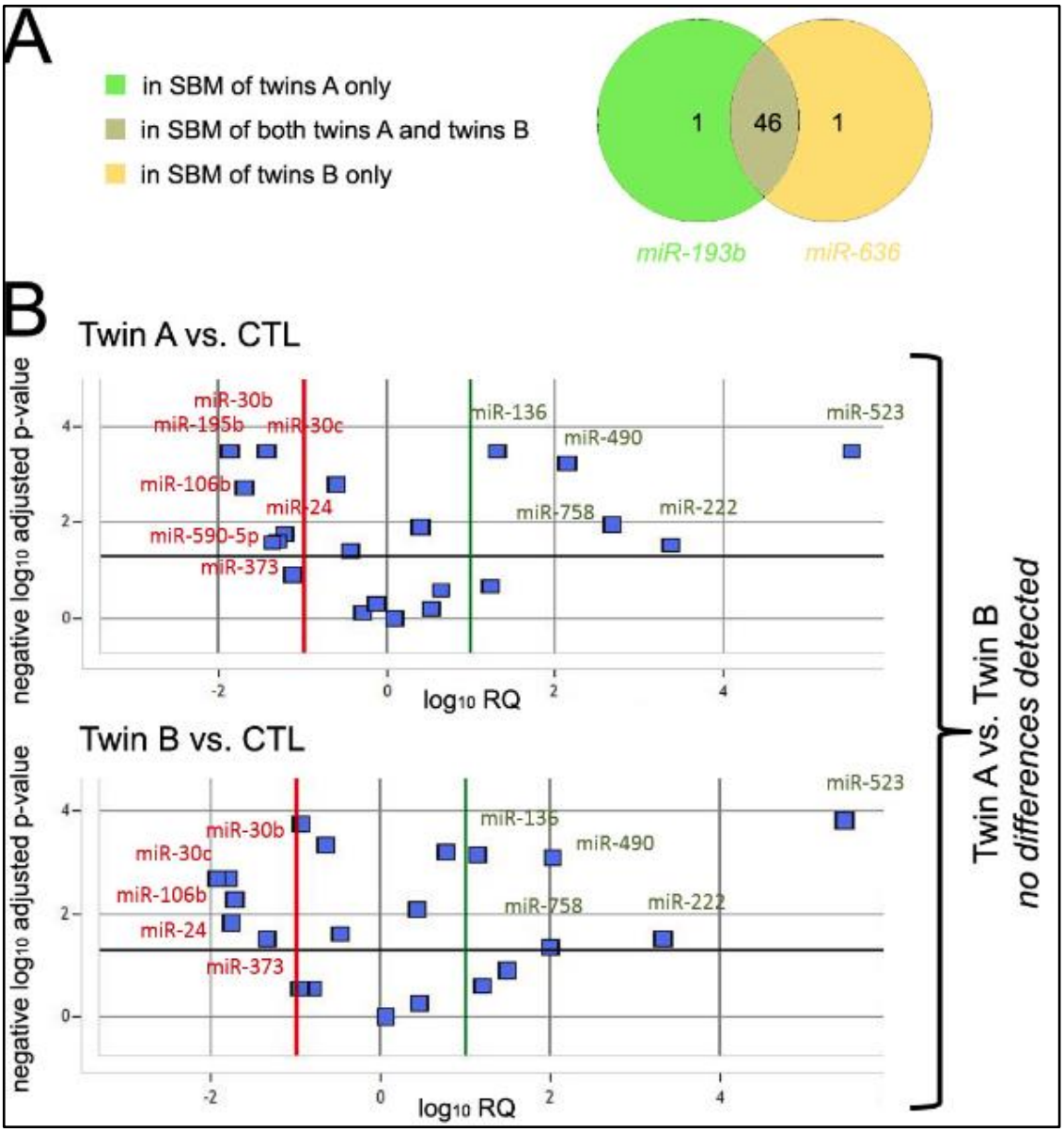


Figure 8.16: Comparison of miRNAs secreted into the culture media by Twin A and Twin B embryos. (A) Greater than 95% (46/48) of the detected miRNAs were commonly expressed by both Twin A and Twin B embryos. (B) Volcano plots comparing the miRNA profiles of Twin A and Twin B embryos, which were nearly completely overlapping. No significant difference was found when Twin A embryos were compared to Twin B embryos. Image reproduced from (Noli *et al.*, 2016a).

A total of 22 miRNAs were overlapping between the SBM miRNA profiles of control and Twin embryos (Figure 8.17 A). A total of 37 miRNAs were found only in the SBM collected from control embryos, and 26 miRNAs were detected only in the SBM collected from the Twin embryos and never detected previously in the media collected from non-manipulated human blastocysts that were created using *in vitro* fertilisation (Noli *et al.*, 2016a). The 59 miRNAs that were detected in the SBM

collected from control blastocysts were also expressed by ICM-free TE cells (Capalbo *et al.*, 2016). However, only 14 of the 26 (53.8%) miRNAs that were secreted by the Twin embryos were also co-expressed in normal blastocyst-derived TE cells. The remaining 12 miRNA were not previously detected in TE cells. Only one out of these 12, miR-374-5p, was expressed in ICM (A. Capalbo, unpublished data). The detection of these 12 novel miRNAs in the Twin embryos suggests abnormal blastocyst development and/or a potential indicator of commitment to a different lineage in the Twin embryos that were formed by embryo splitting.

Of the 22 miRNAs that were expressed in the SBM of both the Twin and the control embryos, a relative quantification analysis was performed and a volcano plot was generated to compare the expression of these miRNAs in SBM that was collected from Twin and control embryos (Figure 8.17 B). The analysis concluded that six miRNAs (miR-203, miR-136, miR-490, miR-758, miR-222 and miR-523) were present at significantly higher levels in the Twin samples, as demonstrated by a fold-change in their expression between 2.7 and 319148, whereas nine miRNAs (miR-193b, miR-30b, miR-106b, miR-30c, miR-373, miR-24, miR-590-5p, miR-25 and miR-27b) were expressed more abundantly in the control samples, with fold-differences as high as -2.8- to -167.6–fold (Noli *et al.*, 2016a).

These results were further supported by single assays specific for miR-30c and miR-203. The mean Twin A Ct±SD was 34.9±1.9 and 31.3±1.1 for miR-30c and miR-203, respectively. The mean Twin B Ct±SD was 35.3±2.2 and 30.8±1.2 for miR-30c and miR-203, respectively. The ΔΔCt analyses showed that there was no difference in the levels of miR-30c when it was normalized to miR-203 levels in Twins A versus Twin B embryos (fold-change: 1.9; p-value: 0.7). When the same analysis was performed to compare the Twins (A and B combined) to the historical control SBM samples obtained from euploid implanted blastocysts, a statistically significant 93.2-fold lower level was detected ($p<0.001$) in the Twins. No miRNA expression was detected in negative controls (Noli *et al.*, 2016a).

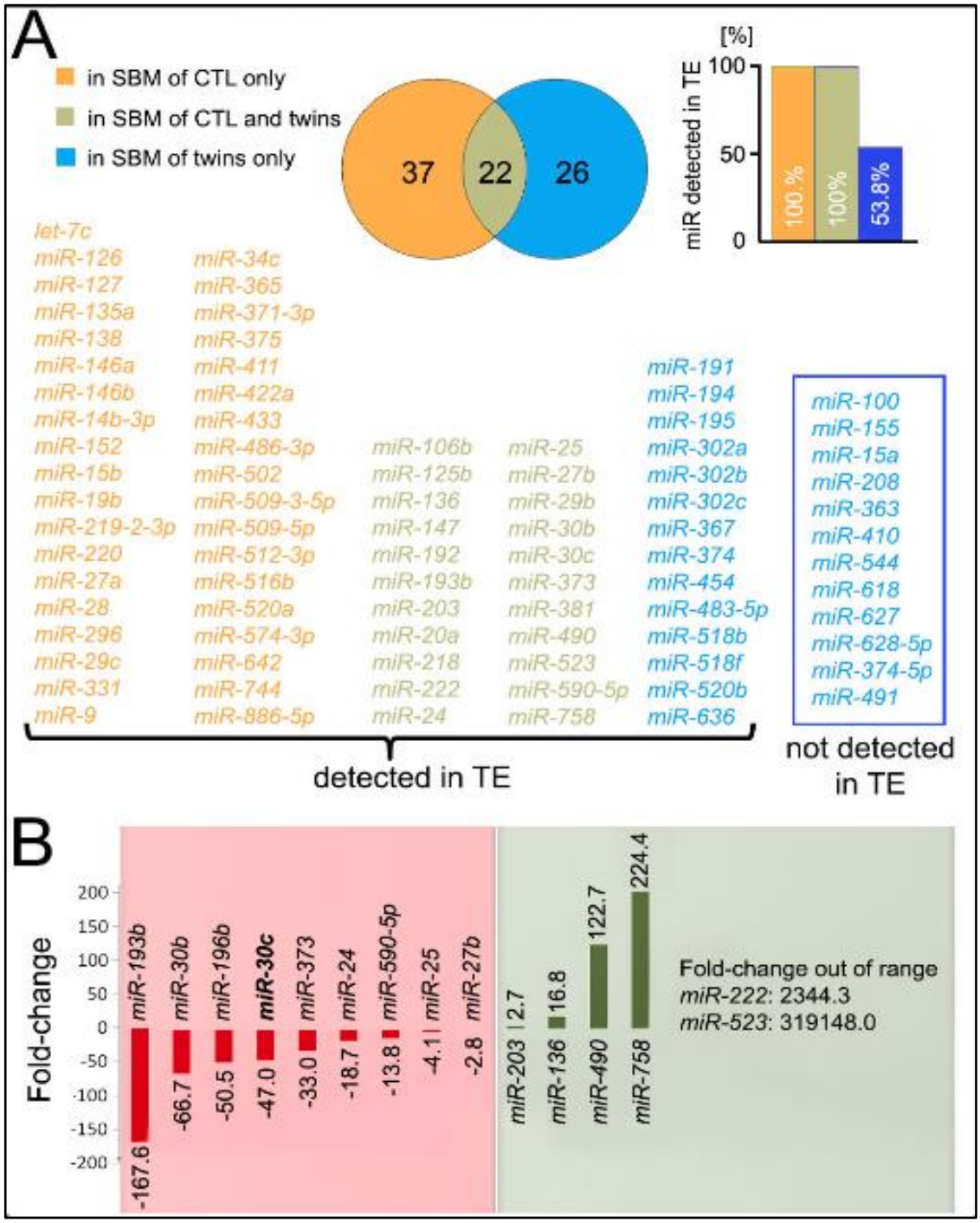


Figure 8.17: Comparison between miRNA expression profiles in Twin and control SBM (A). A total of 37 miRNAs (orange) were expressed only in the SBM of the control blastocysts, and these were co-expressed in TE cells. A total of 22 miRNAs (olive) were expressed by both control and Twin blastocysts and co-expressed in TE cells. A total of 26 miRNAs (blue) were expressed only in SBM of the Twin blastocysts. Of these, only 53.8% (14/26, blue) were co-expressed in TE cells. (B) The volcano plot shows 9 miRNAs were expressed at significantly lower levels in the SBM obtained from Twins when compared to reproductively competent blastocyst controls, with fold changes ranging between -167.6-fold and -2.8-fold. Six miRNAs were expressed at significantly higher levels, with fold-changes ranging between 2.7 and 319148. The miRNA miR-30c, a potential marker of reproductive potential in blastocysts, was identified in the former cluster with a fold-change of -47.0. Image reproduced from (Noli *et al.*, 2016a).

