Human Embryonic Stem Cells - Constitutive Gene Expression and Differentiation Towards Definitive Endoderm and Posterior Foregut Endoderm

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Human Embryonic Stem Cells

Constitutive Gene Expression and Differentiation Towards Definitive Endoderm and Posterior Foregut Endoderm

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Abstract

More than 300 million people are suffering from diabetes worldwide and the numbers of diabetic patients are increasing. Today´s treatments mainly rely on daily insulin injections. This help people to control blood glucose levels but still many patients suffer from the many complications associated with diabetes. Transplantations of islet cells are an alternative treatment that could help patients to become insulin independent and presumably also delay the development of complications. However, the increasing number of diabetic patients would require large amounts of transplantable insulin producing cells. Due to the fact that islet donor material is a limiting factor for transplantations, human embryonic stem cells (hESCs) could be an alternative source of transplantable insulin producing cells. hESCs have the unique potential to self-renew and to differentiate to many cell types, presumably also beta cells. In order to develop protocols that direct differentiation of hESCs along the developmental program of pancreas development, cellular markers and methods that allow the identification and isolation of cells that displays a correct phenotype of each developmental stage are fundamental research tools. In this thesis, we approached single cell gene expression analysis of hESCs differentiated towards DE to provide insight about expression of a panel of DE markers at the cellular level. These data provides novel insight in DE gene expression at the cellular level of vitro differentiated hESCs and illustrates the usefulness of single-cell gene expression analysis in to identify the molecular signature of in vitro differentiated hESCs. Thus, this technique could be of great help to develop protocols that mimics pancreas development in vivo.

Constitutive promoters are useful tools due to their high level of expression in most cell types. Different eukaryotic/mammalian and viral constitutive promoters have been reported to ensure high level and sustained activity in hESCs but a comprehensive study was lacking. In this thesis, we performed a comparative study the activity and stability of five commonly used promoters in undifferentiated hESCs and during differentiation. These data suggested ACTB, EF1a and PGK promoters as the most stable promoters during long term culture of undifferentiated hESCs.Gene expression analysis of EB differentiated hESCs indicated that promoter activities might be restricted to specific cell lineages, indicating the need to carefully select optimal promoters for constitutive gene expression in differentiated hESCs.

Key words:	human embryonic stem cells, diabetes, pancreas, definitive endoderm, foregut endoderm, pancreatic endoderm, constitutive promoter, ACTB, EF1alpha, CMV, PGK, UbC

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Till mamma och pappa

Populärvetenskaplig sammanfattning

Diabetes är en folksjukdom och idag uppskattas antalet diabetessjuka till mer än 300 miljoner och antalet ökar. Diabetes är en grupp sjukdomar som alla har gemensamt att sockerhalten i blodet är för hög. Hos friska personer regleras blodsockerhalten genom att av insulin frisätts vid höga blodsockernivåer och stimulerar upptag av sockermolekyler ut från blodet till vävnader och organ. Insulin produceras av betaceller som finns i langerhanska öarna som finns i bukspottskörteln (pankreas). Om betacellerna slutar att fungera, antingen genom att kroppens eget immunsystem förstör dom i en autoimmun reaktion eller helt enkelt inte kan producera tillräcklig mängd insulin, så höjs blodsockernivåerna och därmed utvecklas diabetes. Diabetes delas vanligen in i två undergrupper; typ I diabetes som orsakas av att kroppens egen insulinproduktion helt eller delvis har slutat att fungera och typ II diabetes som orsakas av att kroppen inte klarar av att producera tillräcklig mängd insulin eller att vävnaderna inte förmår att reagera och ta upp socker ur blodet av den mängd insulin som frisätts. Som en följd av sjukdomsförloppet finns det en stor risk att utveckla en rad följdsjukdomar bla synnedsättningar, hjärt-kärlsjukdomar. Genom att hålla en så bra blodsockerkontroll som möjligt kan man försena uppkomsten av komplikationer. Idag behandlas typ I diabetes genom dagliga insulininjektioner för att reglera blodsockernivåerna. Typ II diabetes behandlas antigen med insulin eller med andra läkemedel vars funktion är att öka upptaget av socker ur blodet. En alternativ behandlingsmetod för typ I diabetespatienter är att transplantera friska betaceller vilket kan innebära att patienter blir helt eller delvis oberoende av dagliga insulininjektioner och eventuellt också minskar risken att drabbas av följdsjukdomar. Ett genombrott i forskningen kring betacells transplantationer av friska betaceller gjordes 2000 när man framgångsrikt transplanterade langerhanska öar vilket resulterade i att patienterna under en tid blev oberoende av dagliga insulininjektioner. Dessa fynd aktualiserade transplantationer som en behandlingsmetod för svårt sjuka typ I patienter. Idag används öar från avlidna donatorer och tillgången på donatormaterial täcker inte det framtida behovet. Därför behövs andra källor av insulinproducerande celler och mycket uppmärksamhet har riktats mot humana embryonala stamceller som har förmågan att dela sig i det oändliga. Humana embryonala stamceller är ospecialiserade celltyper som under rätt förhållanden kan utmogna ett flertal många av alla kroppens celltyper däribland förmodligen också insulinproducerande betaceller. Dessa egenskaper gör att humana embryonala stamceller kan vara en lämplig

källa av insulinproducerande celler som dessutom kan produceras i stora mängder. Denna strategi förutsätter dock att man lär sig att förstå hur man ska styra cellerna så att dom utvecklas till insulinproducerande celler. Den mest framgångsrika strategin bedöms vara att man försöker efterlikna dom olika stadier av specialisering som stamceller genomgår under normal embryonal utveckling. Stamceller odlas i medium som innehåller näringsämnen och andra nödvändiga faktorer för deras livscykel och överlevnad. Genom att odla cellerna under rätt betingelser dvs genom att tillsätta rätt reagenser till cellodlingsmediet kan man påverka och styra utmognaden av stamcellerna till mer specialiserade cell typer. Genom att utnyttja den befintliga kunskapen om embryonal utveckling finns det idag protokoll för att omprogrammera embryonala stamceller så att dom efterliknar definitivt endoderm - det embryonala groddlager som senare bildar bla pankreas, lever, lunga och mag-tarmkanal. Ett problem är dock att kunna visa att enskilda celler i en blandad population verkligen representerar definitivt endoderm och uttrycker en specifik uppsättning gener som är unika för definitivt endoderm. Detta kräver analysmetoder som gör det möjligt att analysera enskilda celler i en blandad population. I den här avhandlingen presenteras ett arbete där förmågan humana embryonala stamceller först har omprogrammerats till definitivt endoderm och sedan har enskilda celler analyserats. Detta arbete indikerar att det finns skillnader mellan definitivt endoderm som är genererat med olika protokoll och som man inte tidigare har känt till eftersom man inte har haft tillgång till tillräckligt finkänsliga analysmetoder. Baserat på dom här resultaten kan vi belysa hur viktigt det är att använda analysmetoder så att man verkligen kan säkerställa likheten av dom omprogrammerade cellerna med embryonala progenitor celler. Genetisk modifiering gör det möjligt att förstå hur gener styr omprogrammering av humana embryonala stamceller till mer mogna celltyper däribland insulinproducerande betaceller. I den här avhandlingen presenteras ett arbete som har utvärderat olika promotorers förmåga att stabilt driva uttryck av gener. Genom att låta olika promoter driva uttrycket av green fluorescent protein (GFP) så kan man märka in celler och därmed följa dess cellers utveckling. Stabiliteten av dom olika promotorerna har undersökts både i humana embryonala stamceller och under omprogrammering till mer mogna cell typer. Detta arbete visar att det finns vissa promotorer som driver uttryck av gener mer stabilt än andra och därför är det viktigt att välja rätt promotor för att långsiktigt och stabilt kunna spåra celler och följa dess utveckling.

Abstract

More than 300 million people are suffering from diabetes worldwide and the numbers of diabetic patients are increasing. Today´s treatments mainly rely on daily insulin injections. This help people to control blood glucose levels but still many patients suffer from the many complications associated with diabetes. Transplantations of islet cells are an alternative treatment that could help patients to become insulin independent and presumably also delay the development of complications. However, the increasing number of diabetic patients would require large amounts of transplantable insulin producing cells. Due to the fact that islet donor material is a limiting factor for transplantations, human embryonic stem cells (hESCs) could be an alternative source of transplantable insulin producing cells. hESCs have the unique potential to self-renew and to differentiate to many cell types, presumably also beta cells. In order to generate therapeutically relevant beta cells, the scientific community focuses on recapitulating the embryonic processes behind beta cell development. In order to develop protocols that direct differentiation of hESCs along the developmental program of pancreas development, cellular markers and methods that allow the identification and isolation of cells that displays a correct phenotype of each developmental stage are fundamental research tools. Identification of definitive endoderm (DE) - from where pancreas originates, requires analysis methods that can detect multiple markers within individual cells, since many markers expressed in DE are also detected in extraembryonic endoderm. In this thesis, we approached single cell gene expression analysis of hESCs differentiated towards DE to provide insight about expression of a panel of DE markers at the cellular level. Some of these markers have conventionally been measured by gene expression analysis at the population level and little information is available about expression at the cellular level in differentiating hESCs. To differentiate hESCs towards DE three different methods of activin A treatment were used. Single-cell gene expression analysis identified distinct gene expression signatures both between the activinA treated populations and within each population. Within the *SOX17+* population, the DE markers *CER1* and *FOXA2* were co-expressed in the majority cells independent of activin A treatment. By contrast, *HHEX, CXCR4, FOXA2, MIXL1* and *LIM1/LHX1* were expressed to various extents within the *SOX17+* populations of each activin A treatment. These data provides novel insight in DE gene expression at the cellular level of vitro

differentiated hESCs and illustrates the usefulness of single-cell gene expression analysis in to identify the molecular signature of in vitro differentiated hESCs. Thus, this technique could be of great help to develop protocols that mimics pancreas development in vivo.

Furthermore, this thesis includes work that test the ability of RA and FGF4 alone or in combination to direct differentiation of hESCs towards PDX1+ foregut endoderm. The rationale for this was that both RA and FGF signaling exhibit a patterning effect during endoderm patterning and also supports pancreas specification. By optimizing the timing and concentration of RA and FGF4, it was shown that RA is required to convert activin A-induced hESCs into PDX1+ cells and that part of the underlying mechanism involves FGF signaling. Characterization of the PDX1+ cells suggests that they represent posterior foregut endoderm not yet commited to pancreatic, posterior stomach or duodenal endoderm.

Directed differentiation of hESCs would greatly benefit from a deeper understanding of the molecular mechanism that regulate growth and differentiation. To approach these questions, efficient genetic engineering techniques are advantageous tools for controlled expression of genes or to introduce fluorescent reporter genes. Constitutive promoters are useful tools due to their high level of expression in most cell types. Different eukaryotic/mammalian and viral constitutive promoters have been reported to ensure high level and sustained activity in hESCs but a comprehensive study was lacking. In this thesis, we performed a comparative study the activity and stability of five commonly used promoters in undifferentiated hESCs and during differentiation. These data suggested ACTB, EF1α and PGK promoters as the most stable promoters during long term culture of undifferentiated hESCs. During EB differentiation, activities of all five promoters were downregulated and EF1α was the most stable promoter although it was downregulated in 50% of the cells. Gene expression analysis of differentiated cells indicated that promoter activities might be restricted to specific cell lineages, indicating the need to carefully select optimal promoters for constitutive gene expression in differentiated hESCs.

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List of publications

This thesis is based in the following papers, which will be referred to by their roman numbering (I-III)

Paper I. **Quantitative Comparison of Constitutive Promoters in Human ES cells**

<u>Karin Norrman</u>, Yvonne Fischer, Blandine Bonnamy, Fredrik Wolfhagen Sand, Philippe Ravassard and Henrik Semb

PLoS One. 2010 26;5(8):e12413.

Paper II. **Distinct Gene Expression Signature at Single-Cell Level in Human Embryonic Stem Cells Differentiated Towards Definitive Endoderm**

<u>Karin Norrman</u>, Anders Ståhlberg and Henrik Semb

Manuscript

Paper III .**FGF4 and Retinoic Acid Direct Differentiation of hESCs intoPDX1-Expressing Foregut Endoderm in a Time- and Concentration-Dependent Manner**

Martina Johannesson, Anders Ståhlberg, Jacqueline Ameri, Fredrik Wolfhagen Sand, <u>Karin Norrman</u> and Henrik Semb

PLoS ONE. 2009;4(3):e4794

Abbreviations

AA	activin A
ADE	anterior definitive endoderm
A-P	anterior-posterior
AVE	anterior visceral endoderm
bFGF	basic fibroblast growth factor, also called FGF2
BMP	bone morphopogenetic protein
DE	definitive endoderm
E	embryonic day
EB	embryoid bodies
EC	embryonic carcinoma cells
EG	embryonic germ cells
eGFP	enhanced green fluorescent protein
EMT	epithelial to mesenchymal transition
ES	embryonic stem cells
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FGF4	fibroblast growth factor 4
GFP	green fluorescent protein
hESCs	human embryonic stem cells
ICM	inner cell mass
iPSC	induced pluripotent stem cells
MEF	mouse embryonic fibroblast
mESCs	mouse embryonic stem cells

PCR	polymerase chain reaction
PE	primitive endoderm
PS	primitive streak
QPCR	quantitative real time PCR
RA	retinoic acid
SCID	severe combined immune deficient
Shh	sonic hedgehog
TGFb	transforming growth factor beta
VE	visceral endoderm
WNT	Wingless-type MMTV integration site

INTRODUCTION

Diabetes

In 2008, the number of diabetic patients have been estimated to 347 million people worldwide (Danaei et al., 2011). The term diabetes refers to several types of hyperglycemic conditions that are either caused by dysfunctional beta cells or impaired sensitivity to the released insulin. The two main forms; type I and type II diabetes are caused by an absolute or relative lack of beta cells. Type I diabetes is an autoimmune disease where beta cells are destroyed by the immune system. This is a lifelong symptom that requires exogenous insulin supply to control blood glucose levels. Type II diabetes is characterized by insulin resistance of the tissues that should respond to the secreted insulin and thereby results in increased blood glucose levels. This symptom is mostly connected to obesity and has previously been observed in older people but becomes more frequently observed in young people. The global increase in obesity is thought to be the main reason for the emerging number of type II diabetes patients (WHO). In addition, diabetic patients suffer from a number of complications of diabetes that collectively affects the heart, blood vessels, eyes and kidneys.

