From the Department of Laboratory Medicine,
Clinical Research Center, Unit for Molecular Embryology
Karolinska Institutet, Stockholm, Sweden

Human Embryonic Stem Cells; A novel model system for early human development

Karin Gertow

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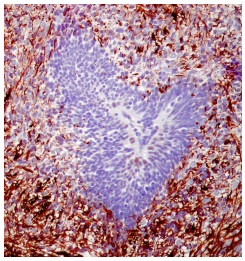

...Brains and big hearts. All of you who have supported me in the becoming of this thesis, have shown great presence of both.

ABSTRACT

Human embryonic stem cells (HESC) have since their first description in 1998 been recognized as a theoretically endless source of cells capable of differentiation into any somatic cell type. Although great attention has been given to their potential use in cell-based therapy, they are equally important as research tools for studies on early human development, both normal and diseased. This thesis provides basic knowledge of HESC and supports their relevant use as a model system for early human development.

HESC are differentiating spontaneously into all three embryonic germ layers (ectoderm, mesoderm and endoderm) *in vitro,* and when xeno-grafted to immunodeficient mice they grow *in vivo* as teratomas. In paper I, the potential of HESC differentiation *in vivo* described and their interaction with the host tissue was for the first time explored. We could show that undifferentiated HESC formed highly organized, even organoid structures, composed of multiple cell types originating from all germ layers. Examples are renal development composed of tubules and glomeruli with associated vascular supply; neural ganglia containing glial cells, and nerves with synapsoid connections. Intestinal structures were found, with basally located proliferative stem cells, goblet cells and smooth muscle layers; as well as skin including keratinized cells and glands. Importantly, HESC derived cells were functionally integrated with the host tissue. Blood carrying compound human/mouse vascularisation was found, concluding that HESC derived vessels anastomosed with the host vascularisation. In the following study (paper II), the kinetic progression of HESC differentiation *in vivo* was followed. Despite the absence of accurate environmental cues, HESC launched a developmental program with many similarities to normal development. For instance, indications were found for gastrulating events and progressive maturation of the tissues, similar to organogenesis. Appearance of human vascularisation (day 20/30) was coupled to a rapid net expansion of the teratoma, suggesting that growth was hampered up to this point. By day 45, more organized structures were apparent; however not until day 60 could for instance mature neurons (NFP) be detected.
Already at the first observation point day 5 after grafting, we observed HESC derived epithelia reminiscent of the epiblast or primitive ectoderm. Strong support for an early neuroepithelial origin of such structures was also found. Although indications point towards remaining pluripotent cells throughout the study, the early and to a great extent dominating finding of neuroepithelia, raises questions regarding the origin of all other non-ectodermal tissues.

The chromosomal integrity of HESC is a concern for future therapeutic interventions but also a possibility for studies of human genetic disease and tumor progression. We studied the chromosomal stability and found the karyotype to be affected by culture conditions. An advertant sub-line selected for feeder independent growth resulted in a variant exhibiting i12p and 7q deletion in 100% of the cells (paper II). This variant showed pluripotency *in vitro*, but formed no teratomas *in vivo*. Variants obtained after bulk expansions were found trisomic for chromosome 12 (paper IV). Such cells appeared pluripotent *in vitro* and *in vivo,* but gave a significantly higher frequency of renal development *in vivo* as compared to the parental diploid line. Interestingly, chromosome 12 changes are frequent findings in germ cell tumours.
All together these studies highlight differences between HESC phenotype *in vitro* and *in vivo* and thereby the importance of studies *in vivo.* Furthermore this thesis supports the relevant use of HESC as a model system for early human development.

LIST OF PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals.

I. **Gertow K,** Wolbank S, Rozell B, Sugars R, Andäng M, Parish CL, Imreh MP, Wendel M, Ährlund-Richter L. Organised development from human embryonic stem cells after injection into immunodeficient mice.
Stem Cells and Development 2004;13:421-435

II. **Gertow K,** Cedervall J, Bogdanovic N, Szöke K, Kärner E, Imreh MP, Ährlund-Richter L. Kinetics of *In Vivo* HESC Development In a Xeno-Graft Model Mimics Human Early Developmental processes.
Manuscript

III. Imreh MP*, **Gertow K*,** Cedervall J, Unger C, Holmberg K, Szöke K, Csöregh L, Fried F, Dilber J, Blennow E, Ährlund-Richter L. *These authors contributed equally. *In vitro* culture conditions favoring selection of chromosomal abnormalities in human ES cells
Journal of Cellular Biochemistry, in press 2006

IV. **Gertow K,** Cedervall J, Unger C, Szöke K, Blennow E, Imreh M, Ährlund-Richter L. HESC with trisomy 12 exhibit growth advantage *in vitro*, but are selected against following transplantation *in vivo*.
Manuscript submitted

V. Imreh MP*, Wolbank S*, Unger C, **Gertow K,** Aints A, Szeles A, Imreh S, Hovatta O, Fried G, Dilber S, Ährlund-Richter L. * These authors contributed equally. Culture and Expansion of the Human Embryonic Stem Cell Line HS181, Evaluated in a Double Color System.
Stem Cells and Development 2004;13:337-343

VI. Hovatta O, Mikkola M*, **Gertow K***, Strömberg A-M, Inzunza J, Hreinsson J, Andäng M, Ährlund-Richter L. *These authors contributed equally. A culture system using human foreskin fibroblasts as feeder cells allows production of human embryonic stem cells.
Human Reproduction 2003;18:1404-1409

VII. Inzunza J, **Gertow, K,** Strömberg AM, Matilainen E, Blennow E, Skottman H, Wolbank S, Ährlund-Richter L, Hovatta O. Derivation of human embryonic stem cell lines in serum-free medium using postnatal human fibroblasts as feeder cells.
Stem Cells 2005;23:544-549

LIST OF ABBREVIATIONS

AFP	Alfa fetoprotein
bFGF	basic fibroblast factor
BIO	Bromoindirubin- 3'-oxime
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
CD	Cluster of differentiation
CDK	Cyclin dependent kinase
C/EBPα	CCAAT-enhancer binding protein alfa
ChAT	Choline acetyl transferase
CIS	Carcinoma in situ
CK	Cytokeratin
CM	Conditioned media
CNS	Central nervous system
CTL	Cytotoxic T-lymphocyte
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
EB	Embryoid body
EC / EG	Embryonic carcinoma / Embryonic germ
E- / N-cadherin	Epithelial- / Neuronal-cadherin
ECM	Extracellular matrix
EMT	Epithelial mesenchymal transition
ESC	Embryonic stem cell
ET	Embryo transfer
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FISH	Fluorescence in sit hybridization
Flt-1	Fms-like tyrosine kinase or (VEGF-R1)
GCT	Germ cell tumour
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescence protein
GSK3β	Glycogen synthase kinase 3beta
HCG	Human chorionic gonadotropin
HE	Haematoxylin and eosin
HESC	Human embryonic stem cell
hFS	Human foreskin fibroblast
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
Hoxb4	Homeobox B4
HSC	Haematopoietic stem cell
hTERT	Human telomerase reverse transcriptase
ICM	Inner cell mass
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL-6	Interleukin-6
Ipf1	Insulin promoting factor-1

ISH	In situ hybridization
IVF	In vitro fertilization
JAK	Janus kinase
LIF	Leukemia Inhibitory Factor
LOH	Loss of heterozygosity
MHC	Major histocompatability
MEF	Mouse embryonic fibroblast
MEK2	Mitogen-activated protein/ERK kinase
mESC	Mouse embryonic stem cell
MOI	Multiplicity of infection
MSC	Mesenchymal stem cell
NC	Neural crest
NCAM	Neural cell adhesion molecule (CD56)
ND-1	NADH dehydrogenase subunit 1
NFP	Neurofilament protein
NOD	Non-obese diabetic
NSE	Neuronal-specific enolase
NTSC	Nuclear transfer ESC
OCN	Osteocalcin
Oct-4	Octamer binding protein-4
OPN	Osteopontin
PBMC	Peripheral blood mononuclear cells
PGC	Primordial germ cell
PI3K	Phosphoinositide kinase-3
Ptc	Patched
RFP	Red fluorescence protein
RTK	receptor tyrosin kinase
RT-PCR	Reverse transcriptase-PCR
SCID	Severe combined Immunodeficient
SCNT	Somatic nuclear transfer
SHH	Sonic hedgehog
SKY	Spectral karyotyping
Smo	Smoothened
Sox-1 /-17	SRY-box containing 1 / 17
SR	Serum replacement
SSEA	Stage specific antigen
STAT	Signal-transduced and activator of transcription
TGF-β	Tumour growth factor beta
TH	Tyrosine hydroxylase
TRA	Tumour recognition antigen
TSG	Tumour suppressor gene
TTF-1	Thyroid transcription factor-1
Tuj1	Beta-III-tubulin
VE-cad	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VGAT	Vesicular γ-aminobutyric acid (GABA) transporter
Wnt	Wingless
WT1	Wilm´s tumour-1

LIST OF CONTENTS

1 INTRODUCTION

Stem cells provide the means of the embryo to diversify, i.e. to give rise to all specified functions of every organ in the body and for homeostasis and regeneration in the adult. As somatic development proceeds, stem cells become increasingly restricted in their potential, i.e. their fate become set for specific tissue. Stem cells are defined by their capacity to self renew, that is, to divide and create additional stem cells or daughter cells, and also to differentiate along a specified developmental pathway. Other cells of the body, so called somatic cells, do not have these abilities.

1.1 STEM CELLS – ORIGIN AND NATURE IN VIVO

Stem cells are present through out life, from the fertilized oocyte to the adult [1,2] (http://stemcells.nih.gov/info/basics). There are three major levels of 'stemness'. The highest level of developmental potential, **totipotency**, is retained only by the zygote and up to the eight-cell stage of the morula. As the embryo grows it forms a hollow sphere containing an aggregate of cells attached to the thin wall. This inner cell mass (ICM) of the blastocyst stage, pre-implantation embryo will develop into all cells of the embryo proper, including germ cells. The ICM cells are **pluripotent** by definition.

1.1.1 Pluripotent Stem Cells

Three types of mammalian pluripotent cells have been identified.

Embryonic Stem Cells (ESC)

Embryonic Stem Cells (ESC) can be derived from the ICM [3, 4, 5]. A detailed description of the derivation of ESC is given in section 1.3.1.

Embryonic Germ Cells (EG) cells

EG cells are derived from Primordial Germ Cells (PGCs) isolated from embryonic gonads of the post-implantation embryo [6, 7]. PGCs are the embryonic precursors of the gametes. Shamblott and coworkers were first to isolate and culture human EG cells isolated from the gonadal ridges of 5- to 9-week-old aborted human foetuses [8].

Embryonic Carcinoma Cells (EC) cells

As opposed to ES and EG cells, EC cells do not play a role in normal development. They are the stem cells of testicular tumours which themselves originate from PGCs [9, 10]. As a distinct cell type, EC cells from such teratomcarcinomas were the first pluripotent cells to be recognized. Teratocarcinomas contain a wide array of tissues such as cartilage, bone, muscle, glandular and squamous epithelia, neuroectoderm and ganglionic structures [11-13]. The remarkable developmental capacity of the EC stem cell became apparent following the generation of chimaeric mice by blastocyst injection of EC cells. However, EC cells are always aneuploid and often limited in their developmental capacity [14, 15].

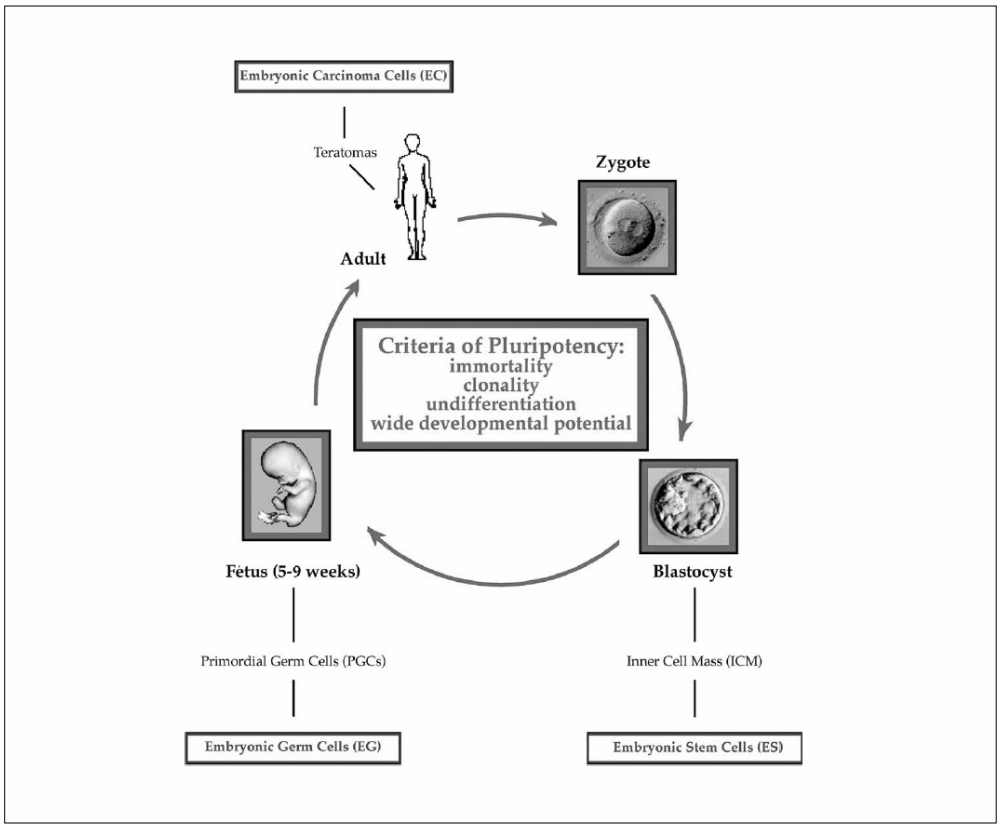

Figure 1. Origin and criteria of pluripotent cells (modified from Eiges and Benvenisty, 2002).

1.1.2 Multipotent Stem Cells

Multipotent stem cells can be isolated from foetal or adult tissue and are more or less committed to specific developmental programs. While foetal stem cells have served their purpose at or shortly after birth, adult stem cells remain throughout life. Adult stem cells are often localized to specific locations, so called niches. Some examples are the epidermis of the skin and the intestinal epithelium. Haematopoietic stem cells (HSCs) are essential in the haematopoietic system as we lose and replace over a billion red blood cells every day. These stem cells differentiate to give rise to progenitor cells of progressively more and more end-differentiated types that together compose all cell components of the particular niche. In some instances more than one stem cell may reside within a niche. This appears to be the case for the bone marrow, which houses not only HSCs, but also mesenchymal stem cells (MSCs). Some adult stem cells do not seem to have a specified niche within their respective tissue. Skeletal muscles for instance are renewed primarily upon injury, when quiescent satellite myoblasts proliferate transiently [16].

1.2 HUMAN EMBRYONIC STEM CELLS (HESC)

In-vitro culture of cells from human blastocysts was first described in the early 1980s, and it was suggested that these cells might have potential in tissue repair [17-19]. Culture of cells from the ICM of human blastocysts and subsequent growth of ESC-like cells for two passages with partial characterization was first described by Bongso et al.1994 [20]. The concept of using human ESC (HESC) in transplantation received greater attention after the first reports of permanent cell lines derived from human blastocysts showing pluripotent developmental capacity [21, 22]. These first reported HESC lines were maintained on the basis of the wide experience since the early eighties with regards to culturing of mouse [3-5], as well as rhesus[23], and marmoset [21] ESC.

By definition, ESCs are capable of growing indefinitely in cultures, keeping a normal diploid karyotype as well as contributing to cells of all three embryonic germ layers; ectoderm, mesoderm and endoderm. The capacity for multi-lineage differentiation is reproduced in culture where ESCs can spontaneously form a wide range of well-defined cell types or after transplantation to an *in vivo* model. In particular, they can participate fully in foetal development when reintroduced into the early embryo. However, such experiments cannot be performed with HESC for ethical reasons.

Since the first description of HESC, they have been recognized as a source of precursor cells that could be differentiated into any cell type, to treat degenerative, malignant, or genetic diseases such diabetes, Parkinson's disease, Alzheimer's, or injury such as spinal cord injury or heart failure. Although great attention has been given to the potential use in such cell-based therapy, HESC are equally important as a research tool to study development, both normal and diseased.

HESC are known to differentiate spontaneously if not kept under optimal culture conditions. Sub-optimal conditions include high cell density, changing the type of feeder layer, removing factors that inhibit differentiation or adding differentiation inducing factors. Spontaneous differentiation can be induced by culturing ESC as aggregates in suspension, so called embryoid bodies (EBs). A wide range of cell types have been recognized, though the structures formed in the three dimensional EBs are more advanced. For instance they differentiate into beating heart muscle cells, neurons, glia, skeletal myocytes, adipocytes, haematopoietic cells and endothelial cells[22, 24].

The fact that HESCs tend to differentiate in culture is not very surprising. After all, when considering the mission *in vivo* of the original ICM, it is evident that their sole purpose is to generate a complete body and that they are not meant to be kept proliferating in an undifferentiated stage. From this argument it is also clear that HESC do not have a direct counterpart *in vivo*, and therefore can be considered as an *in vitro* artefact [5, 25, 26]. Never the less, this does not necessarily impair on their potential usage.

1.2.1 Mouse ESC (mESC) and HESC; alike or unalike?

Until recently, the vast majority of mESC lines were derived from the inbred 129 mouse [27], clearly differing from the situation of HESC lines representing a global outbred population. Given the genetic heterogeneity in embryos used to derive HESC

lines, considerable phenotypic heterogeneity might also be expected between the lines. When grown *in vitro* in a culture dish, ESC forms tight colonies with sharp borders. That clear differences exist between ESCs from the mouse and human is obvious already from the colony morphology. While mESC grow as aggregates several cells thick, HESC instead grow as flat colonies. The population doubling time of HESCs is significantly longer than that of mESCs, possibly reflecting the longer gestational period in humans compared to mice. Furthermore, HESC differentiates more readily and show a much lower cloning efficiency compared to mESC [28, 29]. From an embryological point of view, there are also substantial morphological differences *in vivo* between the species. One example is events during gastrulation, where an egg cylinder is formed in the mouse and turning is a special process, while in the human an embryonic disc is formed [30].
In addition, biochemical pathways as well as pathologies differ between the two species [31]. A great deal of our understanding of the mechanisms that control ESC pluripotency and differentiation has arisen from the study of mESCs and this knowledge have been used to model also the mechanisms of HESC [32-34]. Although HESC lines have not been studied for as long as mESCs and although many signalling pathways are conserved in animal development (including the tumour growth factor- β (TGF-β) superfamily in which BMP4 and TGF- β reside, receptor tyrosin kinase (RTK), Wingless (Wnt), Hedgehog (Hh), JAK/STAT and Notch signalling [35]), several key differences have already emerged, particularly with regard to mechanisms underlying self-renewal. These signalling pathways are described in more detail in section 1.4.2.

Taken together there are basic gross differences pointing out the importance of the developing human systems for the understanding of early human development. In conclusion, differences between mESC and HESC include morphology, cell-surface marker expression, signalling pathways and differentiation ability.

1.3 PROPAGATION OF HUMAN HESC

1.3.1 Derivation

According to Hyslop et al. [36] more than 300 HESC lines have now been reported worldwide since the first derivation in 1998 [29], illustrating how quickly this field of research is expanding.

