FGF signaling in specification of hESC-derived definitive endoderm

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2010

With due permission of the Faculty of Medicine at Lund University, this thesis will be publicly defended on January 29 at 13.00 in Segerfalksalen, BMC, Sölvegatan 19, Lund

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Abstract

Diabetes affects around 200 million people worldwide. Islet transplantation according to the Edmonton protocol have been the most promising therapeutic option for Diabetes type I patients, however, lack of cadaveric donor islets is a major obstacle and new strategies need to be established. Human embryonic stem cells (hESCs) not only offer an excellent source for establishment of strategies for future regenerative cell therapies and drug discovery but also offers en excellent experimental assay for understanding human pancreas development.

Governed by the developmental biological principles that normally control foregut endoderm and pancreas specification, numerous multifactor protocols for directing mature foregut-derived cells from hESCs have been reported. However, to establish less complex and more robust protocols there is a need to further understand the mechanism of action of individual growth and differentiation factors in specification of human pluripotent stem cells towards foregut derived cell lineages.

Here, we describe two studies where we have investigated the role of fibroblast growth factor (FGF) signaling, specifically FGF2 and FGF4, and Retinoic acid (RA) in specification of the hESC-derived DE development. In the first study we show that FGF2 in a dosage-dependent manner specifies hESC-derived DE into different foregut lineages such as liver, lung, pancreatic and intestinal cells. Furthermore, by dissecting the FGF receptor intracellular pathway that regulates pancreas specification, we demonstrate for the first time to our knowledge that induction of PDX1+ pancreatic progenitors in part relies on FGF2-mediated activation of the MAPK signaling pathway. In the second study, we show that FGF4 alone is not sufficient for induction of foregut endoderm but that in combination with RA it efficiently induces PDX1+ cells from hESC-derived DE. Altogether, these observations suggest a broader gut endodermal patterning activity of FGF2 that corresponds to what has previously been advocated for FGF4, implying a functional switch from FGF4 to FGF2 during evolution. Thus, our results provide new knowledge of how cell fate specification of human DE is controlled – facts that will be of great value for future regenerative cell therapies. Finally, we present a method for efficient gene targeting in hESCs, which allows the monitoring of gene expression in living cells.

Key words:	human embryonic stem cells, diabetes, pancreas, definitive endoderm, pancreatic endoderm, FGF signaling, FGF2, FGF4, RA, PDX1, NKX6-1	

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Front cover: Scientific research often requires a group of dedicated and hard working individuals who work together to achieve specific goals. This is illustrated in the upper part of the image, which was initially a part of a photograph taken of the mural at the train station of Riomaggiore, Italy. The mural is originally made by Silvio Benedetto and depicts the hard working unknown workers who constructed the stone walls (muri a secco) throughout the Cinque Terre region. The stony ground in the lower part of the picture is in fact a co-staining of SOX17 (green) with CXCR4 (red). These double positive cells are a hallmark of hESC-derived definitive endoderm.

In Search of the Dream

Those who dare having a project in life, foregoing everything to live their Personal Legend, will end up achieving anything. The important thing is to keep the fire in your heart and be strong to overcome hard moments.

-Paulo Coelho

Till mamma och pappa

ABSTRACT

Diabetes affects around 200 million people worldwide. Curing diabetes would require the prevention of autoimmune destruction of beta cells and restoration of the beta cell mass restored either through regeneration or transplantation of the insulin producing cells. Islet transplantation according to the Edmonton protocol have been the most promising therapeutic option for Diabetes type I patients, however, lack of cadaveric donor islets is a major obstacle and new strategies need to be established. Human embryonic stem cells (hESCs) not only offer an excellent source for establishment of strategies for future regenerative cell therapies and drug discovery but also offers en excellent experimental assay for understanding human pancreas development.

Pancreas originates from the definitive endoderm (DE), one of the three germ layers. The path from definitive endoderm to pancreatic progenitor cells and finally the insulin producing beta cells involves sequential cell-fate decisions characterized by the expression of multiple transcription factors. Governed by the developmental biological principles that normally control foregut endoderm and pancreas specification, numerous multifactor protocols for directing mature foregut-derived cells from hESCs have been reported. However, to establish less complex and more robust protocols there is a need to further understand the mechanism of action of individual growth and differentiation factors in specification of human pluripotent stem cells towards foregut derived cell lineages.

Here, we describe two studies where we have investigated the role of fibroblast growth factor (FGF) signaling, specifically FGF2 and FGF4, and Retinoic acid (RA) in specification of the hESC-derived DE development. Studies in lower vertebrates have demonstrated that FGF2 acts in a restricted manner primarily patterning the ventral foregut endoderm into liver and lung, whereas FGF4 exhibits broad anterior-posterior and left-right patterning activities. Furthermore, whereas FGF2 is not required for ventral pancreas development an inductive role of FGF2 has been shown during dorsal pancreas formation. Whether FGF2 and FGF4 play a similar role during human endoderm development remained however unknown. In contrast, RA has frequently been employed (together with other growth factors) for directed differentiation of hESCs to pancreatic endoderm.

In the first study we show that FGF2 in a dosage-dependent manner specifies hESC-derived DE into different foregut lineages such as liver, lung, pancreatic and intestinal cells. Furthermore, by dissecting the FGF receptor intracellular pathway that regulates pancreas specification, we demonstrate for the first time to our knowledge that induction of PDX1+ pancreatic progenitors in part relies on

FGF2-mediated activation of the MAPK signaling pathway. In the second study, we show that FGF4 alone is not sufficient for induction of foregut endoderm but that in combination with RA it efficiently induces PDX1+ cells from hESC-derived DE. Specifically, FGF4 promoted cell survival in the differentiating hESCs. Hence, in contrast to studies in lower vertebrates we demonstrate that FGF4 neither patterns hESC-derived DE, nor induces PDX1+ pancreatic progenitors suggesting that FGF4 is not responsible for anterior-posterior patterning of the primitive gut during human development.. Altogether, these observations suggest a broader gut endodermal patterning activity of FGF2 that corresponds to what has previously been advocated for FGF4, implying a functional switch from FGF4 to FGF2 during evolution. Thus, our results provide new knowledge of how cell fate specification of human DE is controlled – facts that will be of great value for future regenerative cell therapies. Finally, we present a method for efficient gene targeting in hESCs, which allows the monitoring of gene expression in living cells.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Diabetes är en snabbt växande folksjukdom som redan har drabbat 200 miljoner världen runt. Det finns två typer av sjukdomen: typ I samt typ II diabetes. Den förstnämnda drabbar vanligtvis de unga och beror på en s.k. autoimmun reaktion där kroppens egna immunförsvar av okänd anledning plötsligt inte känner igen insulincellerna och börjar attackera dem. Typ II diabetes uppstår däremot pga. en kombination av insulinresistens, samt otillräcklig sekretion av insulin, och bryter ut i vuxen ålder. Hälsosam kost och motion är av stor vikt för att förhindra typ II diabetes. Det finns i dagsläget inget botemedel för diabetes och båda typerna av sjukdomen behandlas med insulin som antingen administreras i tablettform eller som injektioner. För att bota diabetes måste forskarna dels komma på vad som utlöser den autoimmuna förstörelsen av insulincellerna, dels kunna komma på ett sätt att bilda nya insulinceller som kan transplanteras till de drabbade. Den mest framgångsrika behandlingen av typ I diabetes involverar transplantation av öar med insulinceller. Det finns studier som visar att patienter som genomgått denna transplantation har kunnat leva utan insulinbehandling i flera år. Men tyvärr har denna behandling begränsningar, såsom att det krävs flera donatorer till en transplantation, då ett stort antal av insulinöar krävs för lyckad behandling, och att det krävs livslång behandling med immunosuppressiva medel för att de transplanterade cellerna inte ska stötas bort. Det behövs därmed dels alternativa källor till insulinceller, dels alternativa behandlingsstrategier.

Humana embryonala stamceller har förmågan att bilda vilka celltyper som helst i människans kropp. Av denna anledning är de väldigt intressanta vid behandling av degenerativa sjukdomar såsom diabetes, Alzheimers, Parkinson och MS, där de skulle kunna ersätta den skadade vävnaden. För att kunna styra stamcellerna mot specifika öden eller celltyper krävs det dock kunskap om exakt vilka signalvägar som är av betydelse vid bildandet av de specifika cellerna och hur dessa aktiveras. Genom olika djurmodeller har forskarna lyckats få en inblick i vilka gener och stimuli som är av betydelse för pankreasutvecklingen, men det finns fortfarande många luckor innan vi vet exakt hur en insulincell bildas i människan. Genom att arbeta med humana embryonala stamceller har forskarna även en möjlighet att lära sig mer om människan och hennes utveckling.

Många olika signaleringsvägar är involverade i att styra de omogna stamcellerna till insulinproducerande celler. Idag kan vi genom tillsats av olika tillväxtfaktorer, som har visat sig ha en betydande roll under pankreasutvecklingen i musmodeller, styra utvecklingen av de omogna stamcellerna till definitivt endoderm, det grodlager som ger upphov till pankreas, lever, lunga och tarmarna. Vår fortsatta strategi involverar att hitta nya signaleringsvägar som är av betydelse för att ombilda dessa celler mot

ett förstadium till pankreas.

