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Directed differentiation of HESCs: A model for early bone development

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Directed differentiation of human embryonic stem cells: A model for early bone development

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DIRECTED DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS: A MODEL FOR EARLY BONE DEVELOPMENT

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To my family

ABSTRACT

Research in stem cell biology is an important and necessary requirement for the better understanding of cell differentiation and formation of tissues, while also contributing to the field of regenerative medicine. The establishment of human embryonic stem cell (HESC) lines offers the potential to study the earliest developmental processes and provides an unlimited source of cells which can be used for the differentiation into functional osteoblasts. Bone matrix production and mineralization are guided by complicated mechanisms that differ from other tissues in many ways. There is the initial formation of an organic extracellular matrix (ECM) into which inorganic hydroxyapatite crystals are later deposited. Our first study investigated the molecular processes that occur pre- and post-mineralization within the primary ossification centre during early bone formation using global gene expression analysis. We then continued investigating the osteogenic differentiation potential of several HESC lines. Novel to our studies was the use of commercially available human foreskin fibroblasts to support the undifferentiated growth of the HESC colonies and their propagation in serum-replacement containing culture medium. Two different approaches to differentiate HESCs into the osteogenic lineage were evaluated. Firstly, undifferentiated cells were cultured in suspension, facilitating the formation of embryoid bodies (EB), and secondly in monolayer; both methods were in the presence of osteogenic supplements. Characterization of the osteogenic phenotype revealed that all HESC lines differentiated towards the osteoblastic lineage, demonstrating also that EB formation is not necessary for the initiation of osteogenic differentiation. Mineralization of the ECM occurred through a cell-mediated calcification process. Study of the expression profile of bone-associated genes revealed that the HESC model differs from the standard osteogenesis model, which has been characterized by osteoprogenitor cells. In the redefined model there is first the general cellular proliferation and secretion of pre-maturational matrix stage that is needed for cell migration, and second, the appearance of osteoprogenitors with characteristic ECM synthesis. A gene modification approach to enhance potential osteoblastic differentiation was employed in the fourth and final study. We found that for enhanced osteogenesis originating from *in vitro* cultured HESCs, the correct levels of ectopic transcription factors need to be established. Our data adds additional confirmation of a close relationship between early blood and bone development.

LIST OF PUBLICATIONS

I. Sugars RV., **Kärner E.,** Petersson U.,Ganss B., Wendel M. Transcriptome analysis of fetal metatarsal long bones by microarray, as a model for endochondral bone formation. (2006) *Biochim Biophys Acta.* Oct;1763(10):1031-9.

II. **Kärner E.,** Unger C., Sloan AJ., Ährlund-Richter L., Sugars RV., Wendel M. Bone Matrix Formation in Osteogenic Cultures Derived from Human Embryonic Stem Cells In Vitro. (2007) *Stem Cells Dev.*, Feb;16(1):39-52.

III. **Kärner E.,** Bäckesjö C-M., Cedervall J., Sugars RV., Ährlund-Richter L., Wendel M. Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. *Submitted.*

IV. **Kärner E.*,** Unger C.*, Cerny R., Ährlund-Richter L., Ganss B., Dilber S., Wendel M. * Authors have contributed equally to this study. Differentiation of human embryonic stem cells into osteogenic or hematopoietic lineages: a dose-dependent effect of Osterix over-expression. *Submitted.*

CONTENTS

LIST OF ABBREVIATIONS

AA	Ascorbic acid
ALP	Alkaline phosphatase
AR	Alizarin Red S staining
β-GP	β-glycerophosphate
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
Cbf	Core-binding factor
Dex	Dexamethasone
EB	Embryoid body
EGFP	Enhanced green fluorescent protein
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Flt	Fms-like tyrosine kinase
FTIR	Fourier-transform infrared
GAG	Glycosaminoglycan
GSK	Glycogen synthase kinase
HESC	Human embryonic stem cells
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HoxB4	Homeobox B4
HSC	Hematopoietic stem cell
ICM	Inner cell mass
IGF	Insuline-like growth factor
IL	Interleukin
IVF	*In vitro* fertilization
JAK	Janus kinase
KO-SR	Knockout serum replacement
LIF	Leukemia inhibitory factor
LRP	Low density lipoprotein receptor-related protein
M-CSF	Macrophage colony-stimulating factor
MEF	Mouse embryonic fibroblasts
MSC	Mesenchymal stem cell
NC	Neural crest
NCP	Non-collagenous glycoproteins and proteoglycans
NF-κB	Nuclear factor kappa B
OCN	Osteocalcin
Oct-4	Octamer binding protein-4
ON	Osteonectin
OPN	Osteopontin
OSAD	Osteoadherin
OSX	Osterix
PI3K	Phosphoinositide kinase-3
PK	Protein kinase
RA	Retinoic acid
RANK	Receptor activation of nuclear factor kappa B
RANKL	RANK ligand
RC	Fetal rat calvaria-derived cells
ROCK	P160-Rho-associated coiled-coil kinase
RT-PCR	Reverse-transcriptase PCR
Runx	Runt-related factor
SCID	Severe combined immunodeficient
SIBLINGS	Small Integrin Binding Ligand N-linked Glycoproteins
Sox	SRY (sex determining region Y)-box
Sp	Specificity protein
SPARC	Secreted protein, acidic, rich in cysteine
SSEA	Stage-specific embryonic antigen
STAT	Signal-transduced and activator of transcription
TC	Tetracycline
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRA	Tumour recognition antigen
VEGF	Vascular endothelial growth factor
vitD3	1,25-dihydroxy vitamin D3
Wnt	Wingless

1 INTRODUCTION

Stem cells serve as a fundamental source for tissues throughout the life of every organism. They provide the body with cells for replacement during growth, and are responsible for regeneration following disease or injury. Such cells are found not only during early development, but also in the adult body. Research into stem cell biology is likely to provide useful information to applications such as tissue replacement and drug screening.

1.1 STEM CELLS

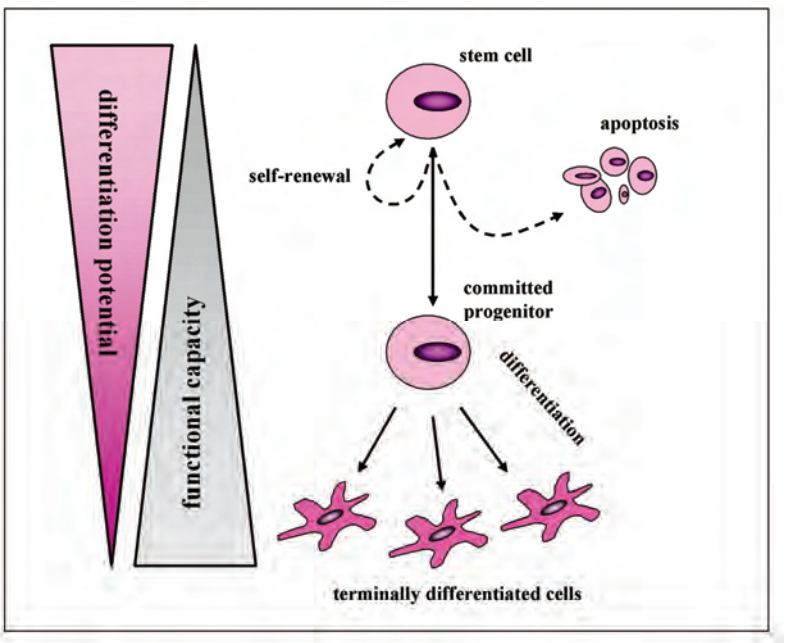
Stem cells are able to differentiate into other types of cells of the organism, and in addition stem cells possess the ability to self-renew. All developing tissues retain cells with stem cell properties; however whether this is the case throughout the entire adult body remains to be clearly demonstrated. Certain tissues, such as skin, muscle, and the hematopoietic system are capable of renewal, although recent medical research demonstrates that tissues previously believed to be non-regenerative, such as brain and heart may possess similar properties [1, 2].

Developmental potency is a functional characterization of stem cells, and does not necessarily describe the range of genes expressed by the cells, their origin and whether they represent an endogenous cell type in the organism. The potency may be revealed experimentally *in vitro*, by **i)** forming aggregates in suspension culture, **ii)** *in vivo* within a teratoma following injection into immunocompromised mice, and **iii)** within an embryo that has had pluripotent cells injected into the blastocyst and results in the birth of chimeras [3]. Different types of stem cells exist, depending on the ability to maintain stem cell–like properties and the variability of derivatives that they give rise to. **Unipotent stem cells** undergo self-renewal and are able to generate only one mature cell type. **Multipotent stem cells** give rise to two or more differentiated cell types. A large number of multipotential cells exist; an example is seen in the adult organism, and during early development, where tissues in the nervous system contain neural crest (NC) stem cells and neural stem cells. Hematopoietic stem cells (HSCs) give rise to lineage restricted stem cells, which can further differentiate into numerous blood cell types [4]. HSCs, together with mesenchymal stem cells (MSCs) reside in the bone marrow. Bone-producing cells, osteoblasts, originate from MSCs along with adipocytes, muscle cells and chondrocytes [5]. The hallmark of **pluripotent stem cells** is the potential to give rise to the representatives of the three germ layers; endoderm,

mesoderm, and ectoderm. This is determined using cell type specific molecular markers, morphological criteria, and functionality. Three types of pluripotent stem cells have been described so far; **i)** embryonic germ (EG) cells of the gonads of a post-implantation embryo, **ii)** embryonal carcinoma (EC) cells, originating from tumorigenic germinal tissue, and **iii)** embryonic stem cells (ESCs) [6]. Recently, new human pluripotent cell lines were induced through a process of reprogramming somatic cells (iPS) [7-9].

Moreover, despite on the above mentioned definitions of pluripotency and multipotency, it is clear that cells with intermediate potencies could exist, for example, the existence of mesoangioblasts has been suggested [10].

Figure 1. A stem cell is able to self-renew and give rise to several differentiated cell types that take on more specialized functions.



1.2 EMBRYONIC STEM CELLS

Embryonic stem cells (ESCs) are by definition derived from early embryos. They are apparently self-renewing cells under *in vitro* conditions while maintaining the potential to give rise to the majority of cell types found throughout the whole body. Intact embryos do not normally maintain proliferation of pluripotent undifferentiated cells, meaning that ESCs could be considered *in vitro* culture artifacts. The mechanisms of ESCs indefinite self-renewing capacity remains incompletely understood, however

within the *in vitro* environment the self-renewal of ESCs is usually obtained by culturing the cells on supportive layers or matrices, and supplementing with certain growth factors to the medium.

Although ESCs are generally derived from the inner cell mass (ICM) of developing blastocyst-stage embryos, they are not directly equivalent to these cells. Isolation of ICM cells, establishment of ESC lines and basic cell culture techniques that we have today facilitate forced selection of these cells. Furthermore, it is incorrect to assume that the ICM cells are the direct precursors of ESCs. Colonies of ESCs differ from the ICM cells in many ways, for example ICM cells retain a memory of axes that enables the cells to have positional relationships [11]. The early stages of ICM growth are extremely vulnerable to cell microenvironment and culture conditions [12]. Thus it is highly possible that during *in vitro* maintenance, the original ICM cells actually give rise to other types of precursor cells. Surprisingly, the origin of ESCs has not been completely clarified after more than 20 years since the first derivation. Some experiments suggest that ESCs closely resemble primitive ectodermal cells [13], whereas others report the close relationship to early germ cells [14]. Moreover, considering that ESCs are derived from the inside of a blastocyst, they are still able to give rise to primordial germ cells [15], and extra-embryonic derivatives [16].

Mouse ESCs were first derived in 1981 using the culture conditions previously described for mouse EC cells, where these cells were mechanically isolated from the ICM of mouse blastocysts [17, 18]. Interestingly, the efficiency in deriving mouse ESCs is strongly affected by the genetic background. Experiments with different mouse strains have demonstrated that mouse ESCs can be easily derived from the inbred mouse strains, particularly 129/ter-Sv, but also C3H/He, while other strains can be less efficient [19, 20]. However, differences in the efficacy of ESC derivation from various mouse strains might have been caused by the suboptimal culture conditions. Indeed, mouse ESCs were successfully derived from some non-permissive strains implementing a continuous removal of differentiated cells by drug selection or modifying the culture with other types of feeder cells, and adding the cytokine leukemia inhibitory factor (LIF) [13, 21].

1.2.1 Derivation of human ESCs

Usually blastocyst-stage embryos with the number of cells and morphology that is appropriate to their age are transferred to the patient in human fertility clinics. To evaluate the quality of the blastocyst, various scoring systems are used. The general

strategy is based on the morphological grading criteria of blastocyst, ICM and trophectoderm used in IVF (*in vitro* fertilization) treatments and described by Gardner and co-workers [22, 23]. Typically, it is the donated low quality blastocyst-stage embryos that are available for the derivation of pluripotent human ESCs (HESCs).

