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Genetic Modification and Mesodermal Differentiation of Human Embryonic Stem Cells

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To my family

Abstract

Background: Pluripotent human embryonic stem cells (hESCs) are a resource of great potential, available for research. Their capability to differentiate into any cell of the human body and an unlimited *in vitro* expansion offers the possibility to study the earliest human development and provides a source of cells for regenerative medicine. However, the ability to generate all cell types also makes it difficult to direct the process. Differentiation often appears to occur at random and the underlying molecular processes are poorly understood. Blood and bone cells are both applied in clinical cell therapies and originate from the same embryonic germ layer, the mesoderm. Although the differentiation of adult stem cells to specific cell types is extensively studied, the hESC model provides a possibility to follow the fate of more primitive cells. In order to steer the differentiation of hESCs, developmental transcription factors (TFs) can be induced through genetic modification. TFs control the expression of multiple genes and can potentially direct the development of blood and bone precursors. Specifically, the TFs HoxB4 and Osterix (Osx) have shown potential to influence the development of embryonic stem cells, albeit the mechanisms in early human mesoderm development are not well defined.

Objective: To establish and examine hESC differentiation into blood and bone derivatives. Genetic modifications were introduced by lentiviral vectors to improve cell traceability and to evaluate the gain-of-function of HoxB4 and Osx.

Results and conclusions: In the first study we achieved effective gene marking of hESCs using lentiviral vectors for transgene expression from a human Ubiquitin C promoter. Using cells marked with fluorescence, interactions between human feeder cells and hESCs could be easily evaluated. As gene marking was stable and retained in any hESC derivatives, it allowed the studies of later developmental stages of cells over-expressing the transgene. In the last two studies, the lentiviral vector construction and the selection of modified cells permitted evaluation of different levels of transgene expression.

In the second study, we established and characterized a basic bone differentiation model using several hESC lines. Two differentiation approaches were compared and both methods showed development into the osteogenic phenotype. Evaluation of the secreted extracellular matrix and deposited mineralized tissue showed that it resembled that found in bone.

In the third study the effects of the transcription factor HoxB4 during early hematopoietic differentiation were evaluated by comparing; GFP, HoxB4^{low} and HoxB4^{high} over-expressing and unmodified hESCs, during embryoid body induced differentiation. HoxB4^{high} cells showed an increased early hematopoiesis, while HoxB4^{low} cells did not. Despite an upregluation of early hematopoietic markers in HoxB4^{high} cells, markers for late blood maturation were absent. It was also determined that transgene expression increased during differentiation, which may have been one reason why the hemato-endothelial marker VE-cadherin was up-regulated instead of blood marker genes. *In vivo* teratoma analysis revealed no proper germ-layer formation from HoxB4^{high} cells.

In the fourth study the over-expression of the TFs Osx and HoxB4 was evaluated during osteogenic differentiation. Osx showed a similar dose-dependent behaviour as HoxB4. Low expression levels of Osx increased osteogenic differentiation but at high levels, surprisingly a more hematopoietic phenotype was induced. Furthermore, higher HoxB4 expression also induced osteogenic differentiation.

The two last studies can be summarized to reveal a dose-effect of the TFs HoxB4 and Osx, whilst also presenting wider effects on multiple cell populations.

LIST OF PUBLICATIONS

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IV. Kärner E*, Unger C*****, Cerny R, Ährlund-Richter L, Ganss B, Dilber MS, Wendel M **"Differentiation of human embryonic stem cells into osteogenic or hematopoietic lineages: a dose-dependent effect of Osterix over-expression"** *accepted in Journal of Cellular Physiology*

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LIST OF ABBREVIATIONS

AA	Amino acids
β-GP	β-glycerophosphate
AGM	Aorta, gonads, mesonephros
ALP	Alkaline phosphatase
AR	Alizarin Red
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
BSP	Bone sialoprotein
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming unit
cPPT	Central polypurine tract
Dex	Dexamethasone
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
EB	Embryoid body
EC	Embryonic carcinoma
ECM	Extracellular matrix
EF1α	Elongation factor 1α
EGFP	Enhanced green fluorescent protein
ES	Embryonic stem
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FLT3-L	FMS-like tyrosine kinase 3 ligand
FTIR	Fourier-transform infrared
GAG	Glycosaminoglycan
G-CSF	Granulocyte colony stimulation factor
GCTM2	Germ cell tumour antigen 2
GFP	Green fluorescent protein
GMP	Good manufacturing practice
hESC	Human embryonic stem cell
HGF	Hepatocyte growth factor
HIV	Human immuno deficiency virus
HLA	Human leukocyte antigen
hMSC	Human mesenchymal stem cell
hOBL	Human osteoblast cell line
HoxB4	Homeobox B4
HSC	Hematopoietic stem cell
ICM	Inner cell mass
IL	Interleukin
IRES	Internal ribosome entry site
IVF	*In vitro* fertilization
KO-SR	Knockout™ serum replacement
LT-HSC	Long term repopulating HSC
LTR	Long terminal repeat
mESC	Mouse embryonic stem cells
NF-Y	Nuclear factor Y
NGF	Nerve growth factor
NK-cells	Natural killer cells
OCN	Osteocalcin
Osx	Osterix
PBS	Phophate buffered saline
PDGFRα	Platelet-derived growth factor receptor alpha

PI	Propidium iodide
PTH	Parathyroid hormone
PVDF	Polyvinylidene fluoride
Q-PCR	Quantitative RT-PCR
RFP	Red fluorescent protein
RNA	Ribonucleic acid
ROCK	Rho-associated kinase
RT-PCR	Reverse transcription-polymerase chain reaction
SCF	Stem cell factor
SIN	Self-inactivating
siRNA	Small interfering RNA
SR	Serum replacement
SSEA	Stage specific embryonic antigen
T-cells	Thymus-cells
TGF	Transforming growth factor
TPO	Thrombopoietin
TRA	Tumor recognition antigen
USF	Upstream factor
VEGF	Vascular endothelial growth factor
VP	Virus particle
WRE	Woodchuck hepatitis virus RNA regulatory element
VSV-G	Vesicular stomatitis virus glycoprotein

1 INTRODUCTION

Human embryonic stem cell (hESC) research is a new dawn of cell therapy. Although cells have been used in many circumstances in the last century, the developmental potential of hESCs is beyond all previous possibilities. The cell types and organs that can be transplanted today are limited to a few therapies e.g. bone marrow transplantations. The derivation and *in vitro* culture of embryonic stem cells is opening up a new area for cell therapy. This cell type, although an *in vitro* artefact, provides theoretically a source for all cells developing in the human body.

Although little is known about the early human development, hESC research is nevertheless already preparing clinical applications. Especially cell types like neurons, which have not been available before for treatment of several neurodegenerative diseases, can now be obtained from hESCs (Chiba et al., 2008; Roy et al., 2006).

However, hESCs are still a young research area, presenting a model system that is still poorly understood. Considering that a whole human being develops from a few of these cells, our limited understanding is not really surprising. The human body is composed of several hundred cell-types, each described in detail in our DNA code, inherited and evolved for billions of years. The human genome consists of about 25,000-30,000 genes (Lander et al., 2001; Pennisi, 2003) and for more than 50% no known function has been presented. Only 2% of the genome is encoding genes and the remaining is non-coding regions, whose functions are still to be fully defined. The stabilization of chromosomes, regulation of transcription and translation are just some general functions in need of definition (Birney et al., 2007).

The studies in this thesis highlight the potential of hESCs and their applicability for establishing new research methods. Furthermore, the thesis recognizes the importance of clear definitions of gene regulation.

1.1 HUMAN EMBRYONIC STEM CELLS (HESCS)

1.1.1 Pluripotency and origin of human embryonic stem cells

Stem cells have the ability to differentiate into more than one cell type as well as the capacity of unlimited self-renewal. Different kind of stem cells appear through the development of the human body, their developmental potential has been chosen to differentiate between three classes – totipotent, pluripotent and multipotent stem cells. A **totipotent** stem cell is able to produce all intra- and extra-embryonic cells and grow an entire human being. Only the zygote and the unspecialized cells until the development of the 8-cell morula retain this highest potential. After the morula stage, the cells start to specialize and form a hollow sphere called the blastocyst. This blastocyst is made up from an outer layer of cells, called the trophoblast, and the inner cell mass (ICM) located in the fluid–filled cavity. Whereas the trophoblast will develop

into extraembryonic tissues, such as the placenta, the ICM has a **pluripotent** potential giving rise to all cells of the embryo and the ultimate fetus. Any further specialized stem cells, still able to develop into more than one cell type is called **multipotent** and can be isolated from fetal or adult tissue. Well known examples for multipotent stem cells are hematopoietic stem cells (HSCs), which will give rise to all red and white blood cells during fetal and adult life.

Embryonic stem cells are pluripotent due to their derivation from the ICM. Considering the short life span of the ICM *in vivo*, human embryonic stem cells are complete *in vitro* artefacts and therefore have, not surprisingly, a high tendency to differentiate throughout propagation.

1.1.2 Derivations

After more than a decade of experience with mESCs (Evans and Kaufman, 1981; Martin, 1981) and primate research (Thomson et al., 1995), the first human ICM cells were cultured for a few passages (Bongso et al., 1994). The first permanently growing hESC lines were finally established a few years later (Reubinoff et al., 2000; Thomson et al., 1998), using conditions similar to derivations of rhesus monkey embryonic stem cells (Thomson et al., 1995).

The number of derived hESC lines was estimated in 2005 to be more than 300 (Hyslop et al., 2005b). Most of these lines have been derived from surplus embryos donated after informed consent in *in vitro* fertilisation (IVF) clinics. Typically they are either evaluated as low quality based on morphology of the blastocyst, ICM and trophoectoderm (Gardner et al., 2000; Gardner and Sakkas, 2003) or found to be genetically defect embryos after preimplantation genetic diagnosis (Peura et al., 2008; Pickering et al., 2003).

The ICM, which is removed from the blastocyst contains roughly 50 cells. It can be isolated by immunosurgery or mechanical dissection (Strom et al., 2007). It has been shown that the efficiency of derivations is highest if derived from the ICM of blastocysts (Lerou et al., 2008). However several lines have been obtained from earlier stages (Strelchenko et al., 2004), from arrested embryos (Zhang et al., 2006) or even single blastomers (Klimanskaya et al., 2006), with the earliest from a 6-cell-stage embryo (Lerou et al., 2008).

The efficiency to obtain a human embryonic stem cell lines from isolated inner cell masses of blastocyst-stage embryos is around 10%, rarely exceeding 20% even in experienced laboratories (Lerou et al., 2008; Strom et al., 2007).

1.1.3 Propagation and storage of human embryonic stem cells

For hESCs to grow and expand, a variety of conditions must be established. The cells do not grow on normal tissue culture-treated plastic wells. The first derivations with a successful outgrowth of a hESC line, was obtained by using mouse embryonic

fibroblast **feeder cells** (Thomson et al., 1998). This is still a widely used method for the research purpose derivation of hESCs (Lerou et al., 2008). As alternative to animal feeder cells, we and many other groups are using human foreskin fibroblast cells as a standard feeder cell type, for the reason of their longer life span and human origin (Amit et al., 2003; Hovatta et al., 2003).

However, the co-culture with feeder cells is labour intensive and enters more variability into the culture system. Therefore, many studies have been successfully establishing feeder-free culture systems, with the most commonly used Matrigel™ (BD Biosciences) as one possible matrix alternative (Klimanskaya et al., 2005; Xu et al., 2001). Although initially conditioned medium from feeder cells was still needed to grow hESCs on Matrigel (Xu et al., 2001), this requirement has now been addressed through the development of several defined media, the first one being the TeSR medium (Ludwig et al., 2006). However, defined culture media are expensive and still in need of optimization and adaptation for each hESC line (Rajala et al., 2007), more studies are devoted to define important culture supplements (Furue et al., 2008). To furthermore develop cells towards clinical applications, an animal-free defined environment needs to be established. Matrigel is a murine sarcoma derived extracellular matrix (ECM) and therefore not xeno-free, however animal-free matrices are still not well-established (Ludwig et al., 2006).

Even though the ongoing development in culture conditions has not come to a final conclusion yet, a protocol for **research purpose hESC expansion** has been well established all over the world and has been used for this thesis. This system is no longer using serum, but instead a commercially available serum replacement (SR) to reduce batch variations (a detailed description can be found in Materials and Methods, chapter 3.1). One ingredient of hESC medium that has been found important throughout all culture protocols, although in different concentrations, is bFGF (Amit et al., 2000; Thomson et al., 1998). Feeder cells usually secrete it, but it is also regularly added to the media to safeguard the availability (Amit et al., 2000; Eiselleova et al., 2008). Increased addition of bFGF directly to the media offers the possibility to omit feeder cells. (Klimanskaya et al., 2005; Levenstein et al., 2006; Li et al., 2005; Ludwig et al., 2006; Xu et al., 2005). To further improve the culture conditions and at the same time reduce the cumbersome cell work, we established human feeder cells stable secreting bFGF (submitted). This could potentially overcome the need for daily changes of medium and therefore reduce the standard workload on researchers. However so far, daily media changes and passaging every 5-6 days are required for hESC maintenance.

In order to **passage** hESCs, either mechanical or enzymatic methods are used. Enzymatic passaging can be done using collagenase, dispase or a Trypsin surrogate (TrypLE, Invitrogen) and promises a higher expansion rate compared to mechanical passaging as well as the possibility to clone cells (Chan et al., 2008; Ellerstrom et al., 2007).

The recently discovered Rho kinase (ROCK) inhibitor (Y-27632) has vastly increased the **cloning** efficiencies normally achieved from hESCs (from ≤1% (Zwaka and

Thomson, 2003) to >20% (Martin-Ibanez et al., 2008; Watanabe et al., 2007)). Increased cloning efficiency can also increase outcome of passaging and cell sorting. We even found that it improved the survival of hESCs after freezing, which means that the ROCK inhibitor has a great impact on hESC research. Low survival of hESCs after freeze-thawing has been another reason for researchers to rather keep cells in culture instead of cryopreservation in-between experiments.