HESC are commonly derived from the ICM of the expanded blastocyst or compacted morula, obtained either from spare donated embryos after *in vitro* fertilisation (IVF) treatment, or from genetically defected embryos found by preimplantation genetic diagnosis [22, 29, 37, 38]. The ICM contains roughly 50 cells and when removed from the embryo and cultured *in vitro,* these cells can generate an ESC line. The ICM is isolated by using immunosurgery or mechanical dissection [34] to remove the trophectoderm (which gives rise to the embryonic side of the placenta) after which it is plated on a feeder layer that provide a suitable environment for attachment and expansion.

Potential contamination of HESC lines by animal-derived ingredients hinder the potential for cell-therapeutic applications why today much effort is put into the

development of xeno-free derivation and culture systems. Originally the immunosurgery technique was used to separate the trophectoderm cells from the ICM and this method is still widely used. Moreover, antibodies raised in animal species are used in this technique. Instead of immunosurgery, pronase treatment of blastocysts have successfully generated HESC lines [39]. Derivations have also been published from later blastocyst stages day-8 using a three-step culture procedure [40]. At this stage the ICM possess more cells than day-6 blastocysts which could be beneficial for the success rate. A HESC line from a mononuclear zygote has also been established [41].

We successfully derived lines from both fresh and frozen–thawed cleavage stage embryos cultured to the blastocyst stage (paper VII). Sjögren et al. [42] reported an overall success rate of 19.3% without a difference between direct application of the blastocysts on feeder cells or the standard immunosurgery method. However, a difference in the efficiency was found in that frozen–thawed embryos gave 3.7 times more cell lines than fresh surplus embryos. The method of using whole embryo culture represents a xeno-free method why this might be the method of choice. Recently the first report on derivation of HESC in defined conditions was reported, in which recombinant proteins were only used, thereby avoiding xeno-components [43].

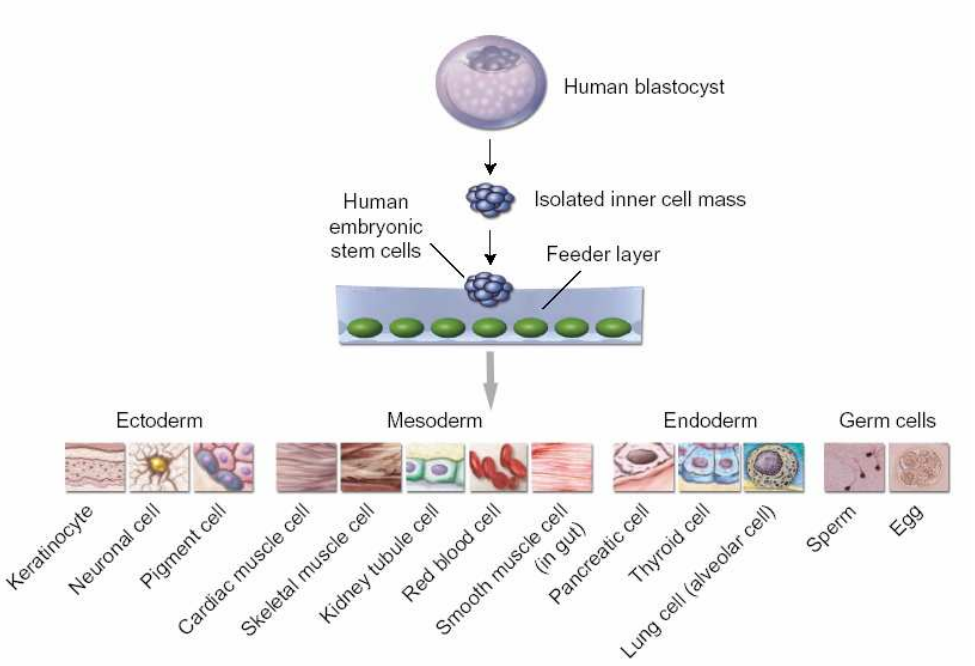

Figure 2. Derivation and potential of HESC (modified from Hyslop, et al., 2005).

1.3.2 Culture conditions

The self-renewal of ESC is influenced by growth factors and feeder cells in culture. As mentioned, at the time of the first HESC derivation, the culture conditions were applied from that of mESC. Most HESC was isolated and maintained on a mitotically inactivated murine embryonic fibroblast (MEF) feeder layer in the presence of 20% foetal calf serum (FCS) in the culture medium. Amit showed that the addition of basic fibroblast growth factor (bFGF; also named FGF-2) was beneficial for the pluripotency as well as improved cloning efficiency [44]. Today most culture conditions include

supplementation with 4-8ng/mL of bFGF. Efforts towards a xeno-free environment have resulted in that several types of feeder cells are used, including diverse human fibroblasts [45] or alternatively, extracellular matrix for the attachment of HESC, along with medium conditioned by feeder cells [46]. Removal of the feeder cells will lead to differentiation, suggesting that the feeder layer provides one or more factors that suppress differentiation or promote self renewal. Several extra cellular matrix components have been shown to possess cell differentiation inducing properties during early embryogenesis and also properties for maintaining cell phenotypes and tissue architecture at mature stages [47]. Efforts to select the optimal extra cellular matrices and to identify the soluble factors present in the conditioned medium are undertaken [48]. Today serum is no longer used, but instead replaced by serum-replacement (SR) components. Since the SR is not entirely free of animal ingredients, considerable efforts are directed at creating chemically defined culture media that contain no animal-derived components. As mentioned above, the first report of successful growth in defined conditions was reported very recently [43]. The panel of human feeder cells include the first reported, foetal muscle and skin cells and human adult fallopian tubal epithelial cells [45]. We described derivation using human foreskin fibroblasts (hFS) with serum or serum replacement (paper VI-VII). Human adult skin fibroblasts [49], human adult endometrial cells and breast parenchyma cells with serum or serum replacement [50, 51], human embryonic fibroblasts from HESC [52, 53] as well as human placental fibroblasts [54] have all been reported to support HESC growth.

In addition to the first described feeder less culture using Matrigel and laminin with conditioned medium from MEFs [46], other matrices include fibronectin with medium supplemented with TGF-β, LIF and bFGF [55] or human serum matrix and conditioned medium from HESC-derived fibroblasts [56]. One HESC line has been derived on extracellular matrix purified from MEFs [57]. Furthermore the medium conditioned by feeder cells have been replaced by high levels of bFGF and noggin [58, 59], and also a combination of activin A, nicotinamide and keratinocyte growth factor (KGF) have been reported [60].

Current culture techniques need to be improved. They are labour-intensive, often involving manual dissection and transfer of HESC colonies either to remove differentiated parts or as passaging method. Bulk enzymatic passaging has been tested even from the very early stages of HESC derivation [61]. However, bulk enzymatic passaging has been suggested to be the major factor behind observed chromosomal abnormalities[62], something that also we have experienced during bulk expansions (paper III-IV).

1.3.3 Freeze storage of HESC

Two major freezing protocols are currently commonly used for HESC, the conventional slow stepwise freezing method using cryovials and storage in LN_2, and a snap-freezing vitrification method using an open pulled straw and storage in LN_2[63]. Slow freezing is commonly used for somatic cell types, but using this method HESC exhibit a lower thaw-survival rate and seeding efficiencies compared to vitrification [63]. The reason for this is likely to be due to ice crystal formation that disrupts cell-cell adhesion. Vitrification on the other hand, induces glass induction instead of ice crystal

formation. Richards reported a xenofree cryopreservation protocol for HESC involving vitrification in closed sealed straws using human serum albumin instead of FCS as the main protein source in the cryoprotectant and long-term storage in the vapour phase of liquid nitrogen [64]. It must be noted that vitrification protocols are extremely labour-intensive and tedious to perform manually. Moreover, for handling bulk quantities of HESC cryostraws are inconvenient.

1.4 PROPERTIES OF ESC; HOW PLURIPOTENCY IS REGULATED AND HOW CAN WE CONTROL IT?

Yet, the understanding of molecular processes behind the self-renewing pluripotent phenotype remains unclear. The relationship between factors that influence HESC propagation *in vitro* and mechanisms of stem cell regulation operative in the embryo is also uncertain, why the mechanisms that control proliferation and differentiation need to be elucidated. Means for achieving this are directed *in vitro* differentiation of HESC and studies *in vivo.*

For historical reasons due to how the research field developed, most markers of pluripotent cells are common between EC and ES cells [65, 66]. However, this also highlights the close relationship between these subsets of pluripotent cells. Some classical markers are alkaline phosphatase, the POU domain transcription factor Oct-4, high telomerase activity and certain cell-surface antigens. The most recently discovered marker is a homeobox transcription factor called Nanog [67].
It should be noted that a worldwide initiative, in which our group is taking part, is currently working to compare and characterize HESC lines. In the International Stem Cell Initiative (ISCI) the objectives are to find correlations between expression patterns of HESC, establish the degree of heterogeneity between lines as well as reach a consensus about the key criteria that should be used to identify HESC. Hopefully ISCI will provide new important knowledge regarding the properties of HESC. Below is a summary of the present views on stem cell markers.

1.4.1 Cell surface antigens characterizing HESC

The stage-specific embryonic antigens 1, 3 and 4 (SSEA-1, -3, -4) are globoseries glycolipids and were first used to distinguish early stages of mouse development. Primate, pluripotent HESC express SSEA-3 and SSEA-4, but express SSEA-1 only upon differentiation [29, 68, 69]. Essentially the reverse is true for mouse ESCs. That EC and ES cells are closely related is furthermore indicted by the fact that it was from studies of EC cells, that the proteoglycans tumour recognition antigens (TRA) TRA-1-60, TRA-1-81, and GCTM-2 were discovered [68]. Although the function of these cell surface antigens is unknown, SSEA-1 recently was shown to mark regionally restricted immature subpopulations of embryonic retinal progenitor cells in the mouse [70].

1.4.2 Molecular markers characterizing HESC

Telomerase activity

An apparent requirement for continuously dividing and self renewing cells is the ability to avoid shortening of the telomeres. The enzyme telomerase is a ribonucleoprotein being involved in maintaining telomere length, and therefore plays an important role in replicative life-span. Telomerase is highly expressed in germ line and embryonic tissue and its expression is correlated with immortality in human cell lines. Since telomerase activity is high in HESC such cells have a replicative life-span exceeding that of somatic cells. Somatic cells do not express telomerase, have shortened telomeres with age, and enter replicative senescence after a finite proliferative life-span in tissue culture [71].

LIF and the JAK/STAT signalling pathway

It is known that the downstream Janus Kinase (JAK)/ Signal-Transduced and Activator of Transcription (STAT) signalling pathway is essential for self-renewal in mESC [72-74], but it does not appear to play an important role in the self-renewal of HESC [22, 75-77]. In this pathway, Leukemia inhibitory factor (LIF) is a pleiotrophic cytokine belonging to the interleukin-6 (IL-6) family [78]. LIF is needed for blastocyst implantation in mice, and may have widespread importance during early pregnancy in other mammalian species. Unlike undifferentiated mESC, which can be cultured using LIF in the absence of a feeder cell layer consisting of MEFs [79], HESC spontaneous differentiate, both in the presence and absence of human LIF [22, 29, 80]. This suggests that other signalling pathways are required for maintenance of pluripotency in HESC.

Oct-4

The best-characterised factor involved in maintaining pluripotency is Oct-4 – a member of the POU family of transcription factors, and encoded by *Pou5f1* [81]. The essential role of Oct-4 in mouse development has been revealed by targeted deletion and via conditional repression/expression in mESCs [82, 83]. These studies revealed that the precise level of Oct-4 governs three different cell fates. In mESCs, an appropriate level of Oct-4 is necessary to maintain pluripotency. Less than a twofold increase in Oct-4 expression causes differentiation into primitive endoderm and mesoderm, whereas loss of Oct-4 expression induces differentiation to trophectoderm [82, 83]. HESC express Oct-4 in stem cell specific manner [22] and expression of Oct-4 is also tightly regulated in human ICM cells whereas it is repressed in trophectoderm [84]. Moreover, downregulation of Oct-4 using RNA interference results in differentiation to both trophectoderm and primitive endoderm, suggesting a similar function in both species. However, Oct-4 is unable to sustain ESC pluripotency on its own [85, 86].

Nanog

Similar to Oct-4, Nanog is a member of the homeobox family of DNA binding transcription factors. Nanog was first found in mESC to be involved in maintenance of pluripotency [67, 87]. A human counterpart, NANOG, has been identified in HESC, EC cells [88] as well as in the ICM [89]. The importance of Nanog was shown when the ICM from mouse embryos mutated in the *Nanog* gene and cultured *in vitro* were composed entirely of primitive-endoderm-like cells. In the same study mESCs with abolished Nanog expression, lost ESC morphology and markers, instead expressing markers for

primitive endoderm [87]. They furthermore suggested that Nanog might regulate differentiation by transcriptional repression of genes that promote primitive endoderm differentiation, such as GATA6.
Similar to Oct-4, quantitative differences in gene expression appear to be important in cell-fate regulation, since a twofold downregulation of Nanog results in endoderm, mesoderm and ectoderm differentiation [83, 90]. Elevated expression of Nanog results in clonal expansion of mESCs, stable Oct-4 expression, and resistance to chemical-induced differentiation [86]. Therefore, Nanog appear to be a key regulator, probably activating genes that are crucial for maintenance of pluripotency. Hyslop et al. showed, using RNA interference, that downregulation of NANOG in HESC resulted in trophectodermal in addition to primitive endoderm differentiation, similarly to Oct-4 [89]. They therefore suggest that also human NANOG acts as a gate-keeper of pluripotency by preventing differentiation to extraembryonic endoderm and trophectoderm. That downregulation of NANOG leads to differentiation of HESC is supported by Zaehres et al. [91].

Recently, overexpresion of *NANOG* was shown to enable the propagation of HESC in the absence of feeder cells or conditioned media (CM), although exhibiting a slower growth. Therefore, it is suggested that an additional pathway is activated by the addition of CM [92]. Interestingly, they also showed that *NANOG* expression in wild-type cells is upregulated during early differentiation, and that its overexpression in HESCs modifies the expression of marker genes to an expression pattern similar to that of primitive ectoderm cells. Taken together, as discussed by Darr et al. [92], this indicates that in both human and mouse, *NANOG* may promote the transition from ICM to primitive ectoderm. Also for NANOG, support is found for different function and regulation in HESC and mESCs.

Signalling pathways investigated by gene-expression analyses

The full understanding of molecular mechanisms underlying self-renewal of HESC remains to be elucidated. However, they are most likely maintained by a highly coordinated signalling network including many signalling specific molecules, of which Oct-4 and Nanog are only two. Considering the technology available today, new knowledge are likely to come from genomics. Gene-expression analysis of stem cells using micro-array technology will and have provided some insight into the signal systems involved. Several groups have made great efforts in trying to elucidate signalling pathways in HESC using microarray technology and today new reports are published with short intervals. Molecular signatures of HESC, mESC and other adult stem cells have furthermore been studied [93, 94], but the data is still insufficient for providing information on the direction of stem cell fate in either self-renewal or differentiation.

Although several signalling pathways are known to be involved in the self-renewal of mESC they are poorly understood in HESC [5, 22, 29]. Following sections summarise what is known for some conserved pathways.

The TGF-β superfamily

Members of the Tumour Growth Factor-β (TGF-β) superfamily signal through two main branches; the BMP–SMAD1/5 pathway and the TGF-β /activin/nodal–SMAD2/3 pathway. The Bone morphogenetic protein 4 (BMP-4) and TGF-β subfamilies regulate a variety of cellular and physiological processes [95, 96].

BMP-4 and FGF signalling pathways in HESC

BMPs have diverse biological functions, including haematopoiesis, mesoderm formation and patterning [97, 98]. Furthermore, BMPs act as antineural factors that inhibit neural differentiation in ESCs and vertebrate embryos, and induce expression of Id ('inhibitor of differentiation') genes via the Smad1/5 signalling cascade [99, 100].

BMP-4 has been shown to induce differentiation of HESC to trophectoderm [101]. Furthermore, inhibition of BMP-4, present in the culture medium by the BMP antagonist noggin and bFGF, is thought to contribute to the maintenance of HESC pluripotency. As mentioned (section 1.3.2), a combination of bFGF and noggin, or high levels of bFGF alone, are sufficient to prevent differentiation of HESC in the absence of fibroblasts or conditioned media [59]. In contrast, BMP-4 in combination with LIF maintains mESC pluripotency in serum free culture [100].

Of the **FGFs**, bFGF was early shown to be a mitogenic factor for HESC propagation [44]. HESC express significant levels of bFGF and three out of the four receptors (FGFR-1, -3 and -4) – contrary to mESCs, which express FGF-4 and very low levels of FGFR-1 [102]. The mechanism underlying bFGF action seems to be inhibition of signalling by BMP-4, presumably via the repression of SMAD1 phosphorylation or prevention of its nuclear translocation [59]. Moreover, several studies showed that FGF-4 and FGFR1 genes were enriched in HESC, as well as BMP-4, BMPRIA and GDF3 [77, 94, 103-105]. Target genes of FGF-4 signalling was also found to be highly expressed in HESC, including c-Kit, essential for the maintenance of pluripotent primordial germ cells and haematopoietic stem cells [77, 106]. Besides, E-Ras was enriched, earlier shown to promote tumour-like growth in mESC [107] why E-Ras might facilitate tumour-like growth also in HESC.

TGF- β signalling pathway in HESC

The TGF-β signalling pathway is involved in patterning of mammalian embryogenesis and organogenesis by the regulation of cell proliferation and differentiation [108, 109]. Recently, it has been suggested that in HESC, the TGF-β/activin/nodal branch is highly active and is downregulated upon differentiation [110]. Most importantly, this signalling pathway is sufficient for the maintenance of markers of the undifferentiated state in HESC and murine ICM but not mESCs [60, 110-112]. In addition, Rho et al. [77] found that follistatin, an antagonist of the TGF-β ligand was highly expressed in HESC, suggesting that an inhibitory regulation is also necessary.

Wnt/β-catenin signalling pathway in HESC

The Wnt signalling pathway, which leads to the activation of the transcriptional regulator β-catenin, mediates somatic stem cell renewal and differentiation [113, 114]. Glycogen synthase kinase 3β (GSK3β) acts as negative regulator of β-catenin, which leads to its degradation. Interestingly, it has been reported that activation of β-catenin via the GSK3β inhibitor bromoindirubin- 3'-oxime (BIO) is sufficient to maintain an undifferentiated state of both HESC and mESCs [115]. Most of the components of the canonical Wnt–β-catenin signalling pathway have been found in both mESCs and HESCs [102] and that Wnt signalling is sufficient was supported in a previous report by Rho et al [77]. TGF-β and Wnt pathways are thought to come together in the phosphoinositide 3-kinase (PI3K)/Akt pathway which can suppress GSK3β activity and thereby activate β-catenin [116]. This pathway appears to play a role in self-renewal of HESC, since when inactivated, differentiation is induced and extracellular matrix components disappear [117]. It appears that stabilization of β-catenin is critical for HESC pluripotency, which might be dependent on the canonical Wnt signalling.

Hedgehog (Hh) signalling pathway in HESC

The Hh signalling pathway is required for the differentiation of ESC into neuroectoderm and extraembryonic endoderm [118, 119], activated by two membrane proteins, patched (Ptc) and smoothened (Smo) [120]. Recently Sonic hedgehog (Shh) was suggested to facilitate the differentiation of HESC as shown by its high expression levels in hEBs compared to low levels in HESC [77]. That Ptc and Smo are expressed in ESCs was also shown [94, 103, 104].

Notch signalling pathway in HESC

The Notch signalling pathway controls cell fate decisions through cell–cell interactions [121, 122]. Expression studies indicate that Notch 3 and Notch 1 are expressed at higher levels in HESC compared to differentiated HESC [77, 94, 103, 104].

