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HUMAN PLURIPOTENT STEM CELLS: EFFECTS OF HANDLING AND MICROENVIRONMENT

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Front cover: Colony of human embryonic stem cells stained for stress fibers using phalloidin which binds to F-ACTIN (red), NANOG (green) and DAPI (blue). Photographed by Pontus Aspenström.

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To my family, with love

ABSTRACT

Pluripotent stem cells hold great promise in regenerative medicine and drug development due to their developmental potential towards multiple lineages. However, there are still hurdles to overcome before these cells are safe enough for clinical use even though attempts have already been initiated.

To generate pluripotent cell populations for specific clinical purposes, it is crucial to understand the regulatory role of microenvironmental cues as well as factors and signaling pathways that control their cellular phenotype. The main purpose of this work has been to study how pluripotent cells may change due to handling and how microenvironmental cues may influence the characteristics of pluripotent cells.

An effective and robust cryopreservation system is necessary in order to store pluripotent stem cells. We compared a standard freezing method with a novel chemically defined xeno-free solution and found that the number of frozen colonies vs. the number of surviving colonies improved significantly with the new method. Using a live/dead assay we obtained 90-96% viability without any impact on proliferation using the new method, compared with the standard freezing procedure (49%). Furthermore, to enable up-scaling of undifferentiated cells, it is important to passage cells with limited effect on cell quality. We investigated whether different passaging techniques can affect the properties of stem cells. We found that cells passaged in the presence of ROCKi displayed alterations in actin organization. We also found decreased expression of genes encoding common pluripotency markers in cells enzymatically passaged compared to mechanical passaged cells, irrespective of the use of ROCKi, but no correlation to histone modification on the promoters of these genes. We found that the effect of enzymatic passaging and ROCKi were at least in part reversible.

It is relevant to investigate differences between early and late passages when it comes to chromatin state and expression of stem cell markers. We analyzed the gene expression and histone acetylation levels in the promoters of well-defined transcription factors associated with pluripotent state. The acetylation analysis was focused on lysines 8 and 12 on histone H4, since acetylation of these residues has been associated with the interaction of the SWI/SNF complex as well as DNA damage. We could detect variations in histone acetylation levels and gene expression between different cell lines. However we found no significant correlation between either histone acetylation and gene expression levels, nor with these parameters alone and passage number. This suggests that other parameters should be evaluated for screening of clinically suitable pluripotent stem cells.

Finally, prolonged culturing of stem cells *in vitro* may select for cells with genetic changes which often result in growth advantages by acquired mutations, called culture adaptation. By performing a comparative *in vivo* and *in vitro* study on human embryonic stem cell lines we were able to identify chromosomal aberrations that had occurred *in vivo*. We also found an amplification of the whole X chromosome in cells differentiated *in vivo*. The potential of precancerous mutations in *in vivo* conditions is important to consider for safety measures, and underlines the necessity to remove all pluripotent stem cells from differentiated populations that will be transplanted.

LIST OF PUBLICATIONS

- I. **Holm F**, Ström S, Inzunza J, Baker D, Strömberg AM, Rozell B, Feki A, Bergström R and Hovatta O.
An effective non-serum xeno-free chemically defined freezing procedure for human embryonic (hESCs) and induced pluripotent (iPS) stem cells.
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- II. **Holm F**, Nikdin H, Kjartansdóttir KR, Gaudenzi G, Fried K, Hermanson O and Bergström-Tengzelius R.
Passaging techniques and ROCK inhibitor exert reversible effects on morphology and pluripotency marker expression of human embryonic stem cells.
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- III. Bergström-Tengzelius R, **Holm F**, Rodriguez-Wallberg KA, Todorova M, Hermanson O and Hovatta O.
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LIST OF ABBREVIATIONS

Ac	Acetylation
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5 (survivin)
BMP	Bone morphogenic protein
Bp	Base pairs
BRG1	Brahma-related gene 1
BSA	Bovine Serum Albumin
CD99	CD99 molecule
CNS	Central Nervous System
CNV	Copy-number variation
CSC	Cancer Stem Cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DMSO-UW	Dimethylsulfoxide in University-of-Wisconsin Solution
DNA	Deoxyribonucleic acid
DSB	Double-strand break
EB	Embryoid body
EIF	Extracellular Ice Formation
ERK1	Extracellular signal-regulated Kinase 1
ERK2	Extracellular signal-regulated Kinase 2
ESC	Embryonic Stem Cell
FA	Formaldehyde
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FGF2	Fibroblast Growth factor 2
FISH	Fluorescent In Situ Hybridization
GDF	Growth Differentiation Factor
GMP	Good Manufacturing practice
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HAT	Histone Acetyl transferase
HDAC	Histone deacetylase
hESC	Human Embryonic Stem Cell
hFF	Human Foreskin fibroblasts
HGDP	Human genome diversity panel
hiPSC	Human induced Pluripotent Stem Cell
ICM	Inner Cell Mass
IGF	Insulin-like Growth Factor
IHC	Immunohistochemistry
IIF	Intracellular Ice Formation
IMDM	Iscoe's Modified Dulbecco's Medium
iPSC	Induced Pluripotent Stem Cell
ISCI	International Stem Cell Initiative

IVF	In Vitro Fertilization
K	Lysine
KO-SR	Knockout Serum Replacement
LIF	Leukemia Inhibitor Factor
LIN28	Lin-28 homolog A
Me1	Mono-methylation
Me2	Di-methylation
Me3	Tri-methylation
mESC	Mouse Embryonic Stem Cell
N2	Liquid nitrogen
NHEJ	Non-homologous end joining
NuRD	Nucleosome remodeling deacetylase
PAR1	Pseudoautosomal gene-rich region
PC1	Principle component 1
PC2	Principle component 2
PEST	Penicillin-streptomycin
PFA	Paraformaldehyde
POU5F1	POU class 5 homeobox 1
Q-PCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
ROCK	Rho-Associated Coiled-coiled Kinase
ROCKi	Rho-Associated Coiled-coiled Kinase inhibitor
RPKM	Number of reads that map per kilobase of exon model per million mapped reads of each gene
RPM	Revolutions Per Minute
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
SC	Stem Cell
SHOX	Short stature Homeobox gene
SNP	Single nucleotide polymorphism
SOX2	SRY(sex determining region Y)-box 2
SR	Serum Replacement
SSB	Single Strand Break
SSEA4	Stage-specific embryonic antigen 4
TGF- β	Transforming Growth Factors-beta
TLDA	TaqMan Low Density Array
Ub1	Mono-ubiquitylation
WNT3A	Wingless-type MMTV integration site family, member 3A
ZBED1	Zink finger BED domain-containing protein 1

1 INTRODUCTION

Stem cell biology became a key player in developmental research, starting with the research of human infertility and *in vitro* fertilization. The wish of the 2010 Nobel Laureate in Physiology or Medicine Robert G Edwards to grow cell lines from fertilized mouse eggs and through these cells produce blood or nerves *in vitro* was a starting point for this field of research.

Stem cell therapy is now being discussed for possible treatments for many diseases where neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and metabolic diseases such as diabetes along with many others are being widely studied.

Human embryonic stem cell lines derived from the inner cell mass of fertilized embryos, and later also human induced pluripotent stem cell lines originating from somatic cells reprogrammed by using four transcription factors, have both the ability to form all cell types within the human body through the process of differentiation.

This introduction will bring to light the possibilities of these cells and how the different influences that they are subject to may impact their quality, and therefore also impact their future use in the treatment of severe diseases.

1.1 EMBRYOGENESIS

Human development begins when a sperm fertilizes an egg and creates a single cell that has the potential to form an entire organism, a process known as embryogenesis.

Embryogenesis is an event that occurs both in plant and animal development and is the process where the embryo forms and develops until it becomes a fetus. In humans, the fertilization takes place in the ampulla of the oviduct when the spermatozoan penetrates through the zona pellucida of the oocyte, resulting in the formation of the zygote (**Figure 1**).

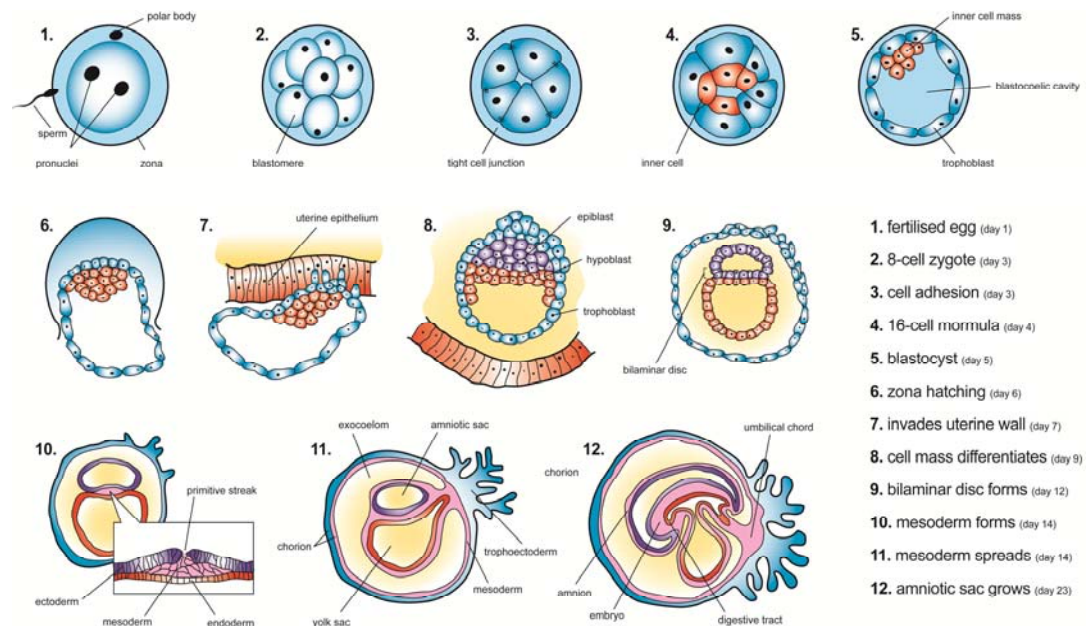


Figure 1. Human embryo development. Embryogenesis starts with the union of a male sperm and a female oocyte, resulting in fusion of their genetic material (1). The zygote undergoes cleavage to increase the number of cells (2). After the 8-cell stage the embryo undergoes compaction where the cells bind tightly together (3). After compaction, the embryo is in the morula stage and cavitation occur where the outermost cell layer- trophoblast secretes fluid into the morula (4). When the number of cells reaches 40-150 the central fluid-filled cavity is formed, called blastocoelic cavity (5). Now the zona pellucida starts to degenerate, allowing the embryo to grow, hatching (6). This stage is reached after approximately 6 days after fertilization and is referred to as pre-implantation stage, and each cell until now is totipotent. Now implantation to the uterine epithelium occurs (8). At day 9 the cell mass starts to differentiate, trophoblast cells will eventually form the placenta, hypoblast (primitive endoderm) will form the extra embryonic tissues such as the yolk sac, and the epiblast also referred to as the primary ectoderm forms all three germ layers of the trilaminar germ disc in a process called gastrulation (8-9). *Illustrated by Emma Nyrell, Modified from www.wikipedia.org.*

1.2 STEM CELLS

A number of functional changes which are important to enhance the mammalian embryo development take place during the short period of implantation in the endometrial wall. Truly naïve embryonic stem cells (ESCs) are found in the transition from morula to blastocyst, and epigenetic reprogramming, such as the re-activation of the paternal X chromosome is essential for its further development¹.

Stem cells (SCs) are non-specialized cells and are found in all multi-cellular organisms, having two distinctive characteristics; they can undergo unlimited cell divisions (mitosis), and they can become more specialized cell types through differentiation. Those specific characteristics make these cells a powerful model system for studies of cellular identity and early mammalian development.

1.2.1 Definition and classification

For a cell to be defined as a SC, there are three major criteria that have to be fulfilled: (1) SCs are unspecialized for any particular function, (2) upon signaling and the right conditions SCs can differentiate into more specialized cell types within the body of a multi-cellular organism, (3) where they can self-renew indefinitely to produce more SCs².

There are four defined types of SCs: two physiological which are present at different stages of life- ESCs and adult stem cells, one engineered or “induced” known as induced pluripotent stem cells (iPSCs), and pathological cells present in cancers having some stem cell properties known as cancer stem cells (CSCs).

ESCs are found in the developing embryo and have the ability to differentiate into all cell types found in the body, but they also maintain the normal turnover of regenerative organs, such as blood and skin. The adult SCs are found throughout the body after development. Unlike ESCs, they can only differentiate to cell types from the organ that they originate, making them multipotent, e.g. a blood SCs can only differentiate to various types blood cells.

There are three stages of differentiation potential among SCs:

Totipotent cells have total potential. They can differentiate into all three germ layers, but also into extra-embryonic tissue (trophectoderm), such as the cells developing into the placenta. The zygote and the first stages of the embryo are the only cells that can be characterized as totipotent³ and it occurs the moment the sperm fertilizes an egg and create a single cell. In the initial hours and days following fertilization, this single totipotent cell divides into more totipotent cells that are exact copies of the original. Evidence of the totipotent nature of these first dividing cells is exemplified by the development of identical twins any time during the first four divisions.

Approximately four days after fertilization, the totipotent cells start to specialize and form a cluster of cells known as a blastocyst (**Figure 1**; illustration 4-5). The blastocyst has yet another group of cells known as the inner cell mass (ICM) and it is these pluripotent cells that will go on to create most of the cells and tissues in the human body.

Pluripotent cells can be isolated, adapted and propagated indefinitely under the right conditions in an undifferentiated state *in vitro*. They are derived from the ICM of a pre-

implantation blastocyst stage embryo or morula stage embryo (**Figure 1**; illustration 5) and have the ability to differentiate into all three germ layers as well as the germ line;

Endoderm forms, e.g., the stomach, liver, pancreas, urinary bladder, epithelial parts of the trachea, lungs and intestines.

Mesoderm forms, e.g., skeleton, skeleton muscles, the dermis of the skin, connective tissue, the heart, blood (lymph cells), kidney and spleen.

Ectoderm forms, e.g., the central nervous system (CNS), the lens of the eye, the ganglia and nerves, the epidermis, hair and mammary glands.

Germ line forms, e.g., oocytes or sperms which carries the maternal or paternal genetic material, having a haploid set of chromosomes.

Unlike totipotent cells, pluripotent cells do not have the ability to form extra embryonic tissue such as trophoctoderm which gives rise to the placenta.

Multipotent cells or adult SCs are found within already specialized tissues and can differentiate into different specialized cell types but are lineage restricted. Hair, skin, bone marrow, central nervous system and male germ cells are examples of where multipotent SC can be found.

1.2.2 Pluripotent Stem Cells

Already in the 1960's stem cells from rabbit cleavage-stage embryos could be grown and the stem cell story began, developed from the beginnings of *In vitro* fertilization (IVF) research when oocytes were matured and fertilized *in vitro*⁴. In **Table 1** a summary of the most important steps of stem cells research can be found in chronological order.

1.2.2.1 *In vitro* fertilization

Fertility is the natural capability to give life, and can be measured by the time taken to achieve pregnancy. In humans, the prevalence of infertility is high, affecting 10-15 % of fertile couples, and infertility is defined as the inability to become pregnant within a year without using contraceptives⁵. The reason for infertility could be caused by either female (one-third) or by male factors (one-third). The last third is due to unexplained causes. Regardless of the cause of infertility these couples can undergo IVF as a treatment in order to conceive children. Fertilized eggs not used in the treatment can be donated to stem cell research.

Table 1. Human IVF and Stem cell development in chronological order.

Modified from Edwards R.G 2001, Nature.

