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OPTIMISATION OF HUMAN EMBRYONIC STEM CELL DERIVATION AND CULTURE – TOWARDS CLINICAL QUALITY

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.

To my family. Together again.

ABSTRACT

For clinical grade human embryonic stem cell (hESC) lines, a robust derivation and culture system without any substances having animal origin would be optimal. The general aims of these studies have been to gradually improve our hESC derivation and cultures.

The first step towards clinical quality was the use of human foreskin fibroblasts instead of mouse embryonic fibroblasts to support the undifferentiated growth of the pluripotent stem cells. This was followed by replacing foetal calf serum as a supplement in the culture medium with the commercially available Serum Replacement, first in the cultures and later also for the derivation of new hESC lines.

The immunosurgery generally used for isolation of the inner cell mass (ICM) involves animal serum and complement. We have been able to replace the surgical method with a mechanical procedure for the isolation of the inner cell mass, and this gives better results.

We have also evaluated whether the morphology of the embryos donated to stem cell research has an impact on derivation success. We have carried out statistical analyses on the early cleavage rate, morphological score of the embryo at cleavage stage and the score for the ICM and the trophectoderm at the time for isolation of the ICM. We have shown that there is no correlation between the morphology and derivation success. All embryos donated for stem cell research should be used for isolation in an attempt to derive new hESC lines. Even embryos with no visible ICM have generated pluripotent hESCs.

In the final study we have been able to culture hESCs on a human recombinant laminin, LN-511, for more than 20 passages (four months) in a well-defined medium devoid of any animal-derived components. The use of a well-defined system is most important in understanding the pluripotent state and being able to direct the differentiation in the desired direction for clinical applications in the future.

We have taken hESC research from a culture system that depended on several animal-derived components to a totally xeno-free system. We hope that these improved culture procedures can be used for the development of cell lineages for use in therapeutic purposes.

LIST OF PUBLICATIONS

This thesis is based on the following articles and a manuscript, which will be referred to in the text by Roman numbers.

I. **Ström S**, Inzunza J, Grinnemo KH, Holmberg K, Matilainen E, Strömberg AM, Blennow E and Hovatta O. Mechanical isolation of the inner cell mass is effective in derivation of new human embryonic stem cell lines. *Human Reproduction. Dec; 22(12):3051-8, 2007*.

II. **Ström S**, Holm F, Bergström R, Strömberg AM and Hovatta, O. Derivation of 30 human embryonic stem cell lines – improving the quality. *In Vitro Cell. Dev. Biol.—Animal. 46:337–44, 2010*.

III. **Ström S**, Rodriguez-Wallberg K, Holm F, Bergström R, Eklund L, Strömberg AM, Hovatta O. The relationship between embryo morphology and successful derivation of human embryonic stem cell lines. *Submitted 2010*.

IV. Rodin S, Domogatskaya A, **Ström S**, Hansson EM, Chien KR, Hovatta O and Tryggvason K. Long-term self-renewal of human embryonic stem cells on human recombinant laminin-511 in a xeno-free and feeder-free environment. *Nature Biotechnology. 28:611-5, 2010*.

CONTENTS

LIST OF ABBREVIATIONS

AFP	Alpha fetoprotein
BSA	Bovine serum albumin
EC	European Commission
ECM	Extracellular matrix
EHS	Engelbreth-Holm-Swarn
EMA	European Medicines Agency
EMEA	European Medicine Evaluation Agency
Erk	Extracellular signal regulated kinase
EU	European Union
FBS	Foetal bovine serum
GAM	Generalised additive models
GMP	Good manufacturing practice
gp130	Glycoprotein 130
GTP	Good tissue practises
Gy	Gray
hESC	Human embryonic stem cell
hff	Human foreskin fibroblast
hpi	Hours post insemination
ICM	Inner cell massResten
ICSI	Intra cellular sperm injection
IL	Interleukin
sIL-6R	Soluble interleukin-6 receptor
iPS cell	Induced pluripotent stem cell
IVF	*In vitro* fertilisation
LIF	Leukaemia inhibitory factor
LN-511	Laminin 511, (consisting of alpha 5, beta 1 and gamma 1 chains)
SR	Serum replacement
MAP	Mitogen-activated protein
MEF	Mouse embryonic fibroblasts
mES cell	Mouse embryonic stem cell
MHC	Major histocompatibility complex
Oct-4	Octamer-binding protein 4
PBS	Phosphate-buffered saline
PGD	Preimplantation genetic diagnosis
ROCK	Rho kinase inhibitor, Y-27632
RT-PCR	Reverse transcription polymerase chain reaction
SOPs	Standard operating procedures
SSEA	Stage-specific embryonic antigen
STAT	Signal transducer and activator of transcription
TRA	Tumour-rejection antigen
qRT-PCR	Quantitative real-time polymerase chain reaction
3PN	Tripronuclear

1 THESIS SUMMARY

Since the first permanent human embryonic stem cell (hESC) lines were described in 1998 (Thomson et al., 1998), the potential applications have been known to be enormous. With their ability to differentiate to any cell type of the body, the hope is that hESCs can be used for clinical cell replacement. To replace cells damaged due to disease or injury would be of revolutionary importance to cure diseases such as Parkinson's disease, diabetes type I and spinal cord injuries.

HESCs are also important as a tool to understand the development and early differentiation of cells, and similarities and differences between healthy cells and cells carrying genetic disorders.

One factor that has limited the clinical application of hESCs has been the threat of zoonoses, diseases transmitted from an animal to humans. Until today, most hESC lines have been cultured and derived in conditions involving xeno-compounds, such as mouse embryonic fibroblasts as a supporting feeder layer, and a medium supplemented with foetal calf serum. By exposing these cells for xeno-compounds, the risk of rejection of any transplant is also higher, because human individuals usually have antibodies against bovine and murine proteins (Martin et al., 2005). Defined cultures are also important for understanding the molecular mechanisms of pluripotency and early differentiation.

In this work, we have been able to exclude the xeno-compounds in the cultures of hESCs both in the isolation of the inner cell mass, and in the culture systems used.

An important finding was also that hESC lines can be derived even form the poorest quality supernumerary embryos.

2 INTRODUCTION

Pluripotent stem cells have the ability to proliferate and differentiate into several derivatives, making them an excellent source to study development, differentiation pathways and diseases. Human embryonic stem cells have an almost unlimited self-renewal capacity and possess the ability to differentiate into any cell type of the human body, given the right signals. The potential for using these cells for clinical purposes has been widely recognised (Edwards, 2001; Keller and Snodgrass, 1999).

The first mouse embryonic stem (mES) cell lines were established in 1981 (Evans and Kaufman, 1981; Martin, 1981). These cells were cultured in a similar manner to embryonic carcinoma cells, on mouse embryonic fibroblasts (MEF) and in a medium supplemented with foetal calf serum (FCS). In 1988, two groups succeeded in replacing both the MEF feeder layer and FCS in the medium by adding leukemia inhibitory factor (LIF) to the culture medium (Smith et al., 1988; Williams et al., 1988). Feeder-free derivation of mES cell lines by adding LIF to the culture medium has also been feasible (Buehr and Smith, 2003; Burdon et al., 1999; Nichols et al., 1990).

LIF functions by activating the STAT3 pathway (Niwa et al., 1998) via a membrane receptor complex that includes gp130, which inhibits differentiation and promotes viability (Yoshida et al., 1994). LIF also activates the Erk pathway, which antagonistically directs the differentiation of ES cells (Burdon et al., 1999). LIF can be replaced by adding IL-6 and a soluble IL-6-receptor, ciliary neurotrophic factor or oncostatin M. All these factors, including LIF, activate gp130. The complex IL-6/sIL-6R, in particular, is thought to act exclusively through gp130 (Nichols et al., 1994).

Differences in the activation of the two pathways, STAT3 and Erk, might be the reason why some strains of mice have been more challenging than others to use for the derivation of ESC. By inhibiting the Erk pathway, Batlle-Morera and co-workers were able to improve the derivation success from several difficult strains of mice, and were able to derive the first rat ES cell lines (Batlle-Morera et al., 2008).

Diapause occurs in rodents to prevent fertilised embryos implanting if the mother is suckling a previous litter. During diapause, the embryos are developmentally arrested. By mimicking this phenomenon, by removal of estrogen, it was possible to synchronise the cells of the epiblast, which gives rise to pluripotent ES cells. The synchronisation seemed to divert the cells of the epiblast from the normal development and differentiation (Batlle-Morera et al., 2008). Further optimisation of the derivation of mES cell lines from several strains was achieved by inhibiting signals leading to differentiation. Inhibition of the extracellular signal regulated kinase (Erk)-activating and the mitogen-activated extracellular-regulated kinase (MEK) pathway have been used to promote ES cell self-renewal and improve the efficiency of ES cell derivation (Nichols and Ying, 2006).

Since the first reports on the derivation of human embryonic stem cells (hESC) (Bongso et al., 1994; Fishel et al., 1984; Reubinoff et al., 2000; Thomson et al., 1998), the field has moved rapidly forward. In the beginning, mouse embryonic fibroblasts (MEF) were widely used to

support the growth, and FCS was used as a supplement in the culture medium. Since then, several different cell types have been used to support the undifferentiated growth of hESCs, including several human cell types (Hovatta et al., 2003; Skottman and Hovatta, 2006). Until today the standard non-FBS-containing culture medium on feeder cells has been supplemented with Serum Replacement (SR) (Inzunza et al., 2005; Koivisto et al., 2004).

HESCs can also be cultured feeder-free. At first, conditioned medium from mouse embryonic fibroblasts (MEF) was used with Matrigel (Xu et al., 2001), but today there are several commercially available culture media for feeder-free culture, such as mTeSR1/TeSR2 (Stem Cell Technologies) StemPro (Invitrogen), NutriStem (StemGent) and hEScGRO (Millipore). Suspension culture of hESCs has also been feasible (Fernandes et al., 2009; Steiner et al., 2010).

The use of xeno-components, undefined human proteins or feeder cells in the cultures can cause problems related to immunogenicity or microbial contamination. They can also cause variability in experimental results (Hovatta et al., 2003; Martin et al., 2005; Xu et al., 2001). A well-defined culture system for hESCs devoid of xeno-components and feeder cells would benefit research and clinical applications.

2.1 PLURIPOTENT STEM CELLS

Pluripotency is the capacity of a single cell to develop into all cell types in the organism. The fertilised mammalian zygote enters the cleavage stage and divides several times, following a pre-set programme, and differentiates into two extra-embryonic lineages: trophoblast and hypoblast, and into the cells of the ICM, giving rise to the pluripotent stem cells. Since 2006, it has been possible to re-programme somatic cells into the pluripotent state, by transduction with regulatory transcription factors into what are known as "induced pluripotent stem (iPS) cells" (Takahashi and Yamanaka, 2006).