All together these data suggest that in these signalling pathways, extracellular molecules such as ligands and antagonists may play important roles in the self-renewal of HESC. In addition the findings support further differences in the self-renewal mechanism between mouse and human ESC.

These transcriptome profiling studies were emphasised at finding genes ubiquitously expressed in all lines in an attempt to identify pluripotency associated loci. The studies have not focused on inter-line variation. However, Rao and Stice reported 75% similarity in microarray profiles between two lines, while in another study 48% of the expressed genes were restricted to one or two lines [123]. Moreover, expression levels of the Oct-4, β-catenin and Nodal genes have been reported to differ between two cell lines tested [77]. Also, gene expression differences for lines derived within the same laboratory have been observed [124], and even within the same HESC lines (38%) after three passages in two different media [125].

The take home message is the understanding of that there might be differences in characteristics between HESC lines and elucidating what these differences are will be very important for understanding how pluripotency is regulated in HESC.

To conclude, one precaution should be taken when interpreting expression studies. Although most studies indicate the same set of core mechanisms, the studies are conducted in different ways, why it is not clear whether the differences between published expression studies are due to genuine differences or to procedures during the expression profiling or bioinformatics analysis.

1.4.3 Immunological properties of HESC

One major concern for potential cell therapy is the immune response launched by the patients' immune system against grafted cells. Therefore it is extremely important to investigate the immune reactions that could be directed against HESC transplants.

This issue was focused upon by Li et al. [126] who showed that HESC injected into the leg muscle of immune-competent (CD-1) mice failed to induce an immune response 48 hours after injection. Though, given the short period *in vivo* of these experiments, it is not possible to comment on whether mature HESC-derived teratomas provoke an immune response. In the same study, *in vitro* analyses showed that undifferentiated HESC and EBs failed to stimulate proliferation of alloreactive primary human T cells. This study indicates that HESC may possess unique immune-privileged properties.

Drukker et al. [127] have studied in detail the immunogenecity of undifferentiated as well as differentiated derivatives using a number of mouse models with different immunological deficiencies. They have been using the Trimera model in which immunodeficient mice are reconstituted with human peripheral blood mononuclear cells (PBMC) in order to regain B, T and NK-cell activity. First they showed that HESC transplanted under the kidney capsule of four different immunocompetent mouse strains fail do develop teratomas as opposed to when injected in Non-obese diabetic/Severe combined immunodeficient (NOD/SCID) mice. To rule out the mechanism of rejection they injected HESC into T-cell, NK-cell and B-cell deficient mice strains respectively, and found that xenorejection of HESCs is T cell-mediated. Furthermore they undertook to investigate how a human immune system might act to reject HESC-derived transplants using the human PBMC-reconstituted Trimera mouse model [128, 129]. Transplantation of undifferentiated HESC, differentiated teratoma fragments, as well as teratoma-derived primary cell lines gave teratoma formation and cell growth regardless of whether the mice had been reconstituted with PBMCs or not. In contrast, human adult skin grafts or a B cell line, were heavily infiltrated by leukocytes or eliminated.

To summarize, Drukker et al. have shown that HESC and their differentiated derivatives have little potential to activate a direct allospecific response by HLA non-matched human lymphocytes. As previously shown, HESC appear not express major histocompatability (MHC)-II molecules, even after *in vivo* differentiation, but show low levels of MHC-I molecules, which are slightly induced by differentiation [130]. Co-stimulatory molecules, such as CD80 and CD86 molecules [131] were not expressed on

undifferentiated or differentiated HESC and neither was FasL ligand (potential to induce apoptosis in T-cells) [132]. The fact that MHC-II-expressing cells or co-stimulatory molecules does not occur in the grafts highlights the ability to transplant purified HESC-derived MHC-II-negative populations that have reduced capacity to activate immune response. It was suggested that the weak immune response towards HESC appear to be due to T-cell ignorance. HESC only rarely differentiate to cells of the immune system with the potential to activate alloreactive T cells. Half of the immune-related genes were not expressed, also supporting the suggestion that immunological maturity appears late during gestation in human embryos [127, 129].

Taken together, immunosuppressive regimens for HESC-based therapeutics could probably be highly reduced compared with conventional organ transplantation. However, as shown by Drukker et al.[127] the MHC-I levels in HESC are sufficient for T cell recognition, as shown by the CTL assays why any haematopoietic cells or progenitors should be removed. What is more to keep in mind is that these studies were not long term experiments. Perhaps more mature HESC derived tissue than the 4-weeks old teratomas used in the expression study would have generated expression of more immune-related genes. Therefore, the immunological properties of HESC-derived tissues should be evaluated in long-term studies.

Strategies to prevent immune rejection

Strategies to avoid and reduce immune rejection reactions of the transplanted cells might be needed. Possibilities include banking HESC with defined major histocompatability complex back grounds, or genetically manipulating HESC to reduce or actively combat immune rejections. Taken into account that HESC derivatives (excluding haematopoietic cells) lack MHC class II antigens, and that MHC I mismatches contribute only slightly to graft rejection, and assumed that human leukocyte antigen (HLA) homozygous cell lines are selected for, might be enough to allow for a bank representing large populations. Genetic manipulation include knocking out the MHC [133], or alternatively deletion or insertion of other genes that can modulate the immune response [134]. In mice this has however not been found successful [133]. The production of isogenic cell lines derived by somatic nuclear transfer (SCNT) to an enucleated oocyte provides still another possibility. These cell lines are genetically identical to the donor of the somatic cell except for the mitochondrial genome, and are often called nuclear transfer ESC (NTSC) lines [135]. One approach in use in the clinic today is haematopoietic chimerism. Here a bone marrow transplant, which prevents rejection, is preceded by transplantation from the same donor [136]. Thereby the creation of lymphohaematopoietic stem cells or dendritic cells (DCs) from the same cell line as the transplant of interest would be a possible route. Recently, Slukvin et al. showed directed differentiation into DC lineage [137].

1.5 THE GERM CELL THEORY

Logically, ESC would be *in vitro* equivalents of the ICM. However, as discussed such cells are not retained *in vivo* (section 1.2) and studies have shown that they transition to a different cell type *in vitro*, of which they first were thought to be primitive ectoderm equivalents. In a recent review by Zwaka and Thomson it is suggested that ESC instead closely resemble early germ cells[138]. This issue is of importance since there in this thesis is support indicative of both theories, and there is today still debate about the origin of HESC. As discussed by Zwaka and Thomson, it is crucial to elucidate the origin of ESC since it may help us to identify genes involved in maintaining a pluripotent state, as well as to compare how accurately ESC differentiation reflects normal developmental events *in vivo*.

When the earliest stages of teratocarcinoma formation in mice was analysed, Stevens et al. observed clusters of polarized epithelial cells surrounding a central cavity that morphologically resembled primitive ectoderm cells [138, 139]. Similarly, as support for the primitive ectoderm theory, we found the first structures formed *in vivo* after HESC grafting being of such morphology, highly reminiscent of primitive ectoderm (paper II). Isolated primitive ectoderm from the mouse gives rise to ESC lines at a higher frequency than does isolated ICM, and also from single cells as opposed to the ICM [140]. Furthermore mESCs do not differentiate *in vitro* to trophoblast cells [141]. The last cells capable of giving rise to trophoblast cells are early ICM cells, why the inability of mESCs to differentiate into trophoblasts is considered strong support for that they are not the equivalent of early ICM cells [142]. Interestingly, HESC do differentiate to trophoblast cells *in vitro* [101]. As discussed by Zwaka and Thomson, this species difference could be explained by that HESC are related to an earlier cell type than mESCs, or that specification of the trophoblast lineage occur differently in human embryos, alternatively that ESC represent a different cell type altogether. Cultured primitive ectoderm cells were shown to spontaneously give rise to early germ cells in culture [143]. BMP-4 appear to be required for the formation of the region that gives rise to PGCs [144], and recently, BMP signalling has been shown to be important for the self-renewal of mESCs [100]. Mouse EG cell lines are in addition remarkably similar to mESC lines [145].

In the same review by Zwaka [138] it is also pointed out that several genes expressed only by PGCs, but not the ICM are expressed also by HESC and mESCs. Tnap (alkaline phosphatase), is strongly expressed by early germ cells and by mESCs, but only weakly expressed by the epiblast and surrounding cells [146]. Fragilis and Dppa3 are recently identified as markers for early mouse germ cell precursors, not expressed by their differentiated neighbouring cells [147]. Germ cell markers were expressed by mESCs themselves, including Dppa3, which help distinguish germ cells from primitive ectoderm [147-150]. Only more mature germ cell markers enabled *in vitro*-derived germ cells to be distinguished from mESCs themselves. Clark et al. studied the differentiation of HESC into germ cells [151], and found that all eight genes that are characteristic of early germ cells was expressed in HESC, but six genes that are characteristic of later germ cells was not detected. Perhaps most importantly, the germ cell specific gene, *DAZL*, was expressed by HESC but not by human ICM.

Together, these data do support the idea that the closest *in vivo* equivalent to ESCs is not the ICM or primitive ectoderm, but an early germ cell. In light of this idea, it is interesting to note that the chromosomal changes we found in paper III and IV can be correlated to changes in germ cell tumours.

1.6 DIRECTED IN VITRO DIFFERENTIATION

Differentiation of HESC into cells of the three embryonic germ layers can be achieved *in vitro* by culture of the cells using nonadherent culture plates. The HESC remain in suspension and form multicellular aggregates of cells called embryoid bodies (EBs) in which precursor cells of different cellular lineages can be identified [152]. However, when HESC are allowed to differentiate spontaneously into multiple lineages, only a small fraction differentiates into one particular cell type. Therefore many protocols have been developed to enrich for desired cell types by directed differentiation. The regimens used for *in vitro* directing HESC vary. Commonly this has been achieved by exogenous addition of soluble factors known to influence development [24, 80] or by spontaneous formation of EBs [152] or both in combination [153].

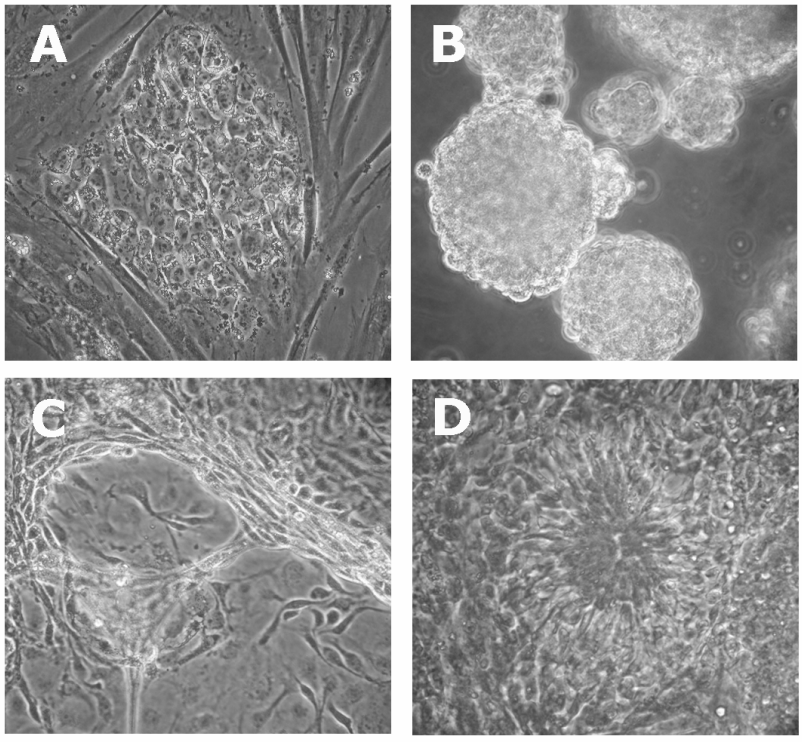

Figure 3. Spontaneous in vitro differentiation of HESC; (A) A small undifferentiated colony. (B) Embryoid bodies (EBs). (C) Endothelial differentiation with 'vessel-like' formations. (D) Neural rosette.

One major limitation currently not yet overcome is the fact that in all protocols used, a selection step is needed to remove undifferentiated cells or cells differentiating into

undesired lineages. Otherwise, remaining undifferentiated cells mount a risk of teratoma formation *in vivo*. The advantage of using *in vitro* cell cultures to study differentiation is provided by the controllable environment, in which it is possible to closely regulate the signals that cells retrieves [154]. *In vitro* differentiation of cultured HESC before transplantation into SCID mice may be used to determine how differentiation *in vivo* can be restricted. Transplantation to an *in vivo* model therefore provide a powerful approach to confirm earlier *in vitro* work and will be useful to test whether the status of grafted HESC influences their ability to differentiate *in vivo*.

1.6.1 Strategies for *in vitro* differentiation

Co-culture with differentiated somatic cells is used to induce differentiation of cells along different developmental pathways. One example is cardiomyocyte differentiation, in which visceral-endoderm-like cells were used as inducer [155]. Another method is **supplementation with growth factors or defined patterning molecules** and has been shown for direction into haematopoietic lineage [156, 157]. Recently, Perrier et al. used a **combination of the two techniques** to derive midbrain dopaminergic neurons from HESC [158]. Neuroectodermal differentiation was triggered by co-culture with stromal feeder cells, followed by addition of sonic hedgehog (SHH) and FGF-8, which are known to be crucial in the specification of midbrain dopaminergic neurons. Some progress has been made moving toward more realistic *in vitro* models of cell differentiation. For instance, culturing HESC on **three-dimensional scaffolds** was shown to markedly influence cell behaviour [159, 160]. The three dimensions provide a possibility for the cells to migrate appropriately and form alternative interactions with neighbouring cells, interactions that are restricted in cultures grown as two-dimensional monolayers. Levenberg et al. used polymer scaffolds coated with matrigel or fibronectin in combination with growth factors such as retinoic acid, TGF-β, activin A and insulin-like growth factor (IGF) [159]. They found, in line with our teratoma findings (papers I-II) that vessels formed within the three dimensional structures could anastamose with the host vasculature upon transplantation. Probably the most invasive method used for directed differentiation is **genetic manipulation** by overexpression or ablation of specific genes. Overexpression of homeobox B4 (Hoxb4) has been shown to enhance the haematopoietic potential of mESCs recipients [161].

1.6.2 Strategies for cell selection

As mentioned, so far no protocols are available in which pure cell populations can be produced. Hyslop et al. review some strategies for how to proceed despite this limitation [36]. Cell sorting of the desired cells can be used when a unique pattern of surface antigens are present [156]. However, this is seldom the case why genetic manipulation instead can be used. The expression of a gene that induces cell death upon activation and placed under the control of an ESC-specific promoter would selectively kill residual ESCs also *in vivo* after a differentiation protocol [162]. The expression of a tissue specific promoter to either drive a marker gene product such as green fluorescence protein (GFP) allows the cells to be separated using fluorescence-activated cell sorting [163]. Or, using a selectable marker such as an antibiotic resistance gene selection with the appropriate antibiotic can be achieved [164].

1.7 CELL TYPE SPECIFIC DIRECTED DIFFERENTIATION

To give an understanding of the potential of HESC, and how the field have progressed with regards to directed differentiation, I have extracted a number of reports on the subject divided into specific cell types.

1.7.1 Neurons

Early phases of human neurogenesis are apparent during HESC gradual differentiation into various neuronal cells. Initially, a proliferating neural progenitor population is derived (such progenitors grow in rosette shapes) similar to the neural tube. This is followed by differentiation of the progenitors, a process that yields a more committed cell population of neurons and glia [165, 166]. When transplanted into the brain of newborn mice, HESC derived neural progenitor cells generated neurons and glia that could be detected in the recipient brain 4 weeks later [166]. The donor cells appeared to have matured and were indistinguishable from the host tissue. Co-culture of HESC with stromal cells guided a differentiation towards development of neural populations with midbrain dopamine characteristics [158, 167]. Heterogeneous populations containing varying degrees of dopaminergic neuron differentiation have also been transplanted into Parkinsonian rats [167, 168]. In these studies, only few human cells could be detected between 5 and 12 wk post-transplantation, but some of the animals showed behavioural improvements [168]. Yan et al.[169] reported on midbrain dopaminergic neurons generated in a chemically defined medium by exposure of bFGF expanded neuroepithelial cells to FGF8 and SHH, or as described by Schulz et al. [170], in aggregate cultures. It has furthermore been shown that HESC can generate motor neurons[171]. Although functional integration has not been demonstrated for those cells it is promising since differentiated EG cells have been reported to partly recover motor function by indirect mechanisms in rats [172].

1.7.2 Endothelial cells

Within EBs, HESC can spontaneously differentiate and organize into three-dimensional vessel-like structures (positive for CD31) in a pattern that resembles embryonic vascularisation. The expression of endothelial markers (such as CD31, VE-cad, CD34 and GATA-2) was shown to increase during EB differentiation and when isolated and grown in culture, these CD31+ cells express endothelial markers and form vascular tubes *in vitro* as well as anastomosed to form bloodcarrying microvessels *in vivo* [173, 174]. When HESC were cultured on collagen IV, a subset of cells expressed specific endothelial markers and gave cord-like organization of the cells, of which 20% was shown to be endothelial cells [175]. Three dimensional culture of the HESC formed massive three-dimensional vascular networks, and upon implantation into SCID mice, the donor endothelial cells within the implants formed vessel structures and anastomosed with the host vasculature [159]. Gerecht-Nir et al. have also shown that HESC cultured on alginate scaffolds form well-vascularised EBs [176]. Consistent with these findings we have moreover reported on the spontaneous differentiation into anstomosed and blood carrying microvessels in teratomas (paper I, II). These studies demonstrate the possibility of using HESC as a model for mechanisms regulating normal endothelial development as well as angiogenesis during tumour formation.

1.7.3 Cardiac muscle

Myocardial infarction results from significant myocardial cell death [177]. Therefore, HESC derived cardiomyocytes might provide a source for treatment of cardiovascular disease. Beating cardiac-like structure that share properties with early stage cardiomyocytes and develop a sarcomeric organization can readily be seen in spontaneously differentiated EBs [46, 178-181], suggesting that HESC undergo cardiogenesis during *in vitro* differentiation. In addition to EB formation, visceral endoderm has also been identified as a source of signals that can induce cardiomyocyte differentiation from HESC [155]. Real-time determination of intracellular Ca^{2+} concentration showed that these cells were electrically coupled [181, 182], although the HESC-derived cardiomyocytes do not undergo maturation to the stage of adult cardiomyocytes [178]. The human cardiomyocytes formed structural and electromechanical connections with cultured rat cardiomyocytes and have been used in xenogeneic transplantation as "biologic pacemakers" for the treatment of bradycardia [180]. Clusters of contracting cardiomyocytes isolated from the differentiation cultures were transplanted to the hearts of swine with complete atrioventricular block, and could rescue pacemaker function as assessed by high resolution electroanatomical mapping.

1.7.4 Bone and cartilage

Regenerated bone tissue from HESC could be used for the treatment of traumas and a range of skeletal defects including developmental abnormalities and degenerative diseases. Sottile et al. also support the usage as a model for investigation of osteogenic differentiation, bone development and repair [183]. The same group was first to describe osteogenic differentiation from HESC. EBs cultured in osteogenic media resulted in nodule formation and mineral deposition in the differentiated cultures. These cultures were immunostained positive for osteocalcin (Bglap). After transplantation of mineralizing cells into SCID mice, regions of mineralized tissue were found after 35 days *in vivo*, suggesting that HESC derivatives underwent further maturation *in vivo* [184]. In comparison with our own study (paper II) on teratoma formation it appears that the formation of mineralized tissue is a slow process, 35 days and 45 days respectively, since transplantation of mineralizing cells does not produce mineralized tissues much faster than uncommitted cells.