I denna avhandling presenteras två olika protokoll för att styra stamcellerna mot ett förstadium till pankreas och insulinceller. I det ena protokollet lyckas vi även få ett förstadium till lever, tarm och lungceller. Dessa nya kunskaper kommer att kunna användas i vårt fortsatta arbete för att styra stamcellerna mot insulinproducerande celler, som i framtiden kan användas för transplantation i diabetespatienter. Slutligen beskrivs även en metod där vi genom genetisk modifiering av stamcellerna lyckas introducera en ny gen som gör att stamcellerna fluorescerar grönt vid uttryck av specifika gener. Dessa nya stamcellslinjer är oerhört viktiga i vårt fortsatta arbete där vi försöker lära oss mer om hur de olika pankreasspecifika generna regleras.

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PAPERS IN SUMMARY

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LIST OF PUBLICATIONS

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

I. **FGF2 Specifies hESC-Derived Definitive Endoderm into Foregut/Midgut Cell Lineages in a Concentration-Dependent Manner.**
Ameri J, Ståhlberg A, Pedersen J, Johansson JK, Johannesson MM, Artner I, Semb H.
Stem Cells 2009 Nov 3. [Epub ahead of print, doi: 10.1002/stem.249]

II. **FGF4 and retinoic acid direct differentiation of hESCs into PDX1-expressing foregut endoderm in a time- and concentration-dependent manner.**
Johannesson M, Ståhlberg A, Ameri J, Sand FW, Norrman K, Semb H.
PLoS ONE. 2009;4(3):e4794.

III. **Gene targeting in human embryonic stem cells using bacterial artificial chromosome-derived targeting vectors.**
Fischer Y, Wu S, Ameri J, Xian X, Johannesson M, Wahlestedt M, Parmar M, Semb H.
Manuscript

ABBREVIATIONS

AA	Activin A
ADE	Anterior definitive endoderm
A-P	Anterior-Posterior
AVE	Anterior visceral endoderm
BAC	Bacterial Artificial Chromosomes
bFGF	Basic FGF, also known as FGF2
bHLH	Basic helix-lop-helix
BMP	Bone morphogenetic protein
CDM	Chemically defined medium
Ct	Cycle of threshold
DAPI	6'diamidino-2-phenylindole
DE	Definitive Endoderm
Dpc	Days post coitum
E	Embryonic day
EB:s	Embryoid bodies
E-cad	E-cadherin
ECM	Extra Cellular Matrix
EGFP	Enhanced green fluorescent protein
EMT	Epithelial-Mesenchymal transition
ESc	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GFP	Green Fluorescent Protein
hESCs	Human embryonic stem cells
HLXB9	Homeo box HB9
HNF6	Hepatocyte Nuclear factor 6
ICM	Inner cell mass
iPS	Induced pluripotent stem cells
IRES	Internal ribosome entry site
KO-DMEM	Knock-out DMEM
KO-SR	Knock-out serum replacement

LIF	Leukimia inhibitory factor
mAb	Monoclonal antibody
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
mESCs	Mouse embryonic stem cells
MEF	Mouse embryonic Fibroblast
NGN3	Neurogenin 3
Neo	Neomycin
PAX6	Paired box gene 6
PBST	Triton X-100 in PBS
PDX1	Pancreatic duodenal homeobox gene-1
PE	Parietal endoderm
PFA	Paraformaldehyde
PH3	Phospho-histone 3
PI3K	Phosphoinositide 3
PrE	Primitive Endoderm
PS	Primitive streak
PTF1a	Pancreas specific transcription factor 1 a
QPCR	Quantitative polymerase chain reaction
RA	Retinoic acid
Raldh2	Retinaldehyde dehydrogenase 2
RAR	Retinoic acid receptor
SCID	Severe combined immuno deficient
SHH	Sonic hedgehog
SOX9	Sex determining region Y (SRY)-box9
SOX17	Sex determining region Y (SRY)-box17
STM	Septum transversum mesenchyme
TG	Thyroglobulin
TGF-β	Transforming growth factor-β superfamily
UTR	Untranslated region.
VE	Visceral endoderm
VEGF	Vascular endothelial growth factor
WNT	Wingless-type MMTV integration site

INTRODUCTION

DIABETES

Diabetes affects around 200 million people worldwide and about 10% of the cases correspond to type 1 diabetes. In diabetes type 1, the pancreatic beta cells producing insulin are destroyed by the immune system, whereas diabetes type 2 is caused by a combination of insulin resistance and inadequate insulin secretion. Both cases require exogenous insulin administration to control the blood glucose level and to avoid secondary complications such as blindness, kidney failure and cardiovascular diseases. Curing diabetes would require the autoimmune destruction of beta cells be prevented and the beta cell mass restored either through regeneration or transplantation of the insulin producing cells [1].

Human islet transplantation according to the Edmonton protocol [2] has been the most promising therapeutic option for Diabetes type I patients as long-term insulin independence have been achieved in some patients. However, high number of islets and life-long immunosuppressive treatment are required to achieve insulin independence and to prevent islet rejection. Due to lack of cadaveric donor islets and the fact that only 10% of the patients maintain insulin independence after a 5 year-period [3]new strategies need to be established and other sources of insulin-producing cells are being sought [1].

Some alternative sources of insulin-producing cells for transplantation therapy include human β-cell lines, transdifferentiation of adult somatic cells or adult and embryonic stem cells. Although established human β-cell lines have generated knowledge regarding the mechanisms involved in β-cell development they tend to be unstable as they gradually loose their differentiated characteristics in vitro [1].

Many adult tissues have adult stem cells or multipotent progenitor cells that can be used to regenerate and repair the specific tissue [1] but whether a pancreatic stem cell exists or not is a debated issue. In addition, while studies have shown that the pancreas has the capacity to regenerate the source of these adult stem/progenitor cells remain unknown. In vitro studies in rodents have shown that β-cell mass/ pancreatic islets can expand both through self-replication of mature beta cells [4]

or through differentiation of progenitor cells either located in the pancreas or in nearby tissues (neogenesis) [1, 5, 6]. However, since the experiments suggesting self-replication of mature beta cells were not designed to rule out contribution by pancreatic progenitors and neogenesis from ductal progenitors, there is strong evidence that endocrine progenitor cells are present in the adult pancreas [1]. Furthermore, it has been proposed that beta cell mass expansion in diabetes type 2 patients occurs primarily through neogenesis [7, 8]. Thus, if autoimmune destruction of beta cells could be prevented, beta cell mass could in theory be restored via beta cell regeneration. However, more knowledge regarding the signals triggering self-replication or neogenesis is required in both humans and rodents before β-cell mass regeneration can be used as an approach for cell-based therapy.

Human embryonic stem cells (hESCs) not only offer a great experimental model for understanding human pancreas development but also represent an excellent source for establishment of strategies for future regenerative cell therapies. In the recent years, there has been great success in generating insulin-producing cells from ES cells through directed differentiation [9-14] but improvements are needed. Furthermore, the emergence of induced pluripotent stem (iPS) cells in 2006 [15] has also attracted a lot of interest as a potential source of insulin-producing cells. As iPS cells are established by adult somatic cells the researchers can obtain pluripotent stem cells without using embryos. In addition, the cells can be matched to the diabetic patient. However, before iPS cells can be used in humans, efficient alternative ways of reprogramming the adult somatic cells are required, more specifically, the current viral delivery system that may potentially trigger oncogenes have to be replaced. In 2009, a new approach involving proteins was presented for deriving iPS cells that could in the future be suitable for therapeutic use [16].

EARLY DEVELOPMENT

Blastocyst formation
The fertilized egg (zygote) undergoes a series of cell division known as cleavage to eventually develop into a morula, which is an early stage embryo. At the 16-cell stage the morula starts to differentiate and form the blastocyst. In humans, the blastocyst is formed five days after fertilization. The blastocyst is composed of a

cluster of cells known as the inner cell mass (ICM) that is surrounded by an outer cell layer called trophectoderm. The fluid-filled cavity separating the ICM from the trophectoderm is known as the blastocoel. The trophectoderm generates the placenta and portions of the parietal yolk sac whereas the ICM gives rise to the embryo proper, the amnion (fluid filled cavity that protects the embryo during pregnancy), yolk sac (responsible for nutrient uptake and circulation) and allantois (responsible for waste removal). The ICM is also the source of embryonic stem cells [17-19].
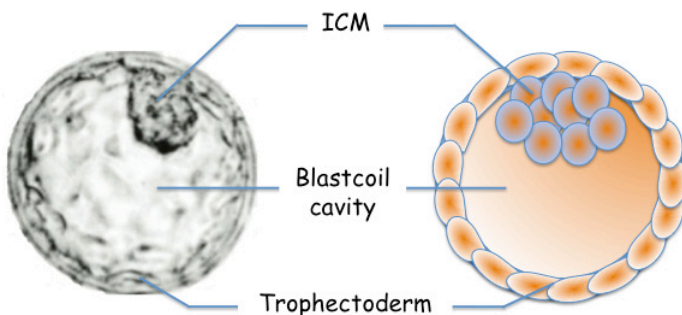
Figure 1. The blastocyst. A five days old human blastocyst is shown to the left (from NIH by Mr J.Conaghan) and a simple schematic to the right. The ICM develops into the embryo whereas the throphectoderm contributes to the placenta. As the blastocyst continue to develop, the embryo expands and the outer shell gets thinner allowing the embryo to finally hatch out of its shell.