Chronology	
1962	Systematic collection of mature oocytes from patient's ovaries
1962	Rabbit embryo stem cells grown and differentiated <i>in vitro</i> from inner cell mass
1963	Rabbit stem cell lines established
1965	Human oocytes matured <i>in vitro</i> to metaphase 2 and first polar body
1967	Cells injected into mouse blastocysts produce mosaic chimaeras
1968	Rabbit blastocysts sex determined successfully
1969	Fertilization <i>in vitro</i> of human oocyte matured <i>in vitro</i>
1970	Mature oocytes aspirated from human follicles after gentle priming of patients with gonadotrophins
1971	Human fertilization and embryo growth <i>in vitro</i> to expanding blastocysts
1972	Human embryo transfers begin
1977	Human embryos grown to day 9, with huge embryonic discs trumpeting news of stem cells
1978	Birth of first IVF baby
1981	First attempt at producing mouse stem cells
1984	First attempt at producing human stem cells
1998	First permanent human embryonic stem cell line
2006	Induction of pluripotent stem cells from mouse fibroblasts using defined factors
2007	Induced pluripotent stem cell lines derived from human somatic cells
2007	Martin Evans receives the Nobel prize in Physiology or Medicine
2010	FDA approves first clinical trial using human embryonic stem cells
2010	Robert G. Edwards receives the Nobel prize in Physiology or Medicine
2012	Shinya Yamanaka shares the Nobel prize in Physiology or Medicine with John Gurdon

1.2.2.2 Embryonic Stem Cells and their derivation

Already in 1981 the first embryonic stem cells (mESCs) were derived from mouse blastocyst⁶ and could be cultured in the same manner as embryonic carcinoma cells on mouse embryonic fibroblasts (MEF)⁷.

After more than a decade since the derivation of mESCs⁶ and the primate research⁸, the first human ICM were successfully cultured for a few passages⁹ and a few years later the first permanent hESC line were established¹⁰, marking a key historical scientific achievement.

hESCs are most often derived from supernumerary blastocysts (5-6 days after fertilization), donated from patients undergoing IVF (**Figure 1**, illustration 5).

Many different techniques have been used during the years to separate the ICM from the trophectoderm cells¹¹, such as immunosurgery¹², laser isolation¹³ whole blastocyst culture¹⁴ and mechanical isolation, using a small flexible metal needle (0,125 mm in diameter). hESCs can be cultured both on MEFs, mitotically inactivated human foreskin fibroblast (HFFs)¹⁵ or feeder free using various matrices¹⁶⁻²² (**Figure 2**).

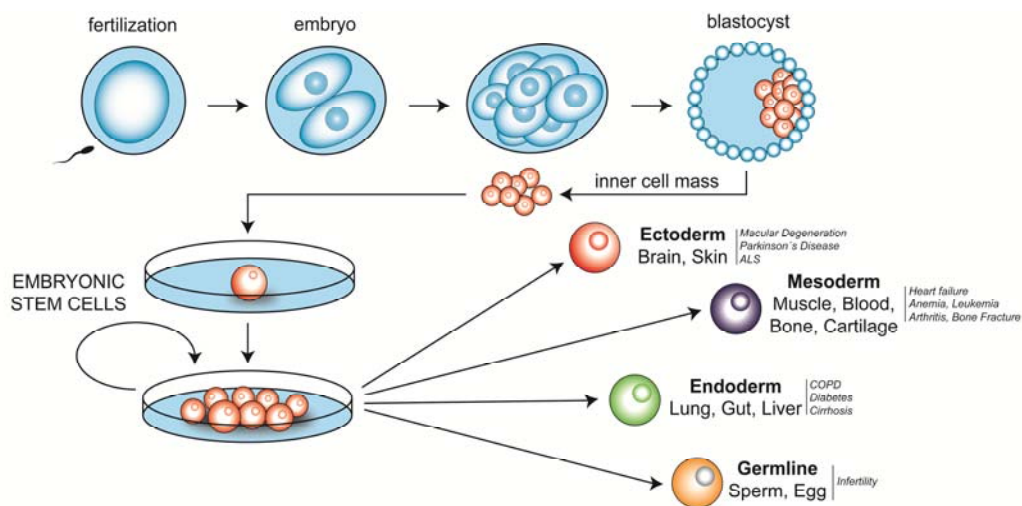


Figure 2. Human embryonic stem cell derivation and their potential. 5-6 days after fertilization the ICM can be isolated and plated on supporting feeder layer or matrix and give rise to a stem cell line with self-renewal potential. These cells can give rise to ectodermal cells, mesodermal cells, endodermal cells as well as the germline. *Illustrated by Emma Nyrell.*

Regardless if the cells are being cultured using supporting cells or a matrix they can be passaged in various ways. Traditionally hESCs, and later hiPSCs, can be passaged mechanically using a surgical scalpel, or enzymatically using trypsin-like solutions. Regardless of their passaging method the cells usually form colonies, each colony consisting of approximately 20 000 SCs. If they are grown on fibroblast the cells tend to follow the fibroblast, resulting in an elongated shape (**Figure 3A**), while cells grown on a matrix have a more circular shape (**Figure 3B**). Typically the morphology of both hESC colonies and hiPSC colonies is flat and compact, with sharp edges, and in high

magnifications it is possible to see that the individual cells have a large nucleus and small cytoplasm (**Figure 3C**).

To control pluripotency in hESC cultures exogenous basic fibroblast growth factor-2 (bFGF2) is supplemented to the culture medium. FGF signaling promotes hESC self-renewal and is involved in blockage of extra-embryonic differentiation²³⁻²⁴. It has also been shown that bFGF cooperate with activin signaling through SMAD2/3 to activate NANOG expression²⁴⁻²⁶.

It has been discussed whether ES cells are trapped in development or if they are simply a result of their culture conditions²⁷. By single-cell RNA sequencing of individual OCT4-positive or OCT4-negative ES single cells, followed by monitoring the progress towards ES cells, signatures of undifferentiated ES cells clearly differ from the cells of the ICM. During the transition from ICM to ES cells, the normal development is changed and the cells acquire the capacity for unlimited self-renewal and the capacity to differentiate towards the three germ layers²⁸.

OCT4, SOX2 and NANOG have been identified as key transcription factors with essential roles in early development and they are also required for the propagation of undifferentiated SCs in culture²⁹⁻³². These key transcription factors have been shown to occupy promoters for many important developmental regulators in human ES cells³³, making it possible to activate or turn off genes to reach a certain developmental state of a cell, e.g. switching off genes involved in differentiation of pluripotent cells, or the other way around, activating genes involved in specific cell types and inactivating genes associated with pluripotency.

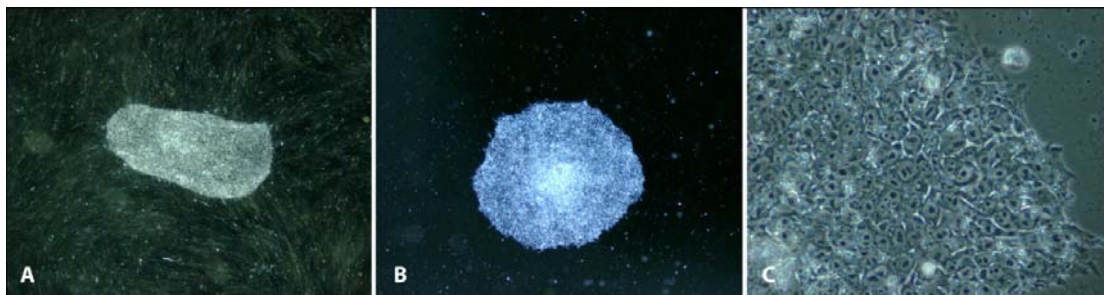


Figure 3. Typical hESC colonies. (A) hESC colony cultured on human foreskin fibroblasts, 40 x magnification. (B) hESC colony cultured on Matrigel, 40 x magnification. (C) Typical morphology of hESC with large nucleus and small cytoplasm, 400 x magnification. *Holm, F unpublished.*

1.2.2.3 Induced Pluripotent Stem Cells

By forced over expression of embryonic transcription factors, most somatic cells in the body can be reprogrammed into a pluripotent state. These stem cell-like cells are called induced pluripotent stem cells (iPSCs). This was first achieved in mouse adult cells³⁴, and later in human cells by two separate research teams³⁵⁻³⁶. To find the optimal set of transcription factors that could reprogram somatic cells into pluripotent cells, twenty-four candidates of transcriptional regulators were transduced into the host genome (MEFs) by retroviral delivery³⁷. The factors were eliminated one by one, and a set of four core genes were identified consisting of OCT4, SOX2, KLF4 and c-MYC (known as the “Yamanaka factors”). Almost simultaneously James Thomson and his team also

succeeded in the generation of iPSCs but by using a lentiviral system comprising a different set of transcription factors, namely OCT4, SOX2, NANOG and LIN28³⁶.

Similar to ES cells, these cells express genes and surface proteins specific for pluripotency, they generate teratomas when injected subcutaneously into immune comprised mice as well as contributing to different tissues of developing embryos upon blastocyst injection³⁴⁻³⁵. These characteristics make them an excellent tool to study developing human diseases and providing an autologous cell source with potential for use in cell-based therapies. iPSCs have been derived from many different somatic cell populations, such as neural cells³⁸, melanocytes³⁹, stomach and liver cells⁴⁰, keratinocytes⁴¹, lymphocytes⁴²⁻⁴³ and pancreatic β cells⁴⁴. This technique has also been applied in various species such as rat⁴⁵⁻⁴⁶, monkey⁴⁷, pig⁴⁸, dog⁴⁹ and rabbit⁵⁰, demonstrating that fundamental features of transcriptional network governing pluripotency remain conserved during evolution⁵¹.

1.2.3 Differentiation

If you look closely at the human body, it is clear that not all cells are alike. For example, cells that make up our skin are certainly different from cells that make up our inner organs. Yet, all of the different cell types in our body are all derived from a single, fertilized egg cell and made possible through differentiation, and each of the different cells in our body contain the same set of approximately 20 000 genes.

Differentiation is the process where an unspecialized cell, such as the embryonic stem cell, becomes specialized into one of the many cells that make up our body, such as a heart, liver or muscle cell. During differentiation, certain lineage-specific genes are turned on, or become activated, while other genes are switched off, or inactivated, such as OCT4 and NANOG. This process is intricately regulated. As a result, a differentiated cell will develop specific structures and acquire qualities to perform certain functions. For example, OCT4, NANOG, SOX2 and KLF4 are part of a core pluripotency network that serves two main purposes, (1) the repression of genes associated with differentiation, and (2) the activation of ESC-specific targets^{33, 52}.

The suppression of pluripotency factors in pluripotent SCs is associated with the recruitment of repressive chromatin modifying complexes, such as the NuRD complex⁵³ and the Polycomb complex⁵⁴, while the activation of pluripotency factors may be associated with chromatin remodeling factors, such as the SWI/SNF complex. These core factors are expressed in ESCs and turned off when the cells differentiate and they are highly enriched for active chromatin marks. The interplay between self-renewal and differentiation in pluripotent SCs is a direct reflection of the levels of active marks such as H3K4me3, and repressive marks such as H3K27me3.

By purposely allowing pluripotent SC to differentiate, both in vitro and in vivo, it is possible to verify whether the cells are truly pluripotent, possessing the ability to differentiate towards all three germ layers.

1.2.3.1 *In vitro differentiation*

By culturing aggregates of pluripotent SCs in suspension without the “anti-differentiation factor” bFGF cells are allowed to undergo differentiation, forming embryoid bodies (EBs). After approximately 3 weeks in culture the cells starts to form three-dimensional spheroid structures. Without the bFGF, this spheroid structures are capable of responding to similar cues that direct embryo development, forming tissues resembling native tissue structures.

EB formation is often used to spontaneously differentiate cells toward all three germ layers, in order to control the pluripotent state of SCs, but can also be used together with specific factors for a more specific and directed differentiation.

1.2.3.2 *In vivo differentiation*

By injecting pluripotent SCs subcutaneously or beneath the testicular capsule of severe immunodeficiency (SCID) mice the SCs will start to form encapsulated tumors, teratomas, which contain tissue components of all three germ layers. The teratomas are usually harvested within a few weeks after implantation of the SCs and analyzed by sectioning and staining.

The ability of pluripotent SCs to form these noncancerous tumors is one of their defining traits, and instead of originating from a single abnormal cell, they originate from a group of normal cells. By comparing gene expression in undifferentiated cells, embryoid bodies and teratomas it was found that the gene survivin (BIRC5) seemed to contribute to tumor formation⁵⁵. This gene is located on chromosome 17, which also is one of the genomic regions that are found to be amplified when ES cells change in culture (culture adaptation) towards being precancerous⁵⁶⁻⁵⁷.

The possibility for tumor formation of transplanted pluripotent SCs is a major safety concern which has to be addressed before using the cells clinically.

1.2.4 Molecular mechanisms of pluripotency

Pluripotency is a complex process established by genome-wide reprogramming during mammalian pre-implantation development, where cells are given the ability to give rise to all the tissues of the adult body. Pluripotency has been called the transitory state of embryonic cells and exists only during a brief window of development. The molecular mechanisms that control pluripotency are important for improving our understanding of development, and therefore understanding defects in development which may cause many diseases. Hopefully this understanding may eventually lead to new therapies.

Pluripotent SCs have a gene expression program that allows them to self-renew but still remain poised to differentiate as a response to developmental cues.

1.2.4.1 Extracellular factors

In human pluripotent SC cultures, one of the most important extrinsic factor is bFGF2 which was the first factor found to be crucial for maintaining hESCs in culture, resulting in the incorporation of this factor in many chemically defined media to improve hESC cultures⁵⁸.

bFGF2 acts through surface receptors on pluripotent SCs leading to activation of Receptor Tyrosine Kinases (RTKs) ERK1 and ERK2 which mediates signals important for pluripotency and self-renewal (**Figure 4**). Inhibition of this pathway leads to differentiation²⁴. The precise biological action of bFGF2 in hESCs is unclear, although there is evidence that it maintain SC phenotype rather than promoting proliferation and cell death. Another role of bFGF2 is to suppress Bone Morphogenic Protein (BMP) signaling and therefore inhibit differentiation⁵⁹.

Another important factor is Transforming Growth Factor beta (TGF- β). The TGF- β super family consists of signaling proteins that mediate a broad range of biological effects by binding to cell-surface receptors, including activin and nodal, growth differentiation factors (GDFs) and BMPs, all of which are involved in maintaining SC state. Activin and nodal, which signal through the same receptors, have been shown to suppress the differentiation of hESCs^{25, 60-61}. These two signaling factors together activate the transcription factors SMAD2 and/or SMAD3 which binds to the promoter of the gene encoding the key pluripotency transcription factor NANOG in hESCs. Therefore, blockage of TGF- β results in differentiation. On the other hand, SMAD1, SMAD5 and SMAD8 which are activated by BMPs have the opposite effect and inhibit NANOG expression by binding to its promoter²⁶ (**Figure 4**).

The Wingless-type MMTV integration site family (WNT)-mediated signaling's main effect on hESCs is to enhance proliferation⁶² and it also allows short-term maintenance of hESCs⁶³, while TGF- β and bFGF2 are required for long-term maintenance^{60, 64}.

1.2.4.2 Intracellular factors

Much effort has been aimed at identifying the genes that are essential for stemness (essential characteristics of a stem cell that distinguish it from ordinary cells). In the pluripotency network, the most important factors are NANOG, OCT4/POU5F1, KLF4 and SOX2. These four transcription factors interact and regulate the expression of multiple genes³³ and have essential roles in early development and are required for the propagation of undifferentiated hESCs in culture.

To further understand how NANOG, OCT4/POU5F1 and SOX2 control pluripotency and self-renewal in pluripotent SCs, a genome-scale location analysis (chromatin immunoprecipitation coupled with DNA microarray) were used to identify the target genes of all three regulators *in vivo*^{33, 52}. The results revealed that these three factors co-occupy the promoters of a large population of genes where many of these target genes encode developmentally important homeodomain transcription factors, and that these regulators contribute to specialized regulatory circuits in hESCs³³. The key concept of how the core transcription factors control ESC state is through positive regulation of their own promoters, where they form an interconnected auto-regulatory loop. They co-occupy and activate expression of the genes which are necessary to maintain ESC state, while contributing to repression of genes encoding lineage-specific transcription factors whose absence keep the cells from undergoing differentiation⁶⁵.