The *in vitro* culture of isolated ICMs from human blastocysts was first reported in 1994 [24], however these cells were kept only for a couple of passages. It was not until 1998 that the first derivation of a HESC line from the ICM of a blastocyst was published [25]. The developmental stages and morphological characteristics of the embryos used to generate the first HESC lines were not well documented [25-27]. Generally, embryos lagging behind in normal development, with poor morphology, or blastocysts without a distinct ICM were discarded by IVF clinics because they lacked full developmental potential. However, such embryos have been used for the establishment of new HESC lines [28, 29]. Recently, it was demonstrated that embryos, which arrested in early development or were highly fragmented seldom yielded cell lines, whereas those that had achieved the blastocyst stage were a good source of normal HESCs [29]. It must be noted that derivation of HESC lines has not followed a common uniform procedure among different laboratories. Moreover, the culture and manipulation of HESCs differs considerably between laboratories and pose several unique challenges. Although similarities in marker expression were observed, different cell lines have a distinct human leukocyte antigen (HLA) profile and blood antigen types O, A and B [30]. Other variabilities among different HESC lines have been reported by several groups, including differences in growth characteristics, differentiation potential, karyotype and gene expression pattern. In fact, such differences might reflect the genetic heterogeneity of the derived HESCs lines, as they are from a genetically diverse, outbread population [31, 32]. Large international networks, such as ESTOOLS (www.estools.org) in Europe, are now formed to compare and share experiences in the HESC research field, and recently 59 HESC lines from 17 laboratories were compared by The International Stem Cell Initiative [33].

To date HESCs have been derived from variety of sources, including earlier morula-stage embryos [34, 35], single human blastomeres [36], and later blastocyst-stage embryos [37]. It is also possible to obtain disease-specific HESCs from embryos with diagnosed mutations by preimplantation genetic diagnosis [38], and such cells could be extremely valuable to study small molecular changes that are characteristic to disease phenotypes.

Interestingly, derivation of two HESC lines in defined conditions were reported [39]. Unfortunately, one developed trisomy 12 and the other had a XXY karyotype. For these lines, a feeder-independent HESC culture system was employed and protein components solely derived from recombinant sources or human material were used. This study described for the first time the now widely used TeSR1 medium (containing basic fibroblast growth factor (bFGF), lithium chloride (LiCl), γ-aminobutyric acid (GABA), pipecolic acid and transforming growth factor beta (TGFβ), and established that the optimal *in vitro* conditions for HESCs are 10%CO_2/5%O_2, and pH of 7.2.

1.2.2 Maintaining undifferentiated HESCs

Undifferentiated HESCs possess a **distinct morphology** when viewed under the light microscope. Individual cells contain a large nucleus, prominent nucleoli and a cytoplasm of relatively small ratio. The undifferentiated cells appear as a tightly packed monolayer [40], forming a colony with a defined border at the periphery. HESC cultures are often heterogenous as they they contain both undifferentiated stem cells and spontaneously arising differentiated derivatives. The single colonies are often surrounded by differentiated cells that appear stroma-like [41] or fibroblast-like [30]. In addition, if HESCs are grown in feeder-free conditions, the HESCs can differentiate into fibroblast-like cells, which surround the undifferentiated cells [12].

Once established, HESCs display an almost unlimited proliferative capacity while maintaining their developmental potential. The long-term stability of HESCs is an important issue and a specialized growth environment is required to retain an undifferentiated phenotype. However, a number of alternative methods exist for the *in vitro* culture of HESCs, and several reviews and protocols have been published regarding the propagation and maintenance of undifferentiated HESCs [42]. HESCs require a growth medium with specific properties to maintain the undifferentiated state. A chemically-defined medium was shown to maintain the characteristic expression of HESC-specific markers, where the cells retained their characteristic morphology, and possessed a normal karyotype *in vitro*, as well as developed teratomas [43]. The **propagation medium** usually contains Knockout Dulbecco´s Modified Eagle Medium (KO-DMEM), approximately 20% commercially available Knockout Serum Replacement (KO-SR), 2mM L-glutamine or its stabilized form GlutaMAX™ (www.invitrogen.com), 0.1μM non-essential amino acids and 0.1μM β-mercaptoethanol. Various concentrations of bFGF have been used successfully to sustain undifferentiated HESCs [44-46]. Even though fetal bovine serum (FBS) is still

used, the use of a defined serum substitute in HESC medium is preferred. KO-SR (patent WO 98/30679) is better defined than FBS, but it must be recognized that it is a proprietary product that cannot be regarded as fully defined [47] and includes proteins like transferrin, which are likely to be from animal sources. This is an important issue, not only for establishing consistent research standards, but also for the eventual development of cell therapies.

In vitro, the first pluripotent EC cells or ESCs were cultured on **feeder cells** or in media conditioned by the cells [18]. The exact biochemical identity of feeder cells remains unclear, however they contribute various factors essential for the maintenance of HESC pluripotency. Interactions, by means of growth factors, cell-surface molecules, the extracellular matrix (ECM), or neutralizers of toxic metabolites produced by the stem cells themselves, exist between HESCs and feeders. As a rule, feeder cells are mitotically inactivated using irradiation or mitomycin C prior to culture with the HESCs. Dissimilarities between HESCs grown on irradiated or mitomycin C-treated feeders have not been reported.

Mitotically-inactivated mouse embryonic fibroblasts (MEFs) have been used successfully to support the growth and maintenance of HESCs. Even medium, which is conditioned by co-culture with fibroblasts is known to sustain HESCs. Several groups have reported that certain human cell lines are capable of supporting the growth and maintenance of undifferentiated HESCs, and changing the type of feeders does not affect the state of HESCs. HESCs can be adapted to cell types other than MEFs including human muscle cells, adult fallopian tubal epithelial cells, adult marrow cells, foreskin fibroblasts, human uterine endometrium cells, breast parenchyma cells and fetal fibroblasts [26, 48-51]. Feeder cells derived from HESCs, as an autogenic system efficiently support the growth and maintenance of pluripotency of HESCs [52, 53]. However, the morphology of HESC colonies grown on human fibroblasts layers was described as slightly different from the ones cultured on MEFs. The cells tended to organize according to the direction of the human feeder layers and the colonies were not so round [54].

In addition to conventional feeder-based cultures, **feeder-free systems** have been established. The very first report of successful culture of HESCs in feeder-free conditions used MEF-conditioned medium and the cells were cultured on Matrigel and laminin coated plates [41]. Matrigel is a basement membrane preparation extracted from a murine Englebreth-Holm-Swarm sarcoma, and conditioning with FBS or KO-

SR containing medium on fibroblasts reduces its bone morphogenetic protein (BMP) signaling activity. However, this method still requires expansion of MEFs for the production of the conditioned medium. In addition, as often is described, the use of MEF-conditioned medium may still expose the HESCs to pathogen transmission and viral infection, such as mouse retroviruses. Thus, methods describing totally cell-free and even serum-free systems for HESC lines have been established [12]. HESCs cultured with animal cells or serum products express Neu5Gc, a non-human sialic acid that would be immunogenic if used for human transplantation [55]. Recently, a study demonstrated that HESCs cultured in serum-free conditions acquired the bovine apolipoprotein B-100 from feeder cell layers and KO-SR [56].

Several alternative methods exist for the culture of HESCs. For maintenance of self-renewal, the HESC colonies are routinely **passaged** by dissociating them and replating onto new tissue culture plates. Enzymatic dissociation with trypsin solution (0.05% trypsin/ ethylene diamine tetraacetic acid (EDTA)) is often used. Advantages of using enzymatic dissociation with collagenase or dispase over trypsin/EDTA include reduced cell death and greater karyotypic stability, but in contrast, disadvantages are the inability to accurately assess cell number and the failure to generate single cell clones. Although **subcloning** is possible, HESC colonies are usually passaged by dissociating into clumps before plating. When plated at low densities, only 1% of individual HESCs survive and form colonies [57]. Undifferentiated HESCs possess gap junctions that express high levels of connexins 43 and 45 [30, 58]. Dissociation of HESCs to single cells causes considerable cell death, and it is highly possible that gap junctional communication is important to the survival of these cells [58]. However, recently it was shown that treatment with p160-Rho-associated coiled-coil kinase (ROCK) inhibitor Y-27632 increased the survival of dissociated HESCs, and the cloning efficiency was about 26% [59].

The long-term stability of HESCs is also an important issue, and despite normal karyotypes being maintained for extended culture times *in vitro*, others have reported the instability of chromosomes 12 and 17 [60, 61]. Thus, it is important to **reassess the karyotypes** regularly for HESC specific cell lines, particularly in those which are passaged into single-cell suspension as they may continue to express pluripotent markers even when they have become aneuploid.

Several **signal transduction pathways** are required for pluripotency. Examination of the conditioned medium from feeder cells revealed the presence of the **cytokine LIF** [62]. LIF, together with related cytokines bind to the gp130 receptor, which dimerizes and forms the LIF/gp130 receptor [63]. This in turn induces the phosphorylation of the transcription factor, Signal-transduced and activator of transcription-3 (STAT3). LIF also activates other signal transduction pathways, such as the cascade of ERK mitogen-activated protein kinases (MAPK) [64]. Interestingly, STAT3 activation alone is enough to maintain pluripotency in mouse ESCs in the presence of serum [65], and thereby LIF is commonly used in mouse ESCs cultures. At the same time, STAT3 is not activated in HESCs, and LIF does not support the undifferentiated growth of HESCs. In serum-free medium, LIF is insufficient to prevent the differentiation of mouse ESCs, but when LIF was combined with BMPs, the undifferentiated state of mouse ESCs could be sustained [66]. Concurrently, the addition of BMPs to HESC cultures induces the differentiation either to trophoblast [16] or primitive endoderm [67] in conditions that otherwise would support their undifferentiated growth.

Contrary to mouse ESCs, **fibroblast growth factor (FGF) signaling** seems to be more important for the self-renewal of HESCs. bFGF permits the clonal growth of HESCs on fibroblasts in the presence of serum replacement. In addition, in the absence of fibroblasts or conditioned medium, bFGF and suppression of BMP signaling with its antagonist noggin supports the undifferentiated proliferation of HESCs [46]. On the other hand, supression of BMP activity alone is insufficient to maintain undifferentiated HESCs, thus bFGF must also influence other signaling pathways. Furthermore, a higher concentration of bFGF allows feeder-independent growth of HESCs cultured in the same serum replacement [46, 68]. The mechanism through which the high concentrations of bFGF function is not completely understood. At higher concentrations of bFGF (40ng/ml), the addition of noggin or other inhibitors of BMP signaling is needed to decrease the background differentiation of HESCs, while at higher concentrations bFGF itself suppresses BMP effects to levels comparable to those observed in conditioned medium, and the addition of noggin is no longer required [46]. At the same time, one should consider that there is a significant production of BMPs by the ESCs themselves.

In HESCs, the inhibition of **TGFβ/activin/nodal signaling** through the Smads is also necessary to maintain pluripotency [69], and Activin A can sustain the undifferentiated state for more than 20 passages without need for feeder cells, or

conditioned medium [70]. Several other factors have been identified supporting the pluripotent growth of HESCs, for example, **pleiotropin**, which is secreted by mouse fibroblasts, and enhances clonal growth of HESCs. HESCs express the receptor for pleiotropin, which is down-regulated upon differentiation [71].

Furthermore, the **Wnt pathway** is represented in HESCs. Signaling downstream of the Wnt/ Frizzled receptor leads to the inactivation of glycogen synthase kinase-3beta (GSK-3), resulting in the nuclear accumulation of β-catenin, which in turn activates the transcription of target genes. Wnt signaling can also be activated by direct intracellular inhibition of GSK-3 function. In short-term cultures, activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor (6-bromoindirubin-3´-oxime (BIO)) has been reported to have a positive effect on HESC self-renewal, as detected by the expression of undifferentiation markers Octamer binding protein-4 (Oct-4), Rex1 (Zfp-42), and Nanog. However, another Wnt inhibitor, LiCl, did not possess similar effect [72].

1.2.3 Markers of HESCs

A large panel of markers are now recognized as important to define HESC pluripotentiality, and include Oct-4, Nanog, SRY (sex determining region Y)-box 2 (Sox2), Forkhead box protein D3 (FoxD3), Rex1, Telomeric repeat binding factor (NIMA-interacting)-1 (TERF1), Growth and differentiation factor-1 (GDF1) receptor, and Stella (reviewed in [11]). In addition, for HESC characterization it is common to report also alkaline phosphatase (ALP) and telomerase activities, the presence of stage-specific embryonic antigens 3 and 4 (SSEA3, 4), Thy1 (also known as CD90), and several keratin sulfate proteoglycans; tumour-recognition antigen (TRA)-1-60, TRA1-81, GCTM2 amongst others [47]. Other stem cells antigens, such as CD117 (c-kit) and CD135 (fms-like tyrosine kinase (Flt)-3 receptor) are also sometimes reported. A comparison study between the common HESC lines cultured in conditioned medium supplemented with 8ng/ml bFGF revealed that undifferentiation markers were expressed similarly between these lines [40]. However, the expression of TRA1-81 and SSEA4 differed between HESC colonies, with some HESC populations expressing higher levels than others [40]. Nevertheless, because early embryonic cells are not maintained as tissue-sustaining stem cells throughout the life of the organism, it is perhaps reasonable to expect that the mechanisms are distinct from those that control adult stem cells [73].

