DERIVATION, CHARACTERIZATION AND DIFFERENTIATION OF FEEDER-FREE HUMAN EMBRYONIC STEM CELLS

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To my grandfather

ABSTRACT

Human embryonic stem cells (hESCs) are pluripotent cells with self-renewal ability, derived from the inner cell mass of a human blastocyst. They have the remarkable potential to develop into different cell types and can thus be used to regenerate and restore damaged tissues and organs in the entire body. Hence, hESCs are of great importance when it comes to future cell-based therapies. In addition, hESCs are also suggested as the ultimate source of cells for drug screening, functional genomics applications and studying early human embryonic development. Despite the recent advances in culture techniques for undifferentiated hESCs, there is a great need for further improvements until hESCs can be applied to human medical conditions. Since hESCs are traditionally cultured on feeder-cells or a coating replacing feeder-cells, some of the issues to address are a less laborious system, cost-effectiveness, culture stability, well-defined components, xeno-free culture conditions and compatibility with good manufacturing practice. In order to use hESCs in clinical applications, it is further highly important to also compare their differentiation capacity towards different tissues to that of other cells sources.

This thesis report an improved culture technique of undifferentiated hESCs in which the cells can be cultured directly on plastic surfaces without any supportive coating. This technique supports the undifferentiated state of the cells, which are denoted matrix-free growth-hESCs (MFG-hESCs). To our knowledge, this is the first study presenting a coating independent culture technique of undifferentiated hESCs. The MFG-hESCs highly resemble feeder-cultured hESCs, retaining the undifferentiated morphology characteristic of hESCs and further grow as colonies in monolayer. In addition, these cells display a high expression of markers for pluripotency like Oct-4, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 and differentiate into tissues of all three germ layers while retaining a normal karyotype. Further characterization and genome-wide expression analysis in comparison to feeder-cultured hESCs revealed that MFG-hESCs have the advantage of increased expression of integrins and extracellular matrix (ECM) proteins, which might be the key factor(s) explaining their attachment and growth on the plastic.

Studying the osteogenic ability of MFG-hESCs compared to human mesenchymal stem cells (hMSCs) revealed a superior ability of the MFG-hESCs to form mineralized matrix. Further results pointed out that these two cell types use different signalling pathways for differentiation into the osteogenic lineage. Microarray analysis revealed that several genes involved in ossification are differently expressed in undifferentiated cells from these two cell types. Quantitative PCR showed that MFG-hESCs had a significantly higher

expression of *OPN* during osteogenic induction while the opposite was true for *ALP, TGFB2, RUNX2* and *FOXC1*. We also report an efficient differentiation method for the generation of chondroprogenitor cells from hESCs. This method is based on direct co-culture of hESCs and chondrocytes. In contrast to hESCs, the co-cultured hESCs can be expanded on plastic. Those cells are further able to produce significantly increased content of cartilage matrix, both in high density pellet mass cultures and hyaluronan-based scaffolds. They further form colonies in agarose suspension culture demonstrating differentiation towards chondroprogenitor cells.

Taken together, this thesis reveals improved culture technique of undifferentiated hESCs avoiding feeder-cells and coating matrix, which promotes stable culture condition of hESCs and facilitates large-scale production, making expansion of hESC less laborious and time-consuming. This thesis also demonstrates the potential of the culture environment to influence differentiation of hESCs towards the mesodermal lineage. In addition, this thesis demonstrates osteogenic and chondrogenic differentiation of hESCs which further can be used in experimental studies like toxicology testing and drug screening, and the differentiation potential demonstrated suggests a potential use of hESCs in future cell therapies.

LIST OF PUBLICATIONS

I. **Narmin Bigdeli**, Maria Andersson, Raimund Strehl, Katarina Emanuelsson, Eva Kilmare, Johan Hyllner and Anders Lindahl. Adaptation of human embryonic stem cells to feeder-free and matrix-free culture conditions directly on plastic surfaces. **J Biotechnol** 2008 Jan 1; 133(1):146-53.

II. **Narmin Bigdeli**, Giuseppe Maria de Peppo, Anders Lindahl, Maria Lennerås, Raimund Strehl, Johan Hyllner, Camilla Karlsson. Extensive characterization of matrix-free growth adapted human embryonic stem cells; a comparison to feeder cultured human embryonic stem cells. **In manuscript.**

III. **Narmin Bigdeli**, Giuseppe Maria de Peppo, Maria Lennerås, Peter Sjövall, Anders Lindahl, Johan Hyllner,Camilla Karlsson. Superior osteogenic capacity of human embryonic stem cells adapted to matrix-free growth compared to human mesenchymal stem cells. **Submitted to Tissue Engineering Part A.**

IV. **Narmin Bigdeli**, Camilla Karlsson, Raimund Strehl, Sebastian Concaro, Johan Hyllner, Anders Lindahl. Co-culture of human embryonic stem cells and human articular chondrocytes results in significantly altered phenotype and improved chondrogenic differentiation. **Stem Cells** 2009;27:1812–1821

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ABBREVIATIONS

AC - adult chondrocyte
ACT - autologous chondrocyte transplantation
ANX - annexin
ALP - alkaline phosphatase
AS - akademiska sjukhuset
ASCs - adult stem cells
ASMA - α-smooth muscle actin
bFGF - basic fibroblast growth factor
BMPs - bone morphogenetic proteins
COMP - cartilage oligomeric matrix protein
Cy3 - cyanine 3
DAPI - 4´-6´Diamidino-2- phenylindole
cDNA - complementary DNA
D-MEM - dulbecco's modified eagle medium
DMEM/F12 - DMEM/nutrient mixture F-12
DMEM-HG - DMEM/ high-glucose
DMEM-LG - DMEM/ low-glucose
DMSO - dimethyl sulfoxide
EBs - embryoid bodies
ECM - extracellular matrix
EDTA - ethylene diamine tetraacetic acid
EGF - epidermal growth factor
EPL - early primitive ectoderm-like
ESCs - embryonic stem cells
EtOH - ethanol
FC - fold change
FACS - flow cytometry
FBS - fetal bovine serum
FCS - fetal calf serum
FISH - fluorescence in situ hybridization
FITC - fluorescein isothiocyanate
FOXC1 - forkhead box C1
GAGs - glucosaminoglycans
GCOS - GeneChip operating software
HBSS - Hank's Balanced salt solution
HCL - hydrochloric acid
hEF - human embryonic fibroblasts
hESCs - human embryonic stem cells
hMSCs - human mesenchymal stem cells
HNF3β - hepatocyte nuclear factor 3β

HSPG - heparan sulfate proteoglycans
ICM - inner cell mass
IGF - insulin-like growth factor
ITS - insulin, transferring, selenium
iPSCs - induced pluripotent stem cells
mEF - mouse embryonic fibroblasts
MFG - matrix-free growth
MSCs - mesenchymal stem cells
MCP - metacarpal phalangeal joint
NC - neonatal articular chondrocyte
NEAA - non essential amino acids
OCPC - ortho-cresolphthalein complexone
Oct-4 - POU transcription factor-4
OPN - osteopontin
PBS - phosphate buffered saline
PCR - polymerase chain reaction
PEST - penicillin-streptomycin
PFA - paraformaldehyde
REX1- reduced expression protein -1
RNA - ribonucleic acid
RT-PCR - reverse transcriptase polymerase chain reaction
RUNX2 - runt-related franscription factor 2
SA - Sahlgrenska University hospital
SCID - severe combined immunodeficient
SR - serum replacement
SRGN - serglycin
SSEA - stage specific embryonic antigens
SSC - sodium chloride-sodium citrate
TDGF-1 - teratocarcinoma-derived growth factor-1
TERT - telomerase reverse transcriptase
TGFβ - transforming growth factor beta
TOF-SIMS - time-of-flight secondary ion mass spectrometry
TRA - tumour rejection antigen
TRITC - tetramethyl rhodamine iso-thiocyanate

INTRODUCTION

Stem cells have over the last decade attracted a lot of attention in several medical research fields. They are considered being the optimal solution to treat several medical disorders and are of great importance when it comes to future cell-based therapies. They have the remarkable potential to develop into many different cell types which can be used to regenerate and restore damaged tissues and organs in the entire body. Such disorders or diseases could for instance be chronic heart failure (after stroke), muscular dystrophy, end-state kidney disease, cancer, fibrosis and hepatitis, osteoporosis, osteoarthritis and burns. Stem cells can also be stimulated to produce different vital hormones and factors such as insulin to cure diabetes and can be beneficial for other autoimmune diseases including rheumatism, lupus and multiple sclerosis. Moreover, the stem cell research provides us with knowledge concerning embryological development in general.

BACKGROUND

Stem cells

Definition of stem cells

Stem cells are unspecialized cells, characterized by their ability of long-term self-renewal through mitotic cell division with the potential to differentiate and give rise to mature cell types that have characteristic morphologies and specialized functions. They can be induced to become a specific cell type, tissue or organ[1]. However, after the embryological differentiation process there are still some stem cells remaining in many tissues, which are able to serve as a sort of internal repair system, dividing and differentiating to replenish or repair the damaged cells in that tissue especially in tissues with high cell turnover such as skin and blood. There are different types of stem cells: embryonic stem cells (ESCs), adult stem cells (ASCs) and the recently developed induced pluripotent stem cells (iPSCc).

Embryonic stem cells

The ESCs are derived from the inner cell mass (ICM) of the blastocyst stage of a fertilized embryo[2]. These cells are known to be pluripotent and able to divide indefinitely giving rise to cells derived from all three germ layers: ectoderm, endoderm and mesoderm to subsequently generate all cell types in the body. During the embryological development, each cell within the embryo proliferates and differentiates and becomes increasingly specialized. When an ESC divides, each new daughter cell has the potential to either remain a stem cell or to become a cell with a more specialized function (figure 1)[1]. Analysis of arrested embryos demonstrated that embryos express pluripotency marker genes[3]. Similar to an embryo, human ESCs (hESCs) express the same markers which are considered as key factors in maintaining pluripotency and are commonly used to designate the identity of hESCs. Some of those genes are; NANOG, POU5F1/OCT-4, SOX2, TDGF-1, ALP, SSEA-3, SSEA-4, TRA1-60, TRA-1-81, TERT and REX1 which are responsible for self-renewal and pluripotent differentiation[3, 4].

Adult stem cells

The ASCs, or somatic stem cells, are undifferentiated cells found in differentiated tissues throughout the body. These cells divide to replenish dying cells and regenerate damaged tissues. They are distinguished from ESCs by being multipotent i.e. they can only produce a limited number of cell types and divide a limited number of times[1]. For several years it has been believed that the ASCs are only represented in tissues with high cell turnover, but recently several studies have demonstrated the presence of ASCs in different kinds of tissues and organs which was believed to be non-mitotic[2, 5, 6]. In addition, there are wide ranges of studies performed regarding ASCs, such as mesenchymal stem cells (MSCs) and stem cells from umbilical cord blood, which can be derived and cultured easier and without any ethical issues in comparison to hESCs.

Mesenchymal stem cells

MSCs are adult stem cells with extensive capacity for self-renewal while maintaining their multipotency. In contrast to hESCs, the MSCs are fibroblast-like cells and morphologically characterized by small cell bodies that are long and thin. It has been shown that MSCs are able to differentiate into osteoblasts, chondrocytes and adipocytes *in vitro*[7-10]. Culturing of these cells in the presence of osteogenic stimuli such as ascorbic acid, inorganic phosphate, and

dexamethasone could promote their differentiation into osteoblasts. In contrast, the addition of transforming growth factor-beta (TGF-b) induced chondrogenic markers and adipogenic inducing factors such as dexamethasone, insulin and isobutyl-methyl-xanthine promotes differentiation toward adipocytes. MSCs can be derived from several tissues but are usually aspirated from bone marrow. However, there is no test that can be performed to characterize true MSCs but there are surface antigens that can be used to isolate a population of cells that express proposed markers such as CD105, CD166 and CD90[10, 11]. The standard test usually performed is to confirm the multipotency of the MSC population by differentiating into osteoblasts, adipocytes, and chondrocytes. However, the degree to which the cells will differentiate varies among individuals, and the capacity of cells to differentiate and proliferate is known to decrease with the age of the donor as well as the time in culture. Only a very small population of the bone marrow derived cells consists of MSCs, but this population can be enriched by standard cell culture technics[12]. MSCs are adherent to tissue culture plastic while red blood cells or haematopoetic progenitors within 24 to 48 hours of seeding do not have this capacity[12]. One can also sort the MSCs by flow cytometry based methods using specific surface markers such as STRO-1. Those cells are generally more homogenous and have higher rates of adherence and proliferation[13].