1.7.5 Haematopoietic cells

HESC have been shown to differentiate into haematopoietic precursors, and could possibly be used to derive erythrocytes and platelets for transfusion as well as cells for haematopoietic cell transplantation. As discussed (section 1.4.3), haematopoietic stem cells may also provide a method to prevent immune rejections [185]. HESC differentiate into haematopoietic precursors when co-cultured with murine bone marrow or yolk sac cell line [185]. BMP-4 and a mixture of haematopoietic cytokines promoted haematopoietic commitment during EB differentiation [156]. The addition of BMP-4, VEGF and cytokines enhanced also development of erythroid cells [186]. Cells expressing B-cell markers have furthermore been described, after co-culture of CD34+ cells on stromal cells (Vodyanik 2005). Recently, Zambidis et al. [187] investigated the stepwise differentiation of HESC to primitive and definitive erythromyelopoiesis from serum free, clonogenic EB cultures. They found that differentiation of HESC models the earliest events of embryonic and definitive haematopoiesis, resembling human yolk sac development. Importantly, Narayan et al. [188] showed that HESC are capable of

generating haematopoietic cells that engraft primary as well as secondary recipient sheep. These cells thereby fulfil the criteria for long-term engrafting haematopoietic stem cells. Interestingly, overexpression of Hoxb4 shown to enhance the haematopoietic potential of mESCs recipients [161] has no effect on the repopulating capacity of HESC-derived haematopoietic progenitors [189]. This suggests that new master controls of HESC haematopoietic differentiation have to be sought.

1.7.6 Pancreatic β-cells

Pancreatic cells are of special therapeutic interest for the treatment of Type I diabetes (caused by an autoimmune destruction of the insulin-producing β-cells) [190]. Initial experiments with HESC showed some characteristics of pancreatic endocrine cells in a small subset of cells [190], and modified protocols from mESC differentiation [191, 192] have allowed for insulin producing clusters of HESC [193]. Segev et al. found a high percentage of insulin and glucagon or somatostatin coexpressing cells in the cell clusters formed, which were considered to be similar to immature pancreatic cells [193]. The response to glucose and antagonists was lower than expected and discussed to be due to an immature stage of the pancreatic-like cell clusters produced, similar to what is shown for foetal pancreatic β-cells [194]. More recently, protein transduction technology, utilizing the TAT-Ipf1 protein, was applied for the induction of insulin-producing cells[195] and co-transplantation under the kidney capsule of differentiated HESC with dorsal pancreas from mouse embryos have furthermore resulted in differentiation of β-cell like cell clusters [196].

That care must be taken before making assumptions on the functionality of ESC directed progenitor cells is illustrated in the attempts to produce insulin producing pancreatic β-cells. In several studies generation of such cells from ESC through an nestin positive progenitor cell have been reported [191, 197-200]. However, two following studies have instead found that using the very same regimen, the insulin finding is due to uptake from the culture media and not by production in the cell itself [192, 201]. The cells weakly expressed insulin-2, they did not express insulin-1 or stained for C-peptide, why insulin positive staining cells were likely to be a result of uptake of insulin from the culture medium. After the report by Rajagopol et al., insulin-producing cells were more carefully investigated, and commonly defined by the parallel synthesis and process of insulin, by the expression of proinsulin and C-peptide. It highlights the importance and difficulties in determining a functional phenotype. While there is clearly more work needed in the development of the pancreatic lineage and β-cell function, progress is encouraging.

1.8 FUNCTIONALITY OF ESC-DERIVED CELLS IN VIVO

mESC are able not only to differentiate into a wide variety of different cell types, but have also in several mouse models of disease been able to affect symptoms [202]. However, while Soria and co-workers[203] reported in 2000 that insulin expressing cells could normalize glycaemia in streptozotocin-induced diabetic mice, Sipione et al. [204], showed that such cells when transplanted *in vivo* in a diabetic model did not reverse a

glycaemic state and that the insulin produced *in vivo* originated from neuronal cells, not β-cells.
In the light of such data, a major question is whether HESC derived cells will behave as normal somatic cells after transplantation. Grafted cells are commonly investigated for their potential to survive and integrate, but there has seldom been provided proof for the functionality. Positive effects have been shown (outlined below), but whether indirect or direct mechanisms are responsible is commonly unknown. Studies on the haematopoietic system have indicated that *in vitro* differentiated cells show an embryonic marker profile [205, 206], and that they do not behave *in vivo* like normal adult somatic cells [187, 205]. Cardiomyocytes have also been shown to exhibit an immature profile although electrically coupled [155, 179, 180, 207]. Kehat et al. [180] showed that *in vivo* transplanted HESC-derived cardiomyocytes integrate and pace the hearts of swine with complete antrioventricular block, but they could not rule out that the improved heart activity was not due to an indirect effect of the transplanted cells on neighbouring host cardiomyocytes. As mentioned above, Ben-Hur et al. [168], reported that HESC derived neural precursor cells transplanted into the striatum of Parkinsonian rats, differentiated *in vivo* into DA neurons, and resulted in functional improvement. Again, considering the mixture of neural and nonneural cells in the graft and the few cells detected in the animal, the reason for these improvements is unclear.
Altogether, these studies highlight the complexity of functionality, and exemplify the progress of *in vivo* analyses as well as their importance. Furthermore they provide that additional functional long-term studies need to be carried out.

1.9 IN VIVO DEVELOPMENT – A MODEL FOR EARLY DEVELOPMENT

Knowledge regarding early organogenesis, how tissues develop and grow, in the human is very limited. Most information assumed for the human has come from studies of other mammals, in particular the mouse. The reason for this is that detailed molecular studies of human development *in utero* are impracticable and only limited studies have been performed, mainly on non-human primates. It remains to be answered how cells during human development "know" where to migrate, divide and specialize and how morphogenesis of organs and the whole body by pattern formation, the spatial organization of cells is achieved. HESC in combination with animal modelling can potentially give new important knowledge of early human development, as suggested in this thesis.
Although the use of HESC as a model system for early human development is becoming increasingly accepted, yet there have been few reports published.

1.9.1 The mouse *in vivo* model system

The use of animal models for studies of transplanted human cells is not a new approach [208] and the immune-deficient *scid* (C.B-17-Prkdc^{scid}) mice were explored already 1983 by Bosma et al. [209]. This discovery have led to the development of even more efficient modified severe combined immunodeficiency (SCID) mice, including the NOD/ShiJic-*scid* with γc^{null} (NOD/SCID/γc ^{null}) mouse that has multiple immunological dysfunctions (Ito 2002) and immune-deficient nude (C57BL/6J-*Hfh11*^{nu}) and *scid* mice

used for studies of graftment and function of transplanted tissues [210-212]. SCID-beige mice (C.B-*Igh-1*^{b} GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7) are commonly used as host for the growth of HESC derived teratomas, also in our lab (paper I) [29]. SCID-beige mice have a double-mutation leading to impaired lymphoid development and reduced natural killer cell activity. Mice with only the *scid* mutation have also been reported to give teratomas [22, 40].

1.9.2 HESC teratomas as model system

The directed differentiation of HESC *in vitro* and the formation of spontaneous EBs have proven useful for studies of mechanisms regulating cellular differentiation. However, with *in vitro* methods it is today not possible to achieve the complexity needed for cellular differentiation into tissues consisting of many different cell types. Furthermore, supported by the discussion in section 1.8, such a functional tissue is even less likely to develop *in vitro*. Although new approaches for cell growth *in vitro* such as three dimensional scaffolds [159, 160] are a step forward, many of the environmental cues that modulate cellular differentiation are absent from such models. As discussed by Przyborski et al. [213] this is not surprising when comparing the characteristics of the growth conditions experienced by cells in the living embryo and those experienced by cells in the artificial world of the Petri dish, as well as the fact that there are more than 200 cell types in the body that HESC could potentially form. Once again, this highlights the importance of investigating cellular differentiation *in vivo*.

The following section describes the potential for teratoma formation as a model system for early human development and summarise what is previously known on non-directed HESC differentiation *in vivo.* This sort of information is so far very limited [213], and this was a reason for the focus of my thesis work.

Tissues identified in HESC-teratomas

Teratomas developed from transplanted HESC into immunodeficient mice form complex tissues consisting of ordered arrangements of different tissues with varying maturation level. Some that in many ways are highly reminiscent of structures found during normal early organogenesis or in the adult. Analyses of such tissues provide a model to study early human development and organogenesis.

Organized tissues that have been reported include; renal structures containing tubules, and associated vascular supply in glomerular formations; primitive epithelium, and neuroepithelium as well as neural ganglia containing glial cells, and nerves with synapsoid connections. Other findings are intestinal structures consisting of a simple columnar epithelium, crypts containing proliferative cells, mucus producing goblet cells and smooth muscle layers. Skin has been found composed of both dermal and epidermal layers, stratum granulosum, keratinized cells, and hair follicles. Moreover respiratory airway composed of pseudostratified ciliated epithelium, smooth muscle, nerves, and supporting cartilage are common as well as skeletal muscle, pigmented retina, glands and both endochondral and intramembraneous bone (paper I) [22, 29, 40, 53].

Cell transplantation has mostly only been followed by standard histological haematoxylin and eosin staining with the sole purpose to test for pluripotency [22, 29, 39, 45, 214-218]. As reviewed by Przyborski, little has been done to further characterize the developmental potential of HESC after transplantation, why very few reports have been published specifically on this subject. Subsequently, information concerning the characterization, regulation, progression, and outcome of HESC after transplantation is lacking.

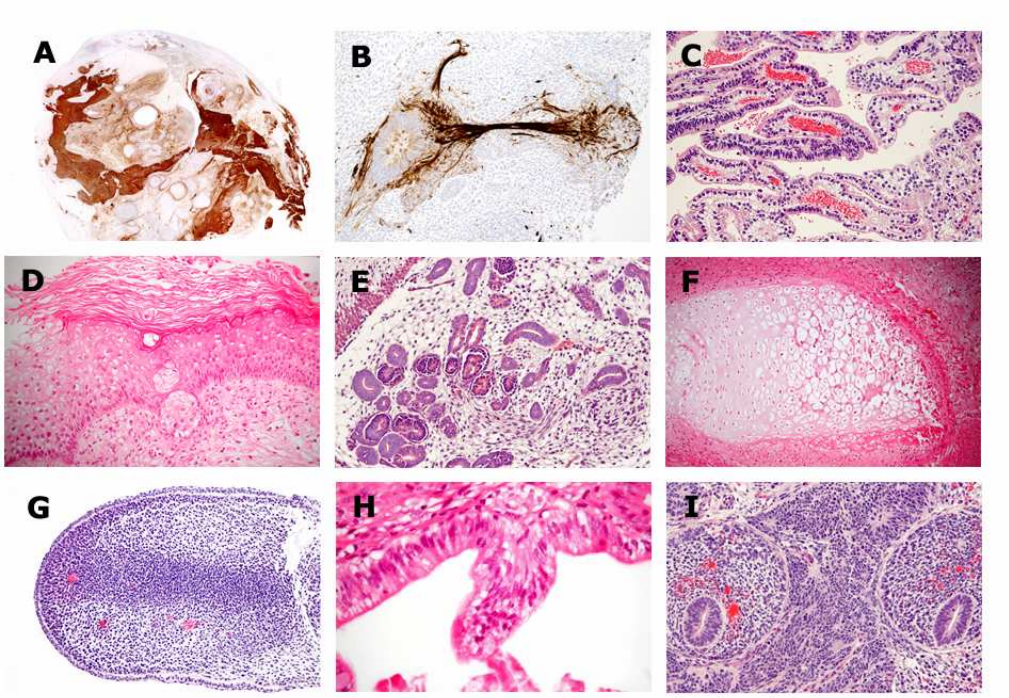

Figure 4. Examples of tissues found in teratomas *in vivo;* (A) CD56 staining illustrating the dominating neuronal contribution in a teratoma. (2x mag.) (B) NFP positive neural connection (20x mag.) (C) Choroid plexus, HE (20x mag.) (D) Keratinized epidermis including a gland, HE (20x mag.) (E) Renal development, HE (20x mag.) (F) Endochondral bone formation, HE (x20 mag.) (G) Limb-bud formation, HE (x10 mag.) (H) Intestinal epithelium including a basally located mitotic cell (stem cell), HE (x40 mag.) (I) Highly organized epithelial structures embedded in a neural region, HE (x20 mag.)

Limitations of the HESC-teratoma model system

It should be noted that this model to study human early organogenesis has limitations. Correct embryo folding and movements of cells for normal development does not occur resulting in spherical and haphazard tissues. The volume of the tissue that can be allowed to develop is limited to what is ethically reasonable for the specific animal model used, and also by vascularisation, why it is more complicated to study later events in teratoma growth. The seemingly haphazard development in the teratomas could be illustrated by the classical experiments by Mangold and Spemann [219], which showed that unorganized or incorrect morphogenetic signals might produce multiple axes or a chaotic development basic body plan, most likely being one reason for the haphazard development found in the teratomas.

Influence of the in vivo microenvironment

An issue to be investigated is whether the site to which HESC are transplanted will influence differentiation. This has to my knowledge so far not been studied or reported. Most investigators have reported teratoma growth after transplantation within the subcapsular compartment of either the testis or the kidney [22, 29, 39, 45, 214-218]. Other sites for transplantation are subcutaneously [173] or in the hind leg muscle [126, 220]. The only evidence indicating that the local microenvironment influence differentiation of grafted ESC is presented in a study using mESC. In this study it was reported that a greater ratio of cartilage was produced in the knee joint compared with the subcutaneous space [221]. However in another study by Deacon et al. [222] where mESC were transplanted to the brain of mice, differentiation to cell types appropriate for the environment was not a consistent finding. No study exist supporting that HESC inoculated beneath the testicular capsule give rise to testicular tissues, nor does it appear as if cells transplanted under the kidney capsule give a higher proportion of renal development. From an ethical point of view, since a knee-joint of a mouse is a very small region, teratomas grown in such a location cannot possibly be grown for long. The same is valid for transplantations to the brain, while subcutaneous growth can be allowed for much longer. If grown only shortly, the composition of teratomas will not reflect the true potential and outcome, why comparison between microenvironmental impacts becomes difficult. Differences in composition can as well be due to the condition of the cells when transplanted, why it is very important for these studies to be performed in parallel. For instance, what also we have experienced, is that the gross morphology of teratomas not always appear the same[39, 213], in that teratomas from one cell line can be more or less compact and occasionally even consisting completely of fluid filled cysts.

Another issue to keep in mind is that the pathways of importance during embryogenesis and organogenesis are changed or even lost in the adult mouse model. Therefore it may be possible that transplantation of HESC into embryonic tissues would provide a more accurate environment to result in more complex and perhaps more organized structures. HESC ability to respond to intrinsic signals when placed in a specific site in the developing embryo has been tested. HESC were transplanted into chick organogenesis-stage embryos, where the cells acquired neuronal fate when placed adjacent to the notochord and neural tube, appropriate to their location, and likely due to regional *in vivo* signals [223]. However, whether differences exist in the developmental potential of HESC engrafted into embryonic or adult chick tissues was not investigated. Methods have been developed and used to study the fate of transplanted cells also in rodent embryos [224]. This approach may be very useful for studying HESC differentiation.

The potential of pre-differentiation

By definition, all the HESC lines reported to date have the ability to form tissues representative of all three germ layers *in vivo* (reviewed by Przyborski [213]) whereas certain EC lineages, such as those derived from the TERA2 parent line, appear more restricted in their capacity for differentiation after transplantation [225, 226]. EC lines that have only been able to produce ectodermal derivatives *in vitro* also produce teratomas consisting only of ectodermal tissues [226-228]. Furthermore, already committed progenitor cells (as outlined in section 1.7) do not appear to form teratomas *in vivo* but can survive and be integrated if transplanted to the corresponding and appropriate location. All those cells are restricted, as opposed to pluripotent undifferentiated cells that posses full

developmental potential and might therefore be less sensitive to the microenvironment at the transplantation site. Instead of *in vitro* pre-differentiation it might also be possible to perform local administration of compounds directly *in vivo*, using for instance alginate gel as carrier.

Do all HESC lines show the same developmental potential in vivo?

One very important question that yet has no answer is whether the developmental potential of different HESC lines *in vivo* are identical, or not. An extensive evaluation of several teratomas from different graftings and independently derived HESC lines will be required to formulate a reliable answer to this question. Considering the large genetic and perhaps also epigenetic variation amongst all existing cell lines, it is likely that some cell lines will be more prone to differentiate into certain lineages than are others. Support for such differences is described in section 5.2.

The current status of the teratoma in vivo model

When in detail studying HESC growth in a teratoma tissue, it is important to distinguish between host and transplanted cells. This has been solved by fluorescence in situ hybridization for a human specific probe, as reported by our lab (paper I-II) or by using immunohistochemistry for human-specific nuclear antigen [39]. As discussed by Przyborski [213] in situ hybridization and immunocytochemistry are particularly useful to determine the maturation level and identity of cells or tissues in a maturation phase, otherwise not recognizable. Although easier to distinguish morphologically, more mature tissues also require multiple markers for their identification, and determination of accurate cell components and position. However, it should be noted that functionality is not confirmed simply by the presence of certain markers.

In paper I, we reported that the maturation level within a teratoma correlated with the time spent *in vivo* and that the vascular system of the host anastomosed with HESC derived vessels. It was furthermore recognized by Przyborski that no study have been aimed at dissecting the progress of teratoma formation, why there has been a lack of knowledge regarding how HESC differentiate to produce differentiated tissues. For instance whether events similar to stages of cell development during normal embryogenesis, such as gastrulation can be identified, or whether more advanced differentiation takes place very rapid into specific tissue types. These questions are some of which we attempted to investigate, in paper II. The study in paper II gives further support to model early stages of human embryogenesis using HESC.

Even so, a vast amount of work will be needed to understand the biological processes driving differentiation of HESC *in vivo*. In addition to provide an opportunity to model early human development, teratoma growth has been used to support human tumour growth and studies of tumourigenic processes and interactions of tumour cells with the surrounding microenvironment [220, 229]. Since there is no experimental system wherein human cancer cells can be grown in the context of a mixed population of normal differentiated human cells, Tzukerman et al. [220, 229] injected cancer cells into HESC derived teratomas in SCID/beige mice. In these studies they describe how the tumour cells invaded the surrounding teratoma tissue and the occurrence of neovascularisation within the carcinogenic tumour.

1.10 CHROMOSOMAL INTEGRITY

It is well established from several laboratories, including our own, that inadvertent genetic HESC variants can be found, including changes in several separate chromosomes, for example 1, 6, 8, 5, 12, 13, 17, 18, 20 or X [61, 62, 230-234]. Even so, HESC are unusually stable under optimal culture conditions in comparison with all other mammalian culture systems [232, 235-237]. The first report on inadvertent chromosomal changes by Draper et al. [230] raised the issue regarding genomic integrity and subsequently safety precautions. However, karyotypic changes can be recognized as a possibility to expand the studies of cellular differentiation also to include mechanisms for tumour progression and birth defects.