There are two main **freezing** protocols applied for hESCs, a slow freezing (~1°C/min) in cryovials or a snap-freezing vitrification method using straws and a final sample storage in liquid nitrogen. Slow freezing is a simple standard method in many labs but hESCs have had a lower survival rate as compared to the vitrification protocol (Reubinoff et al., 2001; Richards et al., 2004). Although vitrification protocols seem to provide better hESC survival, they are also labour-extensive and tedious to perform. Therefore they seem unsuited to handle bulk amounts of cells required for clinical and many research application. Instead many slow-freezing protocols have been optimized over the years and now reach higher survival rates of up to 50% for many hESC lines (Martin-Ibanez et al., 2008; Valbuena et al., 2008). In our studies, we have initially anticipated that a period of 4 weeks would be appropriate to regain growth and satisfactory amount of hESCs to start new experiments, after thawing.

1.1.4 Properties of human embryonic stem cells

As described by Thomson and colleagues in the first publications deriving hESCs, three main properties are defining this pluripotent cell type (Thomson et al., 1998). First, they are derived from pre-implantation embryos, second, they show prolonged undifferentiated proliferation and third, developmental potential to form all three germ layers even after extended culture.

To make sure that the right cell type is obtained and preserved and to provide assurance about the non-cancerogenous character of the cells, several markers and functions need to be evaluated. Human ESCs have a typical **morphology**. The undifferentiated cells form distinct tightly packed colonies growing in a monolayer. This feature is similar under various different culture conditions, although it was found that the feeder cells affect the shape of the colonies (article I). Individual cells contain a large nucleus, prominent nucleoli and a small cytoplasm. However, the culture is usually heterogeneous and contains undifferentiated cells and more mature derivatives. The outgrowth of derivatives can be used to optimize the culture conditions towards less differentiation (article I). Single colonies can be surrounded by differentiated cells, which can be easily seen if hESCs are grown feeder-free (Carpenter et al., 2004; Xu et al., 2001).

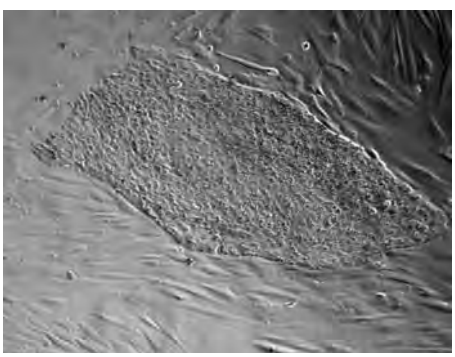


Figure 1 Human embryonic stem cell colony (HS181) on human feeder cells

Next to the morphology, a wide panel of markers is recognized to define hESCs. However, no marker alone is sufficient to identify them. The most widely used markers are equal to embryonic carcinoma cells (Andrews et al., 2005). They express the stage specific embryonic antigens (SSEA) and **surface markers** SSEA3 and 4, but not SSEA1. Essentially the opposite is true in mESCs. Undifferentiated hESCs also express the tumor recognition antigens (TRA) Tra1-60, Tra1-81 and GCTM2, as well as they are alkaline phosphatase positive. Additionally to these surface markers, two **intracellular transcription factors**, Oct4 and Nanog, are commonly evaluated and specific for mouse and human undifferentiated ES cells (Boyer et al., 2005; Hyslop et al., 2005a; Mitsui et al., 2003). Finally a high telomerase activity is expected and kept also after long-term expansion in these cells. Although these markers should be widely expressed on hESC lines, fluctuations and variations have been recognized for SSEA3 and Nanog (Chambers et al., 2007; Enver et al., 2005). In the context of a standard hESC culture, some cells may be occasionally negative for some markers, but still have a pluripotent character. In order to identify general similarities and differences between hESC lines, a larger amount of intra- and extra-cellular markers have recently been evaluated in 59 hESC lines (Adewumi et al., 2007).

In summary, a standard set of hESC markers includes SSEA1 (-), SSEA3 (+), Tra1-60 (+), Oct4 (+) and/or Nanog (+). These markers are sufficient when combined with functional characterization. Additionally, a wider panel of markers is available for specific characterizations.

1.1.4.1 Genetic stability

hESCs seem to have an extraordinary stable chromosomal integrity under optimal culture conditions, in comparison to other mammalian cell culture systems (Buzzard et al., 2004). A normal karyotype is a major requirement for an anticipated safe clinical use in cell therapy. Importantly, the karyotype differentiates between hESCs and embryonic carcinoma (EC) cells. Chromosomal abnormalities are frequently reported with some specific genetic changes being observed more often than others. Draper *et. al*. were the first to report on recurrent gain of chromosomes 17q and 12 in hESC culture (Draper et al., 2004). Chromosomal changes alter the expression of genes that increase proliferation or reduce differentiation. The risk of changes in gene expression is likely to increase during extended *in vitro* culture (Mitalipova et al., 2005). Potentially the upregulation of pluripotency genes such as Nanog, which is located on chromosome 12, is one of many possible reasons leading to the selection of polyploid cells (Draper et al., 2004; Imreh et al., 2006). Considering that only a low percentage (42%, (Hardarson et al., 2003)) of all blastocysts are chromosomally normal, a higher rate of chromosomal changes would also be expected in hESCs. Although many abnormalities may not be selected for *in vitro* (Peura et al., 2008), hESCs are prone to develop chromosomal changes during extended *in vitro* culture (Baker et al., 2007). Abnormalities, induced by derivation or extended culture *in vitro*, may reflect tumorigenic tendencies and manifests the need to carefully confirm a normal karyotype, if hESCs are to be used in clinical applications. Nevertheless, banks of abnormal human embryonic stem cell lines have been established and could be

valuable tools to model diseases (Feki et al., 2008; Verlinsky et al., 2005) or identify key genes for stem cell maintenance and tumoregenicity (Baker et al., 2007).

1.1.4.2 Pluripotency

Although we focused the interest of our studies in certain mesodermal differentiation of hESCs (see also chapter 1.3), many more cell types have been differentiated, providing evidence for the pluripotent character of hESC. Pluripotent cells are able to develop to all cells of the human body. During human embryogenesis, three germ layers are formed from the ICM. The three germ layers, ectoderm, mesoderm and endoderm are a compartimentation of cells that will eventually give rise to all tissues and organs. To provide the evidence for pluripotency, hESCs need to be able to form derivatives of these three germ layers. Since the plasticity of hESCs is so vast, it is hard to understand and control the signalling for directed differentiation. Therefore present differentiation methods employed only achieve to differentiate a small fraction of the desired cells lineages. The detailed molecular mechanisms behind the occurring differentiation are still poorly understood, but many protocols have been developed to increase the number of cell types, *in vitro* differentiated from hESC.

Because the undifferentiated human embryonic stem cell is only an *in vitro* artefact and not a stable cell type *in vivo*, spontaneous differentiation occurs easily under suboptimal culture conditions. However, instead of relying on a slow spontaneous differentiation, culture conditions are usually radically changed to induce a fast differentiation in most of the hESCs within a short time.

Induction of in vitro differentiation

hESCs are typically differentiated in two common ways, as a **monolayer** or in a three-dimensional structure called **embryoid body** (EB). EBs are cell aggregates that are formed from hESC colonies in suspension. Preventing attachment, low attachment plates, spinner cultures or hanging drops can be used to efficiently induce EBs. The three-dimensional structure resembles in vivo development. Although trophoectoderm cannot be formed, virtually every other cell type can be achieved by differentiation.

Defined soluble factors can be added to the culture medium in both methods in order to further control differentiation, and may be analysed for their role in certain cell fate decisions. Similarly, the function of genes/proteins can be evaluated in these model systems. The molecular signalling of differentiation is not well understood, and many co-culture systems have initially been used to provide an appropriate signalling microenvironment to propagate the desired cell type. This was often initiated as proof of principal for the potential of hESCs to differentiate to certain mature cell types. Neuroectodermal differentiation was triggered by co-culture with stroma cells with addition of sonic hedgehog and FGF8 (Perrier et al., 2004). Correspondingly, the induction of hematopoietic stem and progenitor cells was accomplished by co-culture with bone marrow stroma cells (Kaufman et al., 2001).

In vivo differentiation of hESCs in teratomas

As a surrogate measure for pluripotency of hESCs *in vivo*, a teratoma model is widely applied and has been characterized in detail by our network (Gertow et al., 2004). In order to qualify as pluripotent, hESC colonies transplanted into the hind leg or the

testicular capsule of immunodeficient mice should be able to form teratomas consisting of derivatives from the three germ layers. Present *in vitro* methods are not suitable for identification of several different cell types, which complex tissues and organs are composed of. Many environmental signals and the structural support are absent from *in vitro* models and make it less likely that functional tissue can appropriately develop (Przyborski, 2005). In this teratoma model, a wide variety of organized tissues, originating from all three germ layers, have been reported, which assures the developmental potential of the transplanted hESC line. It should be noted that this xeno-transplantation model has limitations, associated with the small volume available in the murine environment and the xeno-character of the surrounding microenvironment. Complex tissues may not fold correctly or receive the wrong signals, limiting a correct development of some cell types or tissues.

The *in vivo* transplantation model has also provided more insight into the immunological properties of hESCs. **Immunogenicity** is of major importance for the use of hESCs or their derivatives in transplantation medicine. In initial experiments of Li et al, the injection of hESCs into immune-competent mice failed to induce an immune response within 48h (Li et al., 2004). However in a later, more detailed study by Drukker et al., teratoma formation was prevented in immune-competent mice after a longer engraftment period. Using different deficient mice strains, T-cells, but not B or NK cells were determined as mediators of this immune response (Drukker et al., 2006). Although hESCs as well as their derivatives can be rejected, an immune response seems weaker. These special *in vivo* conditions may not allow for the maturation of the proper panel of immune markers. hESCs express lower levels of MHC-I molecules and do not express MHC-II as well as co-stimulatory molecules CD80 and CD86 (Drukker et al., 2006; Drukker et al., 2002). Although teratoma conditions seem not able to induce the normal immune phenotype, the use of stroma cells and cytokine combinations induces normal expression (Slukvin et al., 2006). For therapeutical application of hESC-derivatives, strategies such as HLA matching for preventing rejection of the grafts have to be adopted.

1.1.4.3 Therapeutic potential and Clinical applicability of hESCs

Functionality and immunogenicity are the cornerstones in transplantation medicine. As indicated by Taylor et al., a bank of 150 donor hESC line, selected for specific HLA-types, would be beneficial for 84% of patients waiting for kidney transplants in the UK (Taylor et al., 2005). To exclude some concerns of immune rejections, xeno-components should be removed from hESC culture conditions. Uptake of non-human sialic acid, a FBS component, detected by circulating human antibodies may cause rejection of cells (Martin et al., 2005).

Reducing or removing the amount of xeno-components in hESC culture conditions as well as banking a variety of blood type selected lines, are necessary developments to realize the therapeutic potential of hESCs. The recent first derivation of six hESC lines, manufactured in compliance with good manufacturing practice (GMP), has used currently available conditions and verified them, as required for clinical applications (Crook et al., 2007). Although conditions are still not optimal, these cells are currently the most defined hESCs as regards to their “upbringing”. We have summarized the

clinical development of hESCs in a recent review, but also concluded that important steps are still missing to conduct a first clinical trial using hESC-derivatives (Unger et al., 2008b). In regard to the functionality, the authorities have not yet verified hESC differentiation methods. Compliance with GMP is crucial.

1.2 GENETIC MODIFICATION OF HUMAN EMBRYONIC STEM CELLS

Genetic modification implies the change of the genetic information, which can be done by insertion of genes and/or their regulatory elements. Here a focus on eukaryotic cells, and more specifically on hESC, will be presented. For basic research cells are being genetically modified to evaluate the function and regulation of genes. This knowledge can then be applied to track, genetically correct or "improve" cells for research or therapeutic purposes.

Tool to identify function of genes and track cells

A tremendous amount of new knowledge has been achieved throughout the last few years. The human genome is sequenced, but the function and regulation of genes are widely unknown. The development of high throughput technologies, such as microarrays, can monitor expression levels of almost all genes and narrow their potential function to tissues or cell types. To obtain more detailed information about the function of a particular gene, modification methods can specifically induce gain or loss of gene expression, which then might provide evidence for their hypothesized role. Besides the evaluation of novel genes and their functions, reporter genes can be inserted to track cells and give important insight into cell behaviour *in vitro* and *in vivo*.

In hESCs, that differentiate to a number of cell types, identification of target cells can be difficult when changes in gene expression do not manifest visually or when the desired morphology is not easily recognized. In this model system, a number of intermediate progenitor cell types are developing that are difficult to identify by surface markers. They could previously not be obtained from *in vivo* sources and the surface marker expression is not known. Integration of reporter genes to trace and purify these specific cell types would aid their analysis and reduce loss of cells caused by traditional cell selection methods, such as fluorescence activated cell sorting.

Gene therapy

Gene therapy implies a cure for genetic disease. The ability to change genetic information introduced new treatment possibilities for diseases and started the development of gene therapy. Principally, replacement of a defective gene or insertion of a healthy copy could restore the normal function of a cell. This idea was successfully applied in clinical trials for severe combined immune deficiency (SCID-X1). The functional gene for the γc cytokine receptor was retrovirally inserted into transplanted $CD34^+$ bone marrow stem cells, restoring T- and NK-cell differentiation in these patients to normal levels (Cavazzana-Calvo et al., 2000). Although gene therapy is a powerful treatment, adverse events can be equally destructive and in this specific example, caused later the development of leukaemia in some of the treated patients (Hacein-Bey-Abina et al., 2003). Some reasons for these severe adverse events have been identified (Hacein-Bey-Abina et al., 2003), and potential side effects such as insertional mutagenesis need to be studied (Baum et al., 2003).
Apart from introducing a healthy copy of a non-functioning gene, additional approaches include down-regulation of malfunctioning or over-expressed genes by e.g. RNA interference, modification of various cell types in order to survive chemotherapy,

to increase their immunoreactivity against cancer (Guven et al., 2005; Konstantinidis et al., 2005) or remove host-reactive cells that cause GvHD (Rettig et al., 2003; Tiberghien et al., 1994).

hESCs provide a new model system for early human development and a new source of cells for tissue engineering, applied in cell and gene therapy. Because of its novelty and the rather advanced culture conditions, genetic modification methods are still not optimized to the same degree as in better-established cell types. The special growth behaviour in colonies, low cloning efficiencies and high rate of gene silencing are just a few issues that complicate genetic modifications of hESC. However, several approaches have been reported and more are likely to be designed considering the potential of gene transfer in these primitive cells.