The most common treatment of type I diabetes is daily insulin injections that regulate blood glucose levels but the disadvantage with this treatment is that it does not efficiently prevent the many complications. A major progress in diabetes treatment was made in 2000 by Shapiro et al that transplanted cadaveric donor islets to type I patients (Shapiro et al., 2000). After the transplantation, patients were insulin independent with good metabolic control of blood glucose levels. Since then, an increasing number of patients have been transplanted but with time, most of the patients became insulin dependent again Although it is not fully understood why some patients loose the insulin independence and others remain off insulin, the study by Shapiro et al was as proof of principle showing that islet transplantations are an achievable goal in the search for treatments of type I diabetes (Berney et al., 2009). However, considering the amounts of donor islets needed to for transplantations, additional sources of insulin producing cells will be needed to treat an increasing number of diabetic patients. In this context, pluripotent stem cells have got a central role as an alternative source to generate transplantable insulin producing cells. The main objective for this is that pluripotent stem cells can be expanded in vitro in large amounts and thereby offers

an unlimited source of cellular material. An increasing number of reports show that insulin producing cells efficiently can be generated from pluripotent stem cells although these cells still lacks the functional characteristics of normal healthy beta cells. Therefore, research interests are focused on differentiating pluripotent stem cells along the developmental program of beta cell development as illustrated in Fig. 1.
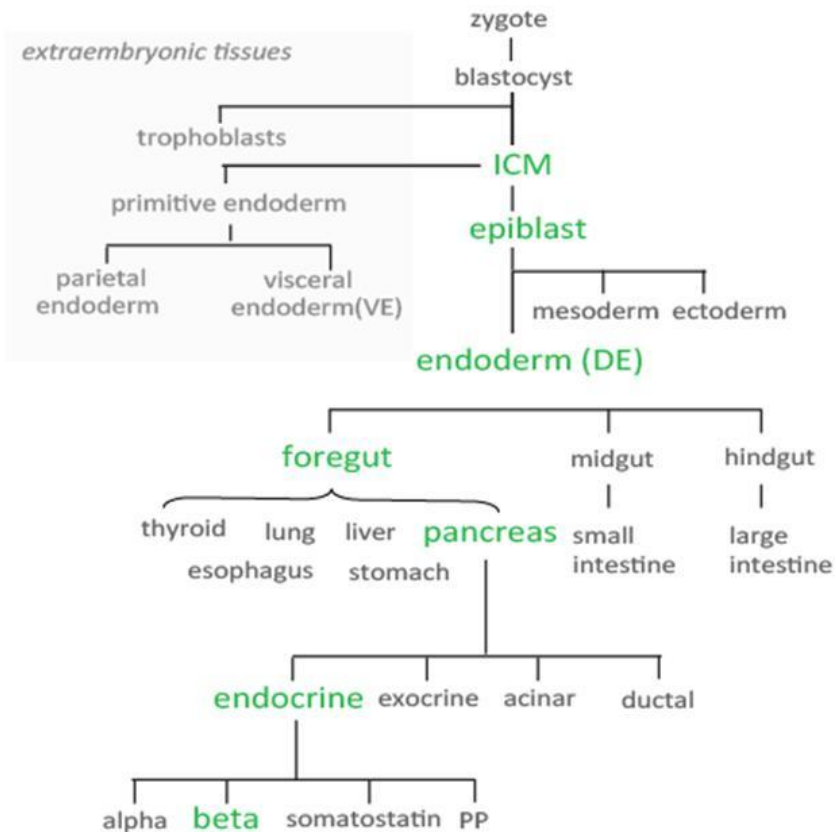

Figure 1. Schematic picture of progenitor stages during betacell development in vivo. Differentiation of pluripotent stem cells towards insulin producing beta cells intends to direct cells to the different progenitor stages of betacell development.

Developmental biology of the pancreas

Anatomy and function of the pancreas

The pancreas has dual functions, both in regulating blood glucose levels and to secret digestive enzymes. The exocrine part of the organs consists of acinar and ductal cells. These cells produce digestive enzymes that are transported to the gut and promote nutrient absorption. The endocrine portion of the pancreas is aggregated in spheroidal cell clusters, the islets of Langerhans that secrete hormones to the blood stream and function in regulating glucose homeostasis. The endocrine portion of the mature organ occupies 1-2% of the organ and is dispersed within the exocrine tissue (Fig. 2). The islets of Langerhans are mainly made up of four principal cell types that each one of them has individual functions in regulating in glucose homeostasis; glucagon producing alpha cells, insulin producing beta cells, somatostatin producing delta cells and pancreatic polypeptide producing PP cells. Regulation of blood glucose levels is controlled by insulin that is released into the blood when blood sugar levels increase ie after food intake. The secreted insulin stimulates other cells like blood cells, muscle cells and fat cells to take up glucose from the blood and use it as an energy source. Thereby, blood glucose levels goes down to normal levels and insulin is no longer secreted. By contrast, low blood glucose levels stimulate the secretion of glucagon that activates gluconeogenesis and thereby blood glucose levels increases. In this way, insulin and glucagon cooperates to maintain normal glucose levels in the blood. The somatostatin and PP hormones have an inhibitory function on endocrine secretion and exocrine secretion, respectively. Another pancreatic cell type is epsilon cells that constitute approximately 1% of islets. These cells produce the hormone grehlin that is involved in metabolic regulation and energy balance (Wierup et al., 2002)

Early embryogenesis

During mammalian development, the fertilized egg undergoes a set of cell divisions or cleavages. The very first event of differentiation towards specialized cell types starts in the 16-cell morula where a group of internal cells are surrounded by an outer cell layer. This outer layer will become trophoblasts that will not give rise to any embryonic structures but forms the embryonic parts of the

placenta that are involved in implantation to the uterus and in oxygen and nutrient exchange. The inner cell layer of the morula will develop into the inner cell mass (ICM). These cells are pluripotent meaning that they have the potential to give rise to any cell type in the body. The ICM express a gene regulatory network that includes Oct4, Nanog and STAT that regulates pluripotency of the ICM. The ICM can be isolated from the embryo and cultured in vitro under conditions that remain expression of Oct4, Nanog and STAT. Under these conditions, these cells are proliferative and can be derived and expanded as embryonic stem (ES) cells. Derivation of ES cells is further described in section Human embryonic stem cells. The ICM will be separated into two cell layers; the primitive endoderm (PE) that forms extraembryonic parts of the embryo and the epiblast cell layer that give rise to all the cells of the embryo.
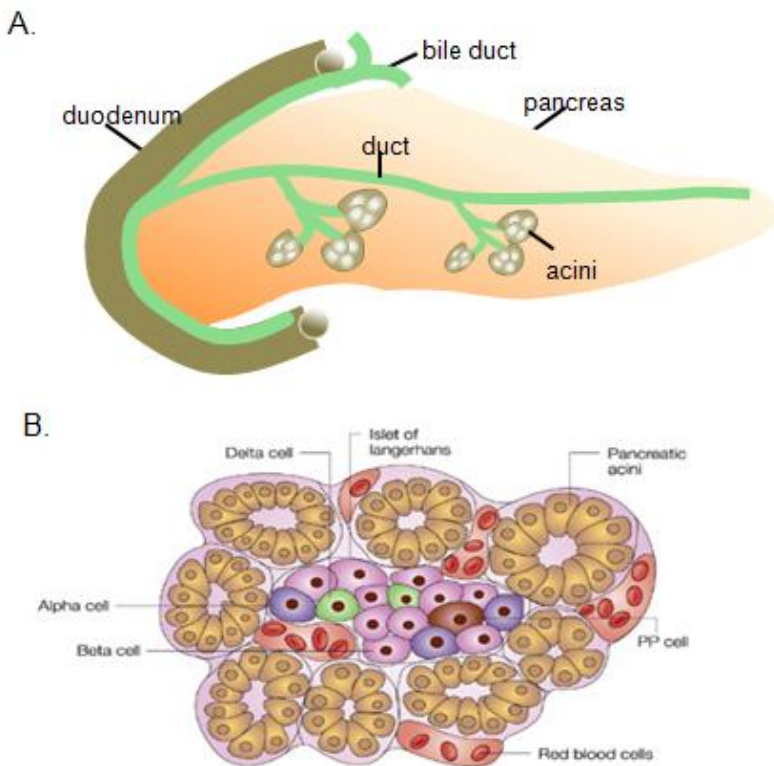

Figure 2.A Schematic picture illustrating the anatomy of the adult pancreatic organ. B. Endocrine cells are organized into islets that are embedded into the exocrine tissue. Fig 2B is printed with permission from Bardeesy, N. and DePinho, R. A, Nature Reviews Cancer 2, 897-909 December 2002.

Gastrulation

PS formation

Before the implantation stage, that in mice occurs at embryonic day 4 (E 4.0), precursors from the ICM migrates away and forms the PE (Fig. 3). At E5.0 PE is specified into parietal endoderm and visceral endoderm (VE). The VE does not contribute to any embryonic tissues but will later on play an important role in A-P positioning of the putative definitive endoderm. The ICM develops into epiblast cells that expand and in the mouse embryo forms a cylinder shaped structure. The VE region is also expanded and will cover the outer surface of the embryo (Fig. 3). At this time, VE is specified into anterior visceral endoderm (AVE). In addition, the primitive streak (PS) is positioned at this time. The site of PS formation defines the future posterior pole of the embryo and correct placement of PS is controlled by the newly established AVE. In mammals, PS appears to be responsible for establishment of all parts of the embryo while AVE and the PS work together to regulate formation of anterior structures.

Formation of the PS is the onset of gastrulation - a series of cell movements and morphogenetic events that forms the germ layers (definitive endoderm (DE), mesoderm and ectoderm) that later on give rise to all cell types of the body. Gastrulation also involves positioning of the body axis plan and allocation of cell lineages according the anterior-posterior, dorsal-ventral and left-right axis. The first sign of gastrulation starts when epiblast cells undergo epithelial-mesenchymal transitions (EMT) and start to migrate through the PS. Depending on the timing of PS migration epiblast cells will differentiate to various types of endodermal or mesodermal progenitors. Cells that first exit the epiblast migrate towards the anterior side of the embryo and gives rise to anterior definitive endoderm (ADE) and axial mesoderm (Lawson and Pedersen, 1987). Cells that exit the PS later contributes to more posterior endoderm, extrembryonic mesoderm, lateral plate mesoderm and paraxial mesoderm. Remaining epiblast cells that do not migrate through the PS will contribute to the ectodermal derived cell types (Fig.4).
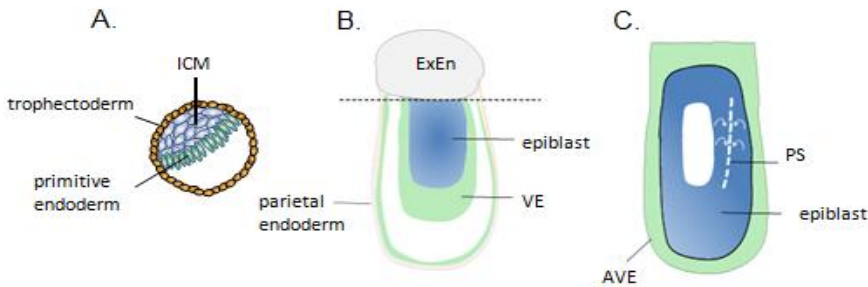
Figure 3 Early development in mouse

A. E4.0 Implanting blastocyst. Precursors of the primitive endoderm migrate away from the ICM. B. E5.0-5.5 Proliferation of the epiblast which derives from the ICM. Primitive endoderm gives rise to visceral endoderm (VE) and parietal endoderm. Thickening of distal visceral endoderm (DVE). Cavitation and epithelial morphogenesis of epiblast begins. C. E6-0-6.5 DVE moves towards the anterior and is specified into AVE that marks the anterior side of the embryo. Positioning of the PS marks the posterior side of the embryo. Parietal endoderm and other extraembryonic structures are not showed in fig 3C. Fig 3B-C are modified from Tam and Loebel, Nature Reviews Genetics 8, 368-381, May 2007.

Patterning along the anterior-posterior axis

As germ layers are formed during PS migration they becomes patterned to get anterior or posterior identities. In the pregastrula embryo, Nodal signaling pathway induces formation of DE and mesoderm and also regulates A-P patterning of the endoderm. Nodal is initially expressed around the proximal epiblast and activates DE and anterior mesendoderm genes. As the AVE migrates towards the anterior, it expresses the Nodal antagonists Cerberus like *Cer1* and Lefty that restricts Nodal expression to the posterior epiblast where expression of posterior genes is activated. In this way, Nodal is regulated by an autoregulatory loop where Nodal originally is expressed in the epiblast and induce ADE formation. Nodal activity is inhibited in the AVE region by the antagonists Cer1 and Lefty1and becomes restricted to the posterior epiblast where mesoderm and posterior endoderm is

induced. Studies in zebrafish, Xenopus and mouse suggest that the levels of Nodal signaling might play a role in segregating DE and mesoderm. A model proposing that high levels of Nodal promote ADE while lower levels promote mesoderm has been suggested. The basis for this is that DE cells develops close to the source of Nodal and requires a sustained period of Nodal activity to be induced. The newly formed DE migrates towards the anterior. This is a coordinated event where AVE moves towards the anterior, reaches the extraembryonic endoderm and is displaced by ADE exiting the PS. At the time of AVE movement a set of genes that regulates A-P patterning of the epiblast or the endoderm is expressed from the AVE. The orthodenticle homeobox 2 *Otx2* regulates expression of Lefty1 and is thereby required for posterior restriction of the epiblast (Perea-Gomez et al., 2001). A model have been proposed where the FOXA2 fork-head family (also called *Hnf3b*) and LIM domain containing gene *Lhx1* in the AVE has a role in restricting PS formation to the posterior side (Perea-Gomez et al., 1999). The homeobox gene *Hex* is one of the earliest markers that regulated anterior identity and is expressed both in AVE and ADE during gastrulation stages.

The anterior endoderm (AVE+ADE) is also suggested to play a role in the A-P positioning of ectodermal derivatives. A gene regulatory network that involves *Cer1, Otx2* and *Hex* among others is expressed in the AVE and is suggested to induce anterior neural structures and head formation (Shawlot et al., 1998; Shawlot et al., 1999).

Definitive endoderm formation

During PS migration, epiblast cells that are exposed to Nodal signaling that activates a number of conserved transcription factors that commits epiblast cells towards an endoderm fate and induce anterior movement of the newly formed definitive endoderm.

The Mixl-like homeobox protein 1 *Mixl1* is expressed in the anterior region of the PS and knock-out studies in mice have shown that *Mixl1* is required for the morphogenetic events associated with movement during gastrulation and also patterning of the epiblast (Hart et al., 2002). Additionally, absence of *Mixl1* causes endoderm cells to remain stationary during gastrulation and results in less recruitment of cells to the gut endoderm and (Tam et al., 2007). Collectively, these

results show that *Mixl1* is involved in anterior expansion of DE and recruitment to the gut endoderm.

The Sry box gene *Sox17* is a downstream target of *Mixl1* and *Sox17* knock-out mice becomes depleted in gut endoderm (Kanai-Azuma et al., 2002). More specifically, this study showed that in the absence of *Sox17*, foregut endoderm developed normally until the neural plate stage (~E7.75) but thereafter the DE population was depleted. At the headfold stage (~E8.0) midgut and hindgut regions of the *Sox17* mutant endoderm was also reduced and failed to expand. In addition, chimeric studies showed that endoderm cells absent of *Sox17* could not colonize the foregut and the midgut/hindgut failed to develop. Taken together, this paper suggested that *Sox17* acts as maintenance factor in the foregut endoderm and more like a differentiation factor in the rest of the endoderm. At E8.5 and onward, *Sox17* mutants normally expressed markers of early liver development but the early pancreatic marker pancreatic and duodenal homeobox 1 *Pdx1* was not detected in the gut epithelium, suggesting that *Sox17* activity may be essential for pancreas development but not for liver and thyroid.

Hnf3b regulates endoderm formation in the early stages of PS formation. If *Hnf3b* is absent the PS does not elongate epiblast cells accumulate in the PS (Ang and Rossant, 1994; Dufort et al., 1998). In *Hnf3b* homozygous mutants lacks both the node and the notochord and thereby endoderm and neural tube formation is severely affected. Moreover, morphogenesis of the gut endoderm is disturbed and most frequent in the anterior regions leading to that foregut and midgut endoderm is not formed but hindgut endoderm is still specified.