Induced pluripotent stem cells

iPSCs are a new type of stem cells derived from somatic cells, and are genetically reprogrammed to assume a stem cell-like state. The iPSCs resemble ESCs in their properties and potential to differentiate into a range of adult cell types. Transgenic expression of only four transcription factors (c-Myc, Klf4, Oct4 and Sox2) is sufficient to reprogram these cells to a pluripotent state[14].

Progenitor cells

Progenitors or precursor cells, are partly differentiated cells, which still have the capacity to differentiate into a specific type of cell. In this point of view, they may resemble to ASCs. But progenitors are in a farther stage of cell differentiation i.e. they are already far more specified. In contrast to stem cells, the progenitor cells can only divide a limited number of times and are stimulated to differentiate into a specialized mature cell type (figure 1).
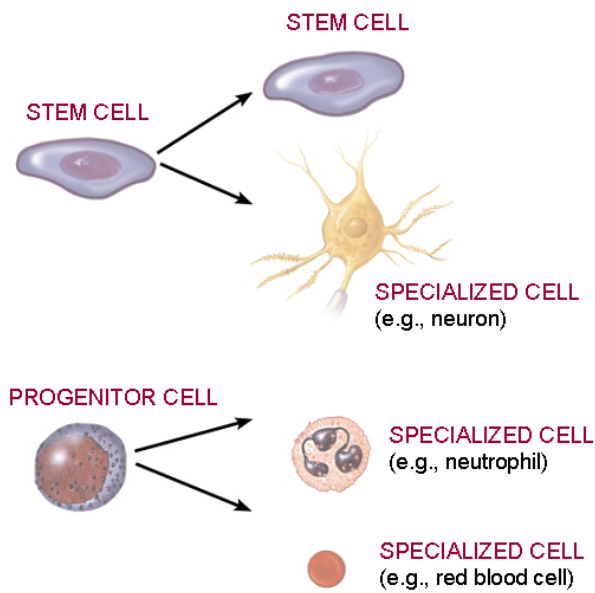
Figure 1. Distinguishing features of stem cells and progenitor cells. The product of a stem cell undergoing division is a specialized daughter cell and a stem cell which has the same capabilities of the originating cell, while a progenitor cell divides to two specialized cells. (Illustration modified from Stem cells: Scientific progress and future research directions, June 2001, with permission from Terese Winslow).

Generation and culturing methods of undifferentiated embryonic stem cells

Derivation of hESCs (feeder-dependent culture)

hESCs are derived from a 4- to 5-days-old fertilized human embryo at the blastocyst stage. A blastocyst possesses three different structures; an ICM, which later forms the embryo and three embryonic germ layers, a cavity known as the blastocoele, and an outer layer of cells, called trophoblast, which forms the placenta and surrounds the blastocoele. The human blastocyst *in vitro* consists of 200 to 250 cells. It is the ICM that is the source of the stem cells, at this stage it is composed of 30 to 34 cells (figure 2). To derive stem cells from a blastocyst, the trophoblast is removed, either by microsurgery or immunosurgery. The isolated ICM is then plated onto a tissue culture dish precoated with mitotically inactivated mouse embryonic fibroblasts (mEF) or human embryonic fibroblasts (hEF) in hESC culture medium[15]. Since the ES cells are not able to attach and grow on non-coated culture dishes, the presence of feeder layer is essential for their attachment and growth. After the cells have been isolated from the ICM, they divide and spread over the surface of the dish. The outgrowth is dissociated into small pieces mechanically, using a Stem Cell Cutting Tool[TM] shaped as a micropipette. The cells are then replated on new mEF or hEF layers in new hESC medium. The small pieces attach to the new feeder layer and grow as individual colonies with undifferentiated morphology

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(figure 3). The same procedure is being repeated every 4 to 5 days. This culture method is known as a feeder-dependent culture of hESCs.

The main advantages of the feeder-dependent culture system and mechanical transfer are the absence of cell-dissociating enzymes and the ability to perform a positive selection at every passage by isolating undifferentiated hESCs from more differentiated cells. It is also a much cheaper system compared to the feeder-free system in which the culture dishes are coated with i.e. Matrigel™, which is usually used for culturing of undifferentiated hESCs. However, the method is very laborious, time-consuming and unsuitable for large scale cell cultures. In fact, the presence of feeder cells in the culture makes it less attractive to stem cell based therapy and regenerative medicine.

Figure 2. Human blastocyst showing inner cell mass, trophoblast and blastocoele. (Illustration modified from Stem cells: Scientific progress and future research directions, June 2001, with permission from Terese Winslow, Photo Credit: Mr. J. Conaghan).
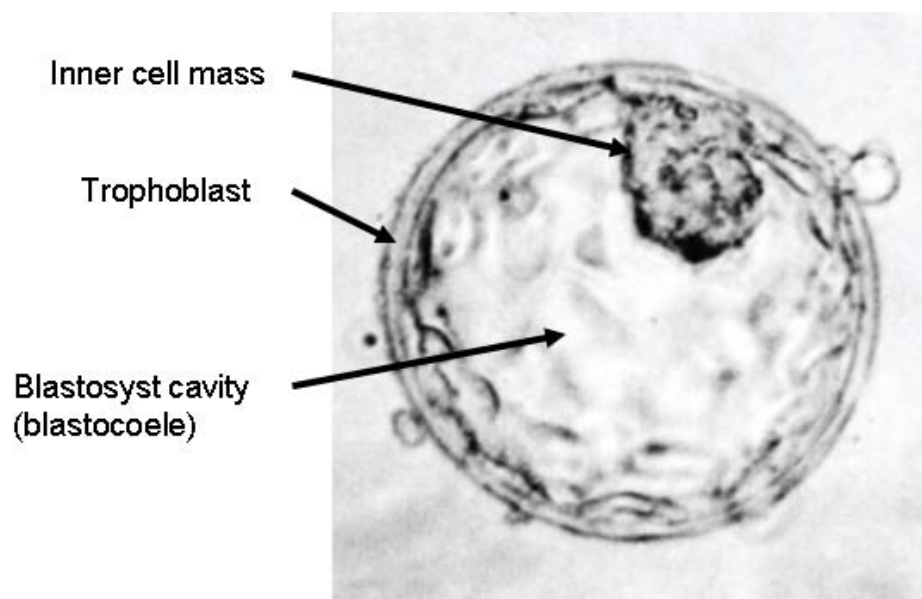
Feeder-free cultures of hESCs

The hESCs can also be cultured feeder-free on coated plates such as Matrigel™, laminin[16] or fibronectin[17]. In this culture system, mechanical dissociation of hESCs has been replaced with enzymatic dissociation and subsequent passage of cell clumps[18] or single cells[16, 17, 19]. Lack of mEF feeder cells in this culture technique makes it more attractive to stem cell therapy, since the risk of viruses or other macromolecules being transmitted to the human cells is eliminated. However, the system is still not a xeno-free culture system which is a prerequisite for transplantation of hESCs. When culturing hESCs without feeder layers on coated surfaces, it is necessary to use conditioned hESC medium. Such

a culture medium is obtained when the hESC medium is incubated on mEF feeder cells, though one can avoid this problem by using hEF cells to make conditioned hESC medium.

Xeno-free culture of hESCs

Culture techniques where hESCs can be cultured under xeno-free conditions in the absence of an animal feeder layer and animal components, are a prerequisite for transplantation of hESCs[19]. To grow hESCs without mEF cells and replace it with human feeder systems[18, 20] can be considered as a xeno-free system. However, the presence of the feeder cells in the culture, still makes it unsuitable for cell-based therapies and limits large-scale production of hESCs. For this reason it has been critical for researchers to develop novel methods for culturing of undifferentiated hESCs which can keep the promise in tissue engineering and regenerative medicine as a source of tissue-specific cells.

Figure 3. Techniques for generating embryonic stem cell cultures. (Illustration is reprinted from Stem cells: Scientific progress and future research directions, June 2001, with permission from Terese Winslow).
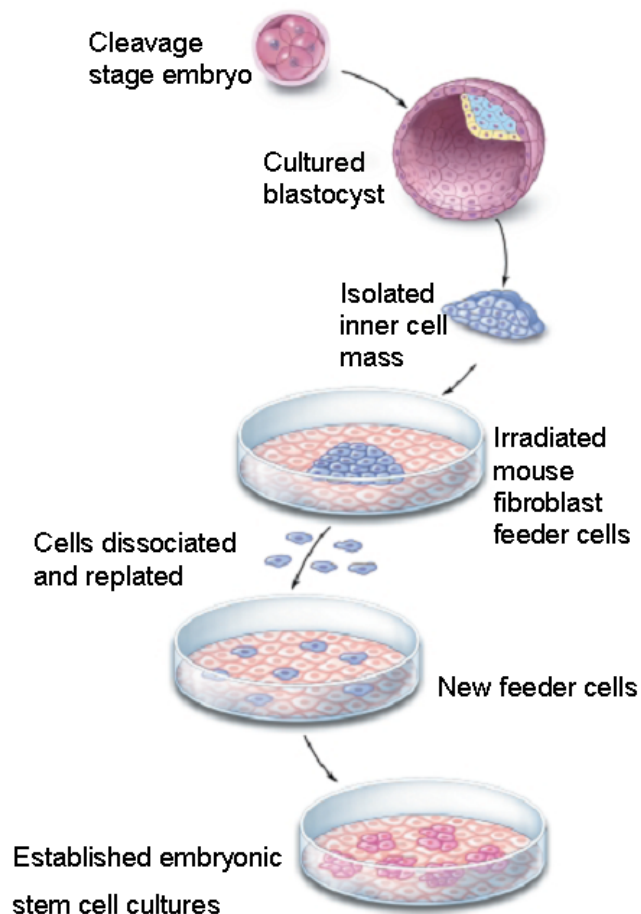
Characterization of undifferentiated hESCs and stem cell markers

So far there is no agreed standard test that demonstrates that stem cells are in their undifferentiated stage, but there are several kinds of criteria that a hESCs shall fulfill to be considered undifferentiated. The cell morphology is one of those criteria. The undifferentiated ESCs have epithelial-like cell morphology and grow in monolayer colonies. The other criterion is the proof of pluripotency and the ability to generate all three embryonic germ layers (mesoderm, endoderm and ectoderm). This can be demonstrated *in vitro* using a three dimensional culture system which triggers spontaneous differentiation of the cells. This system is denoted embryoid body formation (EB). The corresponding *in vivo* system is the test of teratoma formation, i.e. injecting hESCs into immunocompromised mice. Expressing pluripotency markers in monolayer culture is also good evidence. Some of those widely used markers are Octamer Transcription Factor-4 (Oct-4), stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumor-rejection antigen (TRA)-1-60, TRA-1-81 as well as the marker of early differentiation SSEA-1. Finally, retaining a normal karyotype after long-term growth and self-renewal is also a prerequisite for their future clinical use.

Differentiation of stem cells

In vivo/embryonic differentiation

Differentiation is the process by which a less specialized cell such as a stem cell becomes a more specialized cell type. Embryonic development is a three dimensional spontaneous process in which the cells gradually differentiate into more specialized cells. The zygote divides and forms an embryo which differentiates to derivate of the three major embryonic germ layers; endoderm, mesoderm, and ectoderm. These three germ layers subsequently give rise to all cell types and tissues in an organism's body. The ectoderm (external layer) gives rise to skin, neural cells and pigment cells; the mesoderm (middle layer) gives rise to muscle, cartilage, bone and blood cells; the endoderm (internal layer) gives rise to the internal organs (figure 4)[2]. However, the differentiation process is also common in a fully grown organism, as an adult stem cell divides and creates differentiated daughter cell during tissue repair. Differentiation dramatically changes the morphology, size, metabolic activity, and gene and protein expression of a cell. A cell differentiates by internal gene signalling and external signals such as factors secreted by other cells, physical contact with

other cells and exchanging materials and molecules in the microenvironment. The internal gene signalling has a central role in cell differentiation, and is a combination of several different and specific genes which are turned on or off at the right time point to transform a cell into a specific cell type. For instance, the SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and Oct4 are all expressed by pluripotent cells in the ICM. An early differentiation marker is SSEA-1 as well as β-III-tubulin, a primitive ectodermal marker. Hepatocyte nuclear factor 3β (HNF3β) is an endodermal marker and α-smooth muscle actin (ASMA) a mesodermal marker. However, cellular proliferation and differentiation processes are controlled by external signals as well. Such signal molecules, called growth factors, are for example, fibroblast growth factor (FGF), bone morphogenetic proteins (BMPs), epidermal growth factor (EGF), insulin-like growth factor (IGF) and transforming growth factor beta (TGF-β) are some of the most well known growth factors.