Depending on the gene transfer method, genes can be stably integrated into the genome and are therefore replicated into daughter cells, or they are not integrated, i.e. episomal, and are consequently only transiently expressed.

A short summary of these methods in hESCs is described in this chapter, explaining possibilities and drawbacks.

1.2.1 Gene transfer methods into hESCs

1.2.1.1 Non-viral

Although intramuscular injection of plasmid DNA might be adequate for DNA immunisation (Restifo et al., 2000), uptake of naked DNA into mammalian cells is very ineffective. Mechanical and chemical methods have been developed to increase the cellular uptake of DNA and are in fact very potent in cell culture. Electroporation is the most wide spread mechanical transfection method. The cell membrane is temporarily destabilised by an electrical pulse, allowing genetic material to enter the cell (figure 2). However this method can result in a high degree of cell death and requires the detachment of cells, which is why chemical transfection is often preferred where positively charged chemicals form complexes with the negatively charged DNA, facilitating transport across cell membranes and protect the DNA from degradation.

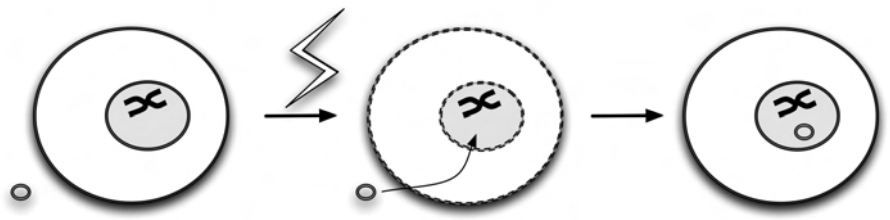


Figure 2 Schematic presentation of electroporation. An electrical pulse temporarily destabilises the cellular membranes, which results in an in flux of material through the membranes.

Eiges and colleagues presented the first report, comparing different chemical transfection reagents and electroporation protocols in hESCs (Eiges et al., 2001). Transfection efficiencies were highest using ExGene500 (Fermentas), but stayed below 10% while the frequency of stable transfections were in the range of 10^{-5}. In a more recent report, the highest transfection rates of up to 50% were obtained by transfection with GeneJammer (Stratagene)(Anderson et al., 2007). Nucleofection (Amaxa) is another optimized electroporation method, using lower amounts of hESCs (Siemen et al., 2005). In one of the latest reports, transient transfection rates of up to 85% have been achieved, mainly through increase of cloning efficiencies (Hohenstein et al., 2008). The efficacy of stable transfection was also increased to 10^{-4}. Currently, nucleofection seems to achieve the highest efficiencies, being possible through new developments and increased survival of cloned hESCs. However, since the reported results vary and new hESC culture conditions are still reported on a monthly basis, it is suggested that transfection may require individual optimization for each cell line and laboratory (Braam et al., 2008; Giudice and Trounson, 2008).

Homologous recombination and site-specific integration

The recombination and targeted integration of genes, occurring between homologous DNA strands, is the "Holy Grail" of gene transfer methods. It allows targeted integration of genes by flanking them with homologous arms to the target region. Very low efficiencies and the difficulties related to sequence design, make this a scarcely reported method in hESCs so far. Electroporation has been most successful to introduce the DNA for homologous recombination in hESCs (Costa et al., 2007; Davis et al., 2008; Irion et al., 2007; Urbach et al., 2004; Zwaka and Thomson, 2003). Another possibility of a more random but stable gene integration had recently been reported using phiC31 integrase combined with lipofection or electroporation (Thyagarajan et al., 2008). Although this method increases integration events, it cannot yet target specific DNA sequences as in homologous recombination.

Transposon-mediated gene integration

One further non-viral gene transfer method with integration is combining nucleofection and the Sleeping Beauty transposon (Ivics et al., 1997). Compared to the random stable integrations during plasmid transfections with a frequency of 10^{-5}, transposons can increase the efficiency to 20% in some cell types. In hESCs the rate of integration was reported to be up to 5% (Wilber et al., 2007). The Sleeping Beauty has also been optimized to target certain regions of chromosomes (Ivics et al., 2007) and can be utilized to insert inducible vectors (Cadinanos and Bradley, 2007).

In summary, transfection and electroporation are efficient methods for transient gene transfer into hESCs but require a degree of individual optimization. One must be aware that a high number of plasmids are transiently delivered into cells, which may cause transgene toxicity. Non-viral methods are not as efficient in obtaining stable integrations as certain viral gene transfer methods, especially if no aiding enzymes are supplied. On the other hand, long-term *in vitro* growth and selection possibility of hESCs makes it easier to select for the few correctly modified clones, especially if site-specific integration is used. Non-viral gene transfer has also the advantage to have less size restrictions than viral vectors.

Protein and mRNA transfection lower the risks connected to stable integration

Protein or mRNA transduction may be alternative methods to genetic modifications. Clinical applications, not in need for a stable gene expression over longer time periods, may certainly be less risky if no permanent gene modification is induced. That reduces the risks of introducing errors into the germ line as well as causing lasting damage in patients. In case of protein transduction, intracellular proteins can be applied externally and taken up by the cell, instead of being internally transcribed and translated from inserted genes. An advantage is the ability to alter protein dose and time of exposure. However, intracellular proteins are usually not secreted or taken up by the cell, which requires their modification for this purpose. The development and production of such proteins is associated with many difficulties. Fusion proteins from transcription factors PDX1 (Kwon et al., 2005) and HoxB4 (Lu et al., 2007) have been used to influence hESC differentiation with some success. They are maybe an effective strategy for future directed differentiation of hESCs, but their application area seems limited to *ex vivo* strategies. Since hESCs are a novel research area, many important developmental genes, such as transcription factors, are not usually commercially available as proteins, and their use is limited.

Instead of modifying proteins, also RNA can directly be transfected into cells, which often achieves even higher efficiencies than DNA transfections. Also here, no genetic material is integrated, but gene expression can be directly introduced and influenced. Efficient GFP mRNA transfection in hESCs was reported (Ponsaerts et al., 2004), but no further reports seem currently available.

1.2.1.2 Viral mediated gene transfer

For millions of years viruses have been optimized by evolution to deliver their genetic material into hosts to secure their continued existence. The evolution produced highly effective/infective particles that are now exploited as gene delivery vehicles. Viral vectors are most effective to introduce genes both *in vitro* and *in vivo*, while previously described non-viral gene transfer strategies largely fail *in vivo*.

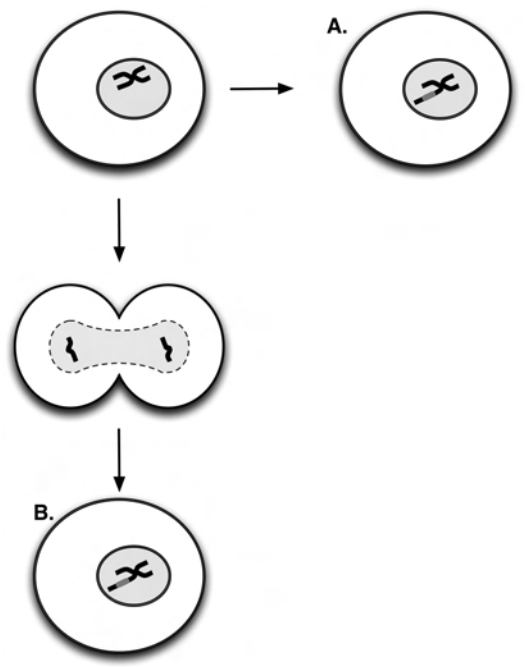
A variety of viral-based gene transfer methods have been reported in hESCs, but retroviral/ lentiviral vectors are used in this thesis and emphasized upon.
Adenoviral and adeno-associated viral vectors have been applied already in 2003, but only reached up to a 10% gene transfer efficiency (Smith-Arica et al., 2003). Both viral vectors are commonly used and allow high gene transfer rates also in non-dividing cells. Whereas adenoviral vectors do not integrate into the genome (Volpers and Kochanek, 2004), adeno-associated viral vectors integrate and may even allow targeting of specific chromosomes (Warrington and Herzog, 2006). Only two later reports described adenoviral vectors in hESCs, likely due to inconsistent applicability reasoned by variable expression of binding receptors (Brokhman et al., 2008). They have been applied for transient high transgene expression during hESC differentiation (Rufaihah et al., 2007).

Recently Epstein-Barr episomal viral based vectors have been reported to deliver large transgenes episomally in hESCs. Epstein-Barr vectors have furthermore been described to provide a degree of stable integration (Ren et al., 2006).

Retroviral vectors

Retroviral vectors are highly efficient gene transfer vehicles and are the most commonly used vectors for gene therapy applications (Cavazzana-Calvo et al., 2000). They are based mostly on the Moloney murine leukaemia virus (MMLV) and integrate transgenes permanently into the genome. A limitation of the common MMLV-based vectors (also called oncoretroviral vectors) is their ability to only deliver genes into dividing cells as they lack the ability to introduce their genetic material into the cell nucleus and thus have to wait for the disintegration of nuclear membrane during cell division. Retroviral vectors are possible to utilize for transduction of activated cells, differentiated from hESCs (Menendez et al., 2004).

Oncoretroviral transduction represents a fast and efficient method for transgene expression and well-established vectors are readily available, providing promising work tools. However, their potential for ES cell work is limited due to their vulnerability to be silenced throughout germ-line differentiation (Lois et al., 2002).



Recently a new efficient retroviral vector has been presented. Foamy virus vectors are integrating and do not have a known pathogenicity as oppose to other retroviruses. Other advantages are their ability to infect non-dividing cells and that the gene expression was found persistent in hESC derivatives. Efficient transduction of hESCs with these vectors has been reported and may be a safer, yet still an effective, alternative to other retroviral vectors (Gharwan et al., 2007). Further development of this system will show if these vectors keep up to their promises.

Figure 3 Schematic presentation of transduction with different viral vectors. Panel A, lentiviral vectors infect non-dividing cells and integrate its transgene. Panel B, oncoretroviral vectors are only able to integrate their transgene in dividing cells.

Lentiviral vectors

In order to circumvent the problems expected from oncoretroviruses during germ-line transmission in ES cells, lentiviral vectors, a subgroup of retroviral vectors were used instead in hESCs. Most lentiviral vectors are HIV-1 based and have been extensively modified to provide safety while sustaining the gene transfer efficiency. Lentiviral vectors are advantageous; in terms of their ability to accept different promoters, are less prone to silencing, and provide a wide range of target cells, including cells that are non-dividing (Miyoshi et al., 1998; Naldini et al., 1996). Although lentiviral vector development has gone through several generations, removing HIV genes responsible for its pathogenicity, they retain most of their infectivity.

Proofing their efficacy, lentiviral vectors were the first viral vectors to introduce stable transgene expression in hESCs (Gropp et al., 2003; Ma et al., 2003; Pfeifer et al., 2002). Recent vectors are able to reach up to 80% transduction. This figure can be increased further by selection of the transduced cells, reaching >99% stably modified cells in a very short time frame (Suter et al., 2006). These vectors are usually pseudotyped with the amphotropic envelope of the vesicular stomatitis virus (VSV) in order to infect human cells and widen their natural host range. Though as compared to ecotropic pseudotyped vectors, only infecting mice, amphotropic envelopes require increased safety precautions. However, the use of ecotropic lentiviral vectors has been described and could potentially reduce the risk associated to amphotropic vectors (Koch et al., 2006). Last generation lentiviral vectors are usually self-inactivating their own viral promoter after insertion into the host cell, therefore providing an additional safety feature.

A limiting feature of most viral delivery systems, as also the lentiviral system, is the limited size of transgenes possibly to deliver (lentiviral vectors ≤8kb). Another risk factor is their undirected integration of transgenes, potentially causing insertional mutagenesis.

1.2.1.3 Altered transgene expression through vector construction

The number of viral insertions/ transgene copies within the genome can change the expression levels of a transgene. The sum of viral insertions per cell can be partially controlled by the ratio of added viral particles to number of cells (Kustikova et al., 2003). However, expression is also dependent on the integration site in transcriptional active regions (Burke and Baniahmad, 2000).

In order to increase or reduce the gene expression in stable or in a controlled manner, viral sequences or different promoters may be added during vector construction. For example, the central polypurine tract of the HIV-genome (Zennou et al., 2000) and the woodchuck hepatitis posttranscriptional regulatory element (Zufferey et al., 1999) are regularly added to increase nuclear import and RNA expression, respectively (Suter et al., 2006).
Additionally, the choice of promoters will strongly influence expression levels depending on their activity in different cell types. Whereas common retroviral vectors are driving transgene expression from their viral LTRs, in advanced self-inactivating (SIN)-vectors, LTRs are inactivated and a chosen internal promoter is additionally

inserted. If a gene should be over-expressed in all tissues, a ubiquitously active promoter is necessary. Such internal promoters have been compared in hESCs and specifically the human elongation factor 1-α (EF1α) is one strong and often used alternative (Clements et al., 2006; Ma et al., 2003; Suter et al., 2006; Xia et al., 2007). In our studies, a human Ubiquitin C promoter was used and provided strong and sustainable expression in hESCs (articles I,III and IV), but it had recently been shown to provide a 2-fold lower gene expression than the EF1α promoter (Zhou et al., 2007).

Another possibility to influences gene expression in bi-cistronic constructs is the insertion of a linker between. Bi-cistronic constructs allow the expression of two genes from a single mRNA. Often a marker gene is fused with a gene of interest, in order to trace or select modified cells. If the two proteins shall not be directly fused, but separated, viral elements such as an internal ribosome entry site (IRES) or a protein cleavage peptide such as the 2A can be cloned in between both genes. Whereas an IRES allows ribosomes to bind and initiate translation, the 2A peptide mediates co-translational cleavage on a specific site, and either way two separated proteins are finally expressed. A different expression for first and second gene is seen for IRES or 2A linked genes (Bochkov and Palmenberg, 2006; Klump et al., 2001; Mizuguchi et al., 2000) and was utilized in article III to achieve different HoxB4 expression constructs.