8.5.4 Significantly lower expression levels of miR-30c were detected in Twins using either normalization strategy

Using either of the two normalization strategies, it was found that significantly lower levels of miRNA-30c were present in the SBM that was collected from Twin embryos than control embryos. This marker was therefore examined closely because miRNA-30c has been suggested as a possible biomarker for the implantation potential of blastocysts when it is secreted at high levels into SBM during IVF cycles (Capalbo *et al.*, 2016). As the next step, the potential roles of miRNA-30c in early development were investigated. Data describing miRNA-gene interactions were provided by the DIANA TarBase v7 (DIANA Tools, 2016), which indicated that miRNA-30c has 1643 putative gene targets. To determine which of these targets are expressed in the TE and/or ICM of human blastocysts, these genes were filtered against the blastocyst transcriptome database, which contained a list of genes that were detected using the RNA-Seq method in isolated TE and ICM samples and described in a previous publication (Noli *et al.*, 2015a). Of the 1643 examined gene targets, 1061 were expressed in ICM, 1166 in TE and 1006 in both ICM and TE (Figure 8.18). Based on pathway information that was furnished by the Kyoto Encyclopedia of Genes and Genomes (Kanehisa Laboratories, 2016) two key pathways were revealed, including ubiquitin-mediated proteolysis and spliceosomes (Figure 8.19) (Noli *et al.*, 2016a).

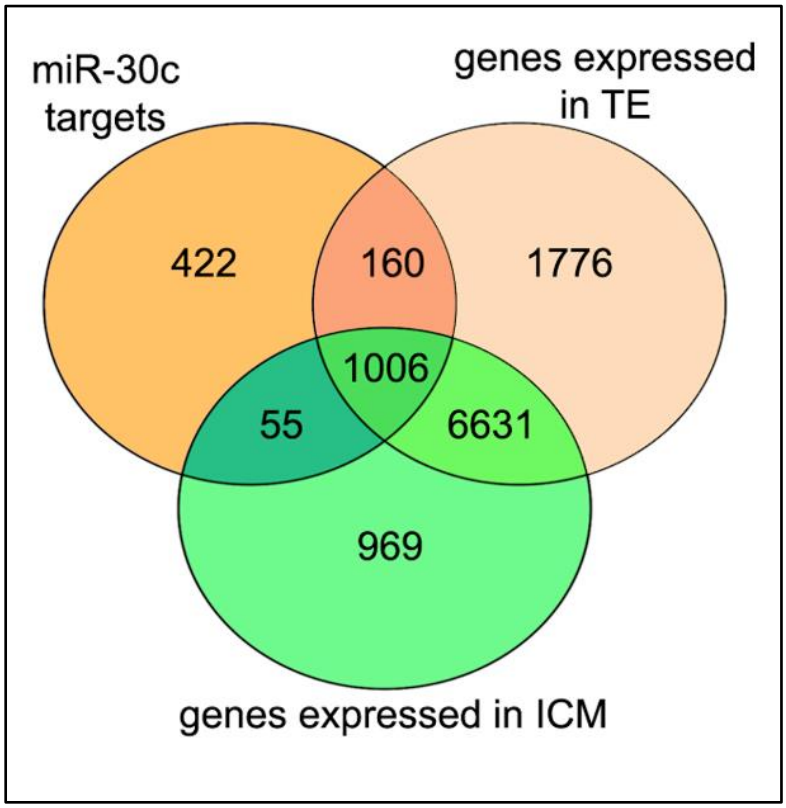


Figure 8.18: Putative roles of miRNA-30c in human blastocysts. From 1643 miRNA-30c target genes, 1061 were expressed in ICM, 1166 in TE, and 1006 in both ICM and TE of the human blastocysts. Image reproduced from (Noli *et al.*, 2016a).

ICM

Pathway Description (KEGG)	Nb Genes in Pathway	Nb Regulated Genes	P-Value	Corrected P-Value
Ubiquitin mediated proteolysis	137	27	7.85E-08	1.17E-05
Spliceosome	126	21	4.25E-05	3.16E-03
Cell cycle	125	19	3.63E-04	1.79E-02
Oocyte meiosis	110	17	6.86E-04	2.52E-02
p53 signaling pathway	68	12	1.97E-03	5.71E-02
Lysine degradation	44	8	1.39E-02	2.94E-01
Wnt signaling pathway	151	17	1.65E-02	2.98E-01
Lysosome	117	14	2.05E-02	3.20E-01
Progesterone-mediated oocyte maturation	86	11	3.00E-02	3.97E-01
Nucleotide excision repair	44	7	4.38E-02	4.87E-01

TE

Pathway Description (KEGG)	Nb Genes in Pathway	Nb Regulated Genes	P-Value	Corrected P-Value
Ubiquitin mediated proteolysis	137	32	7.45E-10	1.18E-07
Spliceosome	126	22	7.17E-05	5.68E-03
Oocyte meiosis	110	19	2.98E-04	1.56E-02
p53 signaling pathway	68	14	4.47E-04	1.76E-02
Cell cycle	125	19	1.42E-03	4.43E-02
Wnt signaling pathway	151	21	2.24E-03	5.77E-02
Lysine degradation	44	9	7.66E-03	1.60E-01
Progesterone-mediated oocyte maturation	86	13	1.09E-02	1.97E-01
Colorectal cancer	84	12	2.26E-02	3.33E-01
Amino sugar and nucleotide sugar metabolism	44	8	2.48E-02	3.29E-01
Thyroid cancer	29	6	4.03E-02	4.48E-01
Lysosome	117	14	4.64E-02	4.67E-01
Regulation of actin cytoskeleton	215	22	4.80E-02	4.52E-01

Figure 8.19: The pathways involving miR-30c targeted genes in ICM and TE according to the KEGG database. Figure 8.19 was reproduced from (Noli *et al.*, 2016a).

Chapter 9

Discussion

In vitro generated monozygotic twin embryos are commonly accepted in research and veterinary medicine. Whereas the mouse is the most commonly used animal in research, veterinary medicine is focused on work with large animals, and successful twinning has been reported in sheep (Willadsen, 1979), cattle (Seike *et al.*, 1989a; Seike *et al.*, 1991; Johnson *et al.*, 1995), goats (Tsunoda *et al.*, 1985), pigs (Reichelt and Niemann, 1994) and horses (Allen and Pashen, 1984). Despite results with both small and large animals, twinning in primates has not worked as expected, and only one live birth has been reported (Chan *et al.*, 2000; Mitalipov *et al.*, 2002). These results have raised the concern that embryo splitting may not be successful in humans. A limited number of studies with human material have resulted in promising observations, though a thorough validation has not been performed (Hall *et al.*, 1993; Van de Velde *et al.*, 2008; Illmensee *et al.*, 2010).

Concurrently, there is a severe shortage of human cleavage stage embryos that have been donated for research purposes due to the shift of clinical practice towards the cryopreservation of blastocysts, and there is an associated potential for future restrictions on human developmental research. Hence, the need to investigate embryo splitting as a suitable methodology that can address this shortage is proposed as the main purpose of this study. An experimental model to assess the safety and efficacy of embryo splitting technology was designed, and the study was subsequently performed to determine whether twin embryos that were generated by splitting donor embryos are suitable as a research tool. The main objective of this project was to validate the developmental, pluripotent and reproductive competence of human twin embryos that were generated using blastomere biopsy. A comparative analysis of embryos that were created by fertilisation was performed as a control.

The expression patterns of lineage markers together with morphokinetic data analyses presented in this study support the hypothesis that the initial stages of human embryonic development are governed by precise temporal control. As such,

an equivalent amount of time is necessary to progress to the blastocyst expansion stage irrespective of the number of blastomeres used to create split embryos (Twins A and B). Furthermore, blastocyst size correlates with the starting number of blastomeres. The data also demonstrate that the cell-cell interactions between blastomeres of cleavage stage embryos do not play an essential role in the development of embryos into blastocysts; no overall statistically significant differences could be found between embryos in which the initial blastomeres remained undisturbed (donors or Twin A) and embryos that were formed as a result of the successive transfer of blastomeres from the donor embryo to the recipient's empty ZP (recipients or Twin B). Another interesting finding was the evidence of discordance of blastulation morphokinetics and lineage commitment in twin embryos; ICM formation was delayed, and the first cells to express NANOG without the co-expression of TE markers, CDX2 or GATA2 were detected only on Day 6 or later, at which point the blastocysts were already undergoing expansion. Importantly, no successful hESC derivation was achieved from the ICM of the twin blastocysts. Finally, fewer and different miRNAs were detected in the SBM from twin embryos in comparison to controls. miR-30c, a putative biomarker of the reproductive competence of human embryos (Capalbo *et al.*, 2016), was also detected at significantly lower levels in the SBM from twin embryos when compared to control embryos.