Mice lacking *Hex* are deficient of DE (Martinez Barbera et al., 2000). *Hex* is also required for normal development of the foregut derived organs liver and thyroid development. In addition, *Hex* is required for normal forebrain development implicating that *Hex* plays a broader role in maintaining anterior identity.

Lhx1 is not required for endoderm formation per se but *Lhx1* mutant embryos exhibit a much smaller domain of anterior gut endoderm while posterior endoderm develops normally (Shawlot et al., 1999). This appears not to be a result of a reduced potency of the epiblast cells to form endoderm but rather by an inadequate allocation of endoderm cells to the anterior region of the gut endoderm. By that, *Lhx1* controls foregut endoderm formation by allocating DE cells to the foregut.

In the attempts of recapitulating embryonic development during DE differentiation of hESCs, a panel of markers needs to be used that collectively illustrates differentiation towards DE of anterior identity, the cell population from which foregut endoderm and later on pancreatic endoderm is formed. There is no marker described that is exclusive expressed in definitive endoderm localized at the anterior PS (ADE). Therefore, a panel of markers could be used to pinpoint ADE according the timing of expression and also the combinatorial expression pattern. In addition, many of the genes that are expressed in DE and important for DE formation are also expressed in VE and in other germ layers and therefore a combination of markers are needed to exclude differentiation to VE.

Brachyuru *T* can be used as a PS marker since it is primarily expressed in PS and in the early mesoderm but is not detected in later endoderm and mesoderm development (Beddington et al., 1992). *T* is expressed in the posterior epiblast and in combination with FGF ligands promote mesoderm formation and repress endoderm formation (Barrow et al., 2007; Rivera-Pérez and Magnuson, 2005). Mesoderm posterior 1 *MesP1* is specifically expressed in mesoderm but not in endoderm and is required for departure of mesodermal cells from the PS and differentiation of cardiac progenitors (Saga et al., 1996; Saga et al., 1999). This expression pattern suggests MesP1 as a marker for mesoderm formation.

In the pre/early-streak embryo, *Sox17* is expressed in the VE but at mid-streak stages, *Sox17* is no longer detected in VE but is instead expressed in the endoderm at the anterior end of the PS, at the site for recruitment of DE. As the DE moves towards the anterior, *Sox17* expression also expands more towards the anterior and remains specifically expressed in DE at this time. This is in contrast to other endoderm markers such as *Hnf3b* and *Hex* that are expressed in VE and DE in parallel. In the gut tube, *Sox17* expression becomes regionalized to the mid- and hindgut while expression in the foregut is reduced. *Cer1* is one of the earliest genes expressed in endoderm and is detected in both VE and DE at the early gastrula stages but is specifically expressed in the anterior endoderm (Belo et al., 1997; Shawlot et al., 1998). Later on *Cer1* expression expands in the anterior lateral portion of the embryo and is detected in the early foregut around E8.0 but expression decreases thereafter.

Hex is expressed in the first anterior DE cells emerging from the PS and remains to be expressed in the ventral foregut endoderm (Thomas et al., 1998) This region is later specified into liver but also into ventral pancreas (Deutsch et al., 2001).

The Sry box gene *Sox7* shows an overlapping expressing pattern with *Sox17* in the extraembryonic endoderm at the gastrula stage but is not detected in the DE or in the embryonic gut tube and could thus be used as a negative marker for DE (Kanai-Azuma et al., 2002).

In the early gastrula, *Otx2* is widely expressed in the epiblast but at the midgastrula stages becomes restricted to the entire anterior part where it is detected in all embryonic germ layers. After gastrulation, *Otx2* is expressed in forebrain structures (Ang et al., 1994).Thus, Otx2 is not an exclusive ADE marker but nevertheless, it is an important marker that is needed to displace the AVE with ADE (Perea-Gomez et al., 2001)

Patterning of gut endoderm

The initial steps of regionalization of the DE along the A-P axis takes place during PS stages in the way that timing of PS migration and exit regulates the final destination of DE cells along the A-P axis. In the late gastrula, DE will form the primitive gut tube (Tremblay and Zaret, 2005). Morphogenesis of gut endoderm begins when the epithelial sheet fold over the anterior and posterior ends forming foregut and hindgut pockets (Fig.4). Endoderm cells that exit the PS first will form the foregut and cells that exit later will form posterior gut regions (Lawson and Pedersen, 1987; Lewis and Tam, 2006). At this time, the embryonic gut tube is specified along the A-P axis into foregut, midgut and hindgut regions before the morphogenesis and budding of gut endoderm derived organs begins. The anterior portion is specified into foregut endoderm. This region is characterized by expression of transcription factors such as NK homobox 1 *Nkx2.1*, transcription termination factor *Ttf*, *Hex*, SRY-box *Sox2* and *Hnf3b* and will later on expand and develop into a thyroid, lung, esophagus, liver and ventral pancreas (Grapin-Botton and Melton, 2000). The foregut/midgut boundary expresses *Pdx1* and will later on give rise duodenum and dorsal pancreas. More posterior of the regions, caudal type homeobox *Cdx* transcription factors are expressed and these posterior most regions of the gut endoderm are specified into small and large intestines.
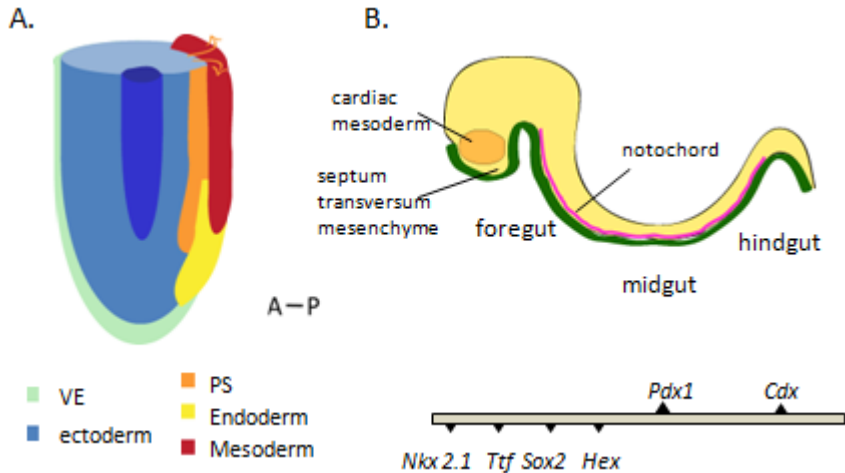
Figure 4

A. Epiblast cells are positioned along the A-P axis and migrate through the PS to form the three germ layers endoderm, mesoderm and ectoderm. **B**. Gut tube formation around E8.25. The gut endoderm is specified into foregut, midgut and hindgut regions that are defined based expression of specific transcription factors. Green indicates foregut, midgut and hindgut endoderm. Fig 3A is modified from Zorn and Wells Annu Rev Cell Dev Biol 2009 and fig 3B is modified from Grapin-Botton Trends in Genetics 2000

Signaling pathways in endoderm patterning

After gastrulation the endoderm receives signals from the surrounding mesoderm that patterns the gut endoderm along the A-P axis and defines the presumptive organ domains. These patterning processes involves a number of signaling pathways like BMP, Wnt, FGF and RA signaling that collectively induce specific expression patterns of transcription factors of each organ domain. Interactions between cardiac mesoderm and the foregut play an essential role in morphogenesis of foregut endoderm where different levels of FGF2 signaling modulate lineage specification of the foregut endoderm (Tremblay and Zaret, 2005).

Retinoic acid signaling

A role for retinoic acid signaling (RA) in patterning of posterior foregut has been suggested from zebrafish studies (Stafford and Prince 2002). The background to

this is that RA is suggested as the mesodermal signal necessary for Pdx1 expression in the posterior foregut. This was shown in RA deficient zebrafishes that did not induce Pdx1 expression and lacked pancreas. This mechanism might be conserved in mammals since mouse endoderm is also exposed to RA signaling from the surrounding mesoderm. However, in mice deficient for RA synthesis enzyme Raldh2, *Pdx1* expression is absent in the dorsal endoderm but is detected in the ventral endoderm. Accordingly, ventral pancreas and liver develops normally but dorsal pancreas is not correctly specified. (Duester, 2008; Molotkov et al., 2005).

FGF signaling

Among the FGF family, FGF4 has a role in patterning the endoderm. FGF4 acts in a concentration dependent manner establishing gene expression boundaries between foregut, midgut and hindgut (Dessimoz et al., 2006). High levels induce more posterior fates (*Pdx1* and *CdxA*) and represses anterior fates (*Hex* and *Nkx2.1*) while lower levels opposes these results. The function of FGF4 in establishing gene expression boundaries in the gut endoderm is active up to early somite stages but in later endoderm development, FGF signaling loses its function to repress anterior endoderm patterning. After establishment of the pancreatic domains, FGF is expressed in the pancreatic mesenchyme and provide instructive signals to the dorsal and ventral pancreatic buds respectively. FGF1, 7 and 10 are expressed in the mesenchyme and induce proliferation of pancreatic epithelium at the expense of differentiation. FGF7 and 10 has also been implicated to promote proliferation of pancreatic epithelial cells in humans (Ye et al., 2005). However, during ventral bud formation FGF signaling from the cardiac mesoderm blocks the pancreatic program. This illustrates the precisely requirement of proper timing and spatial expression of FGF`s in induction of either the pancreatic or hepatic programs.

Notch signaling

The Notch pathway is involved in regulating pancreatic cell differentiation. Inhibition of Notch signaling results in accelerated endocrine differentiation (Apelqvist et al., 1999). This has been shown in mice in which Notch signaling was impaired at the ligand level (Delta) and at intracellular mediator levels (RBP-J_K). In contrast, active Notch signaling inhibits endocrine differentiation indicating that Notch signaling regulates endocrine versus progenitor fates. Endocrine differentiation is mediated by the HES1 that is activated by Notch signaling and

that subsequently blocks transcription of the proendocrine gene *ngn3* (Lee et al 2001).

Wnt/beta-catenin signaling

The gut endoderm is also specified by Wnt/beta-catenin signaling in the way that repression of beta-catenin in the foregut maintains a foregut identity and induce liver and pancreas fates. In the posterior gut, active mesodermal Wnt/beta-catning signaling is required to inhibit the liver and pancreatic fates. Beta-catenin appears to act in part by repressing Hex expression that is one if the earliest markers expressed in foregut endoderm that has not become specified to organ specific domains (McLin et al., 2007). Beyond endoderm patterning, multiple studies have shown that pancreas development is affected by changes in Wnt/beta-catenin signaling but the diverse results obtained in these studies illustrate the complexity of Wnt signaling. Thus, further studies are needed to dissect the temporal and spatial influence of Wnt signaling in pancreas development.

Ventral pancreas

The pancreas is an example of an organ that is developed from two distinct foregut domains that eventually fuse together and create the gland (Slack, 1995). These two distinct domains are located on the dorsal and ventral side of the gut tube and are thus faced by different instructive signaling from nearby mesoderm specifying the two domains. The ventral pancreas and the liver both arise from a lateral domain of the ventral foregut, adjacent to the cardiac mesoderm (Zaret and Grompe, 2008). The default fate of this domain is to develop into the pancreatic fate but FGF from adjacent cardiac mesoderm and BMP from septum transversum mesenchyme blocks this process and induce a hepatic development (Deutsch et al., 2001). Moreover, the authors proposed that hepatic and ventral pancreatic progenitors share a common origin from a bipotent progenitor population that in the right context develops along the hepatic or pancreatic lineage. The mechanism that allows the initiation of the pancreatic program is suggested as a movement of Hex+ endoderm cells escaping away from the FGF signaling (Bort et al., 2004).

Dorsal pancreas

The other domain of pancreatic origin is the dorsal side of the posterior foregut-midgut adjacent to the notochord and later plate mesoderm. Initiation of the dorsal pancreatic program is specifically regulated by inhibition of sonic hedgehog (shh), a member of the Hedgehog family. Hedgehog signaling is involved in several regionalization events of the gut tube and shh is expressed in the presumptive dorsal pancreatic epithelium (Apelqvist et al., 1997; Kanai-Azuma et al., 2002). Specification and morphogenesis of the dorsal pancreas requires secreted factors from the notochord and dorsal aorta, specifically FGF2 and activin (TGFb family member) from the notochord that represses shh in the presumptive pancreatic epithelium (Hebrok et al., 2000).

Molecular markers regulating pancreas development

The specified dorsal and ventral pancreatic endoderm continues to develop along separate pathways regulated by the surrounding tissues. Although the pancreatic organ originates from two different domains of the foregut, exocrine and endocrine cells are developed in both pancreatic domains. After specification of the gut endoderm, the pancreatic epithelium grows and branches into the surrounding mesenchyme forming the pancreatic buds. In this new environment, instructive signals from mesodermal derived endothelial cells ensure blood supply and vascularization and subsequent induces outgrowth of the buds (Lammert et al., 2001). At these stages (E10.5-11.5), the first alpha cells appear. The buds continue to grow and at the secondary transition stage (E13.5-14.5) and a massive differentiation of betacells and acinar cells takes place. At birth, the mature pancreatic organs has formed where the distinct islets of Langerhans is scattered among acinar and ductal cells.

Genetic lineage tracing experiments makes it possible to follow cell fate decisions during differentiation and also to track the origin of differentiated cells. These types of studies have given considerable insight into the processes behind pancreas differentiation and have identified a number of key regulatory genes to follow pancreas development all the way from specification of the foregut, growth and branching of the buds and finally formation of the mature hormone producing gland. Transcription factors mentioned below are also expressed in other tissues but are here discussed from a pancreas development point of view.

Pdx1. The posterior region of the foregut endoderm domain expressing *Pdx1* marks the pre-pancreatic endoderm. *Pdx1* expression is detected at E8.5 in mice in the pre-pancreatic region of the foregut but then expands and can also be detected in the presumptive duodenum, stomach and bile ducts. Lineage tracing experiments have shown that *Pdx1* cells in the foregut are the descendants of the endocrine and exocrine cells in the mature organ (Gu et al., 2002). In addition, mice and humans lacking *Pdx1* do not develop a pancreas (Jonsson et al., 1994; Staffers et al., 1997). *Pdx1* is expressed during all stages of pancreas development but becomes restricted to the beta cells at E15.5. Despite the dramatic phenotype of *Pdx1* ablation, initial pancreatic specification of the foregut epithelium still occurs. This demonstrates that pancreas specification is induced before the onset of *Pdx1* expression. In addition, *Pdx1* expression in dorsal and ventral buds appears to be induced by different transcription factor networks indicating that the two buds are specified by different developmental pathways and signals from the surrounding tissues.

Hlbx9. The motor neuron and pancreas homeobox 1 *Hlxb9* is expressed both in dorsal and ventral foregut endoderm but is crucial for development of the dorsal bud initiation of dorsal *Pdx1* expression. This was demonstrated in *Hlxb9* null mice that failed in specifying the dorsal pancreatic program. Ventral pancreas specification was less severe affected but exhibited a perturbed and delayed endocrine differentiation (Li et al., 1999).

Hnf1b. In ventral foregut endoderm, the HNF homeobox B *Hnf1b* is necessary for bud initiation and activation of *Pdx1* and *Hlxb9* in this domain. Although *Hnf1b* primarily acts in the ventral pancreas it is expressed in both ventral and dorsal foregut endoderm. *Hnf1b* null embryos do form a dorsal bud but only transiently and at E13.5 the pancreas is not longer detected. The expression pattern of Hnf1b is broad and at earlier stages it is expressed in the entire gut endoderm, thereafter in the pancreatic and hepatic primordial. At E13.5 Hnf1b is detected in the pancreatic epithelium and finally gets restricted to the exocrine cells.