In detail, the ICM gives rise to the epiblast and hypoblast. The epiblast generates the amnion, extraembryonic mesoderm and embryo proper. The hypoblast in turn contributes to the formation of the extraembryonic and primitive endoderm (PrE), which eventually gives rise to the yolk sac with its visceral and parietal components. Specifically, a fraction of the PrE cells differentiates into visceral endoderm (VE), while others undergo an epithelial to mesenchymal transition (EMT) to become parietal endoderm (PE) [20, 21]. Visceral endoderm surrounds the epiblast whereas the parietal endoderm surrounds the extraembryonic tissues [18, 22]. Furthermore, both PrE and VE give rise to PE [23] and the expression of GATA4 or GATA6 has been shown to be important for this process [20, 21, 24]. Until placentation, both PE and VE lineages are responsible for nutrient and waste exchange between maternal tissue and the fetus, whereas the yolk sac is responsible for this in lower organisms [20].

Both PrE and the ICM fail to form in the absence of the POU transcription factor Pou5f1 (also known as Oct4), instead only trophectoderm is formed [20, 25]. Fibroblast growth factor 4 (FGF4) also plays an important role during embryogenesis [21, 26]. It is both expressed in the preimplantation mouse blastocysts and in the ICM [27, 28]. Furthermore, PrE is not formed in mice lacking either FGF4 or FGF receptor 2 (FGFR2) [20, 26, 29, 30].

A cascade of transcription factors is suggested to be involved in the differentiation of VE [31]. HNF4, which is initially expressed in PrE becomes restricted to the visceral yolk sac endoderm at E5.25 [32] and is involved in VE differentiation [33]. HNF1b and GATA6 are both required for inducing HNF4 expression in the VE [34-36], and are believed to do so either through, or in parallel to, the BMP pathway. Moreover, BMP2/4 [37], and the activin receptor Alk2 [38] are upstream regulators of HNF4 [20].

Primitive streak formation
After implantation the mouse embryo develops as a cylindrical or cup-shaped structure called the egg cylinder [39]. This differs from humans, chicks and rabbits where the epithelium is flat [40].

The first morphological indication of anteroposterior (AP) asymmetry is the thickening within the distal visceral endoderm (DVE), which occurs between E5.5 and E5.75. This thickening of the visceral endoderm marks the future anterior side of the embryo and is known as the anterior visceral endoderm (AVE) [21]. On the posterior side of embryo a transient embryonic structure known as the primitive streak is formed at E6.5. The appearance of the A-P axis in turn makes the left-right (L-R) axis evident. The formation of the primitive streak is the earliest sign of gastrulation. This domain is characterized by Nodal (a member of the transforming growth factor β (TGFβ) superfamily) and Wnt3 expression [41]. Other primitive streak markers include Brachyury [42] and Mixl1 [43]. It has also been demonstrated that both FGF4 and FGF8 are expressed within the primitive streak and are required for the early embryonic development [21, 22, 44]. Between gastrulation and early somite stages, high persistent expression of FGF4 can be detected in the mesoderm adjacent to the developing midgut and hindgut, whereas low transient expression

can be found in the mesoderm adjacent to the presumptive posterior foregut/anterior midgut endoderm. At the end of gastrulation, FGF4 is exclusively expressed in the posterior mesoderm and ectoderm adjacent to presumptive midgut and hindgut endoderm [45].

Nodal is recognized as the principal factor in establishing the AP-polarity and is expressed symmetrically throughout the ICM/epiblast of the pre-implantation/early post-implantation embryo (EE3.5 to E5.5) [21, 41]. Nodal and Wnt antagonists such as Cer1, Lefty1, Dkk1, Sfrp5 and Sfrp1 direct the migration of the DVE cells towards the AVE is directed by. These antagonists also restrict the expression of posteriorly expressed genes (i.e. Wnt3) to the prospective posterior epiblast [21, 41, 46]. AP axis asymmetry is also reflected in the shape of the egg cylinder itself, which undergoes conformational changes just prior to gastrulation, shifting the long axis of the egg cylinder to align with the AP axis. Wnt3 activity in the epiblast has been shown to be essential for this process as removal of Wnt3a from the epiblast blocks this realignment [41].

Gastrulation
Gastrulation is the morphogenetic process reorganizing the unstructured embryo into a multicellular organism. In humans gastrulation occurs around 16 days after fertilization while in mice it's initiated at E6-7.5, at the time when primitive streak is formed. During this process the following events occur: formation of the three germ layers (definitive endoderm, ectoderm and mesoderm), establishment of the basic body plan, and cell movement to allow inductive interactions [47]. Gastrulation is initiated by the recruitment of the epiblast cells to the primitive streak. During vertebrate gastrulation, EMTs are important for morphogenesis and the migration of the epiblast cells from the primitive streak. Moreover, FGF signaling has been implicated in this process [21, 48]. FGFR1 promotes the EMT and morphogenesis of mesoderm at the primitive streak by controlling Snail and E-cadherin expression [21, 49]. Snail and Twist inhibit E-cadherin expression and are key inducers of EMT [21, 50, 51].
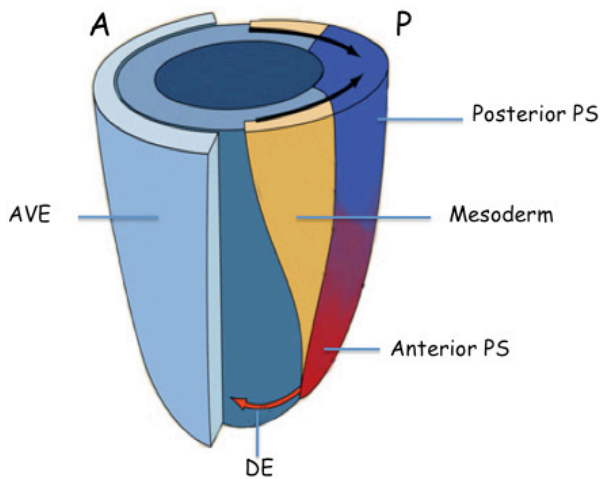
Figure 2. Gastrulation in the mouse embryo. Epiblast cells (shown with black arrows) migrate through the primitive streak to form mesoderm and definitive endoderm. The movement of the early definitive endoderm cells are shown with a red arrow. (Modified picture from Charles E. Murry et al 2009).

Initially the mouse embryo develops "inside-out" with the ectoderm inside surrounded by the mesoderm and endoderm but after gastrulation the ectoderm is brought outside with endoderm located inside and the mesoderm in the middle. The different germ layers consist of specific cell populations that have been brought together and are required for the formation of various tissues or organs in the body [52]. Ectoderm gives rise to the epidermis and nervous system while mesoderm gives rise to skeletal, muscle, blood, bone, and connective tissues. Endoderm forms the epithelial mucous membrane lining of the digestive and respiratory tract and digestive glands [47].

Signals secreted from the mesodermal derivatives are important in patterning and specification of endoderm [21]. Mesoderm differentiates into several transient different sub-structures before forming the mesoderm-derived organs in the body. These structures are: axial mesoderm (which give rise to the notochord and lies under the neural tube), paraxial mesoderm (forms among others the somites), intermediate mesoderm (is located between the paraxial mesoderm and the lateral plate and forms the urogenital system) and the lateral plate mesoderm (located at the periphery of the embryo, it is divided into a somatic layer (forming the future body wall) and the splanchnic layer (forming the circulatory system and the future

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gut wall) [53].

Several families of growth factors have been implicated in the regulation of gastrulation in the mouse, including FGFs and members of the TGFβ superfamily [54-56].

Definitive endoderm formation

The term endoderm refers to both primitive endoderm and the germ layer definitive endoderm. PrE contributes to the formation of the extraembryonic tissues covering the epiblast, whereas DE forms the gut, esophagus, lungs, stomach, intestines, thyroid, liver and pancreas. DE emerges from the anterior segment of the primitive streak 8-10 hours after gastrulation in mouse embryos. Mesendodermal/DE cells [57] are recruited from the primitive streak and to some extent from the epiblast to become incorporated in the visceral endoderm. The recruited anterior DE cell population expands anteriorly and gradually replaces the visceral endoderm [19, 58, 59]. Interestingly, recent data shows that VE cells are not displaced during gastrulation but remain associated with the epiblast in the mouse and contribute to the gut tube [60]. Later, during gut tube formation, the ADE cells migrate ventrally to form the ventral foregut, which contain population of bipotent precursors of the liver and pancreas [59, 61].

Specification of definitive endoderm

The mouse endoderm is a single layered sheet of epithelial cells, which has not been specified along the A-P axis after gastrulation. The first sign of regionalization of the DE is the expression of specific transcription factors along the A-P axis of the DE (E7.5), which eventually transforms the endoderm into the primitive gut tube (E8.5-9) [62]. The anterior segment of DE is characterized by the expression of following transcription factors: Hex1 [59], Cerberus [63], Gsc [64], Foxa2 (formerly called HNF3β) [65], Sox17 [66], and Cxcr4 [67], whereas the posterior portion express Cdx2 and 4 [68].