Nanog is a transcription factor that is essential in early development. The expression is turned on in the morula and is also seen in ES cells and in germline cells of all mammals (Chambers and Smith, 2004; Silva et al., 2009). Over-expression of Nanog is enough to induce self-renewal of undifferentiated ES cells (Chambers and Smith, 2004) but Nanog was not one of the four factors needed in the early induction of somatic cells into pluripotent cells. It does not increase the efficiency when generating mouse iPS cells, either (Takahashi and Yamanaka, 2006).

Direct reprogramming of human fibroblasts to a pluripotent state has been achieved through ectopic expression of the transcription factors Oct4, Sox2, and either cMyc and Klf4, or by Nanog and Lin28 (Takahashi and Yamanaka, 2006; Yu et al., 2007).

Initial methods to derive human iPS cells used genome-integrating viral vectors (Takahashi and Yamanaka, 2006; Yu et al., 2007), but such vectors can result in insertional mutations, and the transgene expression can interfere with the differentiation capacity of the iPS cells (Yu et al., 2007). Different methods to remove the inserted vectors have been reported, such as thr Cre/LoxP recombination (Soldner et al., 2009; Unger et al., 2009) and the excision of piggyBac transposons (Kaji et al., 2009; Woltjen et al., 2009).

Yu and co-workers described in 2009 the establishment of human iPS cells with the use of non-integrating episomal vectors. After removal of the episome, iPS cells are produced that are

completely free of vector and transgene sequences (Yu et al., 2009). Non-integrating, minicircle DNA vectors for the establishment of human iPS cells have also been reported (Jia et al., 2010) as well as high efficiency reprogramming using synthetic mRNA, modified not to induce the innate antiviral immunresponce (Warren et al., 2010).

It has been discussed whether ES cells are trapped in development or if they are a result of culture conditions (Nichols et al., 2009). Recently Tang and co-workers (Tang et al., 2010) have compared gene expression in single cells, following the development from the cells of the ICM through the outgrowth phase to the ES cell. By single-cell RNA-seq analysis of individual Oct-4-positive or Oct-4-negative ES cells from the ICM, followed by monitoring their progression towards ES cells, they found that the molecular signatures of undifferentiated ES cells are clearly different from cells of the ICM. Major changes were found in genes involved in the general metabolism, epigenetic regulators and changes in the expression of microRNAs.

During the transition from ICM to ES cells, the normal development is changed – the cells acquire the capacity for unlimited self-renewal and the capacity to differentiate into all three germ layers (Tang et al., 2010).

2.2 ISOLATION METHODS FOR THE ICM, AND THE DEVELOPMENTAL STAGE OF HUMAN EMBRYOS USED TO DERIVE HESC LINES

Human ESCs are most often derived from donated supernumerary blastocysts, five to eight days after *in vitro* fertilisation. The inner cell mass (ICM) is usually isolated. The most commonly used technique has been immunosurgery, first described by Solter and Knowles (Solter and Knowles, 1975), which involves rabbit anti-human whole serum and guinea pig serum for complement-mediated lysis of the trophectoderm cells. These cells form the ICM and can give rise to pluripotent embryonic stem cells.

Whole blastocyst culture instead of isolation of the inner cell mass has been used by several groups with different results. Tropel and co-workers (Tropel et al., 2010) described high efficiency in hESC derivation from embryos carrying genetic disorders that had been diagnosed by PGD, using the whole blastocyst culture technique (>20%), but others have had less impressive results (Findikli et al., 2005; Heins et al., 2004; Suss-Toby et al., 2004).

Mechanical isolation of the ICM was first described by us (Strom et al., 2007), as shown in this thesis, using flexible metal needles. Laser isolation has also been used (Cortes et al., 2008; Turetsky et al., 2008). Turetsky and co-workers removed both the zona pellucida and the trophectoderm cells from nine PGD embryos carrying a genetic disorder using a laser beam. Three hESC lines were established.

Cortes and co-workers (Cortes et al., 2008) reported in 2008 an optimisation study of mESC derivation. By culturing mouse embryos and comparing whole-blastocyst culture, laser drill technology, and whole-blastocyst culture followed by laser drilling on mouse blastocysts, they concluded that whole-blastocyst culture followed by laser drilling is an effective technique for the establishment of new mouse hESC lines. This technique was then used to derive hESC lines. Batlle-Morera and co-workers showed in 2008 that the most efficient establishment of mouse ES cell lines from different strains was by isolation of the epiblast cells both by

immunosurgery as well as mechanical isolation in defined conditions (Batlle-Morera et al., 2008).

New hESC lines have been derived from embryos at different developmental stages. Strelchenko et al. (Strelchenko et al., 2004) cultured morula-stage embryos and picked up the cells that had stem cell morphology. Apparently arrested embryos at the four-cell-stage or eight-cell-stage have also been used as the origin of hESC lines (Feki et al., 2008; Rajala et al., 2010; Zhang et al., 2006). Rajala and co-workers were able to derive one hESC line from 10 embryos arrested between Day 3 and Day 4 in a newly defined xeno-free culture medium for culture on feeder cells (Rajala et al., 2010). The new hESC line came from an embryo arrested at the eight-cell stage. Feki et al. (Feki et al., 2008) derived a line from the single surviving blastomer of a frozen-thawed four-cell-stage embryo.

HESC lines have also been derived from isolated blastomers of cleavage-stage embryos (Geens et al., 2009; Klimanskaya et al., 2006).

2.3 HESC CULTURES

2.3.1 Feeder cells

Since the first successful establishment of hESC lines in 1998, in which mouse embryonic fibroblasts (MEFs) were used to support the attachment of the cells of the ICM and the growth of hESCs (Thomson et al., 1998), several human cell types have been reported to sustain the derivation and growth of hESCs. Human foetal muscle and skin, human adult fallopian tube epithelial cells and adult muscle cells were described as feeder cells by Richards and co-workers (Richards et al., 2002), In our hands, human foreskin fibroblasts (hff) have proved to work for the derivation and culture of 30 hESC (Hovatta et al., 2003; Inzunza et al., 2005; Strom et al., 2010). Human adult bone marrow stromal cells have also been used for hESCs maintenance (Cheng et al., 2003), while uterine endometrial cells (Lee et al., 2005) and human placental fibroblasts (Genbacev et al., 2005) have been used for the derivation and maintenance of hESCs. All of these feeder cells, however, have been cultured using FBS in the culture medium.

The use of autogenic (hESC-derived) fibroblasts and immortalised fibroblasts derived from hESCs has been reported (Stojkovic et al., 2005; Wang et al., 2005), but the differentiation of hESCs towards fibroblasts, or fibroblast-like cells, has been laborious.

In 2010, Rajala and co-workers described a xeno-free culture medium that could be used together with hff feeder cells. The new xeno-free medium sustained undifferentiated growth of both hESCs and iPS cells over long periods of time, more than 80 passages. The same culture method also proved to work for derivation of four new hESC lines, including one line from a developmentally arrested embryo. The new hESC lines were derived on feeders cultured in medium supplemented with human serum (Rajala et al., 2010).

2.3.2 Feeder-free culture and derivation of hESCs

Feeder-free culture of mouse ES cells has been possible since 1988 (Smith et al., 1988; Williams et al., 1988), by adding LIF to the culture medium. It is also possible to add other

factors such as IL-6 together with sIL-6R (Nichols et al., 1994), which activate STAT3 and the gp130 pathway.

Adding LIF to the culture medium for hESCs, however, did not have the same effect. Instead, the first successful feeder-free way to culture hESCs was on a mouse tumour-derived extracellular matrix, Matrigel, and in a medium conditioned by MEF cells (Xu et al., 2001). Human feeder cells have also been used to condition the culture medium (Xu et al., 2004).

In 2005, Xu et al. showed that unconditioned medium had a high bone morphogenic protein (BMP) activity and that this activity was reduced after the medium had been conditioned. Adding a BMP antagonist, noggin, to the unconditioned medium, together with a high concentration of bFGF, removed the need to condition the medium for the feeder-free culture of hESCs (Xu et al., 2005).

Matrigel is a complex mixture of mouse origin, a soluble basement membrane extract from the Engelbreth-Holm-Swarn (EHS) mouse tumour, consisting mainly of laminins, collagen IV, entacin and heparin sulphate proteoglycan, together with several growth factors (bFGF, insulin-like growth factor-1, epidermal growth factor, platelet-derived growth factor (PDGF), nerve growth factor and transforming growth factor-b1). It has large batch-to-batch variability. In order to replace Matrigel, more closely defined proteins have been used as the supporting substrata. The use of a single substratum would facilitate the understanding of how the pluripotent state is maintained.

Laminin isolated from different tissues has been used by several groups (Beattie et al., 2005; Xu et al., 2001). Fibronectin has also been used (Amit et al., 2004; Noaksson et al., 2005) to maintain hESC cultures. More recently, recombinant vitronectin has been used in the culture of hESCs (Braam et al., 2008). Domogatskaya et al. showed in 2008 that it is possible to culture mouse ESC on several forms of human recombinant laminins, with the best results for proliferation and undifferentiated growth being obtained on laminin-511 (Domogatskaya et al., 2008). In 2010, we described together the maintenance of hESCs on the same human recombinant laminin-511 (Rodin et al., 2010). Some early attempts to derive new hESC lines on laminin-511 in defined culture conditions were also carried out.

Feeder-free derivation of hESCs was first described by Klimanskaya and co-workers, on Matrigel (Klimanskaya et al., 2005). They described the derivation by immunosurgery of one hESC line on a matrix prepared from MEF cells in a medium containing SR and plasmanate. Ludwig and co-workers subsequently described a well-defined xeno-free system used for the derivation of two new hESC lines (Ludwig et al., 2006b). However, also these hESC lines have been in contact with animal components during the ICM isolation, since immunosurgery was used. Both lines proved to be chromosomally abnormal.

2.4 CLINICAL GRADE STEM CELLS

Present European regulations state that all products used in clinical applications must be produced under "good manufacturing practice" (GMP) conditions, see below. This involves complete traceability of all components and substrates used in all steps of hESC handling,

complete record keeping, and quality checkpoints at all steps. GMP also regulates personnel qualifications, sanitation, cleanliness and equipment verification (Hovatta, 2006).

The use of hESCs for clinical purposes would be facilitated if the process of derivation and the culture system could be made free of animal-derived components. A guideline from the European Medicine Evaluation Agency (EMEA) today named European Medicines Agency (EMA) (www.ema.europa.eu) contains a long list of microbes that may contaminate the animal feeder cells or the FBS and BSA used in the standard culture methods for hESCs. This highlights the importance of xeno-free and well-defined derivation and culture systems for hESCs.

The safest option to obtain hESCs of clinical grade would be to derive the lines in a GMP facility. The embryos should also be cultured in GMP conditions according to EC directives and the Swedish Act (2008:286) for standards of quality and safety in use of human tissues and cells. Another possibility would be to transfer current hESC lines from the research laboratory into a GMP facility and perform all the necessary tests and validations there. Animal-derived components can be used in a GMP facility, as long as they meet the standards of GMP regulations.