1.2.4 Transcriptional networks in HESCs

The nuclear factors that regulate pluripotency and convert extrinsic signals into intrinsic cellular responses have been the subject of intense research. Recently three transcription factors have been identified that coordinately regulate the pluripotency program: **Oct-4, Sox2** and **Nanog**. Oct-4 (POU5F1) is a POU domain-containing transcription factor, and interacts with Sox2 to regulate down-stream genes [74]. Target genes for Oct-4 include Rex1, Lefty1 as well as others, and genes that co-operate with Oct-4, such as Sox2. During early mouse development, Oct-4 is activated at the four cell-stage, and is later restricted only to pluripotent ICM and germ cells. Interestingly, exact levels of Oct-4 seem to be important, in that overexpression causes differentiation into endoderm and mesoderm, while lower levels induce the differentiation towards trophoblast [75]. Oct-4 is the most widely used HESC marker for undifferentiated cells, but examination of Oct-4 expression alone may be misleading. This transcription factor does not immediately shut down RNA transcription in differentiating HESCs, taking some time and is also found in other pluripotent cells, as well as in some adult and fetal multipotent stem cells [11]. It has been reported that under certain circumstances differentiating ESCs show a transient burst of Oct-4 expression prior to its down-regulation [73].

As mentioned, Oct-4 binds with Sox2, and in turn Sox2 contributes to pluripotency by regulating Oct-4 levels [76]. Common to Oct-4, Sox2 and Nanog is the ability to **i)** bind to their own promoter and function together to maintain their own expression, **ii)** co-occupy their target genes, and **iii)** target such genes that are actively expressed, or those that are silent in ESCs but are poised for subsequent expression during differentiation [76].

Nanog is needed to maintain pluripotency, but it is not necessary for induced pluripotency following the somatic cell reprogramming [76]. Although, the exact mechanisms of how Nanog regulates stem cell pluripotency remain unclear, it has been proposed that it represses the down-stream genes that are important for differentiation, but at the same time Nanog can activate other genes that are important for self-renewal, such as Oct-4 and Rex1 [74].

However, there are still plenty of other factors and interactions that regulate pluripotency and need to be either identified or studied.

1.2.5 Differentiation of HESCs

Spontaneous differentiation of HESC colonies occurs *in vitro* in prolonged suboptimal cultures and in the absence of active feeder cells. Early differentiation events may be observed in many HESC colonies within a week after the last passage, and heterogeneous expression of pluripotent markers, such as Oct-4, can be observed in early differentiating HESC colonies [11]. When monolayer cultures of HESCs are permitted to overgrow in a two-dimensional system, cells within the multiplying colony begin to pile up and start to differentiate at the central and border areas. A wide range of differentiating cell types can be observed in these flat cultures, including ectodermal neuronal cells, mesodermal muscle, and endodermal organ tissue types [27]. HESCs can also form extraembryonic tissues that differentiate from the embryo before gastrulation [67]. BMP4, for example, induces the differentiation of HESCs to trophoblasts, which even secrete placental hormones, such as chorionic gonadotrophin [16].

Differentiation of HESCs occurs through symmetric cell division suggesting that ESCs more closely resemble transit amplifying cells rather than adult stem cells [73].

1.2.5.1 Basic methods to promote differentiation of ESCs

The physical microenvironment within which cells reside plays an important role [77]. Studies utilizing the **culture of ESCs as monolayers on ECM proteins** demonstrated the role of complex ECMs in tissue-specific differentiation of ESCs, whereas single compartments of ECM such as laminin-1 and collagen type I did not support the growth or morphology of ESCs [78]. A more widely used method is the **culture of ESCs directly on supportive stromal layers**, such as mouse stromal cells that have been used to drive the ESCs towards neuronal fates [79]. Bone marrow stromal cells have been used efficiently to support hematopoietic differentiation [80]. However, such culture systems with stromal cells of animal origin contain still unknown components, and differentiation can be dependent on the culture conditions of the stromal cell line. The **formation of three-dimensional aggregates known as embryoid bodies (EBs)** has been a widely used tool eliminating the need for other cells to support differentiation. It is obvious that the nature of the three-dimensional environment provides a different organization of ECM, thus facilitating the formation of structures that are not otherwise possible on flat surfaces. EBs are spherical structures composed of aggregated ESCs. Aggregation induces ESC differentiation and

the formation of derivatives of the three germ layers [81], for example visceral endoderm was consistently identified in the outer layer of HESC-derived EBs. Moreover, cellular aggregation in mouse ESCs has been shown to induce the repression of Nanog at the outer layer, which occurs independently from LIF/STAT3 or BMP pathways [82]. Most of the early differentiation protocols were based on EBs. EBs can be induced to form by culturing the ESCs in "hanging drops" or in plastic culture dishes that do not favour cell attachment, albeit, cultivation of clumps of HESCs in hanging-drop cultures resulted in considerable cell death [27]. However, HESC-derived EBs possess a consistent appearance and structure with variety of cell types that appeared to develop in a less organized pattern than mouse EBs [83]. Recently, a new reproducible method for production of uniform and synchronously differentiating EBs from HESCs using spinning in low attachment plates was reported [84].

1.2.5.2 Modulation of differentiation in vitro

HESCs provide a potentially unlimited source of specialized cell types for regenerative medicine. One of the key requirements to fulfill this potential is the competence to direct the *in vitro* differentiation of HESCs to selective fates. However, it is the same plasticity that permits ESCs to generate differentiated cell types which makes it difficult to control the very same process. In similarity to all cells, the fate of stem cells is influenced by chemical and physical signals within the surrounding microenvironment. Within *in vitro* conditions, such signals can be manipulated to affect stem cell fate, and it is possible to induce the HESC differentiation towards any specific lineage. On the other hand, the detailed molecular control of this differentiation is poorly understood.

Activation of endogenous transcription factors or **transfection of HESCs with ubiquitously expressed transcription factors** have often been used to manipulate the natural genetic program within HESCs. Traditional techniques are based around homologous recombination, but HESCs have proven more difficult to manipulate compared to mouse ESCs. One reason could be attributable to that the HESCs clonal propagation efficiency is poor, thus making it difficult to screen for induced changes. In addition, the cell size differs, as HESCs are larger (14μm) than mouse ESCs (8μm), and therefore the transfection methods are different. The first report that studied several chemical-based methods and isolated genetically engineered HESCs lines demonstrated that transfection with ExGen500 (Fermentas) delivered DNA into HESCs more efficiently than other reagents ((Lipofectamine Plus (from

Invitrogen), Fugene (from Boehringer Mannheim)). The best chemical reagents yielded stable drug selectable transfectants at rates about 10^{-5} cells [85]. Generally, it is acknowledged that HESCs do not survive electroporation well, however a successful electroporation study used HESCs in clumps and a modified protocol in a protein-rich solution [57]. Therefore, neither chemical transfection nor electroporation are considered as efficient methods to induce stable transgene expression in HESCs, and as a result studies have turned to viral-based gene delivery, in order to achieve long-term transgene expression. Adenovirus-derived vectors have been successfully used in mouse ESC studies [86], however their application in HESCs is still under investigation. Retroviral vectors, including lentiviral vectors which are also derived from retroviruses, are a common and efficient means to transduce HESCs [87-91].

Exposure of HESCs to selected growth factors or their antagonists has become a widely used strategy for directing the differentiation of HESCs. Evaluation of the effects of several growth factors on pre-differentiated HECSs demonstrated that TGFβ and Activin A induced mainly mesodermal differentiation; epidermal growth factor (EGF), FGF, retinoic acid (RA) and BMP4 stimulated ectodermal differentiation; and β nerve growth factor (NGF) and hepatocyte growth factor (HGF) gave rise to all three germ layers [92]. **Co-culture of HESCs with cell types capable of lineage induction** are an interesting field. Mummery et al showed that if HESCs were grown with mouse visceral endoderm cells (END2), they formed beating heart muscle colonies [93].

Despite the progressive interest in developing various differentiation protocols, the **selection of differentiating cells for specific lineages has been difficult** due to the lack of markers for the earliest progenitor cells.

Environmental and epigenetic factors also play an important role in regulating the differentiation of pluripotent HESCs. For example, DNA methylation is required for differentiation, and together with the chromatin regulators, such as the polycomb group proteins, they are important for epigenetic modifications. Among the environmental factors that influence the state of potency, is oxygen concentration. At low oxygen levels, hypoxia has been shown to promote more pluripotent and multipotent cell types at the expense of their differentiated progeny [94].

1.3 BONE TISSUE

The skeleton, composed of cartilage and bone, is essential for providing a scaffold for soft tissues but serves also as a reservoir for calcium, magnesium and phosphate ions that are of critical importance in physiology. Bone is an unique tissue since **i)** it possesses the ability to become calcified by a physiologic mechanism called mineralization, **ii)** it is composed of various cell types within this mineralized matrix, and **iii)** it constantly undergoes a remodeling process. The composition of bone includes 70-90% mineral, and 10-30% is represented by the organic component. Proteins are usually classified as collagenous proteins comprising 90% of the organic matrix, and non-collagenous proteins the remaining 10% [95].

Two types of bone are recognized; **woven bone**, which is highly cellular and formed in response to growth or injury, and **lamellar bone.** Woven bone, eventually is converted into lamellar bone, a mature bone with collagen fibres arranged in lamellae and the principal load-bearing bone of the adult skeleton. Interestingly, the biochemical composition of woven and lamellar bone differs with woven bone being rich in acidic phosphoproteins such as bone sialoprotein (BSP), which are not expressed in lamellar bone. Whereas, on the other hand, lamellar bone contains large quantities of osteocalcin (OCN). Also, mineralization of woven bone occurs faster than in lamellar bone by means of a matrix-vesicle-assisted mechanism [96].

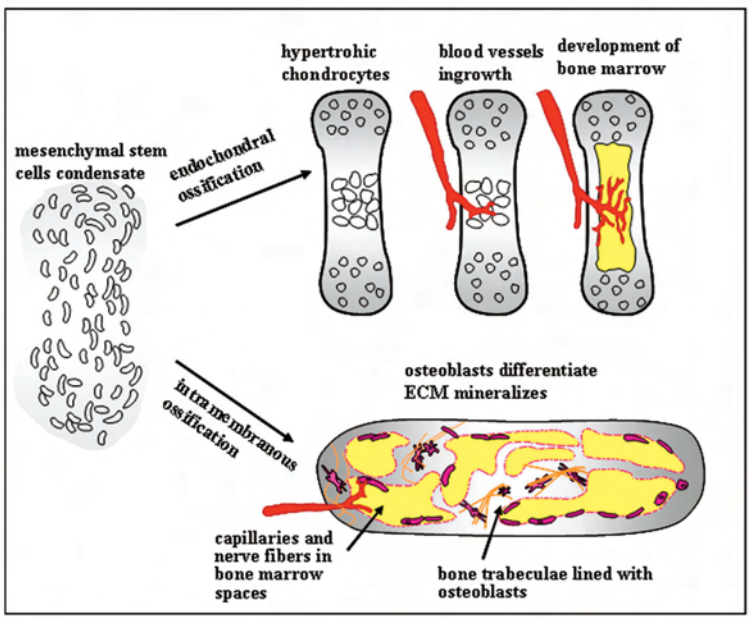
1.3.1 Bone formation

Throughout development, the vertebrate skeleton is **formed by mesenchymal cells** condensing in areas of future bones (patterning phase). The craniofacial skeleton is formed by cranial NC cells, the axial skeleton from paraxial mesoderm (somites), and the limb skeleton is the product of lateral plate mesodermal cells [97].

Throughout embryogenesis, bone tissue forms by two distinct processes. During **intramembranous ossification** clusters of cells adhere through the expression of adhesion molecules, and differentiate into osteoblasts [98]. In regions of **endochondral ossification**, the process first involves cell migration to locations in the embryo where skeletal elements will develop, where they form characteristic mesenchymal condensations of high cell density. This is followed by the differentiation to cartilage producing cells, chondrocytes, and subsequent growth generates cartilage scaffolds for future bones. The cells lay down an ECM particularly rich in collagen type II and aggrecan, and express characteristic chondrogenic transcription factors, Sox5/6/9 [99], stop proliferating, become hypertrophic, and synthesize a distinctive

ECM containing collagen type X. Hypertrophic chondrocytes attract blood vessels through the production of angiogenic factors, they direct adjacent perichondral cells to become osteoblasts, and thereafter undergo apoptotic cell death, creating bone marrow cavity.

Figure 2. Bone is formed either by direct ossification of embryonic connective tissue (intramembranous ossification) or by replacement of hyaline cartilage (endochondral ossification). Intramembranous ossification takes place in the bones of skull, while endochondral ossification is characteristic to the bones of the trunk and extremities.