The primitive gut tube is divided in three parts: foregut, midgut, and hindgut. Depending on the transcription factor profile in the different regions of the primitive gut tube, various endodermal organs will bud and grow [18, 69]. In the anterior

portion of the foregut endoderm regions that are destined to become lung and thyroid express Nkx2-1 or Ttf1, whereas liver and the ventral pancreas develop from a region expressing Hhex1 and Pdx1, respectively. The dorsal pancreas and duodenum originate from the posterior portion of the foregut endoderm expressing Pdx1. The posterior portion of the gut endoderm that expresses Cdx1 and Cdx2 develops into mid- and hindgut, which later give rise to the small and large intestine [70]. Foxa1 and Foxa2 are both expressed in the entire gut tube and are thus important for the development of all gastrointestinal tract-derived organs [71].
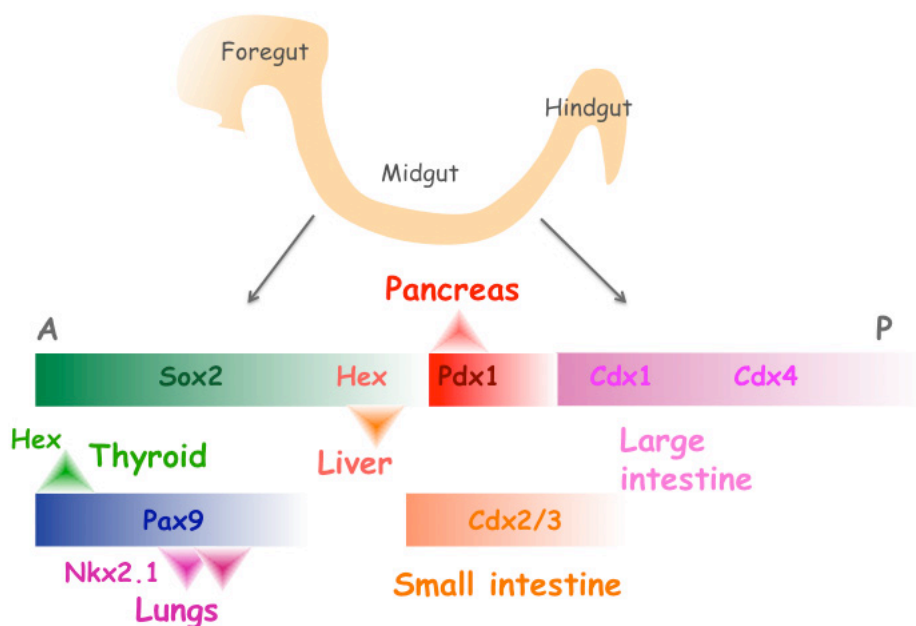
Figure 3. Prior to organs budding from the primitive gut tube, specific gene expression pattern can be distinguished in different regions along the A-P axis. In the anterior foregut where Sox2 is expressed the future stomach, lung and thyroid will develop. In the foregut/midgut boundary pancreas is formed in the region expressing Pdx1. Small and large intestine are generated from the posterior regions of the gut tube characterized by Cdx2, Cdx1 and Cdx4 expression. (Modified picture from Grapin-Botton and Melton, TIG 2000. 16:124-130.)

Although there is a lack of extensive information on how the early endoderm is patterned and how the primitive gut tube is formed in vivo, there are studies showing that instructive signals from the surrounding mesoderm play an important role [62, 72]. Other adjacent tissues that could interact and be involved in the patterning of

the gut tube include the anterior ectoderm, node and primitive streak [22], notochord [73, 74] and the endothelium [75]. Furthermore, following signaling pathways have been implicated in the gut development: Hedgehog [76], bone morphogenic protein (Bmp) [77, 78], FGF [62, 79, 80], Wnt [81], RA signaling [82, 83], and TGFbeta [84, 85]. A more recent study by Reed et al demonstrate also a role for Rho/ROCK/ Myosin II signaling in primitive gut tube morphogenesis in Xenopus [86].

Transcription factor profile of ADE

The path from anterior definitive endoderm (ADE) to the mature hormone-producing islet cell types is complex and involves sequential cell-fate decisions, including formation of pancreatic endoderm, endocrine progenitors, and hormone-producing islet cell types, including ß-cells. Reproducing these events in vitro requires more knowledge about the extracellular cues that are involved in the formation of definitive endoderm and pancreatic cell differentiation. Many of the genetic markers that characterize ADE are also expressed in the visceral endoderm; hence a combination of genetic markers has to be employed to exclude visceral endoderm formation during in vitro differentiation studies. A description of some of these important transcription factors is given below.

HHex (a homeobox-containing transcriptional repressor) is one of the earliest markers of ADE, but is also expressed in the visceral endoderm, more specifically the AVE [59]. Hhex expression in definitive endoderm is essential for development of the forebrain, liver and thyroid gland as Hhex deficient mice all have defects in these tissues and die at mid-gestation (E13.5-E15.5) [87, 88].

Sox17, a member of the SOX family of transcription factors with a high-mobility-group DNA binding domain (HMG box), is detected in the visceral endoderm at 6.0 dpc but at 7.5-8.5 dpc expression is also detected in DE (Kanai-Azuma et al., 2002). In Sox17-null mutant embryos DE is depleted. Foregut expression decreases by 8 dpc while mid- and hindgut expression persists until 8.5 d.p.c. [20]. Hence a sole expression of this marker is not enough to claim that cells in a heterogeneous population are of definitive endodermal origin [66].

Foxa2 is required for the development of the DE in mice [71, 89, 90]. Foxa2 is

continuously expressed from early embryonic stages through adulthood and is required for normal development of endoderm-derived tissues such as the liver, pancreas, and lungs [65]. During early embryogenesis it is expressed in visceral and definitive endoderm, node, notochord, and floor plate [65, 91]. Foxa2-knockout cells can form hindgut but not fore- and midgut [65]. It also plays an important role in the expression of other tissue-specific transcription factors by binding to their promoters e.g. Pdx1 (see below).

Another marker that has been suggested for definitive endoderm is the surface molecule, CXCR4. This marker is not expressed in visceral endoderm or undifferentiated ES cells but in mesoderm [67, 92, 93]. In combination with Hhex [59] or Gsc [93], DE cells can be separated from a heterogeneous cell population.

The TGF-β superfamily (including Nodal and Activin A) has been implicated in formation of mesendoderm and DE both in mice [57, 94] and hESCs [92]. More recently, a role for FGF signaling has also been established in the specification of anterior mesendoderm. Furthermore, it was determined that exogenous addition of FGF4 or FGF2 specifically promoted Hex+/Cxcr4+ ADE as other anterior markers such as Cer1, Gsc, Foxa2, and Sox17 were up-regulated concomitant to down-regulation of mesodermal and primitive streak markers [59]. A role for FGF signaling in early endoderm differentiation has also been shown in another study where FGFR -/- ESCs were unable to contribute to endoderm [95].

PANCREAS DEVELOPMENT

The anatomy and function of the adult pancreas

The adult pancreas is divided into an exocrine and an endocrine part. The exocrine pancreas comprises of 95-99% of the pancreas and includes acinar and the ductal cells [96]. The acinar cells produce and secrete digestive enzymes such as lipases, nucleases and proteases and a bicarbonate rich fluid which are all transported through the pancreatic duct to the duodenum, where these digest fat, proteins and carbohydrates. The endocrine cells secrete hormones into the blood to control blood glucose homeostatis and are located into the islets of Langerhans. The latter are distributed throughout the exocrine tissue, and are of five types in both mice and

humans; insulin-producing β cells (60-70%), glucagon-producing α-cells (15-20%), somatostatin-producing δ-cells (5-10), polypeptide-producing PP cells (2%) [97] and ghrelin cells (1%) [97-99].
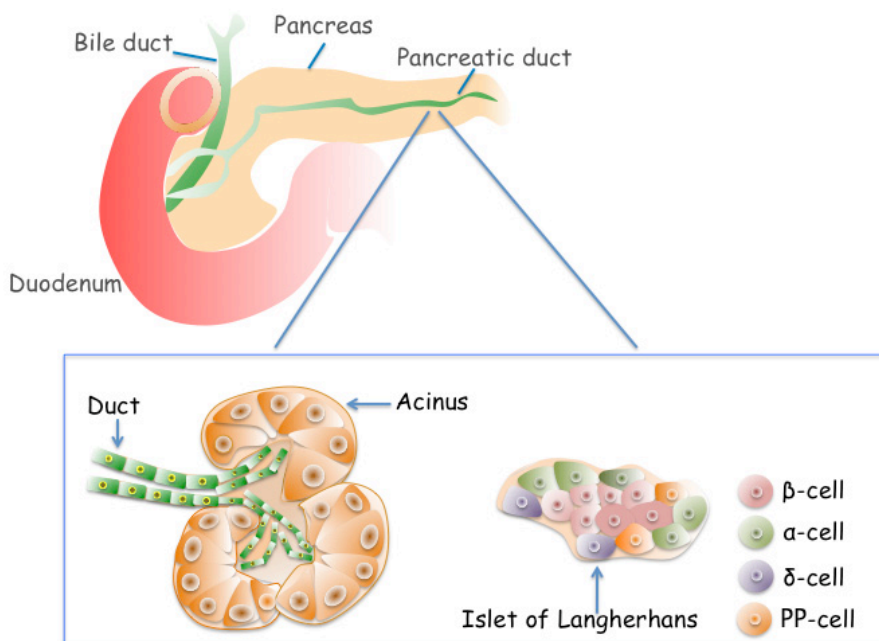

Figure 4. The pancreas is a glandular organ, composed of endocrine and exocrine tissues. The exocrine tissue consists of acini and ducts. The acinar cells produce digestive enzymes that are secreted into the duodenum through the pancreatic duct to digest protein, fat and carbohydrates. The common bile duct (which is connected to the small intestine) connects pancreas to the liver and the gallbladder. The endocrine pancreas secretes various hormones into the bloodstream to regulate blood glucose homeostasis.