In 2007, Crook and co-workers described the derivation of six hESC lines under good tissue practices (GTPs) and GMP conditions (Crook et al., 2007). They described the whole process from embryo procurement to the characterisation and safety testing of the hESC lines, all following GMP, even though the system was not xeno-free. The supporting feeder cells had been cultured in FCS of GMP quality. Any possible contamination by known pathogens of human or animal origin was checked for both in the embryo donors and in the hESC lines.

2.4.1 Regulations for cell therapy products

The use of cells for clinical applications within Europe is regulated by the EMA guidelines and several European Commission directives that lay down rules that govern medicinal products in the European Union. The EU has adopted special regulations that specifically apply to cell therapy products (EC Regulation 1394/2007; EC Directive 2001/83/EC; EC Regulation 726/2004). The standards for the safety of donors, quality, obtaining material, testing, processing, storage, distribution and preservation of human material are regulated under EU Directive 2004/23/EC. Within the EU, GMP inspections are performed by national regulatory agencies.

In the US, cell therapy is regulated by the FDA's Good Tissue Practices Final Rule (US 21 C.F.R., Part 1271; US FDA, 1998).

Both within the EU and the US, the level of manipulation is important, since it is correlated to the degree of risk when using the final cell therapy product. As a result of this, cells that have only been cryopreserved will be subject only to minor requirements and can be handled under defined industrial standards (GTPs), whereas products that have been more than minimally manipulated will require stricter control under GMP conditions. The distinction between these two requirements is the assumed risk. For products with minor requirements, the primary

concerns are contamination and infections. Products that are considered to be more manipulated, in contrast, will raise a broader range of safety issues (von Tigerstrom, 2009).

2.4.2 Good manufacturing practice

GMP is a quality system used in the pharmaceuticals industry. It ensures that the end product meets the high standards and preset specifications, and it covers both the manufacturing and testing of all intermediate products on the way to the end product. It includes traceability of all raw materials and requires that the production follow validated SOPs (Unger et al., 2008b).

The rules governing medicinal products in the European Union are found in EudraLex vol. 4 (http://ec.europa.eu/health), and are guidance for the interpretation of the principles and guidelines of good manufacturing principles for medicinal products for human and veterinary use laid down in Commission Directives 91/356/EEC, as amended by Directive 2003/94/EC, and 91/412/EEC.

A European Union commission (2003/94/EC) presented the principles for GMP in October 2003.

2.5 CHALLENGES FOR THE CLINICAL USE OF HESCS

2.5.1 Immune rejection

Transplantations of allogenic origin may result in graft rejection and immune rejection, and the patients may therefore require lifelong immunosuppression. This may involve serious side effects, including an increased risk of tumorigenicity (Muruve and Shoskes, 2005). It has been shown that hESCs are immunogenic and will be rejected after an allogenic transplantation: they might, however, be less immunogenic than solid organs (Grinnemo et al., 2008).

Several methods have been proposed to avoid or minimize the risk of immune rejection. Engineering the hESCs so that they overexpress genes that suppress the immune system in order to induce immune tolerance (Deb and Sarda, 2008) is one option. Deletion of major histocompatibility complex (MHC) molecules or other immune effectors on the transplanted cells (Hentze et al., 2007) is another. Another proposed method for engineering hESC derivatives has been to induce over-expression of immunosuppressive molecules that will be secreted locally (Murry and Keller, 2008). All these methods would be technically challenging, and some would introduce new side effects: cells lacking MHC molecules, for example, will be targeted by natural killer cells (Hentze et al., 2007).

A more tempting idea that has been proposed is to co-transplant the hESC-derivatives together with hematopoietic cells differentiated from the same hESC line, hopefully inducing lifelong tolerance for the graft (Beilhack et al., 2003).

Another option would be to have sufficiently large numbers of stem cell lines in biobanks to cover the majority of the population's histocompatibility antigen combinations. The estimated need for hESC lines in such banks, however, is unrealistically high (Hyslop et al., 2005; Rao and Auerbach, 2006; Taylor et al., 2005).

2.5.2 Tumorigenicity

The issue of genetic stability is important in all cell-based therapies. The specific concern is whether the cells become unstable or transformed when kept in culture over long periods of time. Normal development is arrested during the derivation from ICM to ES cells. Genetic and epigenetic changes in favour of unlimited self-renewal take place (Tang et al., 2010), and cells maintained in culture may accumulate genetic and epigenetic changes with severe consequences (Carpenter et al., 2009). The prolonged culture of cells may select for cells with genetic changes that give a growth advantage. Recurrent chromosomal abnormalities have been identified in hESC cultures maintained over long time, including amplification at 20q11.21, a mutation associated with cancer (Lefort et al., 2008; Spits et al., 2008). It is well-established that hESCs may undergo non-random gain of chromosomes, particularly gain of chromosomes 12, 17 and X (Baker et al., 2007).

Recent work has shown that hESCs have gained several mutations after as few as 22 passages, and these mutations include gains and deletions of genes related to cancer. A large proportion of human embryos are chromosomally abnormal, especially embryos with poor morphology and developmental delay (Hovatta et al., 2010), and these are the embryos most commonly donated for hESC derivation. Chromosomal abnormalities in hESCs, however, are not commonly reported, at least not in early passages. All 30 hESC lines derived at Karolinska Institutet, for example, have a normal karyotype when analysed by G-banding (Strom et al., 2010; Strom et al., 2007).

Culture adaptation of hESCs and other cells in culture occurs as a successive, selective increase of mutations leading to growth advantages (Blum and Benvenisty, 2008; Harrison et al., 2007). Changes too small to be seen by G-banding occur during culture adaptation and lead to growth advantages (Narva et al., 2010). The results found by Närvä and co-workers show that there is no safe cut-off point for when hESC lines start to pick up chromosomal abnormalities. Both small and large mutations take place at low passages, while some hESC lines are karyotypically normal at passages as high as 142.

The only conclusions that can be drawn are that it is important to screen hESCs for their genetic composition before clinical applications, and it is important to minimize the time in culture. However, there is no true cut-off limit for how long hESCs can be kept in culture before they have gained mutations that might have tumorigenic features.

2.5.3 Large-scale production

The large-scale production, amplification and differentiation of hESCs will be necessary to generate the large quantities of cells that will be needed for the clinical application of tissue replacement. The first automated robot system for plating, feeding and harvesting hESCs was described in MEF co-culture (Terstegge et al., 2007). Several groups have subsequently described the culture of hESCs in suspension (Fernandes et al., 2009; Steiner et al., 2010), and this system seems promising for the future large-scale production of pluripotent stem cells.

3 MATERIALS AND METHODS

3.1 ETHICS

We obtained approval from the Ethics Committee of Karolinska Institutet for the establishment of new human embryonic stem cell lines, obtained from donated supernumerary embryos, and for the characterisation and differentiation of these cells. Embryos were obtained as donations from couples undergoing *in vitro* fertilization at the Fertility Unit at Karolinska University Hospital, Huddinge, Sweden. Both partners signed the informed consent form after both written and oral information. Only embryos that could not be used in infertility treatment were donated for research. No reimbursement was paid to the donors.

3.2 ZYGOTES AND BLASTOCYSTS

The total number of embryos included in this work was 234. Only six of these embryos were used for stem cell derivation before reaching the blastocyst stage. These six embryos had been arrested at the morula stage. The blastocysts were of different qualities, ranging from no visible ICM to extremely high quality. Some blastocysts were fully expanded and hatched while others had not expanded at all.

3.3 EMBRYO CULTURE

Two different culture media have been used for embryo culture, for details see Table 1. After aspiration, the oocytes were collected into ISM1 medium (Medicult, Denmark) or G1 (Vitrolif, Sweden) and incubated for 4-6 hours before insemination by IVF or ICSI. Oocyte and embryo culture was performed in 20 µl drops of culture medium under paraffin oil (Ovoil, Vitrolife) in an atmosphere of 6% CO_2 in air at 37 ºC. Pronuclear formation was evaluated 16-18 hours post insemination (hpi), and only zygotes displaying two pronuclei were selected for embryo transfer. The embryos were cultured from the day of fertilization up to Day 3 in ISM1 or G1 and from Day 3 to the blastocyst stage in ISM2 (Medicult) or G2 (Vitrolife). The embryos that were not to be used for clinical treatment or cryopreservation due to poor quality or developmental rate were, if donated for hESC derivation, transferred to the hESC derivation laboratory. The embryos were usually cultured for one day extra before isolation of the ICM was carried out.

3.3.1 Scoring system

Evaluation of the embryo quality was performed at 200 times magnification using an inverted microscope equipped with differential interference contrast optics, at 42-44 hpi and at 66-68 hpi. A modified scoring system first described by Mohr et al. (Mohr et al., 1985) was used to score and select embryos for transfer and cryopreservation. Briefly, a maximal score of 3.5 was given to the embryo if no factor that reduced the embryo quality was observed. The score was reduced in increments of 0.5 for non-ideal numbers of blastomeres (4 cells at Day 2 and 8 cells at Day 3 were considered ideal), or the presence of more than 10% fragmentation, non-spherical blastomeres, unequal size of blastomers, unevenness of the cell membrane, cytoplasmic abnormalities, or a failure of the embryo to fill the zona pellucida. Embryos with

multi-nuclear blastomers were not used for IVF treatment but they could be donated to stem cell derivation.

Embryos scoring 3.0-3.5 were regarded as being of top quality, those with a score of 2.5 were considered good quality, and 4-8-cell embryos with a score of at least 2.0 were considered suitable for cryopreservation. Embryos with a lower score could be donated for research.

Selection of blastocysts for transfer or cryopreservation was performed at the IVF unit using the system described earlier (Gardner, 2000). The blastocoel was graded from 1 to 6 as follows: (1) early blastocyst with a blastocoel of less than 50% of the embryo volume; (2) early blastocyst with a blastocoel of 50-80%; (3) fully developed blastocoel of at least 80% of the embryo volume; (4) expanded blastocyst; (5) hatching blastocyst; (6) fully hatched blastocyst. The inner cell mass was graded as follows: (A) many cells and tightly packed; (B) average number of cells; (C) few cells and loosely packed. The trophectoderm was graded as follows: (A) many cells equal in size; (B) uneven cells; and (C) few cells. Expanded blastocysts with a good inner cell mass and trophectoderm, i.e. at least 4AB, were considered to be of high quality. Blastocysts scoring at least 3AA were considered to be of good quality.

All blastocysts with a minimum score of 2BB on Day 5 after fertilisation were either transferred or cryopreserved for future fertility treatment. Blastocysts with a lower score could be donated for research, including all embryos with the score C for the ICM. According to Swedish law, embryos for fertility treatment can be stored for a maximum of five years. The embryos can then be donated for research. In the cases where we have performed ICM isolation from embryos with a high score, they have all been slow in development and had a score lower than 2BB on the day of donation. All embryos are now kept at the Fertility Unit for one day extra and cryopreserved for fertility treatment if they develop further.