9.1 Factors affecting morphokinetics analysis

To eliminate variation at the thawing step, the same person carried out the thawing of all donor embryos following the manufacturer's instructions. Upon thawing donor embryos, handling was consistent, ranging from culturing the embryos for the same duration (depending on their developmental stage, 2PN were cultured longer) in the same incubator to using the same type of culture media. In addition, the same person collaborated with an expert embryologist to assign donor embryos containing no obviously degenerating blastomeres, based on morphological assessment, for biopsy (Bolton *et al.*, 1989) to generate Twins A and B. The blastomere biopsy procedure, in which half the number of blastomeres was removed from the donor embryo (to generate Twin A) and placed into an empty recipient ZP (to generate Twin B), was consistently handled by one experienced embryologist throughout the study. Once the blastomere biopsy was completed, the

resultant Twins A and B as well as the controls were exposed to the same culture conditions of 37°C in 6% CO_2 and 5% O_2 in the Embryoscope, a tri-gas time-lapse incubator with TLS. Therefore, there was minimal bias in the experimental procedures prior to video recording of embryonic development.

Monitoring of *in vitro* development of embryos traditionally requires removal of the embryos from the incubator for microscopic examination. Taking pictures would require more time for embryos to be in a suboptimal environment. Exposing the embryos frequently to ambient temperatures and conditions outside the incubator could have deleterious effects on development (Fujiwara *et al.*, 2007; Zhang *et al.*, 2010). Limiting the duration of inspection of the embryos is highly desirable. However, this could result in the loss of meaningful information that can be gained regarding embryonic development, and embryo assessment would become highly dependent on the time required for visual inspection (Scott *et al.*, 2007; Montag *et al.*, 2011). Therefore, for this research, frequent visualization of the *in vitro* development of the twin embryos without disturbing the culture conditions was necessary. Accordingly, time-lapse imaging was employed with acquisition of images automated at 20 minutes intervals.

In previously published reports, embryonic development of human twin embryos was monitored using an inverted microscope, and images were acquired by removing the embryos from the incubator until they reached the blastocyst stage (Van de Velde *et al.*, 2008; Illmensee *et al.*, 2010). This work is the first of its kind to generate data about the development of twin embryos generated by blastomere biopsy and their morphokinetic parameters by time-lapse imaging. However, keeping twin embryos in the optimal environment throughout their development had no significant influence on the outcome. In the next sections, factors that may have affected the morphokinteics analysis that was performed in this study are discussed.

9.1.1 Donor embryo sources

In general, morphokinetic studies were performed using a heterogeneous population of fresh embryos. Only two studies used supernumerary embryos frozen at the 2PN stage, 12-18 hours post-fertilisation (Wong *et al.*, 2010; Hashimoto *et al.*, 2012). No previous studies were identified during the research performed for

this thesis that reported on the morphokinetic parameters derived from time-lapse observation of surplus cryopreserved cleavage stage embryos. All donor embryos used in the splitting study described in this project were surplus embryos that were slowly frozen at either the 2PN or cleavage stage. Post-splitting, both Twins A and B were monitored using a time-lapse system, and thus the morphokinetic analysis of post-splitting cryopreserved embryos were reported for the first time.

9.1.2 Fertilisation methods

Studies have indicated that the fertilisation method can influence the timing of the 1st cleavage, with a higher percentage of ICSI than IVF fertilised embryos having early cleavage (Lundin *et al.*, 2001; Giorgetti *et al.*, 2007; Dal Canto *et al.*, 2012; Kirkegaard *et al.*, 2016), although they reach the 4-cell stage at the same time (Lemmen *et al.*, 2008). Other studies have reported that IVF embryos display a systematic delay in development compared with ICSI embryos, which persists during the cleavage stages (Cruz *et al.*, 2013; Bodri *et al.*, 2015). The difference at the cleavage stage disappeared when the timings were normalized to pronuclear fading rather than time of fertilisation (Cruz *et al.*, 2013; Bodri *et al.*, 2015). During classic IVF, fertilisation occurs similarly to natural fertilisation, but several steps of the spermatozoon-oocyte interaction do not occur when performing ICSI (Fancsovits *et al.*, 2006), such as sperm penetration through the cumulus cells and the ZP towards the oocyte cytoplasm.

Even though the impact of the fertilisation method on the developmental kinetics of embryos is unclear, the method of fertilisation was not taken into account when selecting the donor embryos for the blastomere biopsy. This is because the effects of fertilisation method are mainly exerted on the 1st cleavage, and this kinetic parameter was not accessible for the cryopreserved donor embryos.

9.1.3 Cryopreservation and thawing procedure

The effects of freezing and thawing on developmental kinetics have previously solely been studied with a relatively small sample size of cryopreserved 2PN embryos and compared with images obtained from fresh human triploid (3PN) embryos (Wong *et al.*, 2010). The authors suggested that cryopreserved embryos did not suffer a developmental delay due to the cryopreservation process and

behaved similarly to fresh zygotes that cleaved into two blastomeres. Using the TLS, it was also recently shown that cryopreservation had no significant effect on the kinetic parameters or the average cell number between embryos developed from fresh and vitrified oocytes (De Munck *et al.*, 2015). Others have found that cryopreservation of human oocytes has no effect on aneuploidy incidence (Goldman *et al.*, 2015). Furthermore, recent studies have demonstrated that freeze-all cycles resulted in higher implantation and clinical pregnancy rates than fresh embryo transfer (Roque *et al.*, 2015). Although the authors, in their conclusions, cited the impact of ovarian stimulation on endometrial receptivity to explain this effect, the observed outcome is still reliant on freezing having limited, if any, deleterious effects on developmental competence.

The donor embryos used in these experiments were previously slowly frozen at the 2PN or cleavage stage. Large cohorts of these embryos were obtained from multiple fertility clinics across the UK, and the remaining were donated by Guy's ACU patients. The secondary objective of this study was to run a comparative analysis of embryos resulting from embryo splitting *in vitro* and control embryos created by fertilisation. This necessitated a robust method of manipulation for the split embryos to ensure high viability and continued development to the blastocyst stage. The cryopreserved donor embryos were thawed using the Quinn's Advantage Thaw Kit per the description in the Chapter 3 section 3.4.1. To eliminate variation at the thawing step, the same technician carried out the thawing of all donor embryos following the manufacturer's instructions. However, despite using a consistent thawing procedure with the same commercial thawing kit, some variability between donor embryos may still have been present. This may be the result of two operating variables among the IVF clinics that the embryos were obtained from: the embryo cryopreservation protocols established in-house by the respective clinics and the preferential selection criteria for qualifying the embryos for cryopreservation. Other less significant factors that could have produced variability in this work are variations in donor embryo handling and manipulation techniques, culture media, and culture conditions used prior to the cryopreservation process, which may have affected the viability of the donor embryos post-thawing. All the clinics that provided donated embryos for research under the HFEA license were requested to provide details of the cryopreservation procedure, such as the type of freezing medium and the freezing method used.

However, there may have been circumstances in which these details were not readily available or the participating clinics did not provide the paperwork in full. On the basis of the available information about the donor embryos used in this project, most of the cryopreserved embryos were slowly frozen with a commercially available freezing medium following the manufacturer's protocols. Thus, the criteria for selecting donor embryos to slow-freeze are likely the most significant variables among the different donation centres.

9.1.4 Medium of choice

An external factor likely to affect the timing of embryonic development is the type of medium used for culturing the embryos. A number of previous studies have demonstrated the importance of optimal culture conditions and culture media composition on the development of embryos *in vitro* (Pool, 2002; Behr and Wang, 2004; Pool, 2005). Multiple groups have carried out and reported evaluation of various commercially available and in-house media and their effects on human embryos in culture (Quinn *et al.*, 1985; Gardner and Lane, 1997; Quinn, 2004; Balaban and Urman, 2005; Lane and Gardner, 2007) as well as a comparison of results using different commercially available media for embryo culture (Van Langendonckt *et al.*, 2001; Aoki *et al.*, 2005; Balaban and Urman, 2005; Xella *et al.*, 2010; Basile *et al.*, 2013). The type of culture medium used to culture human embryos has been shown to correlate with the rate of embryo cleavage (Van Langendonckt *et al.*, 2001; Ben-Yosef *et al.*, 2004; Zollner *et al.*, 2004; Sifer *et al.*, 2009).

Although some of these studies reported differences in clinical outcomes and embryonic development, these conclusions were drawn on the basis of evaluating embryos by static observation of their morphology at specific time points. However, embryonic development is a dynamic process, and data collection at specific time points alone may miss information from critical intermediary stages. Furthermore, the process of grading the morphology of embryos is highly subjective and is likely to be prone to substantial inter-observer and moderate intra-observer variations (Baxter Bendus *et al.*, 2006). On the contrary, the utilization of time-lapse imaging technology for monitoring cell division kinetics provides a highly objective method to assess embryonic development.

Three different types of protocols are currently in use for the *in vitro* culture of human embryos: i) culture using a single media, ii) culture using a single medium sequentially, and iii) culture using two media sequentially. Several studies have indicated that a sequential media protocol may be preferable, whereas other studies have reported no difference (reviewed in (Biggers and Summers, 2008). In a prospective cohort study that was performed by Basile *et al.* (2013) to study the morphokinetics of growing embryos that were cultured using two culture media (single step media, Global; sequential medium, Sage Cleavage), the authors observed no significant differences when either media was used in the timing of 2-, 3-, 4- and 5-cell stage embryos or the duration of the second cell cycle. Yang *et al.* (2013) evaluated differences in morphokinetics and chromosome number in human embryos that were cultured in single (Irvine Scientific CSC) versus sequential (Vitrolife G1 and G2) media. No significant differences were observed in the fraction of blastocysts with optimal morphokinetics between embryos cultured in single and sequential culture media. The authors observed a non-significant tendency for more embryos to develop into euploid blastocysts when cultured in a single medium than when they were cultured in sequential media. In contrast, Ciray *et al.* (2012) reported that embryos cultured in a single step medium progressed from the first mitosis to the 5-cell stage faster than their sibling embryos that were cultured in sequential media. However, neither the durations of cell cycle two (cc2 = t3-t2) and s2 (t4-t3) nor the pregnancy and implantation rates differed between the two groups.