In vitro differentiation

Differentiation of hESCs may be directed by controlling cell culture condition to desired cell lineages in unlimited numbers[21, 22]. There are several reports describing *in vitro* differentiation of hESCs into neural[23-25], cardiomyogenic[26, 27], hematopoietic[28], pancreatic[29] and osteogenic lineages[30-34]. Similar to the *in vivo* differentiation, the *in vitro* differentiation requires internal gene signalling and the impact from environmental factors. One can stimulate differentiation of the cells with the assistance of factors such as three dimensional culturing methods, different biomaterials as well as the culture medium and its components such as serum, different chemicals and growth factors and its concentration in the culture medium. Other factors, such as cell-cell contact, co-culture with other cell types, as well as using conditioned medium i.e. factors secreted in the medium by other cells are also effective methods to induce cellular differentiation. Using conditioned medium as an indirect co-culture model can also lead to a better understanding of several molecular pathways leading to specification and terminal differentiation of embryonic cells.

There are several studies investigating the impact and the importance of such factors. For instance, the ability of conditioned media to drive specific differentiation of ESCs from mouse and humans towards e.g. early primitive ectoderm-like (EPL) cells[35] or hepatocyte-like cells[36] has been demonstrated. Directing hepatic differentiation of hESCs has also been demonstrated by co-culture[37]. The potential of the culture micro-environment to influence cellular differentiation has been demonstrated by co-culture to drive stem cells towards required lineages in several studies[38-40]. For instance, differentiation toward

distal lung epithelium by co-culturing EBs with distal embryonic lung mesenchyme[41] or toward hematopoietic cells by co-culture with human fetal liver cells[42] and inducing cardiomyocyte differentiation controlled by co-culture as a differentiation model system initiating differentiation to beating muscle[43]. In fact, using cell-cell interaction as a differentiation model system is central in all of these studies and has not been well investigated in developmental biology. Hence, the effect of direct co-culture is essential to understanding the role of cell-cell interaction.
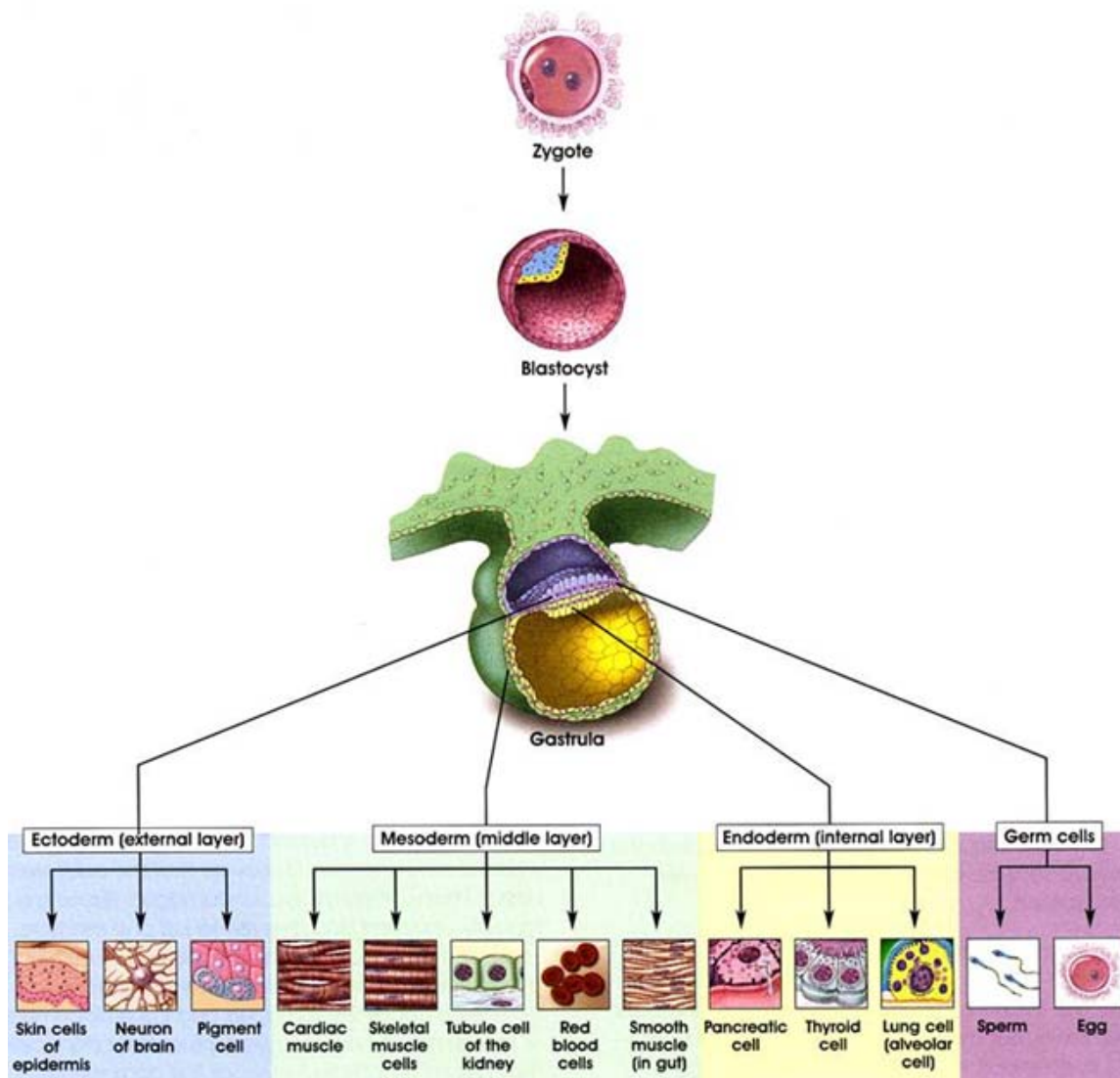
Figure 4. Differentiation to all three embryonic germ layers and subsequently to different tissues. (Illustration is reprinted from Stem cells: Scientific progress and future research directions, June 2001, with permission from Terese Winslow).

Regenerative medicine and stem cell therapy

The goal of regenerative medicine is to replace tissue or organ function lost due to age, damage, disease or congenital defects by functional tissues. Different methods such as organ transplantation or stimulating previously irreparable organs to heal themselves have been experimented through the years. However, problems such as graft rejection and shortage of organs available for donation, are some of the drawbacks to be considered. Since the first successful *in vitro* culture of pluripotent and permanent hESCs[2], the scientists have been empowered by regenerative medicine to grow tissues and organs in the laboratory that can be safely implanted to the body. To date, there are several studies demonstrating the potential of the hESCs to differentiate into different kind of cell types[1]. However, there are still many problems to be solved and considered using hESCs as a therapeutic cell source. Derivation and culture of a xeno-free and feeder-free hESC line, the immunological aspects and the risk and potential of proliferative ES cells developing into cancers cells, are some of the challenges to be addressed.

Articular cartilage histology and matrix composition

The studies presented in this thesis mainly focus on differentiation of hESCs into the osteogenic and chondrogenic lineages. There are three different kinds of cartilage in the human body, the hyaline cartilage which is found in rib cage, joints, nose, trachea and larynx, elastic cartilage in ears and fibrocartilage in spinal columns (discs). Hyaline cartilage is the most common form of cartilage in the body and the studies concerning cartilage in this thesis are focused on hyaline cartilage. The cells which compose the cartilage are called chondrocytes and contribute to 2-5% of the cartilage tissue. Chondrocytes are characterized by their ECM production, which surrounds the cell. ECM in articular cartilage is composed of collagens, proteoglycans, noncollagenous proteins and about 75% water[44-46]. The articular cartilage has a complex molecular organisation with some considerable macromolecules such as collagen type II, collagen type X, Cartilage oligomeric matrix protein (COMP) and aggrecan. There are two different variants of type II collagen, namely collagen type IIA and type IIB. Collagen type IIB is expressed in fully differentiated chondrocytes and type IIA is expressed by non-mature chondrocytes[47,48]. The other noteworthy collagen is type X collagen, which is found in the calcified layers and has a role in mineralization. Aggrecan is the other large macromolecule which has a central core protein that binds chondroitin sulphate and keratin sulphate glucosaminoglycans (GAGs) into hyaluronic acid supported by link protein. The GAGs are negatively charged hence bind water groups and promote osmotic

swelling, which contribute to the compressive stiffness of the articular cartilage[49].

The articular cartilage is divided into several zones depending on its cellular appearance and matrix composition: superficial, transitional, radial and calcified zones[49, 50]. At the surface there is a superficial zone with elongated cells and the matrix consists mainly of collagen type I fibres. This cartilage zone has a frictionless surface due to production of the lubricin protein on the surface layer[51,50]. The transitional zone has the typical morphological features of hyaline cartilage which consists of rounded chondrocytes, and the matrix is rich of proteoglycans and collagen fibres. Then radial zone of the articular cartilage has the lowest cell density with low collagen content in matrix but is rich in proteoglycans such as aggrecan. Beyond that zone there is a layer of calcified cartilage with a matrix rich in type X collagen, lacking proteoglycans and the chondrocytes are in their hypertrophic state.

In contrast to elastic cartilage and fibrocartilage, the articular cartilage tissue does not have any regeneration ability and is totally avascular hence the nutrition of the chondrocytes comes from passive diffusion. Due to this, defects in articular cartilage caused by injuries or diseases have almost no ability to self-repair.

Chondrocyte culture and the effects of monolayer and three dimensional cultures

To access the chondrocytes, a biopsy is harvested by mechanical mincing with a scalpel followed by enzymatic treatment for 24 hours. This procedure releases the cells from the 3D environment and they can be expanded in monolayer culture with support of serum supplemented culture medium. The chondrocyte dedifferentiates when cultured in monolayer with exposure to serum and regains back its pre-chondrogenic phenotype as well as its genetic expression profile. Dedifferentiation is a process in which a partially or terminally differentiated cell reverts to an earlier developmental stage. In this stage the dedifferentiated chondrocytes can divide. Due to the plasticity of chondrocytes, they have the ability to redifferentiate when once again replaced into a 3D environment under the right culture conditions[52, 53]. Some examples of such 3D cultures, which stimulate redifferentiation of the cells are high density pellet mass culture, hyaluronic acid-based 3D scaffold (Hyaff-11 scaffolds) culture and agarose suspension culture[46-48]. The redifferentiation ability of chondrocytes is very important for autologous chondrocyte transplantation (ACT) procedure.

Cartilage and stem cell therapy

Hyaline articular cartilage is one of several subjects in focus on tissue engineering research since it is not able to repair damages caused by injuries or diseases[54]. One of the therapeutic methods which is used today is ACT which was first described by Brittberg, M., et al. here in Gothenburg[55]. In ACT, the patient's own chondrocytes, normally harvested from healthy cartilage at a non-weight bearing area which have been expanded *in vitro*, are implanted into the patient's cartilage defect in combination with a covering membrane. However, there are some disadvantages using this method such as restricted proliferation capacity of the cells in culture, which cause a limitation in size for treatment of the damaged area. The second disadvantage is the need for two surgical procedures for the patient, the harvesting procedure of the healthy cartilage and later on the transplantation of the expanded chondrocytes. Last but not least, some patients lack healthy cartilage. The use of hMSCs is another method worth mentioning. One drawback with these cells is that they tend to differentiate toward hypertrophic cartilage instead of hyaline cartilage, resulting in a tissue that is not adapted to the pressure and shear force that the joint is subjected to[56-58]. Furthermore, the number of hMSCs and their proliferative capacity as well as their synthetic abilities decline with age[59].