Hardarson et al. [238] found that only 42% of all blastocysts are chromosomally normal after investigation of chromosomes 13, 16, 18, 21, 22, X and Y, i.e. only 7 chromosomes. Thus, one would expect a very high rate of chromosomally abnormal HESC lines, if they did not have a disadvantage in the derivation. Cell lines have been found abnormal shortly after derivation (trisomy 13) [39] but the numbers are very low, indicating that mostly chromosomally normal blastocysts are capable of generating cell lines. The cell line was argued to have a growth advantage over occasionally appearing normal diploid cells [239]. It has been suggested that the manner of which HESC are passaged may affect the accumulation of cytogenetic abnormalities. Enzymatic and especially clonal relative to manual passage and feeder-free conditions was suggested to facilitate chromosomal changes during long-term culture [62, 235]. This has not been proven but could be due to the fact that enzymatic treatment makes bulk expansion possible and thereby increases the chance of finding chromosomal changes. Maitra et al.[234] also showed changes in mtDNA in high passage cultures and further argue that also long term culture per se would enhance karyotypic instability.

Changes in chromosome 12 and 17 are the most frequently observed in several HESC lines over time in culture, including in our lab (papers III-IV)[61, 62, 230, 235, 236]. Thus, suggesting a selective pressure for the gain of these chromosomes which provide a growth advantage at the expense of differentiation [230, 240]. Interestingly these changes are common for testicular tumours [241-243], possibly linking HESC to a germ cell origin as discussed above (section 1.5). In addition to the passaging procedure, another hypothesis for the reason of karyotypic changes presented by P. Andrews is that the density to which colonies are allowed to grow may influence the ability to gain karyotypic changes [236]. Within high density colonies, cells are subjected to a greater metabolic stress, which in turn may result in selection of cells that are more resistant to such processes. A shortened population doubling time have been reported for trisomy 12 cells [61] and together with additional changes, supported by reports from us including others (papers III-IV) [233, 239].

Only few HESC variants reported have been investigated for their pluripotent ability *in vitro* and *in vivo* (papers III-IV), [233]. Our data are presented in paper III and IV. The variant described by Plaia et al. [233] retained pluripotent capacity both *in vitro* and *in vivo*. They also found clusters of Oct-4 positive cells in teratomas 8-12 weeks after grafting and report that cells expressing markers for pluripotency could be expanded *in*

vitro from such teratomas. No experiments were performed on normal diploid cells in parallel why it cannot be argued that such cells were retained due to the karyotypic change.

Recently Herszfeld et al.[244] identified a biomarker, CD30, for genetic instability in HESC. CD30 is a member of the tumour necrosis factor receptor superfamily, shown to have a role in apoptosis protection. CD30 was found expressed on transformed but not normal HESC. This marker could possibly be used to facilitate the development of culture methods that protect genomic integrity.

1.10.1 Epigenetic status of HESC

An additional concern is the observed differences in the dynamics of preimplantation DNA methylation programming recently observed between species, with no animal model recapitulating the genome wide changes observed in the human [245, 246]. Wobus et al. [33] points out that other epigenetic modification, such as acetylation, histone modification and chromatin structural changes would also be expected to play an important role in the developmental potential of HESC. Aberrant methylation of the gene promoter region associated with gene silencing is an epigenetic phenomena observed in most human cancers [247], as well as a natural consequence of aging in some tissues [248]. In order to understand the full potential of HESC also epigenetic factors in addition to genetic factors controlling early human development must be defined. This has not yet been well studied in HESC but is a field of intense research at the moment.

Even though HESC could provide a model for epigenetic studies, they are after all derived from a stage in mammalian development when the genome undergoes global epigenetic remodelling [249], why it is unclear whether they retain a stable epigenetic status or not. Maitra et al. studied methylation pattern of 14 genes known for their differential methylation in cancer tissue compared to normal tissue. They found that gene promoter methylation was higher in eight of nine late passage cells compared to early passage cells. Methylation profiles of the promoter or putative regulatory regions of the OCT-4 and NANOG genes was studied by Lagarkova et al.[250], who demonstrated that expression of those pluripotency-maintaining genes correlated with their methylation status. They furthermore propose that DNA methylation underlies the developmental stage-specific mechanisms of pluripotency-related genes expression and reactivation and may have an impact on differentiation potential of HESC lines [250]. In a recent review by Rugg-Gunn et al. [251] data is presented supporting that HESC maintain a substantial degree of epigenetic stability during culture and thereby that HESCs could provide a model for studying epigenetic regulation during the early stages of human cellular pluripotency and differentiation.

2 AIMS AND HYPOTHESES

The overall aim was to investigate the potential of HESC to serve as model system for early human developmental processes, both normal and diseased.

More specifically, the aim was to;

- Evaluate the stringency of the early developmental pathways from HESC following transplantation to *in vivo* conditions.

  And in this way contribute new insights into early human development and the *in vivo* organogenesis, originating from HESC.

- Evaluate the karyotypic stability of HESC and the subsequent consequences *in vitro* and *in vivo*.

Furthermore, in order to fulfil these aims, in an interdisciplinary collaboration at Karolinska Institutet, we first established the conditions for the derivation and analysis of newly derived HESC. My specific role in the network was to develop and evaluate the cell marker profile for differentiation *in vitro,* and histology after xeno-grafting. HESC derived in the network was subsequently used in the projects described in this thesis work.

3 METHODOLOGICAL CONSIDERATIONS

Materials and methods are described and referred to in the individual papers. In this section summarising and additional description is given, and specific methodological issues are discussed.

3.1 DERIVATION (PAPERS VI AND VII)

The derivation, characterization and early differentiation of HESC lines, from donated blastocysts that cannot be used in infertility treatment was performed with approval from the Ethics Committee of the Karolinska Institutet (papers VI-VII).
Human blastocysts were donated after informed consent by couples undergoing *in vitro* fertilization at the IVF unit, Karolinska University Hospital. Each individual embryo was denoted a code number and after the derivation process, the cell cultures from the donated embryos were given a new nomenclature used in the laboratory, for the data bank and for publications regarding the cell lines. The identity of the donors of the embryos from which the human embryo cell cultures were derived can thus be traced only via a coded list.

3.1.1 *In vitro* culture of embryos at the IVF laboratory

Embryo transfers (ET) were in most cases performed on day 2 or 3 post insemination and of embryos not selected for ET, the ones with a score ≥2.0 were cryopreserved for a possible treatment of the couples later on. Embryos not suitable for ET or cryopreservation were cultured up to day 5-7 depending on the speed of blastocyst development and subsequently used for derivation of HESC lines. For evaluating the quality of the blastocysts, the scoring system described by Gardner et al. [252] was used. Briefly, a maximal score of 3.5 is given to the embryo if no factor reducing embryo quality is observed. The score is reduced in increments of 0.5 for non-ideal numbers of blastomeres (4-cells day 2 and 8-cell day 3 were considered ideal), the presence of more than 10% fragmentation, non-spherical blastomeres, unequal size of blastomeres, unevenness of the cell membrane, cytoplasmic abnormalities and when the embryo do not fill the zona pellucida. Embryos with multi-nuclear blastomeres are avoided for ET and cryopreservation and not considered eligible for HESC derivation studies.

Since all really good quality blastocysts embryos are either transferred to the patient or frozen for the couple's infertility treatment in the future, such embryos are generally not used for research. However, cryopreserved embryos of good quality can be made available for HESC derivation, when the couples no long are in need of the embryos. One HESC line was derived after 5 years cryopreservation (paper VII).

3.1.2 Preparation of the ICM

After removing the zona from the blastocyst using 0,5 % Pronase ICM cells were prepared according to the protocol described by Solter and Knowles [34], with minor alterations. In brief; the trophectoderm is removed by immunosurgery using rabbit

antihuman whole serum and guinea-pig complement serum. The ICM aggregate is then placed into a culture dish containing HESC culture medium and feeder cells. Special concern is given to avoid trophectoderm contamination. Initial passaging is performed mechanically without enzyme treatment.
In paper VII whole embryo culture was tested for the derivation of HESC lines. The use of non-human materials bears a risk of transmitting pathogens, and they are not optimal in cultures aimed at cell transplantation in humans. The commonly used protocol for immunosurgery has the disadvantage of containing animal products. Therefore, two blastocysts were at one time subjected to pronase treatment only and placed into culture; neither of them grew out to a cell line (paper VII).

However, since the cell lines are presently only allowed for research use, this is not an issue for the work presented in this thesis.

3.2 CULTURE OF HESC (PAPERS I-VII)

3.2.1 Feeder cells

Up to today, all published routine culturing of HESC includes xeno-components.
The human foreskin fibroblast (hFS) are commercially available (CRL-2429; ATCC, Mananas, USA) and thereby a convenient choice. New batches of feeder cells need to be thoroughly tested for their supporting capacity, but so far 7 different hFS batches supported HESC growth (Imreh et al. unpublished). We have however found that both HESC growth and differentiation rate differ between batches of feeder cells tested. Generally, feeder cells that support a high growth rate will also induce an increased differentiation. Another advantage of using hFS over MEFs cells is that they in our hands can be used for up to at least 22 passages (paper V). MEFs are commonly not used for more than 6 passages. For the studies in this thesis feeder cells were cultured in Iscov´s medium supplemented with 10% FCS. Confluent cultures were mitotically inactivated using irradiation (35 Gy) and commonly seeded at a density of 2.1–2.3x10^4 cells/cm2 (paper V).

3.2.2 HESC culture medium

All cell cultures were kept at 37°C, 6.8% CO^2 with high humidity (95%). Medium was changed daily.

Serum and Serum Replacement (SR)

Our first reported cell line, HS181 (paper VI), was derived using hFS as feeders, but in foetal calf serum (FCS)-containing culture medium. Today all cell lines can be cultured in SR medium with a higher non-differentiated growth rate than in serum-containing medium. Furthermore, in paper VII, we reported the first cell line to be derived both on human feeder cells and in SR medium. It should be noted that SR is not completely xeno-free, but the composition is more defined than FCS, and subsequently vary less between batches.

Additives

The medium used in different laboratories around the world for HESC derivation and propagation also differ in the additives used. In papers I and VI, medium was supplemented with LIF in addition to FCS. The complete medium was the following; KO-DMEM supplemented with 2 mM L-glutamine, 20% FCS, 0.1 mM β-mercaptoethanol, 1% non-essential amino acids, and rhLIF, 1 µL/mL. As discussed (section 1.4.2) LIF is not beneficial for propagation of HESC, but bFGF instead have a proven effect [44]. The medium for derivation using SR (paper VII) has the same composition with the difference of 0.5 mM β-mercaptoethanol, 1% ITS and bFGF (8 ng/mL), but no LIF. In general the derivation medium is richer than the medium used for routine passaging (papers II, III, IV, V) where ITS is excluded and 15%-20% SR, 4ng/mL bFGF and 0.1 mM β-mercaptoethanol is used instead.

3.2.3 Passaging and colony morphology

The initial passages were performed by mechanical dissociation after which the cell lines were gradually adapted to a combination of enzymatic (10ng/mL dispase) and mechanical passaging. Since HESC are known to be very difficult to passage as singular cells all passaging methods depend on the piece of the colonies that can be transferred. Mechanical passaging is very time consuming why enzymatic treatment make it feasible to culture also relatively large numbers of cells. Passaging was performed every 3-6th day depending on seeding density and cell line. For some lines, it is easy to maintain 0,5x10^{6} cells/6-well using the latter. Other HESC lines were more difficult to grow and to keep undifferentiated. Colony morphology was controlled on a daily basis and differentiating regions removed mechanically. Optimal culture methods are yet be obtained, why 10-20% differentiation is not unusual. Some line would have higher frequencies of differentiation. Colonies were considered as good, when the cells were evenly shaped with no space in between. Cells should have a large nucleus versus cytoplasmatic ratio and prominent nucleoli. Furthermore, colonies should have a clear border without signs of differentiation such as heterogeneous cells, neural rosettes or regions with large flattened cells (possibly endothelial). One potential drawback with using dispase for passaging is that enzymatic treatment has been suggested to facilitate chromosomal changes during long-term culture [234](discussed in section 1.10).

3.2.4 Freezing and thawing

In papers VI and VII, vitrification in pulled open straws using ethylene glycol, dimethylsulphoxide (20% each) and 1 M sucrose as cryoprotectants, was carried out as described by Reubinoff et al. [63]. This method allows for small numbers of cells to be frozen and is additionally gentler to the cells. This method is however not feasible to use when large numbers of cells need to be frozen, or retrieved quickly after thawing. Conventional slow freezing using 20% dimethylsulphoxide was therefore successfully used for routine freezing in the research lab (papers I-V). In order to avoid contact with N_2 cryovials were also stored in a -196ºC freezer.

3.2.5 Embryoid Body (EB) formation (papers III and VII)

One method for investigating the differentiation potential and pluripotency of HESC is by formation of EBs (papers III and VII). EBs were formed from HESC using two methods. In paper VII, the so called 'hanging drop' method was applied. Arrays of aggregates were plated in 25-µL drops of 20% SR medium without bFGF as described by Mountford et al.[253]. EBs were moved to fresh medium every second day and cultured for one month. In a second and more convenient method, EBs were formed and cultured by seeding aggregates of cells to a non-cell culture treated dish in above described medium. For routine evaluation of differentiation potential EB formation is a time efficient and cheap way. The alternative method, which is teratomas formation *in vivo,* provide a more realistic and complex environment as described below and in section 1.9.

3.3 EXPRESSION ANALYSES

3.3.1 Immunocytochemistry and histology

HESC are characterised by a set of cell surface molecules; SSEA-3/-4, TRA-1-60/-81, GCTM-2, alkaline phosphatase and telomerase activity and the two transcription factors Oct-4 and the recently discovered Nanog, all discussed in section 1.4. SSEA-1 is commonly used as a sign of differentiation and should thereby not occur in cultures. The TRA and GCTM-2 molecules are keratan sulfate proteoglycans [254], while SSEA-3 and -4 are glycolipids [255], their function although remain unknown. All surface markers were originally described as epitopes on human EC cells, discussed by Badcock et al. [254] and also found on ICM cells [256]. This highlights their close relation. None of the markers are entirely specific for HESC, although their combination together with accurate colony morphology provides a good enough estimate.

It should be noted that about 1% of Caucasians have been reported to lack expression of SSEA-4 [257], why SSEA-4 negative cell lines not necessarily have lost pluripotency. The cell surface markers are not as sensitive a measure as Oct-4 and Nanog, due to their presence also after differentiation has set in, possibly due to a faster turn-over rate of the transcription factors as compared to the large and complex cell surface molecules.

On a routine basis we used a panel consisting of Oct-4, TRA-60/-81, SSEA-4 and SSEA-1, occasionally with the addition of SSEA-3, GCTM-2 and alkaline phosphatase. Characterisation for those markers was always performed in duplicates and in parallel with experiments, to assure the use of good quality cells. As an indication of differentiation *in vitro* (paper V, VII) antibodies for Nestin (embryonic neurons/ectoderm); BMP-4 (mesoderm); AFP (endoderm) and Vimentin (general) were applied. To visualise the epitopes, and estimate their relative amount, indirect immunofluorescent labelling was performed on cell cultures. Immunofluorescent labelling is a subjective method; the intensity of fluorescence can easily be misinterpreted. Therefore, it is important to always include an isotype negative control as a negative baseline. Since immunolabelling methods rely on epitope specific binding, often by a monoclonal antibody, it is furthermore important to be aware of the effect that fixation protocols exhibit on the epitope structure. For internal epitopes, such

as Oct-4 and Nanog permeabilisation of cell membranes by a detergent is required to allow for the antibodies to pass through and reach the epitopes.

Histology is the method used for evaluating the pluripotential capacity *in vivo*, i.e., the presence of tissues representing all three embryonic germ layers. This was performed by conventional haematoxylin and eosin (HE) staining on formaldehyde fixed and paraffin embedded tissue from teratomas (Papers I-IV; VI-VII). Immunohistological studies are not considered necessary for this purpose. In papers I and II, immunohistochemistry was applied to teratoma sections and provided detailed information from combinations of antibodies applied to consecutive sections. The setback is that antibodies are not applied to the same section, which would be optimal for proving co-localisation in the same cell. In paper I however, fluorescent double labelling was performed for neuronal markers on frozen sectioned tissue. Furthermore, immunohistochemistry is feasible only when applied to a representable part of a whole teratoma. These tissues are commonly 1-2 cm in diameter and one section is 5 um thick. Although teratomas are cut in pieces this would roughly mean 500 sections, provided that the tissue is divided in 4 pieces. Immuno staining on paraffin embedded tissue sections were visualise using the ABC-method. This is a technique utilising peroxidase enzymatic reactions and the substrate 3,3'-diaminobenzidine (DAB) for developing a non-fluorescent colour. Many paraffin embedded tissues are auto fluorescent why it can be problematic to perform fluorescent immuno staining on such material.

3.3.2 RT-PCR

mRNA transcripts were detected using reverse transcriptase-PCR (RT-PCR) in cell cultures (papers III, IV, VII) and in tissues from teratomas (paper II). Complementary DNA was synthesized from total RNA and used in the subsequent PCR. Using this technique very low level of RNA can be detected and the expression from several genes can be measured on the same material. The setbacks are that mRNA expression not necessarily reflects a protein expression and that morphological as well as quantitative information is lacking. Comparisons in expression levels between materials were not attempted since conventional RT-PCR is not a quantitative method. In each PCR reaction equal amount of cDNA from all samples were used, and a house keeping gene was studied to assure successful reactions and equal cDNA levels.
One complication is the presence of hFS cells in cultures and mouse tissue in teratomas. It is in principle impossible to completely avoid hFS cells, why the transcriptional profile of those cells must be taken into account. The same is true for teratoma material, regarding possible contamination of mouse cells, though a solution is the usage of human specific primers. However, it can be problematic to find human specific primers for evolutionary conserved genes, a problem that we encountered for genes encoding patterning molecules and other factors involved in early development. A technique that we did not apply, but which add a morphological level and detailed information of the origin of a specific cell or tissue, is laser capture micro dissection (PALM). Using laser, down to a single cell can be cut out from a section and subjected to RT-PCR analysis. This would have been optimal for expression studies in the teratomas. With this method combinations of genes would have more accurately

indicated tissue belonging and cell specification, since genes expressed in a complete teratoma tissue does not reflect the situation in a specific tissue compartment.

3.4 CYTOGENETIC TECHNIQUES

3.4.1 Karyotyp analysis (papers III, IV,VI, VII)

A normal diploid chromosomal constitution is a prerequisite for HESC. It is a well known risk that cells cultured for long periods of time gain chromosomal abnormalities, even though HESC are surprisingly stable. Chromosome banding techniques such as Q- or G-banding of metaphase chromosomes are therefore important in characterizing HESC. G-banding was performed in paper VI, and Q-banding in paper III, IV, VII. In addition, spectral karyotyping (SKY) confirmed the result found by Q-banding in paper III.

Common for those techniques are that the mitotic index (proportion of cells in mitosis) is increased by treating the culture with a spindle disrupting agent such as colcemid. Giemsa, a DNA-binding chemical is used for G-banding. In Q-banding - the chromosomes are stained with a fluorescent dye which binds preferentially to AT-rich DNA. Fluorescing bands are called Q bands and mark the same chromosomal segments as G bands. SKY is based on 'chromosome painting' and fluorescent DNA probes covering an entire chromosome. All chromosomes are marked with different fluorochromes. Limitations with these techniques are the resolution and the number of metaphases. The smallest loss or gain of material visible is about 4 mega bases of DNA and the numbers of metaphases that can be studied seldom exceed 30 cells. Recently a PCR based technique was developed that overcome both these limitations; Representational Oligonucleotide Microarray Analysis (ROMA) [258]. With ROMA a resolution of 30 kb throughout the genome is achieved and high numbers of cells can be investigated. ROMA technology would significantly increase the accuracy of HESC karyotypic analysis.