Inducing gene expression

Of course the preparation of the right construct requires that the needed expression level is known in advance. This may seldom be the case and even be the result in question. Mostly gene expression should be optimized and may even be only required for a certain time frame during cell development, as we suggested in article III. Therefore inducible gene expression would be advantageous to a consistent over-expression. Such systems have been just recently optimized and employed in hESCs, because previous systems were not applicable to hESCs. A Cre-inducible system (Vallier et al., 2007) as well as tetracycline inducible systems have been reported (Vieyra and Goodell, 2007; Zhou et al., 2007). Transcriptional "on-off" regulation of inserted genes or shutdown of gene expression by siRNAs will open a wider window to look at effects of gain and loss of gene functions.

A quantifiable system has still not been reported in hESCs. A recently published tuneable method, fusing synthetic small molecules to regulate protein stability in cells could be an interesting candidate to evaluate in hESCs (Banaszynski et al., 2006) and has recently become commercially available (ProteoTuner™, www.clontech.com).

1.3 DIFFERENTIATION OF HESCS TO MESODERM DERIVATIVES

The hESC differentiation model gives us a unique possibility to study very early human developmental processes. hESCs are also a source that offers the potential to generate virtually any cell type for regenerative medicine. To realize their potential, developmental research is a starting point to understand and control their directed differentiation.

1.3.1 Germ layer development *in vivo*

After extensive cell divisions during expansion of pluripotent cells up to the blastocyst stage, the inner cell mass is forming the bilaminar germ disc, consisting of the epiblast and hypoblast layer. Extensive cell migration is leading to the formation of the three germ layers, ectoderm (outer layer), mesoderm (middle layer) and endoderm (inner layer), in a process called gastrulation. The formation of the three germ layers starts by formation of the primitive streak, which is an ingression of the epiblast cells. These epiblast cells are migrating to the primitive streak, while cells migrating through, will form the mesoderm and endoderm layer.

The ectoderm layer will later form the embryo surface with its epidermis and forms the brain as well as the nervous system. The mesoderm or middle layer will form the tissue of the heart, kidney, gonads, muscles, blood and bone. The endoderm is the innermost layer and forms the epithelium of the digestive organs as well as the lungs.

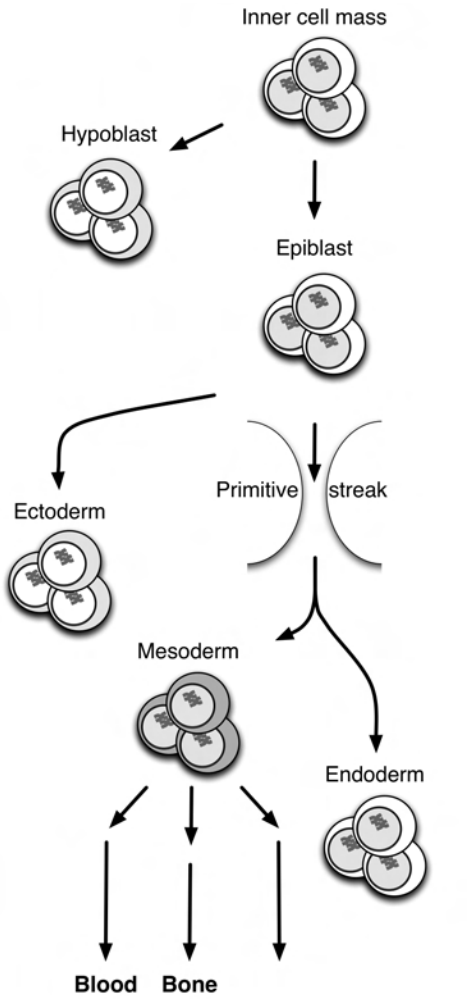


Figure 4 Schematic representation of germ layer development with emphasis on the middle layer (mesoderm), which is the origin of blood and bone.

Although the signaling involved in the regulation of germ layer formation is not fully understood, members of the TGFβ family such as BMP4 (Hogan, 1996) and Nodal

(Conlon et al., 1994) were found to be essential. In a more recent study, two growth factors, nerve growth factor (NGF) and hepatocyte growth factor (HGF) were shown to be involved in commitment of hESCs to all three embryonic germ layers, leading to expression of mesodermal, ectodermal and endodermal markers (Schuldiner et al., 2000). Similarly, Wnt signaling seems to predispose germ layer formation (Yamaguchi, 2001).

1.3.1.1 Development of mesoderm and their derivatives *in vivo*

At day 15-16 of human development, the epiblast cells start to ingress through the primitive streak and start to form mesoderm. By day 17 a thickened layer of tissue has formed, which is called the paraxial mesoderm. The thinner more lateral mesoderm layer is called the lateral plate mesoderm. Due to the development of intracellular cavities the lateral plate mesoderm diverts into somatic and splanchnic layers, while intermediate mesoderm transiently connects paraxial and lateral plate mesoderm. Approximately at day 20 of the embryonic development, the **paraxial mesoderm** evolves into segments called somites and their increase in numbers can be used to determine the developmental age of embryos. Somites then form the cartilage of the vertebrae and ribs, the musculature and dermis of the skin. Some portion of the somites, called the sclerotome, will undergo further mitoses, loose their epithelial and segmented character and become mesenchymal. These will condensate and eventually form the bone of the axial skeleton. The lateral plate mesoderm will eventually give rise to the limb skeleton, but it is better known for the formation of the circulatory system. **Lateral plate mesoderm** will produce heart, vessel and **blood cells**. An emphasis on blood and bone development will be made in further parts of this introduction.

1.3.1.2 Modelling germ layer development with hESCs

Although the molecular mechanisms of early human development are not well defined, several events are well identified and these can be used to evaluate the hESC model. As initially mentioned, hESCs can differentiate into derivatives of all three germ layers. Although organogenesis cannot be directly studied in the human system, the EB differentiation method simulates the three dimensional structure of the germ disc and allows, although random, all germ layers to develop (Itskovitz-Eldor et al., 2000; Schuldiner et al., 2000). Also consistent with the *in vivo* situation, adapting cell culture conditions to each cell maturation stage seem required to differentiate hESCs efficiently (D'Amour et al., 2005). Furthermore several known factors in germ layer development, such as BMP4, have been shown to be crucial for hESCs *in vitro* differentiation strategies (Chadwick et al., 2003; Zhang et al., 2008b). Cytokines can also generate subpopulations of the germ layers *in vitro*, e.g. activin which induces formation of cardiac mesoderm (Laflamme et al., 2007). Taken together with a relative similar temporal differentiation pattern of hESCs *in vitro* compared to the *in vivo* situation, these features indicate that hESCs can be used in functional model systems to explore the molecular mechanisms of early human development.

1.4 HEMATOPOIETIC DEVELOPMENT

Hematopoiesis is the developmental process by which all blood and immune cells are generated. The stem cells of the blood system have been most readily studied as compared to stem cells in other tissues. This so called hematopoietic stem cell (HSC) has been discovered early (Abramson et al., 1977) and shown to give rise to all mature blood cell populations. These cells have been found to arise in the embryonic site of the paraaortic splanchnopleura (AGM region) in association with the intraembryonic blood vessels (Medvinsky et al., 1993; Nishikawa et al., 2001). HSCs are an intermediate part of hematopoiesis and their differentiation in early hematopoiesis is less understood compared to the well-described development to final mature blood cells in late hematopoiesis.

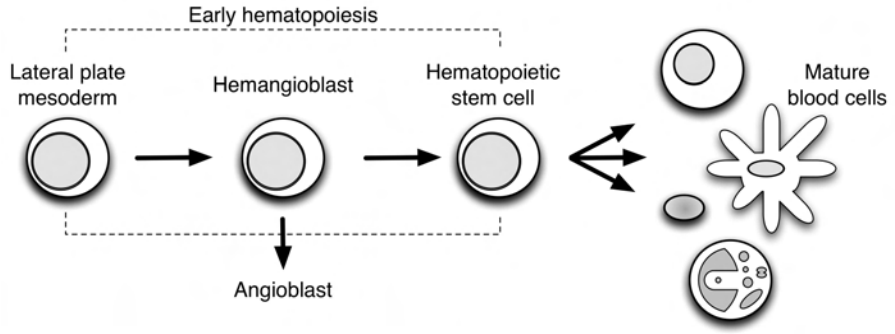


Figure 5 Schematic representation of early and late hematopoiesis.

Hematopoietic stem cells have been in clinical use since the first bone marrow transplantation in 1956 (Thomas et al., 1957). Because HSCs are residing and can be derived from adult bone marrow, peripheral blood and umbilical cord blood, their clinical impact has been driving research on cells from these available sources. They are functionally described by their ability to mediate long-term engraftment and repopulation of all blood lineages after lethal irradiation. Phenotypically HSCs from bone marrow are defined by $CD34^{+}$ $CD38^{-}$ $CD90^{+}Lin^{-}$ surface marker expression (Bryder et al., 2006). However, in mice long term-engraftment has been obtained with one single $CD34^{(lo/-)}$ HSC (Osawa et al., 1996), and many reports are searching for such a cell in humans (Bhatia et al., 1998; Bryder et al., 2006). Even though defining better HSC markers may help to improve engraftment and decrease the number of cells required, it is possible that such cells are scarcely found in adults and may be hardly accessible.

Cord blood may provide a more primitive and rich source of HSCs, but the blood volume that can be obtained is mostly insufficient to engraft in adults (Hofmeister et al., 2007). *In vitro* HSCs expansion protocols could lift this limitation and would also allow an easier scheme for *ex vivo* cell modifications such as gene transfer. Research protocols adding HoxB4 protein (Amsellem et al., 2003), Delta1-Fc chimeric protein (Suzuki et al., 2006) or Angiopoietin-like 5 plus IGFBP2 (Zhang et al., 2008a) to cell culture media have recently been shown to enable HSC expansion up to 20 fold.

However, so far available clinical expansion protocols are just keeping HSC numbers temporarily unchanged (Ivanovic et al., 2006; Mohamed et al., 2006; Sorrentino, 2004; Yao and Hwang, 2007).

hESCs provide an even more primitive source of cells that can be easily expanded and could therefore potentially serve as an unlimited reservoir in the search and growth of potent long term repopulating HSCs (LT-HSCs).

1.4.1 Early hematopoiesis from human embryonic stem cells

The hemangioblast was defined as the first precursor of blood development, a decade ago, using mESCs (Choi et al., 1998; Nishikawa et al., 1998). The hemangioblasts of the lateral plate mesoderm can give rise to both the angioblasts of the vascular system and the pluripotent hematopoietic stem cells of the blood and lymphoid systems (Shalaby et al., 1995; Wood et al., 1997). Cytokines leading to efficient hemangioblast development in mESCs were recently better defined using serum-free medium, emphasizing the importance of BMP4, activin A, bFGF and VEGF (Pearson et al., 2008). However, to differentiate transplantable HSCs has been a goal still difficult to achieve without genetic modification even if using the more established mESCs (Olsen et al., 2005).

Hematopoiesis from hESCs was first modeled in 2001 (Kaufman et al., 2001). Since then more reports have confirmed their potential (Chadwick et al., 2003; Tian et al., 2004) and analysis of defined stages of their development has been started to reveal definitive steps of early hematopoiesis (Wang et al., 2004; Zambidis et al., 2005).

1.4.1.1 The early hematopoietic phenotype

Earliest primitive streak development was recently more defined by application of MIXL1 reporter cell lines, showing MIXL, BRACHYURY and PDGFRalpha expression as early markers for these first mesoderm progenitors (Davis et al., 2008). During continued development of hESCs a hemangioblast stage had been achieved and its phenotype defined by PECAM-1 (CD31), Flk-1 and VE-cadherin but not CD45 (Kennedy et al., 2007; Wang et al., 2004), as was quite similarly defined in mouse development (Fehling et al., 2003; Nishikawa et al., 1998).

As the final step of early hematopoiesis, the HSC phenotype has been defined in several reports specifically trying to identify the LT-HSC. Standard markers for HSCs are CD34 (Brandt et al., 1988; Krause et al., 1996) and CD133 (Handgretinger et al., 2003; Yin et al., 1997), which are commonly used if cells are selected from different adult blood or bone marrow populations. However, these markers are unspecific in the multicellular environment of differentiating hESCs. CD34 is also found on endothelial cells (Oberlin et al., 2002) and also CD133 is not specific and seems more a marker for primitive cells, which can as well be found on fractions of hESCs and their progeny ((Zambidis et al., 2005), see also article III).
Although these markers are commonly used, they should be seen in combinations such as $CD34^{+}CD133^{+}CD90^{+}CD38(-)Lin(-)$.

Because the most primitive LT-HSCs have been suggested to be CD34(-) such as in mice (Osawa et al., 1996) , an earlier cellular marker was needed. CD143 has recently been identified to eventually pinpoint HSCs of the most primitive long term repopulating type (Jokubaitis et al., 2008). It was initially called BB9 (Ramshaw et al., 2001) and seems also able to specifically select LT-HSCs from hESCs (Zambidis et al., 2007). Another novel selective marker for more committed HSCs that are not yet $CD45^+$ may be the recently reported CD43 (Vodyanik et al., 2006). Such new single specific markers would ease detection of HSCs, but more studies are required to prove their usefulness.

Demonstration of maturation from HSCs to all final blood lineages is necessary to differentiate primitive from definitive hematopoiesis, which provide different blood populations as necessary during early or late embryonic development. So far the only feasible way of providing evidence for definitive and full hematopoiesis from ES cells, is to show their lymphoid differentiation potential (Murry and Keller, 2008). NK and T cell differentiations from hESCs have been reported (Galic et al., 2006; Woll et al., 2005). However, the generally low engraftment rates (Narayan et al., 2006), although partially caused by immune rejection (Tian et al., 2006), as well as developmental differences in hematopoietic cell populations (Martin et al., 2008) suggest that the current cell culture microenvironment is still missing important elements.