With this in mind, it has been a challenge to find suitable cell sources that can be used for cell therapy regenerating cartilage tissues. The research concerning regenerative medicine and cell therapy involving hESCs as an alternative cell source has dramatically increased in the recent years due to their pluripotency and immortality. Hence, the hESCs could be the ultimate cell source, which can potentially provide unlimited numbers of chondrocytes or chondro-progenitor cells, and may have significant potential to be used in cartilage tissue engineering.

Musculoskeletal system

The skeleton and bone compositions

The human skeleton functions as an attachment for muscles and supports motions. It also protects vital organs and even serves as the main reservoir of calcium in the body. It is comprised of 213 bones which offer support to the entire body. The adult skeleton consists of (~70%) inorganic material and 20% organic material. Hydroxyapatite stands for the 95% of the inorganic material

while type I collagen and other proteins such as osteocalcin, bone sialoproteins and osteonectin stand for 98% of the organic fraction. The remaining 2% of the organic part is consists of bone cells such as osteoblasts, which have mesenchymal origin and are responsible for bone formation. Another type of bone cells are osteoclasts and osteocytes. The remaining 5-8% of the bone contains water and lipids.

The epiphyseal plate and bone formation

During the early fetal development, the skeleton consists of cartilage, which eventually is replaced by bone. Cartilage replacement process begins in the middle and progresses out toward each end of what will later form the bone. Meanwhile, the bone increases in both length and thickness. After birth, elongation and growth of bone occur in the area called epiphyseal plate. On the leading edge of the epiphyseal plate there are chondrocytes producing cartilage (cell matrix), which eventually surrounds them and gradually becomes calcified. After calcification of cartilage the chondrocytes die and the calcified material begins to erode which gives the osteoblast the opportunity to move in to the area and begin to form bone. As a result, the zone of active bone formation moves outwards from the centre toward the end of the bones.

Osteogenesis and tissue engineering

There are several million surgical procedures on the musculoskeletal system each year. There are skeletal defects arising from tumours, inflammation, developmental abnormalities and degenerative diseases and disorders which can be improved by cell therapy and tissue engineering. The MSCs have been investigated in tissue engineering and transplantation studies for bone and cartilage repair[60-62]. There are several studies trying to apply the MSCs originating from bone marrow which are known to have osteogenic potential[63-65] for therapeutic purpose. However, MSCs limited expansion capacity *in vitro* is a drawback when it comes to skeletal repair and cell therapy in skeletal system since there is often a need for large number of cells to be replaced in skeletal defects. Hence, the hESCs can be more suitable for tissue engineering, since the hESCs can provide unlimited number of cells and have the remarkable potential to develop into many different cell types.

AIMS OF THE THESIS

The overall aim of this thesis was to study derivation of feeder-free hESCs and to investigate mesodermal differentiation of these cells using several models such as conditioned medium, direct co-culture and chemical substances in the culture medium.

Specific aims

I)   To establish hESC lines which are not dependent on any coating support for expansion in their undifferentiated state (paper I).

II) To compare the global gene expression of feeder-cultured hESCs and MFG-hESCs using microarray analysis (paper II).

III) To investigate the osteogenic capacity of MFG-hESCs in comparison to hMSCs (paper III).

IV) To investigate if the co-culture of hESCs with mitotically inactivated human articular chondrocytes *in vitro* results in increased chondrogenic differentiation potential (paper IV).

MATERIAL AND METHODS

Ethical approval

Ethical approval was given for studies of hESC research by the Regional Ethics Committee in Gothenburg (Dnr 376-05). Ethical approval for culture of chondrocytes was given by the Medical Faculty at Gothenburg University (S 040-01). Ethical approval was also given for the study in severe combined immunodeficent SCID mouse model by the Swedish Board of Agriculture (Dnr 231-2007). The donation of bone marrow for studying hMSCs was approved by the ethical committee at the Medical Faculty at Gothenburg University (Dnr 532-04).

Human embryonic stem cell lines

The undifferentiated hESC lines used in the experiments were the hESC line SA167[66] and the hESC sub-line AS034.1[67], derived and characterized at Cellartis AB, Gothenburg, Sweden. These hESC lines were established from blastocysts collected from Sahlgrenska University hospital (SA) and Akademiska Sjukhuset (AS), respectively.

Preparation of conditioned hESC medium

To grow hESCs in feeder-free culture condition, conditioned hESC medium is required. This media is usually obtained by overnight incubation of hESC medium on a confluent monolayer culture of inactivated fibroblasts. Inactivation of DNA occurs by irradiation of the fibroblasts in order to prevent cell division to hold the number of cells per ml conditioned hESC medium constant. This prevents differences in concentration level of secreted factors in the media from one conditioning to the other. The factors secreted in the conditioned hESC media by the fibroblasts seem to be vital to hESCs to obtain feeder-free culture.

Human diploid embryonic lung fibroblast (hEL) cells[68, 69] were used for all studies to prepare conditioned hESC medium. Mitotically inactive hEL cells were expanded and cultured to a confluent monolayer of 59,000 cells/cm^2 in cell culture flasks (Costar, non-pyrogenic polystyrene; Corning Incorporated, NY, USA) in DMEM/F12 (Dulbeco Modified Eagle Medium; Invitrogen, Paisley,

UK) supplemented with L-ascorbic acid (0.025 mg/ml, Apotekets production unit, Umeå, Sweden), 1% penicillin/streptomycin (PEST; 10,000 u/ml, PAA laboratories, Linz, Austria), 2 mM L-Glutamine (Invitrogen) and 10% human serum[70] at 37°C in 7% CO_2. After 24 hours, the hEL cells were washed with phosphate buffer saline (PBS, PH 7, 45) and the culture medium was replaced by hESC medium (0.28 ml/cm^2) for a 24 hour conditioning period. The hESC medium contained 80% KnockOut™ D-MEM (Dulbeco Modified Eagle Medium; Gibco-BRL/Invitrogen, Gaithersburg, MD, USA), 20% KnockOut™ serum replacement (SR; Gibco-BRL/Invitrogen), 2 mM L-Glutamine(Gibco-BRL/Invitrogen), 0.1 mM β-mercaptoethanol (Gibco-BRL/Invitrogen) and 1% NEAA (nonessential amino acids; Gibco-BRL/Invitrogen). Subsequently, the conditioned medium was collected and sterile filtered using Stericup express filter units (250 ml, 0.2 µm; Millipore Corporation, Billerica, USA). 4 ng/ml basic fibroblast growth factor (bFGF; human, recombinant; Gibco-BRL/Invitrogen) was added to hESC medium directly prior to use.

Neonatal articular chondrocytes (NC) isolated from the distal end of the metacarpal phalangeal joint (MCP) were used to prepare the NC conditioned hESC medium[55]. Articular chondrocytes from the knee of a patient undergoing autologus chondrocyte transplantation were used to prepare the adult chondrocyte (AC) conditioned hESC medium[55]. The NC and the AC conditioned hESC media were prepared as described above with the exception of using NC cells and AC cells respectively instead of hEL cells at the medium conditioning step.

Transfer of hESCs to Matrigel™

To avoid the presence of the feeder cells in the culture for further investigation, the feeder-dependent hESCs were adapted and transferred to the coated culture dishes. The hESC lines SA167[66] and AS034.1[67] were transferred from feeder-supported culture to feeder-free culture on Matrigel™ (Matrix Thin Layer; Becton Dickinson, Bedford, MA, USA) as described previously[66]. In brief, the undifferentiated hESC colonies cultured on mitotically inactivated mEF feeder layers, were mechanically cut into small square pieces and carefully detached using a Stem Cell Cutting Tool™ (0,290-0,310mm, Vitrolife Swemed AB; Kungsbacka, Sweden) and transferred to a Petri dish containing collagenase IV for the enzymatic dissociation for a 5-10 minute incubation period. The process was monitored in a microscope until the optimal cluster sizes of hESCs were obtained. The cell suspension was then centrifuged, washed, resuspended in conditioned hESC medium, and transferred to rehydrated Matrigel™ plates. The cultures were then incubated in a humidified atmosphere at 37°C and 5% CO_2.

Passage and expansion of feeder-free Matrigel^{TM} propagated hESCs

The cell cultures were observed visually by using an inverted microscope. When ready for passage, the culture medium was removed and cells were incubated with Collagenase type IV (200 U/ml; Sigma), dissolved in Hank´s balanced salt solution (HBSS buffer; Gibco/Invitrogen) for 10-15 minutes at 37°C. The hESC suspension was transferred to a centrifuge tube, pelleted by centrifugation at 400G for 5 minutes and subsequently washed twice in KnockOut^{TM} D-MEM. The cells were resuspended in conditioned hESC medium and transferred to a rehydrated Matrigel^{TM} coated dish and subsequently cultured in a humidified atmosphere at 37°C in 5% CO_2 .The culture medium was renewed every second or third day and the cultures were passaged every 5 to 7 days as described above.

Derivation of matrix free growth (MFG)-hESCs

Feeder-free Matrigel^{TM} propagated hESC lines AS034.1 and SA167 were used for the adaptation process to MFG culture conditions. To initiate the adaptation process, the hEL cell conditioned hESC medium was replaced by NC conditioned hESC medium one day prior to passage. The above mentioned procedure was used to passaged the hESCs, which in the end was resuspended in NC conditioned hESC medium and transferred to a rehydrated Matrigel^{TM} coated dish and subsequently cultured in a humidified atmosphere at 37°C in 5% CO_2 for a long culture period. The hESCs were cultured for 20 days without passaging, while culture medium was renewed every second or third day. After 20 days in culture with NC conditioned hESC medium, cells were passaged enzymatically as previously described and cultured on regular culture dishes (Costar, non-pyrogenic polystyrene). After 11 days in culture, the MFG-hESCs colonies were passaged to Primaria® dishes (Falcon, surface modified polystyrene non-pyrogenic; Becton Dickinson,Franklin Lakes,USA) and the NC conditioned hESC medium was substituted with hEL cell conditioned hESC medium.

Passage and culture of MFG-hESCs

For passage of the MFG-hESCs the culture medium was removed and cells were treated with Collagenase type IV for 5 minutes at 37°C. The hESC suspension was pelleted by centrifugation at 400G for 5 minutes and subsequently washed

twice in KnockOut^{TM} D-MEM. The cells were resuspended in conditioned hESC medium and transferred to Primaria$^{®}$ dishes and cultured in a humidified atmosphere at 37°C in 5% CO_2. The MFG-hESC cultures were passaged every 4 to 6 days and the medium was changed every second or third day.

Characterization of undifferentiated hESCs

The cell morphology

One of the high- ranking characterization tools is the morphology of the cells which is observed visually by using an inverted microscope. The morphology of undifferentiated hESCs is well-known to resemble epithelia-like cell morphology i.e. small and round shaped cell morphology which grows in monolayer as colonies[1]. The cells in such colonies grow in a very compact manner (figure 5). A differentiated hESC colony consists of much larger cells usually with fibroblast-like morphology i.e. elongated large cells or other different morphologies which no longer grow as a monolayer and prefer to grow in a 3D direction. The undifferentiated hESCs used in this thesis were first estimated by their cell morphology considering above mentioned criterion.
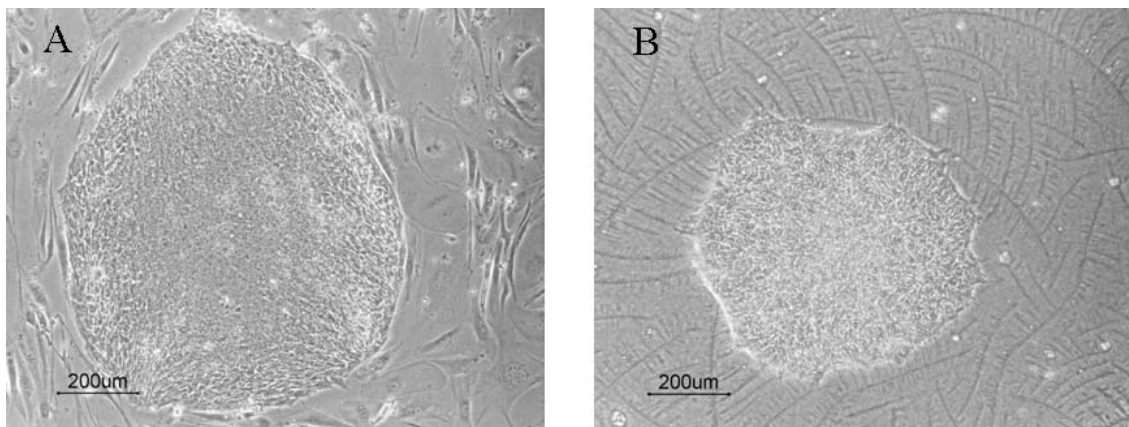
Figure 5. Undifferentiated hESC colony cultured on feeder layer (A) and coating matrix (Matrigel ^{TM}) (B) with epithelia-like cell morphology i.e. small and round shaped cell morphology which grows in monolayer as colonies.