NANOG is a transcription factor known to be important in early embryogenesis and pluripotency^{31, 66-67} and it has been shown that Nanog null mice die before implantation⁶⁶. It has also been used as one of the factors when reprogramming somatic cells towards a pluripotent state, however it does not need to be included as one of the essential exogenous factors which could be explained by OCT4/POU5F1 and SOX2 activation of endogenous NANOG³⁴⁻³⁵.

OCT4/POU5F1 is a transcription factor which is also known to play an important role in pluripotency^{29, 33, 68}. OCT4/POU5F1 has also been used for reprogramming, both alone and together with other transcription factors^{34-36, 69-71}. Mouse embryos lacking the Oct3/4 gene do not develop normal ICM, instead they develop cells with a trophodermal fate⁷².

The transcription factor SOX2 is indispensable for pluripotency in hESCs⁷³ and Sox2 null mouse embryos are incapable of forming normal epiblast and therefore they do not survive³⁰. Together with both NANOG and OCT4/POU5F1, SOX2 is one of the factors used for reprogramming somatic cells towards pluripotency³⁴⁻³⁶.

Proteins which are associated with the core transcription factors OCT4, NANOG, SOX2 and KLF4 include the SWI/SNF complex. This complex of enzymes is found in all cells and has ATPase activity, which means it breaks down ATP (the basic unit of energy currency). It is involved in chromatin remodeling, where its primary function is to aid gene expression. This is achieved by shifting or altering the positions of the nucleosomes, resulting in the exposure of DNA allowing e.g. transcription factors to bind, and therefore increase transcription.

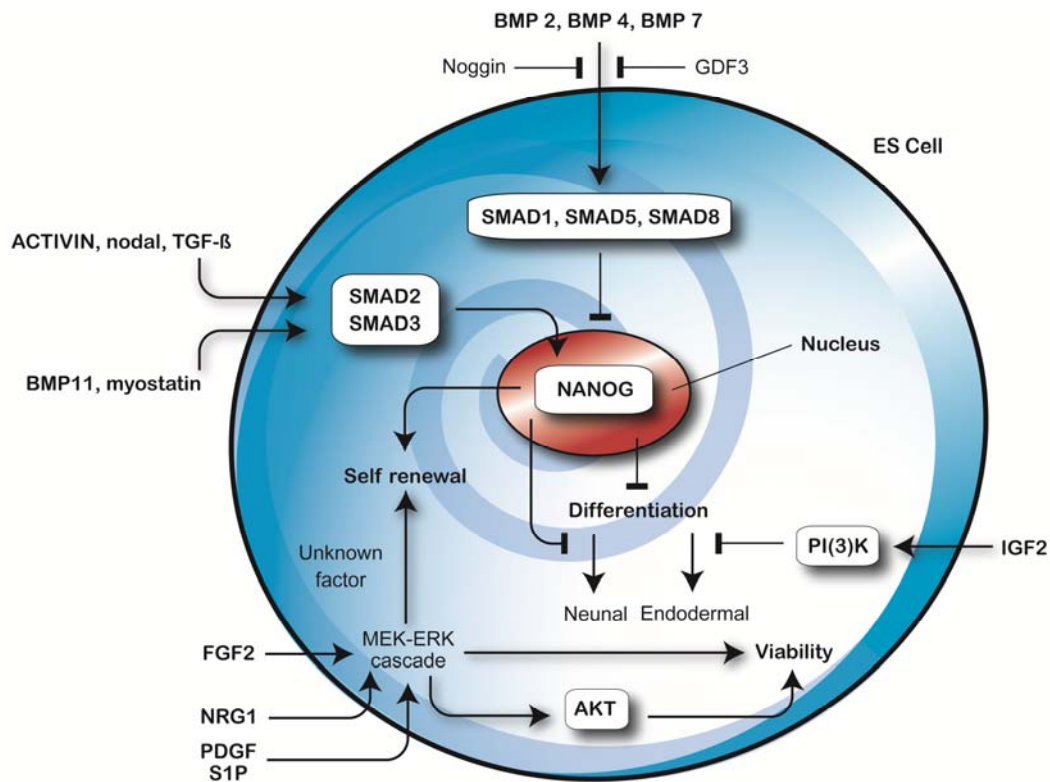


Figure 4. Extrinsic signals affecting self-renewal, differentiation and viability. Signaling mediated by members of the TGF- β family, such as TGF- β , activin and nodal, GDFs and BMPs-converges mainly on NANOG, which maintains ES cells in an undifferentiated state with the ability to self-renew. Signaling activity mediated by the MEK-ERK receptor tyrosine kinase cascade allows self renewal of ES cells and maintains their viability (through inhibiting apoptosis and anoikis). In addition, IGF2-mediated signaling through PI(3)K inhibits ES cells from differentiating into endodermal lineage cells. WNT-mediated signaling might affect these cell-fate decisions, but its role is controversial at present. *Illustrated by Emma Nyrell, Modified from figure 2 Pera, M et al., 2010 Nature.*

1.2.5 Chromatin structure in pluripotent stem cells

In order to fit into the nucleus, the approximately two meters of DNA that each cell in our body contains is packed into chromatin⁷⁴. The packing of chromatin is variable, with euchromatin representing open, gene rich, transcriptionally active regions. In contrast, heterochromatin represents condensed regions with low gene density but high levels of repetitive sequences⁷⁵⁻⁷⁶. The nucleosome is the fundamental unit of chromatin, acting as spools of which 146 base pairs (bp) of DNA is wrapped around, consisting of an octamer of the histones, namely H2A, H2B, H3 and H4, two of each. Between two nucleosomes, the histone H1 acts as a linker, which initiates higher-order chromatin structures (**Figure 5**).

In this way, eukaryotic DNA is packed into highly compacted chromatin. There are three levels of chromatin organization:

DNA wraps around histone proteins forming nucleosomes; the “beads on a string” structure (euchromatin). Euchromatin is less condensed and more accessible.

Multiple histones wrap into a 30 nm fiber consisting of nucleosome array in the most compact form (heterochromatin). Heterochromatin is highly condensed and inaccessible.

Higher-level DNA packaging of the 30 nm fiber into the metaphase chromosome (during mitosis and meiosis).

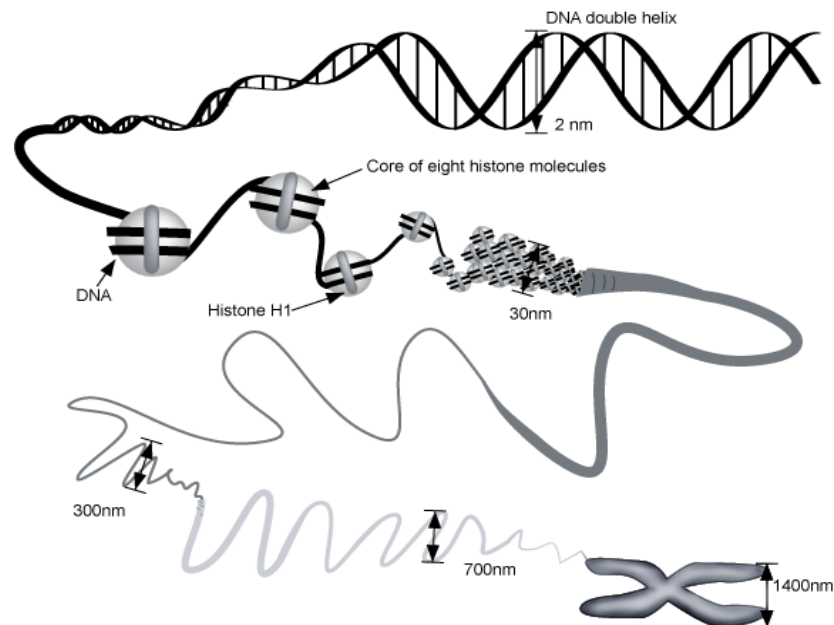


Figure 5. Schematic illustration of DNA packing. The DNA winded around the eight core histones and packed in to the chromosome. Normally each cell in the human body contain 46 chromosomes, 23 paternal and 23 maternal. *Illustrated by Benjamin Tengzelius.*

Chromatin undergoes various forms of structural change to alter DNA packing. Alteration to the chromatin structure that do not affect the sequence of the genome are

called epigenetic modifications, including methylation of DNA, post-translational modifications (PTMs) of histones (often referred to as histone marks), histone variants incorporated into nucleosomes as well as the action of proteins binding to DNA and chromatin remodeling complexes. Also non-coding RNA is involved in epigenetic changes through X chromosome inactivation⁷⁷. Modifications on specific residues, e.g. lysine (K) include acetylation (ac), mono-methylation (me1), di-methylation (me2) and tri-methylation (me3), and mono-ubiquitylation (ub1) which modify chromatin structure and control the access of various enzymes. Acetylation can result in the loosening of the chromatin and lends itself to replication and transcription. Lysine acetylation is generally associated with gene expression, whereas lysine methylation can lead to either gene activation or repression, depending on the residues involved.

Chromatin structure can be distinguished through a microscope by densely packed nucleosomes, heterochromatin, or sparsely packed chromatin, euchromatin.

The chromatin state of undifferentiated stem cells are being recognized as open and hyper dynamic compared to somatic cells, with a progression towards a more compact and repressive chromatin structure upon differentiation.

Heterochromatin is distinguishable molecularly by “repressive” modifications, such as H3K9me3 and H3K27me3⁷⁸.

1.3 MICROENVIRONMENTAL EFFECTS

The maintenance of undifferentiated SCs for prolonged periods and the extensive cultures of SCs need to meet the needs for clinical use. Genetic and epigenetic changes are a direct response to the microenvironment which the cells are exposed to.

Despite the many common characteristics among cell lines, it is now known that there may be large variations between the different cell lines, which could be an effect due to the influence the specific cell line is subject to from its microenvironment.

To enable the use of these cells in the future, one major hitch to overcome has been to cryopreserve SCs in a manner where they can be stored long term and thawed quickly with minimum damage and with minimal impact to the cells pluripotent state. It has been challenging to obtain high survival rates after thawing cryopreserved hESC and later also iPSCs, which have been of importance due to the rapid development within the field suggesting a broad use both in research and therapeutically. Recently there has been clear improvements in regard to techniques and media to cryo-store both small and large amounts of stem cells and the focus has slowly transferred from sole survival of the stem cells to chemically defined and xeno-free cryopreservation systems suitable for GMP making the cells transferrable for clinical use.

Once a suitable protocol exists for preserving cells while awaiting clinical use, another issue is how different culture techniques and long term culturing influences and affect the cells in regard to their pluripotency, especially gene expression as well as acetylation and methylation of the histones, when inhibiting the Rho-associated coiled-coil kinase (ROCK) signaling pathway, which is a pathway involved in many cellular processes, one of them is apoptosis. This led to the interest of inhibiting ROCK in cell cultures to increase the survival rates when the cells are dissociated from each other.

To identify valid hESC lines and hiPSC lines to further clinical use it is important to investigate how shift or altering of the position of the nucleosomes so that the DNA is exposed allowing e.g. transcription factors to bind and therefore increase transcription which can affect the characteristics of the cells, and if it correlates to the gene expression of the same factors.

Another concern when keeping cells in culture for many passages is that it might result in the selection of cells with genetic changes which often results in growth advantages by acquired mutations. These mutations, called copy number variations (CNVs) may range from about 1 kilo base (1,000 nucleotide bases) to several mega bases in size.

The microenvironmental effects on pluripotent SCs, ranging from preserving cells in an effective “stand-by” position ready to be used for patients, to the effect on the cells due to culture techniques, long-term culture and differentiation. This and many other aspects of pluripotent SCs have to be addressed before using these cells clinically.

1.3.1 Cryopreservation

Cryopreservation is the process where cells or whole tissues are preserved by cooling to sub-zero temperatures, $-196\text{ }^{\circ}\text{C}$, which is the boiling point of liquid nitrogen (N_2). These low temperatures are effectively stopping any biological activities within a cell. Two techniques are so far available for cryopreservation of pluripotent SCs: vitrification and slow freezing-rapid thawing. The most challenging and the mostly performed technique is vitrification using open pulled straws, in which a small amount of SCs can be frozen using high concentration of cryoprotectants in order to quickly obtain a glass-like structure⁷⁹⁻⁸⁰. During slow freezing-rapid thawing larger amounts of SCs can be frozen using lower concentrations of cryoprotectants, in cryo vials.

To avoid permanent damage to cells, different cryoprotectant agents are being used, and are presumed to provide an indefinite durability to cells in sub-zero temperatures. Many other biological fields have been using cryo-storage as a possibility to transfer and long term store valuable material, and it became clear that this knowledge could be used also for SCs.

However, living tissues or cells mostly consist of water (H_2O), and when being cooled to below the freezing point water molecules gather together and form ice crystals, damaging the cells by dehydration (migration of water out of the cells) as ice is formed between them.

The ice formation begins in the intracellular spaces, where the osmotic pressure of the ice is lower than the osmotic pressure of the solute water in the surrounding cells and as heat is removed at the freezing point of the solutions, ice crystals grow between the cells, extracting water from them⁸¹ (**Figure 6**).

As ice crystals grow the volume of the cells shrink, and the cells are crushed between the ice crystals. When the cells shrink, the ice also squeezes the solutes inside the cells and concentrates it in the remaining water, increasing the intracellular ionic strength and interfering with the organization of the proteins and other intracellular structures. This eventually lead to the solute concentration inside the cells reaching the eutectic state (mixture of substances having a minimal freezing point) and freezes⁸² (**Figure 6**). The final stage of frozen cells is pure ice in the former extracellular spaces, and inside the cell membranes a mixture of concentrated cellular components in ice and bound water. Without cryoprotectants, this process would not be reversible.

By the addition of a cryoprotectant to the solution it can help prevent water molecules from gathering together to form ice, this way, the molecules start moving slower and slower as they are cooled, instead of freezing altogether. When the temperature reaching below $-100\text{ }^{\circ}\text{C}$ the molecules become locked in place and a solid is formed. Water that becomes solid without freezing is called “vitrified”.

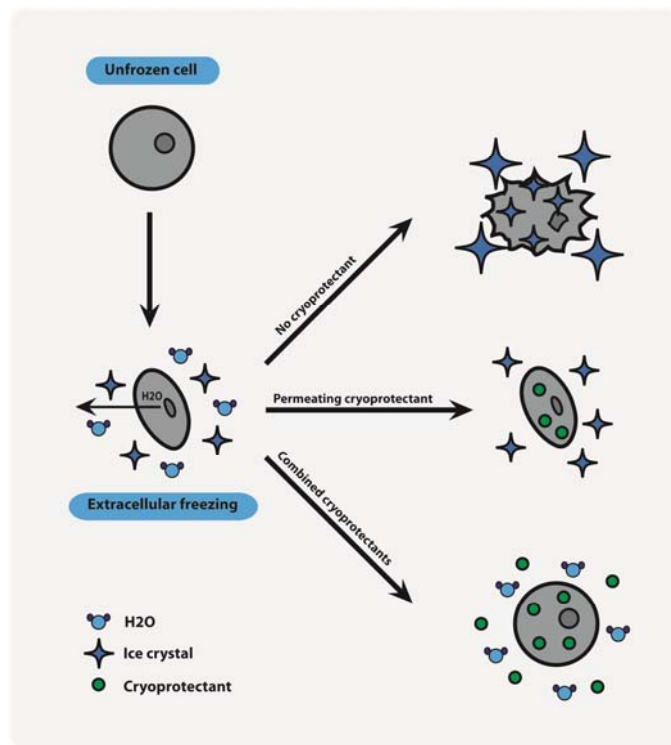


Figure 6. Cryopreservation. Between approx. -5°C and -15°C , ice forms in the external medium, however the cell contents remain unfrozen because the plasma membrane blocks the growth of ice crystals into the cytoplasm. The cooled water in the cells has a greater chemical potential than the water in the partly frozen solution outside. The difference in potential causes water to flow out of the cell osmotically and freezes externally. When no cryoprotectant is added to the cells, the water molecules gather and form growing ice crystals. First ice forms outside the cells, which causes the cells to dehydrate and shrink, leaving the cells to be squashed between ice crystals. When adding a permeating cryoprotectant like DMSO, water molecules surrounding the cells move slower and slower, and finally lock in place to form a solid, with less damage to the cells. By adding both a permeating and non-permeating cryoprotectant, it is possible to control ice crystalization at both extracellular and intracellular level.