1.4.1.2 Signalling and culture conditions

During the process of maturation, the HSC must migrate through several embryonic niches, which provide the signals for each stage of development. In order to drive directed HSC differentiation, it seems most optimal if these signals are recapitulated.
However, undefined conditions using stroma co-culture and cytokines have resulted in highest induction of HSCs also able to mature into substantial amounts of $CD45^+$ cells (Wang et al., 2005a). Most efficient induction of hematopoietic cells was reported within the microenvironment of AGM derived stroma cells (Ledran et al., 2008). A common cytokine cocktail shown to improve HSC differentiation consists of SCF, G-CSF, Flt-3L, possibly also supplemented with IL3 and IL6 (Ji et al., 2008).

Besides the efforts of increasing engraftment potential of ES cell derived HSCs, only genetic modification with the transcription factor HoxB4 has shown a substantial impact.

1.4.2 Enhancing hematopoiesis by genetic modification with HoxB4

HoxB4 is a member of the homeodomain transcription factor family, which are important developmental regulators expressed in precisely coordinated fashion. Class I homeobox (HOX) genes are a family of 39 genes clustered on 4 different chromosomes. Depending on their chromosomal location, they are named A, B, C and D, whereas the human B cluster is located on chromosome 17. Hox-proteins are expressed at different times and stages during development and are known to determine the body patterning such as the limb position. Their expression seems to be controlled in a 3’ to 5’ fashion and control a cascade of target genes important for these

developmental stages (Pearson et al., 2005). The HoxB cluster has also shown a defined expression pattern in hematopoietic cells and pinpointed HoxB4 expression to maturing hematopoietic progenitors (Giampaolo et al., 1994; Kyba and Daley, 2003).

HoxB4 became the dean of new opportunities as it has solved the problems of HSC expansion and engraftment of ESC-derived HSCs. After mapping HoxB4 to the most primitive hematopoietic precursors (Giampaolo et al., 1994; Sauvageau et al., 1994), first gain of function studies in mouse bone marrow (Sauvageau et al., 1995) and mESC-derived hematopoietic cells (Helgason et al., 1996) increased expansion, engraftment and differentiation potential of these Hoxb4 over-expressing cells. HoxB4-HSCs were able to reconstitute the HSC pool to 100%, whereas normal HSCs only reconstitute to 10% of normal HSCs numbers (Thorsteinsdottir et al., 1999). Besides reports showing successful expansion of adult mouse and human HSCs *ex vivo* (Antonchuk et al., 2002; Buske et al., 2002; Krosl et al., 2003a), its functionality also animated the use of HoxB4 in early hematopoiesis from ESCs. HoxB4 was then reported to induce a switch from primitive to a definitive hematopoiesis in ESC-derived HSCs (Kyba et al., 2002).
Earlier studies showed an ease and effectiveness of HoxB4 using retroviral vectors. Also considering the gain of engraftment and expansion was initially not found to relate to oncogenic behavior (Lawrence et al., 1996; Thorsteinsdottir et al., 1999). However, later studies focused on more defined conditions, and it was noticed that these over-expressing HSCs were not only positively affected but there may be a requirement for controlling HoxB4 expression (Klump et al., 2005; Milsom et al., 2005; Schiedlmeier et al., 2003).

Whereas this was shown in human adult HSCs, recent studies in hESC-derived HSCs did not address this issue and presented varying results. Retroviral transfer of HoxB4 resulted in a proliferative advantage of HSCs *in vitro*, but neither engraftment nor blood colony formation was improved (Wang et al., 2005b). However, a later report showed that stable transfection of HoxB4 promoted early hematopoietic development and also strongly improved functional blood cell colony formation capacity (Bowles et al., 2006). We therefore postulated that expression levels might be an important regulation parameter of transcription factors and provided first evidence during the development of hESCs (articles III and IV).

1.4.2.1 HoxB4 targets

How HoxB4 influences HSC development is not well defined. Although many studies have defined regulators and targets of this transcription factor, the sheer variety of them in conjunction with the different developmental cell types makes prediction of cell behavior difficult.
Although initially a few direct target genes, such as c-myc (Pan and Simpson, 1999), Rap1 (Morsi El-Kadi et al., 2002), Irx5 (Theokli et al., 2003) or FLASH (Morgan et al., 2004) have been described, the development of microarrays and high throughput technologies led to discovery of more than 50 HoxB4 target genes in adult or mESC-derived HSCs. In ESC-derived HSCs the number of regulated genes was found even higher than in its adult counterparts (Schiedlmeier et al., 2007). A similar wide array discovering several hundred Hox target genes has been recently reported in Drosophila (Hueber et al., 2007).

1.4.2.2 HoxB4 regulators

Since Hox genes are expressed in an anterior-posterior (3'-5') direction during development, they induce or limit their own expression, in both neighboring and their own cells. Retinoic acid response is higher in more anterior Hox genes and cross regulation of HoxB4 with the retinoic acid receptor was recently shown (Serpente et al., 2005). In summary, HoxB4 is at least partly regulated by other Hox proteins and endogenous retinoic acid. Studying the HoxB4 promoter and its enhancer elements has identified additional transcription regulators, such as upstream stimulation factor 1 and 2 (USF1/2) (Giannola et al., 2000). The nuclear factor Y (NF-Y) was later shown to bind USF1/2 in a complex to fully activate the HoxB4 promoter in HSCs (Zhu et al., 2003). The relative levels of the transcription factors NFY and YY1 may be critical in activation or repression of HoxB4 (Gilthorpe et al., 2002). Finally, thrombopoietin (TPO), the primary regulator of platelet production, was identified to induce HoxB4 expression via stimulation of USF-1. HoxB4 in $tpo^{-/-}$ mice was found to be 2-5 fold lower when compared with controls (Kirito et al., 2003).

There are also several other regulatory elements influencing HoxB4 expression as well as target choice. DNA methylation is one of the elements regulating tissue specific expression (Hershko et al., 2003), but also many different co-factors such as PBX or MEIS (Pineault et al., 2004) play decisive roles on HoxB4 effects on targets. For example a strong negative effect of PBX1 on expansion of HoxB4-transduced HSCs and ultra-competitive HSCs production upon PBX1 down-regulation are shown (Krosl et al., 2003b). The novel discovery of micro RNAs has revealed another regulatory element affecting Hox genes (Chen et al., 2004; Woltering and Durston, 2008).

In summary, orchestrating Hox genes in a defined manner presents a very high complexity. Variations of transcription factor protein (as in articles III and IV) are only one defined piece in the puzzle of these developmental cascades regulating cell fate. In the light of this complexity and recent publications, showing that HoxB4 can increase the risk of leukemia development, clinical use of HoxB4 over-expressing cells seems not likely in the near future (Zhang et al., 2008c; Zhang et al., 2007).

1.5 BONE DEVELOPMENT

As with hematopoietic development, bone development starts from the mesoderm. The close relationship between blood and bone continues throughout adult life. Thus, HSCs reside close to the endosteum of the bone marrow and provide it with many supportive signals (Kiel and Morrison, 2008; Wilson and Trumpp, 2006). This stem cell niche allows proliferation and differentiation of LT-HSCs through the secretion of cytokines by different bone cell fractions. Conversely, hematopoietic cells also provide signals important for bone development (Kacena et al., 2006).

In addition to its role in supporting hematopoiesis, the major functions of bone include the support of soft tissues, to serve as lever for muscle action, to maintain the blood calcium levels and to protect internal organs, including the brain and spinal cord. In order to sustain these functions, bone is constantly renewing itself in a process which is designated remodelling (Harada and Rodan, 2003). For regenerative medicine, an understanding of remodelling and the creation of functional tissue is a major challenge (Griffith and Naughton, 2002). However, when understood, it may fundamentally change the treatment options of bone that has been damaged by trauma or disease through the recreation of new bone tissue.

1.5.1.1 Bone remodelling

Bone remodelling involves a balance between bone formation and bone destruction. On the one hand, osteoblasts are bone-forming cells that produce matrix and coordinate its mineralization. On the other hand, bone destruction or resorption is carried out by hematopoietically derived osteoclasts. An equilibrium between bone formation and resorption, where the destruction is required to release necessary calcium into the blood, maintains the bone mass in adults. Formation and resorption seem to be coupled locally by mechanisms not fully understood, but it is clear that if one process increases the other usually follows to (Harada and Rodan, 2003). Three major regulators influence bone homeostasis: calcium availability, sex steroids and mechanical usage.

1.5.1.2 Bone formation in vivo

There are two major modes of bone formation. Both involve the condensation and differentiation of mesenchymal cells (Hall and Miyake, 2000). In the first process, denoted **intramembranous ossification**, clusters of cells differentiate directly into bone forming osteoblasts. This bone development is mainly taking place in the flat bones of the skull and these osteoblasts secrete a matrix rich in collagen I. However, most bone development occurs according to another differentiation scheme, which is termed **endochondral ossification**. In this process, cells first migrate to locations of skeletal development and differentiate into cartilage-producing cells, so called chondrocytes. Subsequent growth forms cartilage scaffolds of future bones, where the secreted extra cellular matrix (ECM) is particularly rich in collagen type II and the proteoglycan aggrecan. Chondrocytes in the center of the cartilage mould then stop proliferating, they enlarge and subsequently synthesise a distinct matrix containing collagen type X. The production and secretion of angiogenic factors attract blood

vessels. However, such factors also attract perichondral cells, and direct the differentiation of these cells into osteoblasts. In further steps, hypertrophic chondrocytes undergo apoptotic cell death which allows blood vessels to enter. Osteoblasts will then bind to the degenerating cartilaginous matrix and deposit bone matrix (Kronenberg, 2003). Bone formation and growth consist of ordered arrays of proliferating, hypertrophic, and mineralizing chondrocytes.

1.5.1.3 Osteoblasts

Bone forming cells are single nucleated cells, which are nearly indistinguishable from fibroblast *in vitro* (Ducy et al., 2000). Their only typical characteristic is the formation of mineralized ECM. Osteoblasts synthesize and lay down collagen type I, which comprises 90% of the organic matrix of bone. Osteoblasts also produce osteocalcin (OCN), the most abundant non-collagenous protein of the bone matrix. Other matrix proteins are glycosaminoglycans (GAGs), osteonectin, bone sialoprotein (BSP) and cell attachment factor. Mineralisation occurs when osteoblasts mature and requires a high osteoblast alkaline phosphatase (ALP) activity. This enzyme cleaves phosphate groups which act as foci for calcium phosphate apatite deposition. Several matrix components regulate the transport of mineral ions at the site of mineralization. This process seems to be coordinated by osteoblasts.

Osteoblastic differentiation is controlled via both endocrine and local signalling. Two principal but contrasting hormonal factors seem to be involved; parathyroid hormone (PTH) which upregulates bone formation (Bergenstock and Partridge, 2007) and leptin, which downregulates bone formation (Karsenty, 2006). Locally, several signalling molecules regulate osteoblasts. Among these, bone morphogenic proteins (BMPs) are well known to induce bone formation and to be present in the bone matrix (Sakou, 1998; Wozney et al., 1988). Further, latent Transforming growth factor β1 (TGF-β1) can be activated by osteoclasts during bone resorption and is then able to activate osteoblasts (Janssens et al., 2005). Fibroblast growth factors (FGF) are also present in the bone matrix, where they strongly stimulate osteoblasts proliferation and differentiation (Marie, 2003). Finally, several additional factors have been reported to influence bone formation, e.g. Wnt glycoproteins (Hu et al., 2005; Westendorf et al., 2004) and interleukine-6 (IL-6) (Franchimont et al., 2005). In this context, it may also be worth mentioning that matrix elasticity can direct MSC differentiation in humans, which to some extent may explain the effect of mechanical usage on bone formation (Engler et al., 2006).

Two main transcription factors play essential roles in bone formation and are at least partially regulated by BMPs or FGFs. These two specific marker genes are the core binding factor α1 (Cbfα1), also known as Runx2, and Osterix (Osx) which is known to act downstream of Runx2. Runx2 serves as an initial osteogenic marker and activates OCN and collagen type I genes (Komori, 2008). Disruption of Runx2 results in a complete lack of osteoblastic bone formation (Komori et al., 1997). Osx is a zinc finger transcription factor and BMP-2-inducible gene that is necessary for bone formation, as judged from observations in Osx null mice. $Osx^{-/-}$ cells express Runx2, which indicates

a developmentally earlier role for Runx2, while Osx is absent in Runx2 knockouts (Nakashima et al., 2002).

1.5.2 *In vitro* modelling of bone formation from hESCs

Much of the above-mentioned knowledge on bone formation and signalling pathways has been identified *in vitro* using bone tumour cell lines or tissue-derived cells of non-human origin (Bellows et al., 1986; Schmidt et al., 1988; Thomas et al., 2004).

The embryonic stem cell model has provided an attractive test system that can offer an unlimited source of cells while having an intact primary cell type. Initial studies in mESCs demonstrated the possibility to model bone formation from embryonic stem cells (Buttery et al., 2001; Phillips et al., 2001). A few years later the first human osteogenesis model was presented, using hESCs. With this, a possibility to develop a new source of bone tissue for future clinical therapy was actually realized (Bielby et al., 2004; Sottile et al., 2003).

As with hematopoietic differentiation from hESCs, osteoblastic differentiation can be induced either from embryoid bodies (EBs) or in a monolayer. Initially, in studies utilizing mESC and hESCs, differentiation was induced in EBs before the cells were seeded in monolayer. This was believed to promote the most primitive induction of germ layers. However, in more recent studies (including article III in the present thesis), this step was shown to be unnecessary when inducing osteoblastic differentiation (Karp et al., 2006).

The few existing studies that have worked with the ES cell model use a cocktail of culture supplements shown to promote osteogenic differentiation. This cocktail contains ascorbic acid, β-glycerophosphate (β-GP) and Dexamethasone (Dex). Ascorbic acid generally promotes proliferation and differentiation of embryonic cells, while it also induces collagen synthesis (Shin et al., 2004; Takahashi et al., 2003; Tsuneto et al., 2005). β-GP is a precursor of inorganic phosphate and it was reported to promote the nuclear export of Runx2 in osteoblastic cells (Fujita et al., 2001). The glucocorticoid Dex affect bone nodule formation and induces osteoblastic expression of genes (Igarashi et al., 2004).