Immunohistochemistry

Immunohistochemistry is a technique for detection, distribution and localization of antigens (eg. proteins) of interest using antibodies raised against those specific antigens (eg. proteins). Monoclonal antibodies against the pluripotency markers Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 as well as the marker of early differentiation SSEA-1 were used for immunohistochemical characterization (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The procedure used for immunohistochemical analysis has previously been described[71]. In brief, undifferentiated MFG-hESCs were fixed in 4% paraformaldehyde (PFA) and permeabilized using Triton X-100. After washing and blocking the process using 10% dry milk, the cells were incubated with the primary antibody. For detection FITC-, Cy3-, or TRITC-conjugated secondary antibodies were used (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA). Finally, DAPI staining was used to visualize the nuclei (Sigma Diagnostics, Stockholm, Sweden).

Karyotyping and fluorescenece in situ hybridization (FISH)

Karyotyping is a staining used for mapping the full chromosome set of the nucleus of a cell. Usually, a suitable dye such as Giemsa, is used to stain the cells captured during the cell division in metaphase. In paper I the karyotype analyses were performed on MFG-hESC lines and the procedure used for karyotyping in this study has previously been described[72]. In brief, the cells were first prepared for metaphase spreads. The cells were washed in cell culture medium, incubated in Calyculin A, which induces chromosome condensation and washed once again in culture medium. The cells were collected by centrifugation, pre-treated in a hypotonic solution, which results in swelling of the cells and spreading of the chromosomes, and then were fixed using ethanol and glacial acetic acid. Trypsin-Giemsa- or DAPI staining were used to visualize the chromosomes. FISH is a cytogenetic technique that detects and localizes the presence or absence of specific nucleic acid sequences within chromosomes in a cell through the use of specific nucleic acid-labelled probes, in which target sequences are stained with fluorescent dye. Hence, fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes. This staining can be performed during metaphase and interphase. For the FISH analysis in paper I, a commercially available kit (Vysis Inc, Downers Grove, IL,USA) containing probes for chromosomes 12, 13, 17, 18, 20, 21, X and Y were used following the instructions from the manufacturer. Slides were analyzed using an inverted microscope equipped with appropriate filters and software (CytoVision, Applied Imaging).

For the FISH analysis in paper II, the cell lines were analyzed for chromosomes X and Y during the interphase. The cells were centrifuged onto a microscope slide 24 hours prior to analysis. The cells were fixed in Carnoy's fixative and washed twice in 1x Sodium chloride-Sodium citrate (SSC) buffer. The slides were dehydrated by treatment with increasing concentrations of ethanol and air dried. The CEP X/Y DNA Probe kit (Vysis Inc, Downer's Grove, IL, USA) was used and slides were prepared according to manufacturer's instructions. Briefly, the DNA was denaturized and probe hybridization was performed. The slides were washed in 1xSSC buffer followed by DAPI staining (Vysis Inc.). The cells were then observed using a Fluorescence microscope.

In vitro teratoma

In vitro teratoma is a model used for characterization of undifferentiated or pluripotent hESCs, in which a hESC colony detaches from monolayer culture and cultures in 3D suspension culture systems as an aggregated colony for 5-6 days, so called EB formation. The 3D culture system triggers differentiation of the undifferentiated hESCs into all three germ layers. For *in vitro* differentiation, the undifferentiated MFG-hESC colonies were surgically dissected and transferred to a Petri dish containing EB-medium (KnockOut-DMEM; 20% fetal calf serum (FCS; invitrogen), 1% PEST, 1% L-Glutamine, 1% NEAA and 0.1 mM β-merkaptoetanol) to form EBs. The EBs were cultured in suspension for 6 days, then plated in gelatin coated culture dishes and cultured for two additional weeks in EB-medium. The EB derived cells were finally fixed in 4% PFA and were analyzed immunohistochemically for endoderm, mesoderm and ectoderm markers. The procedure used for immunohistochemical analysis in the present study has previously been described in detail[71].

In vivo teratoma (SCID mouse model)

In vivo teratoma is yet another widely used method to investigate the pluripotency of the undifferentiated hESCs, which also takes advantage of the 3D environment in an animal model. For the teratoma formation experiment, the undifferentiated MFG-hESC colonies were surgically dissected and mechanically dissociated into small cell aggregates and were injected under the kidney capsule of 5 weeks old SCID mice, (C.B-17/IcrCrl-ScidBR; Charles River Laboratories, Wilmington, MA, USA). The animals were sacrificed eight weeks after injection and the kidneys were surgically removed and immediately fixed in 4% PFA and paraffin embedded. For histological analysis the resulting

teratomas were sectioned, stained and observed in a microscope to be evaluated as described earlier[71].

Isolation and expansion of hMSCs

hMSCs were isolated after informed consent from bone marrow aspirates from the iliac crest of three patients undergoing spinal fusion[12, 59]. Isolation of hMSCs was performed as described previously[73]. Briefly, 5 ml of fresh bone marrow were transferred into 5 ml of a solution of phosphate buffer containing Heparin E500 (Heparin LEO, Apoteket AB, Sweden) to prevent coagulation. Adipose tissue was removed by centrifugation at 1800 rpm for 5 minutes. hMSCs were then isolated by gradient centrifugation using CPT Vacutainer® tubes prefilled with Ficoll (Pharmacia, Uppsala, Sweden) according to manufacturer's instructions.

hMSCs were expanded in medium consisting of DMEM low glucose DMEM-LG, supplemented with 1% Penicillin-Streptomycin, L-glutamine (2 mM), 10% fetal bovine serum (FBS, Gibco-BRL/Invitrogen) and 10 ng/ml bFGF[59]. Media were changed every 3-4 days and cells were passaged when reaching 80% confluence and cultured at 37°C in 5% CO_2.

In vitro differentiation of feeder-free hESCs into the chondrocyte linage

Isolation of chondrocytes and neonatal chondrocytes (NC)

The chondrocytes used in this study were neonatal articular chondrocytes from the distal end of the metacarpal phalangeal or adult articular chondrocytes harvested from the knee joint of middle-aged donors undergoing ACT[55, 74]. Chondrocytes were isolated from the surrounding matrix by mechanical mincing followed by type II collagenase (0.8mg/ml, Worthington Biochemicals, Lakewood, New Jersey) digestion over night as described earlier[55]. The chondrocytes were seeded at 5 000 cells/cm^2 and expanded in medium containing DMEM/F12 supplemented with L-ascorbic acid, 1% PEST, 2 mM L-Glutamine and 10% human serum in a humidified atmosphere at 37°C and 7% CO_2 (Steri-Cult 200 incubator, Forma Scientific). This medium is referred to as Complete Chondrocyte Medium[70,75].

Monolayer culture

In monolayer culture the cells grew adherent to the plastic on regular culture flasks as a monolayer culture. Single cells of chondrocytes were seeded at a density about 5000 cells/cm^2 during each passage in Complete Chondrocyte Medium and medium was changed twice a week. Cells were expanded by passage to new culture flask when they reached 80% confluence. In order to detach the cells, trypsin-EDTA solution diluted in PBS was used.

Chondrogenic differentiation

Articular cartilage is a tissue with low cell turnover and it is known that chondrocytes in adults do not divide *in vivo*. When a cell differentiates it stops proliferating and changes its morphology dramatically. A non-fully differentiated chondrocyte has a fibroblast-like morphology and is elongated. A chondrocyte differentiates when it begins to produce the surrounding matrix. After this differentiation process the chondrocytes are trapped in its own cell matrix. Hence they are not able to divide any longer and change its morphology from fibroblast-like cell to a rounded cell. However, those chondrocytes still retain their proliferation capacity but are trapped in the cell matrix. To be able to give those cells a new space to grow and a chance to divide one needs to liberate the cells from surrounding matrix. This can be done by using enzymatic treatment such as collagenase which dissociates the collagen fibres. This procedure is used to access chondrocytes for culturing and expansion *in vitro*.

Three-dimensional high density pellet mass culture

It has previously been shown that the 3D pellet mass culture systems act as differentiation systems for chondrocytes[52, 76, 77]. The high cell density and the 3D environment trigger the redifferentiation process of the cells in this culture system. In paper IV the 3D pellet mass culture system was used as one of the model systems to redifferentiate the cells. Briefly, 200,000 cells were placed in a conical polypropylene tube in defined pellet medium consisting of DMEM High Glucose (DMEM-HG; PAA Laboratories, Linz, Austria) supplemented with 10% FCS, 5.0 µg/ml linoleic acid (Sigma-Aldrich, Stockholm, Sweden), insulin, transferring and selenium (ITS-G concentrate no. 41400-045, Life Technologies, Paysley, UK), 1.0 mg/ml human serum albumin (Equitech-Bio, Kerrville, TX, USA), 10 ng/ml TGF-β1 (R&D Systems, Abingdon, UK), 10 ng/ml TGF-β3 (R&D Systems, Abingdon, UK), 10^{-5} M dexamethasone (Sigma-Aldrich), 14 µg/ml ascorbate acid (Sigma-Aldrich) and 1% PEST; 10,000 U/ml. The cells

were centrifuged at 400g for 5 minutes and the medium was changed three times a week. After 21 days of pellet mass culturing, the pellets were fixed, deparaffinized and stained with Alcian Blue van Gieson.

Three-dimensional scaffold mediated culture

Another model to study the redifferentiation ability of the chondrocytes is the use of 3D scaffolds. Chondrogenic differentiation of undifferentiated hESCs and co-cultured hESCs were also studied by culturing the cells in hyaluronic acid-based 3D scaffolds (Hyaff-11 scaffolds, Fidia Advanced Biopolymers, Abano Terme, Italy) as described earlier[78]. This scaffold is a polymer derived from the total esterification of sodium hyaluronate with benzyl alcohol on the free carboxyl group of glucoronic acid along the polymeric chaine[79]. The cells were seeded in Hyaff-11 scaffolds and pre-coated with human serum at $4x10^6$cells/cm^2. The constructs were cultured in the pellet medium, with media changes three times a week. After 4 weeks in culture, the membranes were harvested for histological analysis and fixed, deparaffinized and stained with Alcian Blue van Gieson. The sections were observed in a light microscope (Nikon).

Agarose suspension culture

Cartilage progenitor cells have the ability to divide and form colonies when cultured in 3D agarose culture system. This culture system can be used to study the colony formation efficiency and as a characterization model. In paper II the colony forming efficiency was studied using agarose cultures[80]. These cultures were made according to a modified protocol by Beneya and Schaffer[53]. Culture dishes 50 mm with grids (Nunclone, NUNC, Brand Products, Denmark) were precoated with 1% standard low melting agarose (Bio-Rad laboratories, Hercules, CA, USA). A mixture of 0.75 ml 2% low melting agarose and 0.75 ml DMEM/F12 was mixed with 1.5 ml DMEM/F12 containing $5x10^4$ cells and added to the culture dish[80]. The gels were then allowed to solidify at 4°C. The cells were cultured for 6 weeks in Complete Chondrocyte Medium as described above with the exception of using 10% FCS instead of human serum. Clusters of cells with a diameter larger than 50 μm were then counted in a light microscope (Nikon).

Co-culture experiments

To study the effect of co-culture, high density pellet mass culture was used as a model system using the high density environment and cell-cell contact as a differentiation stimulator. After monolayer expansion and irradiation (25 Gray), ACs or NCs were co-cultured with hESCs in pellet masses as described above. The hESCs and chondrocytes were obtained from donors of different gender in order to be able to investigate the purity of the hESC population by fluorescence in situ hybridization (FISH) analysis after the co-culturing procedure. After two weeks of culture the pellets were treated with collagenas type II in order to liberate the cells for monolayer culture and expansion. As control, three additional high density pellet mass cultures were performed with chondrocytes, irradiated chondrocytes and undifferentiated hESC lines in defined pellet medium, as described above.