1.3.2 Bone-producing cells

Active **osteoblasts** are cuboidal, polarized bone matrix producing cells. In *in vitro* cell culture, osteoblasts are nearly indistinguishable from fibroblasts, and all the genes expressed in fibroblasts are also expressed in osteoblasts [100]. The only morphological feature specific to osteoblasts is the formation of the mineralized ECM. Similar to fibroblasts, myoblasts, chondrocytes and adipocytes, osteoblasts originate from MSCs located in the bone marrow, endosteum and periosteum. During differentiation of multipotent mesenchymal cells into several lineages, the progenitors of these lineages acquire specific phenotypes under the control of regulatory factors of the restricted lineages [99, 101, 102]. Osteoblasts deposit osteoid, the unmineralized

ECM, which subsequently becomes calcified. During this process, a proportion of cells becomes trapped within the lacunae of the matrix and are termed **osteocytes**. Osteocytes are connected by a system of canaliculi, and their proposed function is to regulate the response of bone to mechanical stimuli [103]. The other proportion of osteoblasts becomes **bone-lining cells**, which are flat cells lining the surface of bone.

Osteoblasts also influence the differentiation of **osteoclasts**, bone resorbing cells, which belong to the family of monocyte/macrophage lineage. Osteoblasts express *in vivo* the receptor for activation of nuclear factor kappa B (NF-κB) (RANK) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [104], which in turn activate a number of signaling pathways in osteoclasts, such as NF-κB and MAPK pathways.

1.3.3 Osteoblast differentiation process

The population of cells that is committed to the osteoblastic phenotype are called osteoprogenitors. Such cells divide and differentiate into osteoblasts forming bone. Analysis of fetal rat calvaria-derived osteoblast cultures (RC cells) has indicated that less than 1% of cells are actually destined to form bone [105, 106].

Continuous recruitment, proliferation and differentiation of cells within bone tissue is regulated by the expression of genes providing the characteristics to the bone phenotype. Studies using RC cells have determined a pattern for the expression of marker genes encoding the osteoblast phenotype, which can be subdivided in three chronologically related distinct stages, defined as:

- A growth or proliferation phase,
- A matrix development phase,
- A mineralization phase.

Each stage is characterized by expression of distinctive set of genes and between each growth period there appears to be restriction points to which cells progress but cannot pass without further signals (reviewed in [107], [108]).

The growth or proliferation phase is reflected by a high mitotic activity that is accompanied by the expression of cell-cycle genes, such as those encoding for histones, and cell growth genes, such as C-myc, C-fos, and C-jun. During this period, genes associated with the formation of ECM, such as collagen type I, osteopontin (OPN), and fibronectin are actively expressed, but are then gradually down-regulated. Collagen type I mRNA remains, however, it is expressed at lower levels during the

following stages of osteoblast differentiation. Following the down-regulation of the proliferation genes, an increase in ALP activity is evident. In **the matrix development phase,** the composition and organization of the ECM is greatly modified, providing an environment favourable for mineralization. As the culture matures towards mineralization, all cells possess high ALP activity. The mechanism of **mineralization** is coordinated by the osteoblasts and involves the deposition of a calcium phosphate apatite within an organic framework. Several ECM proteins play role in the mineralization process, and it is generally accepted that the formation of mineral does not occur without a three-dimensional matrix, which consists of collagen together with a number of acidic macromolecules, including proteoglycans, glycoproteins and phosphoproteins. These macromolecules regulate the transport and concentration of mineral ions at the site of mineralization.

1.3.4 Transcriptional control of osteoblast differentiation

Commitment of MSCs to tissue-specific cells is orchestrated by transcriptional regulators (review [109]). A central regulator of bone formation is **Runx2,** also known as Core-binding factor α1 (Cbfα1), a member of the Runx (Runt-related factors) family of transcription factors. The family members, Runx1 (Acute myeloid leukemia gene (AML) -1), Runx2 (AML3), and Runx3 (AML2), are encoded by distinct genes but share a common DNA recognition motif. Runx2 activates the OCN and collagen type I-α1 genes [110], and serves as an initial marker of the osteogenic cell lineage (review in [111]). Runx2 is abundantly expressed in calcified cartilage and bone tissues and is transcribed from two separate promoters. The upstream promoter drives the expression of osteoblast-specific isoforms, whereas the second promoter drives the expression of isoforms that are mainly expressed in T-cells, but they can be found also in osteoblasts and other mesenchymal cells [112-114]. Targeted disruption of Runx2 results in the complete lack of bone formation by osteoblasts, revealing that Runx2 is essential for both endochondral and intramembranous bone formation [115]. Forced expression of Runx2 in skin fibroblasts leads to osteoblast-specific gene expression [116], and *in vivo* ectopic expression of Runx2 leads to endochondral ossification in regions of the skeleton that would not normally ossify [117]. Interestingly, co-cultures with human prostate cancer cells and mouse osteoblasts demonstrated that osteoblast differentiation was induced by tumour cells, which was associated with the up-regulation of Runx2 [118]. Runx2 has been designated as the most pleiotropic regulator of skeletogenesis

[99], it functions as an inhibitor of proliferation of progenitors [119], and is also required for osteoblast function beyond differentiation [120, 121].

A few transcription factors that act up-stream of Runx2 to control its expression have been identified, such as **Msx2** and **Bapx1,** two homeobox-containing transcription factors. Their inactivation in mice causes a marked delay in ossification and an overall decrease in bone volume accompanied by a down-regulation of Runx2 expression, thereby indicating that they directly or indirectly regulate Runx2 expression [122]. **Twist-1**, a mediator of dorsal-ventral patterning and mesoderm formation, is down-regulated for Runx2-induced osteoblast gene expression [122]. **p53 tumor suppressor** plays a pivotal role in preventing cancer, and suppresses osteoblast differentiation by repressing the expression of either Runx2 or Osterix (OSX) [122-124]. **Schnurri-3**, a large zinc-finger protein, was found to control protein levels of Runx2 by promoting its degradation and repressing the Runx2-mediated ECM mineralization [122].

Functioning as a transcription factor, Runx2 protein interacts with a number of co-activators and co-repressors. The most important co-activating protein, essential for enhancement of Runx DNA binding is **Cbfß**, the non-DNA-binding partner of all three Runx proteins. Inactivation of Cbfß causes embryo hemorrhagia and lethality in mice because Cbfß normally dimerizes with Runx1 and Runx3, which are essential for haematopoiesis. Interestingly, transgenic rescue and 'knock-in' experiments demonstrated a delayed ossification phenotype. Other well-characterized co-activators of Runx2 are p300, Creb-binding protein (CBP), Monocytic leukemia zinc finger protein (MOZ), and Mortality factor (MORF). Among co-repressor molecules, **histone deacetylases** have been shown to inhibit Runx2, as well as OPN, BSP, and OCN expression (review in [122]). Another pathway, the **proteosome degradation** pathway decreases Runx2 protein levels and slows down osteoblastic differentiation. Within this pathway, Smurf1, the ubiquitin-protein isopeptide ligase, induces Runx2 degradation, while Smad6 enhances it. Tumour necrosis factor (TNF)-α up-regulates the expression of Smurf1 and consequently promotes Runx2 proteasomal degradation resulting in the inhibition of osteoblast differentiation [122].

Even though Runx2 is essential for osteoblast differentiation, this differentiation program also requires other genes, such as **OSX (Sp7)**, which encodes a transcription factor genetically down-stream of Runx2. OSX, a zinc finger-containing transcription factor and BMP2-inducible gene, was identified as a regulator for the final stages of bone tissue formation [125]. OSX contains a DNA-binding domain, and its C-terminus

shares a high degree of sequence identity with similar motifs in Specificity protein (Sp)-l, Sp-3, and Sp-4. OSX activates OCN and collagen type I-α1 genes, and in mutant OSX-null mice, no endochondral or intramembranous bone formation occurs [125]. The mesenchymal cells in such mutant mice cannot differentiate into osteoblasts, although the cells express normal levels of Runx2. Interestingly, OSX-null osteoblast precursors in the periosteum express chondrocytic markers, such as Sox9 and collagen type II, suggesting that Runx2-expressing progenitors are still bipotential cells, and that OSX acts down-stream of Runx2 [125]. To date there is no evidence as to whether Runx2 and OSX interact [122]. However, it has been demonstrated that OSX gene includes an OSE2 element in the regulatory region, so the OSX promoter might be a direct target for Runx2 [126].

Several studies have implicated additional signaling pathways which may act in parallel to, or independent of, Runx2 during osteoblast differentiation. MAPK and protein kinase D (PKD) signaling pathways mediate the OSX expression upon induction with BMP2 and Insulin-like growth factor (IGF)-I in MSCs [127]. Additionally, **Dlx5**, a homeobox transcription factor, is a BMP2-regulated gene, and has been shown to regulate OSX independently from Runx2 [128]. Koga and co-workers showed that nuclear factor of activated T-cells (NFAT) co-operates with OSX to accelerate osteoblast differentiation and bone formation [129]. In another study, **Activating transcription factor (ATF4)** was identified as being a critically important molecule for the onset of osteoblast differentiation, for osteoblast terminal differentiation, for BSP and OCN synthesis and for post-transcriptional regulation of collagen type I [130].

1.3.5 Regulation of osteoblast differentiation

Factors that are produced by osteoblasts or a range of circulating growth factors are all bound to the proteins of the bone ECM, where they locally influence the osteoblast differentiation process.

Endocrine control of osteoblast differentiation is regulated by two principal hormonal factors, *parathyroid hormone (PTH)* which is synthesized by parathyroid gland, and leptin, produced by adipocytes. Calcium release into the bloodstream requires bone destruction by osteoclasts, and the principal mediators of this process are PTH hormone and its downstream effector vitamin D3. The osteoblasts together with their precursors have a central role in directing the bone resorbing effect of PTH. Continous PTH administration stimulates the expression of RANKL and M-CSF,

molecules that support osteoclastogenesis, in osteoblasts. At the same time, PTH inhibits the expression of osteoprotegerin by osteoblasts, a RANKL-binding receptor which then prevents RANKL binding to RANK. On the other hand, intermittent application of PTH has an anabolic effect on bone, by increasing the osteoblast number and activity. However, the mechanism is complicated and less well understood (reviewed [131]).

Leptin, an adipocyte produced hormone, acts as a physiological inhibitor of bone formation. This inhibition is achieved by leptin action on a subpopulation of hypothalamic neurons, which then act through the sympathetic nervous system, and β2 adrenergic receptors present on osteoblasts. Mice lacking leptin or the leptin-receptor gene have increased bone formation (reviewed [132]).

Figure 3. Signaling pathways in osteoblasts.

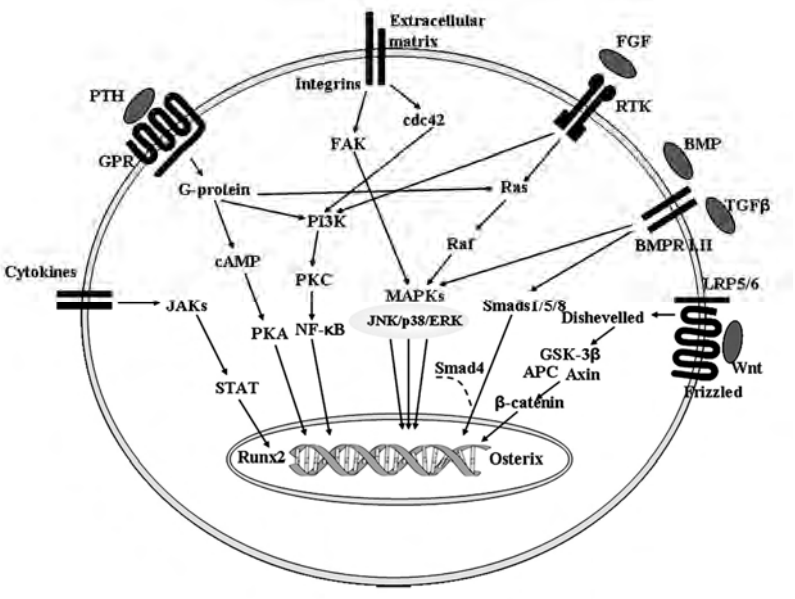


Figure 3 shows a simplified scheme covering the main signaling pathways that are involved during osteoblastogenesis. PTH and many local effectors, such as prostaglandins initiate autocrine and paracrine events through G-protein-coupled receptors, thereafter activating adenylyl cyclase (cAMP) and the protein kinase A (PKA) pathway. ECM signaling through focal adhesion kinase (FAK) activates MAPKs, and a number of growth factors have been identified to be important for the **local control of osteoblast differentiation**. *Transforming growth factor β-1 (TGF-β1)* is a 25kD polypeptide synthesized in an inactive form bound to latent TGF-β binding

protein (latent TGF-β) [133]. It regulates osteoblast proliferation and matrix synthesis including mineralization (reviewed [134]). Osteoclasts activate latent TGF-β during bone resorption to release active TGF-β1 to stimulate new bone formation [135]. However, TGF-β1 cannot initiate the bone formation cascade in extraskeletal sites like the *bone morphogenetic proteins (BMPs)* [136]. BMPs were originally identified as proteins present in demineralized bone matrix that could induce ectopic osteogenesis [137, 138]. At present, over 30 members have been identified, all structurally related to the TGF-β superfamily of secreted signaling molecules. The BMPs are found in the bone matrix and are synthesized by skeletal and extraskeletal tissues as larger precursor molecules, which are processed to around 30kD dimers before their secretion to the cell (reviewed [139]). Both TGF-β1 and BMPs exert their signaling effects via BMP receptors type I and II, and Smad1/5/8 molecules. Smads become phosphorylated by the BMP binding to the receptor, and are translocated to the nucleus in a complex with Smad4, where they regulate the target genes. Another set of inhibitory Smads (Smad 6/7) compete for binding with Smad4, and present a negative regulation of this pathway [139]. Several BMPs (BMP2, BMP4, BMP7) have been shown to induce ectopic bone formation, and are thus called osteogenic BMPs [139]. The regulation of osteoblast gene expression involves the interaction between the Smad1/5/8-Smad4 complex and enhancer-sequences of target genes, the most important being Runx2 and OSX [125, 140, 141]. An additional Smad-independent pathway has been described. BMP2, for example can activate ERK, JNK and p38 in osteoblastic cells, thus providing the evidence of MAPKs are involved as well.