It is unclear whether these findings from morphokinetic studies can be universally extrapolated to all IVF clinics without taking into consideration the methodology of embryo culture. Best *et al.* (2013) demonstrated an inability to equivalently apply a published embryo selection model to a different culture setting. Additionally, the effects of single versus sequential culture media systems on data collected from time-lapse imaging needs to be taken into consideration. To establish and validate the use of time-lapse technology for embryo selection in the clinic, each laboratory must evaluate and characterize the optimal growth patterns in human embryos that are specific to the *in vitro* culture system used in their own facility (Desai *et al.*, 2014).

In this project, two different *in vitro* embryo culture systems were used: two media sequentially (Sage; CM and BM) and a single medium (Sage). Post-thaw, cleavage stage donor embryos were incubated for at least 4 hours in 40-µl droplets of CM, and Twins A and B were transferred into microwells with 25 µl of BM in an Embryoslide dish subsequent to their biopsy. Both CM and BM media were supplemented with 10% SPS under mineral oil at 37°C, in 6% CO_2 and 5% O_2, as described previously in Methods Chapter 3, sections 3.5.3. Control embryos created by fertilisation and resulting in pregnancy and live birth upon single embryo blastocyst transfer were cultured in single step medium, Chapter 4 section 4.2. Given that the precise effects of the culture medium on morphokinetic parameters of embryonic development are unclear, two different culture protocols used in this work were not considered significant variables affecting the kinetic parameters of the twin and control embryos.

9.1.5 Gas environment

The O_2 concentrations that mammalian oocytes and embryos are subjected to in a physiological environment are significantly lower than the O_2 present in the atmosphere. The O_2 tension in the oviducts and uterine horns measured in patients was 3 to 5%, whereas the average intrauterine oxygen tension was 11.8% of air saturation (Ottosen *et al.*, 2006). Throughout gestation and organogenesis, lower oxygen tension values persist. Improved *in vitro* pre-implantation development has been reported in multiple mammals when embryos are cultured in low-oxygen environments, such as mice (Karagenc *et al.*, 2004), sheep, cows (Thompson *et al.*, 1990), goats (Batt *et al.*, 1991) and pigs (Watson *et al.*, 1994). Upon culturing in 5% O_2 saturation, human pre-implantation embryos demonstrated improved morphological grading scores on day 3, as reported by Bahceci *et al.* (2005). However, the authors were unable to find improvement in the on-going pregnancy rate. In another study, embryos cultured in an incubator with 5% O_2 tension ended with a significantly higher number of cells at the blastocyst stage; however, no variation in the rate of pregnancy was determined between embryos cultured in atmospheric O_2 versus 5% O_2 tension (Dumoulin *et al.*, 1999). In a prospective randomized study conducted by Waldenstrom *et al.* (2009), it was reported that culturing embryos under low-oxygen conditions led to the development of blastocysts of higher quality and significantly improved pregnancy

and live birth rates. Another prospective randomized survey on sibling oocytes reported that physiologic oxygen concentrations throughout the culture period improved blastocyst yield at day 5 as well as total blastocyst yield (Ciray *et al.*, 2009).

Embryos were cultured in TLS under 5% O_2 tension in several recently published reports (Azzarello *et al.*, 2012; Dal Canto *et al.*, 2012; Hashimoto *et al.*, 2012; Kirkegaard *et al.*, 2013), whereas other research groups have used atmospheric oxygen culture conditions to study embryo kinetics (Meseguer *et al.*, 2011; Cruz *et al.*, 2012; Hlinka *et al.*, 2012; Kirkegaard *et al.*, 2013). The effects of ambient O_2 concentrations on the development of pre-implantation embryos in humans and mice were documented using time-lapse monitoring. Using atmospheric O_2 conditions to culture mouse embryos in TLS led to significant delays in both the cleavage and the post-compaction stages (Wale and Gardner, 2010) and affected the utilization of amino acids and carbohydrates (Wale and Gardner, 2012). In this work, 5% O_2 saturation was used in the TLS to culture twin embryos (A and B) and control embryos, as a recent study showed that 20% oxygen tension in the TLS to culture human pre-implantation embryos can cause a reduction in the developmental rate and a delay in the pre-compaction stage (Kirkegaard *et al.*, 2013).

9.1.6 Additional factors that may affect embryo morphokinetic parameters

Additional factors that may be introduced due to infertility treatment, such as gonadotropin for ovarian stimulation, have been indicated to affect the timing of embryonic development. Embryos taken from oocyte donors that are exposed to higher doses of gonadotropin, and they progress somewhat faster to the later stages than those exposed to lower doses (Munoz *et al.*, 2012). For embryos used in this research project, information about the doses of gonadotropin given to the patients was rarely known. However, differences observed between embryos could not be ascribed only to gonadotropin. They correlated to patient-specific factors, such as genetic make-up, age and ovarian response as well as differences in stimulation. The developmental competence of human embryos may have been affected by the maternal age of the donor as well as the patient (in case of the control embryos). Control embryos were collected from fresh cycles as described

previously in Chapter 4, section 4.2, and morphokinetic data of those resulting in pregnancy and live birth were subsequently used as a comparative data set against twin embryos generated by embryo splitting. The donor embryos used for splitting (Group 1: 2-5 blastomeres, and Group 2: 6-10 blastomeres) were composed of a heterogeneous population. However, only donor embryos with Grades 3 and 4 and with viable blastomeres were presented following post-thawing, and no obvious degenerate blastomeres were used. Demographic data for both donor and control groups were not taken into consideration when running the comparative analysis for their developmental and reproductive competence. This can be considered a limitation of this study; however, the best category of embryo on both groups was used (controls resulted in live birth, and donor embryos were of high quality Grade 3 and Grade 4). In the event that demographic factors impacted embryonic development, high quality cleavage stage embryos as well as high quality blastocysts that resulted in live birth would not be produced. It is highly unlikely that any differences in the above discussed factors or demographics could fully explain the distinct differences in the developmental competence between control and twin embryos generated by splitting the donor embryos. Thus, such differences can be explained by the side effects of the splitting procedure itself more than any other factor.

9.2 Developmental competence analysis

9.2.1 Human development under strict temporal control

One of the most distinctive features of embryonic development in mammals is the plasticity of embryos in order to adapt to experimental perturbance, a process termed as regulative development. Post-destruction of one blastomere of the 2-cell stage mouse embryo, the remaining blastomere can compensate and develop to term (Nicholas and Hall, 1942; Tarkowski, 1959b). In some cases, even the later stages of development retain this plasticity (Morris *et al.*, 2012). It has been found that, even though cells separated from the 4- or 8-cell mouse embryo are unable to develop into embryos that progress beyond the implantation stage (Tarkowski and Wroblewska, 1967; Rossant, 1976), they are still capable of contributing towards all tissue formation in chimeric animals (Kelly, 1977; Piotrowska-Nitsche and Zernicka-Goetz, 2005). Therefore, in conformity with this finding, repositioning the

cells from these embryos results in a subsequent readjustment in embryonic development (Rossant and Lis, 1979). Even chimeras comprising embryos placed together can adapt themselves to give rise to only a single individual (Tarkowski, 1961; Mintz, 1964). The existence of such plasticity reflects the stochastic nature of early mammalian embryonic development. However, previous work has demonstrated that developmental plasticity is not as universally observed as commonly assumed (Morris *et al.*, 2012). For instance, several blastomeres isolated at the 2-cell mouse embryo stage did not develop into live births despite repeated efforts; the creation of monozygotic twins by this method has been nearly impossible (Tsunoda and McLaren, 1983; Papaioannou and Ebert, 1995). However, the mechanisms underlying developmental plasticity as well as the inability of separated blastomeres to give rise to monozygotic twins have not been fully elucidated (Morris *et al.*, 2012). Research on the spatial and temporal monitoring of mouse embryonic development has suggested the existence of a 'developmental clock' (Morris *et al.*, 2012). In this work, it was found that the landmark developmental events of cell compaction, lineage commitment and cavitation took place at the same time in the manipulated split embryos as well as the controls. Additionally, it was found that blastocyst size and cellularity were directly related to the number and 'age' of the blastomeres used in their creation (Noli *et al.*, 2015b). For manipulated cryopreserved donor human embryos, however, it is possible that the asynchronous status of blastomeric differentiation/pluripotency may result in advanced blastomeres contributing preferentially to the embryo. Additionally, the loss of earlier blastomeres and a contribution from only a small number of blastomeres to the embryo is also possible (Schramm and Paprocki, 2004a). However, if the above-mentioned factors were more applicable, a more pronounced discrepancy between Twins A and B derived from the same parental donor embryo would have been noticed in this study.

Although Twin B developed to a later developmental stage than that of Twin A, the only significant difference between the embryos of Twin A and Twin B was observed during blastocyst formation, as shown in Figure 8.2 B. This result was unexpected, and it may be explained by the splitting methodology used in this work. Twin B was derived from blastomeres that were aspirated one by one and inserted into previously emptied ZPs. With Twin A, the blastomeres remaining in the donor embryos were used; thus, Twin A is not manipulated, and Twin B is

manipulated. However, the sample size is too small to provide definitive conclusions in this regard.

Twins originating from Group 2 tended to display a higher probability of progressing through development than twins from Group 1, as shown previously in Figure 8.2B. Although blastomeres from late cleavage embryos (Group 2) resulted in the formation of blastocysts with larger sizes and higher yields, only 36.84% (14 out of 38) of such embryos had a distinguishable ICM. Conversely, in Group 1, 46.15% (12 out of 26) of the blastocysts demonstrated a clear and distinguishable ICM. Of the ICMs observed, only three in Group 1 and one in Group 2 were Grade B. The remainder of the ICMs were Grade C (Stephenson *et al.*, 2007), as shown in Figure 8.7. Twins from Group 2 were formed from splitting late cleavage stage embryos and therefore contained higher numbers of blastomeres compared to embryos from Group 1, which probably influenced their developmental progression.