Conditioned medium

See description above regarding preparation of conditioned hESC medium.

Other differentiation models

Adipogenic differentiation

To analyze the adipogenic capacity of the cells, the protocol described by Pittenger et al.[59] was used which is based on the cytoplasmatic lipid droplets formation and is typically seen in pre-adipocytes. Briefly, the cells were seeded in control medium consisting of DMEM-LG (PAA Laboratories), 20% FCS, 1% L-Glutamine (2 mM), and 1% PEST at density of 10x103 cells per cm^2. After 24 hours, the media were changed to either control medium or adipogenic-inductive medium consisting of control medium with addition of 1% dexamethasone (10^5 M), 60 µM indomethacin, 5 µg/mL insulin and 0.5 mM isobutyl-methyl-xanthine (all from Sigma-Aldrich). Media were changed three times a week. After at least 3 weeks of culture, the cells were washed with PBS, fixed in Histofix™ (Histolab products AB, Gothenburg, Sweden) and stained with Oil-Red O (Merck, Darmstadt, Germany) solution (in 60% isopropanol) for 1 hour. After repeated washings with water, the lipid content was assessed with microscopic inspection in a light microscope (Nikon).

Osteogenic differentiations

To investigate the mineralization capacity of the cells *in vitro*, well known osteogenic assay protocol was used[81]. The culture medium used in this protocol consist of dexamethasone known to induce mineralization of the ECM[82] and ß-glycerol phosphate, which serves as source of phosphate ions[83]. The cells were seeded in control medium consisting of DMEM Low Glucose (DMEM-LG), 20% FCS, 1% ascorbic acid (5 mM), 1% L-Glutamine (2 mM) and 1% PEST (10,000 U/ml) at a density of 4×10^3 cells per cm^2. After 24 hours, the media were changed to either control medium or osteogenic medium. Osteogenic medium consisted of control medium supplemented with 1% dexamethasone (10^5 M). In order to increase the osteogenic stimulation, 1% ß-glycerol phosphate (2 mM, Sigma-Aldrich) was added to the osteogenic medium after 10 days of culture. After 5-6 weeks of culture, the cells were washed, fixed in Histofix™ and stained with silver based von Kossa staining to analyze mineralization. In paper III mineralization was also analyzed by quantifying the content of calcium and phosphate within the ECM as well as using Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS).

Histological methods

Fixation of material

All pellets, scaffolds and biopsies were fixed using Histofix™ consisting of 4% PFA for 1-24 hours depending on the size of the biopsy, rinsed with PBS and stored in 70% ethanol (EtOH) until further processing i.e. paraffin imbedding and sectioning of the biopsies.

Alcian Blue van Gieson staining

In order to characterize the cartilage depositions in the biopsies, Alcian Blue van Gieson (a well known combined dye) was used. It stains negatively charged proteoglycans such as GAGs which are characteristic of mature hyaline cartilage[84, 85]. Alcian Blue is a cationic dye carrying up to four cationic groups that bind to GAGs while the van Gieson dye stains collagens. Alcian Blue van Gieson stains cartilage blue, connective tissue red and muscle and cytoplasm yellow.

Safranin-O staining

Safranin-O is another staining used to indicate proteoglycans in mature hyaline cartilage which is a monovalent cationic dye, hence binds weaker to GAGs in comparison to Alcian Blue van Gieson staining. Safranin-O binds to sulphate groups and stains the sulphated proteoglycans orange to red, cytoplasm blue greenish and nuclei black[86-89].

Bern score

Bern score is a method for measurement of Safranin-O staining intensity based on visual histological evaluation[90]. The samples were scored by their intensity in colour, uniformity and by the amount of matrix produced between the cells and cell morphology.

Von Kossa staining

Von Kossa staining was used in order to study differentiation ability of the cells towards the osteogenic lineage. Von Kossa staining is a staining which detects deposition of calcium that appears during mineralization. It is a silver-based staining in which the silver cations replace calcium bound to phosphate or carbonate groups, and results in black areas indicating the hydroxyapatite content and mineralization phase of bone development.

For von Kossa staining, the cells were first washed twice in PBS and fixed using Histofix™ for 30 minutes and then rinsed again twice with water. The water was removed and 2% silver nitrate (Sigma-Aldrich) solution was added. The plate was kept in the dark for 10 minutes and then rinsed three times with water before being exposed to bright light for 15 minutes. The cells were rinsed twice and then quickly rehydrated in 100% EtOH prior to microscopic inspection for mineralization as described earlier[91].

Methods for genome studies

RNA isolation

In order to study the expression of specific genes of interest, total RNA for PCR and microarray analysis, was extracted from different cell lines cultured in monolayer. Briefly, in paper III the total RNA was extracted from MFG-hESCs and hMSCs both under expansion and weekly during osteogenic induction using the RNeasy® Minikit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. DNAse treatment was performed in order to eliminate any contamination from genomic DNA according to Qiagen RNase Free DNase Set (QIAGEN GmbH) protocol. In paper IV the undifferentiated hESC colonies cultured on mEF were mechanically dissociated with margins prior to RNA extraction in order to avoid the mEF cells in feeder dependent culture.

Quantitative Reverse transcription polymerase chain reaction (RT-PCR) and Real-time PCR

Quatitative RT-PCR was used both to verify the microarray results and to study gene expression of osteogenic markers during osteogenic differentiation of MFG-hESC and hMSCs in paper III and to verify gene expression results from microarray in paper II. RT-PCR is a method in which an RNA strand is reverse transcribed into its complementary DNA or cDNA using the enzyme reverse transcriptase, and the resulting cDNA is amplified using traditional or real-time PCR.

In both papers reverse transcription was carried out using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA) in a 10 µl reaction, according to manufacturer's instructions. Design of primers was performed using the Primer3 web-based software[92]. Design parameters were adjusted to minimize formation of artifact products and to be able to use an annealing temperature in the PCR at about 60°C. Primers were designed to yield short amplicons (preferably shorter than 200 bp) and to function well with SYBRGreen I fluorescent dye for detection of the PCR products in real-time. Real-time PCR was performed in duplicates using the Mastercycler ep realplex (Eppendorf, Hamburg, Germany) in 20 µl reactions. Cycling conditions were 95°C for 10 minutes followed by 45 cycles of 95°C for 20 seconds, 60°C for 20 seconds and 72°C for 20 seconds. The fluorescence was read at the end of the 72°C step. Melting curves were recorded after the run by stepwise temperature increase (1°C/5s) from 65-95°C.

Quantities of target genes were presented as normalized to the number of cells using the expression of 18S ribosomal subunit. Normalized relative quantities were calculated using the delta Ct method and 90% PCR efficiency ($k*1.9^{\Delta ct}$). Statistical analysis for real-time PCR data was performed using the Mann-Whitney test. P-values ≤0.05 (*) were considered as statistically significant differences.

Microarray and data analysis

Microarray analysis provides the possibility to study expression of several thousand genes, even the entire genome, using different pre-constructed array chips consisting of thousands of microscopic spots of probes (specific DNA sequence). The probes are spotted on solid surface, such as glass or a silicon chip, which usually detects and quantifies by fluorophore-labeled targets. There are two different kinds of microarray analysis, cDNA microarray and Affymetrix microarray. In both paper II and III the Affymetrix microarray analysis was used in order to study the global gene expression comparison between stem cells with different origins.

RNA was subjected to gene expression analysis using the Affymetrix oligonucleotide microarray HG-U133plus2.0 (Affymetrix, Santa Clara, CA) according to manufacturer's recommendations. Briefly, 2 μg of the total RNA was used to synthesize biotin-labeled cRNA. 10 μg of fragmented cRNA was hybridized to GeneChips for 16 hours at 45°C. Washing, staining and scanning of the microarrays were performed using the Affymetrix GeneChip equipment. Raw expression data were normalized and subsequently analyzed with the GeneChip Operating Software 1.4 (GCOS, Affymetrix). Comparative and statistical analyses were performed with the BIORETIS web tool (http://www.bioretis-analysis.de). Functional classification of genes involved in ossification was conducted in paper III with annotations from the Gene Ontology Annotation Database[93]. Only the genes were selected for further analysis, if more than 80% of the single comparisons per group comparison were detected as significantly changed by GCOS and if the genes displayed a mean fold change (FC) ≥3 in paper II and (FC) ≥2 in paper III. The significance level was determined applying the Welch's t-test on log2-transformed signal values.

Hierarchical cluster analysis

Data segmentation or cluster analysis involves grouping or segmenting a collection of objects into subsets or "clusters", in which objects within each cluster are more closely related to one another. The goal of cluster analysis is to notion degree of similarity or dissimilarity between the individual objects being clustered.

Usually in the end of microarray data analysis, in order to find groups of genes which are similar in some ways, a clustering method called hierarchical cluster analysis is used. Such hierarchical cluster analysis was performed on 1000 randomly selected genes in paper II and on genes involved in ossification in paper III, with log2-transformed signals normalized by genes and Pearson correlation as distance measure using Genesis 1.7.2 software[94].

Protein-protein interaction network

Protein–protein interactions analysis is used to discover direct-contact association of protein molecules.

This analysis was used in both paper II and III to investigate the possible interactions among proteins from differentially regulated genes (defined by having a mean FC≥3 in paper II and FC≥2 in paper III) between cell lines of interest. The search tool STRING was used to mine for recurring instances of neighbouring genes. STRING aims to collect, predict, and unify various types of protein-protein associations, including direct (physical) and indirect (functional) associations. A gene of interest was classified as a hub if it had ≥5 interactions with other genes[95]. As default, STRING uses four different sources (genomic context, high-throughput experiments, co-expression, and previous knowledge) to derive protein interaction maps.

Scatter plots

Scatter plots shows the relationship between two variables by displaying data points on a two-dimensional graph. To analyze the similarity in global gene expression between MFG-hESCs and mEF cultured hESCs in paper II, scatter plots were generated between average signals of pairs of samples using standard function in R. The width of the cloud of dots in the dot plot indicates the similarity between the compared samples. Identical samples will give a diagonal line in the plot and samples with very different gene expression pattern will result in a wide cloud of dots, spread across the plotting area.

Other methods

Flow cytometry analysis

Flow cytometry (FACS) is a technique, which can be used on cells in suspension to counting, physically sorting (based on their properties to obtain purify populations of interest) and characterizing by passing the cells one by one through a light beam in an electronic detection apparatus.

Flow cytometry was used to verify the microarray results obtained for cell surface markers. The cells were then stained with antibodies of interest. The FACS Aria flow cytometer with FACS DiVa software (Becton Dickinson) was used to analyze the cells. A 488 nm argon ion laser was used to excite samples, with emission being measured using appropriate band pass filters. The cells were acquired and gated by forward (FSC) and side scatter (SSC) to exclude debris, dead cells and cell aggregates. As control, isotype specific antibodies conjugated to the fluorochromes were used. To calculate the percentages of cells staining positive for each marker, cells with a higher fluorescence than 99% of the cells stained with isotype antibody was considered positively stained.

Time-of-Flight Secondary Ion Mass Spectrometry

Time-of-Flight Secondary Ion Mass Spectroscopy (TOF-SIMS) is a surface-sensitive spectroscopy which uses a pulsed ion beam to remove molecules from the very outermost surface of the sample. The particles are removed from atomic monolayers on the surface (secondary ions) and then accelerated into a "flight tube" and determines their mass by measuring the exact time at which they reach the detector (i.e. time-of-flight). TOF-SIMS is widely used in material science[83].

TOF-SIMS analysis was used in paper III in order to study the mineralization ability and to detect the hydroxyapatite in ECM produced by cells as a token toward osteogenic differentiation. Prior to TOF-SIMS analysis, the samples were rinsed twice and subsequently treated with ethanol (95%) in order to dissolve membranes and fixate the samples.

TOF-SIMS analyses were carried out using a TOF-SIMS IV instrument (ION-TOF, GmbH, Munster, Germany) equipped with a Bi cluster ion source and a C_{60}^{+} ion source. Analysis was performed with the instrument optimized for high mass resolution (m/ m ~5000, beam diameter 3.5 μm) using 25 keV Bi_3^+ primary ions at a pulsed current of 0.1 pA or with the instrument optimized for

high lateral resolution using 50 keV Bi_3^{++} primary ions at 0.04 pA. Depth profiles and 3D maps were recorded by repeated sputtering of the surface using 10 keV C_{60}^+ ions (300x300 μm, 0.6-2.6 nA) and analysis (Bi_3^+ primary ions, high mass resolution, 200x200 μm, 128x128 pixels) in an alternating mode.