Ptf1a. The pancreas specific transcription factor 1A *Ptf1a* is detected in the ventral and dorsal pre-pancreatic region of the foregut (Kawaguchi et al., 2002). In Ptf1a knock-out mice, a dorsal bud is not developed and only a small portion of the ventral bud forms. Ptf1a has been suggested as one of the earliest regulators of a pancreatic fate commitment based on the finding that loss of Ptf1a converts early

pre-pancreatic domain into a duodenal like cell type. Ectopic expression of Ptf1a in liver, duodenum and stomach can divert those cells to pancreatic cells (Afelik et al., 2006). Later in development Ptf1a knock-out mice are absent of exocrine cells. Hence, the role of Ptf1a in endocrine cells development remains unclear but it is nevertheless an important marker of pancreas specification.

Hnf6. Dorsal and ventral pre-pancreatic epithelium does have in common that onecut homeobox 1 *Hnf6* is expressed in these domains. *Hnf6* directly binds to the *Pdx1* promoter and thus acts as main regulator of pancreatic specification of both dorsal and ventral endoderm (Jacquemin et al., 2003).

Sox9. In pancreas development, the SRY box *Sox9* is required for the maintenance of a progenitor population that can give rise to all pancreatic cell types (Seymour et al., 2007). During pancreas development, *Sox9* is expressed in both in the dorsal and ventral buds and expression is restricted to *Pdx1* progenitors. In addition, maintenance of the pancreatic progenitor population seems to be dependent on persistent *Sox9* and also by Notch signaling.

Endocrine differentiation

The initial steps in differentiation of the endocrine lineage is dependent on neurogenin 3 *Ngn3*, that is required and sufficient for development of the four endocrine cell lineages; betacells, alphacells, duct cells and acinar cells (Gradwohl et al., 2000). Lineage tracing studies have shown that NGN3+ cells are the precursors of islet cells (Gu et al., 2002).The expression is first detected in the early pancreatic epithelium e9 and is thereafter expanded and peaks around E15.5 but thereafter declines at E17.5. Endocrine development is also dependent on insulin enhancer protein *Isl1*since *Isl1* knock-out mice lack dorsal bud. *Isl1* is expressed both in the surrounding mesenchyme but also in the pancreatic epithelium and it appears that endocrine development is dependent on the epithelial expression while exocrine development depends in mesenchymal expression (Ahlgren et al., 1997).

Specification of islet cells

Ngn3 is main regulator of endocrine differentiation and a number *Ngn3* downstream target genes have been shown to influence endocrine differentiation in one way or another. The neurogenic differentiation factor 1 *NeuroD* is expressed downstream of *Ngn3* and is not detected in *Ngn3* null mutant mice. In the absence of *NeuroD* all four endocrine cell types do form but the number of endocrine cells are drastically reduced. Overexpression of *NeuroD* result in similar phenotype as Ngn3 overexpression which illustrates a close relationship between *Ngn3* and *NeuroD* in endocrine differentiation (Schwitzgebel et al., 2000). The paired box 6 gene *Pax6* is expressed together with *NeuroD* and *Isl1* in pancreatic epithelial cells committed to an endocrine fate. *Pax6* is not crucial for endocrine development but similar to the *NeuroD* mutant phenotype, fewer endocrine cells are formed and which leads to a severe reduction of the number of alphacells and betacells cells in the adult mouse. *Pax4* is another downstream target of *Ngn3* and has a key role in endocrine differerentiation in the sense beta cells and delta cells do not develop in the absence of *Pax4*. Lineage tracing studies have shown that predominantly insulin+ cells but also the other endocrine lineages originate from PAX4+cells. The aristaless related homeobox gene *Arx* is expressed downstream *Ngn3* and required for alphacell development and a model has been suggested where *Arx* and *Pax4* opposes each other's role in alpha vs betacell differentiation and thus regulates the balance between alpha and betacells (Collombat et al., 2003). NK2 transcription factor related gene *Nkx2.2* is widely expressed in the pancreatic epithelial domain of the gut endoderm but becomes restricted to the endocrine lineage and the *Nkx2.2* mice knock-out mice mostly show endocrine defects such as a severe reduction of alpha and betacells and becomes diabetic (Sussel et al., 1998).

Other members of the NK homeodomain family are *Nkx6.1* and *Nkx6.2* that both have a central role in pancreas development. *Nkx6.1* is a downstream target of *Pdx1* but *Nkx6.2* is not. Both genes are widely expressed in the pancreatic epithelium but *Nkx6.1* becomes restricted to insulin cells while *Nkx6.2* is expressed in glucagon cells and amylase positive cells. *Nkx6.1* expression remains in the mature betacells but *Nkx6.2* is not detected in the pancreas after E15.5. This indicates that *Nkx6.1* has a role in the mature betacells. *Nkx6.1* mutant mice has a severe reduction of betacells but the effect is even larger in *Nkx6.1/Nkx6.2* mutants that are also reduced in the number of alpha cells, indicating that there might be

compensatory roles between the two genes (Henseleit et al., 2005; Pedersen et al., 2005; Sander et al., 2000).

The pancreatic beta-cell-specific transcriptional activator *MafA* is specifically expressed in betacells and appears to be activated by *MafB* that is expressed at earlier stages than *MafA*. It also appears that *MafA* expressing cells are derived from *MafB* insulin positive cells present before the onset of *MafA* expression (Artner et al., 2006). In, *MafA* mutant mice pancreas develops normally but *MafA* is a critical regulator of the insulin gene and play a role in betacell function in the mature animal (Zhang et al., 2005). In contrast, *MafB* mutant display a delayed development of glucagon and insulin cells and in the adult, a reduction of both glucagon and insulin cells. Thus *MafB* acts a regulator of alpha and betacell maturation (Artner et al., 2007)
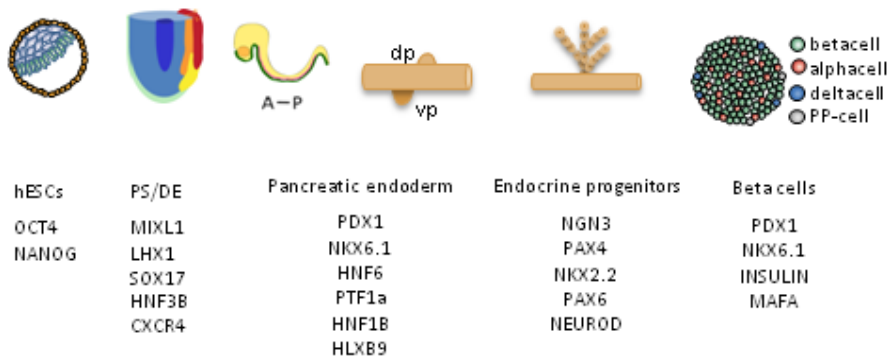

	betacell
	alphacell
	deltacell
	PP-cell

hESCs	PS/DE	Pancreatic endoderm	Endocrine progenitors	Beta cells
OCT4	MIXL1	PDX1	NGN3	PDX1
NANOG	LHX1	NKX6.1	PAX4	NKX6.1
	SOX17	HNF6	NKX2.2	INSULIN
	HNF3B	PTF1a	PAX6	MAFA
	CXCR4	HNF1B	NEUROD	
		HLXB9		

Figure 5. Schematic picture illustrating pancreas development. Molecular markers that are specifically expresseda at each developmental stage are indicated and that are used to identify the cells generated during in vitro differentiation of hESCs towards insulinproducing cells.

Recapitulating pancreas development in vitro

In the efforts of developing protocols for generation of insulin producing cells from pluripotent stem cells, researchers use the approach to mimic pancreas development in vivo. This has been done by using growth factor or small molecule combinations that stepwise differentiate cells to the different stages that beta cells undergoes during development. To ensure that the protocols guide cells through all

the progenitor stages behind beta cell development they are identified based on expression profiles of aforementioned transcription factors. These are either are uniquely expressed in the tissue or are required for specification of the progenitors at each developmental stage as illustrated in Fig 5. A differentiation protocol for efficient generation of DE from hESCs was reported in 2005 (D'Amour et al., 2005). In the following years, this protocol was followed up by a number of protocols that differentiates hESCs towards pancreatic endoderm and insulin expressing cells (Borowiak et al., 2009; Chen et al., 2009; D'Amour et al., 2006). Recently, Zhang et al reported a protocol for highly efficient differentiation hESCs to mature pancreatic insulin producing cells (Zhang et al., 2009) .In this strategy, pancreatic specialization of hESC-derived DE was induced in the presence RA, Noggin and FGF10. This treatment generated PDX1+ progenitor cells that cells co-expressed FOXA2, HNF1b and SOX9. These PDX1+ progenitor was proliferative (Ki-67+) and could be expanded in the presence of EGF. This protocol was further refined by isolating hESC-derived DE cells with the surface marker CXCR4 further enriched the PDX1+ population to near homogeneity (Cai et al., 2009). The isolated cells generated PDX1+ progenitors that also co-express the pancreatic progenitor markers HNF6, SOX9, HNF1b, HNF4a, FOXA2 but not CDX2. For maturation cells were treated with KGF, HGF, Exendin-4 and nicotinamide that differentiated the cells towards to insulin+/c-peptide+ and amylase +cells. The mature cells expressed PDX1, insulin, C-peptide and NKX6.1. This protocol generated 25% insulin+ cells in contrast to previous reports about 7% insulin+ cells.

Although protocols are available for generation of insulin producing cells the cells do not express the entire set of markers that are important for beta cell development in vivo. In addition, functional characteristics of the cells ie regulate blood glucose levels in diabetic animal models are poorly described. However, the report by Kroon et al showed that glucose responsive insulin producing cells can be generated that protected mice against streptozotocin -induced hyperglycemia (Kroon et al., 2008).
In this study insulin producing cell were generated after in vivo maturation of hESC-derived pancreatic endoderm and thus the instructive signals for maturation of pancreatic progenitors were provided by the in vivo environment. The important message of this report is that the hESCs have the potential to mature into functional insulin producing cells.

Another aspect is that protocols should be reproducible and not restricted to a specific cell line or cell culture system. Reproducibility has turned out as an issue in some cases. An explanation for this could be that the differentiation that generates pancreatic endoderm or insulin producing cells is not a direct effect of the added growth factors or chemical compounds but rather an indirect effect caused by the handling of cells or cell culture system itself such as 3D growth, cell densities and so on.

Although protocols for insulin-producing cells have been extensively reported, the stepwise transitions to the different progenitor stages (gut endoderm, foregut, endocrine progenitors) are often not fully investigated. This could be explained by the lack of efficient assays and tools that facilitate analysis of the entire set of markers require for pancreas development. Thus, in order to better characterize cells at the different stages that of pancreas development during in vitro differentiation of hESCs, in vitro and in vivo assays needs to be established that makes it possible to analyze if differentiation protocols progressively direct hESCs through the different stages of pancreas development.

Stem cells

Adult stem cells

All stem cells share the characteristics of self-renewal and potential to differentiate into multiple cell lineages. After the embryonic development, some organs resides adult stem cells or somatic stem cells that have the capacity to self-renew and to differentiated to some or all of the specialized cell types of that organ. The function of adult stem cells is to regenerate new cell types in the post-natal life in order to maintain and repair damaged tissue. Adult stem cells have been found in a number of tissues and organs such as brain, skin, teeth, peripheral blood, bone marrow, mammary glands, gut and liver. Clinical applications of adult stem cell types involve bone marrow transplantations to treat hematopoietic cancers or also to treat cancer in other organs where blood system is severely affected by high dosage chemotherapy. The existence of pancreatic stem cells is a controversial question that from a diabetes treatment point of view is of great interest. Expansion of mature beta cells could offer an alternative source of transplantable insulin producing cells in diabetic patients. However, the adult pancreas is a

relatively quiescent population with low turnover. The beta cells are known to have a low proliferative rate and in the adult normal pancreas, beta cells regeneration and maintenance relies on proliferation of terminally differentiated beta cells rather than by self-renewal of endocrine progenitors (Dor et al., 2004). Thus, these data point to the fact that pancreatic stem cells do not exist to the same extent as for example hematopoetic stem cells. Interestingly, facultative endocrine progenitor cells have been shown to exist in adult mouse pancreas but only after the organ have been damaged by partial duct ligation (Xu et al., 2008). This study shows that the adult mouse pancreas contains progenitors that revert to an embryonic-like mode of differentiation towards betacells. These and other results (reviewed in Guo and Hebrok, 2009) point to the plasticity of adult betacells in differentiation potential, a property that could be used to regenerate functional beta cells.

Embryonic stem cells

The history of pluripotent stem cells originates from mouse embryonic carcinoma cells (EC) that were derived from teratocarcinomas developing in the gonads of some inbred mouse strains (Kleinsmith and Pierce, 1964). The teratocarcinomas were found to contain multiple cell types and blastocyst injections of EC cells generated chimeric mice. These experiments indicated the value of EC cells as models to study development. However, the EC cells had chromosomal abnormalities and their ability to differentiate to multiple cell types was limited. Pluripotent cells were also observed when blastocysts were ectopically transplanted in mice and thereby raised the question if pluripotent cells could be derived directly from blastocysts. In 1981, Martin, G.R. reported that diploid pluripotent cells could be derived from mouse blastocysts (Martin, 1981). These embryonic stem cells (ES) had the capacity to differentiate to a wide variety of cell types. Pluripotent stem cells can also be isolated from the primordial germ cells of the blastocyst. These embryonic germ cells (EG) give rise to multiple cell types of the body but appears to have different gene regulatory network that maintains pluripotency. Human EC have been reported but they have a limited capacity to differentiate to all cell types of the body. This limits the use of human EC cells as model to study human development (Hogan et al., 1977). In 1998, Thomson et al reported the derivation of human ES cells (Thomson et al., 1998). These cells had a normal karyotype, expressed high levels of telomerase activity, expressed surface markers typical for primate ES cells and could be differentiated to

derivatives of all three embryonic germ layers. In addition, they could be proliferated as undifferentiated cells for long periods. These characteristics suggested human ES cells (hESCs) as model system to study human development.

Induced pluripotent stem cells (iPSCs)

In the last years, a number of studies have shown that it is possible to reprogram terminally differentiated cells to pluripotent stem cells by overexpressing transcription factors regulating pluripotency. The Yamanaka lab initially made a screen of transcription factors and identified that overexpression of four transcription factors; Oct3/4, Klf4, Sox2 and c-myc was necesssay to reprogram mouse and human fibroblasts into pluripotent cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). This groundbreaking research has opened up the possibility to design patient-specific cell lines that may circumvent the problems of immunological rejections during transplantation and therefore are of great use in cell replacement therapies. Initially, generation of iPSC relied on lentiviral mediated gene transfer but since then a number of iPSCs lines have been generated using different combinations of reprogramming genes and different methods for gene transfer. TypeI diabetes patient specific lines were generated with by retroviral infection with Oct, Sox2 and Klf-4 (Maehr et al., 2009). Thus, iPSC not only offers a patient specific source of transplantable cells but also serve as disease model of type I diabetes. This tool could potentially provide new insight to the genetics factors behind diabetes.