1.5.2.1 Bone functionality

When analysing bone-formation in *in vitro* models, mineralisation is the most distinct marker. Most commonly, labelling of calcium deposition is detected through alizarin red or von Kossa staining. However it must be noted that these methods are not specific for bone mineral. Positive identification of biomineralisation requires additional techniques, such as fourier transform infrared (FTIR) analysis (Bonewald et al., 2003; Karp et al., 2006).
However, in addition to *in vitro* marker analysis mouse and human ESCs should also show *in vivo* engraftment and mineralization, which has been reported using stroma co-culture and biodegradable scaffolds (Bielby et al., 2004; Jukes et al., 2008; Kim et al., 2008).

1.5.3 Enhancing osteogenesis by genetic modification with Osterix

Bone development is regulated by the Runx2 transcription factor, but Runx2 is also expressed in chondrocytes and is therefore not specific for osteoblasts. However, recently a new osteoblast-specific transcription factor, Osterix (Osx) has been identified. Further, it was shown that Osx null mesenchyme could not differentiate into osteoblasts (Nakashima et al., 2002). The human Osx is located on chromosome 12 and belongs to the zinc finger gene family, which is the largest class of transcriptional regulators in the mammalian genome. The zinc finger motif has a high degree of homology to the murine SP family and was therefore assigned the symbol SP7 (Gao et al., 2004). Because of its recent discovery, regulator and targets are not well defined. However, it was found that Dlx5, a homeobox transcription factor that can be induced by BMP2, is directly targeting the Osx promoter (Lee et al., 2003). Osx activates OCN and collagen I, and a conjunction with nuclear factor of activated T cells (NFAT) seems to be important for its proper transcriptional activity (Koga et al., 2005; Nakashima et al., 2002).

Few studies inducing the gain of function of Osx have been conducted in mouse embryonic stem cells and mesenchymal stem cells (Kim et al., 2006). Hypothetically, Osx overexpression in these cells could increase the yield of osteoblastic cells. In one study, it was noted that osteogenic differentiation from stably Osx-transfected mESCs was increased, as judged from the upregulation of several osteoblastic markers (Tai et al., 2004). Osx has been studied in hESCs, but without any analysis of its effect on bone differentiation (Tai et al., 2005). Therefore article IV of this thesis is the first study, which evaluates bone-inducing effects of Osx in differentiating hESCs.

2 AIMS OF THE THESIS

The overall aim of this thesis was to establish and analyse specific differentiation of human embryonic stem cells (hESCs) into blood and bone lineages, thus evaluating effects of important developmental transcription factors on early differentiation.

Specific aims of this thesis:

I. To establish lentiviral gene transfer to hESCs in order to stably integrate and express genes and using fluorescence marked cells to evaluate our existing culture protocol.

II. To establish and characterize a basic bone differentiation model using a number of hESC lines, and evaluating two methods with a wide panel of osteogenic markers.

III. To analyze the gain-of-function effects of the transcription factor HoxB4 on early hematopoietic differentiation from hESCs, thereby characterizing lentiviral constructs and the previously established gene transfer method to introduce the HoxB4 gene.

IV. To evaluate the gain-of-function effect of the transcription factor Osterix on bone and blood development using the above established differentiation protocol.

3 MATERIALS AND METHODS

This section contains our reasoning and some general considerations on materials and methods used throughout this work. These may not always be described in standard protocols but were found important during the completion of this thesis. More detailed descriptions can be found in the appropriate sections of the individual papers.

3.1 HUMAN EMBRYONIC STEM CELL LINES AND CULTURE METHODS

3.1.1 hESC lines

hESC lines HS181 (Hovatta et al., 2003), HS207, HS306 used in our studies, originated from derivations at the Karolinska University Hospital Huddinge and H9 was obtained from the WiCell Research Institute (www.wicell.org). Each hESC line is derived under special conditions and has a donor origin. Although available lines are equal in many aspects, such as morphology or surface marker expression, their properties and behaviour can vary (Adewumi et al., 2007). It is therefore important to compare data from several hESC lines when drawing general conclusions. Furthermore a detailed check of derivation and culture conditions is necessary if data should be compared to other studies. Therefore, in essence considerations should be made on the feeder cell or matrix type, the medium used and the passaging method.

HS181, is the mostly published hESC line from our Karolinska consortium and grows equally well compared to H9, in comparison HS207 and HS306 have been less widely used due to their later derivation dates. HS181 was also the first hESC line that was derived on human foreskin fibroblast feeder cells instead of the commonly used mouse embryonic fibroblast feeder cells (Hovatta et al., 2003).
The H9 line is one of the earliest derived and most widely spread hESC line. Results can be easily compared to other published studies. We found these cells easy to handle in many aspects, such as expansion and differentiation. However one needs to consider that hESCs may have specific properties connected to the donor origin or derivation procedures.

3.1.2 Culture medium

The standard culture medium was comprised of a commercially available serum replacement-containing medium:

Amount	**Name**	**Provider**
	Knockout™ DMEM	Invitrogen
15-20%	Knockout™ Serum Replacement (KO-SR)	Invitrogen
1%	Non-essential amino acids (100x)	Invitrogen
2mM	L-Gluthamine or GlutaMAX™	Invitrogen
0.1mM	β-mercaptoethanol	Invitrogen
4-6ng/ml	bFGF	Biosource/ Invitrogen

3.1.3 Feeders

In order to exclude animal components from our culture system and improve our culture conditions, human foreskin fibroblasts became quickly our preferred feeders cells. They can be used for longer periods of time than mouse feeders and do not require the pre-coating of wells with gelatine. Besides the use of commercially available human fibroblasts from the American tissue and cell culture collection (ATCC, catalogue no. CRL-2429), we also established our own human fibroblast derivations to have better control over culture conditions of the hESCs (Unger et al., 2008a). For research purposes, the human feeder cells were commonly grown using Iscoves modified dulbeccos medium (IMDM with GlutaMAX™) containing 1% non-essential amino acids and 10% FBS (all Invitrogen).

3.2 LENTIVIRAL VECTORS

To effectively and stably integrate genes into hESCs, we chose to use lentiviral vectors as the transfer method. Lentiviral vectors have been successfully used to transduce human embryonic stem cells *in vitro* (Gropp et al., 2003; Ma et al., 2003). They have been employed to efficiently transfer the enhanced green fluorescent protein (EGFP)-gene into hESC lines. In parallel, to the first reports we utilized a lentiviral vector, provided by the lab of David Baltimore (Lois et al., 2002) (article I). Our system in articles I and III is based on similar self-inactivating (SIN) vectors, containing additional sequences such as the central polypurine tract (cPPT) and the woodchuck posttranscriptional regulatory element (WRE). However, we employed a different human promoter, equally known to drive transgene expression throughout germline transition an approach that had not been previously applied to hESCs (Lois et al., 2002; Qin et al., 2003). The first studies used the elongation factor 1α (EF1α)-promoter, however we were able to drive stable transgene expression with the human Ubiquitin-C promoter.

In article IV, we modified the backbone plasmid to the commercially available pLenti6/UbC/V5-Dest Gateway® vector (Invitrogen) because of the chemical selectivity, as well as the cloning advantages incorporated by the Gateway® system. These vectors also drives transgene expression from the human Ubiquitin-C promoter but are not equipped with the cPPT and WRE enhancer sequences, which as a result express lower amounts of transgene.

3.2.1 Lentiviral backbone plasmids

Article I: FUGW (Lois et al., 2002);
Article III: FG12 (Qin et al., 2003);
Article IV: pLenti6/UbC/V5-Dest (Invitrogen) + EGFP = pLenti6/UbC-EGFP

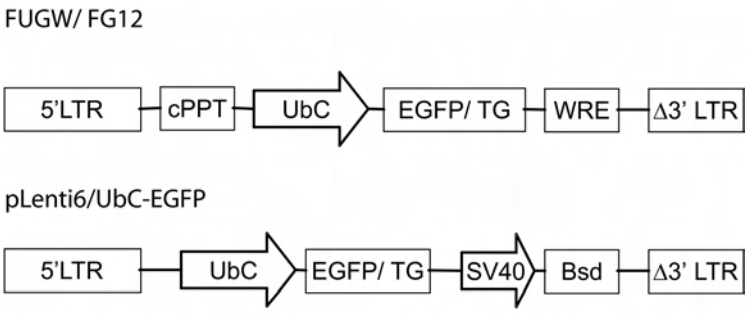


Figure 6: Schematic representation of lentiviral backbone plasmids;
TG: Transgene, WRE: Woodchuck hepatitis virus posttranscriptional regulatory element, cPPT: Central polypurin tract, UbC: Human Ubiquitin C promoter, SV40: Simian virus 40 promoter, Bsd: Blasticidin selction marker, LTR: Long terminal repeat

3.2.2 Transgenes

The EGFP transgene was subcloned from commercial plasmids (Clontech), whereas the Osterix (Osx) transgene cDNA was subcloned from a plasmid obtained from Bernhard Ganss (Gao et al., 2004) and HoxB4 was PCR-cloned from human buffycoat DNA. In articles III and IV using bicistronic constructs the transgenes were cloned in frame with EGFP. The extended 2A linker sequence from pSTA1/33 (Donnelly et al., 2001) was obtained as an oligonucleotide.

3.2.3 Lentiviral particle production

Production and concentration of lentiviral particles was achieved by transient co-transfection of three plasmids into 293FT cells. These three plasmids were (1) the amphotropic envelope plasmid pMDG harbouring the gene encoding vesicular stomatitis virus glycoprotein (VSV-G), (2) pCMV-R8.91 expressing the lentiviral gag and pol genes, and (3) our transfer vector (backbone + transgene). Viral particles were collected 48 and 72 hours after transfection, filtered through a 0.45μm filter, concentrated using centrifugation (50,000g, 2h) and frozen at -80°C.

3.3 DIFFERENTIATION OF HESCS

3.3.1 Hematopoietic differentiation of hESCs (Article III)

Hematopoietic development in a monolayer system without stroma cell support was initially found to be impaired in ESCs (Dang et al., 2002). Therefore, to induce hematopoietic differentiation, a modified standard **EB formation** protocol was used (Tian and Kaufman, 2005).

hESCs were removed from the culture dish by incubation with collagenase NB5 and mechanical scraping. Cell aggregates were transferred to low attachment bacterial culture dishes with differentiation media consisting of DMEM low glucose containing GlutaMAX™, 15% FBS and 1% non-essential amino acids (all from Invitrogen). The modification concerns the use of DMEM containing physiological glucose concentrations that were found to be better for growth of EBs (Khoo et al., 2005). No additional cytokines were used.
To minimize variation during the differentiation experiments, one FBS batch was applied throughout the study (article III).

3.3.2 Osteogenic differentiation of hESCs (Articles II & IV)

Differentiation experiments were initially set up using both monolayer conditions and the formation of embryoid bodies (EBs). After we found no need for EB initiation in article II, only monolayer differentiation was used in the following article IV.

To initiate cell differentiation in **monolayer**, the hESCs were released from the culture dish by incubation with collagenase solution and mechanical scraping. Approximately five colonies of 400 cells each (1000 cells/cm^2) were seeded onto gelatine-coated plates.
EB formation was induced in the hESC colonies by enzymatically detaching the cells with collagenase and transferring them to bacterial non-adherent culture dishes. On day six, the EBs were dissociated and subsequently plated on gelatine-coated tissue culture plates.
Osteogenic differentiation was induced by adding 20% FBS instead of KO-SR to the basal medium and supplementing with 10mM β-glycerophosphate (β-GP), 50 μg/ml ascorbic acid, and 1 μM Dexamethasone (Dex). The cultures were maintained for up to 25 days and the medium was changed every second day. In order to analyze the effect of cellular density in gene-modified hESC osteogenic differentiation cultures (article IV), the cells were split in a 1:2 ratio after seven days, and the cultures continued as described.

3.4 CHARACTERIZATION

3.4.1 Semi-quantitative and quantitative RT-PCR (Q-PCR)

In article II, total RNA was collected from hESC lines HS181, HS237 and HS306 after 4, 8, 15, and 25 days in osteogenic culture using RNeasy Mini Kit. In article III, total

RNA was extracted from undifferentiated and differentiating H9 cells. To obtain unaltered gene-expression levels from EB cells, the complete EBs were directly lysed. The obtained gene expression levels were normalized to the percentage of gene-modified cells as determined by flow cytometry. In article IV, total RNA was extracted from the undifferentiated and differentiated H9 cell line, and human osteoblast (hOBL) cell line, modified and unmodified HeLa cells, and purified CD34+ cells from peripheral blood. Human umbilical cord CD31+/CD34+ endothelial cells were kindly provided by Narinder Gautam (Department of Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden). cDNA from primary hMSCs was kindly provided by Mehmet Uzunel (Department of Clinical Immunology, Karolinska University Hospital Huddinge, Stockholm, Sweden). cDNA from human primary osteoblasts was kindly provided by Thomas Lind (Department of Medical and Physiological Chemistry, Uppsala University, The Biomedical Center, Uppsala, Sweden).

Semi-quantitative RT-PCR was performed using PCR Core Kit (Roche Diagnostics Scandinavia AB), and Q-PCR was carried out using human TaqMan Gene Expression Assays from Applied Biosystems (Foster City, CA). Amplification of bonesialo protein (BSP) and osteocalcin (OCN) in article IV was carried out using the SYBR® GREEN Master Mix (Applied Biosystems) in the reactions with similar specific primers as described in article II. The comparative cycle threshold (Ct) method was used to analyze data, and hydroxymethylbilane synthase (HMBS) was used to standardize the Ct values.

3.4.2 Flow cytometry (Articles III & IV) and cell sorting (Article III)

Flow cytometry was used to quantitatively evaluate the amount of flourochrome marked single cells. For a clear analysis, it was important to optimize several parameters and include proper controls.