Cryopreserving pluripotent SCs has resulted in low survival rates and loss of SC identity such as proliferation, differentiation potential and also pluripotency, an important feature of ESC.

The results did not improve despite that all the other fields in SC research quickly moved forward. Just as having optimized clinical grade cultures, it is essential to obtain efficient clinical grade cryopreservation systems for hESCs and hiPSCs, which led to a combined effort within the cryo-storage field and which progressed during the 20th century.

Since the beginning, cryopreservation of these cells included culture medium containing 10% dimethylsulfoxide (DMSO) as the only cryoprotectant (permeable) where large amount of cells have been cryo stored in one vial. This method has been improved during the years using various solutions⁸³.

Both hESCs and iPSCs are highly sensitive to cryo-injury caused by cryopreservation which has limited its utility and thus led to extensive studies recent years^{80, 83-88}.

By using a medium that is completely serum and animal substance free, containing DMSO, anhydrous dextrose and a polymer as cryoprotectant instead of using only one cryoprotectant, DMSO, the survival rates have increased. The use of combinations of cryoprotectants in hESC banking has not been reported earlier in the SC biology field and this procedure can be conducted without dedicated instrumentation which will facilitate its use.

1.3.1.1 Cryopreservation using STEM-CELLBANKER

STEM-CELLBANKER is a defined cryopreservation medium which contains 10% DMSO, glucose and a high polymer working as a secondary extracellular cryoprotectant, as well as NaCl, KCl, Na₂HPO₄, HK₂PO₄, NaHCO₃ as pH adjustors to maintain the cell function. These ingredients are all dissolved in phosphate-buffered saline (PBS).

For optimal dehydration and minimal ice crystal formation, the medium contains both permeating and non-permeating cryoprotectants. The cells can be frozen directly at -80 °C, without a programmed freezer. After 24 hours the cells should be transferred to N₂ tanks.

This system using a defined freezing–thawing system offers an excellent simple option for banking of hESCs and iPSCs, allowing large cell numbers to be frozen in one vial, resulting in outstanding cell survival after freezing. When comparing this novel method with the standard method (10% DMSO), the cells had a high viability (90–96%) without any impact on proliferation and differentiation, compared with the standard freezing procedure where viability was much lower (49%)⁸⁹.

1.3.1.2 Thawing using CELLOTION

When a cell has avoided lethal damage during cooling to low sub zero temperatures, they must still survive series of physical-chemical challenges associated with warming/thawing. The rate of thawing can exert effects on survival which is dependent on whether the prior rate of cooling has induced intracellular damage or dehydration. If the cells have not been killed outright, the ice crystals tend to be small, and therefore fuse to form larger crystals during thawing, the process is called re-crystalization and is often damaging to the cells if the crystals are too big from the beginning, resulting in the cells breaking.

CELLOTION™ is a chemically defined washing solution containing NaCl used when cells frozen with STEM-CELLBANKER are being thawed. It is a unique solution free from serum, proteins and sugars, which enables cells to sink to the bottom when being centrifuged and therefore minimizes the cells lost during washing steps.

1.3.2 Passing human pluripotent stem cells

Pluripotent SCs can be expanded essentially for an indefinite period in culture and kept in a pluripotent state. In order to keep the cells from undergoing spontaneous differentiation they need to be passaged every fourth to seventh day, meaning that colonies are divided into smaller pieces and transferred to fresh culture dishes. Passaging of pluripotent stem cells can be compared to manual “cutting and pasting” of colonies. Various passaging techniques for hESCs and hiPSCs have been established^{15, 90-94} to facilitate their use, for example mechanical passaging using a surgical scalpel or cell scraper¹⁶, and enzymatical passaging using trypsin-like solutions to dissociate cells into single cell suspensions.

1.3.2.1 Mechanical passaging

Mechanical passaging is usually performed either with a surgical scalpel, or a cell scraper¹⁶. This method is one of the most widespread methods for passaging of pluripotent SCs. With this technique undifferentiated SC colonies are cut into smaller pieces, separated based on their morphology, making it possible to avoid any differentiated cells. This manual procedure is very laborious and time-consuming, but most importantly it is difficult to obtain high enough numbers of cells needed for many of the techniques used in research.

1.3.2.2 Enzymatical passaging

Enzymatical passaging techniques using solutions such as TrypLE Select/Express⁹⁵, Trypsin-EDTA⁹⁶⁻⁹⁸ or Accutase⁹⁹ to dissociate the cells into single cells have been widely used. Enzymatical passaging give rise to larger amount of cells in a shorter time frame, compared to mechanical passaging⁹⁵. However, pluripotent SC have shown to lose their cell-cell interactions when being dissociated from each other, resulting in higher cell death during passaging. Groups of cells communicate with each other by cell-cell interaction through the Notch signaling pathway. The Notch protein has a large extracellular domain and a smaller intracellular domain, and ligand proteins bind to the extracellular domain and after proteolytic cleavage the intracellular domain is released and enters the nucleus where it engages other DNA binding proteins and can modify gene expression¹⁰⁰. Almost all ligands are trans-membrane proteins; therefore the receptor can only be triggered through cell-cell contact making it possible for groups of cells to influence one another to make large structures.

To overcome these problems which occur when cells lose their cell-cell interaction, using a ROCK inhibitor (ROCKi), Y-27632 was necessary¹⁰¹.

1.3.2.3 Rho-associated coiled-coil kinase (ROCK)

Rho is a member of the superfamily Ras and act through a number of different proteins, most importantly ROCK. Activation of the Rho pathway occurs when G-protein-coupled receptors (GPCR), growth factor receptors (GFR) and integral membrane proteins (e.g. integrins) transport signals into the cytoplasm through the membrane. In the membrane GDP is exchanged for GTP in order to be activated, this process is catalyzed by RhoGEFs. GTP-bound Rho activates the kinase domain of ROCK by binding to it, allowing ROCK to regulate effectors located downstream, including myosin light chain (MLC) and LIM (**Figure 7**).

To inhibit the signaling cascade, Rho-GAPs enhance the conversion of GTP-GDP and thereby inactivate Rho and the pathway, the alternation between the active and inactive state is regulated by guanine exchange factors (GEFs) and GTPase-activation proteins (GAPs). The ability of Rho to alternate between GDP-bound inactive state and a GTP-bound active state allows Rho to control downstream signal transduction events.

SCs undergo high cell death when they are dissociated from each other which are believed to be due to the loss of their cell-cell interactions. To solve this issue ROCKi, has been widely used¹⁰¹, where it is added to culture prior to passaging, as well as the first 24 hours after passaging.

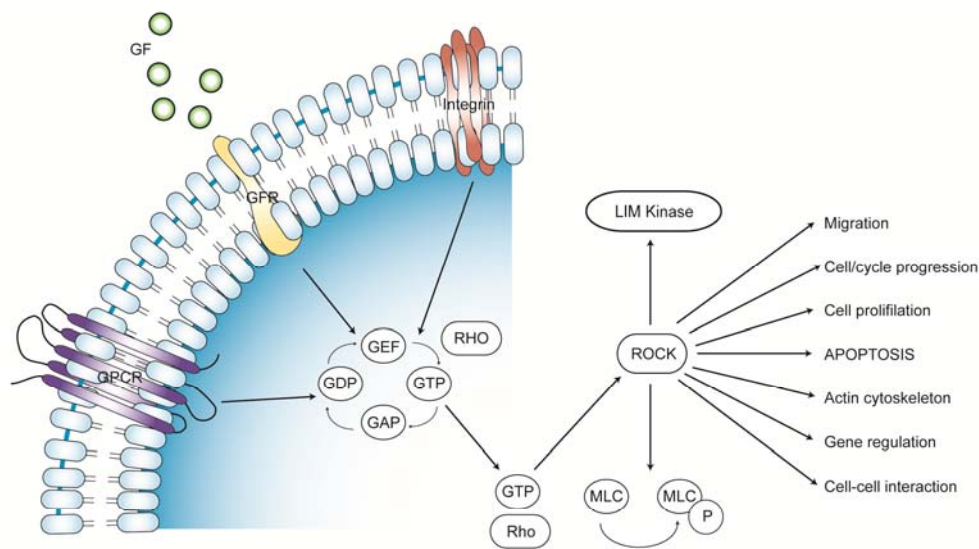


Figure 7 Rho/ROCK signaling cascade. GPCR, GFR or integrins can activate the Rho pathway. When the signal for activation reaches Rho, it is transported to the plasma membrane where it is activated by exchange of GDP to GTP, catalyzed by RhoGEFs. Rho with the GTP binds to and activates the kinase domain of ROCK allowing ROCK to regulate effectors such as MLC and LIM kinase. This signaling cascade can be inhibited by RhoGAPs convert GTP-GDP and thereby inactivating Rho and its pathway. *Illustrated by Emma Nyrell.*

1.3.3 Histone acetylation and methylation

The N-terminal tails of histones extend outwards from the nucleosome particle (**Figure 8**), and are the sites for regulatory modification. Histone methylation and acetylation typically takes place on arginine or lysine amino acid residues in the protein sequence. Histones that for example are methylated on certain residues can act epigenetically to repress or activate gene expression. Histone modifications that are associated with active transcription such as acetylation of histone H3 and histone H4 and di- and trimethylation of lysine 4 on histone 3, are commonly referred to as euchromatin marks, whereas modifications that are associated with inactive genes, such as methylation on lysine 9 and lysine 27 on histone H3 (H3K9, H3K27) are referred to as heterochromatin marks.

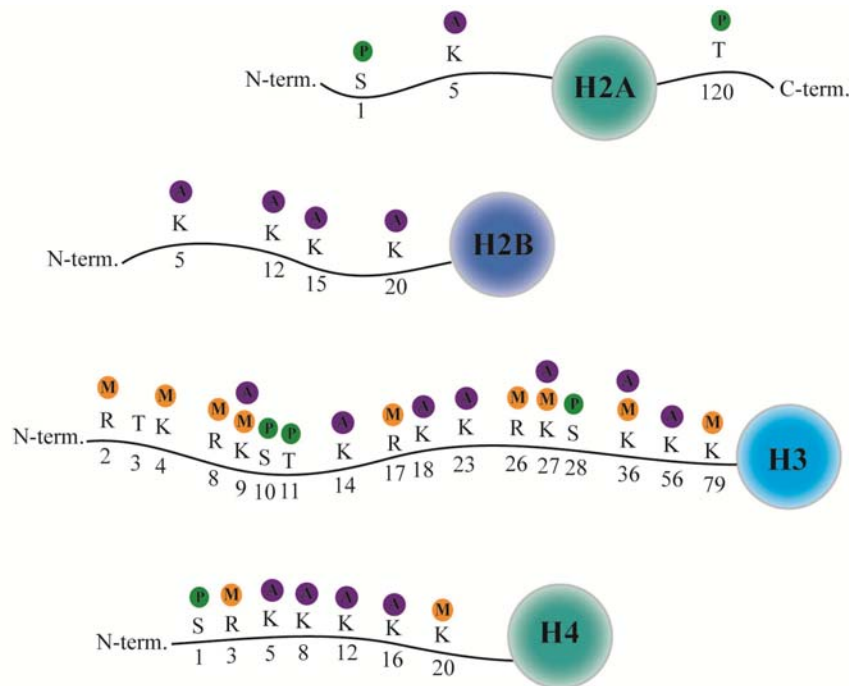


Figure 8. Schematic representation of the N-terminal tails and C-terminal tails on histone H2A, H2B, H3 and H4. Posttranslational modifications of histones include acetylation (A), methylation (M) and phosphorylation (P). Some lysine residues can be either acetylated or methylated.

Acetylation of histones has two well-characterized functions; (1) causes local changes in the charge between DNA and the histone proteins that disrupt the chromatin structure. The addition of acetyl groups neutralizes the positive lysine residues and therefore reduces the histone interaction with DNA which is negatively charged. (2) Serves as platforms to bind proteins.

Histone acetylation is mediated by histone acetyltransferase complexes (HATs)¹⁰². A rule of thumb is that acetylation of histones leads to an open and transcriptionally permissive chromatin, and increased acetylation of histones has been correlated with increased gene expression¹⁰³⁻¹⁰⁴.

Histone acetylation in gene promoters is reversible which can be achieved by the recruitment of histone deacetylase (HDAC) that remove the acetyl groups, limiting access to DNA and therefore repressing transcription¹⁰³⁻¹⁰⁴.

Unlike acetylation, methylation has no electrostatic activity. Instead it alters the properties of the nucleosomes and affects the interaction with proteins. There are three forms of histone methylation, and it is the modification of certain amino acids on the histone proteins, caused by the addition of one (mono), two (di) or three (tri) methylgroups. This leads to transcriptional activation or repression of genes.

1.3.3.1 Chromatin remodeling complex

The ATP-dependent nucleosome remodeling complex SWI/SNF, was the first chromatin remodeling proteins to be identified in two independent screens of mutations affecting mating-type switching and growth on sucrose, and the name SWI/SNF comes from switching defective or sucrose nonfermenting¹⁰⁵⁻¹⁰⁶.

SWI/SNF is recruited by transcription factors and modified histones to the promoters of genes, where they enhance or reduce the access of transcriptional components to DNA sequences with resulting positive or negative effects on gene activity.

BRG1, is the catalytic subunit of SWI/SNF and have been shown to interact with γ -H2AX nucleosomes by interacting with acetylated histone H4. Histone H4 acetylation is required for the binding of BRG1 to γ -H2AX nucleosomes.

1.3.3.2 Double-strand break

Double-strand breaks (DSBs) are the most challenging lesions to repair, because they physically cleave the DNA strand. They arise through both replication errors and exogenous events such as exposure to ionization radiation. The mechanism of DSB repair is the phosphorylation of histone H2AC (γ -H2AX) and loading of DNA repair proteins onto the chromatin adjacent to the DSB. The open relaxed chromatin structure is created through the coupled action of SWI/SNF ATPase and histone acetylation by HATs

For a number of intracellular processes, such as DSB repair, it is necessary for proteins to access the chromosomal DNA. This is regulated by two general chromatin modifier mechanisms, including histone modification and alteration of the nucleosomal position¹⁰⁷.

SWI/SNF (among other chromatin complexes) has been shown to be recruited to a DSB in such a way to facilitate DNA repair and/or modulate checkpoint activation¹⁰⁸.

Mammalian SWI/SNF has been shown to target the chromatin at DSB sites and facilitate DNA repair¹⁰⁹.

Acetylation of histones located at DSB sites by HATs is a critical chromatin modification required for DSB repair. In particular, the N-terminal lysine residues of Histone H3 and H4 are acetylated during DSBs. In addition to HATs, SWI/SNF chromatin remodeling complexes are recruited to DSBs. ChIP analysis showed that DSB-induced acetylation of lysine 8 and lysine 12 within histone H4 occurs in mammalian cells¹⁰⁸.

1.3.4 Culture adaptation

The value of hESCs and hiPSCs in research and therapeutic applications highly relies on their genomic integrity and stability. The moment the hESCs are transferred to a culture dish they are subject to new influences from their new environmental conditions and the stability of the cells during long term culture cannot fully be guaranteed⁵⁷. And even if differences between individual DNA sequences provide the basis for human genetic variability, forms of genetic variations such as SNPs, insertions/duplications, deletions and inversions/translocations may provide changes in hESCs and hiPSCs that favor cells with characteristics which are not optimal for further use.

Extensive cultures of stem cells can select for cells with genetic changes. These acquired mutations often result in growth advantages, called culture adaptation. By using karyotypic analysis these changes have been detected and often involve non-random gains of various chromosomes or fragments of chromosomes¹¹⁰⁻¹¹³.