The mineralized bone matrix also contains heparin-binding *fibroblast growth factors (FGFs)*, which are powerful mitogenic stimulants for osteoblasts [142] and important during chondrogenesis and osteoblast differentiation [143]. The FGF ligands are usually between 20-35 kD and bind to the FGF receptor's extracellular ligand binding domain to induce FGF signaling. Upon ligand binding, the FGF receptors (FGFRs 1-4) dimerize and subsequently cause autophosphorylation of the intrinsic kinase residues, setting off the FGF signaling cascade through the MAPK pathways [144]. The FGF signaling has been shown to activate ERK, p38, JNK, PKC, and PI3K pathways to transduce cell signaling in osteoblasts [145]. The role of FGF signaling during osteogenic differentiation from mouse ESCs has been demonstrated [146], and bFGF was shown to induce ALP activity in rat bone marrow precursor cells, and to induce the expression of Runx2 [146]. Furthermore, other members of the FGF-family can induce OCN expression and are important in matrix mineralization [145].

However, conflicting evidence exists regarding the effect of FGF on osteoblast proliferation and the expression of the osteoblast markers, and this discrepancy appears to be a result of the stage-specific effect of the FGF signaling [145].

Wnts, a family of secreted glycoproteins bind to the Frizzled receptors (FZD). Wnts signal through several pathways, however it is the Wnt/β-catenin (canonical) pathway that appears to be important for bone biology [147]. Wnt signaling functions downstream of Indian hedgehog (Ihh) in development of the osteoblast lineage [148], and is activated by the formation of a complex with FZD/low density lipoprotein receptor-related proteins 5/6 (LRP5/6) at the cell surface. The following signals are generated through the protein Dishevelled, which inhibits a protein complex of Axin/ adenomatous polyposis coli (APC) /GSK3. In the absence of a suitable ligand, β-catenin becomes phosphorylated and is degraded. Free cytosolic β-catenin is translocated to nucleus where it activates target gene transcription, such as Runx2 in the developing osteoblasts [149]. Although it is known that pathologically high levels of Wnt signaling result in higher bone density, the exact function of Wnt in bone biology remains unclear [150]. LRP5 deficient mice were viable but postnatally developed a low bone mass phenotype because of reduced osteoblast proliferation and function [151], and an activating mutation has been linked in two cases to individuals with high bone density [152, 153]. Several studies have shown that Wnt proteins inhibit the ability of human MSCs to differentiate to osteoblasts [154, 155], while others show the opposite [156, 157]. In the absence of β-catenin, osteoprogenitors fail to express OSX and instead differentiate into chondrocytes [158], thus β-catenin seems to be required for osteoblast differentiation at a very early stage.

1.3.6 Extracellular matrix of bone

Collagen fibers play critical roles in maintaining the structure and function of bone tissue. Collagens, in general, cover a large family of proteins with up to 38 genes giving rise to more than 20 different collagens [159]. They can be subdivided into:

- fibrillar collagens (types I, II, III, V, XI),
- non-fibrillar or basement membrane collagens (types IV, VI, VII, XII),
- fibril associated collagens with interrupted triple helices (FACIT) (types VIII, IX, X, XIII).

It is the fibrillar collagens that have been suggested to be of primary importance in the process of mineralization, providing the framework for crystal nucleation and

environment for cellular migration and differentiation [160]. The FACIT collagens are found for example in the developing cartilage, where they may serve as molecular bridges that are important for the organization and stabilization of the ECM [161].

An important part of the ECM is composed of the **non-collagenous glycoproteins and proteoglycans (NCP),** secreted by osteoblasts into the surrounding millieu. The highly anionic complexes have a high ion-binding capacity and are thought to play an important part in the calcification process and the fixation of hydroxyapatite crystals to collagen fibers. The most important NCPs are:

- **Small Integrin Binding Ligand N-linked Glycoproteins (SIBLINGS)** (phosphoproteins osteopontin, bone sialoprotein),
- **Cell-matrix mediating proteins:** osteonectin,
- **Proteoglycans (PGs):**
    - small leucine-rich PGs (SLRPs) (biglycan, decorin, osteoadherin),
    - large aggregating PGs (aggrecan, versican),
    - cell-surface PGs,
    - CD44, glypican,
    - basement membrane PGs,
    - intracellular PGs,
- **GLA-carboxylated** (osteocalcin, matrix Gla),
- **Other specialized proteins:** fibronectin, laminin, tenascin, vitronectin, integrins, serum proteins.

One of the most abundant NCPs in bone is a phosphorylated glycoprotein **osteonectin (ON)**, an important molecule for cell-matrix interactions and encoded by the SPARC (secreted protein, acidic, rich in cysteine) gene. ON is expressed in a wide variety of adult and embryonic tissues, such as developing bone, odontoblasts, kidneys and lining epithelium. As an acidic protein it has a high affinity for binding collagen and calcium ions [162]. Several other functions have been proposed; ON inhibits cellular proliferation, modulates cell-matrix interactions, and binds and regulates negatively apatite crystal growth in hard tissues as well as at sites of ectopic calcification [163, 164].

The proteoglycan (PG) family contain more than 30 proteins that are post-translationally modified by glycosylation or the addition of negatively charged

glycosaminoglycans (GAGs) [165]. Small leucine-rich PGs (SLRPs) constitute of a core protein and contain either dermatan/chondroitin, heparan or keratan sulphates. Several of SLRPs have been shown to bind to collagen and regulate mineral crystal formation [166, 167]. **Osteoadherin (OSAD)** is currently believed to be an osteoblast-specific SLRP, and has a role in inhibiting actively proliferating cells. It was found to possess a similar distribution to BSP in rat long bone and calvaria [168].

At an early stage of osteogenic development, osteoblasts secrete **OPN**. Expressed by cells in numerous tissues throughout the body [169], OPN is also found in body fluids like plasma, urine, bile and milk. During bone developmnt OPN mediates cellular interactions and is expressed by proliferating osteoprogenitors prior to other matrix proteins including BSP and OCN [170, 171]. The early expression of OPN has been linked to its role in cell attachment and the control of relationships between cells and the ECM [172, 173]. Owing to its overall acidity, OPN binds to calcified matrices and has been proposed to link organic and inorganic phases [174]. Indeed, OPN is abundant in mineralized tissue and has therefore been implicated both in bone formation and remodeling [175].

OPN and **BSP** are both phosphorylated sialoproteins containing tyrosine sulphates, regions enriched in acidic amino acids and possessing an Arg-Gly-Asp (RGD) cell attachment sequence. BSP is involved in the nucleation of hydroxyapatite at the mineralization front of bone [176], whilst **OCN** delays and OPN inhibits nucleation [177]. OCN, a member of the Gla-protein family, is a small, highly conserved molecule only associated with the mineralized matrix of bone. As OCN is only expressed at the later stages of the osteoblast development sequence, it provides an ideal marker for the mature osteoblast.

The current belief is also that mineralization of the matrix is initiated by the expression of the membrane-bound glycoprotein **ALP**. ALP is expressed in large amounts by osteoblasts *in vivo* [178], and has also been found in differentiation studies with osteoblast-like cell lines *in vitro* [179]. Although ALP is assumed to play a role in the early stages of osteogenesis, the role of this enzyme in bone development is still uncertain.

Bone also contains large amounts of some **serum proteins**, including albumin and α_2-HS-glycoprotein that accumulate in bone because of their affinity for hydroxyapatite [180].

1.3.7 Mechanisms of mineralization

Biomineralization is the process by which mineral crystals are deposited in an organized fashion in the matrix.

Matrix-mediated mineralization is the generally accepted mechanism for the formation of hard tissues. Two functions must be synchronized in order to provide a structurally hard tissue, first the matrix must provide a highly ordered scaffold possessing a suitable reactive surface for nucleation and crystal deposition, and second the matrix must provide means for the transport of calcium and phosphate ions to the appropriate site.

It is proposed that the ECM controls the formation of initial mineral deposits (nucleation) and orientation of the resulting crystals (crystal growth). The ECM proteins stabilize the smallest mineral crystals (nuclei) and/or bind to the crystal surfaces and regulate their morphology. The inorganic fraction of the matrix consists of plate- or spindle-shaped mineral crystals of hydroxyapatite that are deposited on the collagenous framework. It appears less likely that the framework itself is directly responsible for mineralization, but plays role in the orientation of crystal nucleators and in the regulation of crystals size. Before nucleation, the clusters of mineral continuously form and dissolve. However, needed is the formation of stable clusters, known as the critical nucleus.

Another mechanism for mineralization is termed as **matrix-vesicle mediated mineralization**, in which enzyme-rich membrane-bound organelles, matrix-vesicles, regulate the process. Such vesicles contain phosphatases that are involved in the nucleation process. Moreover, they are also likely to provide a protective microenvironment for crystal growth. It has been proposed that matrix-vesicle mediated mineralization occurs in dystrophic mineralization [181], calcifying cartilage [182], and in intramembranous bone [183]. However, the precise mechanism of this type of calcification remains unclear.

1.3.8 *In vitro* models for osteogenesis

Bone generating, osteogenic culture conditions were first established using bone tumour cell lines or tissue-derived cells of non-human origin [184-191]. The traditional bone nodule assay, a standard culture model originating from early studies using RC cells has contributed significantly to the increased understanding of osteoblast differentiation [192]. However, models based on animal cells express inappropriate

signaling systems regarding humans, and tumour cell lines often have an impaired cell cycle, and thus do not exhibit the true phenotype of the bone tissue [191].

Many **growth factors and cytokines** have been shown to promote the differentiation of osteoblasts *in vitro*, including IGF-1 [193], melatonin [194], and interleukin (IL)-6 related cytokines [195]. Furthermore, BMP family members have been shown to exert particularly strong osteoinductive effects [196, 197]. Osteoblasts display also receptors for LIF [198-200]. Cadherin-11 was identified as a cell adhesion molecule constitutively expressed by osteoblasts, particularly during the early stages of their differentiation [201]. The authors demonstrated that a cadherin-11-positive cell population, following subculture in the presence of osteogenic stimuli including dexamethasone (Dex) and BMP2, differentiated into osteoblastic cells. The cadherin-11-negative cell fraction, which was subcultured under identical conditions, showed no evidence of bone nodule formation or expression of other osteoblastic markers suggesting that this population had few or no cells with osteoblast lineage potential. This suggests that cadherin-11 is a potential marker for osteoblastic precursors.

When osteogenic cells are maintained for extended periods in the presence of serum, the cells develop into well defined three-dimensional structures termed **" bone-like nodules"** (reviewed [186]). In the presence of β-glycerophosphate (βGP) such nodules become mineralized, and stain with von Kossa reagent and Alizarin Red S (AR) dye. A nodule consists of a central collagenous unmineralized ECM region, which is covered by a continuous layer of cuboidal osteoblast-like cells and resembles woven bone.

1.3.9 Differentiating ESCs to osteoblasts

Several studies have investigated the osteogenic differentiation potential of ESCs, and the most important of these are presented in Table 1.