Specification of the pancreatic anlagen

Pancreas forms via the fusion of the dorsal and ventral bud that appear as outgrowths from the gut endoderm between E8.5-E9.5. The dorsal pancreas appears at E 8.5 in the mouse while the ventral pancreas appears shortly thereafter. The buds grow independently and communicate with the foregut through a duct. By E10.5 the buds undergoe branching morphogenesis into a ductal tree that finally fuse to form the pancreas between E13-E14, at this stage secondary transition starts [100]. Although few endocrine cells (specifically insulin and glucagon cells) are seen during the first transition between E9.5-E10.5 these cells do not contribute to the endocrine

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cells formed at later stage [101]. During E14-E16 endocrine cells undergo extensive proliferation. At E16 (the beginning of third transition) the endocrine cells organize themselves into islet-like clusters. The mature islets are however not formed until E18-E19 and continue to remodel and mature for 2-3 weeks after birth [100].

Gene activation studies have shown that different sets of transcription factors are required for the formation of each bud [102-104] (see below). Moreover, different inductive stimuli are required for dorsal and ventral bud formation. Signals from the mesodermal derivatives, notochord [73] and the dorsal aorta [75] are important for dorsal pancreas differentiation whereas signals from the lateral plate mesoderm (LPM), cardiac mesoderm (CM), and septum transversum (STM) are required for ventral pancreas [72].
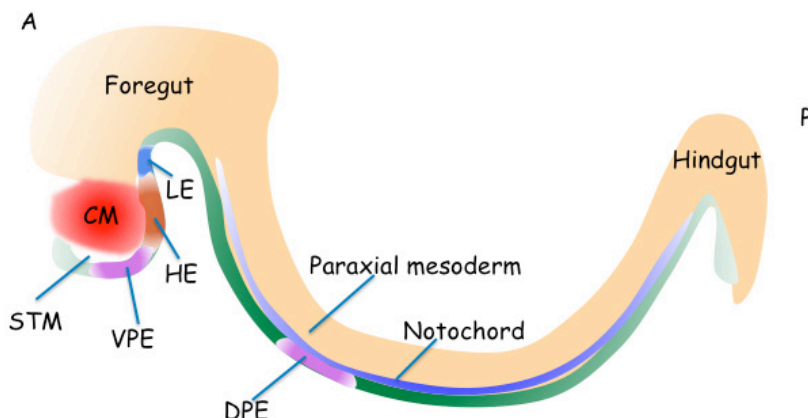
Figure 5. Various signals from adjacent tissue specify the gut tube endoderm. FGF and BMP signaling from the cardiac mesoderm (CM) and the septum transversum mesenchyme (STM), respectively, are crucial for induction of liver (HE), lung (LE) and ventral pancreas (VPE) from the ventral foregut endoderm. Retinoic acid signaling from the paraxial mesoderm is also important for pancreas and liver development. Activin and FGF secreted from the notochord inhibit Shh signaling and allow dorsal pancreas (DPE) to form.

The role of notochord signaling in dorsal pancreas induction

At the 15-somite stage the dorsal gut epithelium is in direct contact with the notochord, which is thought to induce the onset of the Pdx1 expression and dorsal pancreatic epithelium evagination [105, 106]. Later, around E9, the notochord becomes separated from the dorsal pancreatic epithelium (now expressing high levels

of Pdx1) by the dorsal aorta initially, and later by the accumulated mesenchyme [105, 107]. Signaling factors such as Activin-beta B and FGF2 are secreted from the notochord to repress of Sonic hedgehog (Shh) in the pancreatic anlage [73, 108, 109]. The type II activin receptors (ActRIIA and ActRIIB) are both expressed on the pancreatic islet cells of the adult mice. The type II activin receptor-mediated signaling, occurs through restriction of Shh expression, and is required for endocrine differentiation in the pancreas and the stomach. Since several ligands e.g. Activin A, Activin B, Nodal, BMP4 can bind to both receptors, it is believed that multiple ligands are involved in this process [85]. In addition, the dorsal aorta (separating the pancreatic epithelium from the notochord) mediates inductive signals to the pancreatic epithelium [75] (see below).

The role of lateral plate mesoderm in ventral pancreas induction
The part of endoderm giving rise to the ventral pancreas is initially in contact with the LPM. Signals from the LPM more specifically the splanchnic mesoderm are involved in inducing the ventral pancreatic domain. These signals belong to the BMP, Activin and RA families of growth factors. Furthermore, these signals are considered as instructive in contrast to the notochord secreted factors, which are believed to be permissive. This is due to the fact that LPM secreted signals are able to induce pancreas differentiation in endoderm that would not normally form the pancreas [72].

The role of cardiac mesoderm and septum transversum mesenchyme
In addition, it has been shown that the ventral foregut endoderm consists of a bipoptential progenitor cell population that can give rise to both liver and ventral pancreas in the presence of the right signals. Furthermore, in absence of FGFs secreted by the cardiac mesoderm the ventral foregut endoderm forms the pancreas by default [61], whereas specific concentrations of FGFs (specifically FGF2) are required for induction of liver and lung [80].
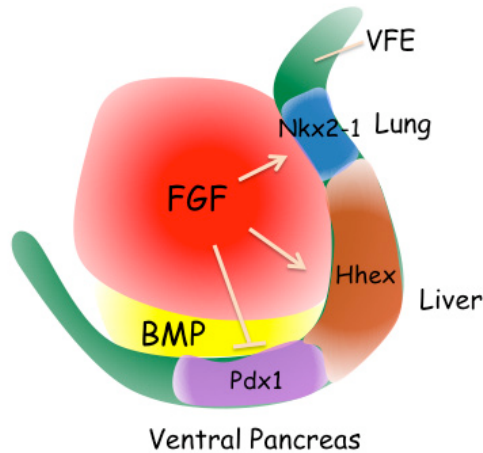
Figure 6. Induction of foregut derived organs. Depending on the level of FGF signals secreted from the cardiac mesoderm (shown in red) different regions of the ventral foregut endoderm (VFE, shown in green) differentiate into the liver (brown), lung (blue) and pancreas (purple). The presence of high FGF levels promotes a pulmonary cell fate whereas low levels of FGF together with BMP signals from the septum transversum mesenchyme favor a hepatic fate. Ventral pancreas is promoted in the absence of cardiac mesoderm or FGF. The presumptive pancreas, liver and lung are marked by Pdx1, Hhex, and Nkx2-1 expression, respectively.

Simultaneous signaling from another mesodermal source, the septum transversum mesenchyme is also required for hepatic induction. STM is in close contact with the ventral foregut endoderm at the time of hepatic specification and is a source of BMPs [110]. It is suggested that the BMPs pattern the endoderm so that it can induce liver gene expression in response to FGFs secreted from the CM. Although treatment with the BMP inhibitor noggin activates the pancreatic marker Pdx1 in explants, BMP signaling per se is not considered to inhibit pancreas specification. This is due to the fact that when pancreatic program is initiated by the ventral foregut endoderm in the absence of cardiac mesoderm/FGFs it is surrounded by a BMP-expressing mesenchyme [110].

The role of blood vessels

Signals from the blood vessel endothelium are involved in the induction of endocrine pancreatic differentiation. By recombining pre-patterned dorsal endoderm from mouse with aorta endothelium, notochord or neural tube, Lammert and colleagues

showed that endocrine pancreatic differentiation (marked by insulin expression) only occurs when aorta is present. Furthermore, morphological changes such a pancreatic bud outgrowths were also seen. They also showed that signals from endothelial cells were specifically required for endocrine pancreatic development. This was based on the observation that endocrine gene expression was not initiated in pancreatic endoderm when endothelial cell precursors of the dorsal aorta were removed in frog embryos, while gene expression in other tissues was normal. Finally, by using VEFG-A transgenic mice (driven by the Pdx1 promoter), they showed that endothelial cells are involved in differentiation of PDX1-expressing foregut epithelium into pancreatic cells [75].

In an attempt to identify some of endothelial signals involved in the endocrine differentiation, Nikolova G et al demonstrated that beta cells unlike exocrine cells do not form a vascular basement membrane. The beta cells attract endothelial cells by expressing high levels of vascular endothelial growth factor VEGF-A. The endothelial cells in turn form a vascular basement membrane next to the beta cells (around the capillaries). Furthermore, they identified the components of the basement membrane (laminin, fibronectin and collagen IV) as endothelial signals. More specifically, laminins were shown to be involved in beta cell proliferation through β1-integrin [111].