3.4.2 In situ Hybridization

Papers I-V include techniques in which sequence specific DNA probes labelled with fluorescent markers were utilised to distinguish between cell types (papers I, II, V) or study aneuploidies (papers III-IV). This is a highly sensitive method by which singular copies of DNA or mRNA can be detected. Provided that a specific chromosomal segment of interest is known, thousands of cells compared to tenths of cell by G- or Q-banding can be studied. Using a mixture of 'male' and 'female' total human genomic DNA probes, human cells were distinguished from mouse cells in teratoma sections (papers I-II). In situ hybridization (ISH) for Wilm´s tumour-1 (WT1) mRNA was conducted in paper I. Here sense and antisense probes were hybridized to the tissue sections and detected by anti-DIG antibody and a subsequent substrate for the colour reaction. Centromere-specific probes were used to verify the copy number of chromosome 12 in papers III and IV and a yet another probe to verify the copy number of 12p in paper III. When assaying for a chromosome probe in cell culture it is important to avoid false positive results by superimposed cells. Therefore only distinguishable cells were evaluated. In tissue sections the situation is further complicated. In some tissues cells can be tightly adhering making them difficult to

distinguish and it is not possible to know how large fraction of the nuclei that are present within the section. However, the likelihood for cutting off chromosomes is equal for diploid and triploid cells, why it is still possible to evaluate the ratios in between.

3.5 *IN VIVO* DEVELOPMENT - TERATOMA FORMATION

A key criterion for stem cell pluripotency is the formation of cell types representing the three germ layers after injection into immunodeficient mice. Implantation of HESC beneath the testicular capsule or kidney capsule or intramuscularly into immunodeficient mice results in a growth that often contain well organized and differentiated tissues (section 1.9). To evaluate pluripotency and study developmental potential *in vivo*, we used SCID-beige mice (C.B-*Igh-1*^{b} GbmsTac-*Prkdc*^{scid}-*Lyst*^{bg} N7), commonly used as recipients of HESC grafts also by others [29] (papers I-IV, VI-VII). This is a strain of double-mutant mice with impaired lymphoid development and reduced natural killer cell activity. We chose the testicular capsule as grafting site for several reasons. The testis is not a vital organ, conveniently located and well encapsulated keeping the graft in position. Moreover, the testis is an immunopreviliged site. Yet, no study have been performed to evaluate whether difference in tissue development appear between grafting sites. Possibly, the tissue environment directs differentiation of a HESC graft. In the earlier reports, (papers I, VI, VII) intra peritoneal injections with avertin was used as anaesthetics. The dose is very critical and animals recover relatively slow, why we changed to isoflurane anaesthesia. Isoflurane is an inhalation anaesthetics and thereby easy to regulate, and the animals recover momentaneously after removal of the anaesthetics. As mentioned, the seemingly haphazard development in the teratomas is not surprising, since for instance axes determination is not organized. There has been no effort in trying to apply morphogenetic signals to the system, but this might be a possibility in order to refine the system in the future.

3.6 FLOW CYTOMETRY

Flow cytometry is based on the same principle as immunocytochemistry (section 3.3.1), but instead of microscopic analysis singular cells are subjected to a laser beam and subsequent light emission is recorded. Moreover, this technique enables purification of living cells, such as performed in paper V. Here, GFP-HESC and RFP-hFS were sorted on a FACS Diva cell sorter to retrieve pure populations of cells. FACS analysis was performed in paper III to quantify the number of SSEA-4 and TRA-1-60 positive variant cells. The technique is very sensitive, small quantities of cells can be detected rapidly. Furthermore, flow cytometry can be used to analyse co-localisation of several antibodies on large quantities of cells. Morphology is lost which is a drawback, but quantisation is achieved as compared to the more qualitative immunocytochemistry. One precaution when sorting for rare cells is the possibility to clonally expand cells with growth advantages, and thereby a risk for selecting mutated clones.

3.7 TRANSDUCTION AND LENTIVIRAL VECTOR CONSTRUCTS (PAPER V)

Lentiviral vectors were used to transduce HESC and hFS with GFP and RFP respectively. Lentiviral vectors have been demonstrated to be efficient transfer vehicle for genes into ESCs [259, 260]. They reach high efficiencies and provide also gene expression in differentiating cells [261]. Lentiviral vectors were produced by transient co-transfection of three plasmids. One envelope plasmid (pMD-G), a packaging plasmid and the transfer vector FUGW were used. Retroviruses were produced by transiently transfect Phoenix GP cells using Fugene 6 reagent. Transduction of HESC and hFS was performed in the presence of Polybrene and a lentivirus MOI of 10. GFP and RFP expressing populations were expanded and FACS-sorted.

4 RESULTS AND DISCUSSION

The work in this thesis can be divided into three categories. Since the vast majority of the stem cell research at the time was focused on directed differentiation *in vitro* and yet little was known about the situation following *in vivo* transplantation, as well as chromosomal stability of HESC, these two latter issues became the main focus of this thesis. In the section below summarizing and additional discussion is given.

4.1 *IN VIVO* DEVELOPMENT AND POTENTIAL (PAPER I AND II)

4.1.1 HESC grafted *in vivo* differentiate to form highly organized tissues (paper I)

Paper I presents a detailed analysis of the early developmental outcome and potential of HESC after transplantation to an *in vivo* system. We have used immunodeficient (SCID/beige) mice as the transplantable host. As reviewed by Przyborski [213], this is the first thorough descriptive study of its kind. **The aim** was to provide important insights into the developmental potential of HESC in forming organoid structures and the integration these tissues have with the host. Five separate teratomas, all derived from injections of the HS181 cell line, were subjected to analysis. One teratoma was analysed after 22 weeks and four teratomas after 6-8 weeks *in vivo.* A large panel of tissue specific antibodies were used and human specific FISH to study integration with the host. All teratomas were composed of both solid areas, showing complex cellular organization and polarization, and cystic components. FISH analysis with the human-specific probe showed the teratomas to be mainly of human origin, with the most organized areas being exclusively human. TRA-1-60, TRA-1-81, SSEA-4 and Oct-4 for the undifferentiated, pluripotent stage of HESC could not be detected and no signs of malignant regions seen. Differentiation along a neuronal lineage, the formation of bone/cartilage and epithelia was predominant. The 22 week old teratoma contained the most mature structures identified, clearly suggesting a correlation in line with time *in vivo*. Organoid structures in this teratoma were reminiscent of tissues in the adult.

Neuronal differentiation

Both early and late stages of neural development were found, neural epithelium being the dominating component. Early stages were identified by immunofluorescent staining using antibodies for nestin, Tuj1 (βIII-Tubulin), and GFAP (Glial Fibrillary Acidic Protein). Double-labelling experiments showed co-expression of Tuj1 and vesicular γ-aminobutyric acid (GABA) transporter (VGAT), thus indicating that GABAergic neurons is a dominating feature. Cholinergic neurons as seen by choline acetyl transferase (ChAT) appeared to be absent. Tyrosine hydroxylase (TH) staining was detected only in fibres, but never in cell bodies, most likely sympathetic (adrenergic) and originating from the mouse's testicular innervation. Mouse/human cooperation was additionally seen in forming choroid plexus-like structures. The mature neuronal marker neurofilament protein (NFP), which stains neurites, was found overlapped with synoptophysin staining, in a fine synapsoid network. Ganglion-like structures were negative for Ki67, indicating post mitotic cells, and were found strongly positive for

NFP and S100 (glial cells) as well as neuronal-specific enolase (NSE). GFAP confirmed the presence of glial cells. The expression of nestin and Tuj1, but not SSEA-4, in the neuronal structures indicated the presence of neural precursors or progenitors rather then stem cells. Also, pigmented cells were found in simple epithelium, resembling early retinal epithelium.

Formation of bone and cartilage

Cartilage condensation with the resemblance of the initial stages of endochondral bone formation [262, 263] as well as intramembraneous bone was observed. Bone sialoprotein (BSP) and osteocalcin (OCN), showed staining within the hypertrophic chondrocytes and extracellular matrix of the mineralized tissue. Osteopontin (OPN) was detected in chondrocytes and osteoblasts, and OSAD, a small leucine-rich proteoglycan, was found to be present in the matrix of bone and the supporting cells. Mineralization was further shown by von Kossa staining for calcium phosphate deposition. Of particular interest, NCAM, a polysialated cell adhesion molecule known to be highly expressed in adrenal glands, central nervous system (CNS), and early during kidney development [264-266] was observed in the surrounding prechondrocytes and osteocytes embedded within the mineralized matrix. NCAM has been reported in the initiation of mesechchymal condensations [267].

Epithelial development

The anti-cytokeratin antibodies CK5, Cam5.2, and MNF116 were used to detect subunits expressed in ectodermal and simple epithelia. The phenotype suggested organoid development into bronchial or intestinal-like structures. Co-expression of CK8 and CK18 (Cam 5.2) together with thyroid transcription factor-1 (TTF-1) indicated early endodermal development. Because TTF-1 is expressed during lung, forebrain, and thyroid gland development [268], it is possible that these features replicate the early morphogenesis of thyroid or lung development. More mature endodermal development was clearly seen in the 22 week old teratoma, in which intestinal formations were found composed of an epithelial duct with mucus producing goblet cells, suggestive villous and a proliferative cell basally located in a crypt formation. All in accordance with mature intestine. Some of the cystic cavities were lined with a bilayered epithelium, structurally compatible with the formation of periderm. As support, strong staining for CK5 was detected, a marker known to be expressed in such structure [269] as well as Cam 5.2, as would be expected for simple epithelia. In the 22 week old teratoma more advanced maturation was found indicating complete formation of epidermis. This structure with the phenotype of mature skin was composed of a cornified, stratified squamous epithelium including the basally located dermis with epiderm above and a keratinized surface. An excretory duct was incorporated in this tissue.

Vascularisation with functional mouse and human co-operation

FISH analysis as well as CD34+/CD31+ staining showed compound mouse/human endothelial lining of vessel lumens interpreted as functional co-operation between mouse and human cells in establishing the teratoma vasculature. Erythrocytes in the lumen further suggest that microvessels had formed and anastomosed with the mouse vasculature, becoming functional blood-carrying microvessels. However, in the 22

week old teratoma regions of cell death and necrosis was found, most likely due to an insufficient blood supply in fast growing regions.

Renal development

Renal development is dependent upon reciprocal interactions between the metanephric mesenchyme and the ureteric buds [270]. In two of the teratomas, formation of primitive nephrons was seen. These consisted of metanephric blastema in close apposition to tubular structures as well as glomerular formations partly being vascularised. Mitotic cells as shown by Ki67 staining were common within the mesenchymal aggregates and also seen within the tubules, though only few cells of the more differentiated parts were positive. In addition, the mesenchyme was found to be strongly positive for NCAM. In situ hybridization showed that WT-1, a hallmark of renal development [271], was expressed. Thus, a full nephron differentiation capacity was indicated, although it was not possible to identify the proximal tubules as such, and it remains unclear if the vascularisation arose from human or mouse cells in this area.

The question arises as to what stimulates such a differentiation pattern de novo. The origin of glomerular capillaries has been debated, why it is possible that this *in vivo* model can be used to address this problem in the future. The induction mechanism governing nephron development in the teratomas is also unclear, however recombination of metanephric mesenchyme with dorsal neural tubes in culture yields development of renal tubules [272, 273]. Because neural tissues, at different stages of development, are dominating components of the teratomas, reciprocal interactions between the metanephric mesenchyme and neural cells might results in renal differentiation and development. These findings also strongly infer that equivalents of the intermediate mesoderm are formed during growth and differentiation.

In conclusion, this investigation extends previous published reports regarding *in vivo* development of HESC [22, 29, 37, 45, 214-217], in that for the first time cells derived from the mouse host and the implanted HESC are distinguished. A close correlation between proliferation and immaturity was indicated, and also suggests that the cells do differentiate along the predictable developmental pathways. The teratomas developing from the HS181 cell line resulted not only in derivatives from all three germ layers, but also in multi-lineage development from each germ layer. Altogether, these findings support the relevant use of this model for studies of early human development**.**

4.1.2 Kinetics of early development from HESC (paper II)

In paper I, support was found for the presence of a maturation process between weeks 7 to 22 after injection. In this follow up study we aimed at dissecting the kinetics of the early differentiation processes and developmental pathways from HESC *in vivo*. Again, this was to our knowledge the first such study performed.

Thus, i**n paper II**, the kinetics of early development was studied after transplantation *in vivo*. The analysis was mainly focused on stages prior to more mature structures, as these have been described in paper I. **The aim** was to evaluate HESC teratoma formation as a model for early human development and to provide new insights of cellular interactions in early developmental stages.

Using the HESC cell line HS181, six time points after HESC transplantation were analysed; DAY5, 10, 20, 30, 45 and 60. A net growth was not observed before DAY30, but analysis of sectioned material revealed at DAY5 the formation of epithelial structures representing the dominant feature up to DAY30. Together with evidence for NESTIN expression [274, 275] both by immunohistochemistry and RT-PCR, in combination with the morphology and growth pattern, the presence of HESC derived neuroepithelium could be clearly demonstrated. Expansion of the neuroepithelia was seen between DAY5 and DAY30 at which stage the epithelium making up the tubular structures attained a clearly multilayered structure, resembling the actively proliferating neuroepithelium seen during and after neural tube closure.

The proliferation rate stayed high at all timepoints, but declined slowly, as seen by Ki67 staining. DAY30 to DAY60, the numbers declined from 80-90% to 50% in the tissues formed. Loose mesenchyme stayed 10-20% proliferative at all timepoints. After an initially very high apoptotic rate, possibly representing an adaptation period to the change from *in vitro* to *in vivo* conditions, or being a part of normal epiblast development, apoptosis declined around DAY20-DAY30 and this paralleled with both a rapid net growth and the first appearance of human vasculature. Similarly to paper I, all teratomas appeared grossly well encapsulated, expanding rather than infiltrating the testicular capsule. The neuronal compartment was a highly dominating part of all teratomas until DAY60 and at this stage the teratomas contained tissues and structures similar to what we reported in paper I. However, neither cartilage nor bone formation was observed until DAY45, along with renal structures containing partially vascularised primitive glomeruli and tubules forming in association with a condensing mesenchyme of nephrogenic character.

h-TERT and NANOG was expressed at all timepoints, but NANOG showed a dip during DAY20-30. SSEA-4 was found by IHC at DAY5 and DAY30. While still labelling discrete cell populations at DAY30, this was limited to structures made up of a simple single layered epithelium. Together, these findings indicate the presence of immature possibly pluripotent cells, at all timepoints throughout the study, as opposed to what we reported in paper I.

Development from DAY5-DAY60

For a summery of the RT-PCR results, see Table 3 in paper II.
5-10 DAYS post grafting, Wnt3 and WNT5a, SHH, Brachyury and BMP-4, all known to be expressed during gastrulation, were found expressed in some of the specimens. The expression of these genes appeared more prevalent in DAY10 teratomas, but was still restricted to a few. Expression of CD90, Flt-1 and CD45 is commonly linked to post gastrulation and mesodermal tissue, however individual samples were found positive at this early stage.

At **DAY20-30** proliferation was a hallmark of the organized structures. Notably genes associated with both gastrulation; e.g. Shh, or early organogenesis; e.g. CD45, CD90, CD34, AFP, MyoD was expressed. CK18 could be found in epithelial structures, some also expressing p63 characteristic of both ectodermal differentiation as well as

development from the endodermal foregut. AFP, another endodermal marker was co-expressed with CK18, but not with p63. Since p63 is normally expressed in the developing lung, this would suggest that AFP-positive cells represent an intestinal-like pattern of differentiation. NFP was not found to be expressed at this stage, but the neuronal marker CD56 (NCAM) was clearly so.

Throughout the development of the HESC teratomas vascularisation was evident, most likely formed from preexisting mouse testicular vessels. However, in line with our previous findings in paper I, discrete populations of human CD31 and CD34 positive endothelial cells were found from DAY30. CD31 was seldom positive, indicating immature vessels. This may explain why expression of VE-cadherin or Flt-1 [276, 277] could not be demonstrated before DAY30 using RT-PCR, and strengthen our findings of vascularisation coinciding with net growth of the teratomas around DAY30. The lack of vascularisation possibly hampered growth up to this point even though an early recruitment of host vasculature was observed DAY10.

Surprisingly, DAY30, a unique structure resembling a limb bud was found. Normal development of limb buds consists of a central core of condensing mesenchyme lined by an immature epidermis. Immunohistochemically the epidermal equivalent expressed p63 [278, 279], peripheral cells were elongated and stained with the CK18 antibody [280, 281] and all epithelial cells were E-cadherin positive. This pattern is compatible with the situation in early differentiation of the mammalian epidermis. The mesenchyme was highly proliferative, with the central part showing condensating cells expressing CD56, characteristic of early precartilaginous development [282].

The process of ossification was indicated by markers for bone development such as SP7 and Bglap (former OCN) and parallel expression of Spp1 (former BSP), all showed initial expression early and strong expression was seen from DAY20 and onwards. As mentioned no bone formation was evident morphologically until DAY45. It appears that muscle formation is a later event in the teratomas preceded by MyoD (determined myoblasts [283]) expression from DAY20 as a triggering factor, while Myogenin (mature myoblasts [283]) was not found until DAY60. The limb bud development found DAY30 did not show Myogenin expression.

The two teratomas analysed at **DAY45**, were mainly limited to RT-PCR analysis, however, all genes analysed at this stage were expressed. Ipf1 (Insulin promoting factor 1) which is of importance for the endoderm derived pancreas [284] was first expressed at DAY45. Renal development could at this stage also be confirmed by IHC for WT1. While no hCG protein was detected by IHC, RT-PCR revealed hCG gene transcription in a few samples from DAY20 and onwards, all specimen positive at DAY60. No morphological observations supported formation of extra embryonic tissue.

DAY60, multilayered neuroepithelium was composed of both proliferative compartments as well as post mitotic neuronal cells stained by NFP, doublecortin and βIII-tubulin, clearly demonstrating a neuronal fate. CD56 strongly stained both these neuropeithelial compartments. In addition, NESTIN and PAX6, the latter known to be associated with development of the ocular and nervous structures was highly expressed. Ganglion-like structures expressed NFP, particularly in the fibres. Moreover,

doublecortin antibodies strongly outlined the fibre tracts. The neuroepithelium of the cerebral hemisphere normally undergoes a process of proliferation, migration, and differentiation by which the cortex is produced. Cells of the ventricular layer proliferate to produce neuroblasts, which migrate peripherally and establish the different layers of the cortex in a lower to peripheral manner [285]. The segregation of a proliferative and a post mitotic zone in neuroepithelia suggest that maturation appears to reach a pre stage of cortical plate development. Larger well circumcised areas were observed with NFP and CD56 positive cells reminiscent of CNS nerve nuclei. Peripheral neurons of the cranial nerve pathways situated in ganglia are commonly derived from neural crest (NC) cells, lying outside of the CNS, while central neurons reside in a CNS nucleus. Taken together, our data suggest that neuronal maturation from HESC *in vivo* require up to 8 weeks from the start with NESTIN positive cells at DAY5. There seem to be a developmental path from neural tube formation up to the beginning of a cortical plate formation. Most neuronal compartments were devoid of human vasculature even at DAY60, though clearly being vascularised. Likely, since those epithelia are the first structures to develop they become vascularised by recruiting mouse vasculature and therefore do not need to produce human vessels at later times, instead keeping the mouse vascularisation.