An intriguing question is how similar are iPSCs to the hESCs and are they fully reprogrammed to an embryonic-like state? It could be hypothesized that if they carry a memory of the originating donor cells is this related to the pluripotent differences observed between iPSCs and hESCs. In a recent study, gene expression profiles of human hESCs, iPSCs and the originating donor cells revealed that there is a persistent donor cell gene expression in iPSCs lines and that this contributes to the differences in pluripotency observed between hESCs and iPSCs (Ghosh et al., 2010).Therefore, this study concluded that reprogramming not completely de differentiate somatic cells to an ESC-state. It remains to be known whether the persistent memory influences the capacity of reprogrammed cells to differentiate to the originating cell type more easily than to other cell types. Therefore, a better understanding of the mechanisms behind the incompleteness of reprogramming would be helpful to understand the link

between complete reprogramming and potential to differentiate into cell types with the same functional characteristics as their in vivo counterparts. In summary, there are a number of pluripotent stem cells types that all have the possibility to self-renew but level of pluripotency varies. Among these, hESCs exhibits pluirpotentcy both in vivo and in vitro and can be produced indefinitely. These characteristics make hESCs suitable a model system to study human embryonic development and possibly also for the generation functional insulin producing beta cells or progenitors thereof that can be used for transplantation of diabetes patients. With that in mind the work in this thesis were based on hESCs as a model system to study early development in human cellular system.

Human embryonic stem cells

The hESCs originate from the inner cell mass (ICM) in the blastocysts of preimplanatation embryos (Fig. 5). At this early stage of development, the blastocyst is composed of the trophoblasts and ICM and this stage represents the first sign of differentiation since a cell fate decision has been made towards trophoblasts or ICM (Fig.1). This distinction is regulated by a key regulatory network of genes that maintains the pluirpotent capacity of the ICM. This network involves Oct3/4, Nanog and Stat that function in repressing ICM cells from becoming trophoblasts and controls self-renewal. The ICM are pluripotent cells that will give rise to the embryo and its associated allantois, amnion and yolk sac. hESCs are derived from the ICM of embryos as illustrated in Fig. 6. Embryos used for hESC derivation have been produced for in vitro fertilization purpose and after informed consent from donors and approval of the local ethics committees. To isolate ICM, blastocysts are hatched from the zona pellucida by pronase treatment or immunosurgery (Thomson et al., 1998). Thereafter, trophoblasts are separated from the inner cell mass by immunosurgery that includes a treatment with human specific antiserum that binds to the trophoblasts. The isolated inner cell mass is then taken into cell culture by plating on mouse embryonic fibroblasts (MEF) in a supportive cell culture medium. The MEF cells are mitotically inactivated cells that secrete growth factors that support proliferation of hESCs. After the hESCs have been isolated, they are characterized according the criteria of pluripotency, expanded and cryopreserved in liquid nitrogen. hESC lines used in this thesis work includes SA121and SA181 derived at Cellartis AB,Sweden and HUES-1,3,4 and15 derived at the Melton laboratory, Harvard University, USA (Cowan et al., 2004; Heins et al., 2004).
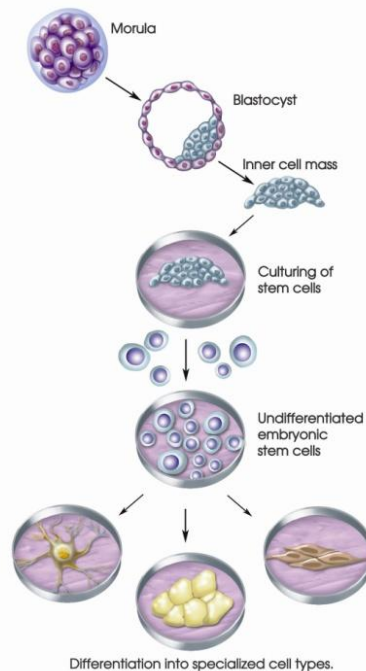
Figure 6. Derivation of human embryonic stem cells (hESCs)
The inner cell mass (ICM) are pluripotent cells that later on will give rise to the embryo proper. These cells can be isolated and maintained in cell culture as hESCs. hESCs have a high proliferative rate and can either be expanded as undifferentiated cells or differentiated to more mature cell types.

Characteristics of undifferentiated hESCs

The criteria that define hESC lines are all based on the characteristics of the originating pluripotent ICM. They should maintain pluripotency and a high proliferative rate over long periods, and they should maintain a stable karyotype (Amit et al., 2000). The proliferative capacity is determined as a population doubling time that is usually around 36h that but this is variable between lines. Another criterion is telomerase activity that is highly connected with immortalization of human cell lines. Telomerase adds telomere repeats at the chromosome ends and if reintroduced into some somatic cell lines it can extend the replicative life-span. Therefore, high telomerase activity indicates a high

replicative and proliferative capacity of hESCs. hESCs show a high nucleus to cytoplasm ratio and when maintained on MEF cells the colony morphology is more similar to that of primate ES cells than to mouse ES cells. In addition, a hESC line should possess a normal karyotype of 46 XX or XY chromosomes. hESC lines are also characterized for the expression of a set of surface markers; stage specific embryonic antigen 3(SSEA-3), SSEA-4, TRA1-60, TRA1-81 and alkaline phosphatase. These markers were originally identified as characteristic of non human primate ES cells and human EC cells (Thomson et al., 1995).

There are a number of methods to test pluripotency and the capacity of hESCs to differentiate to cell types derived from the three embryonic germ layers endoderm, mesoderm and ectoderm. In vivo experiments test the capacity of hESCs to form teratomas after transplantation to immunodeficient mice, usually SCID mice. The teratomas should consist of cell types that are dervided from all three germ layers. Pluripotency is also tested in vitro by spontaneous differentiation that is induced by removal of bFGF from the culture medium and in many cases by embryoid body (EB) formation where the cells are cultured as in suspension cultures as three dimensional aggregates.

Propagation of hESCs

Conventionally hESCs are maintained and propagated on feeder cells in a supportive medium. An essential component of the medium is bFGF that is required to maintain hESCs in an undifferentiated state (Smith, 2001). MEF cells are commonly used as feeder cells but human derived feeder cells are also used. hESCs grown on MEF cells usually form distinct colonies. To maintain the cells in an undifferentiated state, the cultures are passaged when they reach confluence or at appropriate colony size. hESCs can be passaged either by mechanical dissociation or by enzymatic dissociation. Mechanical dissociation is done by cutting the colonies into smaller pieces using stem cell knife that facilitates cutting of colonies in appropriate sizes. It has turned out that mechanically dissociated hESCs do not acquire an abnormal karyotype to the same extent as when cells are enzymatically dissociated to single cells. Enzymatic dissociation offers the possibility to dissociate the colonies into single cells and to isolate subclones from a culture. Experiments in this thesis have been based on hESCs maintained on MEF cells but also under feeder free cell culture conditions. The MEF cultured cells were originally mechanically dissociated but were adapted to enzymatic

dissociation with trypsin similar to the HUES lines. The primary reason for this is that expansion of mechanically dissociated cells is time consuming and therefore it is difficult to produce sufficient amounts of cells needed for a proper experimental design.

Feeder free hESC culture and chemically defined cell culture conditions

Feeder based hESC culture relies is based on that feeder cells secrete factors supporting cell growth and survival. However, this also results in an instability of the cell culture system that may interfere with self-renewal and differentiation (Keller, 2005). To circumvent this, chemically defined hESC culture conditions have been established where cells are maintained on without feeder cells and in a medium based on chemically defined components. In feeder free hESC culture systems cells are often maintained on Matrigel™ as an adhesive surface and in a cell culture medium that have been conditioned on MEF cells. However this surface contains a mixture of extracellular matrix molecules derived from mouse sarcomas and is therefore not defined which may results in inconsistent stability of the hESCs quality due to batch-to batch variations of the Matrigel. To further refine the methods for feeder free hESC culture, new cell culture systems have been developed that are based on completely defined surfaces and culture medium. These are often based on the recombinant proteins and synthetic biomaterials (Melkoumian et al.; Rodin et al.; Villa-Diaz et al., 2010).

Xenofree cell culture

Although chemically defined culture conditions offer stable hESCs culture systems they may still contain animal derived reagents and are therefore unsuitable for clinical applications. Transplanting xeno-contaminated hESCs may cause graft rejections and transfer of nonhuman pathogens and therefore hESC culture systems that have not been exposed to reagents of animal origin will be needed in clinical applications. Aspects that needs to be considered in developing xenofree systems includes finding a surface, either human feeder cells or xenofree matrices and a cell culture medium free of animal derived componentes. In a true xenofree hESC culture, the hESC line should also be derived under xenofree conditions. Traditionally, immunosurgery is used to isolate the ICM from trophoectoderm by incubation in human serum antibodies and guinea pig complement. A method for truly xeno-free derivation of hESC lines were developed by Ellerström et al that

included xeno-free derivation of the hESC line, culturing cells of human foreskin fibroblasts and in a xenofree medium composition (Ellerström et al., 2006).

Applications of hESCs

Regenerative medicine

One of the great promises of hESCs is to generate cells that can replace damaged or dysfunctional cells. However, issues regarding safety and graft rejections are important questions that need to be considered. One main concern is the persistence of remaining undifferentiated hESCs that may cause tumour formation following transplantation. Transplantation of undifferentiated hESCs into animals develops into teratomas that are benign tumours consisiting of cell types derived from all three embryonic germ layers (Shih et al., 2007). Teratoma formation has been reported after transplantation of pancreatic progenitor populations and teratomas were concluded as a result of remaining undifferentiated cells (Kroon et al., 2008).

Immune rejections of transplanted cells are other important aspects in regenerative medicine. Type I diabetes patients have an autoimmune reaction that destroys the beta cells highlighting the need for suppression of the autoimmune reaction. Additionally, the recipient´s immune system may recognize transplanted cells as foreign and therefore reject the grafted cells in a host-graft response. Strategies to circumvent immune rejection of transplanted cells mostly focus on encapsulation of cells. These ideas originate partially from experiments where islet cells are encapsulated in permeable matrices and transplanted into the hosts. Thereby, the transplanted cells exerted insulin secretion without being attacked by immune system. Diabetes patients that are transplanted with encapsulated islets cells are not fully recovered and have to continue on insulin. Therefore, establishing robust methods for encapsulation of islets cells are important aspects for in using hESCs regenerative medicine.

Basic tools to study hESC biology

In vitro differentiation

In directed differentiation, growth factors and other chemical compounds are added that specifically target certain developmental pathways. This strategy is

approached in the efforts of developing differentiation protocols for generation of insulin producing cells.

In spontaneous differentiation, cells are allowed to differentiate without the presence of stimulating growth factors. This is basically performed by withdrawal of bFGF from the cell culture media resulting in that cells spontaneously differentiate towards more mature cell types. Culturing cells under 3 dimensional structures as embryonic bodies (EB´s) commonly generates cell types that are derived from all three embryonic germ layers. Spontaneous differentiation as EB´s is a common method that used to test the potential of new hESC lines, new hESC culture systems or genetically modified hESC lines to differentiate to the three embryonic germ layers

In vivo differentiation

Another strategy to mature hESCs is to transplant undifferentiated cells into immunodeficient mice. In these conditions, undifferentiated pluripotent cells form teratomas that are tumours composed of tissues that resemble derivatives from all three embryonic germ layers.

Differentiation of cells in vivo also offers a possibility of study differentiation in an environment resembling the normal in vivo situation and that may provides instructive signals needed for maturation of progenitors (Brolen et al., 2005). This strategy is particular useful for establishing functional tests that verify the potential of hESC-derived progenitor cells to mature and display the properties of functional cell types in an in vivo environment.

Genetic engineering

To investigate the molecular mechanisms behind growth and differentiation of hESCs, genetic engineering is a fundamental tool that makes it possible to alter expression of key regulatory genes or label cells with fluorescent reporter genes such as enhanced green fluorescent green (eGFP). Reporter cell lines, in which a reporter gene is linked to an endogenous promoter facilitates gene regulation studies of hESCs cultures. Reporter cell lines can also be used in genetic lineage tracing studies. The basic principle for genetic lineage tracing is to constitutively label cells with a reporter gene and induce reporter gene expression by Cre-recombinase. The Cre-recombinase can be driven by a tissue specific promoter

and thereby it is possible to follow cells along the developmental pathways. Genetic lineage tracing studies in mice have among other things shown that all islet cells originates from a NGN3+ progenitor population and thereby revealed an important marker. In this context, lineage tracing could potentially be used to identify cell fate decisions during differentiation of hESC towards insulin producing cells.

There are several methods available for genetic modification of hESCs such as viral transduction, chemical transfection or electroporation(Costa et al., 2005; Gropp et al., 2003). Retroviral mediated gene transfer has turned out as an efficient method for stable integration of transgenes into hESCse (Yao et al., 2004; Zufferey et al., 1998). Downregulation of transgene expression as a result of transcriptional silencing of retroviral vector have been reported both in undifferentiated hESCs and in their differentiated state. However, lentivirus that is a subfamily of retrovirus has been shown to resist silencing in mouse ES cells. Lentiviral vectors that are based on the human immunodeficiency virus type 1 (HIV-1) have successfully been used in hESCs for generation of stable transgenic cell lines (Ma et al., 2003; Naldini et al., 1996; Suter et al., 2006). The basic principles for lentiviral mediate gene transfer are illustrated in Fig. 7.

Another advantage of lentivirus is that they transduce both dividing and non-dividing cells and therefore can be used for genetic modification of a wide variety of cell types including both undifferentiated and differentiated hESCs. Using viral vector mediated gene transfer for clinical applications bio-saftey is an issue that needs to be addressed at several levels. Nevertheless, lentivirus continues to function as an easy and efficient method to deliver transgenes into hESCs and is thus appropriate for experimental research.-

Recently, virus-independent methods for stable integration of transgenes into the human genome have been reported. These methods are based on the piggyBac transposons where insertion/excision is catalyzed by transposase. Combining this system with a multiprotein expression vector facilitates insertion of multiple transgenes into a single site of the genome that also could be excised (Kaji et al., 2009).
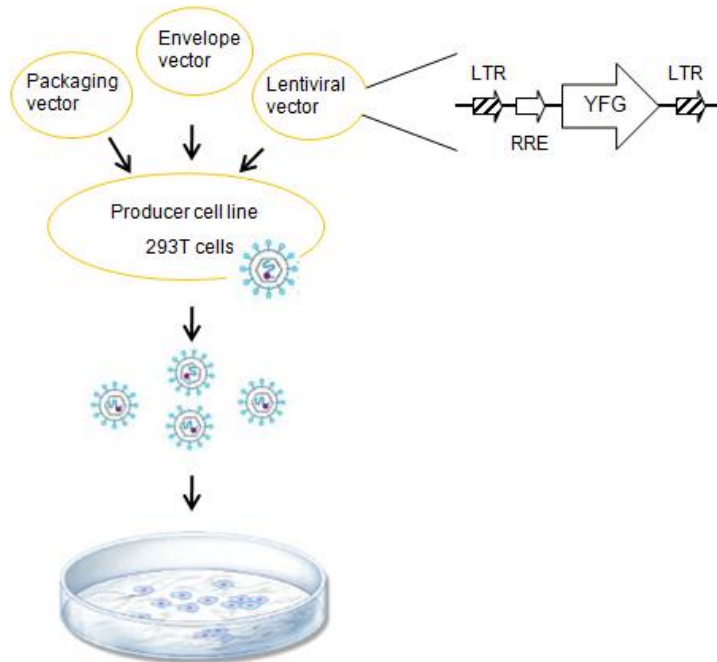

Figure 7. Basic principle for using viral vectors for gene delivery.
The viral genome contains genes for reproduction and infection that are flanked by LTR (promoters and enhancers).The entire genome is deleted and replaced by your favorite gene (YFG). The genes needed for generation of infectious viral particles are kept on packaging and envelope vectors that are separate from the viral vector harboring YFG. Co-transfection of the three vectors into a packaging cell line gene generates virus that infect other host cells but cannot replicate in the host.