A few *general considerations* are summarized below:
A set-up must be performed for each cell type and it is specific for each flow cytometer.
Antibodies must be tested, the number of cells, concentration of antibody, time, temperature of incubation and washing steps all need to be optimized. For optimization a positive and a negative (isotype) control is required to achieve good signal separation. Controls must be included during acquisition of final samples in order to account for experimental variation. For detailed instructions on flow cytometry, "Practical flow cytometry" by H.M. Shapiro (Ralph, 1995) can be consulted.

hESCs

The analysis of alive, undifferentiated hESCs requires the inclusion of a dead-cell marker (such as propidium iodide) because of the high sensitivity of the cells. To reduce the amount of dead or apoptotic cells during flow cytometric analysis, fast handling and a high amount of FBS (5-10%) in the suspension-medium is recommended. A single cell solution, necessary for flow cytometric analysis, was

obtained by incubating undifferentiated hESCs with TrypLE for 5min at 37°C. EBs were instead TrypLE-treated for 20min at a slowly circulating wheel and subsequently the single cell suspension was centrifuged and resuspended in medium containing 2-5% FBS.

A wide choice of commercial **primary antibodies** for SSEA1, 3, 4 and for example Tra1-60 is available. However they are expensive and required high concentrations (1:10) for good signal separation. Therefore, we switched to concentrated antibodies kindly provided from the lab of Peter Andrews (University of Sheffield, Sheffield, UK). These antibodies are available for low costs, are well quality-controlled and found of similar or higher quality as the commercially available ones.

For **fluorescence activated cell sorting** (FACS) of hESCs, it was difficult to obtain highly pure cell populations, since these cells do not like to grow as single cells. Assumingly, the recently published ROCK inhibitor can be used to strongly increase the survival and outgrowth after single-cell sorting (Watanabe et al., 2007).

3.4.3 SDS-PAGE and Western blot (Articles III and IV)

Cells were lysed using TRIzol reagent and protein extracts were quantified. Each sample was electrophoresed on SDS-PAGE and the proteins were electroblotted onto nitrocellulose membranes. After blocking, the membranes were probed with primary antibodies followed by a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (DAKO, Glostrup, Denmark). Proteins were detected with ECL Plus Western Blotting Detection System (GE Healthcare). For the detection of human Osx in paper IV, the primary antibody was kindly provided by Bernhard Ganss (Toronto University, Toronto, Canada).
For the detection of HoxB4 in modified and unmodified cells, total protein was extracted from the corresponding cell populations, and electrophoresed on a 10% pre-cast gel (BioRad Laboratories). Gels were blotted onto PVDF membranes (BioRad Laboratories), which were probed with rat anti-HoxB4 hybridoma supernatant (Developmental Studies Hybridoma Bank, Iowa, USA) overnight, followed by secondary anti-rat IgG antibody conjugated to HRP. Bound antibodies were detected using SuperSignal West Pico Chemoluminescent Substrate (Pierce, Rockford, IL, USA).

3.4.4 Histology and immunocytochemistry

As an indicator of mineralization within the hESC cultures, calcium deposition was analyzed by Alizarin Red S (AR) staining (articles II and IV). The calcium salt crystals within the bone-like nodules stained dark red, while the collagenous extra cellular matrix (ECM) turns yellow. It is important to distinguish between mineralizing nodules, and fibrous nodules, which also are three-dimensional structures, but do not mineralize. AR is often preferred to another staining method, von Kossa, which can detect calcium phosphate sediments within the cell culture. The synthesis of glycosaminoglycans (GAGs) was analyzed by Alcian Blue staining in article II, which

is a widely used method based on copper. The GAGs stain blue; however specificity can be manipulated by modifying the pH to selectively identify neutral, sulphated, and phosphated mucopolysaccharides. pH 2.5 is commonly used to detect GAGs within the cartilaginous matrix. Lipid droplets in developing adipocytes were stained with Oil Red O in article II. However, no positive signal was detected in osteogenically differentiated hESCs cultures under these experimental circumstances. For the detection of BSP and OCN, the cells were incubated with the HRP-conjugated secondary antibody, and the signal was detected with freshly prepared DAB (DAKO) solution activated with 0.1% H_2O_2. The sections were mounted with Pertex. Controls for primary and secondary antibodies revealed neither non-specific staining nor antibody cross-reactivity.
The degree of hESC differentiation was inspected by examining the expression of human Oct-4 (Chemicon), Nanog (ab21603, Abcam, www.abcam.com), Tra1-60 and SSEA1 (both antibodies provided by Mark Jones from the laboratory of Peter Andrews, The University of Sheffield, Sheffield, UK).

3.4.5 Colony forming assay (Article II)

The cells were treated as described above to obtain a single cell suspension, and the cell number was determined. Human hematopoietic progenitor assays were performed by plating single cell suspensions of EB cells into Methocult GF+ media (Stem Cell Technologies) consisting of 1% methylcellulose, 30% FBS, 1% BSA, 50 ng/ml stem cell factor, 20 ng/ml granulocyte-macrophage colony-stimulating factor, 20 ng/ml interleukine (IL)-3, 20 ng/ml IL-6, 20 ng/ml granulocyte colony-stimulating factor, and 3 units/ml erythropoietin. Cells were aliquoted in duplicate samples at a density $2.5x10^5$ alive cells per plate, and maintained at 37°C in 5% CO^2 for 15 days in a humidified atmosphere. After that differential colony counts were performed based on morphological characteristics. To identify specific cell types, individual colonies were aspirated from the plates, washed once in PBS and then resuspended in PBS containing 1% human albumin before cytospin. Slides were fixed in Methanol and stained with May-Grünewald/ Giemsa.

3.4.6 Teratoma formation and detection (Article III)

To analyze effects of HoxB4 expression on germ layer differentiation and pluripotency on modified hESCs *in vivo*, xenografting of unmodified and modified H9 cells into immunodeficient mice was perfomed.
Male C.B.-17/GbmsTac-scid-bgDF N7 mice (MTC, Karolinska Institutet, Stockholm, Sweden), six weeks of age, were kept under isolator conditions with access to water and food ad libitum. H9 cells passages 82/83 were harvested mechanically prior to implantation and $1x10^5$ cells were inoculated beneath the testicular capsule, as described (Gertow et al., 2004). The animals were sacrificed after eight weeks; the teratomas were fixed in 4% neutral buffered formaldehyde overnight, prior to dehydration through a graded series of alcohols to xylene. The tissues were embedded in paraffin, serially sectioned at 5µm and stained with Hematoxylin-Eosin. Normal non-injected testes served as controls.

3.4.7 Fourier-transform infrared spectroscopic analyzes (Article II)

The crystalline structures of the calcium phosphate deposits were analyzed by Fourier-transform infrared (FTIR) spectroscopy. This method was important in order to establish whether the deposited mineral resembled that of hydroxyapatite. We compared two hESC lines, HS181 and H9 after osteogenic induction. The cells were fixed, washed with Tris buffered saline (TBS), treated with the buffer containing 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, and 0.1% Triton X-100. The cells were centrifuged, the supernatant was removed, and then the cells were incubated 48h in 0.1 M Tris-HCl, pH 7.5 and 10 mM $CaCl_2$ containing 10 mg/ml non-specific protease at 55°C. The pellet was centrifuged again and washed with TBS. Thereafter, the mineral crystals were lyophilized and combined with dried spectroscopic grade potassium bromide in the ratio 1:200. The samples were resuspended in acetone and thoroughly dried. Spectra were obtained using a Thermo Nicolet Avatar 360 FTIR.
This method was performed in the laboratory of our collaborator Alastair Sloan.

3.4.8 Karyotyping and FISH analysis

To evaluate the genetic stability of our hESCs we generally performed Q-banding in at least 20 metaphases. It is suggested to test cells before, during and after the experiments.

Briefly, cells were cultured with 16ng Colchicine per ml medium (United States Biochemical Corporation, Ohio, USA) for 12h, washed and trypsinized for approximately 5min with TrypLE Express. Subsequently, cells were resuspended in PBS, centrifuged and then incubated for 10min in pre-warmed 0,56% KCl at room temperature, then centrifuged again and finally fixed in Methanol and acetic acid (mixed in ratio 3:1). After fixing, cells were spread out on glass slides and stained with Quinacrine mustard (Sigma-Aldrich) (Caspersson et al., 1972). Metaphase spreads were captured and analyzed using an Olympus BX60 fluorescence microscope connected to a Cytovision imaging system (Applied Imaging, Newcastle, UK). Karyotypes were defined according to the international system for human cytogenetic nomenclature (ISCN 2005).

To evaluate more cells and possibly quantify a certain genotype, fluorescence in situ hybridisation (FISH) analysis was performed. It is a useful technique to separate the male feeder cell metaphases from the female hESC metaphases, through staining of the sex chromosomes. For that, slides were de-paraffinated in xylene, and then treated with proteinase-K for 50min. Before the probe (Vysis XY, Abbott Molecular Inc, Des Plaines, USA) was applied, the slides were de-hydrated in alcohol series and air-dried. Preparation and probe were simultaneously denatured in a HYBrite (Abbott-Vysis) at +73ºC and hybridized at +37ºC for 15h. Post hybridization washes were done in 0.4xSSC/0.3% Igepal CA-630 (Sigma Chemical Co, MO,USA) at +72ºC, 2min and then cooled at room temperature for 30s in 2xSSC/0.1% Igepal CA-630. Slides were air dried protected from light and then mounted in VectaShield antifade solution (Vector

laboratories, Burlingame, Ca, USA). Analysing was done on an Olympus fluorescence microscope BH60 with the appropriate filterset equipped with a CCD camera and connected to a CytoVision image analysis system (Applied Imaging Corp, Ca, USA), in which the results were documented. Alternatively, the human chromosome X probe was labeled with cyanine 3 (Cy3) (CAMBIO, Cambridge, United Kingdom) and the Y probe with biotin (CAMBIO, Cambridge, United Kingdom).

Experts in our haematology lab, Birgitta Stellan, Monika Jansson and Ann Wallblom, helped with and performed most of the karyotyping and FISH procedures explained above.

3.5 ETHICAL PERMISSIONS

The experiments conducted in this thesis were covered by the following ethical permissions; Dnr 402/99, Dnr 114/00, Dnr 514/00, Dnr S-172/03, Dnr N-105/07 and Dnr 151/00

4 RESULTS AND DISCUSSION

4.1 GENETIC MODIFICATION OF HESCS USING LENTIVIRAL VECTORS

4.1.1 Effective genetic marking to trace cells in mixed cell populations

In article I we established a new lentiviral vector system, to mark hESCs with EGFP. We decided to use a SIN-lentiviral vector provided by the lab of David Baltimore (California Institute of Technology, Pasadena, USA). This vector includes the EGFP gene under the control of the human Ubiquitin-C promoter. The Ubiquitin-C promoter has been previously applied in mESCs (Lois et al., 2002), with reliable expression in a variety of differentiating cell types. At the time, few publications had used lentiviral vectors to express GFP in hESCs (Gropp et al., 2003; Ma et al., 2003; Pfeifer et al., 2002) and our work was the first to show stable lentiviral-transgene expression from the human Ubiquitin-C promoter in hESCs and their derivatives (Article 1).

Labelling with EGFP enabled an easy identification of hESC and their derivates in culture.
Similarly, to positively identify the feeder cells a retroviral vector with the RFP gene was used to transduce a human foreskin feeder cell line.

4.1.2 Shedding light on interactions between human feeder cells and hESCs

Thus, a combination of EGFP labelled hESC and red fluorescence protein (RFP) labelled feeder cells could be used to evaluate parameters for keeping undifferentiated hESC cultures. Detection of green cells located outside the borders of hESC colonies was interpreted as an indication of differentiated migrating hESC and thus suboptimal culture conditions.

The hESCs, although seeded on top of the feeder cells, were found to pushing the fibroblast cells aside, and attaching to the underlying matrix. The occurrence of fusion between feeder cells and hESC did not seem to be significant, as indicated by the extremely low detection of cells co-expressing EGFP and RFP.

We found that while mitotically inactivated (by irradiation) human feeder cells re-attached in subsequent cell culture passages (6,5% after the first passage), low amounts of feeders could still be detected after three passages (0,5%). This is important knowledge when evaluating the stable gene-modification of hESCs, whilst they are kept on feeder cells at the time of transduction. Transduction of hESCs with lentiviral vectors would transduce also many of the surrounding feeder cells, and thus hESC should be kept under feeder-free conditions at the time of transduction.

4.1.3 Gene marking can reveal growth and differentiation behaviour of cell populations expressing a transgene

In articles III and IV, we over-expressed the HoxB4 transcription factor together with EGFP in bi-cystronic vectors. This type of constructs enables the tracing of transgene over-expressing cells in mixed populations by following their EGFP-expression induced from the same RNA.
In article III, we followed the behaviour of HoxB4-overexpressing cells side by side with non-modified cells during EB differentiation. Although a transient increase of these cells was seen in the beginning of their differentiation, they were slowly subsequently lost. The finding of a slow loss of modified cells was found to be reproducible and strong for two separate hESC lines. The EGFP from the control vector population did not decrease during EB differentiation, which suggests possible transgene-silencing a less likely reason for the loss of EGFP expression. The induced HoxB4 expression, which appears to have contributed to this limited expansion in EBs is discussed in the section on HoxB4 and hematopoiesis (4.3).

4.1.4 Effects from various transgene expression levels

We compared the effects of transgene expression dosage on the differentiation of hESCs, in articles III and IV. In article III, we chose to construct two different lentiviral vectors with the aim to achieve separate levels of transgene expression. We found a lower expression if the fusion gene was longer and contained more gene elements. Although this is not surprising to find that a more complex fusion gene is expressed lower, the main contributing factor to this difference in transgene expression in the hESCs could be most likely attributed to a variation in the number of insertions per cell. Therefore, in article IV only one vector was used for the over-expression and we selected cell populations from different transductions with different expression levels. In both papers it would have been valuable to identify the transgene copy numbers. However, the polyclonal character of our populations makes it difficult to verify this on the population level.