The described renal development, including primitive glomeruli and ducts, was prevailing also at DAY60. Renal development is a process of reciprocal induction of the ureteric buds and metanephric blastema [270]. Morphologically, these structures now appeared to arise from a nephrogenic mesenchyme, and confirmed by staining with antibodies against WT1 [271], in some cases also being expressed in structures compatible with early glomerular development. WT1 is expressed in the metanephric mesoderm before the ureteric bud is formed. Its expression is required here for the sprouting of the ureteric bud. CD56 was also intensely expressed in the mesenchyme as expected [264-266]. Furthermore, CK18 could be detected in glomeruli and Bowmans capsule [286] and also rare events of p63 expression [287]. While HE stained sections clearly showed the glomeruli to be vascularised, we only detected scattered CD34 positive cells associated with the vascular structures. This indicates host origin and argues against glomerular endothelial cells being derived from the nephrogenic mesenchyme. Proliferation was preferentially found in the immature metanephrogenic mesenchyme. However, the question remains as to what cells or tissues within the teratomas that can induce such a development.

AFP could still be found in some of the CK18 positive structures. p63 and TTF1 did not co-segregate in the same cells, and co-expression was only found in a few scattered cells indicating differential developmental capabilities even in cohesive cellular structures. More mature epidermal epithelium was strongly supported by the expression pattern of p63, being highly expressed in the peripheral cells. CK18 was found to be expressed in periderm-like cells associated with the p63+ area. DAY60 we found regions exclusively expressing CK18, strongly suggesting the formation of a choroid plexus equivalent [288]. However, we failed to demonstrate human endothelial cells here, suggesting the vascular network to be purely of host origin.

Discussion and possible interpretations

Markers indicative of primitive streak formation and subsequent early post-gastrulating events, such as Wnt3, Brachyury, BMP-4, Wnt5a and Nodal [289-292] were found by RT-PCR. The continuous expression of Brachyury might indicate that notochordal tissue or primitive streak formed, or that development in the teratomas is not synchronized over time, i.e. that signals for gastrulation are present at all time points. FGF9 expression, a possible sign of intermediate mesoderm, from which kidney develop [293] could not be found until DAY20. Early markers for definitive endoderm and derivatives of the foregut [294] such as TTF-1, Wnt-2, GATA4, Sox 17, C/EBPα and AFP, [102, 268, 295-297] were investigated. Taken together, markers for definitive endoderm appear late, indicating that other developmental features precede, and that specification of endoderm appear later.

Haematopoietic stem cells (HSC) co-express the endothelial-specific marker VE-cadherin, the pan-leukocyte antigen CD45 and the stem cell marker Kit [298]. CD45 was surprisingly found DAY5, though only in 1 of 5 teratomas, while VE-cadherin did not appear until DAY30. c-Kit was found only rarely by DAY60. Although inconsistent and highly speculative, these data could indicate differentiation in the teratomas into haematopoietic lineage, but not from a common progenitor to endothelial cells as suggested.

NANOG, hTERT and SSEA-4, as well as NESTIN indicated the early cells to be pluripotent and such cells appeared to be present throughout the study. However, multipotent progenitor cells have also been shown to express NANOG [299] why NANOG expression could originate from such progenitors. Darr et al. [92], recently showed NANOG levels to be highest during early differentiation of HESC, decreasing thereafter, and suggest such cells to be primitive ectoderm. The pattern of NANOG expression described herein support the findings from Darr et al. in that the expression strongly increased by DAY10 and dropped at DAY20-30. Furthermore, SSEA-4 has also been shown on neuronal stem cells [300], and together with NESTIN and PAX6 expression, this further supports our notion of the early epithelia being mostly of neuroepithelial origin. It furthermore concurs with early developmental stages during and after gastrulation; when the neural plate forms the neural tube leaving a sheet of epidermal ectoderm above. In support for the presence of neural tube formation and closure, E-cadherin revealed a mosaic pattern of expression [301]. Moreover p63, a marker for epidermal development, could be found co-expressed with E-cadherin in such epithelia being negative in neighbouring neural epithelia. CK18 was detected in parts of some neuroepithelia DAY30 and is known to be down regulated upon neural tube fusion [288]. Consistent with our findings, by DAY20 when the tissue started to expand, co-expression of both BMP-4 and Shh, both needed for neural tube closure, occur and imply that neural tube fusion and subsequent expansion was made possible. At DAY30 polarized growth was seen in the neuroepithelia and it is tempting to speculate around the similarities to development when the neural tube starts expanding.

By the time the neural tube fuses, neural crest (NC) cells are generated at the border between the neural plate and non-neural ectoderm, where they undergo an epithelial mesenchymal transition (EMT), and delaminate from the neuroepithelium. NC cells

migrate away and will give rise to most parts of ganglia and peripheral nervous system as well as melanocytes and some cartilage and bone, tissues that we find in the teratomas partly from DAY30 and fully from DAY60. It is possible that NC cells are progenitor cells for those structures in the teratomas. The presence of ganglion areas, most likely derived from NC derivatives, support the hypothesis that NC cells might be responsible for other non-neuronal tissues formed. Considering the dominating neural compartment initially and the appearance of non-neural tissues at later time points, it is of interest to speculate around the origin of such tissues. Either there are remaining pluripotent stem cells that later give rise to remaining tissues or, in fact what appears to be neuronal stem cells, expressing NESTIN and SSEA-4, might have this multipotential ability?

Interestingly there are indications that the development follows a similar path in time and space as expected for normal development. Markers for gastrulation appear earlier than markers for more mature differentiation. For instance, limb bud development occur *in utero* from the fourth to eight week, consistent with our findings for such tissues; periderm, mesenchymal condensation to form bone, and muscle. It is during the 4th week the neural plate folds into a tube, in line with morphological as well as gene expression data from the teratomas. Choroid plexus develop first DAY60 in the teratomas, while *in utero* from week 10 of development. Renal differentiation is yet an example found from DAY45 in the teratomas, and formed *in utero* from week 5 to 15 [302].

In conclusion, this study of HESC temporal development following *in vivo* transplantation revealed an early expansion of mainly neuroepithelia followed by a gradual differentiation leading to more diversified mature structures 8,5 weeks post grafting. Net growth was paralleled by human vascularisation DAY20, and while the growth up to 30DAYS was almost completely dominated by neuroepithelium, DAY45-60 displayed other types of differentiation. This raises the issue of the origin of later cell populations producing e.g. primitive glomeruli and associated cell formations, or endothelial cells forming functional vessels. Most importantly the development follows a scheme reminiscent of normal developmental processes, and thereby supports the relevant use of HESC-teratomas as model system for early human development.

Further discussion paper I and II

If unorganized or incorrect signals for axes definition are launched, multiple axes and a chaotic body plan is the result, most likely being the reason for the haphazard development found in the teratomas. Taking this fact into account we have not found any indications for that the development *in vivo* would be in any way restricted. The limiting factor as today appears to be the size of growth allowed and a sufficient blood supply. As a continuation of these studies it would be of interest to follow also later events. We have seen that the maturation of teratoma tissue follow a quite adequate time line as compared to normal early development and furthermore we know that the oldest teratomas are the most developmentally advanced (22 weeks *in vivo*). It would be important to test how far developed the tissues can be, in order to set the time frame of relevant use of HESC as a developmental model system. This is however problematic from an ethical point of view (section 1.9.2). Moreover, the relatively

small size of the tissue formed compared to what might be needed for larger tissues, together with our finding of necrosis most likely due to comprised vascularisation by week 22, suggest that it will be of importance to further investigate the vascularisation process in tissues formed from HESC. It might be possible to stimulate neoangeogenesis by administration of angiogenic factors such as VEGF in an alginate gel from which the proteins slowly are released. One hypothesis for the seemingly insufficient blood supply is as described in paper II that the initial growth mainly, not to say only, consists of neural ectoderm. A smaller portion of other ectodermal derivatives can be identified, as well as mesodermal and endodermal derivatives, implying a suppression of those lineages. Thus, it can be suggested that there is an imbalance between inductive and suppressive signals for an equal distribution of the lineages, assumed that the neuroectoderm is not due to restrictions gained already from *in vitro* conditions, which of course can not be dismissed. A balance might be achieved by administration of appropriate factors. However, as the main message of this thesis imply, not enough is known about how the early events during development is regulated. Thereby it will not be possible to calculate what signals and factors to apply, this has to be tested empirically. In doing so, we have a good opportunity to gain new important knowledge also on these early processes during development *in utero*.

4.2 GENETIC INTEGRITY; SELECTIVE PRESSURE AND POSSIBLE CORRELATION TO HUMAN DISEASE (PAPER III AND IV)

4.2.1 Culture conditions favouring chromosomal changes (paper III)

Although HESC under stringent conditions have been shown to exhibit a stable chromosomal constitution during extended *in vitro* culture [235, 237, 303], it is also well established from several laboratories, including our own, that inadvertent genetic HESC variants can be found [62, 230-232, 236, 304, 305]. The occurrence of growth adapted mutants offer the advantage of HESC being particularly well suited also as a potential model for tumour progression, extending the preceding models of human EC cell lines. **The aim** of this study was to investigate how a selective pressure could influence chromosomal constitution and to study the phenotype of the subsequent variant cells.

In paper III we report a premeditated functional adaptation of HS181 cells with a specific chromosomal change in 100% of the cells; 47,XX,del(7)(q11.2),+i(12)(p10). Those cells, referred to as HS181$^{7q-,i(12p)}$, showed increased survival and growth on plastic and also maintained expression of HESC markers, but showed a decreased pluripotency, as demonstrated by results from EB formation *in vitro*. The finding of reduced pluripotency may not be totally unexpected since the variant cells during the adaptation to growth on plastic were selected for self renewal and proliferation, not differentiation. However, the mutated HESC variant failed to grow as a xeno-graft *in vivo,* which was surprising and needs a further mechanistic analysis for its explanation.

For HS181 cells grown on a 'post-feeder ECM' similar to previously described for mouse ESC [57], optimal attachment was obtained only using 100% conditioned medium, suggesting that components in the conditioned medium was required for the attachment. After 6 passages on post feeder matrix some of the HS181 cells were

transferred to untreated plastic culture dishes. Also here 100% conditioned medium was found to be necessary and resulted in that a small number (< 1%) of the seeded cells could attach and grow. The HS181$^{7q-,i(12p)}$ cells grown on plastic showed a heterogeneous cell morphology and the growth rate on plastic was much reduced (half) compared to the HS181 cells when cultured at our regular conditions [306]. Interestingly, the morphology and also the doubling time of HS181$^{7q-,i(12p)}$ cells could be reversed back to the original HS181 state, after only one passage on hFS. After 11 passages on post-feeder ECM followed by culture directly on plastic the karyotype was 100% 47,XX,del(7)(q11.2),+i(12)(p10).

The HS181$^{7q-,i(12p)}$ cells grown on plastic were found to exhibit a marker profile for undifferentiated pluripotent cells (Oct-4, SSEA-4, TRA-1-60/-81, CD9, Nanog, hTERT) but also for differentiation (SSEA-1, CD90, CD34, Brachyury, Flt-1, VE-cadherin, vimentin). In addition, flow cytometry analysis on mutant cells grown on feeder cells revealed 89% SSEA-4 and 88% TRA-1-60 positive cells, while the same analysis on cells grown on plastic showed 81% SSEA-4 and 80% TRA-1-60 positive cells. Thus suggesting a low (≈10%) frequency of spontaneous differentiation on feeder cells, being slightly higher (≈20%) on plastic. A possible de-differentiation could be observed after returning the variant cells to feeder cell support, in that the HS181$^{7q-,i(12p)}$ cells lost the expression of VE-cadherin and SSEA-1.

It has recently been suggested that the inner cell mass cells of the blastocyst transition to a different cell type during ESC derivation, i.e. adaptation to *in vitro* growth, and that the closest *in vivo* equivalent of HESC is an early germ cell [138]. The concept finds strong support from marker studies performed by Clark et al. [240]. In this context, it is of interest that the specific chromosome 12 change observed in the present study, i(12p), has been repeatedly linked to clinical findings in germ line tumours [243]. Gain of 12p is a consistent finding in invasive germ cell tumours (GCT) and up to 80% have i(12p) (reviewed in [241-243]). Carcinoma in situ (CIS) is a precursor of such tumours but lack gain of 12p, leading to a suggested model where gain of 12p is associated with invasiveness (reviewed in [243]). In this model, loss of dependence of CIS cells from Sertoli cells, (i.e. cell growth independent of ECM) is related to resistance of a phenomenon known as anoikis and is crucial for invasiveness of GCT. Sertoli cell independence and suppression of apoptosis is associated with gain of 12p. When escaping anoikis, cells escape the microenvironment without going into the spontaneous apoptotic suicide of anoikis (reviewed in [307]). Several candidate genes on 12p have been proposed as responsible [151]. This is one hypothesis for the explanation of the phenotype showed by the variant cells.

Long term culture per se would also seem to enhance appearance of mutations [308]. Our data suggests that under routine passage of HS181 cells, using our most stringent culture conditions [306] amplification of chromosome 12 could not be detected (frequency of $<10^{-3}$) when using FISH analysis (probe detecting centromere). However, following two bulk expansions of HS181 cells, with the aim to achieve larger numbers of cells ($>10^7$ cells), a selective amplification of the whole chromosome 12 (mean 38% and mean 66%) was detected. These results could indicate a low percentage of trisomy latently present already at earlier passages, though not detected with the analysis strategy previously used i.e. not enough cells analysed. It is also possible that in line

with the above findings, the HS181 cell line is prone to amplify chromosome 12 when being pushed into a more expansive growth phase. It should be noted that the i(12p) mutation detected in the plastic growth adapted variant is not identical to the trisomy 12 detected in cultures aimed for maximal expansion, though it is possible that the isochromosome could arise from the trisomy.

Interestingly, the selective growth advantage of HS181$^{7q-,i(12p)}$ cells when propagated on plastic *in vitro* did not seem to have an effect when the cells were transplanted *in vivo*, since no growth of the variant was observed. This was somewhat surprising. In contrast, trisomic HS181 cells exhibit pluripotent *in vivo* growth under identical conditions (reported in paper IV). The HS181$^{7q-,i(12p)}$ cells have in addition to amplified 12p also a deletion of 7q. It has been suggested that a tumour-suppressor gene involved in cell-cell contact and adhesion reside on 7q31 [309, 310]. The loss of heterozygosity (LOH) of a putative tumour suppressor gene (TSG) on chromosome 7q may be part of an explanation for the enhanced *in vitro* growth potential of the HS181 variant, but is not in line with the *in vivo* findings of the variant. Several genes important for proliferation (Wnt2 in the canonical Wnt-pathway [311]), survival pathways (involving P13K, MEK2, caveolin-1, caveolin-2) [309] or potentially important to invasive growth (c-Met) [312] can be found on 7q. One could speculate that the findings with HS181$^{7q-,i(12p)}$ *in vivo* might reflect e.g. lower activity of c-Met and its downstreams effectors such as PI3K and that this effect overcomes the effect from loss of a putative TSG. Additionally there is a link between i(12p) and the 7q deletion considering cell cycle regulation. Cyclin D2 and the CDK inhibitor, p27Kip1 lies on 12p while a catalytical partner, CDK6, reside on 7q [313].

In conclusion this work highlights the possibility to study the importance of specific genetic anomalies in tumour progression by production of advertant HESC variants. Our data support that HESC appear to be prone to gain chromosome 12 changes. Importantly, the study moreover highlights the differences between *in vitro* and *in vivo* phenotype of such cells. Investigation of genetic integrity and characterization of variants could reveal important knowledge on the process of tumour transformation and progression.

4.2.2 Characteristics of trisomy 12 cells *in vitro* and *in vivo* (paper IV)

In paper IV we followed the proliferation and differentiation of variant sublines of the HESC line HS181, exhibiting low or high frequencies of trisomy 12. **The aim** was to investigate manifestation of amplification of chromosome 12 *in vitro* in HESC and to investigate the subsequent impact for *in vitro* proliferation, as well as the effects on survival and pluripotency following *in vivo* transplantation to an immunodeficient xeno-graft model.

FISH analysis for a chromosome 12 specific probe of HS181 cultures containing trisomy 12 (tri-12) cells revealed a random distribution in colonies *in vitro*. Everything in-between entirely transonic colonies to colonies completely devoid of transonic cells was detected. The thawing of frozen stored HESC is a well known risk in that the initial low number of cells may enforce a selection of cells with adaptation to enhanced *in vitro* growth. Using FISH, two out of four thawing of cells (all with 46,XX karyotype

according to G-banding; paper III) yielded chromosome 12 diploid cells (n=1400 and 1700, respectively). In two other thawing a low frequency (1-4 %) of trisomy 12 (n=791 and 741, respectively) was detected. In line with previous findings [306], expansion of HS181 cultures to larger number of cells (10^7 or more) resulted in increased frequency of transonic cells. Thawing from p28 yielded after only 5 passages of expansion 30% cells (884/2973) with trisomy 12. Thus, the expansion to larger number of cells at higher concentrations led to increased frequency of tri-12 cells. This could indicate a selective growth advantage during the expansion process of this particular chromosomal aberration. Culture at lower cell densities without expansion reduced already after 4-5 passages the frequency of tri-12 cells significantly. This leads us to hypothesize that at higher HESC density, cells with trisomy 12 are favoured, while at lower density, the growth advantage of trisomy 12 is reduced. This hypothesis is also in line with the findings that continued passage of cultures with low frequencies of trisomy 12, showed only a weak trend for increasing numbers of trisomy 12 in two out of three experiments. For a mechanistic explanation, chromosome 12 includes several genes of interest for a possible gene dose effect explaining the *in vitro* growth advantage [230, 240, 314, 315].

In contrast, when HS181 cultures with defined frequencies of tri-12 cells were injected under the testis capsule of immunodeficient mice and the resulting teratomas analysed for abundance of various tissues; the proportion of tri-12 cells was significantly reduced and thus these cells were selected against *in vivo*. From the *in vivo* data collected, the contribution of tri-12 cells to various tissues (**I**; *Bone/Cartilage,* **II;** *Loose mesenchyme,* **III;** *Neuronal,* **IV;** *Epithelial/Gut,* **V;** *Kidney* and **VI;** *Muscle*) in teratomas seem to be random and no special preference regarding differentiation pathway of tri-12 HS181 cells could be observed. Interestingly, teratomas generated from HS181 cultures with even a low frequency of tri-12 cells show an increased abundance of kidney formation (8/9 teratomas). Renal development has been a rare finding from diploid HS181 cells and 1/9 teratomas from diploid HS181 cells contained few renal areas. This is similar to what has been shown by others [24, 29, 39, 213]. WT1 staining supported the morphological appearance of renal development and the presence of CD34, but absence of CD31 within glomerular areas indicated immature vascularisation of the nephronic structures.

Four teratomas generated from HS181 cultures with tri-12 cells were subjected to a detailed FISH analysis regarding the proportional contribution of trisomic cells *in vivo,* in which at least 4000 cells were evaluated for each teratoma. There was a marked reduction of the proportion of tri-12 cells, relative to the frequency of tri-12 cells in the HESC population used for injection. After injection a HS181 population with 68% tri-12 cells exhibited an overall *in vivo* frequency of tri-12 cells of 23,6%, 12,8% and 26,8%, respectively and similarly a HS181 population with 30% tri-12 cells yielded an *in vivo* total frequency of 11.0% tri-12 ($p<0.001$, Chi2). However, such areas of renal formation included no increased relative frequency of tri-12 cells, suggesting indirect mechanism(s) for the enhanced formation. The proportion of tri-12 cells in areas with renal formation ranged between 11,0–26,8% and had a mean±SD of 18,3±11,7%, compared to a range of 10,9- 31,8% and a mean±SD of 18,8±7.3% for all tissues analysed (N.S. Chi2). Thus, in spite of the increased abundance of renal formation no significant difference in the frequency of tri-12 cells was found for kidney forming areas, compared to other tissues.