AIMS

The objective in this thesis was to unravel strategies and analysis methods for in vitro differentiation of hESCs, more specifically towards definitive endoderm and posterior foregut endoderm.

The specific aims were;

Paper I   To analyze activities of constitutive promoters that drives high and sustainable gene expression in hESCs, both during long term culture of undifferentiated cells and during spontaneous differentiation.

Paper II   To characterize hESC-derived definitive endoderm at the cellular level by single-cell gene expression analysis.

Paper III   To examine the role of FGF4 and RA in directing differentiation of hESC-derived definitive endoderm into foregut endoderm-the origin of pancreatic endoderm.

Papers in summary

Paper I

Rationale

Constitutive promoters that ensure sustained and high levels of gene expression are basic research tools that have a wide range of applications in hESCs research. The original reports for generation iPSCs were based on constitutive expression driven by the *EF1α* promoter overexpress the four transcription factors;*Oct4*, *Sox2*, *Klf-4* and *c-MYC* in order to reprogram somatic cells to iPSCs (Takahashi and Yamanaka, 2006). Additionally, constitutive expression driven by the CAG promoter has been used to generate hESC-derived endodermal progenitors by overexpressing the endodermal genes *SOX17* and *SOX7* (Séguin et al., 2008). Another application of constitutive promoters is to drive the expression of fluorescent reporter genes. This makes it possible to follow differentiating hESCs in animal grafting experiments without using time consuming species-specific antibody labeling systems or *in situ* hybridizations. It is important to verify that the promoter of choice ensure sustained and high level gene expression depending on the application of constitutive expression ie both in undifferentiated hESCs and during differentiation. A comprehensive study of the functionality of commonly used promoters in hESCs has been lacking and much information has relied on experiences from the mESC system. Therefore, we conducted a study to compare the activity of commonly used constitutive promoters in hESCs, both in undifferentiated cells and during differentiation to the three embryonic germ layers endoderm, mesoderm and ectoderm.

Experimental design

Lentivirus was used as gene delivery system to introduce promoter-eGFP constructs. Lentivial mediated gene transfer efficienctly transduces hESC and ensures stable integration of transgenes into the genome. Therefore, this gene transfer system was considered as appropriate to study stable gene expression in hESCs. hESC line SA121 was transduced with lentiviral particles harboring *ACTB-, CMV-, EF1α-, PGK-* and *UbC*-eGFP constructs, respectively. hESCs was transduced with viral vector particles at concentrations that were previously optimized to generate low copy number integration of viral vectors. To

quantitatively compare activity between the different promoters, we isolated eGFP expressing cells after transduction by FACS sorting. This generated pure *ACTB-, CMV-, EF1α-, PGK-* or UbC -eGFP expressing populations. After FACS sorting, all cells were maintained under feeder free conditions to facilitate quantification of percentage eGFP+ cells of the total population. Promoter activity during long term culture of undifferentiated hESCs was evaluated as percentage of eGFP+ cells and was measured 15, 30 and 50 days after FACS sorting. FACS sorting referred to as day0 and represents 100% eGFP+ cells. Promoter activities and intensities were also quantified in hESCs differentiated to the three embryonic germ layers. Undifferentiated promoter-eGFP+ cells were spontaneously differentiated as embryoid bodies (EB) and decrease of promoter activity and intensity of each promoter was quantitatively measured after 22 days of differentiation. To address if promoter activities show any preference to the three germ layers, eGFP+ and eGFP- populations of the EB differentiated cells were separated by FACS sorting and gene expression levels of markers representative for each germ layer were compared between eGFP+ and eGFP- populations. To test the reproducibility of promoter activities, experiments were performed at three independent experiments. Additionally, promoter activities during long term culture of undifferentiated hESCs were repeated in an additional hESC line, Hues-4.

Results

Lentiviral transduction and viral vector copy number integration

Genomic integration of lentiviral vectors harboring the eGFP constructs was measured by qPCR on genomic DNA from FACS sorted *ACTB-, CMV-, EF1α-, PGK-* and *UbC*-eGFP+ populations. All promoter constructs integrated with average <5 copies to the hESC genome in both hESC cell lines tested. Copy numbers of integrated vectors remained stable during long term culture of undifferentiated cells up to 50 days for all promoters except for the UbC promoter. Promoter-eGFP+ cells expressed pluripotency markers OCT3/4, NANOG and hES-Cellect during long term culture of undifferentiated cells as shown by immunostainings.

Promoter stability in undifferentiated hESCs and during differentiation
The ACTB, EF1α and PGK promoters were found to be more efficient than CMV in driving gene expression during long term culture of undifferentiated hESCs.

Equal activities were detected for the ACTB, EF1α and PGK up to 30 days of culture but thereafter EF1*α* promoter activity decreased whereas ACTB and PGK ensured sustained eGFP expression up to 50 days. The ACTB was the most superior promoter in undifferentiated cells by maintaining eGFP expression up to 50 days of culture in both cell lines tested. eGFP expression driven from the CMV promoter were rapidly down regulated at day7.Thus this promoter was concluded as the less efficient than the other analyzed promoters.

During EB differentiation, promoter activities were less stable than in undifferentiated cells. Of the analyzed promoters, EF1α was the most stable promoter during differentiation. Nevertheless, it was down regulated in approximately 50% of the differentiated cells. To test if promoter activities showed any preference to specific cell lineages, differentiated cells were separated into eGFP+ och eGFP- populations. Gene expression analysis of eGFP+ and eGFP- populations was performed for relative quantification of mRNA levels of markers representing the three embryonic germ layers. Interestingly, in cells were the EF1α promoter was active, gene expression levels for all three embryonic germ layers markers were similar to or higher in eGFP- cells, indicating that this promoter does not show any preference to endodermal, mesodermal or ectodermal hESC derivatives. The EF1α, PGK and UbC promoters were active in *SOX17+* early endoderm and in cells differentiated towards ectodermal lineages. The EF1α promoter was also active cells *ALBUMIN+* late endoderm cells/hepatoblasts and in *CD31+* and *PPARγ+* late mesoderm. In contrast, the ACTB promoter was not active in these populations.

Discussion

Here, we provide data on the characteristics of constitutive promoters in hESCs using eGFP as a reporter gene. Thus, results presented here are representative for constitutive promotes activities detected as eGFP expression. Lentivirus was used as gene delivery system since it is ensure stable integration to the genome compared to other gene delivery systems such as chemical transfection or adenovirus. Lentiviral vectors are more efficient tools for stable genetic modification since they are known to be less prone to gene silencing than other retroviral vectors.

The ACTB promoter were found to be the most stable promoter in undifferentiated cells up to 50 days of culture, as observed in two different hESC lines. This is in

line to a previous study showing that ACTB is active both in undifferentiated cells and in cell differentiated to all three embryonic germ layers (Costa et al., 2005).

When generating reporter cell lines that are supposed be used to follow cells fate decisions it is essential to use promoters that ensures stable expression of its downstream gene but also to rely on strong promoter activity. The latter is to ensure that the promoter drives detectable levels of reporter gene expression. The fluoresecent signal from the promoter-eGFP transduced populations showed that the ACTB and PGK promoters expressed eGFP at stable intensity levels during the 50 day culture period. In relation to the observation that ACTB was active up to 50 days of culture, these data suggests ACTB as a strong and stable promoter in undifferentiated cells.

During EB differentiation, the promoter activities decreased compared to undifferentiated cells. Downregulation of transgene expression in ES cells transduced with viral vectors is often related to gene silencing. Silencing have mainly been studied in gamma retrovirus MLV transduced ES cells. Regarding lentivirus, it has been shown that silencing is more pronounced in single-copy transduced ES cells compared to cells harboring multiple viral vector copies. Since we observed promoter specific differences in the degree of promoter activity during differentiation, we conclude that the lentiviral mediated transgene expression is at least partially dependent on the applied promoter.

This study indicates that promoter activities may be restricted to specific cell lineages during EB differentiation and thus careful selection of promoters during differentiation is important. Future studies will have to resolve the activities of constitutive promoter during directed differentiation to specific cell lineages. To conclude, this study provides a guideline for choosing suitable promoter in undifferentiated cells and when in vitro differentiation to certain cell lineages is desired.

Paper II

Rationale

In the efforts of developing protocols for differentiation of pluripotent stem cells to insulin producing cells, research tools that allow analysis of multiple markers at the single-cell level are needed. The background to this is that the different progenitor stages of beta cell development can only be characterized as cells co-expressing specific set of markers. Definitive endoderm (DE) is the origin of pancreatic endoderm and today there is no single marker that is exclusively expressed in DE but a combination of marker could be used to identify DE. Therefore, methods that facilitate analysis of hESCs at the single cell level are needed to identify DE cells among heterogenous populations. In this study, we approached single-cell gene expression to characterize the expression pattern of a panel of DE markers at the cellular level during DE differentiation of hESCs.

Experimental design

Initially, temporal expression pattern of primitive streak (PS) and DE markers was analyzed measured in hESCs differentiated with different activin A treatments. Activin A is a ligand for Nodal and is conventionally used to differentiate hESCs towards DE and to mimic Nodal signaling that induce gastrulation and endoderm development in vertebrates. Time course studies were measured on the global population by gene expression analysis for markers specific for PS and endoderm formation. At the endpoint stage of DE differentiation, DE marker expression was confirmed by immunostainings. Immunostainings were performed for markers SOX17, OCT4, FOXA2, CDX2, AFP and SOX7 where antibody staining were resulted in staining with a specific staining pattern. To characterize expression of the additional PS/DE markers at the cellular level, single-cell gene expression was performed at the endpoint stage of the different acitivinA treatments. This enabled comparison of results obtained from the entire population to results from individual cells.

Results

Analysis of DE differentiation over time

Time course gene expression analysis of the global population showed that two of the three activin A treatments, AAB27 and AANaB, showed similar gene expression pattern of PS/ADE genes as during vertebrate gastrulation and early endoderm development. More specifically, PS markers were transiently upregulated before endoderm markers were upregulated. Genes that defining a pluripotent state (OCT4 and NANOG) were downregulated with time. The third acitivinA treatment, AAFBS, showed less upregulation of DE genes and downregulation of pluripotency genes to a less extent compared to the two other activinA treatments.

hESCs differentiated towards DE and had not yet become gut endoderm

Gene expression results were confirmed by immunostainings showing that AAB27 and AANaB treatments efficiently differentiated hESCs towards DE, characterized by expression of endoderm markers SOX17 and FOXA2, and down regulation of pluirpotency marker OCT4. Efficiency of DE differentiation appeared as similar between the AAB27 and AANaB treatments while AAFBS was less efficient as observed by poor induction of SOX17 and poor down regulation of OCT4 expression. Qualitative analysis of protein expression was also performed for a panel of markers that covers differentiation to extraembryonic endoderm and later specification of DE to gut endoderm. Immunostainings showed that AAB27 and AANaB cells expressed DE markers SOX17 and FOXA2 but not SOX7 that is specifically expressed in extraembryonic endoderm, thus indicating differentiation towards DE rather than VE. Moreover, to assess if hESC-DE populations have differentiated towards later stages of the endodermal lineage, gut tube markers were also analyzed by protein expression. Neither CDX2 nor AFP could be detected among these populations suggesting that cells have not yet become gut endoderm.

hESCs differentiated towards DE were analyzed by single-cell gene expression analysis in order to characterize the molecular signature of hESC-DE generated by the different methods.

Heterogeneous marker expression was observed within each of the hESC-DE populations generated from the different activin A treatments. This indicates asynchronous differentiation within a population consisting of both hESC-DE and undifferentiated cells. To specifically resolve the molecular signature of hESC-DE cells, cells that expressed *SOX17*mRNA was specifically analyzed in AAB27 and AANaB treated cells. Among the *SOX17* positive cells, expression of other ADE markers was identified to the same extent between AAB27 and AANaB cells. Notably, the *SOX17* positive population generated from AAB27 differentiation co-expressed *LHX1* and *MIXLl1* to a higher extent than *SOX17* expressing cells generated with AANaB differentiation. In addition, higher levels of relative gene expression were detected among the *MIXL1*positive and *LHX1* positive cells generated with AAB27 differentiation compared to *MIXL1*positive and *LHX1* positive from AANaB differentiation. From these experiments we could extract the information that there are distinct molecular signatures of hESC-DE generated with different activin A treatmens.

Discussion

The results obtained here illustrate the usefulness of single-cell gene expression analysis to characterize expression of multiple markers at the cellular level. Moreover, the distinct gene expression signatures that were identified in hESC-DE generated from different activin A treatments illustrates the importance of establishing molecular markers and techniques that specifically identify and analyze subpopulations of interest within heterogeneous cell cultures.

The observation that *SOX17* positive cells co-express *MIXL1* and *LHX1* to a larger extent inAAB27 treated cells compared to AANaB treatments, raises the questions if this is timing related difference between the two treatments, or alternatively, if AANaB treatment does not generate *SOX17* positive co-expressing *MIXL1* and *LHX1* to the same extent as during AAB27 treatment. To answer these questions, time course analysis of the *SOX17* populations of each treatment would be needed. In mice, *Mixl1* is expressed in the PS and is required for DE formation and

suppression of mesoderm (Hart 2002, Tam 2007). In mice, *Mixl1* and *Lhx1* are involved in movement of DE that is followed by regionalization and patterning of the primitive gut tube into foregut, midgut and hindgut endoderm. Thus, it would be interesting to test if the *MIXL1* and *LHX1* positive and negative hESC-DE populations respectively, have the potential to progress further in differentiation. To test this, in vivo assays addressing the potential to respond to posteriorizing cues from the embryonic environment would be needed. Such assay was successfully used to test the functionality of mESC DE that after transplantation in chick endoderm incorporated to the pancreatic region of the developing endoderm (Hansson 2009).

Paper III

Rationale

Retionic acid (RA) and fibroblast growth factor (FGF) signaling are both involved in patterning of gut endoderm along the anterior-posterior axis. Studies in zebrafish have shown that RA is required for pancreas and liver specification but not for more posterior endodermal organs. Increased levels of RA results in expansion of pancreas and liver domains at the expense of more anterior organs such as thyroid and pharynx and thus RA is suggested to acts as an posteriorizing agent. mESCs that are differentiated in the presence of RA induce expression of PDX1+ pancreatic endoderm. RA has been used for hESC differentiation towards pancreatic cell types but not without defining its exact role. Also, the optimal timing of RA addition has been not been addressed and the expression pattern of retinoic acid receptors (RAR) is unknown in hESCs.

FGF signaling is also responsible for patterning of the gut tube endoderm along the anterior-posterior axis.FGF4 that is expressed in the vicinity of posterior endoderm in the gastrula promotes posterior endoderm and inhibits anterior endoderm cell fates. Exposing endoderm to recombinant FGF4 repressed anterior cell markers *Hex1* and *Nkx2.1* and disrupted foregut morphogenesis. Inhibition of FGF signaling by SU5402 showed that FGF signaling is necessary for maintaining gene expression boundaries between midgut and hindgut, from gastrulation to somitogenesis. In this study, the ability of RA and FGF4 to direct differentiation and patterning of hESC-derived definitive endoderm towards gut endoderm was addressed.