These culture adaptations that leads to growth advantages in pluripotent SCs occurs as a successive, selective increase of mutations¹¹⁴. It has been shown that there is no safe cut-off point when hESC lines start to pick up chromosomal abnormalities, and even changes that are too small to be detected by G-banding can lead to growth advantages¹¹⁵. DNA-sequence variations can occur in our genome and are called single-nucleotide polymorphisms (SNP). This occurs when two sequences of DNA fragments from different individuals contains a difference in one single nucleotide, for example AAGCCTA to AAGCTTA.

Both small and large mutation can take place in low passages, while some hESC lines are karyotypically normal after hundreds of passages.

1.3.4.1 Chromosomal stability and instability

hESCs have been cultured for extended periods, while retaining a diploid karyotype, however they are typically aneuploid with distinct chromosomal abnormalities. Up until recently, the genome of pluripotent SCs has been characterized mainly by G-banding metaphase karyotyping, a technique that cover relatively low resolution of mutations from 2-10 Mb.

Chromosomal instability of pluripotent cells is a major obstacle for a safe clinical usage of these cells, and since one of the characteristics of SCs is to adapt to alternative fates (self-renewal, differentiation or death). It might not be surprising that the cells kept in a pluripotent state may favor variants that enhance the probability to self-renew through selection¹¹⁶ and it is known that almost all hESC lines suffer from mutations as a result of extended cultures as a response to its microenvironment¹¹⁷. Many chromosomal alterations have been identified, but the most common mutations, known to be a result of the phenomena culture adaptation, has been narrowed down through several studies to chromosomes 12, 17, 20 and X^{56-57, 110-111, 113, 118-121}. Extra copies of fragments of these specific chromosomes might drive the cells to an increased exposure of one or several genes that promote self-renewal⁵⁶.

There are variations between human populations, so a SNP allele that is common in one geographical or ethnic group may be more unusual in another. This was for example used when the International Stem Cell initiative analyzed 125 hESC lines and 11 hiPSC lines from 38 laboratories worldwide and could reveal, by the use of SNP arrays, that the cell lines included represented most of our major ethnic groups (**Figure 9**). 112 cell

lines passed the quality control criteria and were further analyzed to determine whether specific structural variants could be found in particular cell lines and could be narrowed down to the population that they were derived from, or if the variants were common to all hESC lines with no ethnic correlation. By using an ethnically diverse set of reference samples it was possible to cluster the cell lines to their ethnic origin, and it was found that the most commonly cited hESC lines originated from northern, northwestern and central Europe, Han Chinese, Indian and Middle Eastern populations.

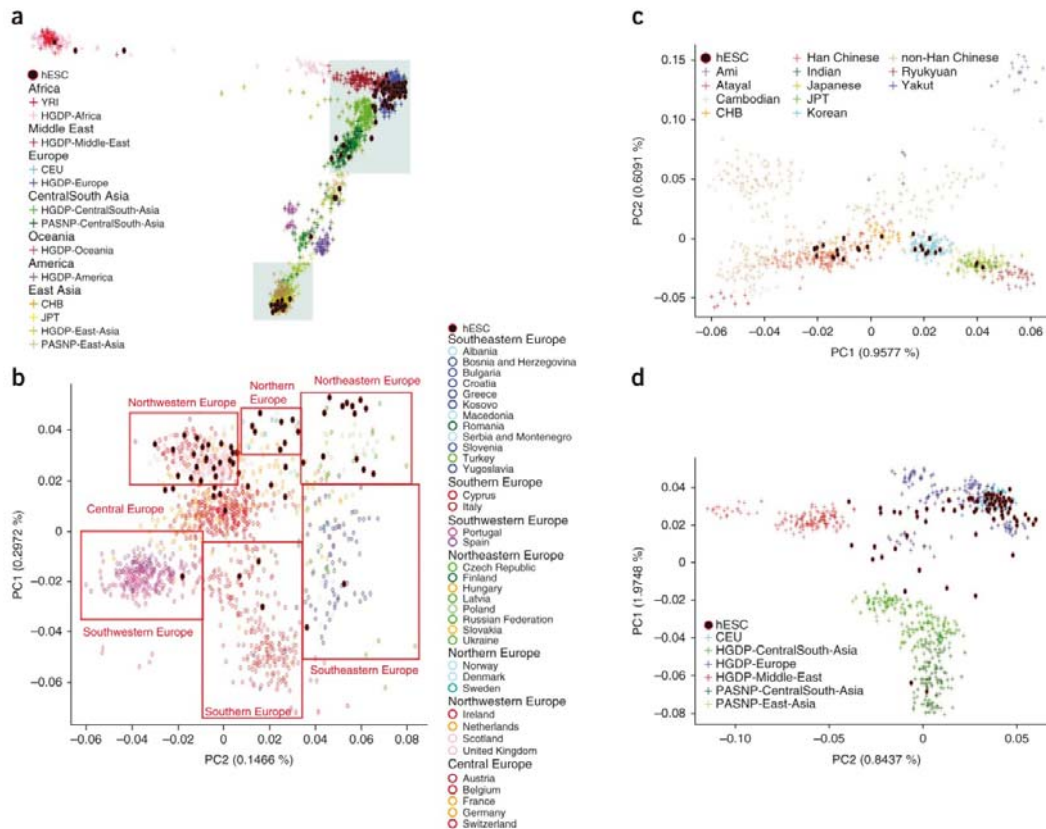


Figure 9. Population structure of the human ES cell lines analyzed. Principal component (PC) analyses were conducted on the entire final merged data set. PC1 and PC2 are plotted on the y and x axes, respectively. **(a)** The overall distribution of the human ES cell lines studied compared to the major ethnic groups identified in the HapMap study (REF!), the human genome diversity panel (HGDP) and the Pan-Asian SNP initiative (REF). **(b-d)** The cell lines were further subdivided to show their relationships to European **(b)**, East Asia and Indian **(c)** and Middle East-European-Central South Asian populations **(d)**. Amps, K et al., *Nat Biotechnol* 2011 Nov 27;29(12):1132-44. Figure was reproduced with permission from publisher.

To further demonstrate the impact of long term culture of pluripotent SCs, ISCI found a structural variant gain within chromosome 20 in 22 of the analyzed cell lines, which were karyotypically normal. The gain was within the minimal amplicon 20q11.2. When comparing the cell lines in early and late passage, there were five cells lines where this gain could be detected in both low and high passages, but 17 of the 22 cell lines gained the structural variant in the late passage, demonstrating that the gain may be a response of long term culture, resulting in a positive selective growth advantage⁵⁶

(Figure 10). The least difference between low and high passage number in the same cell lines where this gain was found only in late passage, was 22 passages difference.

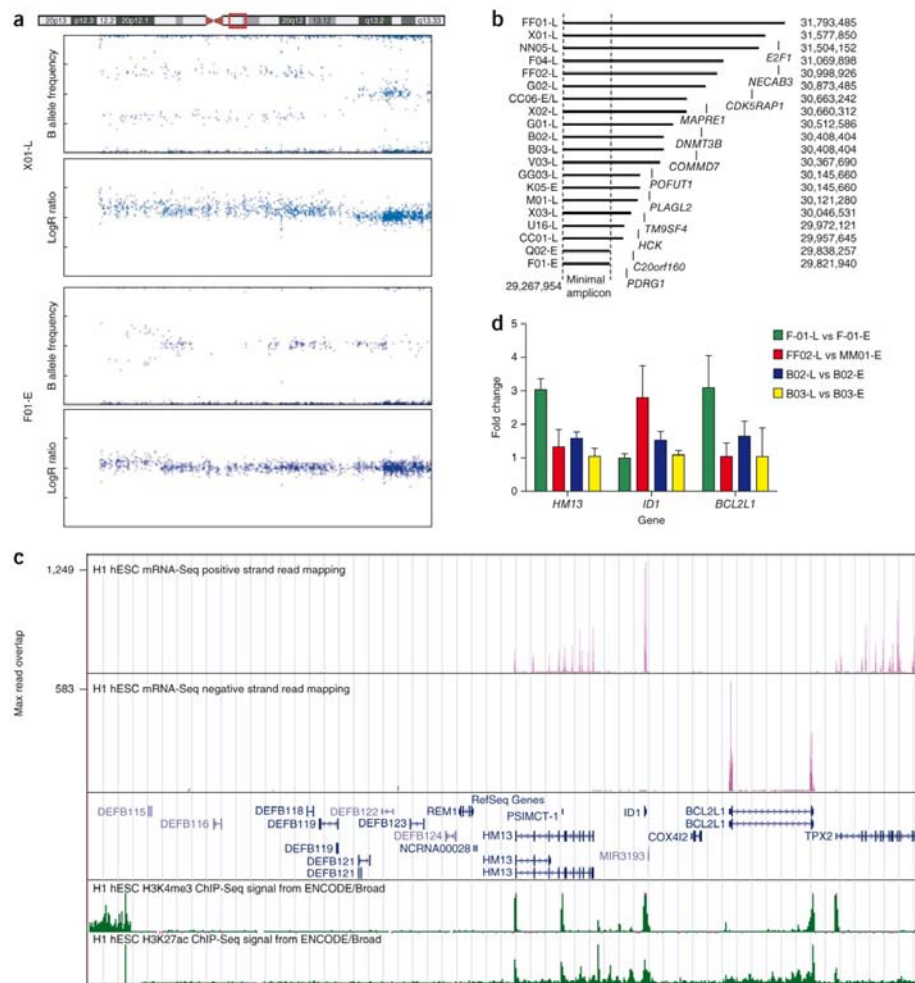


Figure 10. Copy number variation occurrence in human ES cell lines during prolonged passage. **(a)** 20q11.21 gain. The region on chromosome 20 frequently found to experience gain over extended human ES cell culture is indicated by the red boxed region in the chromosome ideogram. Also shown are the B allele frequency and logR ratio plots representing instances of one of the longest and one of the shortest 20q11.21 structural variants. **(b)** Length representation of all individual occurrences of gains in the 20q11.21 region. Samples from which the structural variants were derived are indicated on the left-hand column. The invariant 5' region and the variable 3' positions are indicated. Positions of the genes outside the minimal amplicon that show greater than 20 RPKM level of expression in human ES cells are shown (RPKM = number of reads that map per kilobase of exon model per million mapped reads of each gene). **(c)** Expression, Refseq gene, and regulation tracks in the minimal amplicon. Positive and negative strand mRNA-Seq data from H1 human ES cells indicating polyA RNA transcripts expressed within the minimal amplicon region (chr20:29,267,954-29,853,264) are shown together with H1 human ES cell ChIP-Seq data of histone modifications considered universal predictors of enhancer and promoter activity. **(d)** Comparison of expression levels of three genes (HM13, ID1, BCL2L1) contained within the identified minimal 20q11.2 amplicon between early- (normal) and late-passage (20q11.2 CNV carrying) samples. MM01 and FF02 are genetically identical sub-lines from two separate laboratories, MM01 has no amplification at 20q11.2, whereas FF02 possesses a copy number change at 20q11.2 that includes the identified minimal amplicon **(b)**. *Amps, K et al., Nat Biotechnol 2011 Nov 27;29(12):1132-44.* Figure was reproduced with permission from publisher.

1.3.4.2 DNA copy number variations

Ideally hESCs should preserve their genomic integrity while in culture to avoid abnormal development or tumorigenesis. DNA copy-number variations (CNVs) are alterations in DNA in the genome, and it contrasts with SNPs which affect only one single nucleotid base. These alterations may be inherited or caused by de novo mutations. When CNVs have occurred, larger regions of DNA have been deleted or duplicated, resulting in abnormal numbers of copies of one or more sections of DNA. CNVs can be a result of replication stress and the repair of DSBs by NHEJ. They also tend to cluster in regions with complex architecture such as fragile sites, subtelomeric regions and centromeres¹²².

CNVs include amplifications and deletions that can range between a few kb up to 1 Mb and can therefore be studied by using e.g. SNP-arrays (Affymetrix SNP 6.0 array). The SNP-array provides copy number estimate for more than 1.8 million markers on a single array. The low resolution of karyotyping cannot accurately determine most CNVs, which is estimated to involve 10-20% of the genome, due to the large mutation coverage. The degree of mosaicism found in samples must be determined by other methods, such as fluorescent in situ hybridization (FISH).

When using interphase FISH it is possible to detect X chromosome aberrations and the Y chromosome by using DNA probes specific for the X centromere and the SHOX gene on Xp/Yp. The SHOX gene is a homeobox gene and is important in development and was first identified during the search for short stature in women with Turner syndrome, a syndrome defined by the loss of genetic material from the X chromosome or loss of one entire X chromosome (46:X). By studying the SHOX gene it is possible to disclose extra chromosomal material on the X chromosome, which is known to be involved in culture adaptation.

1.4 THERAPEUTIC POTENTIAL OF PLURIPOTENT STEM CELLS

Both hESCs and hiPSCs hold enormous potential for revolutionizing current medical understanding and practice, and these cells have for many years been studied by researchers who have been exploring their unique capabilities. Not only are these cells an exciting research tool to probe mammalian development or use as a disease model, they also have a future in custom-tailored cell therapy⁵¹.

Pluripotent SCs provide a chance to obtain a renewable source of healthy cells and tissues and they have a vast potential to treat a wide range of diseases, namely because they give rise to all cells within the human body. Despite the success and fast progress in research using both hESCs and hiPSCs, these cells are not yet ready for transplanting into patients. The main issue for using these cells therapeutically is the safety concerns, one of them being that both hESCs and hiPSC tend to form teratomas (encapsulating tumor tissue components resembling normal derivation of the three germ layers) when transplanted, and the current differentiation protocols cannot eliminate residual undifferentiated cells¹²³.

However, a key advantage of iPSCs is the possibility to use the patient's own somatic cells for reprogramming, an approach where rejection of the graft could possibly be avoided. But to date, there are still hurdles to overcome, such as the methods used for reprogramming.

There have been promising reports in mice where iPSCs have been used to treat genetic disorders. It has for example been shown that iPSCs can be used in the treatment of Sick cell anemia, where the disease causing mutation was repaired by gene targeting and the progenitors were transplanted back into the mice and the healthy cells started to produce normal red blood cells¹²⁴.

Drug research is another area that pluripotent SCs may prove useful. Animals are a commonly used model to assess the safety and use of drugs. Instead of initially testing drugs on animals, they can be evaluated though testing on cells grown from pluripotent SCs. Those drugs that appear tolerant and safe can then progress to testing on animals, and finally humans.

Even if differences among hESCs and hiPSCs shown by others as well as discussed in this thesis, such as passage number, viral integration, culture conditions and line-to-line variations influence the epigenetic and functional properties of these cells, it is, at this point, still important to keep studying these two types of pluripotent SCs in parallel.

2 AIMS OF THE THESIS

General aim

The overall aim of the work presented in this thesis was to investigate effects on pluripotent stem cells caused by microenvironmental influences, such as cryopreservation, passaging techniques and culture adaptation.

Specific aims listed according to each paper:

Study I

To evaluate a chemically defined xeno-free cryopreservation system for hESC lines and hiPSC lines, and its effect on the gene expression, karyotype and proliferation caused by cryopreservation and thawing.

Study II

To evaluate the effects on morphology and gene expression in correlation with epigenetic marks due to different techniques when passaging hESCs

Study III

To investigate putative correlations in hESC and hiPSC lines between gene expression and SWI/SNF complex related histone acetylation on lysine 8 and 12 on H4 on the promoter region of genes associated with pluripotency.

Study IV

To investigate chromosomal stability of three hESC lines. We compared in vivo differentiated pluripotent SCs with in vitro differentiated SCs, using high-resolution array to study DNA-copy number variations and FISH.

3 MATERIALS AND METHODS

This section gives a brief overview of a few of the methods used to obtain the results presented in this thesis. It mainly focuses on derivation and culture of pluripotent SCs.

The ethics that is necessary to conduct research on SCs derived from fertilized eggs will be discussed, and what motivate the donation of some embryos for research instead of using them for infertility treatment.

Some of the techniques used to characterize the pluripotent SCs, such as gene expression and chromatin immunoprecipitation will be described briefly.

More detailed protocols, such as concentrations, primer sequences and other in-depth details can be found in the materials and methods section of the included studies.

3.1 DERIVATION OF HUMAN EMBRYONIC STEM CELLS

Fertilization of oocytes and embryo culture is performed by embryologists at the Fertility Unit at Karolinska University Hospital.