All embryos that were not fertilized normally or carried a genetic disorder could be donated for research.

3.4   ISOLATION OF THE INNER CELL MASS

3.4.1   Immunosurgery

Isolation of the inner cell mass using immunosurgery was carried out for the first 20 hESC derivations using 126 blastocysts (Strom et al., 2007). The zona pellucida was first removed using 0.5% pronase (Sigma-Aldrich Co., USA) and the trophectoderm was removed by immunosurgery as described earlier (Solter and Knowles, 1975), using rabbit antihuman whole serum (Sigma) and guinea pig complement serum (Sigma).

3.4.2   Mechanical isolation of the ICM

Mechanical isolation of the ICM was performed by using specially made flexible metal needles, 0.125 mm in diameter, made for us at the Helsinki School of Micromechanics (Espoo, Finland) and now commercially available from Hunter Scientific (Essex, UK). One needle was used to hold the blastocyst while opening the zona pellucida with the second needle. This was followed by isolating the ICM from the trophectoderm cells (Strom et al., 2007). The ICM was allowed to grow on human skin feeder cells for 12-15 days before the first transfer to a new feeder plate.

3.5 HUMAN EMBRYONIC STEM CELL CULTURE

3.5.1 Culture medium

The medium used for culture and derivation of the stem cells consisted of Knockout Dulbecco's Modified Eagle's Medium supplemented (20%) with Knockout SR, 2 mM glutamax, 0.5% penicillin-streptomycin, 1% non-essential amino acids (all from Gibco Invitrogen Corporation, UK), 0.5 mM 2-mercaptoethanol (Sigma-Aldrich) and bFGF (R&H Systems, UK) at 8 ng/ml.

The medium for culture and expansion of the fibroblasts consisted of IMDM supplemented by 10% FBS and 0.5% penicillin-streptomycin (all from Gibco Invitrogen). The medium of the feeder plates was exchanged on the day after irradiation to medium supplemented with 10% SR. Three days after irradiation the prepared feeder plates sustained the undifferentiated growth of hESCs best and before transfer of hESCs to the feeder plates the medium was changed to complete stem cell medium.

mTeSR1, a commercially available culture medium (Stem Cell Technologies, VA, USA), was used together with Matrigel (BD) according to the manufacturers' description.

O3-medium was a variant of mTeSR1, which was prepared in the laboratory. A stock "A" was prepared by adding 165 mg of thiamine and 50 mg of reduced glutathione to 500 ml of distilled water (GIBCO Invitrogen) as described by Ludwig et al., (Ludwig et al., 2006a) but without l-ascorbic acid. The solution was then sterile filtered (0.22-μm filter), divided into aliquots and frozen at -20 °C. Stock "B" was prepared by adding 67g BSA to 500 ml distilled water as described previously (Ludwig et al., 2006a), but without selenium, insulin and holo-transferrin. 6 mg of phenol red was then added per 100 ml. The stock was carefully stirred and sterile filtered. Stock B could be stored at 4 °C for up to 2 months. Stocks of transforming growth factor β1 (TGF-β1), pipecolic acid, GABA (γ-aminobutyric acid) and LiCl were prepared and stored in aliquots, as described previously (Ludwig et al., 2006a).

To prepare 100 ml of O3 medium, DMEM-F12 medium was supplemented with 20 ml of stock B, 200 μl of TGF-β1 stock, 13 μl of pipecolic acid stock, 200 μl of GABA stock, 200 μl of LiCl stock, 1 ml of MEM non-essential amino acid solution (GIBCO Invitrogen), 1 ml of 200 mM l-glutamine solution (GIBCO Invitrogen) and 2 ml of insulin-transferrin-selenium supplement (GIBCO Invitrogen). To compensate for the salt balance and to adjust the pH of the medium, 145 mg of NaCl and 56 mg of NaHCO$_3$ were added. The solution was thoroughly mixed and the pH of the medium was adjusted to 7.4 using 10 M NaOH, at room temperature. The solution was filtered through a 0.22-μm filter, and 200 μl of chemically defined lipid concentrate (GIBCO Invitrogen) was added. O3 medium could be stored at 4 °C for up to 1 month.

Before use, the medium was supplemented with 96 ng/ml recombinant human basic FGF (R&D Systems) and 40 μg/ml ascorbic acid (Sigma-Aldrich).

TeSR2 (Stem Cell Technologies), a xeno-free, commercially available medium for feeder-free cultures, was used together with Matrigel (BD), according to the manufacturer's description. This medium has been previously described (Ludwig et al., 2006a).

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We prepared a variant of this TeSR2, called H3. Stock A was prepared as described above for O3 medium. Human albumin solution (Albuminativ) was purchased from Octapharma AB. The solution was dialyzed three times against cell culture PBS for 3 hours each time, using a dialysis membrane that passed molecules of molecular mass 12-14 kDa (Spectrum Laboratories), and then once against DMEM-F12 medium. We measured the optical density to assess the final concentration of the protein in the solution. Stock B was mixed using the appropriate volume (depending on the concentration) of the dialyzed human albumin solution to achieve the same concentration of albumin as in stock B for O3 medium (described above). Stock of TGF-β1 was prepared (Ludwig et al., 2006), but the dialyzed human albumin was used instead of BSA. All other stocks were prepared as described above. H3 medium was mixed as described for O3, but NaCl was not added.

Before use, the medium was supplemented with 96 ng/ml carrier-free recombinant human FGF basic (R&D Systems) and 40 µg/ml ascorbic acid (Sigma-Aldrich).

3.5.2 Feeder cells

Human foreskin fibroblasts (hff) (CRL-2429; ATCC, Manassas, VA, USA) were used as feeder cells (Hovatta et al., 2003). They were mitotically inactivated by irradiation (40 Gy), 100,000 cells were plated onto 2.84-cm^2 dishes (Falcon) and left overnight to form a confluent monolayer.

3.5.3 Passaging

HESCs were passaged mechanically by scraping off small pieces of the colonies with a surgical scalpel and transferred using a pipette. With mechanical passaging the hESCs can be expanded 1:3-1:4 with a five to seven day interval.

Enzymatic passaging was performed by adding a Rho-kinase inhibitor (ROCK) Y-27632, (Merck Chemicals Ltd., Nottingham, UK), diluted 1:500 for 1 hour in the culture medium (Watanabe et al., 2007). The culture plates were washed once with PBS and then incubated with TrypLE™ Express/Select for five minutes at 37 °C. After incubation, the TrypLE™ Express/Select was removed and warm culture medium added. Single cell suspension was achieved by flushing the culture medium over the colonies until the hESCs had detached. With enzymatic passaging using ROCK and TrypLE™, the hESCs could be expanded 1:5-1:8 and passaged with a five to seven day interval.

Colonies with uniform morphology were chosen for transfer to feeder-free culture. The colonies were transferred by mechanical passaging in small aggregates. Before passaging, the medium was changed from SR-containing medium to the desired feeder-free medium. The hESC colonies were lifted in small aggregates from the feeder layer and transferred to the prepared and pre-warmed matrix-coated plate.

Colonies could also be passaged enzymatically from feeders to feeder-free culture by first washing the plate with D-PBS, and adding dispase or collagenase type IV for five minutes, at 37 °C, until the edges of the hESC colonies started to curl up. The plate was gently washed to remove the enzyme before adding fresh culture media and scraping of the colonies in small aggregates.

For xeno-free, enzymatic splitting, TrypLE Select was used as above, but the incubation time was between 45 s and 2 minutes from feeder layer and 30-45 s when passaging from feeder-free culture. The hESCs were not allowed to become single cells.

3.5.4 Cryopreservation

Early lines were frozen in small cell numbers at early passages by a vitrification method first described by Reubinoff et al., in open pulled straws (Reubinoff et al., 2001). Small plastic straws were heat-softened over a hot plate and manually pulled until reaching the optimal diameter. After cooling to room temperature, the straws were gamma irradiated (40 Gy) in order to sterilize them. Two vitrification media were used, VS1 and VS2. Both were made from the same base of holding medium (HM), which included DMEM that contained HEPES buffer supplemented with 20% SR. VS1 contained 10% DMSO and 10% ethylene glycol, and VS2 contained 20% of both DMSO and ethylene glycol as well as 0.5 mol/l sucrose. Cell aggregates were incubated in VS1 for 1 minute, followed by 25 seconds of incubation in VS2. The cell clumps were then washed in droplets of VS2 and placed into a droplet of 1-2 μl of VS2 from where the colonies where loaded by capillary force into the narrow end of the straw. The straw was then immediately submerged into liquid nitrogen for long-term storage.

For larger quantities of hESCs, slow freezing was used. Undifferentiated hESC colonies (~25 cells) were cut out from feeder layer using a scalpel. Colonies were added into cryo-vials and the freezing medium containing cold hESC culture medium without bFGF but supplemented with 10% DMSO (Sigma-Aldrich) was added. The vials were placed into a Nalgene Cryo1°C Freezing Container "Mr. Frosty" (Nalgene, Nunc International, Rochester, USA) containing isopropanol and immediately stored at -70 °C. The next day, cryo-vials were removed from the -70 °C storage and placed into a liquid nitrogen tank.

Colonies of hESCs could also be frozen by slow freezing using StemCELL Banker as the freezing medium. The procedure was the same as for 10% DMSO-freezing medium, but with a higher survival rate (Holm et al., 2010).

HESCs can be frozen as single cells by first adding ROCK (1:500) (Martin-Ibanez et al., 2008; Watanabe et al., 2007) to the culture medium followed by incubation for 1 hour. Cells were harvested by enzymatic passaging using TrypLE™. The cell suspension was resuspended in cold freezing medium, either in 10% DMSO in SR-medium or in StemCELL Banker (Holm et al., 2010).

3.5.5 Extracellular matrix coating

3.5.5.1 Matrigel

Plates coated with Matrigel (BD Biosciences, Bedford, MA) were prepared according to the manufacturer's instructions by diluting one aliquot of Matrigel in 25 ml DMEM/F12 (Stem Cell Tehnologies) followed by incubation of the culture plates with diluted Matrigel at 4°C overnight. The Matrigel-coated plates were equilibrated in room temperature before use, and excess Matrigel was removed just before adding the cell suspension.

3.5.5.2 LN-511

Laminin was thawed slowly at 4 °C and diluted in D-PBS without calcium or magnesium (Gibco). For a 12-well plate, 19 µg LN-511 was diluted in 500 µL D-PBS per well, and for a 6-well plate, 48 µg LN-511 was diluted in 1 ml D-PBS. The plate was wrapped in parafilm and incubated at 4 °C overnight. Before use, the plate was pre-equilibrated at 37 °C for one hour, and washed twice with warm medium without growth factors, before adding the cell suspension.