4.1.5 Conclusion

We conclude that lentiviral vectors with an internal Ubiquitin C promoter are a good means for efficient and stable transgene expression in hESCs. Together with a marker gene, cells can easily be followed to evaluate cell behaviour. Our data highlights a dose-dependent effect of the tested transgenes in differentiating hESCs, illuminating the strong need for analysing the expression level and correlating this to the resulting phenotype. Considering this, a need for regulated transgene expression becomes evident, requiring the use of inducible and quantifiable systems.

4.2 **BLOOD AND BONE DIFFERENTIATION FROM HESCS**

The derivation and establishment of hESCs gave us the possibility to model parts of human mesodermal development *in vitro*. Differentiation to bone producing cells was

studied in articles II and IV, whereas early hematopoiesis was the focus of article III and contributed to article IV.

4.2.1 Osteoblastic differentiation

In article II, we evaluated the capacity of several hESC lines to differentiate to an osteoblastic phenotype. Three hESC lines derived and cultured on human feeder cells and H9 hESCs derived and expanded on mouse feeders were differentiated comparing two methods. First by plating them in a monolayer on gelatine-coated wells, and second, to initiate their differentiation as EBs during the first five days, and after that seed them on the gelatine coated wells. Cells were allowed to differentiate in the presence of Dex, ascorbic acid and β-GP.
We followed marker expression from early mesoderm to fully differentiated osteoblastic cells.
hESCs differentiated in a monolayer or EB-derived, showed a similar induction of early mesoderm expressing T-Brachyury, Flt1 and BMP4. However, in EB-derived cultures T-Brachyury declined faster in H9 than in HS181. Immunohistochemical stainings against BMP4 also showed that the signal was localized to areas that eventually become bone-like nodules. Screening for osteoblast-specific gene expression, the bone-matrix markers were detected in all cell lines and in both monolayer and EB-derived cultures after 25 days of differentiation. However, it was apparent that in three of four cell lines it was the monolayer cultures that expressed a higher degree of the bone-specific marker set. AR staining demonstrated calcium deposition within the mineralising ECM and this was further confirmed by FTIR. The spectroscopy profiles showed that the deposited mineral resembled a poorly crystalline biological calcium hydroxyapatite

4.2.2 Hematopoietic differentiation

In article III, we specifically aimed to improve early hematopoiesis of human embryonic stem cells by the over-expression of the homeobox transcription factor HoxB4. We used a basic EB-based differentiation method, known to induce hematopoietic cells from the studied H9 cells. Without the use of extra cytokines in the differentiation medium, we obtained a low base line differentiation to $CD34^+$ cells (<2% from which 50% were $CD34^+/CD31^+$) that also translated into typical colony-forming units in a standard hematopoietic colony-forming assay.

4.2.3 Conclusion

We established a differentiation protocol to the osteoblastic lineage with our cell lines and proved their character with a wider marker set than previous studies. Furthermore, we found monolayer differentiation equally effective and therefore continued with this method in article IV. Hematopoietic differentiation of unmodified hESCs was established in article III and provided a basic experimental set-up in order to evaluate positive transgene effects on hematopoiesis.

4.3 TRANSCRIPTION FACTORS AS REGULATORS OF DIFFERENTIATION

In context of the two established mesodermal differentiation models, evaluation of gain-of function of single transcription factors was the focus of our further studies (articles III and IV).

4.3.1 Overexpression of transcription factors to increase hematopoiesis

In article III, we aimed to increase and direct the outcome of hematopoietic precursor cells appearing during EB-differentiation by over-expression of the HoxB4 transcription factor. At the time of our study, HoxB4 had been reported to increase expansion and maturation of mESC-derived and adult HSCs. Accounting for a possible dose effect for transcription factors, we constructed lentiviral vectors that expressed differential levels of HoxB4. HoxB4 was stably integrated in hESCs and expressed on two different levels. Consistent with the undisturbed expansion over more than 20 passages, no effect on pluripotency markers SSEA3 and Oct4 was noticed. Seemingly no HoxB4 target genes are available in undifferentiated cells and therefore, make it possible to expand modified cell populations over long time periods undisturbed.

Ubiquitin C promoter driven transgene expression increased during cell differentiation

We induced stable HoxB4 expression on two different levels and studied the behaviour of our vectors during germ-layer transmission. We then followed their expression throughout differentiation, and uncovered a steady increase of transgene expression reported in article III, and was confirmed in article IV. In both studies the same promoter was used to drive transgene expression, and thus we concluded that the Ubiquitin-C promoter activity increased in more mature cell types.

Hematopoietic characterization of HoxB4 effects

Analysis of hematopoietic markers during EB-differentiation revealed distinct changes in cells expressing high or low HoxB4 levels. The number of $CD34^{+}$ hematopoietic progenitor cells was significantly increased if HoxB4 expression was high. This increase was seen up to day 14 of EB-differentiation and dropped at day 21. No change was noticed if HoxB4 was expressed on a lower level. CD34 is not a specific marker for hematopoietic stem cells alone, therefore we analysed a wider collection of surface and intracellular markers to define the development of the cells. We followed CD117 and CD133, which are both expressed partially in hESCs and are found on HSCs. However, we could not find a significant difference between EGFP, $HoxB4^{low}$ and $HoxB4^{high}$ cells. Analysis of markers of more differentiated hematopoietic cells revealed an increase in percentage of cells expressing CD38, although an expected increase of the pan-hematopoietic marker CD45 did not occur. This would have indicated a normal hematopoietic differentiation route initially expected from the increased $CD34^{+}$ numbers in $HoxB4^{high}$ cells. Concurrent with the lack of $CD45^{+}$ cells, we could not detect a higher number of blood colony-forming cells in standard methylcellulose assays.

Additionally analysing the gene expression pattern of these cells, the early hematopoietic transcription factor SCL was upregulated, but the later erythroid transcription factor Gata1 did not change significantly.

HoxB4 effects on other cell types

Instead of later hematopoietic markers, we could however detect a strong up-regulation of the vascular endothelial (VE)-cadherin. Furthermore, a high HoxB4 expression was found to prevent normal teratoma formation of *in vivo* differentiating hESCs. Separately found in article IV, increased HoxB4 lead to an increase of osteoblast markers during osteogenic differentiation.

In summary, only the HoxB4^{high} H9 cells were showing an increased early hematopoiesis, while HoxB4^{low} cells were not. Although early hematopoietic markers were upregulated in HoxB4^{high} cells, markers for late blood maturation were absent. Besides the initial difference in HoxB4 expression, also the constantly increased availability in more differentiated cells, might have prevented CD34^{+} cells from normal blood cell maturation. Endogenous HoxB4 is known to be downregulated in differentiating blood cell populations and the forced expression in more developed cell types may direct cells into other lineages. Interestingly, we could detect HoxB4 expression in umbilical cord endothelial cells (article IV), which together with the HoxB4-induced endothelial VE-cadherin expression may indicate some HoxB4 function in endothelial development. In context with the *in vivo* data obtained, HoxB4 expression affects not only hematopoietic cells, but presents a much wider target range. This is in line with recent reports revealing a high number of HoxB4 target genes.

4.3.2 Over-expression of transcription factors to increase osteoblast differentiation

In article IV, we aimed to evaluate whether the over-expression of the bone-specific transcription factor Osx could enhance hESC differentiation towards osteoblasts. The transcription factor Osx has been identified as a crucial regulator of osteogenesis and is predominantly expressed in early osteoblastic cells. We applied a similar lentiviral vector as in articles I and III, to drive the expression of an Osx-fusion gene from the human Ubiquitin-C promoter. Additionally, this lentiviral vector also contained a chemical selection marker, enabling easier assortment for gene-modified cells. In this study (IV), we also included the over-expression of HoxB4 that we found previously to effect more than hematopoietic development. However, the differentiation conditions were aimed to differentiate towards the osteoblastic lineage as established in article II.

Possible effects of transcription factors on hESC expansion

For the first time we detected an effect of the transgene over-expression on some of the pluripotency markers (SSEA3 and Nanog), which was not reported in the previous study with HoxB4. A partial loss of these markers was seen in Osx-over-expressing cells, which was also reflected by noticable differentiation during every day cell culture. However, these effects were variable and could not be confirmed at all times, as shown by standard immunocytochemistry on modified hESCs colonies (article IV).

Furthermore, no difference in proliferation was noted for cells with or without transgenes. We concluded that a partial explanation for this variability might be the outgrowth of different cell clones from our initial polyclonal population.

Osteogenic characterization of Osx effects

To evaluate effects of transgene expression during osteogenic differentiation we first selected transduced cells that expressed significantly different RNA levels of the transgenes. Therefore, we intended to account for the dose-dependent effects found in article III. In similarity to article III we also found the expression of transgenes increased during differentiation, and initially different Osx expression levels resulted in different developmental behaviour. We showed that low expression of Osx, and not high amounts induced an upregulation of mineralization-associated mRNAs, such as collagen I, BSP and OCN, also confirmed by western blot. In addition these low amounts of Osx also increased calcium deposition, shown by AR staining, suggesting that functional osteoblasts had formed. Interestingly high levels of HoxB4 induced osteoblastic markers, possibly indicating a role of HoxB4 during pathological mineralization, such as found in blood vessels.

Unexpectedly, within the osteogenic monolayer environment, the high Osx expression increased endogenous HoxB4 and the number of $CD34^+$ cells, which suggests an increase in hemato-endothelial development. Furthermore, an increase of the later hematopoietic transcription factor Gata1 was detected. The effect on CD34 expression seemed to be density dependent, which adds another regulatory element effecting cell fate decisions.

In summary, the action of the Osx transcription factor showed a similar dose-dependent effect as that observed with the transcription factor HoxB4. For enhanced osteogenesis, it is apparent that the expressed levels of protein are important and need to be evaluated to ensure differentiation towards the required cell type.
In general, higher levels of transgenes showed a tendency to influence development of other lineages. High HoxB4 expression increased osteogenesis whereas high amounts of Osx increased hematopoiesis, while in both cases lower levels affected their initially anticipated lineage. Furthermore, our findings support the notion of possible cell-cell interactions between pre-osteoblasts and HSCs, as found on the bone marrow endosteal surface.

4.3.3 Conclusion

With the current methodology being stable over-expression, effects on cells are difficult to predict and need to be evaluated for each vector and cell type. In addition to effects on hematopoiesis or bone development, the transcription factors also affect other cell types, which may depend on its developmental state and microenvironment. There is a wide variety of functions worth to be defined in further studies and emphasizes the regulatory role of transcription factors such as HoxB4 or Osx.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 CONCLUSIONS

The results in this thesis have emphasised the potency of hESCs as model system for research as well as a potential source for cell therapy. Testing the notion that hESC differentiation may reflect human development demands the recapitulation and understanding of a complex molecular environment. It is concluded that a use of genetically modified hESCs can facilitate the evaluation and constitute a means for studies of the effects of important genes upon differentiation and development.

Specific conclusions are as follows:

- Lentiviral vectors with an internal Ubiquitin C promoter are a good means for the efficient and stable transgene expression in hESCs. Together with a marker gene, cells can easily be followed to evaluate cell behaviour.

- Several hESC lines are able to undergo osteogenic differentiation as shown from their osteogenic marker profile. However, their capacity and temporal behaviour appears to be dependent on the specific cell line. An initial embryoid body formation step seems not necessary to induce bone differentiation. The deposited mineralized tissue detected in this osteogenic model resembles that formed by cell-mediated mineralization as found in the *in vivo* situation.

- Stable HoxB4 over-expression, through a wide range of differentiating hESCs, can be achieved using lentiviral vectors driving gene expression from the human Ubiquitin C promoter. Our data highlights a dose-dependent effect of HoxB4 during early human hematopoiesis; however, the dose established in our study did not allow for the final maturation of blood cells. In accordance with the *in vivo* data obtained, HoxB4 affects not only hematopoietic cells, but presents a much wider target cell range.

- Over-expression of the bone-specific transcription factor Osterix demonstrates a dose-dependent behaviour during development. Low levels of Osx resulted in increased osteogenic differentiation, whilst higher amounts increased hematopoiesis. Interestingly, we also found the converse is true with HoxB4, in that high levels of HoxB4 increased osteogenesis, while low levels increased hemato-endothelial markers. For an enhanced osteogenesis, it is apparent that regulated levels of the protein are important and should be evaluated if differentiation to a certain cell type shall be achieved.

In summary, we found that the amount of transcription factors available in developmental cells is an important regulatory element. With stable gene expression, cells can be efficiently marked and their behaviour could be evaluated. However, a need for regulated transgene expression of at least some transcription factors becomes

evident, and future studies should ideally include the use of inducible and quantifiable systems.

5.2 FUTURE PERSPECTIVES

Taking into account the results from our studies it is very clear that the expression level of transcription factors should always be considered for each vector, cell type and developmental stage. Unphysiologically high expressions of transcription factors are likely to destroy their regulatory capacity or cause unpredictable cell behaviour. Also very small changes in availability of a specific transcription factor may start a cascade of downstream events and thus affect cell behaviour. Few studies have so far addressed the dose-dependent functional effects of transcription factors, and further research is needed.

In order to get a further grip on transcriptional regulation operated by transcription factors, such as HoxB4 or Osterix, target genes and regulators need to be identified. The sheer number of targets requires high throughput technologies to get an overview and identify key genes responsible for specific cell functions. However, it is most likely that a whole set of genes or signals are required for adequate regulation of cell differentiation into specific cell types. Current knowledge indicates that in the field of hematopoietic differentiation of hESCs, several steps are needed to efficiently mature the correct cell type. As presented herein, the over-expression of HoxB4 seems to be only transiently helpful and should rather be silenced at later blood cell stages.

Technology development

With the development of inducible and targeted gene transfer technologies, in future studies dose effects could be easier addressed. In order to reduce risks associated with integrating viral vectors, such as insertional mutagenesis or cancerogenous expansion of modified cells, vector/ transgene removal after treatment would be a valuable construction feature. Also delivery of recombinant transcription factor proteins would be a safe and versatile strategy. However, this would require extensive knowledge on necessary protein alterations to allow correct secretion and uptake into target cells. Also methods of efficient *in vivo* delivery would be an important area for extensive research. The use of the hESC model systems now provides experimental access to the earliest human cell decisions.

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