Alkaline phosphatase activity

Increased alkaline phosphatase (ALP) activity manifests the osteogenic differentiation in the cells. Hence, the ALP activity was measured after lysis of the cells using M-PER (Fisher Scientific, Gothenburg, Sweden) in paper III. The ALP activity was assayed by using p-nitrophenylphosphate as substrate. The quantity (in alkaline solution) of the p-nitrophenol produced, which exhibits an absorbance maximum at 405 nm, was considered directly proportional to the alkaline phosphatase activity. The analysis was performed at the accredited laboratory of Sahlgrenska University Hospital.

Ca/P measurement

In order to quantify the degree of mineralization, the content of calcium and phosphate ions within the ECM was measured. Briefly, samples were rinsed twice with culturing medium (without serum) and fixed in Hisotfix[TM] for 30 minutes. After rinsing with distilled H_2O, the samples were demineralised by incubation in HCl (0.6N) on an orbital shaker for 24 hours at room temperature. The calcium content was then measured using the ortho-cresolphthalein complexone (OCPC) method and phosphate was determined by colorimetry of phospho-vanado-molybdic acid. The analysis was performed at the accredited laboratory of Sahlgrenska University Hospital.

SUMMARY OF RESULTS AND CONCLUSIONS

Paper I: Adaptation of human embryonic stem cells to feeder-free and matrix-free culture conditions directly on plastic surfaces

A protocol was derived for adaptation of hESC lines to feeder-free and matrix-free culture, in which hESCs could be cultured directly on plastic surfaces without any supportive coating in an undifferentiated state. This coating independent culture method is fully comparable to hESCs cultured on feeder cells with regard to differentiation and growth rates as well as to maintaining all the normal hESC features. Not only does this method facilitate propagation of cells without laborious and time-consuming pre-coating with feeder cells and the manual cutting and transferring of colonies, but also avoids the presence of the feeder cells in culture. Furthermore, this system avoids all coating materials in general such as Matrigel^{TM,} which is an expensive animal-based product commonly used in feeder-free culturing of hESCs in spite of disadvantages such as large variation between different batches resulting in unstable cell culture conditions. In this paper we adapted two different feeder dependent hESC lines into coating independent culture, denoted MFG-hESCs followed by characterization before and after the MFG-adaptation. This coating independent culture method promotes more stable culture condition of hESCs and facilitates large-scale production of hESCs and makes hESCs culture less laborious and time consuming. Moreover, the purity of this culture makes it suitable for several other studies which couldn't be preformed until now, due to the presence of feeder cells or coating matrix as an interrupting factor in the culture. Therefore, it can be used for experiments in which feeder-free and matrix-free hESC culture is an advantage or fundamental e.g. medium development, comparative studies of the effect of different substrates, animal studies which usually require large amounts of cells, and also in developing xeno-free culture systems for cell-based therapies and tissue engineering.

Paper II: Extensive characterization of matrix-free growth adapted human embryonic stem cells; a comparison to feeder cultured human embryonic stem cells

In this paper we compared the MFG-hESCs derived in paper I to feeder-cultured hESCs by subjecting them to genome-wide microarray analysis. The results demonstrated that MFG-hESCs highly resemble feeder-cultured hESCs.

Moreover, data revealed that MFG-hESCs have the advantage of increased expression of integrins and other RGD-binding proteins, which is a prerequisite for attachment and growth on non biologic material such as plastic. Further investigation verified that presence of RGD peptides in culture results in 65% less attachment of MFG-hESCs compared to cells cultured in RGES peptides, confirming the role of integrins for attachment of these cell lines. Our extensive comparison demonstrated that the protocol for the adaptation of hESC lines to matrix-free growth results in cell lines retaining the characteristics of undifferentiated hESCs.

Paper III: Superior osteogenic capacity of human embryonic stem cells adapted to matrix-free growth compared to human mesenchymal stem cells

In this paper the osteogenic differentiation capacity of MFG-hESCs was compared to that of hMSCs using a simple monolayer culture protocol commonly used to differentiate hMSCs. Von Kossa staining, TOF-SIMS analysis and measurement of calcium and phosphate in the ECM produced by the cells, demonstrated a superior ability of the MFG-hESCs to produce a mineralized matrix compared to hMSCs. Beyond the superior ability of the MFG-hESCs to form mineralized matrix, the results pointed out that these two cell types use different signalling pathways for differentiation into the osteogenic lineage. Microarray analysis revealed that several genes involved in ossification are differently expressed between these two cell types. RT-PCR showed that MFG-hESCs had a significantly higher expression of *SPP1* during osteogenic induction while the opposite was true for *ALP, TGFB2, RUNX2* and *FOXC1*, and the activity of the ALP enzyme demonstrated different signalling pathways as well. Due to MFG-hESCs capacity in large-scale production and superior ability to form mineralized matrix, this cell line can be a promising alternative to the use of adult stem cells in future bone regenerative applications.

Paper IV: Co-culture of human embryonic stem cells and human articular chondrocytes result in significantly altered phenotype and improved chondrogenic differentiation

An efficient differentiation method for the generation of chondroprogenitor cells from hESCs was devised. The method is based on direct co-culture of hESCs and chondrocytes in high density pellet mass culture system in chondrogenic

medium supplemented with TGF-β for 14 days. In contrast to hESCs, the co-cultured hESCs can be expanded on plastic with a morphology and expression of surface markers similar to mesenchymal stem cells. Those cells are able to form a more homogenous pellet with significantly increased cartilage matrix production, both in high density pellet mass cultures and hyaluronan-based scaffolds. They further form colonies in agarose suspension culture demonstrating differentiation towards chondroprogenitor cells. Our results confirm the potential of the culture micro-environment to influence hESCs morphology, expansion potential and differentiation abilities over several population doublings. These chondroprogenitor cells can be used in experimental studies like toxicology testing and drug screening, and might be used as a potential source of cells for cartilage regeneration in future cell therapy studies. Furthermore, the co-culture method in particular can also be used as a model system for studying chondrogenic differentiation in developmental biology.

GENERAL DISCUSSION

Improved culture techniques of undifferentiated hESCs takes future cell-based therapies one step further

During the last decades, the interest in tissue engineering has increased exponentially and as a consequence, stem cell research has gained a lot of attention and has come into focus. After successful culture of pluripotent and permanent hESCs lines[2] in the late 90's, the interest for these cells has grown enormously. The ability to maintain the pluripotency of hESCs during long-term culture and yet induce differentiation into multiple lineages is a prerequisite for toxicological screening, *in vitro* modeling of genetic disorders or therapeutic cell replacements[96, 97]. However, still after a decade of research, substantial advances in basic cell biology and clinical techniques are required until these endpoints can be fully realized at industrial scale and stem cells can be applied to human medical problems. A less laborious system and ease of handling, cost-effectiveness, culture stability, well-defined components, xeno-free culture condition and compatibility with good manufacturing practice (GMP) are some issues to address. Furthermore, use of hESCs will require large-scale culture of the cells in order to reproduce the amount of cells replacing missing or defected cells or tissue in the body. Traditionally, hESCs were cultured on mEF feeder layers in serum containing media[2]. However, due to concerns over mouse–human transfer of viruses[17] and immunogenic epitopes, such as N-glycolylneuraminic acid (Neu5Gc) non-human sialic acid[98] a variety of human tissues has been investigated in order to find suitable feeder layer replacement[99]. For instance, human foreskin fibroblasts have been used to isolate xeno-free and clinical-grade hESC lines[100]. However, there is batch-to-batch variability of isolated feeder cells which remains a concern[101]. As an alternative to feeder cells, the ability of ECM components to support hESC attachment and proliferation has been evaluated. One popular matrix which has been used to support pluripotency in many hESC lines[16, 102] is mouse sarcoma based Matrigel[TM], which is rich in laminin, collagen IV, heparan sulfate proteoglycans (HSPG) and nidogen (enactin)[103]. Other matrixes such as fibronectin, laminin, enactin, and vitronectin have been used successfully to culture hESCs[17, 104, 105]. However, all ECM preparations are usually expensive and often require cold storage. They can also lose their biological properties when dehydrated, which limits the life expectancy of those products. For the above mentioned reasons, development of a feeder-free and matrix-free system is desirable for the culture of hESCs.

In paper I we present novel hECS lines denoted MFG-hESCs which do not require feeder cells or any other coating material supporting the adhesion and

proliferation in their undifferentiated state. These cells are capable of growing on regular plastic culture dishes. In other words, this trait makes hESCs culture less laborious in comparison to feeder-dependent or matrix-dependent culture systems, which are to date the only existing culture system for hESCs. In contrast to feeder-dependent hESCs, these cells do not require mechanical transfer since enzymatic dissociation can be used for passage of the cultures. Hence, culturing these cells generates increased number of cells and moreover, by virtually eliminating the feeder cells, the purity of cultures improves. In addition, this superior ability makes the culture system inexpensive, thus expensive coating materials are no more essential. Additionally, the pure undifferentiated cultures are more useful for further applications such as genetic analysis (microarray) and differentiation experiments, where the presence of feeder cells or coating matrix could interfere.

The impact of NC conditioned hESC media in maintenance of pluripotent MFG-hESCs

Pluripotency and maintenance of the MFG-hESC lines under feeder-free and matrix-free conditions were demonstrated and compared with previous results for mEF cultures of their respective cell lines[66, 67] in paper I. The characterization was performed by examining the morphology, expression of pluripotency markers, karyotype, and differentiation *in vitro* and *in vivo*. We were able to show that these cells are eminent in their undifferentiated state and can give rise to all three embryonic germ layers. However, this study was actually designed to induce chondrogenic differentiation using indirect co-culture of hESCs with chondrocytes as a differentiation model in which both ACs and NCs were used to condition media. The effect of indirect co-culture i.e. external signals such as different soluble factors produced by chondrocytes, were not sufficient for differentiation of the hESCs towards chondroprogenitor cells but it had substantial impact on adhesion ability of hESCs to non-coated surfaces. One could speculate that the chondrocytes have this impact due to their assignment to produce ECM and proteins attaching cells to this ECM, which might stimulate the hESCs to produce such proteins thus promoting the cell adhesion. On the other hand, it was only the NCs which had this impact on the hESCs resulting in derivation of MFG-hESCs. The neonatal origin of these cells might in fact be of major importance to MFG-adaptation of the cells. The NCs may still have signalling pathways and communication patterns similar to embryonic cells, which can facilitate the connection and interaction between these two cell types and the influence of the chondrocytes. Such signalling patterns and the ability to communicate might be lost in ACs due to their increased distance with differentiation stage. Another alternative can possibly be

that the conditioned media from NCs included higher concentration of secreted matrix components and adhesion molecules due to the NCs viability, which can be a key factor for MFG-adaptation.

However, the mechanism behind and the effect of indirect co-culture of hESCs with chondrocytes as well as the influence of several different factors secreted in the NC conditioned hESC media, is the key factor behind the MFG-adaptation which still remains unanswered. Investigating the proteins and factors secreted in NC conditioned hESC media as well as genomic studies of the MFG-hESCs, can take us a step further to understand this mechanism.

The role of integrins in regulation of cell adhesion, survival and proliferation

To explain this remarkable ability of MFG-hESCs, we attempted to uncover the underlying reasons through extensive characterization and genomic comparison to feeder cultured hESCs in paper II. Microarray analysis revealed remarkably higher expression of several receptor proteins called integrins and collagen family of ECM proteins and moreover, FACS analysis confirmed the presence of adhesion markers on the surface of the cells.