3.6 CHARACTERISATION

All new hESC lines were characterised as soon as sufficient cells had been banked.

3.6.1 Immunocytochemistry

The hESC colonies were fixed with 4% paraformaldehyde for ten minutes. For surface markers, blocking of unspecific staining was performed by incubation with 10% FBS in PBS for 30 minutes followed by incubation with primary antibody diluted in 10% blocking solution overnight at 4 ℃.

For staining of nuclear markers, blocking and permeabilisation were performed by incubation in 2% donkey serum, 0.1% Triton-X and 0.1% BSA diluted in PBS for two hours at 4 ℃.

Secondary antibodies were diluted in either 10% blocking solution (surface markers) or 0.1% Triton-X and 0.1% BSA in PBS for nuclear markers and incubated for two hours, in the dark at 4 ℃.

DNA staining was performed by 10 minutes incubation with 1x Hoechst in PBS.

3.6.2 FACS analyses

Cells were removed from the culture dish with trypsin-EDTA, dissociated into a single-cell suspension, and then resuspended in ice-cold FACS buffer (2% (vol/vol) FBS, 0.1% (wt/vol) sodium azide in Hank's buffer). Cells were incubated with primary antibodies against SSEA-4, SSEA-1 (both from R&D Systems), Tra1-60 or Tra1-81 (both from Millipore) for 1 hour on ice, then washed three times with ice-cold FACS buffer. They were then probed in FACS buffer with 1:400 dilutions of Alexa Fluor mouse secondary antibodies (GIBCO Invitrogen) for 30 minutes in the dark and washed four times. Control cells were incubated with mouse immunoglobulins and then with the secondary antibody as described above.

To analyse Oct-4 expression, cells were removed from the culture dish using Trypsin/EDTA (Invitrogen) containing 2% chick serum (ICN) for 10 minutes at 37 °C and resuspended in 1 ml FACS buffer [PBS -/- (Invitrogen) containing 0.1% sodium azide (Sigma) and 2% foetal bovine serum (Gibco)]. Cells to be probed for the internal marker Oct-4 were fixed with 0.1% paraformaldehyde (Electron Microscopy Sciences) for 10 minutes at 37 °C then permeabilised with 90% methanol (Fisher Scientific) for 30 minutes on ice. $1\text{-}5 \times 10^5$ fixed. Cells were then probed for 30 minutes at room temperature with a 1:100 dilution of the specific monoclonal antibody (Santa Cruz Biotechnology, Inc.) in FACS buffer + 0.1% Triton-X100. Cells were then washed and probed in FACS buffer + 0.5% Triton-X100 with 1:1000 dilution of an

alexaflour anti-mouse secondary antibody (Invitrogen) for 30 minutes in the dark at room temperature.

All treatments were performed in duplicate. Cells were analyzed in a FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed with the CellQuest software (Becton Dickinson).

3.6.3 RT-PCR

In order to test for pluripotency *in vitro*, EBs were cultured for three weeks (see below) and analysed with RT-PCR, for the three markers, Nestin, BMP-4 and AFP, representing the three germ layers. Total RNA was extracted from EBs using the Qiagen RNeasy Mini Kit protocol. Total RNA was calculated using a Nano-Drop spectrophotometer and ND-1000, v.3.2.1 software. One microgram of total RNA was reverse-transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Applied Biosystems) in a 20 ml-reaction volume containing the manufacturer's buffer supplemented with 4 mM dNTPs, 20 U RNase inhibitor and 2.5 mM random hexanucleotides. The RT-PCR mixture was then diluted to 100 µl and PCR was performed with 10 µl of complementary DNA, 1.5 mM $MgCl_2$ and 0.4 mM dNTPs in PCR buffer (reaction volume 50 ml) using 2.5 U Taq DNA polymerase (Promega). PCR cycle parameters were 94 ℃ for 3 minutes, followed by 30 or 35 cycles at 94 ℃ for 30 seconds, annealing at the primer-specific temperature for 30 seconds, and 72 ℃ for 30 seconds. Final extension was at 72 ℃ for 7 minutes. The sequences of primers used in Articles I and II were as follows: BMP-4 (sense, 5´-TTTGTTCAAGATTGGCTGTC-3´ antisense, 5´-AGATCCCGCATGTAGTCC-3´) AFP (sense, 5´-CTTTGGGCTGCTCGCTATGA-3´; antisense, 5´-TGGCTTGGAAAGTTCGGGTC-3´) and nestin (sense, 5´-AAACTCAAGCACCAC-3´; antisense, 5´-TTTTAAACTCCAGCCATCC-3´).

3.6.4 qRT-PCR

Total RNA was isolated using the Absolutely RNA Microprep kit (Stratagene) according to the manufacturer's instructions. cDNA was synthesized using 0.2 µg of total RNA in a 20-µl reaction mixture containing oligo (dT)₁₂₋₁₈ primers and Superscript II reverse transcriptase (GIBCO Invitrogen), according to the manufacturer's instructions. To compensate for variable cDNA yields, the amount of cDNA for each PCR reaction was calibrated using the expression level of *GAPDH* as a standard. Amounts of cDNA yielding an equivalent amount of *GAPDH* PCR product (at 20 cycles, data not shown) were used for subsequent PCR reactions. All PCR reactions were run for 30 cycles (including those *GAPDH* PCRs that are shown in the figures in Article IV) and were performed in 20 µl under standard conditions using 1 U of recombinant Taq DNA polymerase (GIBCO Invitrogen). The PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide. For each RNA sample, RT-PCR without reverse transcriptase was performed to confirm that no genomic DNA was isolated. For primers used in Article IV, see Table 1.

Gene name	Forward primer	Reverse primer
GAPDH	GAAGGTGAAGGTCGGAGTCA	TTCACACCCATGACGAACAT
Oct-4 (Pou5)	CGACCATCTGCCGCTTTGAG	CCCCCTGTCCCCCATTCCTA
Nanog	AGCATCCGACTGTAAAGAATCTTCAC	CGGCCAGTTGTTTTTCTGCCACCT
Brachyury	GAAGGTGGATCTCAGGTAGC	CATCTCATTGGTGAGCTCCTT
AFP	CTTTGGGCTGCTCGCTATGA	TGGCTTGGAAAGTTCGGGTC
Sox1	CTCACTTTCCTCCGCGTTGCTTCC	TGCCCTGGTCTTTGTCCTTCATCC
Pax6	AACAGACACAGCCCTCACAAAC	CGGGAACTTGAACTGGAACTGAC

Table 1. Primer sequences used for RT-PCR in Article IV.

3.6.5 Karyotyping

Karyotyping of new cell lines was carried out using the G-banding technique. Samples of hESCs were treated with colcemid KaryoMAX (0.1 µg/ml, Gibco) for 5 hours. The colcemid-treated cells were placed into a centrifugation tube and centrifuged at 1,300 rpm, for 7 minutes. In the meantime, cell cultures were treated with trypsin for 5 minutes. The supernatant was removed after centrifugation, and the trypsin-treated cells were added to the tube and resuspended. Pre-warmed hypotonic solution (0.0375 M KCl) was added to the tube and the cells were incubated for 10 minutes at 37 °C. Following centrifugation, the cells were resuspended in a fixative composed of methanol and acetic acid (3:1). Metaphase spreads were prepared on glass microscope slides, G-banded by brief exposure to trypsin and stained with 4:1 Gurr's/Leishmann's stain (Sigma-Aldrich). A minimum of 10 metaphases were analyzed.

3.6.6 Embryoid body formation

By culturing aggregates of hESCs in suspension without bFGF for 3 weeks, embryoid bodies (EBs) were formed. The presence of tissue originating from the three embryonic germ layers was demonstrated using RT-PCR and immunohistochemistry for marker genes of the three embryonic germ layers: AFP for endoderm; Nestin, Tuji, and Pax 6/Sox-1 for ectoderm; and BMP-4 for mesoderm.

3.6.7 Teratoma formation

The pluripotency of a new hESC line together with earlier control lines were tested *in vivo* as previously described (Inzunza et al., 2005). In brief, exponentially growing hESC lines were harvested from the culture plates using mechanical splitting. Five colonies (10^3 to 10^4 hESCs) were washed twice in phosphate-buffered saline (PBS) and put into a 1.5-ml collection tube containing 80 µL of culture medium, and subsequently implanted beneath the testicular capsule of a young (7-week-old) severe combined immunodeficiency/beige male mouse (C.B.-17/GbmsTac-scid-bgDF N7, M&B, Ry, Denmark). Teratoma growth was followed by palpation weekly, and the mice were sacrificed (cervical dislocation) 8 weeks after implantation. The teratomas were fixed, and sections were stained with hematoxylin and eosin. The presence of tissue components of all three embryonic germ cell layers was analyzed from the stained sections (Inzunza et al., 2005).

All animal experiments were performed at the infection-free animal facility of Karolinska University Hospital in accordance with Ethics Committee approval.

3.6.8 Cell contact area measurement

MaxiSorp 96-well plates (Nunc, Rochester, NY) were coated with ECM proteins (LN-511, LN-332, LN-111, Matrigel and Poly-ornithine) and blocked with 1% heat-denatured bovine serum albumin (BSA) solution in water. Undifferentiated ES cells were plated at a cell density of 800 cells per mm^2 on ECM-coated plates and were left to adhere for 1 hour at 37 °C. Non-adherent cells were washed away, and adherent cells were fixed for 20 minutes with 5% glutaraldehyde, washed, and stained with 0.1% crystal violet (Kebo Lab, Stockholm, Sweden). Crystal violet was extracted after 1 hour from the cells with 10% acetic acid and quantified by measuring the optical density at 570 nm.

3.6.9 Adhesion-blocking assay using integrin antibody

Plates were coated with LN-511 and blocked by 1% heat-denatured BSA solution. An ES single-cell suspension was incubated with function-blocking antibodies to integrin (concentration as recommended by the supplier) for 30 minutes, plated on LN-511-coated plates and allowed to adhere for 1 hour at 37 °C. Unattached cells were removed and the remaining adherent cells were fixed for 20 minutes with 5% (vol/vol) glutaraldehyde, washed and stained with 0.1% (wt/vol) crystal violet. After 1 hour, crystal violet was extracted from the cells using 10% (vol/vol) acetic acid and quantified by measuring optical density at 570 nm.

3.6.10 Assay of cell adhesion to surfaces coated by integrin antibodies

The assay was designed to identify integrin receptors that were expressed in sufficient amounts to retain cells attached to a surface coated with integrin-specific antibody. MaxiSorp 96-well plates (Nunc) were coated with purified integrin antibodies at a concentration of 10 μg/ml at 4 °C overnight and later washed and blocked with 1% (wt/vol) BSA solution. ES cells were plated onto antibody-coated plates and allowed to adhere for 1 hour at 37 °C. Unattached cells were removed, and the remaining cells were fixed, stained and quantified as described above.