For full developmental competence, mouse embryos need to have developed an epiblast with at least four pluripotent cells before undergoing implantation. However, single blastomeres derived from 4-cell mouse embryos have thus far been unable to demonstrate this epiblast formation. Altogether, the evidence for the presence of a 'developmental clock' as well as the necessity for an epiblast of a specific size in higher animals, including humans, has been up for debate. Single blastomeres derived from 4-cell embryos in cows have led to the production of identical calves (Johnson *et al.*, 1995), and it was reported that a child was born subsequent to the transfer of a 4-cell human embryo, in which only a single blastomere survived post-thawing (Veiga *et al.*, 1987). The formation of small blastocysts containing NANOG-positive cells upon splitting a 4-cell stage human embryo has been reported (Van de Velde *et al.*, 2008). Additionally, single blastomeres of 4- and 8-cell embryos have led to the successful derivation of hESCs (Klimanskaya *et al.*, 2006; Chung *et al.*, 2008; Geens *et al.*, 2009; Ilic *et al.*, 2009). However, the successful derivation of ESC lines has also been reported from single blastomeres of 2-, 4- and 8-cell mouse embryos (Chung *et al.*, 2006; Lorthongpanich *et al.*, 2008), thereby implying that the ability to generate pluripotent stem cell lines is not a thorough indicator of developmental competence and embryonic plasticity (Noli *et al.*, 2015b).

Early implantation has been extensively investigated to elucidate the mechanisms involved in temporal control. Following ovulation, the newly released oocyte moves through the oviduct aided by ciliated epithelia of the Fallopian tube. Fertilisation can also be referred to as stage I of implantation. Stage II begins with the onset of cell division in the newly fertilised oocyte. At stage III, the morula, or small collection of embryonic cells, is transported into the uterus. The blastocyst is formed following further cell divisions. Approximately 20% of human embryos are thought to develop to this stage and progress to the activation of the genome required for successful implantation (O'Rahilly and Muller, 1987). Together, these early stages of development occur within a short window of time, as the embryo reaches the uterine cavity within 72–96 hours of fertilisation taking place (Croxatto *et al.*, 1978).

The endometrium is an extremely dynamic tissue that exhibits physiological changes triggered by steroid hormones. This response renders the endometrium receptive in synchrony with the entry of the implanting blastocyst into the uterine cavity during the window of implantation (WOI). This occurs between days 19 and 21 (Diaz-Gimeno *et al.*, 2011). During this period, the phenotype of the endometrium switches to receptive, and the epithelial cells of the endometrium exhibit plasma membrane transformation (Diaz-Gimeno *et al.*, 2011).

Once the embryo enters the uterine cavity, the embryo attaches to the endometrium following an interval of three days. This period allows the commencement of communication between the embryo and mother prior to attachment to the endometrium. Human chorionic gonadotropin (hCG) is produced by the early embryo before hatching from the ZP takes place, thereby allowing the mother to recognise the pregnancy (Fishel *et al.*, 1984; Hay and Lopata, 1988).

This progression of pre-implantation events in the embryo is essential for implantation, as outlined, and it occurs within a narrow time frame during the mid-secretory phase. However, these events are not sufficient for the development of a successful pregnancy, which requires synchronization with the uterine cycle (Singh *et al.*, 2011). In addition, the success of pregnancy development in humans is further restricted due to the high probability of chromosomal abnormalities in preimplantation embryos (Teklenburg *et al.*, 2010).

Furthermore, endometrial receptivity in humans represents a narrow window of opportunity that must coincide with the differentiation of EnSCs. These cells develop into highly specialized decidual cells to facilitate implantation. Without the coincident presence of a pregnancy, menstruation will be triggered (Teklenburg *et al.*, 2010).

The role of cyclic decidualization of the endometrium in the implantation process and the nature of the decidual cytokines and growth factors that mediate the crosstalk with the embryo are important factors in determining whether implantation occurs (Teklenburg *et al.*, 2010; Singh *et al.*, 2011; Cha *et al.*, 2012)}. Delayed development disrupts this process, thereby causing the developing embryo to miss the window for implantation.

The donor embryos used in this research were all cryopreserved by slow freezing at either the 2PN or cleavage stage. The total number of hours that the embryos were in culture prior to cryopreservation was not known and could only be estimated. It is possible that this culture time varied significantly even for two embryos of a similar stage. No previous studies were identified during the research performed for this thesis that indicated that pre-implantation development is under strict temporal control in humans. Therefore, the splitting strategy used in this work was devised according to the methods of (Tsunoda and McLaren, 1983). In their work, the authors overcame the developmental clock limitation via the mechanical division of blastomeres of the 8-cell mouse embryo into two equal groups. In spite of this, the morphokinetic results from this project also suggest the presence of a developmental clock during human pre-implantation development (Noli *et al.*, 2015b).

9.2.2 The role of cell-cell interactions in fate specification and Hippo signalling

The unanswered question of how spatial cues 'inside' and 'outside' of cells are incorporated into the transcriptional programmes of the developing embryo exposes a significant gap in what is known about the mechanisms that contribute to embryonic lineage specification (Boroviak and Nichols, 2014). In mice, lineage-specific master regulators, such as CDX2 and GATA3 in the TE and POU5F1 (OCT4) as well as SOX2 and NANOG in the ICM, are responsible for regulating

these transcriptional programmes (Chambers *et al.*, 2003; Niwa *et al.*, 2005; Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008; Guo *et al.*, 2010). Mouse embryos lacking *Cdx2* are able to develop the TE specification, but they subsequently require *Cdx2* to maintain morphological features, further development and implantation (Strumpf *et al.*, 2005). In mice, Hippo signalling is one of the key regulatory pathways involved in lineage specification (Nishioka *et al.*, 2008). In addition, the Hippo pathway controls cell proliferation, organ size, and tumourigenesis in both mammals and *Drosophila* (Jia *et al.*, 2003; Dong *et al.*, 2007; Bando *et al.*, 2009), and in mouse pre-implantation embryos, it specifically plays a key role in establishing cell fates at the morula stage during the formation of the inner/outer cell types (Nishioka *et al.*, 2009). In the outer cells of the morula, the Hippo pathway is dormant, and the YAP1 moves to the nucleus where it binds to TEAD4 to induce *Cdx2* expression and enhance commitment towards the TE lineage. In the inner cells, significant cell-cell contact causes the Hippo signalling pathway to activate, resulting in the cytoplasmic retention of Yap1, where it undergoes phosphorylation by large tumour suppressor 1/2 (Lats1/2) kinases (Nishioka *et al.*, 2009).

Phosphorylated Yap1 is then removed from the nucleus and degraded. As a result, Yap1 is unable to act as a co-activator for Tead4, thereby resulting in failure of the induction of the TE programme mediated by the expression of Gata3 and Cdx2 (Nishioka *et al.*, 2009; Ralston *et al.*, 2010). Preferential development towards the TE-like lineage occurs due to loss of Lats1/2 kinases, as Cdx2 is no longer restricted to the TE of the blastocyst and the outer cells of the morula (Nishioka *et al.*, 2009; Lorthongpanich *et al.*, 2013). In accordance, the loss of Lats1/2 in the early stages of the pre-implantation embryo prevents commitment towards the ICM lineage (Lorthongpanich *et al.*, 2013). Studies have indicated that the decline in Tead4 levels causes the failure of the formation of the blastocyst cavity, thereby indicating that TEAD4 is placed upstream of TE transcriptional regulation (Yagi *et al.*, 2007; Nishioka *et al.*, 2008). The function of Tead4 is dependent on its intracellular localization as regulated by the Hippo signalling cascade rather than the specific expression of Tead4 itself (Nishioka *et al.*, 2009; Boroviak and Nichols, 2014).

Previous research in the murine system has supported the importance of

continuous cell-cell interaction in the regulation of blastomere fate, as biopsied blastomeres tend to re-establish cell-cell interactions subsequent to their transfer into recipient ZP (Johnson and Ziomek, 1981; Lorthongpanich *et al.*, 2012). Converse to a pre-patterning model (Piotrowska *et al.*, 2001; Piotrowska and Zernicka-Goetz, 2001), which posits that the ICM and TE lineages undergo predetermination due to the asymmetrical localization of molecular determinants in the oocyte, the *inside-out* (Tarkowski and Wroblewska, 1967) and *cell polarity* models (Johnson and Ziomek, 1981) hypothesise a decision-making process dependent on position.

Similarly, the results of this work, have suggested that lineage determination in human embryos takes place through the *inside-out* or the cell *polarity model*. In the pre-patterning model, the number of blastomeres used for the creation of twin embryos is not a governing factor; therefore, the probability of forming twin embryos with better quality ICMs is higher. Recently completed studies have provided insights into the fact that the Hippo pathway is responsible for the translation of positional information to lineage specification, acting primarily through the downstream mediator proteins YAP1 and TEAD1-4 (reviewed in (Lorthongpanich and Issaragrisil, 2015). The immunostaining of twin embryos on day 6 showed that YAP1 co-localizes with CDX2 as in the control blastocysts, implying that the mechanism may also be conserved in human embryonic development (Figure 9.1). In a recent case study, immunostaining pre-implantation embryos of different stages of development for YAP1 showed that YAP1 is localized in the nuclei of ICM cells only during the early stage of blastocyst formation. During the late blastocyst stage, the nuclear localization of YAP1 is restricted to TE cells (Noli *et al.*, 2015a).