Before using the fertilized egg for treatment of infertility all embryos undergo an evaluation of their quality following a modified scoring system¹²⁵. Briefly, a maximal score of 3.5 is given to embryos where no factor that reduced the embryo quality is observed. The score is reduced in increment of 0.5 for various non-optimal variations found in the morphology of the embryo, such as non-ideal numbers of blastomeres or non-spherical blastomeres, presence of more than 10% fragmentation, unevenness of the cell membrane, cytoplasmic abnormalities e.g.

Embryos scoring 3.0-3.5 are regarded as top-quality embryos and are always used in first hand for the couples. A score of 2.5 is considered good quality and 4-8-cell embryos with a score of at least 2.0 are considered suitable for cryopreservation. Embryos with lower scores can be donated to research.

All embryos with a score of 2.0 or more are stored in liquid nitrogen in case of any sibling treatment. After storing cryopreserved embryos for five years, all patients get to decide whether the embryos should be discarded or donated for research if more children are not desirable. This make it possible to derive cell lines from embryos with better quality, however a correlation between embryo morphology and successes rate in deriving new cell lines have not been detected¹²⁶.

A high proportion, 92 % of all couples that went through infertility treatment in Sweden, preferred donation their supernumerary embryos to stem cell research instead of discarding them¹²⁷.

3.1.1 Isolation of inner cell mass

Isolation of the ICM is mostly performed using two different methods, immunosurgery or mechanical isolation. When performing immunosurgery the zona pellucid is first removed by using 0.4% pronase followed by removing the trophoderm using rabbit anti-human whole serum and guinea pig complement¹².

Mechanical isolation of the ICM is performed by using two small flexible metal needles with a diameter of 0.125 mm. One needle is used to hold the blastocyst, and the other to open the zona pellucida and remove the ICM from the trophectodermal cells¹¹. The isolated ICM grow on hffs for 12-15 days before the first passage to new feeder cells or matrix.

3.1.2 Ethics

To derive and establish hESC lines, ethical approval from the Ethics Committee of Karolinska Institutet has been obtained. Donated supernumerary embryos are used for derivation, donated from couples undergoing IVF treatment at the Fertility Unit at Karolinska University Hospital, Huddinge, Sweden. To donate embryos, both parents need to sign an informed consent form after receiving both written and oral information. Only embryos that are not used in the treatment of infertility can be donated for research. Fertilization of eggs is not allowed to obtain embryos solely for research. No reimbursement is paid to the couples.

3.2 CULTURE AND CHARACTERIZATION OF PLURIPOTENT STEM CELLS

3.2.1 Culture medium

At Karolinska Institutet the medium used for derivation and culture of pluripotent SCs consists of Knockout Dulbecco's Modified Eagle's basal Medium (DMEM) supplemented with 20% knockout serum replacement (SR), 2 mM glutamax, 0.5 % penicillin-streptomycin (PEST), 1 % non-essential amino acids, 0.5 mM 2-mercaptoethanol and 8 ng/ml bFGF. The media are changed every day.

mTeSR1 is a commercially available culture medium that is used when cells are cultured feeder-free on a matrix called Matrigel, according to manufacturers description^{18, 128}.

3.2.2 Feeder cells

Commercially available hff's are used as feeder cells¹⁵. They have been mitotically inactivated by irradiation (40 Gy).

The medium for the fibroblasts consist of Iscove's modified Dulbecco's Medium (IMDM) supplemented with 10 % fetal bovine serum (FBS) and 0.5 % PEST. The day after plating irradiated fibroblast the medium is changed to a medium supplemented with SR instead of FBS. Three days after plating new irradiated fibroblasts they are ready to be used for the culture of pluripotent SCs. Before transfer passaged SCs to plates the fibroblast medium is changed to the medium based on DMEM (described above).

3.2.3 Extracellular matrix coating

Plates coated with Matrigel (BD Biosciences, Bedford, MA) are prepared according to the manufacturers recommendations by diluting one aliquot of Matrigel in 25 ml DMEM/F12 (Stem Cell Technologies). Coated plates are incubated in room temperature followed by either storage in 4 °C, or a second incubation of 1 hour prior to use in 37 °C. Excess Matrigel is removed just before adding pluripotent SCs.

3.2.4 Passaging

The different passaging techniques have been discussed in the introduction part.

3.2.5 Karyotyping

Karyotyping is carried out using the G-banding technique. Cells are treated with 0.1 µg/ml colcemid KaryoMAX to stop the cell cycle in metaphase. The colcemid treated cells is removed by enzymatical passaging and centrifuged at 1,300 revolutions per minute (rpm) for 7 minutes. The centrifuged cells are re-suspended with pre-warmed hypotonic solution (0.0375 M KCl) and incubated in 37 °C for 10 minutes. After the incubation the cells is re-suspended in fixative composed of methanol and acetic acid (3:1). Metaphase spreads are prepared on glas slides, G-banded by brief exposure to trypsin and stained with 4:1 Gurr's/Leishmann's stain. A minimum of 10 metaphases are analyzed.

3.2.6 Embryoid body formation

To form EBs, pluripotent SCs are cultured as aggregates in suspension without bFGF. After 3 weeks in culture the cells have formed spheroid balls containing tissues originating from the three germ layers. This is demonstrated by RT-qPCR using markers specific for genes within the germ layers. The EBs can also be sectioned and stained by immunohistochemistry using antibodies specific for antigens on the surface, or nuclear staining can be performed.

3.2.7 Teratoma formation

Pluripotent SCs are harvested from culture plates either mechanically or enzymatically. The cells are washed with PBS and re-suspended with a small amount of culture medium. The cell suspension is implanted beneath the testicular capsule of a young (7-week-old) beige SCID mouse (C.B.-17/GbmsTac-scid-bgDF N7, M&B, Ry, Denmark). The teratoma growth is monitored by weekly palpation and the mouse is sacrificed by cervical dislocation after 8 weeks. The presence of tissue components of all three germ layers are analyzed from stained sections¹²⁹.

All animal experiments are performed at the infection-free animal facility of Karolinska University Hospital, Huddinge, Sweden, in accordance with Ethics committee approval.

3.2.8 Live-dead assay

The ratio between live and dead cells can be assessed by using a calcein-esterase based live-dead assay (Molecular Probes, Eugene, USA). Membrane-permeable calcein is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, and membrane impairment ethidium homodimer-1 labels nucleic acids of membrane-comprised cells with red fluorescence. The ratio between red and green cells can be seen and calculated using a fluorescence microscope.

3.2.9 RT-qPCR

Total RNA from pluripotent SCs are isolated by using the commercially available Qiagen RNeasy kit, following the manufacturer's instructions. The concentration of the RNA is quantified spectrophotometrically at 260/280 nm.

To obtain first-strand complementary DNA (cDNA) the RNA is reverse transcribed using the enzyme reverse transcriptase (RT). The single strand cDNA was then used as a template for PCR amplification. The TaqMan protocol utilizes a probe which has a reporter dye in the 5' end and a quencher in the 3' end. While the probe is intact, the reporter dye will be quenched (**Figure 11**). The probe is specifically design to anneal downstream of one of the primer start sites and when reverse transcription starts it will break up the probe and release the reporter dye. This is repeated every cycle and thus the reporter signal is proportional with the amplified product. This method only detects specific amplification products. The SYBR Green protocol is based on small fluorescent molecules that can bind to any double stranded DNA products, such as primer dimmers. It is not dependent on a specifically designed probe for each reaction which reduces the running costs. For primer or probe sequences see materials and methods section in respective manuscripts in the appendix. PCR amplification with

H₂O instead of cDNA is used as a negative control. As endogenous controls to compensate for variable cDNA yields, GAPDH can be used for SYBR Green reactions, and 18S for TaqMan reactions. The platform used is ABI 7500 Fast real-time PCR system.

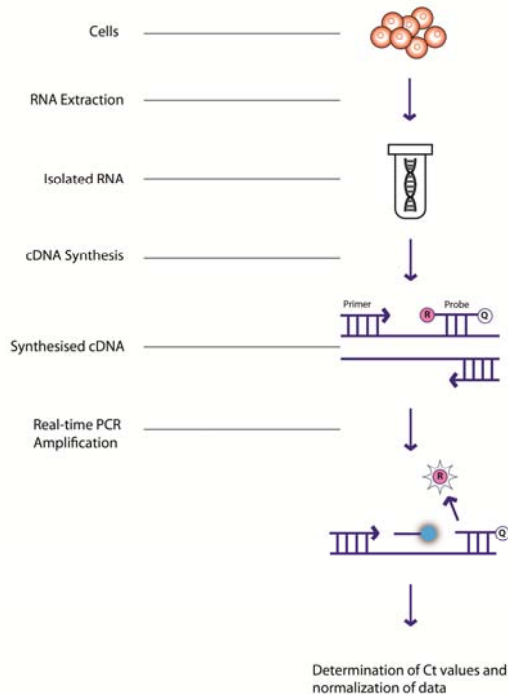


Figure 11. RT-qPCR. RNA is extracted from pluripotent SCs and cDNA synthesis is performed. Sequence of interest is detected either by using a primer pair, or by using a probe with an attached reporter dye. The amount of fluorescent released when the probe break-up is determined in Ct values, which are proportional with amplified product.

3.2.10 Immunohistochemistry

Immunohistochemistry (IHC) is used to determine the presence and cellular distribution of different proteins.

Pluripotent SCs are fixed using paraformaldehyde (PFA). For surface markers, blocking of unspecific staining was performed by incubating the fixed cells with 10 % FBS diluted in PBS for 30 minutes followed by incubation with primary antibodies, diluted in 10 % blocking solution over night in 4 °C.

For nuclear markers, blocking and permeabilization of the nucleus is performed by incubation in 2 % donkey serum, 0.1 % Triton-X and 0.1 % bovine serum albumin (BSA) in PBS for 2 hours at 4 °C.

The secondary antibodies are diluted in 10 % blocking solution for surface markers or 0.1 % Triton-X for nuclear markers and incubated for 2 hours at 4 °C in dark.

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

3.2.11 FACS

Pluripotent SCs should be harvest enzymatically to obtain single cells, and dilute them in 5 % FBS in PBS. Dilute primary antibody according to manufacturer's recommendations and add cells. Incubate for 30 minute in 4 °C. After incubation wash cells with 5 % FBS in PBS. Add diluted secondary antibody and incubate for 30 minutes in 4 °C and wash again to remove any unspecific binding of the antibodies.

Use a negative control antibody to set a threshold for scoring the proportion of cells that are positive for each test antigen. Typically this threshold should be set at the inflexion point on the negative control histogram, such that only 1-5 % of the cells stained with the negative control is scored “positive”. Using this threshold, the % cells scored positive with each antibody is recorded.

A second region is set on the histogram, encompassing all cells – from this gate the “mean fluorescent intensity” of the cells stained with each antibody is recorded (**Figure 12**).

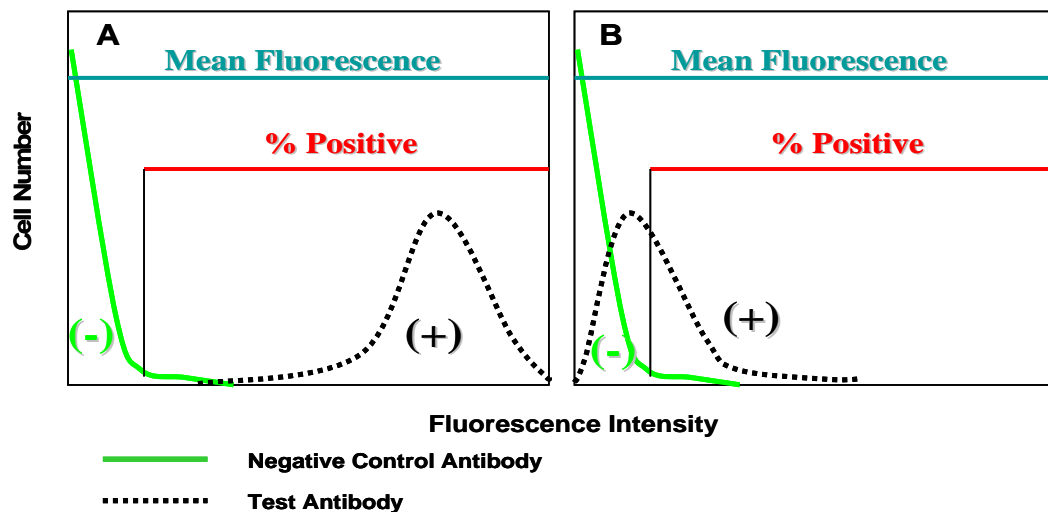


Figure 12 FACS. (A) The positive antibody stains cells substantial brighter than the negative control, nearly 100 % of the cells should be scored as antigen positive. (B) The positive histogram overlaps significantly with the negative control histogram, rather less than 100 % of the cells should be scored positive for this antigen. The mean fluorescence of cells stained with the test antibody in panel B will be substantially less than the mean fluorescence of the cells stained with the test antibody in panel A. The two numbers; mean fluorescence and % positive cells, provide two complementary numbers for comparing results between cells and antibodies.

3.2.12 Chromatin immunoprecipitation

For ChIP analysis the commercially available kit LowCell#ChIP kit from Diagenode is being used, detailed protocol can be found in the kit.

Cross-linking of the cells is performed using formaldehyde (FA).

Chromatin shearing is performed by adding a buffer containing protease inhibitor to cross-linked cells. After incubating, the cells are sonicated for 10 minutes to obtain smaller fragments of DNA. Centrifuge sonicated samples and transfer supernatant to a new tube. A small amount of sample is used to determine the size of DNA fragments by running an agarose gel.

To bind antibodies to magnetic beads, a buffer mixed with protein-A magnetic beads is prepared. The specific antibodies are added to beads and incubate for 2 hours in 4 °C.

Immunoprecipitate is performed by spinning tubes with antibody-coated beads and place in a magnetic rack before aspirating the supernatant. Add diluted sheared chromatin in each tube in the rack. Save chromatin for input. Remove tubes and incubate on rotator over night in 4 °C. Wash and capture beads on magnetic rack. Aspirate the buffer and add DNA isolation buffer (also to input sample) and remove tubes from magnetic rack. Incubate all tubes in 55 °C for 15 minutes and then 100 °C for additional 15 minutes. Centrifuge and transfer supernatant to new tubes and analyze by qPCR (**Figure 13**).

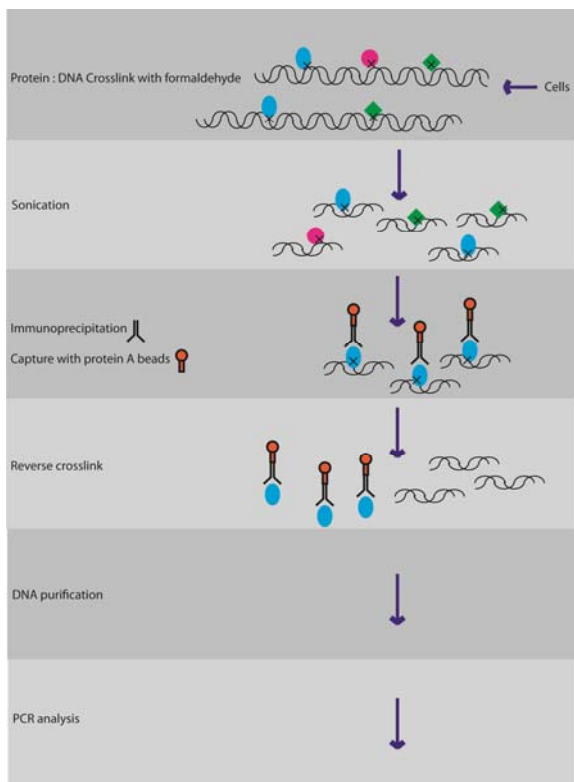


Figure 13 Chromatin Immuno-precipitation. Crosslinking of harvested pluripotent stem cells is performed using formaldehyde. For smaller DNA fragments the fixated cells are sonicated. After sonication antibodies against modified histones are added and captured by protein-A beads. Reverse crosslinking is performed to remove antibodies and beads from modifications of interest. After DNA purification, PCR against the DNA sequence is performed.