3.6.11 Western blot and densitometry analysis

HESCs were collected, counted and pelleted by centrifugation, mixed with non-reduced SDS-PAGE sample buffer to equal concentrations of 2,000 cells/μl and sonicated five times for 15 s. Gradient 4-12% gels were used for SDS electrophoresis, and the proteins were transferred to PVDF membranes. Membranes were blocked using 5% (wt/vol dry) milk solution in PBS buffer with 0.1% (vol/vol) Tween for 2 hours. Primary antibodies against Oct-4 and SOX2 (both from Millipore) in 5% milk solution in PBS buffer with 0.1% Tween were incubated with the membranes overnight at 4 °C. After being washed four times, HRP-conjugated secondary antibodies in 5% milk solution in PBS with 0.1% Tween buffer (diluted 1:1,000) were incubated with the membranes for 40 minutes at room temperature and washed five times with PBS. Chemoluminescent horseradish peroxidase substrate from Amersham Biosciences was used for visualization. Films were scanned at 2,400 d.p.i. and analyzed with the ChemiImager 5500 programme (using the 1D-multi line densitometry mode). HESCs cultured on Matrigel and on feeder cells were used as positive controls.

3.7 LAMININ-511

Human recombinant LN-511 was produced in human embryonic kidney cells (HEK293; ATCC CRL-1573) sequentially transfected with full-length laminin γ1, β1 and α5 constructs. For

protein production, the HEK293 cells were cultured in DMEM supplemented with GlutaMax I and 4.5 g/l glucose (GIBCO) for up to 6 days. The LN-511 molecules were affinity-purified using anti-Flag matrix (Sigma) as previously described (Domogatskaya et al., 2008) and then characterized using 3-8% and 4-15% gradient SDS-PAGE under reducing and non-reducing conditions. The proteins were visualized using SYPRO Ruby (Bio-Rad) protein staining and immunostaining of the chains on polyvinylidene difluoride membranes. To further characterize the protein, Western blot analyses were performed with antibodies against the laminin α5, β1 and γ1 chains. Human recombinant LN-411 was produced in the same way as LN-511. All other extracellular matrix proteins were obtained as described previously (Domogatskaya et al., 2008).

3.8 STATISTICS

Logistic regression analysis was performed in Article III to assess the association between the number of cells on Days 2 and 3, morphological scores on Days 2 and 3, ICM score, trophectoderm score and expansion score against the success of establishing a new hESC line. As prior knowledge was sparse regarding the chosen predictors and their relationship to the success of establishing a new hESC line, a backward selection procedure was performed. P-values less than 0.1 were used as the exclusion criterion. Generalised additive models (GAM) were used to evaluate the functional form of the relationship between each predictor and the probability of successfully establishing a new hESC line. All variables displayed a nonlinear relationship to the outcome and were therefore categorised prior to the logistic regression analysis. Cells on Days 2 and 3 were classified as (fewer than 4 cells, 4 cells, more than 4 cells) and (fewer than 8 cells, 8 cells, more than 8 cells).

Morphological scores on Day 2 and 3 were both classified as less than 2, 2-2.5, greater 2.5. ICM score, trophectoderm score and expansion scores were classified as (0-1, 2, 3), (0-1, 2, 3) and (1-3, 4, more than 4), respectively. The statistical analysis was performed in SAS Version 9.3 (SAS Institute inc., Cary, NC, USA). Descriptive statistics was done in SPSS 18.0 (SPSS Inc., Chicago, IL).

Statistical significance was determined in Article IV using Student's two-tailed t-test for unequal variances.

4 RESULTS

4.1 MECHANICAL ISOLATION OF THE INNER CELL MASS

During 2005-2006, 19 blastocysts were received and used for mechanical isolation of the inner cell mass. From these 19 blastocysts we were able to derive five new hESC lines with the new method. The cells of the ICM attached for 18 of the 18 ICM isolations, and nine of them started to grow. A stem cell colony-like outgrowth was seen in five of the outgrowths and all five were transferred by mechanical passaging to Passage 1, 12-15 days after ICM isolation.

Each cell line was characterised as soon as sufficient cells had been obtained and the line had been cryopreserved.

All five lines expressed Oct-4, Nanog, SSEA-4, TRA-1-60 and TRA-1-81 and were negative for SSEA-1, as revealed by immunostaining. All five hESC lines formed EBs that expressed AFP, BMP-4 and Nestin, as revealed by RT-PCR and by immunohistochemistry. All five lines formed teratomas when injected into SCID mice, and the teratomas contained tissue components from all the three germ layers. Cell lines HS415 and HS429 had a normal female 46;XX chromosome constitution and cell lines HS420, HS422 and HS426 had a normal male 46;XY karyotype.

The efficiency of the derivation was difficult to compare with immunosurgery because the methods were not carried out in parallel. During 2003-2005 we received 100 blastocysts that resulted in 16 new hESC lines, derived by immunosurgery. Five new hESC lines from 19 blastocysts (26%) derived by mechanical isolation appears to be at least as effective as 16 lines out of 100 blastocysts (16%). Since then, only mechanical isolation of the ICM has been used in our laboratory.

4.2 DERIVATION OF 30 HESC LINES – IMPROVING THE QUALITY

Between 2002 and 2009, 236 blastocysts have been used for hESC derivation, resulting in 30 permanent hESC lines. Four of these lines have been derived in a medium containing FCS and the remaining 26 hESC lines have been derived in a medium supplemented with SR. Mechanical isolation has been used for isolation of the ICM for ten of the 30 hESC lines.

All the lines that have been analysed expressed Oct-4, Nanog, SSEA-4, TRA-1-60, TRA-1-81 and were negative for SSEA-1 when analysed by immunohistochemistry. They all formed EBs *in vitro* when aggregates of cells were grown as spheres in SR-medium without bFGF for three weeks. They also differentiated *in vivo* into cells representing all three germ layers after subcutaneous injection into SCID/beige mice, see Table 2.

hESC line	Day of ICM isolation	ICM isolation method	Score before ICM isolation	Medium supplement	Karyotype	Teratoma formation
HS181	6	Immunosurgery	4BB	20% FCS	46, XX	Yes
HS207	7	Immunosurgery	2CB	20% FCS	46, XY	Yes
HS235	6	Immunosurgery	3CB	20% FCS	46, XX	Yes
HS237	6	Immunosurgery	3BB	20% FCS	46, XX	Yes
HS293	6	Immunosurgery	4BB	20% SR	46, XY	Yes
HS306	5	Immunosurgery	3BB	20% SR	46, XX	Yes
HS346	7	Immunosurgery	4BB	20% SR	46, XX	Yes
HS351	6	Immunosurgery	4AB	20% SR	46, XX	-
HS360	6	Immunosurgery	4CB	20% SR	46, XY	Yes
HS361	7	Immunosurgery	4CB	20% SR	46, XY	Yes
HS362	6	Immunosurgery	4BB	20% SR	46, XY	Yes
HS363	6	Immunosurgery	4BB	20% SR	46, XY	Yes
HS364	6	Immunosurgery	4AB	20% SR	46, XY	Yes
HS366	6	Immunosurgery	4BB	20% SR	46, XX	Yes
HS368	7	Immunosurgery	3BB	20% SR	46, XY	Yes
HS380	6	Immunosurgery	4CB	20% SR	46, XY	Yes
HS382	6	Immunosurgery	4BB	20% SR	46, XY	Yes
HS400	6	Immunosurgery	4BB	20% SR	46, XX	Yes
HS401	6	Immunosurgery	4AB	20% SR	46, XY	Yes
HS402	6	Immunosurgery	3AB	20% SR	46, XY	Yes
HS415	6	Mechanical	5CB	20% SR	46, XX	Yes
HS420	6	Mechanical	4AA	20% SR	46, XY	Yes
HS422	6	Mechanical	4BB	20% SR	46, XY	Yes
HS426	8	Mechanical	4BC	20% SR	46, XY	Yes
HS429	6	Mechanical	4CB	20% SR	46, XX	Yes
HS475	7	Mechanical	5BB	20% SR	-	Yes
HS480	6	Mechanical	6BC	20% SR	-	Yes
HS481	6	Mechanical	3AB	20% SR	-	-
HS491	6	Mechanical	4BC	20% SR	-	-
HS539	5	Mechanical	5BB	20% SR	-	-

Table 2. All hESC lines derived at Karolinska Institutet

4.3 NO RELATIONSHIP BETWEEN EMBRYO MORPHOLOGY AND SUCCESSFUL DERIVATION OF HESC LINES

4.3.1 Scores and derivation results

We have derived 30 pluripotent hESC lines, and a further 29 early lines that were lost between Passages 2 and 15. All these lines have been derived from embryos at the blastocyst stage from both poor and high quality embryos. Six embryos have been used in attempts to derive hESC lines before reaching the blastocyst stage, none of these embryos resulted in hESC lines. Only one embryo with Expansion Score 1 has been used in an attempt to isolate cells from the ICM. These cells attached and started to grow and were passaged twice before the line was lost. One

hESC line was derived from a total of 11 embryos with the expansion score two. This gave a derivation success of 9.1%, see Table 3.

The highest derivation success has been with embryos with the expansion score three. We have managed to establish seven hESC lines out of 38 donated blastocysts with expansion score three. This gave a derivation success of 22.3%.

A total of 117 blastocysts with expansion score four (fully expanded) have been used for the isolation of the ICM. This resulted in 54 outgrowths. Thirty-two were passaged at least once. Three early lines were lost between Passages 2 and 15, while 15 permanent lines were established. This gave a derivation success of 12.8%.

Thirty-eight blastocysts with expansion score five (hatching) were used for ICM isolation. From these, 22 resulted in outgrowths and 13 could be passaged at least twice more (34.2%). We have obtained five permanent hESC lines from donated embryos with Expansion Score 5, 13.2% of all embryos at Expansion Score 5.

Twenty-tree blastocysts had expansion score six, fully hatched. Of these 23 embryos, 13 (56.5%) resulted in outgrowth and four were passaged further. One early line was lost at Passage 4, but we managed to derive two permanent hESC lines (8.7%) from all hatched blastocysts.

Expansion score (%)	0	1	2	3	4	5	6
No. ICM isolations	6 (2.6)	1 (0.4)	11 (4.7)	37 (15.8)	120 (51.3)	36 (15.0)	23 (9.8)
No. outgrowths	0 (0)	1 (100)	5 (45.5)	15 (40.5)	57 (47.5)	22 (61.1)	13 (56.5)
No. hESC lines (permanent)	0 (0)	0 (0)	1 (9.1)	6 (16.2)	18 (15.0)	3 (8.3)	2 (8.7)

Table 3. The table shows the total number of attempted ICS isolations/derivations from embryos with expansion score 0-6, total number of outgrowths and total number of established permanent hESC lines. Percentages are given within the brackets and show how many of the embryos had the given expansion score of the total number of embryos used. Line 2 shows how many outgrowths and Line 3 shows how many hESC lines were obtained from all attempted derivations from a given expansion score.