As mentioned earlier, ECM products such as Matrigel[TM], laminin and fibronectin coating are generally used in today's feeder-free culture of hESCs. The major components in Matrigel[TM] are ECM proteins, such as collagen type IV, laminin and HSPG. Collagen substrates enhance the attachment and growth of several different cell types[106]. Type IV collagen is a major structural protein of basement membranes and is chemically and genetically distinct from widely distributed collagen type I and III and cartilage collagen type II[107, 108]. The performed microarray analysis showed that collagen type IV was highly expressed in MFG-hESCs displaying a FC of 5, which may play a major role in the adhesion of MFG-hESC to plastic surface. The collagen families of ECM proteins play a fundamental role in the cell adhesion process and bind to several cell adhesion receptors. Integrins are the largest family of cell adhesion receptor proteins and are composed of two transmembrane proteins, one α and one β subunit[109, 110]. Fibronectin is an ECM glycoprotein which binds to integrins. It also binds to ECM components such as collagen, fibrin and heparan sulphate[111]. In general, fibronectin mediates the attachment of various cell types to collagen substrates[112]. For instance, FCS used in cell culture is a rich source of fibronectin[106]. One crucial step for the regulation of cell adhesion, survival and proliferation, is the activation of the cell surface integrins upon binding to ECM proteins. Integrin α1 has a unique role among the collagen receptors in regulating cell proliferation in collagenous matrices both *in vivo* and *in vitro*[113].

Moreover, the hESCs expanded with or without feeder cells, express integrin α6 and β1, which may form a laminin-specific receptor[16]. MFG-hESCs express several integrin groups such as integrin β1, β3, β5 and β6. For instance, Integrin β1 expression was 16 times higher in MFG-hESCs. Additionally, MFG-hESCs further displayed a three times higher expression of fibronectin, which supports cell adhesion in hESC cultures. The MFG-hESCs had also highly expressed CD44 which is a cell-surface glycoprotein involved in cell-cell interactions as well as cell adhesion and migration. It is a receptor for ECM hyaluronan molecules, and can also interact with other ligands, such as osteopontin (OPN) and collagens. CD44 has previously been described to bind to collagens I and VI[114]. Moreover, the MFG-hESCs showed high expression of cell adhesion molecules such as CD58 and CD47.

The high expression of ECM proteins and collagen families as well as several integrines and cell adhesion molecules are perhaps the key factors for MFG-hESCs adhesion ability on non-coated surfaces. In fact, the role of integrins for attachment of MFG-hESCs to tissue culture plastic was demonstrated using RGD peptides, which resulted in approximately 65% less cell attachment in the RGD treated cultures.

However, the most up regulated integrin related gene in MFG-hESCs was *OPN* displaying a FC of 54. OPN is an extracellular structural glycoprotein and functions as an adhesion protein which first was identified in osteoblasts. *OPN* is expressed in bone but is also biosynthesized by a variety of tissue types. Many functions have been described to OPN, including the regulation of mineralization and mediation of cell attachment[115]. OPN binds to several integrin receptors e.g. α4β1, α9β1, and α9β4. These receptors have been well-established to function in cell adhesion, migration, and survival. In paper I we characterized the MFG-hESCs regarding its pluripotency The MFG-hESCs possessed the undifferentiated morphology of the hESCs and grow as colonies in monolayer. The MFG-hESCs colonies clearly expressed Oct-4, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81, whereas they were negative for SSEA-1, a marker associated with early differentiating hESCs[116] and showed normal stable karyotype. Teratoma formation proved the capacity of the MFG-hESCs to differentiate into all three embryonic germ layers, which are usually are being used as a proof of pluripotency in other studies. The hESC lines showed similar results in characterization before[66, 67] and after the MFG-adaptation. On the other hand, further characterization and genomic comparison to feeder cultured hESCs in paper II, revealed not only several similarities but also some differences. The microarray analysis demonstrated that MFG-hESCs highly resemble mEF-cultured hESCs. In accordance with earlier results in paper I, the MFG-hESCs displayed an increased expression of genes involved in the establishment and maintenance of pluripotency, such as *NANOG* and *OCT4*[74, 117]. However, beside

the above mentioned differences in gene expression regarding integrins and ECM proteins there are several other genes which are highly expressed in MFG-hESCs e.g. genes involved in cellular processes such as chromatin remodelling, gene transcription, RNA processing and export, as well as protein synthesis, several ribosomal genes, genes involved in cell growth and hESC proliferation as well as tumor suppressor genes. Hence, the MFG-hESCs are not fully identical to the mEF-cultured hESCs but still possesses the important characteristic trails of undifferentiated stem cells, such as pluripotency and expression of stem cells markers. In conclusion, the MFG-hESCs can still be used as a cell source in tissue engineering and hESC research, since it retains the characteristics of undifferentiated hESCs such as self-renewal and pluripotency with the advantage of being coating independent hESC.

As mentioned above, the influence of several different factors secreted in the NC conditioned medium could be the key factor behind the MFG-adaptation which makes the study of this medium attractive. In order to identify and quantify the proteins secreted by the ACs and NCs we have used the stable isotopic labeling by amino acids in cell culture (SILAC^{TM}) technology. In the SILAC study, AC and NC cell populations grow in identical cell culture media deficient in essential amino acids lysine and arginine. One cell population was cultured in medium with heavy (isotopic-lysine and arginine.) amino acids while the other cell population was cultured in medium with light (normal-lysine and arginine.) amino acids. Since this labelling results in isotopically distinct peptides, they can easily be distinguished by using mass spectrometry (MS) analysis after equal volumes of medium from ACs and NCs has been mixed. One can also quantify differential protein expression based on the relative peak intensity of the isotopic peptide pairs between different samples. Hopefully, this set-up will increase our knowledge about which factor(s) secreted by the NCs resulting in MFG-adaptation. One could then possibly by adding this factor(s) to unconditioned medium induce plastic growth in hESCs.

MFG-hESCs display higher osteogenic capacity compared to hMSCs thus having a high potential in tissue engineering applications

hMSCs obtained from adult bone marrow are known to have high differentiation capacity toward osteogenic cells, thus presenting a promising source of cells for bone tissue engineering. However, hMSCs have restricted clinical utility due to their low frequencies and limited proliferation, which inspires researchers to search for other suitable alternatives. hESCs can be an alternative cell source due to its potential to provide an unlimited supply of different cell types. In

paper II, microarray analysis of MFG-hESCs revealed 54 times higher expression of *OPN* in comparison to mEF-cultured hESCs, which prompted us to investigate their osteogenic potential. OPN is a protein expressed in bone and has been implicated as an important factor in bone remodeling[118]. Many functions have been described to OPN during the mineralization process, including the capability to anchoring osteoclasts to the mineral matrix of bones[119]. Furthermore, the *ANX* expression was 23 times higher in MFG-hESCs in comparison to mEF-cultured hESCs. *ANXs* are a family of proteins with the ability to bind to acidic phospholipids in the presence of calcium[120]. Annexins are abundant in bone matrix vesicles, and are hypothesized to play a role in calcium entry into vesicles during hydroxyapatite formation[121]. In paper III we investigated the MFG-hESCs osteogenic capacity in comparison to hMSCs. Superior ability of the MFG-hESCs to produce a mineralized matrix compared to hMSCs was illustrated by Von Kossa staining and TOF-SIMS analysis. Microarray analysis of genes involved in ossification revealed differential expression of several genes between these two cell types. The results from RT-PCR also showed that these two cell types differentiate into the osteogenic lineage using different signalling pathways. *BMP4* is known to regulate skeletal development and several *BMPs* are expressed by early osteogenic cells[122]. *SRGN* has been demonstrated having an inhibitory effect on hydroxyapatite crystal growth in vitro[123]. The high expression of *BMP4* and *OPN* in undifferentiated MFG-hESCs, as well as the high expression of *SRGN* in hMSCs, might be some of the factors explaining the significantly increased mineralization ability of these cells compared to that of hMSCs. Moreover, it is known that undifferentiated hESCs express high levels of ALP activity, which consequently may increase osteogenic differentiation since ALP activity is necessary to initiate mineralization during osteogenic induction[124]. In conclusion, the MFG-hESCs not only can be differentiated toward osteogenic cells but also have a superior ability to form mineralized matrix compared to hMSCs, demonstrating that this cell line can be a promising alternative to the use of adult stem cells in future bone regenerative applications.

Stem cells in cartilage

Articular cartilage is a tissue with low cell and matrix turnover. Consequently, when damaged, it has low capacity for self-repair. As an effort to repair cartilage defects, there have been several studies made in transplantation of autologous chondrocytes into the damaged area. Taking advantage of MSCs originating from bone marrow has also been tested as alternative in cartilage repair using techniques such as drilling or microfracturing, owing to the chondrogenic differentiation potential of MSCs. However, the quality of MSCs differs enormously between different individuals, which influence the degree of

proliferation and differentiation of the cells. Beyond that, the MSC population decreases with the age of the patient. In addition, MSCs are prone to differentiation into the fibrocartilage lineage instead of articular cartilage which is yet another drawback. Due to the pluripotency of hESCs and their potential to provide an unlimited supply of cells, it can be considered as a universal donator and an excellent alternative cell source in future cartilage tissue treatments. Hence, we investigated the chondrogenic potential of hESCs.

In paper I, we attempted to differentiate hESCs into the chondrogenic lineage using indirect co-culture with chondrocytes by culturing the hESCs in conditioned media from chondrocytes. However, indirect co-culture did not result in chondrogenic differentiation. Hence, in paper IV, we instead attempted to differentiate the cells through direct co-culture with chondrocytes, hypothesizing that direct contact between the cells in high density culture may lead to chondrogenic stimulation of the hESCs. Our results showed that co-cultured hESCs differentiated into chondroprogenitor cells demonstrating several abilities which characterize chondrocytes. Unlike hESCs, the co-cultured hESCs could be expanded on plastic with a morphology and expression of surface markers similar to MSCs. Direct co-culture resulted further in a more homogenous pellet and significantly increased cartilage matrix production, both in high density pellet mass cultures and hyaluronan-based scaffolds. They also formed colonies in agarose suspension culture demonstrating differentiation towards chondroprogenitor cells. Our results confirmed the potential of the direct co-culture to influence hESCs toward chondrogenic differentiation. This study demonstrated the potency of cellular contact and the ability of exchanging information through direct cell-cell contact to accomplish chondrogenic differentiation. However, these cells were not fully differentiated into mature chondrocytes. One reason may be that the period of the co-culture or the co-culture, method itself, is not sufficient for fully differentiation of the hESCs. Another possible theory is the fact that the chondrocytes were irradiated in order to inhibit cell proliferation prior co-culture, which to some extent resulted in less matrix production preventing total redifferentiation in the pellet mass culture. Other consequences of irradiation beside inhibition of proliferation have not been investigated, so this treatment may result in other effects in the cells which can prevent terminal differentiation of the cells toward chondrocytes when co-cultured with irradiated cells. Yet another conceivable issue is the time of investigation of the chondrogenic ability of the cells, which were tested in high passages due to need of quantifying the cells for several different analyses. It is known that monolayer expansion dedifferentiates the cells and gives progenitor properties back into the cells cultured *in vitro* [125, 126]. Due to very high plasticity of the hESCs, several cell doubling after co-culture processes may trigger the dedifferentiation of the co-cultured cells giving back some embryonic properties to the cells.

PERSONAL COMMENTS AND FUTURE ASPECTS

Improved culture techniques of undifferentiated hESCs are not only important to facilitate hESC culture, but are also essential for regenerative medicine and tissue engineering which is the main object of hESC research. This thesis has contributed to an improved culture technique for hESCs which could be cultured directly on plastic surfaces without any supportive coating due to their increased expression of integrins. However, to date there are various issues making the use of hESCs non-evident in human medical conditions, and the goal to achieve a GMP quality system for hESC culture lies far in the future. However, if all the problematic issues regarding hESC culture were accomplished there are still issues such as immunological rejection and possibility of tumour formation to be considered.

The increased knowledge of osteogenic and chondrogenic differentiation is important for developing cell-based therapy and tissue engineering application of hESCs. This thesis has also demonstrated the superior ability of hESCs to differentiate towards the osteogenic lineage and the potential of the culture micro-environment and cell-cell contact to influence hESCs differentiation toward chondroprogenitor cells. Due to low cell turnover in cartilage, and the previously described limitations using hMSCs as an alternative cell source for the treatment of both bone and cartilage, it is of great importance to increase the knowledge regarding how to direct hESCs into these lineages in order to be able to use hESCs as universal cell donors in tissue engineering applications. Since articular cartilage is avascular, the possible immunological rejection after a cell theraphy treatment with hESCs is not considered an issue while it can be more problematic in bone tissue. This thesis adds further information concerning how to induce differentiation of hESCs using direct/non-direct co-culture as a differentiation model which can be of interest both in order to understand cartilage development, but also to improve the possibilities of a future clinical application for hESCs.

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