4 RESULTS

The studies included in this thesis investigate effects on pluripotent Stem Cells due to handling and microenvironmental cues, such as cryopreservation and thawing, passaging methods, long term culturing and mutations caused by differentiation and culture.

All four studies, two of them which have been published in peer-reviewed journals and one which is under revision, can be found in their entirety in the appendix.

This section gives a brief overview of the results.

4.1 AN EFFECTIVE NON-SERUM XENO-FREE CHEMICALLY DEFINED FREEZING PROCEDURE FOR HESCS AND IPSCS

Traditionally, DMSO ($(\text{CH}_3)_2\text{SO}$) is the most commonly used cryoprotectant for long-term storage of pluripotent stem cells. It is an organosulfur compound which have relatively high freezing point (18,5 °C) meaning that it, at lower temperatures is solid which can limit its utility in chemical processes, in this case, crystallization with cooling. It can permeate the cell membrane without damaging the cell. It is mostly used in a 10% dilution with cell culture media. However, DMSO can be toxic to stem cells and may be one of the reasons for the low survival rates post freezing.

STEM-CELLBANKER also contain DMSO, but a second non-permeable cryoprotectant in the solution helps even out the ice-formation outside of the cells, while DMSO penetrate the membrane, crystallizing the water inside the cells. This way, the cells are less likely to burst due to the ice-formation.

We have used two hESC lines (HS293 and HS306 both derived at Karolinska Institutet) and one hiPSC line. The hiPSC lines CHiPS A have been established in collaboration with University of Geneva⁹² by transducing human skin fibroblasts with OCT4, NANOG, SOX2 and LIN28. The experimental design was to characterize these three cell lines before cryo-storage, and after thawing, to look for any indication of affected properties of the cells. Both hESCs and iPSCs are highly sensitive to cryo-injury caused by cryopreservation which has limited their utility^{80, 83-88}.

All cell lines were cultured in the same manner prior to cryopreservation. The undifferentiated colonies were mechanically harvested and frozen either with the conventional method using 10 % DMSO in culture medium (20% knockout SR, 2 mM glutamax, 0,5 % penicillin-streptomycin, 1 % non-essential amino acids, 0,5 mM 2-mercaptoethanol and 8 ng/ml basic FGF) or using the ready-to-use freezing solution STEM-CELLBANKER.

We found that there were differences between the two different methods, 10 % DMSO and the method using STEM CELLBANKER.

By counting cells using the trypan blue exclusion method in a haemocytometer, we found significant differences between the two methods in the number of surviving cells after freeze-thawing. hESC line HS293 had 93 % survival after thawing and HS306 displayed 96 % survival when using the new method, compared to 49 % survival of both cell lines frozen with the standard method. The hiPSC line CHiPS A also displayed better survival after thawing with the new method, 90 % survival compared to 50 % in standard method. The number of colonies that survived freezing was fewer in the standard method compared to the new method using STEM-CELLBANKER, this was also verified using calcein-esterase based live-dead assay. In order to control the recovery of the cells, growth curves during the first passages after thawing were performed.

The freeze-thawed cells from both methods were also stained positive for antibodies against the pluripotency markers OCT4, NANOG, TRA1-81 and SSEA4. The level of pluripotency in frozen-thawed cells was also controlled by Real time-quantitative PCR

(RT-qPCR) for OCT4 and NANOG. We did not find any significant differences in the pluripotency markers expression between the two methods

Lastly, the cells were karyotyped to ensure that they had not acquired any chromosomal changes due to the cryopreservation. The teratoma formation showed that the cells did form tissues represented in the three germ layers (Endoderm, Mesoderm and Ectoderm).

The advantages using STEM-CELLBANKER instead of 10 % DMSO:

Significantly increased cell viability while maintaining cell pluripotency (HS293 $P=0.0019$ and HS360 $P=0.0030$), normal karyotype and proliferation ability after freeze-thaw procedure.

Safer cryopreservation with medical graded ingredients, no risk of contamination of animal-derived components.

Save time/cost/steps with the complete formulation, no further preparation, direct freezing at -80°C without having to use a programmed freezer.

4.2 PASSAGING TECHNIQUES AND ROCK INHIBITOR EXERT REVERSIBLE EFFECTS ON MORPHOLOGY AND PLURIPOTENCY MARKER EXPRESSION OF HESCS

Previous reports have shown that the use of the ROCKi decreases the cellular stress response and apoptotic cell death in pluripotent stem cells when dissociated from each other during passaging¹⁰¹ and even cryopreservation⁸³. These observations resulted in a wide usage of this inhibitor among scientists.

By studying the morphology of hESCs we found differences between cells that had been passaged mechanically, to those passaged enzymatically with addition of ROCKi to escape the dissociation-induced apoptosis. Mechanically passaged cells kept growing in typical SC colonies, with a time frame of approximately 5-6 days between each passage. Enzymatically passaged cells pre-treated with ROCKi produced more colonies in a shorter time frame than those passaged mechanically. Cells which were not treated with the inhibitor, but passaged enzymatically grew slower and produced fewer colonies. This could be an indication of selection of cells with a growth advantage when adding the inhibitor, where enzymatically passaged cells without ROCKi are the truly pluripotent ones.

ROCK was initially discovered as a downstream target of the small GTP-binding protein Rho which is involved in the regulation of many cellular processes such as growth, adhesion, migration and apoptosis by controlling actin cytoskeletal assembly and therefore cell contraction¹³⁰. By staining cells passaged using these three passaging methods it was discovered that both mechanically and enzymatically passaged cells had organized and dynamic organized actin filaments indicating that the cells were under stress. Cells passaged with ROCKi did show a much more disorganized structure of the actin filaments, in order with previous suggestion that the addition of ROCKi during passaging reducing the amount of stress in cells caused by passaging.

However, cells from all three passaging techniques expressed positive immunoreactivity for the pluripotency marker. The staining also demonstrates an enlargement of the nucleus in enzymatically passaged cells, which was confirmed by measuring nuclear area.

The main purpose of passaging pluripotent SCs, besides expanding cells, is to maintain their pluripotent state and therefore avoid spontaneous differentiation. When analyzing the cells with RT-qPCR we found a dramatic decrease in gene expression of the most common pluripotency markers; OCT4/POU5F1, NANOG, SOX2, KLF4 and IGF2, using passaging methods where cells had been dissociated from each other, and treated with ROCK inhibitor. C-MYC which is a marker associated with cancer cell characteristics were not expressed in any of the analyzed cell lines. Also no expression levels of H19 were detectable. H19 is a gene for long non-coding RNA, exclusively expressed on one parental allele in imprinting, which seems to play a role in some cancers.

The differences in the gene expression profile of different passaging methods correlated poorly with an analysis of histone modifications in the promoters of the same genes as assessed by chromatin immunoprecipitation (ChIP).

Due to the increased area of the nucleus found in the staining we speculated whether there could be a general effect on the chromatin structure that obscured more specific differences in histone lysine methylation between the different passaging techniques. Due to this we included a modification influencing the histone-histone interaction, higher order chromatin structure and nucleosome organization; H4K16ac. However, there were no significant differences detected between the different passaging techniques.

We also included another epigenetic mark associated with transcription start site of active genes; H3K4me3, and to marks associated with repressed genes; H3K9me3 and H3K27me3. We could not find any correlation between the decrease in gene expression and histone modification, suggesting that the effects are only located in the mRNA.

To evaluate whether these effects on the morphology and gene expression were permanent, cells passaged enzymatically with and without ROCKi were now passaged mechanically instead. After the transition between the different passaging methods the cells were cultured for several passages in the standard method (mechanically) and then re-analyzed. We found that the earlier decreased levels in gene expression of the most commonly used pluripotency markers were back to normal levels. Cells passaged with ROCKi had a mean expression between the three hESC lines used of 93 % of OCT4/POU5F1 and 104,1 % of NANOG. Cells enzymatically passaged without ROCKi had a mean expression between the three different hESC lines of 125,6 % for OCT4/POU5F1 and 108,4 % for NANOG. All compared with 100 % expression of the markers for cells passaged mechanical throughout the study. This indicates that the effects were reversible and a direct response to the different passaging techniques.

4.3 HISTONE H4 LYSINE ACETYLATION AND RELATIVE GENE EXPRESSION OF PLURIPOTENCY MARKERS IN HESC AND HIPSC LINES

Earlier studies show that the ATP-dependant chromatin remodeling complex SWI/SNF is associated with histone modification, especially histone acetylation of lysine 8 and 12 on histone H4¹³¹. Due to this, we wanted to investigate if we could correlate gene expression of OCT4, SOX2, NANOG and KLF4, with histone acetylation on the promoters of the same factors. We focused the histone acetylation analysis of H3K8 and H4K12 since acetylation of these two specific residues have been associated with the SWI/SNF complex as well as DNA repair of DSBs.

To see whether high passage numbers correlate with gene expression and histone acetylation, our study aimed at investigating differences in gene expression levels and the levels of histone acetylation between low and high passage numbers. Different cell lines have distinct differentiation potential, which may be affected by high passaging numbers of pluripotent SC, exhibiting increased DSBs and DNA repair.

In this study we compared two hiPSC lines; CHiPS A and CHiPS W, and three hESC lines; HS346, HS361 and HS401. As a negative control line we included the fibroblast line (hff), from which the hiPSC lines were reprogrammed.

RT-qPCR was performed to detect mRNA levels of depicted pluripotency factors associated with stemness; OCT4, NANO, SOX2 and KLF4. RT-qPCR was performed on three hESC lines and two hiPSC lines of different passaging numbers, with the parental fibroblast line as a negative control.

We could identify differences in relative expression levels between the different pluripotency markers. However no clear difference between the different passages was found, suggesting that cells which have been in culture for a long period of time do not differ significantly between low-passage counterparts. We could also not find any differences in gene expression levels between hESC lines compared with hiPSC lines. However, there were differences in levels of pluripotency marker expression between the hiPSC lines CHiPS A and CHiPS W. CHiPS W show constant lower levels relative gene expression of all four studied pluripotency markers.

When analyzing the relative acetylation levels of lysine 8 and lysine 12 on histone H4 on the promoters of the pluripotency markers using ChIP, we expected to find that cell lines with a higher passage number would have more signs of DSBs, and therefore have increased levels of acetylation. We could not detect any such correlation between passage number and the relative acetylation levels of DSB-associated residues H4K8 and H4K12 on the promoters of the studied pluripotency markers.

We could on the SOX2 promoter of both hiPSC lines CHiPS A and CHiPS W detect lower levels of relative acetylation of both residues, H4K8 and H4K12, compared to the hESC lines, which all have similar levels of acetylation. In the promoter region of KLF4 a small, but not statistically significant, differences between the two hiPSC lines was found. CHiPS W had a higher level of relative acetylation on both residues,

compared to CHiPS A, however the levels of both residues are similar in the individual hiPSC lines.

The studied lysine residues 8 and 12 on histone H4 has been associated with DSBs and also to BRG1, which is one of the SWI/SNF components. Due to the lack of correlation between passage numbers and the relative acetylation levels, we hypothesized whether there would be a correlation in relative acetylation levels and gene expression between the two different lysines on histone H4 on the promoters of the pluripotency markers OCT4, SOX2, NANOG and KLF4. No significant correlation was however detected.

The gene expression levels and promoter acetylation of lysine 8 and lysine 12 of the most studied markers have poor correlation to each other, but also to pluripotency potential. Epigenetic modifications other than H4K8 and H4K12 are most likely involved in the regulation of characteristics of hESCs and hiPSCs.

4.4 IN VIVO DIFFERENTIATED HESCS CAN ACQUIRE CHROMOSOMAL ABERRATIONS MORE FREQUENTLY THAN IN VITRO DURING THE SAME PERIOD

Three hESC lines, HS346, HS366 and HS368 were used to study chromosomal stability during long term culture. hESCs can be affected of microenvironmental conditions which may lead to chromosomal alterations. *In vitro* and *in vivo* conditions were studied using high-resolution Affymetrix SNP 6.0 array DNA copy number variations (CNVs) and fluorescent in situ hybridization (FISH) analyses.

All three hESC lines cultured and maintained on a feeder layer of human foreskin fibroblasts were analyzed at passage 27 (HS346), 42 (HS366) and 28 (HS368). The cells were karyotypically normal, expressed immunocytochemical surface markers (SSEA-4, TRA-160 and TRA-180) and nuclear markers (NANOG and OCT4). The mRNA expression levels confirmed that the cells were in a pluripotent state using quantitative RT-PCR. The cells also formed solid teratomas with components of 3 primitive embryonic germ layers when implanted beneath the testicular capsule and subcutaneously of a young SCID mouse.

SNP arrays for HS364 at starting passage 27 revealed 9 variations on different chromosomes, after 7 additional passages this cell line did not gain any additional mutations. However, after being differentiated *in vivo* this cell line showed an amplification of a whole X chromosome, and had a higher degree of mutations with 67 variations (compared to the 9 variation from *in vitro* cultures), most variations were gains and mostly clustered on chromosome X, suggesting an extra X chromosomal material.

The FISH analysis of the same *in vivo* differentiated cells showed normal pattern of XY in 58 % of the cells (one X centromere and 2 SHOX signals), whereas 24 % showed two X centromere signals and 2 SHOX signals. A pattern consistent with duplication of the X chromosome, suggests mosaicism for XY and XX (with lost Y chromosome) cells. This confirms the result of extra X chromosome material from the SNP array.

SNP arrays for cell line HS366 at passage 42 show a total of 6 alterations, after growing for 7 more passages, a loss registered on chromosome 17p11.2 had disappeared from the cells.

Cell line HS368 had 10 variants at passage 28. After 8 weeks in the teratoma (testis) a new gain appeared on chromosome X encompassing about 150 kb of the pseudoautosomal gene-rich region PAR1 (ZBED1, which may have a role in cell proliferation) and CD99 which may act as a oncosuppressor. In another teratoma we found 8 more mutations gained after 8 weeks of *in vivo* differentiation.

Interphase FISH analysis showed that 74% of the cells had normal XY content, whereas 21% showed X centromere signal and 3 SHOX signals. This implicated a gain of the pseudoautosomal regions, consistent with the results of the SNP array.

In summary, a total of 12 solid teratomas were generated from the 3 hESC lines used in this study. DNA was extracted from the teratomas after harvest and analyzed by the high-resolution Affymetrix SNP 6.0 array. Cells at the same passage as the injected ones were cultured in vitro up to 8 weeks, until teratomas were extracted, to compare their developmental adaptation capacity with the in vivo developing cells. We assessed the genomic variations by in vitro and in vivo growth. We observed increased mutation coverage of about 30% (median).

Table 2. Mutation coverage (in kb) in teratomas compared to in vitro cell growth (from paper IV)

<i>Line</i>	<i>Tissue</i>	<i>Passage</i>	<i>Mutation coverage (kb)</i>	<i>Change in mutation coverage in respect to in vitro growth</i>
HS364	Cells in vitro	34	3,609	
HS364	Teratoma		4,702	30% (median)
HS366	Cells in vitro	49	714	
HS366	Teratoma		1,581	121% (median)
HS368	Cells in vitro	35	3,298	
HS368	Teratoma		4,239	29% (median)
			Total	30% (median)

The results from this study where we found that structural variants exist in 3 hESC lines which are otherwise karyotypically normal have also been addressed in a study published by the International Stem Cell Initiative (ISCI)⁵⁶. By analyzing 125 hESC lines and 11 hiPSC lines from 38 laboratories in 19 different countries we searched for common genetic changes that could occur during prolonged culture, some cells of which maintained in a pluripotent state for many passages might be subject to strong selection, and variants that would enhance the probability of self renewal would be favored.

5 DISCUSSION

Genetic stability and stable stem cell characteristics of pluripotent SCs is important for both clinical applications and scientific research. Abnormalities occurring in sensitive areas of the genome are likely to result in marked changes in functional and biological effects. This leads to loss of application value, aiming towards using cells with as little effect on their characteristics as possible. The benefit to culture pluripotent SCs without influencing their characteristics requires well-established and risk-evaluated techniques. In the future this knowledge will be invaluable to this field where the hope is to direct the production of tissue specific cells to replace part or whole damaged or diseased organs.