All 30 hESC lines have been derived between Day 4 and Day 8 after fertilisation. Fourteen blastocysts have been used on Day 5, resulting in 11 outgrowths (78.6%) and two permanent hESC lines (14). Most of the ICM isolations have been carried out on Day 6: 163 blastocysts, resulting in 137 outgrowths (84%) and 22 of the 30 hESC lines come from six-day-old blastocysts. In addition we obtained two early lines that were lost at Passages 9 and 13, respectively. Fifty-six isolations were carried out on seven-day-old blastocysts and 48 (85.7%) resulted in outgrowths. Five new hESC lines were established (8.9%) (for all numbers see Table 4).

ICM isolation-day (%)	Day 5	Day 6	Day 7	Day 8
No ICM isolations	14 (6.0)	163 (69.7)	56 (23.9)	1 (0.4)
No. outgrowths	11 (78.6)	137 (84.0)	48 (85.7)	1 (100)
No. hESC lines (permanent)	2 (14.3)	22 (13.5)	5 (8.9)	1 (100)

Table 4. The table shows on what day the ICM isolations were carried out, how many outgrowths and established hESC lines were obtained from different ICM isolation days.

Not all embryos used for establishment of new hESC lines were given a score for the ICM/trophectoderm; only the embryos that had reached the blastocyst stage. Of all included attempts, 226 embryos had a complete score for the ICM. Forty had the maximum score A, (17.7%), 122 had score B (54.0%) and 64 blastocysts had score C (28.3%). Fifty percent of all blastocysts with the maximum score A resulted in an outgrowth, and 13 (30%) were passaged further. Seven of these ICM isolations resulted in hESC lines, one was lost in Passage 4, but the remaining six (15% of all blastocysts with score A for the ICM) have been fully characterised.

Fifty-nine (48.4%) of the 122 blastocysts with score B for the ICM resulted in outgrowths. Three early lines stopped growing between Passages 5 and 15. Eighteen (14.8%) of our permanent hESC lines have been established from blastocysts with score B for the ICM.

Sixty-four (27.4%) blastocysts had score C for the ICM (very few cells, perhaps not visible). Thirty-two (50%) resulted in outgrowths and were passaged at least once. From these 64 blastocysts, we were able to establish six permanent hESC lines (9.4% of all "C"), see Table 5.

ICM score (%)	A	B	C
No ICM isolations	40 (17.7)	122 (54.0)	64 (28.3)
No. outgrowths	20 (50.0)	59 (48.4)	32 (50.0)
No. hESC lines (permanent)	6 (15.0)	19 (15.6)	5 (7.8)

Table 5. Total number of blastocysts with score A-C for the ICM. Total number of outgrowths and permanent hESC lines from all embryos with score A, B or C for the ICM.

Twenty-five (10.7%) of all blastocysts had the maximum score A for the trophectoderm, 173 (73.9%) had score B and 28 (11.7%) had score C. Twelve (48%) of all blastocysts with score A resulted in outgrowths and seven were passaged further, six were lost early and two new hESC lines were established (8% of all "A").

Eighty-five (49.1%) of all blastocysts with trophectoderm score B resulted in outgrowths, 46 (26.6%) were passaged further and 24 permanent hESC lines were established, 13.9% of all blastocysts with score B for the trophectoderm layer.

Twenty-eight of the blastocysts used for ICM isolation had the lowest score, C, for the trophectoderm. Of these 28, 14 (50%) resulted in an outgrowth, five were passaged at least once and four (14.3%) resulted in new hESC lines. See Table 6 for details.

Trophectoderm score (%)	A	B	C
No ICM isolations	25 (11.1)	173 (76.5)	28 (12.4)
No. outgrowths	12 (48.0)	85 (49.1)	14 (50.0)
No. hESC lines (permanent)	2 (8.0)	24 (13.9)	4 (14.3)

Table 6. Total number of blastocysts with score A-C for the trophectoderm layer. Total number of outgrowths and permanent hESC lines of all embryos with score A, B or C for the trophectoderm.

4.3.2 Three pronuclear zygotes

We have received two embryos discarded at the IVF unit for having three pronuclei (3PN) on the day following insemination. Both attached to the feeder layer after mechanical isolation of the ICM and one continued to grow. This 3PN embryo had four cells on Day 2 after fertilization and a score of 3.0. On Day 3 the embryo had eight cells and still scored 3.0. The embryo was still a compacted morula on Day 5, but had reached the blastocyst stage on Day 6. This derivation resulted in the cell line HS429, which is a karyotypically normal (46XX) hESC line that grows well.

4.3.3 Statistical analysis

Only embryos with complete data for the number of cells on Days 2 and 3, scores on Days 2 and 3, ICM-isolation day, ICM-score, trophectoderm-score and expansion score were statistically analysed. This data was available for 206 embryos. Only one variable showed statistical significance for the probability of successful derivation of new hESC lines. This was whether the embryo had had less than four cells on Day 2, $p=0.018$. No other variables included in this statistical model showed any significance, see Table 7.

Differences of number of cells Day 2, least square means					
No. cells on Day 2	No. cells on Day 2	Pr |t|	Odds ratio	Lower bound of odds ratio	Upper bound ofodds ratio
< 4 cells	4 cells	0.0181	0.274	0.094	0.800
< 4 cells	> 4 cells	0.2540	0.468	0.127	1.731
4 cells	> 4 cells	0.3369	1.708	0.571	5.111

Table 7. The only variable showing a significant p-value for the probability of success in deriving new hESC lines was to have less than four cells on Day 2.

4.4 LONG-TERM SELF-RENEWAL OF HUMAN PLURIPOTENT STEM CELLS ON HUMAN RECOMBINANT LAMININ-511

We have cultured three hESC lines: HS207, HS401 and HS420, and two iPS cell lines: BJ#12 and LDS1.4, on human recombinant LN-511 in O3 medium, a variant of the chemically defined mTeSR1 medium first described by Ludwig et al. (Ludwig et al., 2006b), containing BSA as the only animal-derived component. We have also cultured these cells in H3-medium, a variant of the xeno-free TeSR2 medium (Ludwig et al., 2006b) containing human serum albumin. Control cells were cultured in O3 medium on Matrigel or on hff-feeders in SR-medium.

When comparing adhesion properties of hESCs on LN-511, LN-332, LN-411, LN-111, Matrigel and on poly-D-lysine, the average cell contact area was about 1.6 times larger on LN-511 than for cells plated on Matrigel, and about 1.2 times larger than for cells plated on LN-332. Spreading on the other substrata was significantly smaller than it was for LN-511.

RT-PCR was performed in order to find out if our hESCs express laminin chains. HS207, HS401 and HS420 all express laminin α5, β1 and γ1 as well as α1, α2 and β2, demonstrating that all three lines were expressing LN-511. α3 and β3 chains were not detected, suggesting that LN-332 is not expressed by pluripotent stem cells.

Experiments using functional blocking antibodies against integrins showed that α6 and β1 integrins were the most important for LN-511 binding, while β2, β3 and β4 antibodies bound with 19% efficiency or less. Immunofluorescence staining was performed in order to confirm the expression of α6 and β1 integrin in undifferentiated, SOX-2 positive hESCs.

The hESC lines HS207, HS401 and HS420 were cultured for at least 28 passages (5-6 months). The cells were passaged in small aggregates every 6-7 days in 1:2-1:6 ratio. Growth curves were calculated during five passages both on LN-511 and on Matrigel in O3 medium, and the hESCs proliferated similarly on both substrata. The hESCs had similar morphology in O3 and H3 media and expressed the pluripotency markers Nanog, Oct-4 and SOX-2, as revealed by immunostaining. FACS analyses of Oct-4, TRA-1-60, TRA-1-81, SSEA-3 and SSEA-1 expression were performed on HS207 cells after 25 passages on LN-511 in O3 medium. 97% of the cells were positive for Oct-4, 99% were positive for SSEA-4, 85% were positive for TRA-1-60 and TRA-1-81 and no cells were positive for SSEA-1. After 10 passages on Matrigel in O3 medium, 93% of the cells were positive for Oct-4 and 94% of the HS207 cells were positive for SSEA-4. After 16 passages, 94% of H1 cells were positive for SSEA-4, and after 12 passages 97% of H9 cells were positive for SSEA-4. 88% of LDS 1.4 cells were positive for TRA-1-60 after six passages on LN-511.

Quantitative RT-PCR was performed on HS207 cells grown on LN-511 in both O3 and H3 medium, and cells grown on Matrigel in O3 medium. It was also carried out on line HS207 grown on hff feeders in SR-medium. Similar expressions of both Oct-4 and Nanog were seen in all conditions. Western blot was performed in order to see the protein expressions of the pluripotency markers, Oct-3 and SOX-2. The highest expression was seen on LN-511, but with some variability.

The hESC lines H1 and H9 and the two iPS cell lines BJ#12 (Maherali et al., 2008) and LDS1.4 have also been cultured on LN-511 in O3 or mTeSR1 medium. H1 and H9 had similar proliferation rates and morphologies as HS207, HS401 and HS420, and the immunofluorescence analysis revealed that H1 and H9 maintained the expressions of pluripotency markers Oct-4, Nanog, Sox-2 and TRA-1-60. The level of Nanog expression was compared between LDS 1.4 cells cultured on Matrigel and cells cultured on LN-511, and was similar in both conditions after six passages.

Spontaneous differentiation was measured for HS207 after 20 passages on LN-511, four passages on Matrigel and on hff feeders by qRT-PCR for the differentiation markers Pax6,

Sox17 and Sox7. The highest level of expression for both Pax-6 and Sox-7 was seen on hff feeders in SR-medium and the lowest level of expression for these markers was seen on Matrigel with LN-511 as an intermediate. The opposite was seen for Sox-17.

All three hESC lines formed teratomas after culture in O3 medium on LN-511 for 15-20 passages and HS207 cells formed teratomas after 23 passages in H3 medium. All teratomas consisted of tissue compartments from all three germ layers. All three hESC lines formed embryoid bodies after *in vitro* differentiation for three weeks, expressing markers for the three germ layers, and for smooth-muscle actin, nestin, MAP-2 and AFP, as revealed by immunostaining. All cell lines used had a normal karyotype after culture on LN-511 when analysed after 20 passages.

Isolation of the ICM was performed to explore whether LN-511 can be used for the establishment of new hESC lines. Mechanical isolation was performed on blastocysts 6-7 days after fertilisation and the cells of the ICM was plated onto LN-511 in H3 medium, devoid of any xeno-components or in mTeSR1 medium supplemented with 1 µg/ml. In H3 medium, 10 out of 12 plated ICMs attached and five started to grow. In mTeSR1 supplemented with LN-511, all nine ICMs attached and started to grow, but no hESC lines were obtained.