Experimental procedure

To test the effect of FGF4 and RA, differentiation was performed in different steps. Initally, hESCs were differentiated to definitive endoderm (DE) using a previously published protocol for DE differentiation (D'Amour et al., 2005). In this protocol, cells are induced to DE by Activin A(AA) and Wnt3a treatment that is added the first day of differentiation and thereafter is AA alone added for an additional 2 days. After AA-induction, cells were treated with FGF4 and RA alone and in various combinations. Growth factors (GF) were added in a temporal and concentration dependent manner. The medium basal medium composition was mainly based on the Damour protocol (Damour 2005+2006). AA-induction is performed in RPMI in low medium serum (FBS) and day4 to 7 in RPMI supplemented with 2% FBS. From day 8 and onward DMEM supplemented with 2%FBS is used as basal medium.

The effect of added GFs was monitored by analyzing markers that are specifically expressed in different domains of the gut endoderm. Marker expression was measured as up regulation of relative gene expression and protein expression was confirmed by immunofluorescent stainings with antibodies. As control for FGF4 and RA treatments, cells were differentiated to DE and thereafter cultured in basal medium without GF.

Results

The protocol used for DE differentiation induced expression of DE markers. This was analyzed as increased relative gene expression levels that were verified by immunostainings for protein expression. After the AA-induction, FGF4 was added alone and in combination with RA. In the absence of RA, FGF4 was unable to induce *PDX1* expression. However, *PDX1* expression increased when FGF4 was added directly after AA-induction and after four days replaced by RA, that was further added for an additional 4 days. During this treatment, *PDX1* expression was dependent on the concentration of FGF4. Control samples showed that endogenous *FGF4* expression only was detected in undifferentiated cells and not at later time points.

This finding led to the speculation that timing of RA addition might improve the differentiation protocol. Previous reports have not addressed the timing of RA

more than that is should be added after AA-induction. Therefore, of the expression of the retinoic acid receptor β (*RARβ*) was analyzed at different time points after AA-induction in the absence of any GF. Notably, at day4 immediately after AA-induction, *RARβ* expression increased and remained upregulated until day 8 when gene expression levels declined. Based on timing of *RARβ* expression, various combinations and time points of RA and FGF4 were tested for improved expression of *PDX1,* during a 13 day period of differentiation. Adding RA at day4 increased *PDX1* expression compared when RA was added at day 8. Of the combinations tested, the highest PDX1 expression levels were obtained when RA was added the entire differentiation period. Prolonged RA/FGF4 treatment resulted in higher relative PDX1 levels but after 12-13 days cultures started to deteriorate possibly due to high confluence.

FGF4 in combination with RA for 12 days differentiation resulted in increased number of cells. However, relative *PDX1* expression was not induced compared to cells that were treated with RA alone. This observation was supported by the cell viability assay Alamar blue showing that FGF4 treatment resulted in more viable cells. Thus, these results indicated that the role of FGF4 in this treatment is to promote cell survival by reducing cytotoxicity effects possibly caused by RA.

To further determine if RA was required for PDX1 expression, inhibition of RA signaling was performed with RA antagonist AGN. The RA antagonist blocked expression of PDX1 demonstrating that RA is required for PDX1 in this treatment. Blocking FGFR signaling in the presence of RA reduced relative PDX1 expression that may indicate that RA signaling acts partially via the FGFR pathway.

To further characterize the identity of the PDX1+ cells obtained with RA/FGF4 treament, markers representative for pancreatic foregut endoderm, non-pancreatic foregut endoderm or posterior gut endoderm were examined. Consistent with *PDX1* induction, *HNF6* and *SOX9* were also upregulated. These markers are expressed in the foregut endoderm. By contrast, *NKX6.1* and *PTF1A* that are specifically expressed in pancreatic foregut endoderm were not upregulated during this treatment. Expression of *CDX2* varied between experiments. Immunostainings showed that the majority of PDX1+ cells also co-expressed HNF6 and SOX9 indicating that these cells represent multipotent foregut endoderm potential with the potential to become pancreatic, duodenal or stomach endoderm.

The RA/FGF4 protocol was repeated with consistent outcome in independent experiments within the same cell line and in an additional cell line. This showed that the RA/FGF4 protocol is reproducible and cell line independent.

Discussion

These results conclude that RA and FGF4 induce PDX1 expression in hESCs in a time-and concentration -dependent manner. The function of RA and FGF4 in patterning gut endoderm is well known in vivo but the role in differentiating of hESCs towards gut endoderm has been poorly investigated. Previous publications have used RA and FGF4 to direct hESCs to endodermal cell types but GFs are often added in a multifactorial manner that makes it hard to define the specific role of each GF. The experimental strategy used here was designed to address the function of RA and FGF4 alone and in combination during differentiation of AA-induced hESCs into PDX1 expressing cells. By testing out the most optimal combination of RA and FGF4, AA-induced hESCs could be differentiated to PDX1 expressing cells and that on average 32% of the cell population expressed PDX1. From these data it could also be concluded that RA is responsible for converting AA-induced hESCs into PDX1 expressing cells, and that FGFR signaling is partially involved in this. Moreover, FGF4 exerts a role in cell viability during RA treatment and does not seem to induce PDX1 expression. This is in contrast the role of FGF4 in patterning gut endoderm in mouse and chick. In these experiments FGF4 did not exhibit any posteriorizing effect on gut endoderm as reported from chick experiments. Furthermore, it cannot be excluded that FGF4 might exhibit additional effects on cell differentiation.

RA has been used in previously published protocols for directed differentiation of AA-induced hESCs towards gut endoderm but the timing of addition was not investigated. Temporal analysis of RA receptor *RARβ* showed that, RA should be added immediately after AA-induction rather than at later time points, under conditions tested here. Adding RA after AA-induction generated higher PDX1 expression compared to when added at day 8 of differentiation.

Characterization of PDX1+ cells generated with the FGF4/RA protocol showed that PDX1+ cells also co-expressed SOX9 and HNF6 and therefore we speculated that they represented a multipotent foregut endoderm population with the potential to become pancreatic endoderm, posterior stomach/duodenal endoderm. Gene

expression of *NKX6.1* and *PTF1A* that specifically distinguishes pancreatic endoderm from other gut endoderm cell types was very low. Thus, we speculated that RA/FGF4 treatment generated cells that either represents posterior stomach/duodenal endoderm or pre-pancreatic endoderm not yet expressing marker specific pancreatic endoderm. Interestingly, gene expression of hepatic markers was up regulated in control cells induced with AA and thereafter cultured in absence of any GFs.

In summary, these results show that RA and FGF4 direct differentiation of AA-induced hESC to PDX1+ foregut endoderm in a robust and efficient manner.

CONCLUDING REMARKS

Diabetes is caused by a dysfunctional insulin production having the effect that normal blood glucose homestasis is disturbed that in turn results in elevated blood glucose levels. The disease is broadly classified into type II diabetes that is caused by insulin resistance in the tissues or a reduced production of insulin by the beta cells. Type I diabetes is an autoimmune reaction resulting in a destruction of beta cells and thereby the insulin production is reduced or completely absent. As an alternative treatment to daily insulin injections, islet transplantation have been suggested as a promising strategy that potentially could help people to get insulin free. In addition, transplantations could also potentially prevent an early onset of the many complications associated with diabetes. The lack of cadaveric donor material has drawn the attention to hESCs due to the fact that they can self-renew and thus can be cultured in large amounts. Additionally, under the right signals hESCs can differentiate to many cell types of both fetal and adult tissues and presumably also to insulin-producing beta cells.

Today, protocols that progressively direct differentiation of hESCs to pancreatic endoderm and further to insulin producing cells are available. Although, insulin producing cells can be generated in vitro they do not seem to control blood glucose in the same manner as normal healthy beta cells. The lack of functionality indicates that the in vitro differentiation procedures do not recapitulate the developmental program of pancreas specification, morphogenesis and maturation of beta cells that takes place during embryonic development. In order to generate therapeutically relevant insulin producing cells, differentiation protocols that translate developmental biology to in vitro differentiation conditions needs to be established. The most important aspect in this work is to identify combinations of growth factors or chemical compounds that progressively guides differentiation along the developmental program of pancreas development. Equally important is to establish molecular tools and markers that ensure analysis of single cells within mixed cell cultures.

In order to understand the basic mechanisms behind growth and differentiation of hESCs, genetic engineering techniques are advantageous tools that make it possible to control expression of genes. Constitutive promoters should ensure high and stable gene expression in most cell types. Thereby, they have a wide range of applications for example in genetic lineage tracing experiments where it is

possible to follow cell fate decisions during differentiation. However, there are a number of constitutive promoters available but a comprehensive study in hESCs has been lacking. In the present investigation, we compared the stability of five commonly used constitutive promoters in undifferentiated hESCs and during differentiation. This study showed that ACTB and EF1α are active during long term culture of undifferentiated hESCs. In contrast, the activity decreased during EB differentiation for all promoters where EF1α were the most stable promoter, although the activity decreased in approximately 50% of the cells. Moreover, this study also indicated that promoter activities might be restricted to specific cell lineages suggesting the need to carefully select promoters for constitutive expression in differentiated hESCs. For future studies, a deeper knowledge of constitutive gene expression during differentiation of hESCs towards the endoderm lineage opens up the possibility to specifically follow the differentiation of one cell. Furthermore, the possibility to label cells with reporter genes that are constitutively expressed in many cell types can be used to unravel cell fate decisions during hESC differentiation. This will be helpful in the efforts of designing differentiation protocols that recapitulate the developmental biology of pancreas development.

Another aspect that would faciliatate development of differentiation protocols that generate therapeutically relevant insulin cells is to establish methods that ensure analysis of individual cells among heterogenous populations. The rationale beind this is that hESC cultures in many cases consists of heterogenous populations and thereby demands analysis at single cell level. In the present investigation we provide results highlighting the importance of analyzing hESCs differentiated towards DE at the cellular level. Single cell gene expression analysis was performed to further investigate the expression of a broad panel of markers at the cellular level of hESC-derived DE obtained from different activin A treatments. Immunolocalization and gene expression analysis at the population level showed that two of the activin A treatments resulted in seemingly similar cell populations. However, single cell gene expression revealed differences in marker expression that was not possible to detect at the population level. In many cases, progenitor cells are identified by co-expression of multiple markers within the same cell and in these cases methods that facilitate analysis at the cellular level are needed to identify these cells. This is the case for DE and therefore single-cell gene expression analysis could serve as a method for characterization of hESC-derived DE.

In summary, the work behind this thesis focused on constitutive promoters that are useful tools to drive high level and sustained gene expression in hESCs. Knowledge about promoters that constitutively drive gene expression is a basic tool for genetic lineage tracing studies where it possible to follow hESCs during differentiation. Moreover the importance of using methods that allow analysis at the single cell level have also been addressed and highlight the need to identify and isolate specific subpopulations among heterogeneous cultures. The combinatorial role of FGF4 and RA was investigated and showed that timing of RA addition regulates PDX1 expression of activin A induced hESCs. For future studies, functional assays that test the potential to develop into more mature cell types would provide information about the potential of hESC-derived DE and posterior foregut endoderm to further mature into functional insulin producing cells.

REFERENCES

Afelik, S., Chen, Y., and Pieler, T. (2006). Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue. Genes & Development 20, 1441-1446.

Ahlgren, U., Pfaff, S.L., Jessell, T.M., Edlund, T., and Edlund, H. (1997). Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature 385, 257-260.

Amit, M., Carpenter, M.K., Inokuma, M.S., Chiu, C.-P., Harris, C.P., Waknitz, M.A., Itskovitz-Eldor, J., and Thomson, J.A. (2000). Clonally Derived Human Embryonic Stem Cell Lines Maintain Pluripotency and Proliferative Potential for Prolonged Periods of Culture. Developmental Biology 227, 271-278.

Ang, S.-L., and Rossant, J. (1994). HNF-3[beta] is essential for node and notochord formation in mouse development. Cell 78, 561-574.

Ang, S.L., Conlon, R.A., Jin, O., and Rossant, J. (1994). Positive and negative signals from mesoderm regulate the expression of mouse Otx2 in ectoderm explants. Development 120, 2979-2989.

Apelqvist, Å., Ahlgren, U., and Edlund, H. (1997). Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. Current Biology 7, 801-804.

Artner, I., Blanchi, B., Raum, J.C., Guo, M., Kaneko, T., Cordes, S., Sieweke, M., and Stein, R. (2007). MafB is required for islet β cell maturation. Proceedings of the National Academy of Sciences 104, 3853-3858.

Artner, I., Le Lay, J., Hang, Y., Elghazi, L., Schisler, J.C., Henderson, E., Sosa-Pineda, B., and Stein, R. (2006). MafB: An Activator of the Glucagon Gene Expressed in Developing Islet α- and β-Cells. Diabetes 55, 297-304.

Barrow, J.R., Howell, W.D., Rule, M., Hayashi, S., Thomas, K.R., Capecchi, M.R., and McMahon, A.P. (2007). Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis. Developmental Biology 312, 312-320.

Beddington, R.S.P., Rashbass, P., and Wilson, V. (1992). Brachyury - a gene affecting mouse gastrulation and early organogenesis. Development 116, 157-165.

Belo, J.A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M., and De Robertis, E.M. (1997). Cerberus-like is a secreted factor with neuralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. Mechanisms of Development 68, 45-57.

Berney, T., Ferrari-Lacraz, S., Bühler, L., Oberholzer, J., Marangon, N., Philippe, J., Villard, J., and Morel, P. (2009). Long-Term Insulin-Independence After Allogeneic Islet Transplantation for Type 1 Diabetes: Over the 10-Year Mark. American Journal of Transplantation 9, 419-423.

Borowiak, M., Maehr, R., Chen, S., Chen, A.E., Tang, W., Fox, J.L., Schreiber, S.L., and Melton, D.A. (2009). Small Molecules Efficiently Direct Endodermal Differentiation of Mouse and Human Embryonic Stem Cells. Cell Stem Cell 4, 348-358.

Bort, R., Martinez-Barbera, J.P., Beddington, R.S.P., and Zaret, K.S. (2004). Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. Development 131, 797-806.

Brolen, G.K.C., Heins, N., Edsbagge, J., and Semb, H. (2005). Signals From the Embryonic Mouse Pancreas Induce Differentiation of Human Embryonic Stem Cells Into Insulin-Producing {beta}-Cell-Like Cells. Diabetes 54, 2867-2874.

Cai, J., Yu, C., Liu, Y., Chen, S., Guo, Y., Yong, J., Lu, W., Ding, M., and Deng, H. (2009). Generation of Homogeneous PDX1+ Pancreatic Progenitors from Human ES Cell-derived Endoderm Cells. Journal of Molecular Cell Biology 2, 50-60.

Chen, S., Borowiak, M., Fox, J.L., Maehr, R., Osafune, K., Davidow, L., Lam, K., Peng, L.F., Schreiber, S.L., Rubin, L.L., et al. (2009). A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat Chem Biol 5, 258-265.

Collombat, P., Mansouri, A., Hecksher-Sörensen, J., Serup, P., Krull, J., Gradwohl, G., and Gruss, P. (2003). Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes & Development 17, 2591-2603.

Costa, M., Dottori, M., Ng, E., Hawes, S.M., Sourris, K., Jamshidi, P., Pera, M.F., Elefanty, A.G., and Stanley, E.G. (2005). The hESC line Envy expresses high levels of GFP in all differentiated progeny. Nat Meth 2, 259-260.

Cowan, C.A., Klimanskaya, I., McMahon, J., Atienza, J., Witmyer, J., Zucker, J.P., Wang, S., Morton, C.C., McMahon, A.P., Powers, D., et al. (2004). Derivation of Embryonic Stem-Cell Lines from Human Blastocysts. N Engl J Med 350, 1353-1356.