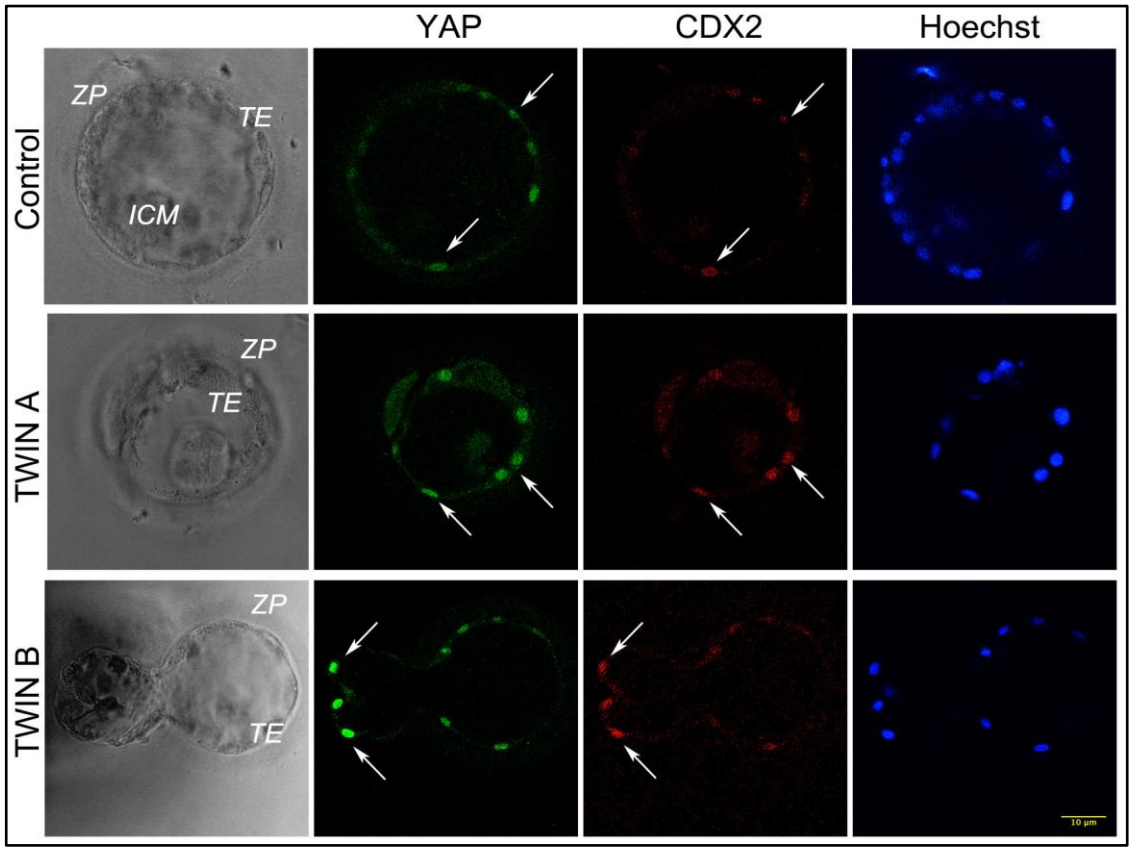


Figure 9.1: Immunostaining of TE markers in control and twin blastocysts at day 6. ICM, inner cell mass; TE, trophectoderm; ZP, zona pellucida. Arrows: Cells that demonstrate strong co-expression of YAP and CDX2. The image was taken from (Noli *et al.*, 2015b).

9.2.3 Lineage commitment

The first embryonic cell fate commitment is towards ICM and TE lineage segregation. This event begins at the compaction/morula stage, when asymmetric cell division pushes one cell inwards and the other daughter cell remains outside (Bruce and Zernicka-Goetz, 2010; Lorthongpanich *et al.*, 2012). This decision, however, is not ultimate. The outer cells from the morula and the early blastocyst tend to retain their plasticity for a short period of time. These cells express the pluripotency markers POU5F1 (OCT4), SOX2 and SALL4 and the TE markers HLA-G and KRT18 but not CDX2 (Cauffman *et al.*, 2009; Chen *et al.*, 2009; Verloes *et al.*, 2011). NANOG expression has also been reported in the polar TE cells of the early blastocyst (Cauffman *et al.*, 2009).

Upon isolation from fully-developed human blastocysts and their subsequent re-aggregation, TE cells were able to develop into blastocysts expressing the pluripotency marker NANOG (De Paepe *et al.*, 2013). Furthermore, most of the isolated TE cells did not restore towards their original position when placed in the

centre of the embryo; instead, they integrated into the ICM with subsequent expression of NANOG, indicating that the TE cells at that stage of embryonic development were not yet fully committed (De Paepe *et al.*, 2013).

By Day 5, the twin embryos in this work expressed NANOG almost universally, with NANOG-positive cells co-localizing with the TE markers CDX2 and GATA2 (Figure 8.9). To eliminate false positive NANOG signals in the twin immunostaining data, twin and control embryos were processed in the same drop in the majority of experiments. Twin and control embryos were easily distinguished visually due to their different sizes. By day 6, TE cells that demonstrated co-localization of NANOG with CDX2 and GATA2 were reduced in number, but they still constituted a significant fraction of the TE cells. However, only 1 to 4 cells lost the expression of CDX2 and GATA2, indicating the initial formation of the ICM (Figure 8.9). SOX17, a marker of primitive endoderm, was also detected in twins with the larger ICM on day 6 (Figure 9.2). This indicated that the molecular events responsible for first and second fate commitment of the embryos took place in the split embryos; however, they lagged behind the control blastocysts obtained by fertilisation (Noli *et al.*, 2015b). Irrespective of these findings, any twins that contained an ICM on day 6 had one of small size and poor quality. This may suggest that the epiblast possessed an insufficient number of cells to continue the post-implantation development of the conceptus (Balbach *et al.*, 2010; Morris *et al.*, 2012). This also may explain the poor results reported from twinning experiments in non-human primates (Chan *et al.*, 2000; Mitalipov *et al.*, 2002).

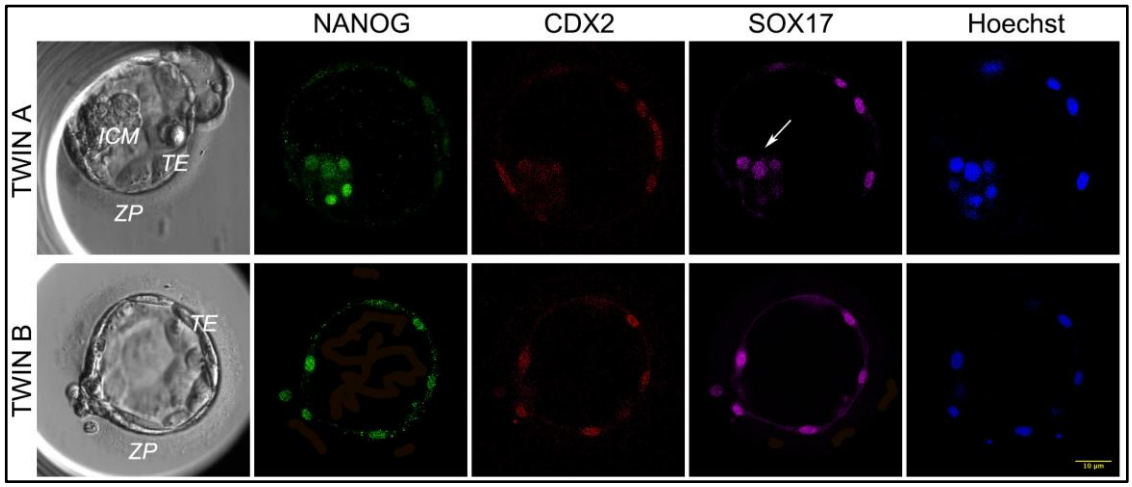


Figure 9.2: TE immunostaining in control and twin blastocysts at day 6. On day 6, Twin A, possessing the larger ICM, was found to express SOX 17, a primitive endoderm marker. Image was taken from (Noli *et al.*, 2015b).

9.2.4 The necessity of an 'inside' allocation for inner cell mass specification

The Hippo signalling pathway is not exclusively responsible for the first lineage decision. In Mouse, overexpression of neurofibromatosis 2 (Nf2) is unable to modify the localization of Yap, possibly due to the absence of other components in the outside cells (Cockburn *et al.*, 2013). Ectopic expression of Cdx2 in the ICM occurs due to the knockdown of Lats1/2. However, this is concurrent with the continuous expression of Oct4 and Nanog, thereby signifying only a partial conversion of the inner cells into bona fide TE (Lorthongpanich *et al.*, 2013). Therefore, more information must be obtained to determine ICM fate additionally to the absence of an apical domain (Lorthongpanich *et al.*, 2013). For example, inside cells may utilize adherens junction- and gap junction-mediated intercellular communication, which may lead to alterations in cytoskeletal structures and signalling pathways via focal adhesion kinases. Additionally, inside cells may be positioned such that they preferentially receive signalling molecules. Given the minor amount of intracellular space, even small quantities of ligands are experienced at high concentrations within the cells. Lastly, the asymmetrical localization of proteins in the outside cells results in the inside cells being exposed to a specific 'basal' environment (Boroviak and Nichols, 2014). Evidence supporting the requirement for the 'inside' in conjunction with the Hippo signalling pathway was derived from mouse blastomeres grown in isolation (Lorthongpanich *et al.*, 2012). Blastomeres were separated after being cultured for the initial five cell divisions (1/32), and their lineage marker expression was profiled and subsequently compared with ICM

and TE cells. Although their lineage marker expression patterns were different from both ICM and TE cells, they more closely matched the pattern of TE than ICM, thereby supporting the necessity for an inside environment for ICM specification (Lorthongpanich *et al.*, 2012). It was also shown that individual blastomeres were responsible for preferential contribution towards TE formation in morula aggregations (Lorthongpanich *et al.*, 2012). The induction of Hippo signalling takes place in individual blastomeres, indicating that the loss of apical-basal polarity is insufficient for the adoption of ICM fate (Figure 9.3).

Corroborating this data, it has been reported that mouse blastomeres are capable of generating functional TE when implanted as single cells into a female recipient, but they are unable to give rise to embryonic tissues (Rossant, 1976). Mouse embryos at the 4-cell stage with the ZP removed can reorganize their cells into different configurations in culture. Embryos that adopt a linear configuration with minimal intracellular interactions result in blastocysts with significantly fewer ICM cells (Graham and Lehtonen, 1979), thereby resulting in inferior development upon implantation in the uterus. In contrast, embryos with tetrahedral configurations, allowing maximal intercellular interactions, result in blastocysts with more ICM cells, which tend to develop better upon implantation in the uterus (Suzuki *et al.*, 1995). Single blastomeres from the 4- and 8-cell stages lead to the concurrent formation of blastocysts at frequencies of 40% and 15%, respectively; there is an increase in the number of empty trophoblastic vesicles (Tarkowski and Wroblewska, 1967). In summary, these data suggest that blastomeres grown individually, despite losing apical-basal polarity, preferentially move towards the TE lineage and fail to progress to the embryonic lineage.