The role of mesenchyme
Signals from the mesenchyme (loose cells of mesodermal origin) are also involved in the proliferation of the progenitor cells and their differentiation into endocrine and exocrine tissues [112-114]. Studies where they have looked at the role of the mesenchyme in beta cell differentiation have shown that it exerts an inhibiting effect on the beta cell differentiation. In fact, beta cell differentiation occurs faster without the mesenchyme [115, 116]. This delay is thought to be important for the expansion of the pancreatic progenitor cells (that proliferate at a faster rate than the beta cells) and the generation of a sufficient number of beta cells. Jensen et al [117] did in fact show that an accelerated beta cell differentiation in mice would lead to pancreatic hypoplasia as a result of inadequate growth of the pancreatic progenitor cells. Furthermore, the inhibitory effect of the mesenchyme on beta cell develop-

ment seems to be mediated by repression of Ngn3 (an important transcription factor for endocrine cell development in pancreas) [115, 118, 119].

Members of the FGF family, especially FGF10 and to some extent FGF-7, have also been suggested to have a part in this mesenchymal epithelial signaling. When analysing the expression pattern of FGF7 and FGF10 in human embryonic pancreatic mesenchymal cells, it was shown that these cells express both these factors and that both are involved in the proliferation of epithelial cells [120] as observed in rodents.

CRUCIAL TRANSCRIPTION FACTORS DURING PANCREAS DEVELOPMENT

The role of Pdx1 in pancreas formation

As beta cell mass reduction and β-cell dysfunction are hallmarks of both type 1 and 2 diabetes, it is necessary to understand the molecular mechanisms responsible for regulating death of β-cells [121]. The transcription factor Pancreatic duodenal homeobox 1 (Pdx1) is essential for pancreas development and beta cell function [121]. Several studies propose that Pdx1 marks a multipotent cell population from which endocrine and exocrine cells arise [118, 122, 123]. In fact, lineage tracing analysis has shown that all types of pancreatic tissues are derived from Pdx1 expressing progenitors [119]. Furthermore, the importance of Pdx1 in the pancreas is underscored by the development of pancreatic agenesis in mice [124, 125] and humans [126, 127] that are homozygous for mutations in PDX1. As the mesenchyme in these mice develops normally independent of the epithelium, the observed pancreactic defect is restricted to the epithelial cells. In addition, whereas Pdx1 is essential for pancreas formation it is not required for initial bud formation [105] as this occurs in the mutants while the morphogenesis and the differentiation of the buds is arrested.

Pdx1 expression and regulation

Pdx1 is a master regulator of pancreas [124, 125] and beta cell development [128]. It is first detected at E8.5 and expressed in the vertebrate posterior foregut endoderm that is destined to become the antral stomach, pancreas and rostral duodenum.

During early stages of mouse embryogenesis, Pdx1 is expressed in the entire pancreatic epithelium (including both endocrine and exocrine cells) and in subpopulations of duodenal and gastric enteroendocrine cells [129] but never observed in the pancreatic mesenchyme during the pancreas development [105]. At E10.5 Pdx1 expression decreases and remains low in the proliferating pancreatic epithelial cells until the beta cells have formed (E13) where it reappears. In the adult mouse pancreas, high Pdx1 expression is restricted to nuclei of insulin producing beta cells, where Pdx1 is crucial for the function of the mature β- cell [106, 128].

In different studies researchers have tried to elucidate which transcription factors are of importance in regulating the Pdx1-expression in beta cells. Using sequence homology analysis, four different regions within the Pdx1 5´-flanking region, named area I, II, III and IV, have been implied to be of importance in regulating the Pdx1 transcription in mice and humans. Furthermore, it has been concluded that Foxa2/Hnf3beta has a role in regulating the Pdx1 expression by binding to areas I [130], II [131, 132] and IV [133] in both mouse and human [130, 133, 134]. Pdx1 itself can also bind to area I [134] and IV [133]. Unfortunately, HNF3beta homozygous null mutant mice cannot be used to further understand the role of Hnf3beta in transcriptional regulation of Pdx1 in vivo, since the animals die before pancreatic endoderm is formed [91, 130, 135]. However, by using mouse embryonic stem cells, Gerrish et al were able to show that Hnf3beta is required for Pdx1 expression in embryoid bodies (EBs). In humans, HNF1α is also suggested to be involved in regulation of the PDX1 gene in the adult beta cells [136].

Here, a short description of some of the most important transcription factors involved in the Pdx1 regulation and endocrine cell differentiation will be given.

Hlbx9 or Motor neuron and pancreas homeobox 1 (Mnx1)
Hlxb9 is expressed at the 8-somite stage in the dorsal and ventral endoderm and similar to Pdx1 it is down-regulated by E10.5 until E13.5 when its expression reappears in β-cells where it is maintained. Hlxb9 deficient mice fail to express Pdx1 in the dorsal epithelium resulting in dorsal pancreatic bud agenesis [103, 104] while the ventral pancreas forms and express Pdx1. Hence, Hlxb9 expression is

required for Pdx1 expression in the dorsal pancreas, while expression of Pdx1 in the ventral pancreas is regulated by other transcription factors.

Hepatocyte nuclear factors (HNFs)

Members of the HNF family, including Hnf1a, Hnf3α, Hnf3β, and Hnf6, are all expressed in the developing pancreas. Hnf1β mutants suffer from pancreatic agenesis and a number of the pancreas specific markers such as Pdx1, P48 and Hlxb9 are also severely affected [137]. Hnf6 (Onecut1) is believed to act upstream of Pdx1 (by binding to the Pdx1 promoter and stimulating transcription of Pdx1) by controlling expression of Pdx1 in the ventral and dorsal pancreatic endoderm and during later stages of pancreas development. Hnf6 deficient mice display hypoplastic pancreas as a result of a delayed onset of Pdx1 that in turn leads to fewer Pdx1 expressing endodermal cells [138]. It was also shown that Hnf6 stimulate Ngn3 expression and that Hnf6 is critical for islet development and function [139, 140].

Nkx6-1 and Nkx6-2

Pedersen et al showed that expression of one member of the Nkx family, Nkx6-1 is also absent in Pdx1 mutants, suggesting that Nkx6-1 lies downstream of Pdx1 and that Pdx1 expression is required for induction of Nkx6-1 expression in the mouse pancreas [141]. Nkx6-1 has been detected in mice at E10.5 in the early pancreatic epithelium [141-143]. Later in pancreas development, Nkx6-1 expression (similar to Pdx1) is restricted to beta cells [142, 143]. Another member of the Nkx family, Nkx6-2 is very similar to Nkx6-1 and both are suggested to be expressed in early pancreatic progenitor cells [141]. Although, Nkx6-1 expression is significantly diminished in Pdx1 null mutants, no change is observed in the number of Nkx6-2+ cells, suggesting Nkx6-2 is not downstream of Pdx1 [141]. Nkx6-1 deficient mice have an 85% reduction in β-cells, whereas the Nkx6-2 null mutant has a normal pancreas. The Nkx6.1/Nkx6.2 double null mutant mice however has a 65% reduction in α-cells, suggesting that Nkx6-2 is crucial for development of α-cells and that Nkx6-1 can compensate for Nkx6-2 in its absence [144]. As Pdx1 and Nkx6-1 are normally co-expressed in both mouse and human pancreatic epithelium, true pancreatic progenitor cells should co-express these markers.

Pancreas transcription factor 1 a (PTF1a)

The basic helix-loop-helix (bHLH) transcription factor Ptf1a (also known as P48) is initially expressed in pancreatic progenitors, whereas later it becomes later restricted to the exocrine cell lineage [145]. Ptf1a is expressed in the ventral pancreas at E8.5 and in the dorsal pancreas at E8.75 [146]. Pancreatic progenitors characterized by Pdx1/Nkx6-1/Ptf1a co-expression are abundant at E9.5 in the dorsal pancreas and at E10.5 in the ventral pancreas, while from E14 onwards Ptf1a expression is restricted to acinar cells [146, 147]. In the Ptf1a knockout mice, the acini and ducts are not formed, whereas the endocrine cells develop and migrate to the spleen [144]. Humans with PTF1a non-functioning mutation are born without any pancreas, and have neonatal diabetes[144, 148]. Ptf1a expression is induced when the dorsal aorta comes in contact with the Pdx1-expressing endoderm [149]. Ectopic expression of Ptf1a and Pdx1 convert posterior endoderm to pancreatic tissue [150], suggesting true pancreatic progenitor cells should co-express Pdx1 and P48. Moreover, lack of P48 expression in the ESC-based strategies could be explained by the lack of specific signals emanating from the dorsal aorta.

Sex determining region Y (SRY)-box 9 (Sox9)

Sox9 (a member of the Sox gene family) is first detected in both Pdx1+ dorsal and ventral pancreatic progenitor cells at E9.5 [151]. Later in development it becomes restricted to the ductal cells and is absent in hormone expressing cells [152]. Targeted deletion of Sox9 in the Pdx1+ pancreatic progenitor cells confirms that Sox9 expression is crucial for keeping the pancreatic progenitors in a proliferative state and to prevent premature differentiation [97, 153, 154].