D'Amour, K.A., Agulnick, A.D., Eliazer, S., Kelly, O.G., Kroon, E., and Baetge, E.E. (2005). Efficient differentiation of human embryonic stem cells to definitive endoderm. Nat Biotech 23, 1534-1541.

D'Amour, K.A., Bang, A.G., Eliazer, S., Kelly, O.G., Agulnick, A.D., Smart, N.G., Moorman, M.A., Kroon, E., Carpenter, M.K., and Baetge, E.E. (2006). Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotech 24, 1392-1401.

Danaei, G., Finucane, M.M., Lu, Y., Singh, G.M., Cowan, M.J., Paciorek, C.J., Lin, J.K., Farzadfar, F., Khang, Y.-H., Stevens, G.A., et al. (2011). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2•7 million participants. The Lancet In Press, Corrected Proof.

Dessimoz, J., Opoka, R., Kordich, J.J., Grapin-Botton, A., and Wells, J.M. (2006). FGF signaling is necessary for establishing gut tube domains alongthe anterior-posterior axis in vivo. Mechanisms of Development 123, 42-55.

Deutsch, G., Jung, J., Zheng, M., Lora, J., and Zaret, K.S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development 128, 871-881.

Dor, Y., Brown, J., Martinez, O.I., and Melton, D.A. (2004). Adult pancreatic [beta]-cells are formed by self-duplication rather than stem-cell differentiation. Nature 429, 41-46.

Duester, G. (2008). Retinoic Acid Synthesis and Signaling during Early Organogenesis. Cell 134, 921-931.

Dufort, D., Schwartz, L., Harpal, K., and Rossant, J. (1998). The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. Development 125, 3015-3025.

Ellerström, C., Strehl, R., Moya, K., Andersson, K., Bergh, C., Lundin, K., Hyllner, J., and Semb, H. (2006). Derivation of a Xeno-Free Human Embryonic Stem Cell Line. Stem Cells 24, 2170-2176.

Ghosh, Z., Wilson, K.D., Wu, Y., Hu, S., Quertermous, T., and Wu, J.C. (2010). Persistent Donor Cell Gene Expression among Human Induced Pluripotent Stem Cells Contributes to Differences with Human Embryonic Stem Cells. PLoS ONE 5, e8975.

Gradwohl, G.r., Dierich, A.e., LeMeur, M., and Guillemot, F.o. (2000). neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proceedings of the National Academy of Sciences 97, 1607-1611.

Grapin-Botton, A., and Melton, D.A. (2000). Endoderm development: from patterning to organogenesis. Trends in Genetics 16, 124-130.

Gropp, M., Itsykson, P., Singer, O., Ben-Hur, T., Reinhartz, E., Galun, E., and Reubinoff, B.E. (2003). Stable Genetic Modification of Human Embryonic Stem Cells by Lentiviral Vectors. Mol Ther 7, 281-287.

Gu, G., Dubauskaite, J., and Melton, D.A. (2002). Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129, 2447-2457.

Guo, T., and Hebrok, M. (2009). Stem Cells to Pancreatic Beta Cells: New Sources for Diabetes Cell Therapy. Endocrine Reviews 30, 214-227.

Hart, A.H., Hartley, L., Sourris, K., Stadler, E.S., Li, R., Stanley, E.G., Tam, P.P.L., Elefanty, A.G., and Robb, L. (2002). Mixl1 is required for axial mesendoderm morphogenesis and patterning in the murine embryo. Development 129, 3597-3608.

Hebrok, M., Kim, S.K., St Jacques, B., McMahon, A.P., and Melton, D.A. (2000). Regulation of pancreas development by hedgehog signaling. Development 127, 4905-4913.

Heins, N., Englund, M.C.O., Sjöblom, C., Dahl, U., Tonning, A., Bergh, C., Lindahl, A., Hanson, C., and Semb, H. (2004). Derivation, Characterization, and Differentiation of Human Embryonic Stem Cells. Stem Cells 22, 367-376.

Henseleit, K.D., Nelson, S.B., Kuhlbrodt, K., Hennings, J.C., Ericson, J., and Sander, M. (2005). NKX6 transcription factor activity is required for alpha- and beta cell development in the pancreas. Development 132, 3139-3149.

Hogan, B., Fellous, M., Jacob, F., and Avner, P. (1977). Isolation of a human teratoma cell line which expresses F9 antigen. Nature 270, 515-518.

Jacquemin, P., Lemaigre, F.P., and Rousseau, G.G. (2003). The Onecut transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of Pdx-1 in the specification cascade. Developmental Biology 258, 105-116.

Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 371, 606-609.

Kaji, K., Norrby, K., Paca, A., Mileikovsky, M., Mohseni, P., and Woltjen, K. (2009). Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature 458, 771-775.

Kanai-Azuma, M., Kanai, Y., Gad, J.M., Tajima, Y., Taya, C., Kurohmaru, M., Sanai, Y., Yonekawa, H., Yazaki, K., Tam, P.P.L., et al. (2002). Depletion of definitive gut endoderm in Sox17-null mutant mice. Development 129, 2367-2379.

Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R.J., and Wright, C.V.E. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 32, 128-134.

Keller, G. (2005). Embryonic stem cell differentiation: emergence of a new era in biology and medicine. Genes and development 19, 1129-1155.

Kleinsmith, L.J., and Pierce, G.B. (1964). Multipotentiality of Single Embryonal Carcinoma Cells. Cancer Research 24, 1544-1551.

Kroon, E., Martinson, L.A., Kadoya, K., Bang, A.G., Kelly, O.G., Eliazer, S., Young, H., Richardson, M., Smart, N.G., Cunningham, J., et al. (2008). Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotech 26, 443-452.

Lammert, E., Cleaver, O., and Melton, D. (2001). Induction of Pancreatic Differentiation by Signals from Blood Vessels. Science 294, 564-567.

Lawson, K.A., and Pedersen, R.A. (1987). Cell fate, morphogenetic movement and population kinetics of embryonic endoderm at the time of germ layer formation in the mouse. Development 101, 627-652.

Lewis, S.L., and Tam, P.P.L. (2006). Definitive endoderm of the mouse embryo: Formation, cell fates, and morphogenetic function. Developmental Dynamics 235, 2315-2329.

Li, H., Arber, S., Jessell, T.M., and Edlund, H. (1999). Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. Nat Genet 23, 67-70.

Ma, Y., Ramezani, A., Lewis, R., Hawley, R.G., and Thomson, J.A. (2003). High-Level Sustained Transgene Expression in Human Embryonic Stem Cells Using Lentiviral Vectors. Stem Cells 21, 111-117.

Maehr, R., Chen, S., Snitow, M., Ludwig, T., Yagasaki, L., Goland, R., Leibel, R.L., and Melton, D.A. (2009). Generation of

pluripotent stem cells from patients with type 1 diabetes. Proceedings of the National Academy of Sciences 106, 15768-15773.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proceedings of the National Academy of Sciences 78, 7634-7638.

Martinez Barbera, J.P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D., and Beddington, R.S. (2000). The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. Development 127, 2433-2445.

McLin, V.r.A., Rankin, S.A., and Zorn, A.M. (2007). Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. Development 134, 2207-2217.

Melkoumian, Z., Weber, J.L., Weber, D.M., Fadeev, A.G., Zhou, Y., Dolley-Sonneville, P., Yang, J., Qiu, L., Priest, C.A., Shogbon, C., et al. Synthetic peptide-acrylate surfaces for long-term self-renewal and cardiomyocyte differentiation of human embryonic stem cells. Nat Biotech 28, 606-610.

Molotkov, A., Molotkova, N., and Duester, G. (2005). Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Developmental Dynamics 232, 950-957.

Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., and Trono, D. (1996). In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector. Science 272, 263-267.

Pedersen, J.K., Nelson, S.B., Jorgensen, M.C., Henseleit, K.D., Fujitani, Y., Wright, C.V.E., Sander, M., and Serup, P. (2005). Endodermal expression of Nkx6 genes depends differentially on Pdx1. Developmental Biology 288, 487-501.

Perea-Gomez, A., Lawson, K.A., Rhinn, M., Zakin, L., Brulet, P., Mazan, S., and Ang, S.L. (2001). Otx2 is required for visceral

endoderm movement and for the restriction of posterior signals in the epiblast of the mouse embryo. Development 128, 753-765.

Perea-Gomez, A., Shawlot, W., Sasaki, H., Behringer, R.R., and Ang, S. (1999). HNF3beta and Lim1 interact in the visceral endoderm to regulate primitive streak formation and anterior-posterior polarity in the mouse embryo. Development 126, 4499-4511.

Rivera-Pérez, J.A., and Magnuson, T. (2005). Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. Developmental Biology 288, 363-371.

Rodin, S., Domogatskaya, A., Strom, S., Hansson, E.M., Chien, K.R., Inzunza, J., Hovatta, O., and Tryggvason, K. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotech 28, 611-615.

Saga, Y., Hata, N., Kobayashi, S., Magnuson, T., Seldin, M.F., and Taketo, M.M. (1996). MesP1: a novel basic helix-loop-helix protein expressed in the nascent mesodermal cells during mouse gastrulation. Development 122, 2769-2778.

Saga, Y., Miyagawa-Tomita, S., Takagi, A., Kitajima, S., Miyazaki, J.i., and Inoue, T. (1999). MesP1 is expressed in the heart precursor cells and required for the formation of a single heart tube. Development 126, 3437-3447.

Sander, M., Sussel, L., Conners, J., Scheel, D., Kalamaras, J., Dela Cruz, F., Schwitzgebel, V., Hayes-Jordan, A., and German, M. (2000). Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development 127, 5533-5540.

Schwitzgebel, V.M., Scheel, D.W., Conners, J.R., Kalamaras, J., Lee, J.E., Anderson, D.J., Sussel, L., Johnson, J.D., and German, M.S. (2000). Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development 127, 3533-3542.

Séguin, C.A., Draper, J.S., Nagy, A., and Rossant, J. (2008). Establishment of Endoderm Progenitors by SOX Transcription Factor

Expression in Human Embryonic Stem Cells. Cell Stem Cell 3, 182-195.

Seymour, P.A., Freude, K.K., Tran, M.N., Mayes, E.E., Jensen, J., Kist, R., Scherer, G., and Sander, M. (2007). SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proceedings of the National Academy of Sciences 104, 1865-1870.

Shapiro, J., Lakey, J., Ryan, E., Korbutt, G., Toth, E., Warnock, G., Kneteman, N., and Rajotte, R. (2000). Islet Transplantation in Seven Patients with Type 1 Diabetes Mellitus Using a Glucocorticoid-Free Immunosuppressive Regimen. New England Journal of Medicine 343, 230-238.

Shawlot, W., Deng, J.M., and Behringer, R.R. (1998). Expression of the mouse cerberus-related gene, Cerr1, suggests a role in anterior neural induction and somitogenesis. Proceedings of the National Academy of Sciences of the United States of America 95, 6198-6203.

Shawlot, W., Wakamiya, M., Kwan, K.M., Kania, A., Jessell, T.M., and Behringer, R.R. (1999). Lim1 is required in both primitive streak-derived tissues and visceral endoderm for head formation in the mouse. Development 126, 4925-4932.

Shih, C.-C., Forman, S.J., Chu, P., and Slovak, M. (2007). Human Embryonic Stem Cells Are Prone to Generate Primitive, Undifferentiated Tumors in Engrafted Human Fetal Tissues in Severe Combined Immunodeficient Mice. Stem Cells and Development 16, 893-902.

Slack, J.M. (1995). Developmental biology of the pancreas. Development 121, 1569-1580.

Smith, A.G. (2001). Embryo-derived stem cells Of Mice and Men. Annual Review of Cell and Developmental Biology 17, 435-462.

Staffers, D.A., Ferrer, J., Clarke, W.L., and Habener, J.F. (1997). Early-onset type II diabetes (MODY4) linked to IPF1. Nature Genetics, 138-139.

Sussel, L., Kalamaras, J., Hartigan-O'Connor, D.J., Meneses, J.J., Pedersen, R.A., Rubenstein, J.L., and German, M.S. (1998). Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development 125, 2213-2221.

Suter, D.M., Cartier, L., Bettiol, E., Tirefort, D., Jaconi, M.E., Dubois-Dauphin, M., and Krause, K.-H. (2006). Rapid Generation of Stable Transgenic Embryonic Stem Cell Lines Using Modular Lentivectors. Stem Cells 24, 615-623.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell 131, 861-872.

Takahashi, K., and Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell 126, 663-676.

Tam, P.P.L., Khoo, P.-L., Lewis, S.L., Bildsoe, H., Wong, N., Tsang, T.E., Gad, J.M., and Robb, L. (2007). Sequential allocation and global pattern of movement of the definitive endoderm in the mouse embryo during gastrulation. Development 134, 251-260.

Thomas, P.Q., Brown, A., and Beddington, R.S. (1998). Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. Development 125, 85-94.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic Stem Cell Lines Derived from Human Blastocysts. Science 282, 1145-1147.

Thomson, J.A., Kalishman, J., Golos, T.G., Durning, M., Harris, C.P., Becker, R.A., and Hearn, J.P. (1995). Isolation of a primate embryonic stem cell line. Proceedings of the National Academy of Sciences 92, 7844-7848.

Tremblay, K.D., and Zaret, K.S. (2005). Distinct populations of endoderm cells converge to generate the embryonic liver bud and ventral foregut tissues. Developmental Biology 280, 87-99.

Wierup, N., Svensson, H., Mulder, H., and Sundler, F. (2002). The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. Regulatory Peptides 107, 63-69.

Villa-Diaz, L.G., Nandivada, H., Ding, J., Nogueira-de-Souza, N.C., Krebsbach, P.H., O'Shea, K.S., Lahann, J., and Smith, G.D. (2010). Synthetic polymer coatings for long-term growth of human embryonic stem cells. Nat Biotech 28, 581-583.

Xu, X., D'Hoker, J., Stangé, G., Bonné, S., De Leu, N., Xiao, X., Van De Casteele, M., Mellitzer, G., Ling, Z., Pipeleers, D., et al. (2008). Beta Cells Can Be Generated from Endogenous Progenitors in Injured Adult Mouse Pancreas. Cell 132, 197-207.

Yao, S., Sukonnik, T., Kean, T., Bharadwaj, R.R., Pasceri, P., and Ellis, J. (2004). Retrovirus Silencing, Variegation, Extinction, and Memory Are Controlled by a Dynamic Interplay of Multiple Epigenetic Modifications. Mol Ther 10, 27-36.

Ye, F., Duvillié, B., and Scharfmann, R. (2005). Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. Diabetologia 48, 277-281.

Zaret, K.S., and Grompe, M. (2008). Generation and Regeneration of Cells of the Liver and Pancreas. Science 322, 1490-1494.

Zhang, C., Moriguchi, T., Kajihara, M., Esaki, R., Harada, A., Shimohata, H., Oishi, H., Hamada, M., Morito, N., Hasegawa, K., et al. (2005). MafA Is a Key Regulator of Glucose-Stimulated Insulin Secretion. Mol Cell Biol 25, 4969-4976.

Zhang, D., Jiang, W., Liu, M., Sui, X., Yin, X., Chen, S., Shi, Y., and Deng, H. (2009). Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res 19, 429-438.

Zufferey, R., Dull, T., Mandel, R.J., Bukovsky, A., Quiroz, D., Naldini, L., and Trono, D. (1998). Self-Inactivating Lentivirus Vector for Safe and Efficient In Vivo Gene Delivery. J Virol 72, 9873-9880.

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