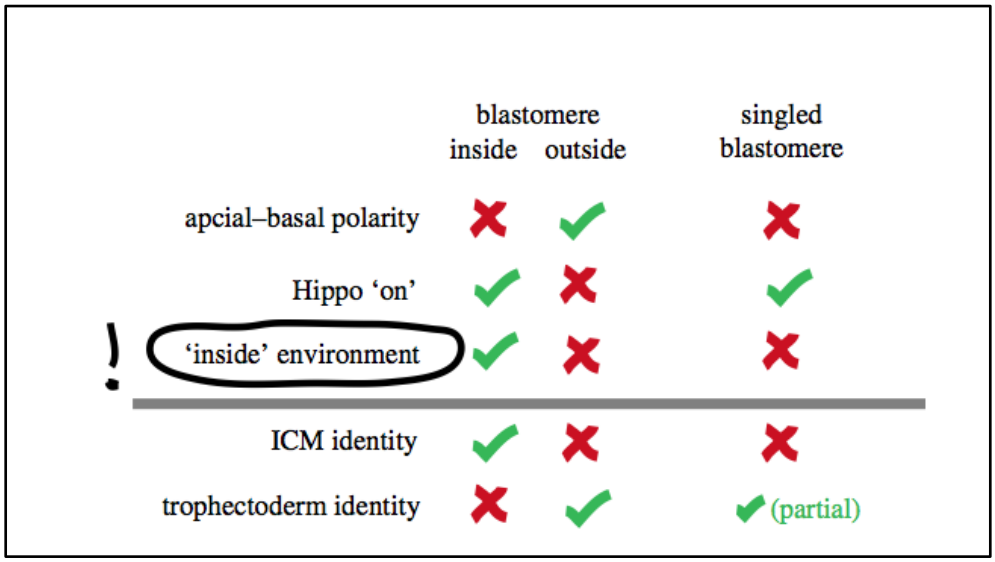


Figure 9.3: Summary of the cellular characteristics of blastomeres within the embryo and isolated single blastomeres grown in culture (Lorthongpanich *et al.*, 2012). Single blastomeres displayed an absence of apical–basal polarity and active Hippo signalling (identical to 'inside' cells destined to become ICM); however, they partially recapitulated the trophectoderm lineage. This implied a catalysing role for the 'inside' environment in embryonic lineage establishment, which was absent in single blastomeres. Image was reproduced from (Boroviak and Nichols, 2014).

9.3 Pluripotency competence analysis

9.3.1 hESC derivation

The first hESC line was derived from the ICM blastocyst in 1998 (Thomson *et al.*, 1998). Since then, more than 1000 hESC lines have been derived around the world. Over time, it has emerged that derivation is successful from both high and poor quality embryos. The lines can be derived from embryos in which no real ICM is distinguishable and from a single blastomere of an arrested embryo (Zhang *et al.*, 2006; Feki *et al.*, 2008; Strom *et al.*, 2010). Furthermore, several groups have reported derivation of hESC lines from a single blastomere of a cleavage stage embryo (Klimanskaya *et al.*, 2006; Chung *et al.*, 2008; Geens *et al.*, 2009; Ilic *et al.*, 2009).

The hESC-derivation team at ACU optimized the protocols, and the success rate for derivation using frozen embryos is now between 50-60%, which is higher than most quoted efficiencies in the literature (Ilic *et al.*, 2012; Stephenson *et al.*, 2012). Therefore, given such a high efficiency of derivation, the twin embryos should be being able to derive hESC line if they had normal pluripotent stem cells. However, no outgrowth was observed post isolated and plated the ICM of the twin embryos. The number of embryos used for derivation was relatively small (5 pairs, n=10),

and one cannot exclude the possibility that the derivation hESC line(s) may happened if using a higher number of embryos. The attempts for hESC derivation was not continue because immunostaining for TE and ICM markers did not suggest normal distributions; rather, the cells expressed both TE and ICM markers. Such dual expression suggested that the developmental ability of the embryo might be compromised.

9.4 Reproductive competence analysis

9.4.1 Analysis of the miRNA secretion profiles in SBM

One of the most common functions of miRNAs is the post-transcriptional repression of target genes. The role of miRNAs in the early embryonic development of a large number of species has been identified. However, there is limited knowledge of the miRNA regulatory network in mammalian pre-implantation embryos (Mineno *et al.*, 2006; Yang *et al.*, 2008; Suh *et al.*, 2010; Goossens *et al.*, 2013; Maraghechi *et al.*, 2013; Rosenbluth *et al.*, 2013). Anomalous miRNA expression of transferable blastocysts has been observed in male factor infertility and polycystic ovaries (McCallie *et al.*, 2010). As the secretion of miRNAs has been reported (Valadi *et al.*, 2007), studies have been carried out to connect specific miRNAs detected in the SBM with the ploidy status of the embryo and its reproductive competence (McCallie *et al.*, 2010; Kropp *et al.*, 2014; Rosenbluth *et al.*, 2014; Capalbo *et al.*, 2016).

One of the potential sources of miRNAs in the spent medium collected from early embryos is the paracrine pathways of constituent cells, which reflect the normal communication between cells present in healthy embryos. Alternatively, miRNAs can also be components of the communicative pathway between the developing embryo and the surrounding uterine epithelium, affecting the ability of the uterine epithelial lining to position and implant the embryo. The third possibility is that these miRNAs detected in the SBM are products of degrading cells (Noli *et al.*, 2016a).

In this work, significantly lower levels of miR-30c in twins resulting from embryo splitting could potentially indicate the poor developmental prognosis of the twin embryos. miR-30c has been demonstrated to disrupt the epithelial-to-mesenchymal

transition (EMT) in breast cancer via the regulation of *TWF1 and IL11* (Bockhorn *et al.*, 2013). Additionally, miR-30c has been reported to act as a putative marker of reproductive competence (Capalbo *et al.*, 2016). EMT is a fundamental part of the first lineage fate decision, when one of the daughter cells is pushed inwards during asymmetric cell division, losing polarity and forming ICM (Johnson and Ziomek, 1981; Bruce and Zernicka-Goetz, 2010). Whether the role of miR-30c lies in the embryo-maternal dialogue at the time of implantation or in the formation of the ICM is still under investigation (Noli *et al.*, 2016a).

In this project, the significantly decreased miRNAs identified in the SBM from the twin embryos as determined by the mean normalization strategy are part of several developmental and differentiation processes. For example, miR-373 promotes mesendoderm differentiation (Rosa *et al.*, 2014), miR-30b plays a role in the development of embryonic ectoderm (Song *et al.*, 2011), and miR-24 is necessary for haematopoietic differentiation (Roy *et al.*, 2015). However, only miR-24 (Kropp and Khatib, 2015) and miR-25 (Kropp *et al.*, 2014) were found in culture media collected from pre-implantation embryos.

Recent studies have indicated that miRNAs are likely to be involved in embryo-endometrium cross-talk during implantation (Dior *et al.*, 2014; Galliano and Pellicer, 2014). Microarray profiling showed that six miRNAs were differentially expressed in the human endometrial epithelium during the implantation window and were subsequently secreted into the endometrial fluid. The most differentially regulated miRNA identified in this study, miR-30d, has been shown to become internalized by embryonic TE as an exosome-associated molecule, thereby causing the upregulation of genes involved in adhesion (Vilella *et al.*, 2015). Other miRNAs detected in follicular fluid, such as miR-320, have been suggested to affect embryo quality (Feng *et al.*, 2015). However, embryonic development is under the influence of miRNAs from the surrounding tissues, as well as the miRNAs present in the early embryos themselves. For example, the miRNA Let-7a is involved in the post-transcriptional regulation of ribonuclease type III Dicer 1. This leads to an alteration in the miRNA profile, and therefore, the competency for implantation of the activated blastocysts (Cheong *et al.*, 2014). On the other hand, miR-29b is responsible for the negative regulation of DNMTA3A/3B expression, causing

changes in DNA methylation levels in the transition from the morula to blastocyst stage (Zhang *et al.*, 2015).

The most interesting finding from this work was that 25% (12 out of 48) of miRNAs secreted by twin embryos were never encountered in normal, reproductively competent blastocyst controls (Figure 8.17). The immunostaining data showed that the TE cells of twin embryos were found to express dual markers of both ICM and TE (Figure 8.8 and 8.9) (Noli *et al.*, 2015b); therefore, the difference in miRNA profiles is not completely unexpected. miR-155, found particularly in the SBM of twin embryos, has been reported to inhibit the proliferation and migration of trophoblast-derived cell lines (Dai *et al.*, 2012). However, no information is yet available about the putative roles of the other 10 "twin-specific" miRNAs in the development of the human pre-implantation embryo. Despite prior knowledge regarding their roles in cancer diagnosis, prognosis and therapy, it is currently too speculative to extrapolate the existing data to infer their roles in embryonic development (Noli *et al.*, 2016a).

Additionally, these findings support the use of miRNA analysis from collected SBM to study the biological variability between embryos of differing quality. The miRNA analysis of SBM as detailed by this study was able to confidently differentiate between low quality manipulated embryos resulting from splitting different cleavage stage embryos and non-manipulated high quality embryos. Therefore, these results support the hypothesis that miRNA analysis from SBM possesses enough resolution to capture biological variation between reproductively competent and non-competent blastocysts, thereby functioning as a novel, non-invasive biomarker of embryo selection (Noli *et al.*, 2016a).

In conclusion, this work provides a preliminary indication of the involvement of miRNAs in the development of human pre-implantation embryos. Although the overall implications of these results are not yet entirely known, they may provide a valuable addition to the currently limited understanding of these mechanisms.

9.5 Limitation and biases that were introduced by the study design

In the section below, the limitation of each validation method used in this work will be discussed. There were two main objectives for this project:

1. To assess the developmental, pluripotent and reproductive competence of twin embryos generated by embryo splitting using blastomere biopsies with four different validation methods.
2. To compare the quality of the twin embryos against the quality of control embryos created by fertilisation.

Four different validation methods were used to achieve the objectives above.

9.5.1 Limitations of the morphokinetic study

Validation method I was a comparative analysis of the morphokinetic parameters of 176 twin embryos that were created by splitting 88 human embryos from either early (2 – 5 cell stage, n = 43) or late (6 – 10 cell stage, n = 45) cleavage stages, as recorded using a time-lapse imaging system. Then, these data were compared with the morphokinetic data obtained from embryos that were created by fertilisation and that resulted in pregnancy and live birth upon single blastocyst transfer (n = 42).

9.5.1.1 Major limitations related to donor embryos

1. The exact duration that the donor embryos were cultured prior to cryopreservation was unknown and therefore estimated.
2. Because the embryos were cryopreserved at the cleavage stage and as a result of their developmental behaviour post-splitting, no early morphokinetic parameters were available to calculate the time interval that occurred between different embryonic developmental stages.
3. No demographic data, such as age and genetic profile, were collected from donors. Furthermore, if such data had been collected, the number

of variables, in combination with an insufficient number of embryos available for splitting, would have made it impossible to obtain meaningful data.