5 DISCUSSION

Most of the existing human embryonic stem cell (hESC) lines have been in contact with animal-derived components either during the process of isolation of the ICM by immunosurgery or by contact with animal cells or derivatives in the cultures. For research aiming at therapeutic purposes, the use of animal-derived components is not optimal. It not only introduces the risk of transmitting viruses and prions between species but also increases the risk of rejection of transplanted cells (Unger et al., 2008a).

In Article II we describe how we have improved the derivation and culture system between 2002 and 2010, first by introducing human foreskin fibroblasts as the supporting feeder cells (Hovatta et al., 2003), followed by replacing FCS in the culture medium with Serum Replacement. This replacement was first carried out for the culture medium for existing lines (Koivisto et al., 2004), and later in the derivation medium (Inzunza et al., 2005).

The next step in improving the cultures is described in Article I, by replacing the commonly used method for isolation of the ICM when establishing new hESC lines. Immunosurgery is a method that includes both rabbit anti-human serum and guinea pig complement serum (Solter and Knowles, 1975). We were able to establish a novel method in which two flexible metal needles are used to isolate the cells of the ICM with a higher success rate than when using immunosurgery. The new system is both simpler and much faster. It was not possible to carry out a statistical analysis of the difference between the new method and the old method, because the cultures were not prepared in parallel and other factors may have influenced the results. Since 2007, when Article I was published, we have been able to derive five new hESC lines by mechanical isolation, as described in Article II. Other forms of mechanical isolation system that have been described include laser-assisted isolation (Cortes et al., 2008; Turetsky et al., 2008), but our system is less costly and does not cause any heat, which might harm the sensitive human pluripotent cells of the ICM.

Another method described for derivation of hESCs is plating of the whole blastocyst after removal of the zona pellucida (Cortes et al., 2008; Ellerstrom et al., 2006; Heins et al., 2004). This method has not been as successful in our hands (Article III). For mouse ES cells, the most effective way to derive new lines appeared to be isolation of the ICM, instead of whole blastocyst culture (Batlle-Morera et al., 2008).

The embryos that were donated for hESC derivation were in most cases regarded as not being suitable for IVF treatment, due to poor morphological quality and delays in development. We have received high-quality embryos in a few cases. These embryos had either been frozen for more than five years, which is the legal limit for their use in reproductive therapy in Sweden, or they had had a good morphology at the time of ICM isolation, but subsequently developed slowly. When comparing the relationship between successful derivation of hESC lines with the morphology and developmental rate of the donated embryos, we found no significant correlation to success in derivation of new hESC lines. This work is described in Article III. Only a few, maybe only one, pluripotent cell appears to be sufficient for the establishment of a hESC line (Tang et al., 2010). By single cell RNA-sequencing analysis, Tang and co-workers were able to trace the pluripotent cells of day 3.5 and 4.5 cell of the early mouse embryo,

through the outgrowth phase to the self-renewing ES cell. The molecular signature of the pluripotent ES cells was clearly distinct from the earlier cells of the ICM. All cells of the ICM that were analysed had high expression of Oct-4, Sox2 and Nanog but seven out of 18 cells in Day 3 outgrowths had lost the expression of Nanog indicating loss of pluripotency (Tang et al., 2010). They were also able to establish a new ES cell line from one single isolated cell of a Day 5 outgrowth, supporting our theory that only one pluripotent cell in the ICM could be enough to establish a hESC line from donated 'poor' quality embryos III.

Several of the ICMs resulting in the hESC lines that had been regarded as being of "good" quality on the day of ICM isolation were regarded as being of "poor" quality on Day 5 after fertilization. Both hESC lines, HS181 and HS364, when observed by embryologists on Day 5 were still compacted morulae and were therefore not regarded as suitable for transfer or cryopreservation. They were then donated to the stem cell laboratory. When left in culture for one more day, the embryos developed to fully expanded blastocysts with a score of 4BB. Two more cell lines, HS293 and HS400, were given a score of 1 on Day 5, but developed in culture and were given a score of 4BB (HS293) or 4AB (HS400) on Day 6. The policy at our fertility unit has now been changed due to these findings, and the embryos are kept in culture for one day extra at the IVF laboratory. If they develop to the blastocyst stage with a minimum score of 2BB, they are cryopreserved for fertility treatment in the future. Embryos with a score lower than 2BB can still be donated to stem cell derivation, and this includes all embryos with a score of C for the ICM. We have received a total of 64 blastocysts with a score of C for the ICM, and five (7.8%) of these resulted in pluripotent hESC lines, as described in Article III.

A statistical comparison of embryo quality has been challenging due to the high variability and the number of factors that influence the result. We received during this period 12 embryos regarded to be of top quality by the IVF unit with scores between 4AA and 6AA on the day of ICM isolation. Only one new hESC line, HS475, was derived from these embryos. We conclude that the top quality embryos did not result in new hESC lines more often than the "poor" quality embryos.

This derivation success with very poor quality embryos has not been described earlier. The other materials have been smaller and similar statistical comparisons have not been possible (Feki et al., 2008; Lerou et al., 2008; Rajala et al., 2010; Zhang et al., 2006).

Embryos that have not been normally fertilized and have either 1PN or more than 2PN can be donated to stem cell research since they are not used for IVF treatment. We have been able to establish a karyotypically normal hESC line, HS429, from a 3PN embryo. Six days after fertilization, this embryo had developed to a fully expanded blastocyst and mechanical isolation of the ICM was performed. The result was a hESC line that grew well and had a normal karyotype, 46XX, when analysed at Passage 8.

We can only speculate how this embryo with 3PN at Day 1 could have developed to a karyotypically normal hESC line. One reason could be that one polar body had not been extruded when observed on Day 1 and that this process happened after the observation. It is also possible that the embryo had corrected itself by exclusion of the extra set of chromosomes during metaphase, and these chromosomes were then discarded by the developing embryo. It

has been described how microsurgical correction on 3PN zygotes was used for the rescue of the embryos to heteroparental form (Escriba et al., 2006; Ivakhnenko et al., 2000). It has also been described how human dispermic embryos can develop to the blastocyst stage (Sathananthan et al., 1999). Kattera and Chen have described how microsurgical enucleations of one pronucleus from three 3PN zygotes were performed, and the corrected embryos were transferred to a woman for fertility treatment. The result was a normal healthy baby (Kattera and Chen, 2003).

A high proportion of human oocytes are affected by chromosome abnormalities, but the vast majority of these embryos are unable to develop to blastocyst stage, fail to implant or culminate in a miscarriage (Wells, 2010). All our 30 hESC lines have been derived from embryos at blastocyst stage. A recent study came with the conclusion that embryo morphology screening might be a better tool than genetic screening for at least a part of the patients (Finn et al., 2010). It is interesting to note that large chromosomal abnormalities such as aneuploidy in hESC lines at low passages are not common.

We also described a new feeder-free culture system on human recombinant LN-511 in a defined xeno-free culture medium, Article IV. Both hESCs and iPS cells retained their capacity to self-renew and differentiate into all three germ layers, both *in vitro* and *in vivo*. We cultured five hESC lines and two iPS cell lines on LN-511 for at least four months (20 passages), after which they still had normal karyotypes and could form teratomas when injected into SCID mice. The teratomas contained cells of all three germ layers. HESCs cultured on LN-511 spread to form a uniform mono-layer and almost all cells were positive for the pluripotency marker Oct-4. The results support previous results (Miyazaki et al., 2008) that $\beta1$ integrins, mainly $\alpha6\beta1$, are the major binding site for hESCs to the extracellular matrix. HESCs that express $\alpha6\beta1$ integrin attach quickly and can migrate easily on LN-511-coated dishes, facilitating their self-renewal. Hence we believe that LN-511 provides focal adhesion contacts for the hESCs.

The well-defined cultures also provide an excellent tool to study pathways involved in differentiation and development, since all components are known and can be altered. Well-defined cultures have several advantages for the standardisation of human pluripotent stem cell cultures. The use of feeder cells or the use of poorly defined substrata such as Matrigel involves batch-to-batch variations, due to variations in their composition and the constitution/secretion of bioactive molecules, such as growth factors, cytokines and other unknown molecules. This use also involves a risk of transmitting microbial and viral contaminations.

Our cultures have evolved using FCS in the medium (Hovatta et al., 2003) together with hff feeder cells to support the undifferentiated growth of pluripotent stem cells. It is now possible to isolate ICMs without any contact of animal-derived components by the use of the mechanical isolation system developed at our unit (Articles I and II). We were able to culture these pluripotent stem cells in chemically defined cultures devoid of any xeno-components. We believe that we will soon have hESC lines derived and cultured using feeder-free techniques in well-defined xeno-free media on either a human recombinant laminin or in suspension culture (Steiner et al., 2010). We will be able to provide cell lines suitable for clinical therapy. The well-defined cultures also provide an excellent tool for the investigation of molecular mechanisms of differentiation, and in designing reproducible protocols fo the differentiation of human pluripotent cells.

6 CONCLUSIONS

The aim of this thesis was to improve the methods for derivation and culture of hESCs, in order to obtain reliable research results and material suitable for cell transplantation. We also wanted to determine whether the quality of the donated embryos is related to the success of derivation of new hESC lines.

- We managed to reduce the variability in the cultures and the risk of infection, first by replacing FCS in the culture medium, later also in the medium for deriving new hESC lines (Articles I and II).

- With mechanical isolation of the ICM for deriving new hESC lines, we were able to withdraw several xeno-components to which hESCs are exposed. The new technique also improved the success rate of derivation of new hESC lines (Article I).

- When investigating how important the embryo morphology and developmental rate were to the success of deriving new hESC lines, we found no correlation. Derivation of hESC lines succeeded from poor quality and good quality embryos to the same extent. In several blastocysts, no real inner cell mass (ICM) was seen, but permanent hESC lines could be established that grew well (Article III).

- One 3PN zygote that developed to the blastocyst stage gave rise to a karyotypically normal hESC line that grew well (Article III).

- We also established a feeder-free culture method on human recombinant laminin, LN-511. As a component of the natural niche of hESCs, this laminin allowed the hESCs to grow in a monolayer on the surface of the matrix. Approximately 97% of the cells expressed the pluripotency marker Oct-4 (Article IV).

- Cultures on LN-511 are devoid of animal products and support the non-differentiated growth of both hESCs and iPS cells in well-defined culture media. HESCs proliferate with normal karyotype for at least four months, with the ability to form teratomas that express markers from all three germ layers (Article IV).

- We have now a xeno-free and feeder-free culture system that enables the use of hESC lines for clinical purposes. HESC cultures based on human recombinant proteins may be more acceptable to regulatory authorities in many countries.

- Well-defined cultures for pluripotent stem cells help in studying the mechanisms involved in differentiation and early development.

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