Table 1. List of published studies on the *in vitro* osteogenic differentiation potential of ESCs, their experimental set-up and results

ECS line and type of feeders	Induction of differentiation	Results	References
MESC: CEE **F:** MEF	**EB:** 10%FBS 5days,	**protein**: OCN, Col I **AR**	Buttery et al, 2001 [202]

MESC: CGR8	**EB:** HD 2 days, then cultured w RA 3 days. **OG**: AA, βGP, compactin, BMP2	**mRNA**: OCN, OPN, ALP **vonKossa, AR**	Phillips et al., 2001 [203]
MESC: D3 **F:** MEF	**EB**: 5 days, **OG**: AA, βGP, vitD3;	**mRNA**: OCN; ON, BSP, OPN, Col I, ALP, Runx2; **protein**: ALP, OCN; **vonKossa, AR, TC**.	zur Nieden et al., 2003 [204]
MESC: CEE **F:** SNL (LIF+)	**EB**: 10%FBS 5days, **SD**: $5x10^4$/6w plates, after 14 days added AA, βGP, Dex	**mRNA:** Oct-4, Runx2, OCN, OPN, IGF-II, STRA13, cadherin11. **Microarray**.	Bourne et al, 2004 [205]
MESC: CGR8, E14/Tg2a, EFC1	**EB**: HD 2 days, **OG**: 3 days w/wo RA, then 2days wo RA, then AA, Dex, βGP, BMP4	**mRNA (Q-PCR)**: several, **ALP, AR**	Kawaguchi et al., 2005 [206]
MESC: D3 **F:** MEF	**EB**: 5 days, OG: BMP2, AA, vitD3, TGFβ1, insulin.	**mRNA**: chondrocytic markers, OCN, BSP, Runx2, ALP, OPN, ON, Col I. **prot**: Col II, **AB**	zur Nieden et al., 2005 [207]
MESC: E14/ TG2a	**EB**: 1,3,5 days **SD**: $3x10^4c/cm^2$, **OG**: AA, βGP, after 14 days added 1μM Dex, **CC**: HepG2	**protein**: cadherin11, tested also cardiogenic markers. **CPA**, **AR.**	Hwang et al, 2006 [208]
MESC: R1	**EB**: HD method, then **i)** cultured 35 days, **ii)** single cells 10 days. **OG**: AA; Dex, vitD3	**mRNA**: ALP, OCN, Runx2. **AR**	Duplomb et al, 2007 [209]
Monkey ESC: CMSA2 **F:** MEF	**EB**: HD 15%FBS 3 days, then RA 2 days, **OG**: 100nMDex, AA, βGP, BMP2	**mRNA**: Col I, OPN; Runx2, OCN; **protein**: OCN; **calcium** (quantified)	Yamashita et al., 2005 [210]
HESC: H1, H9 **F:** Matrigel + MEF CM	**EB**: 48h in CM + 4 days 10%FBS, **OG**: AA, βGP, 100nM Dex	**mRNA**: Oct-4, PTHR, OPN, Runx2, Col I, OCN, BSP, ALP. **protein**: OCN, **AR**, **calcium** (quantified), **x-ray diffraction**	Sottile et al., 2003 [211]
HESC: H1 **F:** MEF	**EB:** 15%FBS 5days, dissociated; **OG:** AA 50μg/ml, βGP 10 mM, Dex 1μM	**mRNA**: Oct-4, Runx2; **protein**: Oct-4, SSEA4, OCN; **in vivo** 35 days, **HE**, **AR**	Bielby et al, 2004 [212]

HESC: H9 **F:** MEF	**EB**: 20%SR 5days, direct plating, after 2 days **OG**: AA, βGP, 100nM Dex.	**mRNA**: ALP, Runx2, OCN **protein**: Stro1, ALP, OCN	Cao et al, 2005 [213]
HESC: CHA-hES3 **F:** STO cells	**EB:** 5%SR 3 days, **CC:** hOBL prim	**mRNA**: Col I, Runx2, BSP, ALP, OCN, Oct-4; **protein:** Col I, OCN. **FACS**: OCN, **AR**	Ahn et al, 2006 [214]
HESC: H9 **F:** MEF	**SD**: 10^5c/cm^2, 10%FBS, **i)** **EB:** 20%SR 5days, OG 35 days, **ii)** OG: AA, βGP, Dex.	**protein: EM** for collagen fibers and mineral; ALP, OCN; **vonKossa**, **TC, FTIR**	Karp et al., 2006 [215]

Abbreviations: AA – ascorbic acid, AB – Alcian Blue staining, AR – Alizarin Red S staining, βGP – β-glycerophosphate, CC – co-culture, CM – conditioned medium, CPA – cell proliferation assay, Dex – dexamethasone, EB – embryoid body, F – feeders, FACS – fluorescence activated cell sorting, HD – hanging drop, HE – hematoxylin-eosin staining, hOBL – human primary osteoblasts, MEF – mouse embryonic fibroblasts, OG – osteogenic induction, RA – retinoic acid, SD – seeding density, SR – serum replacement, TC – tetracycline staining, vitD3 – 1,25-hydroxy vitamin D3, vonKossa – von Kossa staining.

The first study investigating the osteogenic differentiation of ESCs was based on mouse ESCs and was published in 2001 [202]. One of the initial strategies to derive differentiated tissues from mouse ESCs was the formation of EBs [203-205, 212]. Two methods have been used for EB induction, the hanging drop method and in suspension culture on non-adherent plates. Routinely, after culture for 4–6 days, the EBs were plated onto tissue culture dishes and subsequent differentiation was often promoted in monolayer conditions by supplementing the medium with FBS. In particular, it has been shown that the addition of supplements such as βGP, ascorbic acid (AA), Dex, and 1,25-dihydroxy vitamin D3 (vitD3) resulted in the increased differentiation along the osteogenic pathway, whereas the supplementation with BMP2 and TGFβ1 directed the differentiation along the chondrogenic pathway. Besides the traditional osteogenic supplements, retinoic acid (RA) treatment during the EB phase has been used, and the inclusion of compactin was demonstrated to increase the number of mineralized nodules [203]. More recently a study aimed at defining some key factors that drive mouse ESCs into specialized mesenchymal fates. The approach was based on classical RA treatment, followed by BMP and TGFβ3 exposure [206]. The authors demonstrated that Dex/βGP/AA were necessary for calcium deposition in EB outgrowths. Moreover, the differentiation of osteoblasts from mouse ESCs without the generation of EBs was lately reported [209]. Microarray studies on mouse ESCs, which had been stimulated

with serum-containing culture medium supplemented with βGP, AA, and Dex revealed a combination of up-regulated genes involved in osteoblast differentiation (OPN, IGF-II), and a down-regulation of those genes that were involved in the differentiation of other phenotypes, such as neuroectoderm [205]. In addition, using an antibody against cadherin-11, the authors purified a subpopulation of cells with osteoblastic characteristics.

Another approach to differentiate ESCs has come from using co-culture systems. In order to promote potential osteoblast formation, the mouse ESCs were cultured together with fetal murine osteoblasts [202]. Recently, a study with HESCs by co-culture with primary human osteoblasts was reported [214].

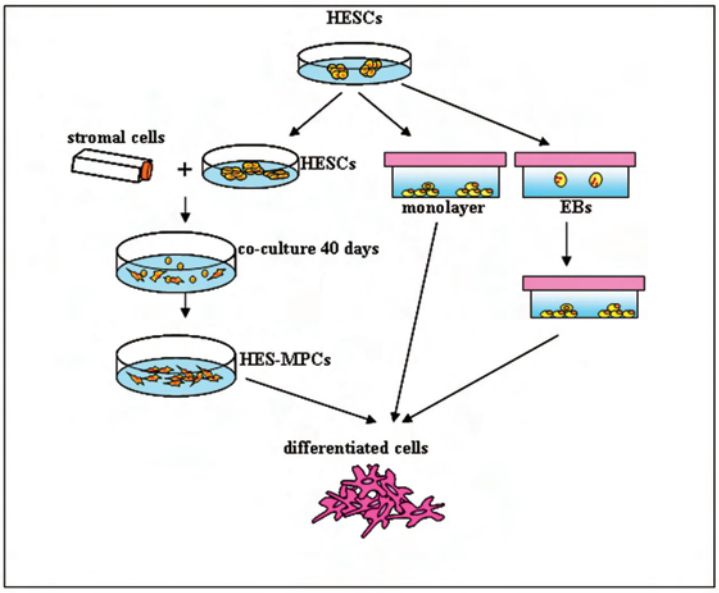
In most stem cell osteogenic *in vitro* models, bone-like nodules tend to develop after a certain time in culture, yet, there is no agreement if all HESC lines are able to form bone-like nodules. Conflicting reports exist as to whether, for example human MSCs form nodules or if instead the matrix represents a diffusely mineralized network [5, 216].

In general, only a few studies have been published with HESCs. In these experiments the differentiation of HESCs was induced by EB formation in 10% FBS, however differentiation by omitting the EB generation step was also recently reported [215]. Osteogenic induction was often performed with AA, βGP and Dex, three supplements that have been extensively used in studies investigating the formation of bone-like mineralized matrices *in vitro* (for review [186]). It is generally accepted that AA promotes the proliferation and differentiation of cells, and induces the synthesis of collagen, whereas βGP is a precursor to inorganic phosphate and has been shown to induce the nuclear export of Runx2 and lower the expression of OCN in mouse osteoblastic cells [217]. Glucocorticoids, such as Dex, affect both the nodule formation [186] and induce the osteoblastic gene expression [218]. However, the stimulatory effect of these three factors is not limited just to osteoblasts, and when the process of osteogenesis is induced using HESCs, cells from other lineages can be generated, too [219-221].

Following cell culture, in order to detect successful osteogenesis, the most common method seems to be the histochemical staining for the detection of calcium deposition using AR and von Kossa staining. Several studies have used tetracycline (TC) incorporation into the developing bone-like nodules. However, it must be noted that neither ALP activity nor calcium deposition is an exclusive feature of osteogenic cells and can be misleading in studies with ESCs.

Taken together, all these models still lack defined conditions for the differentiation and isolation of pure osteogenic precursor populations.

Figure 4. Schematic overview of currently used differentiation techniques to drive osteogenic differentiation of HESCs. (HES-MPCs – human embryonic stem cell derived mesenchymal progenitors).



1.3.9.1 Differentiating ESCs to osteoblasts through mesenchymal precursors

Two distinct strategies have been followed in the field of osteogenic differentiation from ESCs; first and the most often followed approach is the induction of differentiation directly from ESCs, and the second approach involves a pre-differentiation step into mesenchymal progenitors prior to the induction of the desired cell lineages. Some authors state that the length of time that the differentiating ESCs are maintained in culture (~ 21 days) is in accordance with the time scale for the osteogenic differentiation pathway of murine and human primary osteoblasts, and MSC cultures [202]. However, these are more committed progenitor cells than the ESCs. Thus the strategy based on the initial isolation of multipotent mesenchymal precursor cell populations rather than specific mesenchymal derivatives has gained attention.

Induction of mesodermal fates from mouse ESCs follows a highly reproducible and stereoptypic time-frame. In that the undifferentiated ESCs reproduce the

developmental stages during *in vitro* differentiation, an example was shown by the appearance of neuronal, hematopoietic and cardiac mesoderm (reviewed [222]). A pure population of mesenchymal precursors can be isolated using fluorescence activated cell sorting (FACS)-based isolation of CD73+ progeny, [223], or other conditions [224], and the cells could then be further expanded as mesenchymal precursors or differentiated into various mesenchymal derivatives. Interestingly, cells isolated under these conditions support the undifferentiated growth of HESCs [224]. In order to obtain pure mesenchymal precursors from HESCs, the cells require pre-differentiation in co-culture with the stromal cell line (OP9) for 40 days (Figure 4). Thereafter, the mesenchymal precursor cells could be isolated by FACS based on the expression of CD73, a marker routinely used for the isolation of adult bone marrow-derived MSCs. The resulting CD73+ population could be proliferated or differentiated further. Such cultures do not express detectable levels of ESC-specific markers, such as Nanog and Oct-4, and work with severe combined immunodeficient (SCID)/Beige mice suggested that they do not form teratomas *in vivo* [222].

2 AIMS OF THE PRESENT INVESTIGATION

The overall aim of the project was to study the process of osteoblastic differentiation in HESCs.

Specific aims:

- To investigate the molecular processes that occur during bone formation in the primary ossification center *in vivo*, using global gene expression analysis;

- To characterize bone matrix formation within the HESC model of osteogenesis by:
  - comparing various differentiation methods,
  - comparing more than two HESC lines,
  - examining the expression of both early and late osteoblast-related genes that are generally acknowledged as marker genes,
  - characterizing the synthesis of bone matrix proteins into bone-like nodules,
  - investigating whether the mineralization mechanism is occurring through a cell-mediated processes,

- To further elucidate the dynamics of bone marker gene expression, in order to establish a time-frame for supplementation with growth factors, so that a more defined/directed differentiation model can be established.

- To investigate the gain-of-function effects of an early osteoblast-related gene in order to enhance the osteogenic differentiation potential of HESCs.

- To establish a protocol for stable transgene expression for mineralized tissue formation within the HESC model.

3 MATERIALS AND METHODS

A more detailed description of Materials and Methods can be found in the individual papers. Here, only a summary is given and specific issues are discussed.

3.1 *IN VIVO* MODEL FOR OSTEOGENESIS (PAPER I)

The key to successful characterization of any cell type is the information obtained from *in vivo* conditions. In order to establish the global gene expression profile during early ossification processes, we studied two specific time-points during endochondral long bone development, representing the conditions before and after the onset of mineralization. For **tissue preparation,** mice from NMR1 strain (Scanbur BK AB, Stockholm, Sweden) were used. Central mouse metatarsals were dissected free under sterile conditions on embryonic days 15 (E15) and 19 (E19), and **total RNA** was extracted. **Microarray gene expression analysis** was performed using the GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Inc., Santa Clara, CA, USA). The analysis, including RNA processing, labeling and hybridization to the GeneChips®, was performed at the Bioinformatics and Expression Analysis core facility at NOVUM, Karolinska Institutet, Sweden.