From the perspective of future therapeutic applications, the banking of pluripotent SCs requires optimized cryopreservation systems which are defined, xeno-free, economic and can be performed under good manufacturing practice (GMP).

So far low efficiency and laborious techniques have limited the procedure where pluripotent SCs can be stored for long periods of time in liquid or saturated nitrogen.

It is important with an effective system to store large amount of cells for research, but most importantly for clinical use, where large amount of cells may be necessary to use for patients with an urgent need of cell therapy, where culture of new cells for each patient may have a limited time-frame. For that reason a freezing and thawing medium to cryopreserve and facilitate long-term storage of large quantities of pluripotent SCs is important without losing cells when thawing is crucial.

We have shown in *Study I* that a combination of both permeating and non-permeating cryoprotectants has limited the direct cell injury induced during freezing by reducing both the extracellular ice formation (EIF) and intracellular ice formation (IIF). This unique combination of a non-permeating cryoprotectant that keeps the water molecules from producing ice crystals causing the cells to dehydrate and shrink, and a permeating cryoprotectant that keeps the water molecules within the cells to freeze and therefore keeping the cells from bursting due to the expansion that crystal formation causes.

Unlike cells frozen slowly, vitrified colonies have consistently shown very high survival and re-attachment rate^{80, 85, 132}. Vitrification transforms the intracellular medium into a glasslike structure which prevents the formation of IIF. However, the cooling rates needed for successful vitrification require that the sample volume is kept low, resulting in that only few hESC or hiPSC colonies can be cryopreserved in one straw which is originally designed for small quantities such as oocytes and embryos. In 2008 a bulk vitrification system was described for the first time¹³³. In addition to the technical difficulties, another major problem with these vitrification methods is that they are open systems. For clinical grade cryostorage, sterile liquid nitrogen would be needed, requiring a specific vacuum filtration system for the liquid nitrogen. The open-pulled straws that are being used can of course be stored in closed vials in the gas phase of liquid nitrogen but this does not prevent the original contact during the vitrification procedure. Also, handling of different vitrification solutions in various concentrations, together with several incubation steps, is more likely to have technical drawbacks with the bulk vitrification method, rather than the new easy and safe method presented in *Study I*, which has fewer crucial steps. Previously, freezing stem cells with only permeating cryoprotectants has been used, but the two cryoprotectants combined in this novel cryopreservation system have been shown to not only give good survival in pluripotent cells, but we have also found that this system give better results in somatic cells, such as human hepatocytes ($P < 0.01$) compared to DMSO in the University-of-Wisconsin solutions (DMSO-UW)¹³⁴.

To further enable the use of pluripotent SCs in the future; it is important to keep the cells from undergoing spontaneous differentiation. In *Study II* we have shown that cells being passaged mechanically using a surgical scalpel or cell scraper have minimum impact on the expression levels of the pluripotency markers OCT4, NANOG, KLF4 and SOX2. The organized stress fiber formation found in these cells may however indicate that the cells are under stress, which might be due to mechanical-induced

damage that the cells are subject to when cutting in a cell colony by hand using a scalpel.

On the other hand, cells that undergo an enzymatic treatment in order to be passaged, display a different morphology, decreased level of expression of pluripotency markers and an enlargement of the nucleus, quantified by area size. As the mechanical passaged cells, they exhibit organized stress fiber formation which indicate stress.

Enzymatical passaged cells with ROCKi also had a different morphology and decreased expression levels of pluripotency markers. However, unlike cells without ROCKi, the cells did not display organized actin filaments. This indicates that even though levels of pluripotency markers are decreased, the cells show no sign of stress as a result of passaging, in line with earlier data about ROCKi decreasing cellular stress¹⁰¹. The precise regulation of the structure and dynamics of the actin cytoskeleton is essential for many vital developmental and physiological processes, and abnormalities in actin dynamics are associated with many pathological disorders¹³⁵. The large amount of impact on cells due to passaging techniques could be an indication that, in our hands, dissociation of cells into single cell suspension has a clear negative effect on the cells pluripotency, but ROCKi keep the cells in a state with less stress.

Earlier published work has suggested that the addition of ROCKi would overcome problems such as apoptosis, with no impact on hESC characteristics¹⁰¹. However our cells seem to react differently with ROCKi. Even though ROCKi reduces levels of stress when being dissociated, their characteristics are affected in regard of their pluripotent state with a drop in pluripotency marker expression. In this study we can also see a difference between the enzymatically passaged cells with ROCKi added, and the cells that were enzymatically passaged without the inhibitor. Cells passaged without the inhibitor did seem to undergo programmed cell death to a larger extent than the cells with the inhibitor, known as an apoptosis inhibitor. Also, cells passaged without ROCKi exhibit lower proliferation rates, leading to less colony formation. Together with organized stress fiber formation, comparable with those found in mechanical passaged cells, this suggest that ROCKi may have led to the survival of cells which without the addition of the inhibitor would have been excluded by apoptosis, due to their lost stem cell characteristics.

The loss in expression of pluripotency marker could potentially be an indicator that the cells were heading towards cancerogenic properties, but the oncogene c-MYC were not detected in these cells, neither at gene expression level nor on the promoters of these genes.

The fact that these cells with the decrease in expression of pluripotency markers did not have any correlation with the acetylation or methylation of epigenetic marks for active or repressed genes, may be one of the explanations why these effects were reversible, where the effect only seemed to be measurable on a mRNA level and not in the chromatin state of the promoters. The reversible passaging may therefore have led to the selection and survival of the truly pluripotent cells for each further passage. These finding demonstrate the importance of caution when cells are being passaged using different techniques.

During the revision of this manuscript we are performing FACS and western blot in order to determine if we can detect differences also on the protein level.

Not only passing techniques influence the pluripotent SCs. The occurrence of genetic and epigenetic changes in hESC lines and hiPSC lines in early or late passages are also important in respect to their use in regenerative medicine. In *study III* we hypothesized that cells kept in culture for many passages exhibit larger amount of DSBs, than found in cells from early cultures.

Downs and co-workers showed by ChIP analysis that DSB in mammalian cells induce acetylation of several lysines, namely 5, 8, 12 and 16 within histone H4¹⁰⁸.

The chromatin remodeling complex SWI/SNF was found at DSB sites in budding yeast¹³⁶⁻¹³⁷ and was therefore believed to have an important role in DSB repair by altering nucleosome positions at the site of DSBs. The complex contains a core ATPase, either BRG1 or BRM, plus seven or more non-catalytic subunits¹³⁸. SWI/SNF-type chromatin remodeling complex is present in all eukaryotic cells, where they act in an ATP-dependant manner to disrupt chromatin structure and facilitate the binding of transcriptional regulators at nucleosomal sites. The activity of SWI/SNF complex is crucial for proper differentiation and development¹³⁹.

It was found when studying a major repair pathway for DSBs called non-homologous end joining (NHEJ) where two broken DNA ends are joined together. Acetylation of histones located at DSBs by the HATs CBP/p300, results in the recruitment of BRG1 to facilitate for the chromatin remodeling complex SWI/SNF to alter the nucleosomes¹³¹. Particular lysine 18 on histone H3, but also on lysines 8 and 12 (among others) on histone H4 are acetylated during DSBs¹⁴⁰.

Our study investigating epigenetic changes in cells from early and late passages both in hESC lines and hiPSC lines, looking at lysines 8 and lysine 12 on histone H4 did not indicate that these specific marks were involved in the acetylation of histones on sites of DSBs. We have been looking at the promoter region of genes associated with pluripotency, but also the oncogene c-MYC. c-MYC is one of the four factors used to reprogram the hiPSC lines used, and it has been shown that the regulation of c-MYC expression is profoundly dependent on the activity of the SWI/SNF complex. It is therefore believed to play an important role in reprogramming where SWI/SNF promotes reprogramming by interacting with c-MYC. First SWI/SNF activates the expression of c-MYC and thereafter recruits SWI/SNF to the promoter of the pluripotency factors to enhance their reprogramming. At the same time, the SWI/SNF complex is recruited on the promoters of differentiation genes and maintain these genes in a repressed state, and finally the somatic cells are induced into iPSCs¹⁴¹. To investigate whether DSBs are more common in cells in late passage compared to early, there are other possible genes to investigate, such as BRG1. BRG1 is, as mentioned, one of the subunits of the SWI/SNF complex having enzymatic activity and also as a bromodomain, that recognizes acetylated lysine residues such as those on N-terminal tails of histones.

Staining for γ -H2AX, a gene coding for H2A, which becomes phosphorylated on serine 139 as a reaction of DSBs, could therefore be measured to evaluate its activity in damage response.

Due to the lack of correlation between gene expression and acetylation of the two studied lysines, it is possible that other epigenetic modification play a more important role in the regulation of stem cell characteristics of pluripotent SCs, for example acetylation on histone H3. It has been suggested that SWI/SNF stimulates H3 acetylation by facilitating S139ph and by studying knock-down BRG1 it was found that acetylation at K9, K14, K18 and K23, but not at K27, were largely decreased by

SWI/SNF knockdown, but methylations at most residues were not affected by SWI/SNF knockdown¹⁴².

Other studies have linked the enzyme DNA topoisomerase II β -dependent DSBs and the components of the DNA damage and repair machinery in regulated gene transcription¹⁴³, it could therefore be of interest to study this enzyme in correlation with promoter regions of the genes associated with pluripotency.

It is possible that methylation of the DNA and not methylation of histone tails could be more important when looking at epigenetic changes in late passage, comparing hESC lines and hiPSC lines. Recent studies have identified differences in DNA methylation between hESCs and hiPSCs¹⁴⁴⁻¹⁴⁵. By studying iPSCs generated from somatic cells from all three germ layers and comparing them with their original un-reprogrammed cells it was found that early passage iPSCs retain a transcriptional memory of the original cell, which could be explained by incomplete promoter DNA methylation¹⁴⁶. It is possible that the hiPSC lines used in *Study III* in early passage exhibit more DSBs originating as a transcriptional memory from the fibroblasts that they originally reprogrammed from, which might be lost in later passages. By studying DNA methylation this could possibly identify differences between hiPSCs and hESCs, and early and late passage which could be correlated with differences in gene expression.

hESC lines destined for therapeutic use should have a normal genetic composition, however, keeping cells in culture for prolonged periods may result in genetic instability of the cells, and the possibility that these cells may possess the ability to produce tumors *in vivo* present a major challenge.

Our findings in *study IV* where larger chromosomal changes in teratomas *in vivo* was discovered compared with the same cells cultured *in vitro* in parallel with the teratoma growth may influence the potential use of these cells. The three different hESC lines used in this study revealed that the mutation rate differ between different cell lines, suggesting that there are variations to what extent the cells are prone to undergo mutations. As pointed out, the increased mutation rate of up to 30% in the teratomas suggest that while adaptation to the *in vitro* environment is already achieved, and the rates of new mutations are small, when injected into mice, the stem cells have to re-adapt to their new environmental conditions, which may cause the increased mutation coverage. The period of 8 weeks that the teratomas grew in the mice is a relatively short period, and it remains unclear whether the mutation coverage would increase with time and also be of more malignant types, or if the mutations are acquired within the first cell divisions and thereafter remain stable. To assess safe clinical use of these cells more studies are necessary. The tissues seen after an 8-week period did not contain any malignant features and were not invasive or metastatic, however it is not possible to study whether this would change after a longer period, since it is, of ethical reasons, not possible to keep the teratomas in the mice for longer periods. This result shows the importance of awareness when transplanting these cells, and the risks of not fully removing pluripotent cells from the cell populations aimed to be transplanted. As known to date, differentiated cells no longer have a teratoma-forming capacity, but our results underline the importance of more detailed genetic testing of pluripotent SCs before their clinical application in regenerative medicine, and not only cytogenetic testing.

It is known that a large portion of early human embryos are chromosomally abnormal, often due to the fact that embryos with poor morphological or developmental delays are

the ones donated to research and are used to derive new lines, whereas the normal ones are used for the patients undergoing fertility treatment in first hand. The large mutation coverage in one of the three lines in this study could potentially be due to this, since it has been shown that there is no correlation between embryo morphology and success in deriving new hESC lines¹²⁶. However it is most likely that the vast majority of the mutations found are due to culture adaptation and microenvironmental conditions when being xeno-grafted, and that for clinical applications it will be important to minimize the time in culture.

Another mechanism that could potentially contribute to the genomic instability of hESC lines could be the abnormal DNA repair system found in pluripotent SCs but not in somatic cells whereby the mitotic spindle assembly checkpoint is functional but does not initiate apoptosis as it does in somatic cells¹⁴⁷.

These observations of the extent of CNVs in the genome in hESCs leaves an open question regarding the extent to which CNVs account for hESC genomic variability and whether individual hESC lines have characteristic gains or losses of genetic material that may influence their replication, proliferation, differentiation or functional potential. It is known that extra copies of chromosome X may lead to an increased dosage of a gene or genes that favor self-renewal. Most of the 67 variations found in the teratoma cells from HS364 were clustered on the X chromosome, suggesting that the cells have extra X chromosomal material. Recently, it has also been shown that iPS cells can acquire mutation during their derivations, although many of these mutations are lost on subsequent passaging¹⁴⁸.

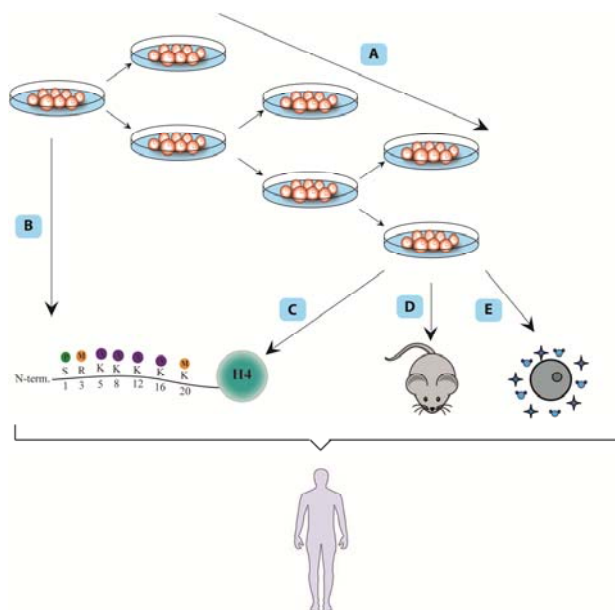


Figure 14. Thesis summary. There are many aspects to consider before pluripotent SCs can be used for patients in a safe manner. (A) We have shown that passaging techniques influence the stem cell characteristics when using different techniques, and (B, C) there are variations in gene expression and acetylation profiles in different cell lines, but no detected correlation between them (D). Pluripotent SCs gain more genetic mutations in vivo, than in cells cultured in parallel in vitro. The mutations gain were especially located on chromosome X, known to be involved in growth advantages. (E) To keep pluripotent SCs cryopreserved and ready for patients we identified a freezing system with minimal effect on SC characteristics.

6 CONCLUSION

Over the past few decades, the scientific community has continued to build a remarkable understanding of the complex nature of SCs. Through the four studies in this thesis we have clarified a few of the obstacles of using pluripotent SCs.

The cryopreservation system using CELLBANKER together with the thawing solutions results in better survival of pluripotent SCs compared to the more laborious techniques using only one cryoprotectant.

Different passaging methods do affect the gene expression of the most commonly used pluripotency markers, however the effects seen are not permanent and can therefore be reversed by using a non-enzymatical technique.

Acetylation of lysine 8 and lysine 12 on histone 4 which have previously been described to be important in the recruitment of the SWI/SNF complex at DSB sites does not seem to be the main acetylated lysines involved in regulation of pluripotency in both hESC and hiPSC lines.

Larger chromosomal changes were found by SNP analysis in *in vivo* cultured cells within teratomas, than in *in vitro* cultured pluripotent SCs. This was probably due to mosaicism for XY and XX cells present in teratomas, which was revealed by FISH analysis.

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