There are so far no data published suggesting a strong link between the early urogenital development and genes on chromosome 12. Alternatively, we hypothesize an indirect mechanism of gene dosage from chromosome 12 to promote the development of intermediate mesoderm, thus favouring urogenital development and for some reason kidney development in particular. As earlier discussed recombining metanephric mesenchyme with dorsal neural tubes in culture is known to yield development of renal tubules. Since neural tissues are among the dominating components of HESC derived teratomas, we would currently favour a model where reciprocal interactions between the metanephric mesenchyme and neural cells results in kidney differentiation and development. This finds support in that the expression of NANOG was recently reported to increase upon the early primitive ectodermal differentiation [92], leading possibly further enhancement of the neural compartment and the supposed inductive effect by neural cells on metanephric mesenchyme.

Amplification of chromosome 12 has been repeatedly linked to clinical findings in germ line tumours [316]. This could show the way to another events explaining the increased kidney development, linking amplification of chromosome 12 and urogenital development. The teratomas in the present study were harvested at week 6 after injection of the HESC. We have in a recent kinetic study found the early time scale for tissue formation in the HESC teratomas to be very similar to early normal human development (paper II). In normal early human development, germ cells migrate from the yolk sac endoderm at week 5 and the following separation of testes and kidney development occurs at weeks 8-17.

In conclusion, a density dependent growth advantage *in vitro* for HESC tri-12 cells was found, however such cells were selected against *in vivo*. Moreover, a highly increased frequency of renal development appeared *in vivo*. Altogether, the study supports the conclusions from paper III, i.e. differences between *in vitro* and *in vivo* phenotype are apparent and investigation of genetic integrity and characterization of variants could reveal important knowledge on the process of tumour transformation and progression as well as other genetic defects.

Further discussion paper III and IV

It is uncertain whether the above described changes would appear specifically in the current cell line, HS181, or if they are more general. With this in mind it will in future studies be of importance to investigate additional cell lines. Some preliminary data already exists: Cell line (HS346) has been shown to carry trisomy 12 cells. However there is so far insufficient teratoma material to conclusively dissect the contribution of trisomy 12 cells in the tissues. The mechanisms behind the inability of HS181$^{7q-,i(12p)}$ cells to grow *in vivo* and the enhanced renal development from tri-12 sublines need further investigation for their explanation. As discussed in paper III and IV many candidate genes are present on chromosomes 12 and 7q. Although highly speculative, a few hypotheses can be presented.

One hypothesis is linked to the correlation of i(12)p with germ cell tumours. Germ cells are bound to Sertoli cells by N-cadherin [317]. Assumed that CIS cells loose their

dependence of N-cadherin, and combined with the finding in paper II of neuroectoderm as the first structure formed, and the fact that neuroectoderm need expression of N-cadherin, it might be possible that growth is inhibited *in vivo* due to the inability to form these first structures.

Unpublished results from Western Blot experiments indicate that the MAPK pathway is downregulated in HS181$^{7q-,i(12p)}$, as preliminary seen both in a reduction of pErk and total Erk. The proto-oncogene and RTK (receptor tyrosin kinase) c-Met and its ligand HGF (Hepatocyte Growth Factor) activate signal transducers such as PI3K, ERK and Ras. c-Met has been shown to be important in mammalian development, tissue maintenance, and repair and also have a important role in the formation of tubular structures *in vivo*, such as vasculature. Insufficient blood supply and insufficient inhibition of apoptosis are known to suppress *in vivo* growth. Interestingly, inhibition of c-Met function has reversed cancer associated phenotypes *in vivo* [312, 318, 319]. Again, highly speculative, the inability to grow *in vivo* could be a reflection of the dosage due to a missing allele (7q deletion) and thereby lower activity of c-Met and its downstreams effectors such as PI3K. This might hinder vascularisation from the HESC and subsequently growth. Furthermore CDK5 together with PI3K both present on 7q are involved in neuronal survival and death [320], further supporting a dysregulation of the first structures formed *in vivo*.

4.3 DERIVATIONS AND IMPROVED CULTURE CONDITIONS TOWARDS A XENO-FREE ENVIRONMENT (PAPERS V-VII)

4.3.1 A dual colour system for studies of in vitro growth (paper V)

In **paper V** a dual-colour system was applied to detect interactions between hFS and HESC, using RFP-transfected human foreskin fibroblasts (hFS-RFP) to support the growth of GFP expressing HESC (hES; HS181-GFP). **The aim** was to provide a system for studies of *in vitro* growth and behaviour after changes of e.g. culture conditions. After overnight culture, the HESC colonies were "pushing away" the underlying feeder cells. This phenomenon occurred with both a low and high density of feeders. The density of the feeder cell layer, however, influenced the growth pattern of HESC colonies. At a high feeder cell density, the HESC colonies were more pointed and aligned with the direction of the fibroblasts, whereas less dense feeder layers allowed a more rounded and flat HESC colony formation. Irradiated feeder cells were shown to be passaged along with HESC for three passages (0,3%). FISH using chromosome X- and Y-specific painting probes demonstrated the ploidy in the hFS population. Fusion events were extremely rare, only one potentially fused cell was found after screening approximately $10x10^6$ HESC.

The HESC growth-supportive effect of hFS cells was evaluated at densities ranging between $0.5x10^5$ and $2.5x10^5$ irradiated feeder cells per well (six-well plates). In this interval, a less than 10% variation of growth (doubling time) of the HESC was observed, but seeding efficiency was lower on low density feeder. The highest feeder density showed a "crowding effect" by piled-up feeder cells around the colonies. An hFS cell concentration of $1.8–2.0x10^5$ cells per well, corresponding to $2.1–2.3x10^4$

cells/cm^2, was found to be optimal for good plating efficiency and growth of the HS181 cells. We further found that a density of 1–2x10^4 HESC/well (corresponding to a 10–20% confluent layer when assayed 16 h after plating), is a good seeding density, resulting in a passage time of 3–5 days. The appearance of differentiated stroma-like cells is a known phenomenon when HESC are maintained in feeder-free conditions [46, 237]. Such phenomena were easy to follow in the cultures when using GFP-expressing HESC.

In conclusion we found that HESC colonies do not grow on top of the feeder layer, but instead push the hFS cells aside, and that stroma-like cells grow out from the colonies. This dual-colour system allows analysis of colony formation and also helps to identify and follow the differentiation of cells.

4.3.2 Derivation using a human feeder layer (Paper VI)

In **paper VI**, as a first step toward a xeno-free and optimal culture system, **the aim** was to use post-natal, commercially available human foreskin fibroblasts (hFS) as feeder cells [321, 322]. Others have previously used Matrigel- and laminin-coated dishes, and conditioned MEF medium, and human foetal muscle, skin, and adult Fallopian tube epithelial cells [45, 46]. ICMs were isolated from five supernumerary blastocysts, obtained as donations from couples undergoing IVF treatment. Two lines were obtained in this publication, HS181 and HS207. The HS181 line, used in several of the studies in this thesis was derived from a blastocyst scoring 4BB (expanded, small cell numbers in the ICM and trophectoderm).
The HS181 line was shown to express the stem cell markers alkaline phosphatase, Oct-4, SSEA-4 and TRA-1-60. The karyotype was 46,XX. Pluripotency was demonstrated by teratoma formation after injection of passage 20 of these cells under the testicular capsule of SCID/beige mice. The teratomas were analysed 18 weeks after the injection and were well circumscribed tumour-like structures, containing cystic cavities and solid areas.

Finding optimal techniques to culture HESC without a feeder layer and culturing them without xeno-components is important for future cell transplantation to be possible.
In conclusion, in this report human foreskin fibroblasts were for the first time successfully used as feeder cells for derivation and continued undifferentiated growth of HESC.

4.3.3 Derivation using a human feeder layer and serum replacement (Paper VII)

In **paper VII** the HESC lines HS293 and HS30 were derived using SR medium instead of conventional FCS and hFS as feeder cells.
Since all animal-derived components, nonhuman sera, and feeder cells in the cultures represent a risk of transmitting pathogens to HESC, such materials should be avoided for future clinical purposes. In this paper **the aim** was to extend on paper VI in that cell lines was derived on human foreskin feeder cells and using SR-medium. These were the first described HESC lines derived and propagated under such conditions.

The karyotype of HS293 was 46,XY, and that of HS306 46,XX. All markers of pluripotency were expressed by the two cell lines (Oct-4, SSEA-4, TRA-1-60, TRA-1-81, GCTM-2, and alkaline phosphatase). Pluripotency was shown in embryoid bodies *in vitro*, and the pluripotency of line 293 was shown *in vivo* by teratoma formation in SCID/beige mice. The embryoid bodies formed from lines HS293 and HS306 expressed SOX-1, ND-1, alpha-cardiac actin, and AFP in RT-PCR and Nestin, BMP-4, and AFP using immunofluorescent staining.

Derivation of a HESC line on MEF feeder cells but using SR was reported earlier [323]. Furthermore, Amit et al. [324] described serum-free culture conditions for a line that had originally been derived using the conventional FCS and mouse fibroblast system. They used SR medium and postnatal human fibroblasts as feeder cells. The same group also described a system involving culture of HESC on fibronectin [55]. Today, we are using SR medium for the culture of all HESC lines, also those originally derived in FCS-containing medium, e.g. in paper I-VI.

Another source of factors of animal origin in customary culture systems is the enzymes used for passaging the cells. It is possible to keep the HESC lines using mechanical isolation to keep the line free in this respect. The feeder cells, hFS, were cultured in FCS-containing medium and even though good quality FCS was used, it is not optimal for possible future human cell transplantation. Fully serum-free conditions for establishing and propagating feeder cells need to be obtained, but the use of human serum might be an alternative. The SR medium used also contains some animal-derived purified proteins, which in an optimal culture system should be replaced by human recombinant proteins. Feeder-free serum-free derivation and culture have proved successful in mouse cell lines [256] and this would, of course, also be important to establish for HESC. For this, the factors regulating self-renewal of human ES cells should be identified.

The efficacy of derivation in SR, two permanent lines out of ten blastocysts, seemed to be at least as good as or better than that during the period when FCS was used (four permanent lines from 67 blastocysts, plus 9 early lines which faded off). 10 early lines were growing from 27 blastocysts. A prospective comparative study using embryos in derivation in either FCS or SR has not been feasible. Though, our existing lines originally derived in FCS containing medium grew better in SR medium [325](paper V).

In conclusion HESC lines can be successfully derived using SR medium and postnatal hFS as feeder cells. This was a step toward xeno-free and better defined conditions.

Further discussion paper V-VII

Cell line HS181 was our first well growing and characterized cell line, and the one we have most data on. Thus, HS181 have been the main focus in this thesis. Today also line HS207 is growing well and most of 10 additional early lines in paper VII including HS306 are fully characterized. New cell lines are continuously derived and there are at this date 17 cell lines partly or fully characterized. The karyotypes have initially been found normal with the exception for HS237 containing an isodicentric X chromosome [231].

5 GENERAL DISCUSSION

5.1 CULTURE CONDITIONS

There is debate as to whether ESC are an *in vitro* artefact that do not correspond directly to any developmental equivalent *in vivo,* but arise only from selection and adaptation to culture [32]. Pluripotent cells of the embryo respond *in vivo* to a tightly regulated developmental program and differentiate in a very short period of time. In contrast ESC are adapted to culture and are exposed *in vitro* to different stimuli from the *in vivo* environment [326].
As described above (section 1.4.2) gene expression studies have shown differences between cell lines and also within cell lines. Variation in the kinetics of differentiation has furthermore been reported between cell lines [123]. Such differences could be the result of intrinsic genetic differences but are perhaps more likely a consequence of epigenetic modifications (discussed in section 1.10.1) that each line has acquired since the time of derivation. Consequently as suggested by Ginis et al. [124] differences may be reduced by optimized culture conditions. Together, this point out the importance of finding defined culture conditions and full understanding of their impact. Moreover, it highlights the importance of dissecting HSC fate *in vivo*.

5.2 TERATOMA FORMATION

From our experience the number of cells needed for teratoma growth is very low, $10x^2$-$10x^3$ and the outcome of successful grafts does not change significantly as compared to grafting $10x^4$-$10x^5$ cells. The percentage of successful grafts is about 90%, why the system in not oversaturated. This is important for relevant assessment of the pluripotency of a HESC line. Therefore we inject $10x^3$-$10x^4$ cells for characterization of a new cell line, in order to avoid an oversaturation of the system. We have found that the number of cells injected, ranging from $10x^3$-$10x^5$ cells, does not appear to affect the range of tissues formed or the tissue size, which could be a possibility considering the size limitation of the teratomas formed after a certain time *in vivo*. Only the HS181 line has been subjected to a deeper *in vivo* analysis. Since we have enough material from over 10 lines it would be valuable to compare them all. In general using evaluation of HE sections, they are very alike. Exceptions are e.g. lines HS235 and HS360, which repeatedly only resulted in large cystic growths, despite presence of all markers *in vitro*. Cystic teratomas could possibly represent a trophectodermal or early endodermal development or epiblast/primitive ectoderm surrounding a putative proamniotic cavity. The same finding has been reported by Heins et al.[39]. However, the HS235 and HS360 cell lines do often differ morphologically from cell lines generating solid teratomas. The colonies exhibit a central cavity of very flat cells surrounded by densely packed cells.

As discussed teratomas from the HS181 cell line develop similarly *in vivo*. At one occasion though, have the composition differed from the normal pattern. High passage

HS181-GFP cells were grafted and the subsequent teratomas were highly dominated by bone and cartilage. Likely, those cells had been clonally expanded after FACS sorting and possibly already partly committed. Another explanation, as described in paper III and IV, might be that those cells had gained an abnormal karyotype affecting the differentiation capacity, or epigenetic changes. This is today not known.

Since the xeno-graft mouse model allow a very limited teratoma size to develop, it is difficult to study later stages. To come around this limitation, one possibility could be to retransplant tissue from a teratoma to a new host. Our initial experiment from retransplanting cell suspension was not successful, although being a positive finding considering the safety aspect. Grafting a larger piece of the teratoma, which already contains vascularisation and rigid cell-cell and cell-matrix contacts could possibly be a better choice. For the purpose of studying more advanced maturation of an existing tissue, this is even a requirement. The best option for such studies is probably a larger *in vivo* model, in which tissues can be allowed to grow larger, given that the vascularisation will be sufficient.

5.3 CHROMOSOMAL STABILITY – PRECAUTIONS OR POSSIBILITIES?

We and others have shown that karyotypic changes do occur in HESC [62, 230-232, 236, 304, 305]. We have shown that these can be specifically selected for and affect the phenotypic behaviour of the cells *in vitro* and *in vivo*. Even though no signs of invasiveness have been observed, one must not forget that the teratomas grow only for a limited time *in vivo*. To leave the animals for a longer time period is however not optimal either, due to two facts. The tissue can grow too large and make the animal suffer in addition to that the tissue eventually become necrotic most likely due to compromised vascularisation. Also for such studies a larger xeno-graft animal model would be needed.

As discussed by Liew et al.[327], terminally differentiated cells are known to have none or very limited proliferative capacity, and cells derived from karyotypically abnormal cells may not necessarily evoke any greater tumourigenic reaction in transplanted patients than normal cells. Support for this as well as for future clinical usage of HESC is the reports of *in vitro* differentiated NTERA-2 EC-cells used in clinical trials on stroke patients. The studies showed neurological improvement and no signs of neoplasm after 27 months [328-330]. Therefore, as long as sublines with chromosomal changes behave as their parental line, there is no reason for such cells to be excluded at least from basic research.

Although promising, a clinician's hope for HESC cells to be used in cell therapy is yet a matter for the future. Optimal culture conditions are to be developed but as long as accurate precautions are taken, this is a problem that will be possible to control for. I do not find the genetic abnormalities worrying. After all, the cells are more resistant to changes than most other mammalian cell lineages. However, first and foremost, this appear to be an excellent model for understanding malignancies appearing in early life as well as for understanding genetic defects leading to spontaneous abortions and birth defects.

5.4 FUTURE PERSPECTIVES – INDUSTRIAL OR CLINICAL USAGE?

This is still a field in its infancy. The first cell line was published in 1998 [29] and not until the last few years have the research accelerated. To expect that the cells should be in clinical usage shortly is not very probable. The hopes for cell therapy is based on the assumption that developmental pluripotency can be unleashed at will under controlled conditions. The full nature of HESC needs to be understood, first then will it be possible to direct them in a controlled manner, into a specific lineage. Currently much effort is put into differentiating the cells into a wide range of more or less mature cell types, this with cell therapy hopes in mind. Many groups have as discussed been more or less successful using this approach, but no one have to my knowledge been able to actually control the process and gain a pure population of the desired cell type. The end differentiated cell type is not necessarily the optimal source for cell therapy. Perhaps would a progenitor stem cell capable of producing the desired cell type(s) and at the same time remain as a stem cell pool be the optimum. For such approach one must be able to stop the differentiation of the HESC at a specific stage, i.e. in a 'controlled' manner. If stem cells are to be used to treat a wide variety of human diseases, we will need to overcome also other formidable challenges. Stem cells will be needed in large quantities, and histocompatible with an individual. From an industrial perspective HESC both *in vitro* and as teratomas *in vivo* provide a system for toxicological studies already today.

More realistic is the main message of this thesis, that HESC offer a system for modelling the early phases of human development, both normal and diseased. It is apparent from comparisons of murine and human ESC that a human model system is vitally important, due to large interspecies variation.

6 CONCLUDING REMARKS

The work presented in this thesis gives support to the hypothesis that HESC can serve as a model system for early human development. The overall aim, to investigate the possible potential of HESC to serve as a model system for early human developmental processes, both normal and diseased, was thus fulfilled.

- **Paper I** describes in detail the *in vivo* potential of HESC. The composition of HESC teratomas, including organoid-like and highly organized structures containing cellular derivatives from multiple germ layers is presented. Integration and cooperation with the host tissue was shown, most notably in the formation of the vasculature. A correlation of tissue maturity and time spent *in vivo* was found.

- **Paper II** presents in detail the HESC teratoma progress over time. The findings support that HESC differentiation *in vivo* follow a developmental scheme similar to processes during normal embryogenesis. Growth appeared by day 5 after grafting and there is strong support for this tissue to be neuroectodermal. Net growth was detected day 20/30 after grafting and coupled to the appearance of human vascularisation. Thereafter more mature tissues also of other kinds were developed. Interestingly NANOG and NESTIN were expressed at all timepoints. Together these findings raises the issue of the origin of the later non-ectodermal cell populations.

- **Paper III** shows how *in vitro* culture conditions can be utilized to select for changed *in vitro* characteristics of HESC and interprets the resulting karyotype and the *in vivo* characteristics of the resulting variant cells. This work highlights the possibility to study specific genetic anomalies in tumour progression, by the generation of advertant HESC variants. Importantly, the study moreover highlights the differences between *in vitro* and *in vivo* phenotype of such cells. Investigation of genetic integrity and characterization of variants could reveal important knowledge on the process of tumour transformation and progression as well as other genetic defects.

- **Paper IV** describes the influence of trisomy 12 on the *in vitro* and *in vivo* phenotype of HESC. A density dependent growth advantage was observed *in vitro*, however such karyotypically abnormal HESC cells were selected against *in vivo*. Moreover, a significantly increased frequency of renal development appeared *in vivo*. Altogether, the study further supports the conclusions drawn in paper III.

Furthermore, conditions for the derivation and analysis of newly derived HESC were established in **papers V-VII.** Herein the aim to derive and establish new HESC lines was achieved, and initial studies were performed towards a xeno-free culture system. Cell marker profiles *in vitro* and xeno-grafting procedures *in vivo* were developed and applied.

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