Neurogenin 3 (Ngn3)

Ngn3 is a basic helix-loop-helix factor that is expressed in epithelial pancreatic progenitor cells and is required for endocrine cell differentiation [118]. While Ngn3 mRNA is detected as early as E9.5 in Pdx1 expressing cells [155], the protein is detected at E11.5. Expression of Ngn3 peaks at E15.5 after which it is down-regulated with very few cells seen at E17.5 [156] and none in the adult pancreas [157]. Lineage tracing and analysis of Ngn3-deficient mice [118] demonstrate that Ngn3 is required for the formation of all endocrine cells [119]. Ngn3 is believed to lie upstream of NeuroD1 and although it is necessary for the formation of islet

cells, it is not expressed in hormone expressing cells [158], suggesting that it is turned off prior to final differentiation. Ngn3-deficient mice lack endocrine cells and expression of early endocrine markers such as NeuroD, Pax4 and Pax6, adding further support for Ngn3 as a pro-endocrine gene [118]. Forced Ngn3 expression results in endocrine differentiation with few detectable exocrine markers [156, 157], emphasizing the importance of Ngn3 in endocrine cell fate specification.

The mesenhyme seems to have a dual role regarding Ngn3 expression. It delays Ngn3 induction by acting upstream while it inhibits beta cell differentiation through Ngn3 expressing cells by acting downstream of Ngn3 [115]. The role of Ngn3 in human pancreas formation however, remains unclear, although Ngn3 deficient mice develop diabetes [118] patients with mutations giving rise to lack of function of NGN3 are not born diabetic [159].

Paired box 4 (Pax4) and 6 (Pax6)

Another important family of transcription factors involved in endocrine differentiation includes members of the paired-box homeodomain family, more specifically Pax4 and 6 [160, 161]. Pancreatic buds express Pax4 at E9.5 and expression continues in endocrine precursors with a peak around E14-E15 [162]. Pax4 is co-expressed with both Ngn3 and Isl1 suggesting a role in endocrine cell specification and differentiation. Alpha and PP-cells increase in Pax4 mutant whereas beta and delta cells fail to develop [161-163].

Pax6 is expressed and maintained in all differentiated endocrine cells, [160, 164]. Pax6 regulates the expression of glucagon, insulin and somatostatin by binding to the respective promoters. Decreased number of endocrine cells is observed in Pax6 null mutants [160, 164].

v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) and B (MafB)

The Maf gene family is also important for maturation of the endocrine cells. MafA expression starts at E13.5 and is restricted to the beta cells [165, 166]. Whereas beta cell differentiation is unaffected in MafA-deficient mice, beta cell function is impaired [167]. MafB is initially expressed in endocrine progenitors and early alpha and beta cells, but later becomes restricted to alpha cells [168]. MafB mutant mice

show a reduced number of alpha and beta cells [169]. In conclusion, whereas MafA is crucial for β-cell function, MafB appears to be important for both α- and β-cell maturation.

SIGNALING PATHWAYS REGULATING PANCREAS DEVELOPMENT

The role of FGF signaling in pancreas development

The FGF family

FGFs constitute a large family of heparin binding proteins involved in many aspects of development including cell survival, proliferation, growth, and differentiation. 22 members have been identified in humans where 18 of them bind and activate FGF receptors (FGFRs), while the other four do not bind to the FGFRs [170]. The FGFs that activate the FGFRs are divided into six subfamilies based on their sequence homologies:

Group 1: FGF1, FGF2
Group 2: FGF3, FGF7, FGF10, FGF22
Group 3: FGF4, FGF5 and FGF6
Group 4: FGF8, FGF17and FGF18
Group 5: FGF9, FGF16 and FGF20
Group 6: FGF19 (FGF15 in mouse), FGF21 and FGF23

All FGFs have a heparan sulfate glycosaminoglycan (HSGAG)-binding domain [171]. Based on recent studies it has been shown that tissue-specific HSGAG sequences mediate the specificity of a given FGF ligand binding to its cognate receptor (FGFR) [170].

The first five subfamilies are considered to be paracrine factors that have high affinity for HSGAG, which in turn allows them to act near the source of their expression. The last subfamily (group 6) is however considered as endocrine FGF ligands with low HSGAG affinity. The decreased affinity leads to an increased diffusion of these FGFs from their source, but it also reduces the ability of HSGAGs to promote the

binding of these FGFs to their receptors. The endocrine FGF ligands are instead dependent on klotho proteins (type I transmembrane glycoproteins) to signal in the target tissues. The klotho proteins increase ligand-receptor affinity by binding both to the endocrine FGFs and their corresponding FGFR [170].

The FGFR-non-binding FGFs (FGF11-FGF14) or fibroblast homologous factors (FHFs) are not considered as members of the FGF family although they bind heparin and share high sequence and structural homology with the other FGFs [170]. In addition, they lack signal sequences and are thought to remain intracellular [171]. FGF1 and FGF2 also lack signal sequences but are secreted independently, potentially by an exocytotic mechanism [171]. All the remaining FGFs have signal peptides and are secreted through the endoplasmic reticulum (ER)–Golgi secretory pathway [170]. This is also the case for FGF9, 16, and 20, which however lack signal peptides [170, 171].

There are at least four different tyrosine kinase receptors (FGFR1-FGFR4) that bind different FGF ligands with varying affinities. In addition, alternative splicing of FGFR1-FGFR3 generates b and c isoforms of FGFR1, 2 and 3, which display distinct expression patterns and ligand specificities [172]. In total there are seven different FGFR subtypes. The alternative splice isoforms of FGFRs are tissue specific, the b isoform is usually expressed in epithelial tissue whereas the c isoform is expressed in mesenchymal tissue [173]. Most of the FGFs produced in the epithelium will activate a receptor in the mesenchymal tissue and vice versa except for some ligands that have the capacity to activate both the b and c isoforms of certain FGFRs (e.g. FGF1) [170]. By binding to their respective receptors (FGFRs), FGFs activate several signal transduction pathways, including phosphatidylinositol-3 kinase (PI3K) and ERK1/2 mitogen-activated protein kinases (MAPKs) [170].
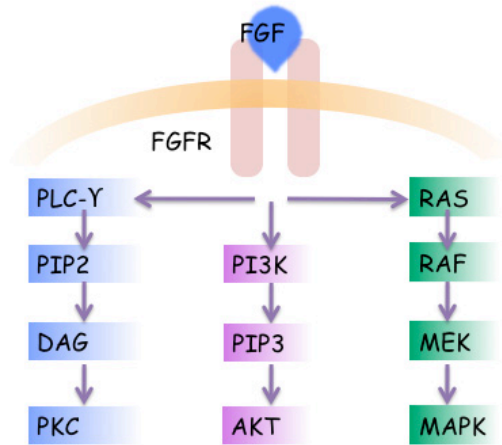
Figure 7. Schematic view of the intracellular signalling pathways activated by FGFs.

Different inhibitors of FGF signalling exist. These include the sprouty proteins, which inhibit FGF stimulation via the MAPK pathway by interacting with GRB2 (growth factor receptor bound protein 2), the mAPK phosphatase 3 (mKP3), which acts by dephosphorylating extracellular signal regulated kinase (ERK), and the transmembrane protein SEF [170, 174].

FGF signaling in pancreas specification

Multiple FGFs (FGF1, FGF2, FGF4, FGF7, and FGF10) and their receptors (FGFR-1 and -2) are expressed and involved in gut tube patterning [70] and pancreas development [120, 175-179].

FGF2 acts mainly via the IIIc isoforms of FGFR1, 2, and 3, but it also binds with lower affinity to FGFR1b and 4 [171]. Mouse studies have shown that FGF2, which is secreted from the cardiac mesoderm, patterns the adjacent multipotent ventral foregut endoderm in a concentration-dependent manner into liver and lung [80, 180]. In the absence of cardiac mesoderm and FGFs a ventral pancreatic fate is pro-moted [61, 80]. In contrast, an inductive role of FGF2 (secreted from the notochord) has been demonstrated in mouse and chick during dorsal pancreas formation [84, 181].

FGF4 (similar to FGF2) mainly binds to the IIIc splice forms of FGFR1-3 while it binds with low activity to IIIb splice forms of FGFR-1 to -3 [172, 182]. FGF4 acts as a posteriorizing growth factor with broad anterior-posterior and left-right patterning activities [62, 70, 183]. FGF4, patterns cultured e7.5 mouse endoderm in a concentration dependent manner. High concentrations promote a posterior/ intestinal cell fate and low concentrations induce a more anterior/pancreas–duodenal cell fate [70].

FGF7 and FGF10 are secreted from human embryonic pancreatic mesenchymal cells and mediate proliferation of pancreatic progenitors in vitro [120]. This activity is mediated by signaling through the FGFR2-IIIb expressed on epithelial cells [184, 185]. Furthermore, mice deficient in FGF10 or the FGFR2-IIIb isoform show impaired pancreatic development [175, 186]. Specifically, Bhushan et al showed that although initial bud formation occurs in FGF10 mutant mouse embryos, proliferation of Pdx1 positive progenitor cells was reduced and differentiation and morphogenesis of the pancreatic epithelium were inhibited [175]. Moreover, FGF10 signaling has been shown to be important for maintaining Ptf1a expression in pancreatic progenitor cells in the dorsal pancreatic bud [187]. Furthermore, ectopic expression of FGF10 in the pancreatic epithelium resulted in pancreatic hyperplasia characterized by increased number of Pdx1+/Nkx6-1+ pancreatic progenitor cells and perturbed endocrine and exocrine differentiation [188].

In summary, whereas FGF2 and 4 display a patterning activity in the mouse endoderm and can induce a pancreatic cell fate, FGF7 and 10 are crucial for the proliferation of the Pdx1+ pancreatic progenitors.