In addition, entire mouse paws from E15 and E19, were stained with **Masson's Trichrome** in order to identify the synthesized collagenous matrix, and to show the presence of preosteoclasts and osteoclasts, sections were stained for tartrate-resistant acid phosphatase (TRAP) with **fast red violet**.

3.2 OSTEOGENIC DIFFERENTIATION OF HESCS *IN VITRO* (PAPERS II, III, IV)

3.2.1 HESC culture maintenance

HESC lines HS181, HS237, HS306 (from Karolinska Institutet Stem Cell Network, Stockholm, Sweden), and commercially available H9 cell line (from WiCell Research Institute, Madison, WI) with a normal karyotype were used. The HESCs were maintained in medium that consisted of KO-DMEM containing 17% KO-SR, 1 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β- mercaptoethanol and 4-6 ng/ml of bFGF. Cells were kept in an undifferentiated state at 37°C in a humid 6.8% CO_2 atmosphere. The cultured HESCs were checked visually and the culture medium changed daily. HESCs were passaged by incubation in collagenase (1 mg/ml) for 7–10 min at 37°C every 5–6 days or when colonies revealed signs of differentiation. HS181,

HS237, and HS306 cell lines were derived and maintained on human foreskin fibroblasts. In comparison, the H9 line was propagated on a layer of MEFs (paper II) and switched onto human fibroblasts (paper IV). **Human foreskin fibroblasts** were obtained from ATCC (CRL- 2429; ATCC, Manassas, VA), and **MEFs** were kindly provided by S. Teglund, (Institute for Biosciences, Karolinska Institutet, Sweden). The human fibroblasts were kept up to 20 passages, mouse cells up to 9 passages, and both were mitotically inactivated by irradiation at 35-40 Gy before use. The seeding density for feeder cells was ~200 000 cells/well in a six-well plate (Corning, NY), and the culture medium consisted of Iscove's modified Dulbecco's medium (IMDM) and 10% FBS. The cells were allowed to attach to the plate 3–5 days prior to use as feeder layers.

3.2.2 Control cell lines and culture conditions

Human mesenchymal stem cells (hMSCs) (paper II) and **human osteoblasts** (hOBL) (papers II, III and IV) were purchased from Cambrex Bioscience (Walkersville, Inc., MD). The epithelial line **HEK293-EBNA** and the human osteosarcoma cell line **Saos-2** (HTB-85, ATCC, Manassas, VA) were used in papers III and IV. The **293FT** (Invitrogen) lentivirus packaging cell line and **Hela** cells were used in paper IV. All cell lines were kept in medium consisting of DMEM (high glucose) containing 1 mM L-glutamine and 10% FBS. The **K562** cell line (LGC Promochem/ ATCC) used in paper IV was cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS.

The cells were incubated at 37°C in a humid 5% CO_2 atmosphere, and the medium was changed twice a week.

For differentiation purposes the cells were dissociated to single cells and ~5000 cells/cm^2 were seeded onto tissue culture plates. Basal medium was supplemented with βGP, AA, and Dex. The control cultures were maintained for 15 days, because by day 25 true osteogenic cells had become fully mineralized, thus making it impossible to extract RNA.

3.2.3 Osteogenic differentiation *in vitro*

To initiate cell differentiation in **monolayer**, the HESCs were removed from the culture dish by incubation with collagenase solution and mechanical scraping from the culture plate. Approximately 5 colonies of 400 cells each (1000 cells/cm^2) were seeded onto gelatin-coated 24-well-plates. **EB formation** was induced in the HESC colonies by again enzymatically detaching the cells with collagenase and transferring

them to bacterial non-adherent culture dishes. On day 6, the EBs were dissociated and plated on gelatin-coated tissue culture plates. Osteogenic differentiation was induced by adding 20% FBS instead of KO-SR to the basal medium and supplementing with 10 mM βGP, 50 μg/ml AA, and 1 μM Dex. The cultures were maintained for 25 days and the medium was changed every second day.

For treatment with growth factors (paper III), recombinant human (rh)-BMP2 (100ng/ml) and rhVEGF (vascular endothelial growth factor) (25ng/ml) were used. The concentrations were based on data published in the literature. Both growth factors were added to the cultures along with the osteogenic differentiation media from day 10.

In order to analyze the effect of cellular density in gene-modified HESC osteogenic differentiation cultures (paper IV), the cells were split in a 1:2 ratio after 7 days, and the cultures were continued as described.

3.2.4 Cellular proliferation and metabolic activity (paper III)

Cell number was counted after dissociating the differentiating cells into single cells on days 3, 7, 10, 17, 21 and 25 of the experiments, and 4 wells of a 24-well plate were assessed. **Cellular metabolic activity** was determined by measuring with MTT colorimetric assay (Roche Molecular Biochemicals) at days 0, 7, 13, 19 and 25. Cellular activity of cells that had been osteogenically-treated was normalized to the undifferentiated HS181 cells, which were considered as 100% active.

3.2.5 Assessment of osteogenic phenotype

Osteoblastic differentiation potential of HESCs was assessed as presented in Table 2.

Table 2. Methods for assessment of *in vitro* osteogenic differentiation potential of HESCs

Phenotype detection	Methods	Study
RNA level	**Sq-RT-PCR:**	
	a) Flt-1, T-Brachyury	Paper II
	b) BMP4, Runx2, OSX, OCN, BSP, OPN, Col I, PTHR1	Paper II

	Q-RT-PCR:	
	a) OSX, OCN, BSP, OSAD, PTHR1, Col I, OPN, ON	Paper III
	b) OSX, Col I, BSP, OCN	Paper IV
Protein level	**Western blot:**	
	OSX, Col I, BSP, OSAD	Paper III
	IHC:	
	BMP4, BSP, OCN	Paper II
Mineralization	**FTIR**	Paper II
	Alizarin Red S	Paper II, IV
	Alcian Blue	Paper II

3.2.5.1 cDNA synthesis, semi-quantitative reverse transcriptase polymerase chain reaction (Sq-RT-PCR), and quantitative RT-PCR

In paper 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 paper III, the total RNA was extracted from undifferentiated (day 0) and differentiating HS181 cells (after 36h, 72h, and every other day from day 5 to 25 in culture). Total RNA was also extracted from irradiated human fibroblasts, hOBL at day 0, hOBL and hMSCs grown with and without osteogenic supplements at day 15, and Saos-2 and HEK293-EBNA cells. In paper IV, total RNA was extracted from the undifferentiated and differentiated H9 cell line, and hOBLs, modified and unmodified Hela cells, purified CD34+ cells, and human umbilical cord CD31+/CD34+ endothelial cells. cDNA from primary hMSCs was kindly provided by Dr M.Uzunel (Department of Clinical Immunology, Karolinska University Hospital Huddinge, Stockholm, Sweden). cDNA from human primary osteoblasts was kindly provided by Dr T.Lind (Department of Medical and Physiological Chemistry, Uppsala University, The Biomedical Center, Uppsala, Sweden).

Sq-RT-PCR was performed using PCR Core Kit (Roche Diagnostics Scandinavia AB), and quantitative RT-PCR (Q-PCR) was carried out using human TaqMan Gene Expression Assays from Applied Biosystems (Foster City, CA). Additionally human HoxB4 and human Gata1 were analysed in paper IV. Amplification of BSP and OCN in paper IV was carried out using the SYBR® GREEN Master Mix (Applied Biosystems) in the reactions with similar specific primers as described in paper II.

The comparative cycle threshold (Ct) method was used to analyze data, hydroxymethylbilane synthase (HMBS) was used to standardize the Ct values, and undifferentiated HS181 (paper III) or H9 (paper IV) were used to calibrate the values of differentiating HESCs.

3.2.5.2 SDS-PAGE and Western blot (paper III and IV)

Cells were lysed using TRIzol reagent and protein extracts were quantified. Each sample was electrophoresed on a 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 HoxB4 modified and unmodified Hela cells in paper IV, the total protein was extracted from unmodified K562 and Hela cells, 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).

3.2.5.3 Histological and immunocytochemical studies

As an indicator of mineralization within the HESC cultures, calcium deposition was analyzed by **AR** staining in papers II and IV. The calcium salt crystals within the bone-like nodules stain dark red, while the collagenous 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 sediments within the cell culture.

The synthesis of glycosaminoglycans (GAGs) was analyzed by **Alcian Blue** staining in paper II, which is a widely used method based on copper. The stained parts are blue; however the specificity can be manipulated by 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 paper II. However, no positive signal was detected in osteogenically differentiated HESC cultures under these experimental circumstances.

For **immunocytochemical analysis**, the cell cultures described in paper II were fixed, rinsed and treated with 0.2 M HCl and 3% H_2O_2 to clean the epitopes. The antibodies were non-specifically blocked with 4% normal goat serum (DAKO) and the cells were incubated with primary antibodies directed against human tissue diluted in 4% normal goat serum at 4°C overnight followed by incubation for 1 h at room temperature with the corresponding secondary antibody prepared in blocking solution. After washing with Tris buffered saline (TBS), the samples were mounted with Vectashield containing DAPI (Vector labs Inc., Burlingame, CA). For the detection of BSP and OCN antibodies, 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 glycerol. Controls for primary and secondary antibodies revealed neither non-specific staining nor antibody cross-reactivity.

To investigate if pluripotent cells remained within the differentiating HESC cultures in paper III, the cultures were fixed, rinsed, treated with 0.2M HCl and 3% H_2O_2. The antibodies were non-specifically blocked with phosphate buffered saline (PBS), 3% BSA (Fraction V, Sigma-Aldrich), 0.1% Tween-20, 0.1% bovine serum albumin (BSA)-c (Aurion) for 40min. After blocking, the cells were incubated with primary antibody diluted in PBS, 0.1% Tween-20, 0.1% BSA-c for 1h. The cells were washed with PBS, 0.01% Tween-20, 0.01% BSA-c and incubated for 1h at room temperature with fluorescent labeled secondary antibody. After washing again with PBS, the samples were mounted with Vectashield containing DAPI.

3.2.5.4 Fourier-transform infrared spectroscopic analyzes (paper II)

The crystalline structure 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 TBS, treated with the buffer containing 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, and 0.1% Triton X-100. After centrifugation, the supernatant was removed and 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.

3.2.6 Lentiviral transgene expression (paper IV)

3.2.6.1 Construction and production of lentiviral vectors

OSX cDNA, originally cloned into the TOPO vector was PCR-amplified, and inserted into pEGFP-N1 vector (Clontech, Palo Alto, CA) in order to create the fusion with EGFP (enhanced green fluorescent protein). Thereafter, the OSX-EGFP fusion sequence was cleaved from the pEGFP-N1-OSX construct and ligated into the same sites of the Gateway plasmid pENTR4 (Invitrogen). In order to create the final lentiviral vector, the construct was transferred into the lentiviral backbone pLenti6/UbC/V5-DEST (Invitrogen). The EGFP-control plasmid was constructed by subcloning the EGFP gene from pEGFP-N1 into the Gateway plasmid pENTR4, and was recombined with the lentiviral backbone pLenti6/UbC/V5-DEST.

Human genomic HoxB4 was cloned from extracted donor buffycoat DNA, subcloned into pEGFP-C3 (Clontech), E2A sequence was inserted between EGFP and HoxB4, and subcloned into the Gateway pENTR4 plasmid. To obtain the final lentiviral transfer vector, the EGFP-E2a-HoxB4 fusion gene in pENTR4 was recombined with pLenti6/UbC/V5-DEST using the Gateway LR reaction. The DNA structure in the individual steps was tested by restriction enzyme analysis and the final constructs were verified by sequencing.

Virus was produced by transient co-transfection of three plasmids into 293FT cells; an envelope plasmid pMDG harbouring the gene encoding vesicular stomatitis virus glycoprotein (VSV-G), pCMV_R8.91 expressing Gag and Pol, and our transfer vector constructs.

Viral titers (TU/ml) were determined by transduction of Hela cells with serial dilutions of the viral supernatant and flow cytometric analysis of the percentage of EGFP-expressing cells. For transduction with lentiviral vectors, passages between 35-50 H9 HESCs were used. Blasticidin resistant feeder cells were specifically prepared for this study by transducing the human fibroblasts with pLenti6/UbC/V5-GW-lacZ control vector (Invitrogen) followed by chemical selection with blasticidin.

3.2.6.2 Flow cytometric analysis

The cells were washed with PBS and dissociated with TrypLE Express (Invitrogen). Fluorescent-labeled monoclonal antibodies: CD34-Allophycocyanine (APC), CD31- Phycoerythrin (PE) and their corresponding isotype controls (BD Biosciences) were used to detect the hemato-endothelial phenotype. For detection of pluripotency SSEA3, and TRA1-60 (kindly provided by Mark Jones from the lab of Peter Andrews, The University of Sheffield, Sheffield, UK) were used. All antibodies were previously optimized in terms of their concentration. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA). Acquisition and analysis was performed using BD CellQuest™ Pro (BD Biosciences) and FlowJo (Tree Star, Ashland, OR, USA) software.