4. The method of fertilisation and the culture media were not considered variables in this study.

9.5.1.2 Donor vs control embryos

1. Fresh PGD embryos were used as controls, and slow-frozen donor embryos were used for splitting in the study group.
2. The control embryos were cultured in a single medium, while the donor embryos were cultured in a sequential system.
3. When recording the controls, the morphokinetic parameter used as the start point for recording was the 2PN stage, whereas when recording twins, recording was begun post-splitting. The presence of an expanded blastocyst was used as the endpoint for both twins and controls.

Ideally, frozen-thawed non-manipulated embryos at the 2PN or cleavage stage would have been used as additional controls for a morphokinetic study. However, the number of available 2PN embryos was a limiting factor. The number of the cleavage-stage embryos that are available for research purposes has been reduced due to the shift of clinical practice in assisted reproduction towards blastocyst cryopreservation and the introduction of the blastocyst transfer policy. Had this study used 2PN embryos for controls as well as for the study group, there would have been an insufficient number of embryos for the study.

Finally, the morphokinetic data obtained during the experiments performed for this thesis cannot be directly compared to the data in other previously published studies because most of those studies have used fresh/frozen embryos at the 2PN developmental stage as a starting point. The adoption by HEFA of a policy supporting blastocyst transfers to reduce the multiple-pregnancy rate and the more recent adoption of time-lapse imaging technologies by IVF clinics have collectively resulted in research studies that do not typically use cleavage-stage cryopreserved embryos.

9.5.2 Limitations of the developmental competence analysis

Validation method II was a comparative analysis of the expression patterns of early lineage-specific transcription factors in twin blastocysts that were derived from split embryos and non-manipulated Day 5 and Day 6 blastocysts using immunocytochemistry.

Too few twin embryos (n= 21 pairs) were used for validation method II to draw a solid conclusion due to the aforementioned limited supply of embryos for research. Had there been a sufficient number of embryos, staining at least 2 or 3 embryos from each of subgroups (2, 3, 4, or 5 blastomeres in Twin A and the same in Twin B) would have been preferable.

9.5.3 Limitation of the pluripotency competence analysis

Validation method III was a derivation of hESCs from twin embryos and a comparison between these hESCs and hESC lines that were previously derived from non-manipulated embryos on Day 5 and Day 6 at the assisted conception unit (ACU) at Guy's Hospital, King's College, London.

Again, a small number of embryos was used for derivation (n= 5 pairs). By using more embryos, the derivation of hESC line(s) may have been possible. Due to the low number of twin embryos with visible ICM as well as due to the dual expression of both ICM and TE markers, the attempted derivation of hESC was not continued.

9.5.4 Limitation of the reproductive competence analysis

Validation method IV was a comparative analysis of the miRNA profiles of SBM obtained from twin embryos that were created from blastomere biopsies (n=7 pairs) and control blastocysts that were generated by fertilisation and resulted in live births upon single embryo transfer (n=7).

9.5.4.1 Embryos selection

1. SBM previously collected from vitrified blastocysts from GENERA, the Centre for Reproductive Medicine, Clinica Valle Giulia, Rome, that resulted in a healthy pregnancy and a live birth following single embryo transfer were used as the control group. SBM was collected from twin embryos created from slow frozen donor embryos.

2. The control embryos and twin embryos were cultured in a sequential system at 37°C in 6% CO2 and 5% O2. However, different medium supplements were used (10% SPS *vs.* 5% HSA), see Chapter 3 (3.4.2 and 3.5.3) and Chapter 7 (7.2.2).

It would have been preferable to use control embryos donated from Guy's hospital for this study. However, doing this may have introduced an additional variable; at the time of running this validation method, the unit had already started to use a single step medium.

Given the circumstantial limitations, the control group used in this validation was deemed to be acceptable. The profile of miRNA secretion *in vitro* culture media consistently distinguished between twin and control embryos. This can be explained by the abnormal development of the twin embryo or as a result of differential lineage commitment in the twin embryos, rather than the study design limitations described above.

9.5.5 Technical limitations

9.5.5.1 Splitting strategy

The splitting strategy employed in this project (Chapter 3, section 3.5.2) may result in some biases. Firstly, in the case of donor embryos with an even number of blastomeres, the methodology resulted in differential manipulation. For example, to create Twins A and B, half the number of blastomeres from donor embryos were aspirated and inserted into a previously emptied ZP to create twin B. This resulted in Twin A not being manipulated and Twin B being manipulated. Subsequently, the split embryos were not treated in the same way. It would have been preferable to remove all blastomeres from the donor embryos and divide them equally across

two previously emptied ZPs, so that all of the blastomeres would have been aspirated and squeezed.

In this work, introducing such differences between Twin A and B was intentional to assess whether the disruption of cell orientation and communication had any effect on the quality of the twin embryos. In this scenario, we would have expected Twin A to have better outcomes developmentally and morphologically, although this was not the case. Twin B actually developed to a later developmental stage than Twin A did, although the only significant difference between the Twin A and Twin B embryos was found in blastocyst formation, as shown in Figure 8.2 B. This result was not expected and may explained by the splitting strategy used in this work, since Twin B originated from blastomeres aspirated one by one and inserted into previously emptied ZPs. Since the blastomeres remaining in the donor embryos were used for Twin A creation, Twin A was not manipulated, whereas Twin B was manipulated; however, the sample size is too small to conclude that the differential splitting strategy caused the results shown. However, it can be safely concluded that cell orientation communication did not influence the outcome.

Secondly, in the case of donor embryos with odd numbers of blastomeres, the bigger blastomeres exhibited delayed development, whereas the smaller blastomeres were the more developmentally advanced. In the splitting strategy used in this project, the bigger blastomeres were randomised between A and B in order to prevent any bias in development between the twins as well as to avoid selecting more advanced/delayed blastomeres for either of the twins. Consequently, any differences between the developmental competences of Twins A and B as well as between Group 1 and Group 2 were not related to manipulation.

9.5.5.2 Diameter of the biopsy pipettes

In this work, two different ages of donor embryos were used: the earlier age (or Group 1) consisted of cleavage stage embryos containing 2-5 blastomeres, and the later age (or Group 2) consisted of cleavage stage embryos containing 6-10 blastomeres.

The diameter of the pipette used for blastomere biopsy for both groups was 35 µm. This might have had some effect on the aspirated blastomeres from Group 1, which were bigger in size than the blastomeres of Group 2. However, the aspiration of the blastomeres required a similar amount of time and effort, regardless of their size. No blastomeres were obviously physically damaged during the procedure (if so, the blastomere was excluded). Therefore, it is unlikely that the size of the pipette affected the outcome. If we had noticed damage to the larger blastomeres during the procedure or if it was more difficult to aspirate them, we would have used pipettes with a bigger lumen.

Consequently, the pipette size alone cannot explain why twins from Group 2 generally exhibited a higher probability of progressing during development than twins originating from Group 1, as outlined in Figure 8.2B.

Although they resulted in blastocysts with larger sizes and higher yields, only 36.84% (14 out of 38) of these embryos had a distinguishable ICM. On the other hand, in Group 1, 46.15% (12 out of 26) of the blastocysts were shown to have a clear and distinguishable ICM. Of the ICMs observed, the majority were poor quality, with only three in Group 1 and one in Group 2 being Grade B. The remaining ICMs were Grade C (Stephenson *et al.*, 2007), as shown in Figure 8.7.

Therefore, it is not possible to conclude that the pipette size affected the development outcome for Group 1, as it yielded blastocysts with a higher quality ICM.

9.6 Conclusion

Overall, the analyses established that the quality of the human embryos generated by twinning *in vitro* was not comparable to the quality of the embryos created by fertilization. Together, the data from morphokinetic analyses, immunocytochemistry, hESC derivation and miRNA expression profiles in spent culture media all suggested that human twin embryos created *in vitro* are unsuitable not only for clinical use but also for research purposes (Noli *et al.*, 2015b; Noli *et al.*, 2016a; Noli *et al.*, 2016b).

Morphokinetic data indicated that human preimplantation development is subject

to strict temporal control according to a set 'developmental clock'. The size of twin embryos generated in the study was directly proportional to the starting cell number of the embryos used in their genesis. Furthermore, the first commitment decision in terms of cell fate was delayed, with the ICM becoming distinguishable later in the study group than that in the normal control blastocysts produced through fertilization. The ICM, if present at all, was small in size and of low quality. Furthermore, most cells in the twin embryos concurrently expressed both ICM and TE markers.

The nature of the miRNA secretion in the *in vitro* culture media consistently varied between the twin and control embryos. Using the global geometric mean approach, six miRNAs were found at a significantly higher levels in the SBM from twin embryos, while and nine were significantly higher in the SBM from euploid implanted blastocysts. Notably, miRNA-30c, one of the nine, has been previously reported to indicate blastocyst implantation potential. Finally, using two different statistical approaches, miRNA-30c was found to be significantly lower in SBM compared to that in twin embryos.

The exact mechanism of 'developmental clock has yet to be ascertained. Additionally, the timing for specific events as set by the developmental clock could potentially perturb the sequence of events in lineage commitments and the molecular events in twin embryos created with blastomere transfer, thereby resulting in decreased developmental competence. Creation of twins by blastocyst bisection (Mitalipov *et al.*, 2002) may potentially avoid the restrictions of the 'developmental clock.' However, as in the case of mouse epiblasts, a minimum number of pluripotent cells in the embryo at the time of implantation (Morris *et al.*, 2012) may also be required for development to birth in humans. This may be responsible for rendering embryo twinning for both clinical and research purposes. The potential role of miR-30 in the first lineage commitment is intriguing, and further work is necessary. However, due to the shift towards blastocyst transfers in the clinical setting, the supply of cleavage-stage embryos donated for research is decreasing, and dissecting the regulatory mechanisms of pre-implantation development in the human system may become increasingly difficult, if not impossible, in the near future.

Since very little information was previously known about the effects of embryo splitting on the development and reproductive competency of human embryos, this study has provided a valuable contribution to our understanding of the therapeutic and research potential of this technology.

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