4 RESULTS AND DISCUSSION

4.1 PAPER I

An individual gene expression pattern is demarcating to each phase during endochondral bone formation: condensation of mesenchymal progenitors, chondrocyte differentiation with the eventual vascular invasion followed by osteoblast differentiation. In order to obtain the best overview of such dynamic changes in gene expression pattern we used microarray analysis. Two time points and mouse metatarsal bones were chosen to model the formation of the primary ossification center *in vivo*. At embryonic stage, E15 an avascular cartilage anlagen and pre-hypertrophic chondrocytes in the diaphysis were detected, whereas by E19 the formation of the primary ossification center and a primary marrow cavity could be seen.

Gene expression analysis of the total RNA isolated from mouse metatarsals embryonic stages E15 and E19 identified 1285 genes, of which 543 were up-regulated on E19 compared to E15, and 742 were down-regulated (selection criteria: 2-fold change, P value of <0.005). Analysis of the data followed the gene ontology categories **Biological Process**, **Cellular Component** and Molecular Function, however this study focused on the two first categories. In summary, the gene expression data followed the expected scheme for developmental progression of osteogenesis. We found that Hoxd genes 10–12, Gli2 and Noggin were down-regulated post-mineralization (E19). No change in gene expression was identified for BMP2,-4,-5 and -7. TGF-β1 and BSP were highly up-regulated from E15 to E19, as well as OPN and DMP1. There was a 7.8-fold increase in OCN levels, a marker for terminally differentiated osteoblasts.

However, within the Cellular Component classifications, a large number of genes related to bone remodeling predominantly featured. They included a number of proteases, such as matrix metalloproteinases, TIMP 1 (tissue inhibitor of matrix metalloproteinase), and cathepsin K. The presence of these enzymes demonstrates the full differentiation and activation of osteoclasts, which was also observed in the TRAP positive cells at E19. Structural molecules like the SLRP family; fibromodulin, biglycan, asporin, and decorin were up-regulated. Using the metatarsal long bone model we were able to identify and examine the genes associated with the formation of the primary ossification center in an *in vivo* system.

4.2 PAPER II

The derivation and establishment of culture systems for HESC lines provided us with a novel model system by which investigate the process of osteogenesis within a distinct environment. The focus of the study II was to examine the capacity of HESCs to differentiate towards the osteoblastic lineage and their subsequent ability to form a mineralized ECM. A selection of marker genes defining osteogenesis, which were identified from study I were used, reaching from the earliest progenitor cells to the differentiated osteoblasts. Four pluripotent HESC cell lines were studied and two methods were used to initiate differentiation, first by plating the HESCs in monolayer onto gelatin-coated plates, and, second, initiating the differentiation within EBs. The cells were allowed to differentiate further in the presence of Dex, AA, and βGP. Novel to our study was the use of HESC cell lines (HS181, HS237, and HS306) derived and maintained on commercially available human foreskin fibroblasts to support the undifferentiated growth of the HESC cell colonies.

We ensured that the HESCs followed a typical differentiation pathway from early mesodermal progenitors to the fully differentiated osteoblastic phenotype. Monolayer cultures exhibited similar levels of T-Brachyury expression examined in the two cell lines (H9 and HS181). However, following growth within EBs, the levels of T-Brachyury declined in the H9 line earlier than the HS181 cells. Immunohistochemical staining against human BMP4 in the osteogenic-induced monolayer cultures demonstrated that the signal was specifically localized to the cells aggregating to form eventual bone-like nodules. In the HS181 monolayer culture, the levels of BMP4 gene expression increased earlier compared to H9, correlating also with the formation of larger bone-like nodules. Screening for the osteoblast-specific gene mRNAs demonstrated that the markers were detected in all HESC cell lines, and within both monolayer and EB-derived cultures. It was observed in our study that the highest levels of OSX expression were accompanied by raised levels of BSP and OCN. The SqRT-PCR analysis also showed that BSP and OCN were expressed to a higher degree in monolayer cultures, whereas the EB-derived cultures revealed more variable expression levels.

It is known that in *in vitro* cultures, it is often hard to distinguish between cell-mediated calcification and dystrophic calcium depositions. In the current study, mineral deposition in the ECM was assessed by AR staining, and positive staining was detected in all the cell lines examined. In order to further examine whether the deposited calcium phosphate is similar to the biological apatite crystalline form, as found in *de*

novo bone, the samples were also analyzed by FTIR spectroscopy. This method provided confirmatory information at the biochemical level that indeed the mineral phase within the osteogenic cultures resembled a crystalline apatite, which had been formed by a cell-mediated calcification process.

Taken together, we were able to show that the cultures differentiated towards the osteogenic lineage, however some differences were apparent between the gene expression patterns for the bone matrix markers, which were dependent on the method used to induce differentiation and between the cell lines. Overall, cells cultured in monolayer conditions revealed higher levels of osteoblastic markers, whereas the EB-derived cultures displayed generally lower levels of expression. We concluded that lineage potential is not dependent on the mode of differentiation induction but on a cell line itself.

4.3 PAPER III

In the third paper, we tried to analyze further the standard model system for osteogenesis of HESCs in order to establish the expression profile of bone-related genes during differentiation triggered by supplementing the medium with AA, β-GP and Dex, three factors which are widely used to trigger osteogenesis from HESCs. Based on our pilot studies and previously published work (paper II), we established that the initial cell density plays an important role in differentiation. The optimal seeding density for osteogenic HESC cultures (HS181 cell line) was about ~1000 cells/cm^2. Such cultures reached confluency 7-8 days after seeding, followed by the up-regulation of the bone specific transcription factor, OSX. We believe that such density provides the cells with enough space to proliferate until reaching cell-cell contact at confluency, followed by the interaction with the produced ECM to switch on the optimal signaling pathways. We show that the experimental period needed to induce the expression of the latest osteoblast marker, OCN, was 25 days. In addition, we show that "osteogenically" treated cultures retain a potentially undifferentiated population of cells.

Osteoblastic development is usually subdivided into certain developmental stages: proliferation and differentiation of cells, and ECM synthesis, maturation and mineralization. In this study, we used an alternative approach to the HESC osteogenic model, and considered separately the cellular compartment activity on one side, and matrix formation and mineralization on the other. We believe **the first regulatory transition,** triggering the initiation of osteoblastic gene expression, takes place after the active proliferation step even though several ECM-associated gene mRNAs were

expressed in actively proliferating immature cells. We show that at the end of active proliferation, the osteoblast-specific transcription factor OSX was up-regulated suggesting that its expression was regulated by the onset of contact-inhibition and its function preceedes matrix maturation. ON, a major non-collagenous component of bone was up-regulated straight after the end of the proliferative phase. Another currently believed mineralizing tissue-specific NCP, OSAD was expressed at the beginning of the culture period, supporting the possibility that it has a role in inhibiting the actively proliferating cells. However, OSAD is also associated with the terminally differentiated osteoblastic phenotype and to our knowledge it is so far considered as osteoblast-specific. **The second regulatory transition** mediates the initiation of gene expression for ECM formation, maturation and mineralization. OPN gene expression was progressively down-regulated towards the end of the culture, which is in agreement with the reports that low OPN levels are required for apatite crystal growth.

Q-PCR analysis revealed that OCN was expressed at the end of matrix maturation, being rapidly down-regulated before mineralization, but thereafter increased again. PTHR1, receptor for PTH and parathyroid hormone-like hormone, was up-regulated during matrix maturation. PTHR1 has been described as a "globally" expressed marker for osteoblastic cells, whereas OPN, BSP, and OCN can be differentially expressed at mRNA and protein levels in only a subset of osteoblasts, depending on the maturational state of the cells.

The direction of differentiation towards osteogenic lineage with growth factors are essential to either increase the outcome of osteoblastic cells or decrease the presence of other cell types. Due to the specificity of HESCs as an undifferentiated and pluripotent system, the timing is of utmost importance. Here, our results showed that HESCs seeded at 1000cells/cm^2, reached confluency around day 7-8, followed by the up-regulation of OSX.

VEGF-treated cells demonstrated down-regulated levels of known osteoblast associated mRNAs. However, we also show that inclusion of BMP2 rescued expression, which could be due to the fact that during osteogenic lineage progression, in addition to the BMP pathway, several other signal transduction pathways mediate osteoblastic gene expression. The combined addition of both growth factors demonstrated that BMP2 decreased the inhibitory effect of VEGF on most of the bone-related gene mRNAs. OSX, OCN and OSAD all showed increased expression levels compared to levels in the VEGF-treated cells. Addition of BMP2 induced an earlier significant up-regulation of BSP compared to "osteogenically"-treated cells. The

finding that OCN was not significantly increased by BMP2, could be because OCN is expressed at low levels in the young bone, where BSP along with other acidic phosphoproteins are expressed at high levels. The overall higher expression of OSX and BSP, indicative of immature mineralized tissue formation confirms that assumption. Perhaps, continuation of the culture period would have exposed an increased expression level for OCN. Interestingly, the combination of growth factors had an inhibitory effect on BSP expression throughout the culture time. A similar observation was reported in another study where a cross-communication between the two pathways was suggested.

4.4 PAPER IV

The aim of the paper IV was to study whether ectopic expression of an early bone-specific gene could enhance HESC differentiation towards the osteoblastic lineage. We used a lentiviral vector-based system, which has previously been reported to be less affected by gene silencing during HESC differentiation, and evaluated the effects of gain of function of OSX, currently recognized as the earliest bone-specific transcription factor. The transcription factor OSX has been identified as a crucial regulator of osteogenesis and is predominantly expressed by early osteoblastic cells. OSX-deficient mice show a complete lack of osteoblast differentiation, and no endochondral or intramembranous bone formation can be detected. To evaluate the effects of the forced expression of OSX, we established a HESC line stably expressing the transgene under the control of the Ubiquitin promoter to enhance the directed differentiation into osteoblasts. However, it was not the main aim of the study to focus on the analysis of osteogenesis. Within the study, we also included the analysis of another transcription factor, HoxB4, which is an early hematopoietic transcription factor. This factor was ectopically expressed in a similar lentiviral system. The transduction of HESCs resulted in two HESC populations exhibiting different levels of expression, which were compared to naturally occurring levels. We show that the expression of OSX at low levels induced the transcription of endogenous HoxB4. Furthermore, the up-regulated levels of mineralization-associated gene mRNAs, such as collagen I, BSP and OCN, by high HoxB4 could also indicate a role for HoxB4 during pathological mineralization, perhaps similar to that found in blood vessels. Our findings support the notion of cell-cell-interactions between early preosteoblasts and HSCs on the bone marrow endosteal surface, required for hematopoiesis. We concluded that for an enhanced osteogenesis originating from *in vitro* cultured HESC,

the correct levels of ectopic transcription factors need to be established. Our data also highlights the notion of a close relationship between early blood and bone development.

5 CONCLUSIONS

In this thesis my goal was to study the osteoblastic differentiation potential in HESCs and to establish the model of ostegenesis in HESCs. The specific conclusions are the following:

Paper I

- We concluded that the metatarsal long bone model is a valuable and reliable tool for examining the genes associated with the formation of the primary ossification center.

Paper II

- All HESC lines are able to express osteoblast-related gene mRNAs, but the differentiation capacity towards the osteoblastic lineage is dependent on the cell line.
- Initiation of the differentiation process through EB formation is not necessary in HESCs.
- The mineralization of the ECM is a cell-mediated calcification process.

Paper III

- We characterized the step-by-step expression profile of bone-associated genes.
- We identified the time-frame for further supplementation with growth factors.
- We established that two distinguishable phases occur during osteogenesis within the HESC model that differ from the standard osteogenesis model characterized by progenitor cells. Firstly, there is the cellular proliferation and secretion of pre-maturational matrix stage that is needed for cell migration, and second, the appearance of osteoprogenitors with characteristic ECM synthesis.

Paper IV

- Lentiviral expression system is an efficient method to study osteoblast-associated transgene expression in HESCs.
- We found that for enhanced osteogenesis originating from *in vitro* cultured HESCs, the correct levels of ectopic transcription factors need to be established.
- Our data adds additional confirmation of a close relationship between early blood and bone development.

6 FUTURE PERSPECTIVES

In summary, the methods described in this thesis clearly demonstrate that HESCs can be differentiated towards the osteoblastic lineage. However, it is important to compare the derivation and differentiation potential towards osteogenic cells among a larger numbers of HESC lines. The effect of growth factors, either alone or in combination with the three classical osteogenic supplements is important to investigate. Furthermore, the effect of a total ECM extract or its single substances on osteoblastic gene expression needs to be further examined.

One interesting future perspective is the use of HESC lines in functional studies *in vivo*, exploiting various animal models of musculoskeletal diseases.

We have shown that cells with a potential pluripotent phenotype remain present within the osteoblastically differentiated HESC cultures. An important concern for clinical applications of HESC-derived progeny in regenerative medicine is the risk of teratoma formation due to the presence of residual undifferentiated ESCs among the differentiated progeny. Thus, more studies are needed in order to sort the osteoprogenitor cells and/or eliminate the potential multipotential cells.

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