RA signaling in pancreas specification
Retinoic Acid (RA) is a vitamin A derivative that is available in two forms: all-trans-RA (at-RA) and 9-cis-RA that bind different nuclear receptors. In contrast to FGFs, which bind to cell surface receptors, RA enters the nucleus and binds directly to target genes via nuclear receptors and retinoic acid response elements (RAREs) [189]. Both forms of RA activate the retinoic acid receptors RARα, β and γ, whereas the retinoid X receptors RXRα, β and γ are only activated by 9-cis-RA [190, 191].

Retinoic acid (RA) signaling is another signaling pathway involved in pancreas specification. Inhibition of RA signaling results in impaired expression of pancreatic and hepatic markers while increased levels leads to expansion of the domains from where pancreas and liver develops. Moreover, the absence of Pdx1 expression in RA mutants suggests that RA specifies pancreas before Pdx1 expression is initiated [83]. Furthermore, it has been shown that Raldh2, one of the enzymes converting vitamin A to RA and is expressed in the dorsal pancreatic mesenchyme, is important for dorsal pancreas development. Raldh2 deficient mice never develop a dorsal pancreatic bud and lack Pdx1 expression in the dorsal endoderm [82, 192]. Moreover, it has been shown that HLXB9 expression, albeit decreased; persist in the dorsal epithelium of the Raldh2 mutant [192].

Notch signaling in pancreas development
Notch signaling is involved in the proliferation and differentiation of the pancreatic progenitor cells. In Delta (the Notch ligand) expressing cells where Notch is activated, Ngn3 expression is suppressed and the cells are kept in a proliferating progenitor state. In contrast, when Notch activation is inhibited Ngn3 is turned on and subsequent differentiation into endocrine cells occurs [156, 193]. Hes1 mediates the repression of Ngn3 and subsequent endocrine differentiation [194]. Notch ligand deficient mice show increased Ngn3 expression and consequently premature endocrine cell differentiation at the expense of proliferation [156].

STEM CELLS AND THERAPY

Derivation and characterization of hESCs

hESCs are derived from the inner cell mass of a preimplantation blastocyst, that are usually around 5 days old. The blastocysts are acquired from in vitro fertilized eggs that would otherwise have been discarded [195].

The most common method to derive hESCs from a blastocyst is to remove the zona pellucida with pronase and isolate the ICM through immunosurgery. The trophectoderm cells are eliminated by complement-mediated cell lysis. This method requires high quality embryos and the isolated hESCs cannot be used therapeutically as they have been exposed to animal products [196]. An alternative way to remove the zona pellucida is with Tyrode's acid and through mechanical isolation of the ICM. The advantage with this method is that the blastocyst will not be exposed to animal-derived pronase, mouse antibodies, and complement factors. Since, it is not possible to eliminate all trophectoderm cells without damaging the ICM, the remaining trophoblasts are mechanically removed after plating. The zona pellucia can also be removed by assisted natural hatching or by using recombinant enzymes. The isolated ICM is then placed on mitotically inactivated mouse embryonic fibroblasts (MEFs) also called feeder cells and later dissociated and further passaged [197]. The MEFs that secrete to date unidentified soluble factors are required to keep the hESCs in an undifferentiated state [198]. Although many laboratories are successfully maintaining their hESCs in xeno-free conditions [199, 200] nearly all hESC lines available today are derived using pronase and immunosurgery [201]. Hence, the biggest obstacle remaining here is finding efficient xeno-free ways of removing the zona pellucida and to establish chemically defined medias for hESC propagation.
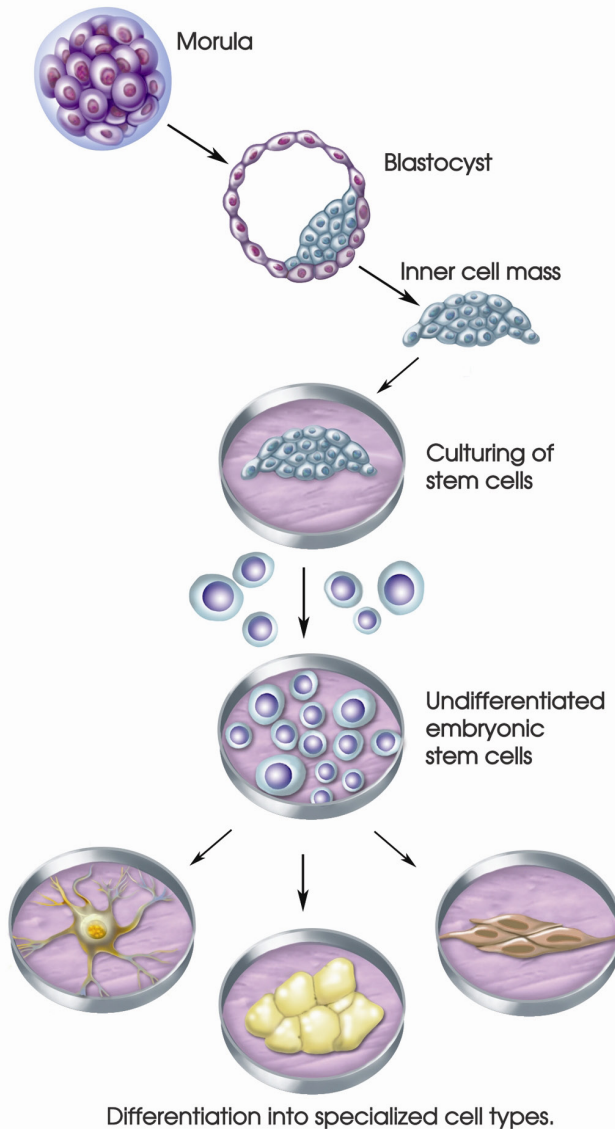
Morula

Blastocyst

Inner cell mass

Culturing of stem cells

Undifferentiated embryonic stem cells

Differentiation into specialized cell types.

Figure 8. Derivation of hESCs. The fertilized egg undergoes a series of cleavages to form the early stage embryo (morula), which after reaching the 16-cell stage starts to differentiate into the blastocyst. hESCs are derived from the inner cell mass (ICM) of a 5 days old pre-implantation blastocyst. The isolated ICM is plated onto mitotically inactivated mouse embryonic feeder cells (MEFs) and passaged and replated until a hESC line has been established. The hESCs can then be differentiated into various cell types.

hESCs are characterized by their capacity to self renew, indicating that the hESCs can divide for unlimited number of times without differentiating. The hESCs are also defined by their pluripotency, meaning they have the potential to become any cell type derived from the three germ layers [202, 203]. It is these characteristics that make the hESCs not only a great experimental model for understanding human development but also present an excellent source for establishment of strategies for future regenerative cell therapies.
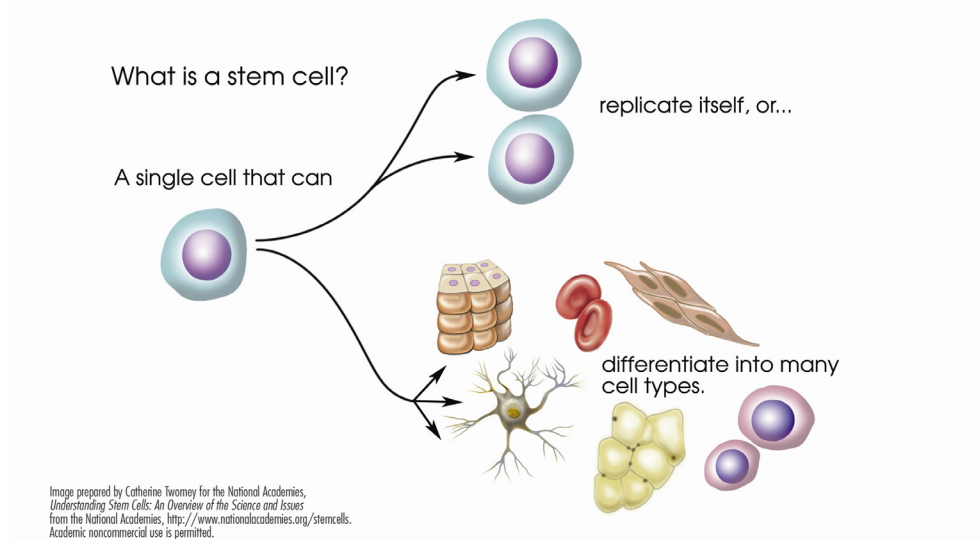

Figure 9. Characteristics of a stem cell. Stem cells can regenerate and differentiate to specific cell types in the body. Embryonic stem cells are characterized by their capacity to self-renew and generate any cell type in the body whereas adult stem cells (found in differentiated tissues) have a limited differentiation and self-renewal capacity.

Morphologically the hESCs are characterized by high nuclear to cytoplasmic ratio. hESCs are also defined by the presence of several transcription factors and cell surface proteins such as; OCT4, NANOG, SSEA 3 and 4, and TRA 1-60 and 81. In contrast to mESCs, where SSEA 1 is expressed in undifferentiated cells, SSEA 1 is expressed by differentiated hESCs. To validate pluripotency hESCs are either analyzed after spontaneous differentiation in culture or after transplantation, e.g. under the kidney capsule, in SCID mice where they form teratomas that normally consist of cells from all three germ layers [200, 203].
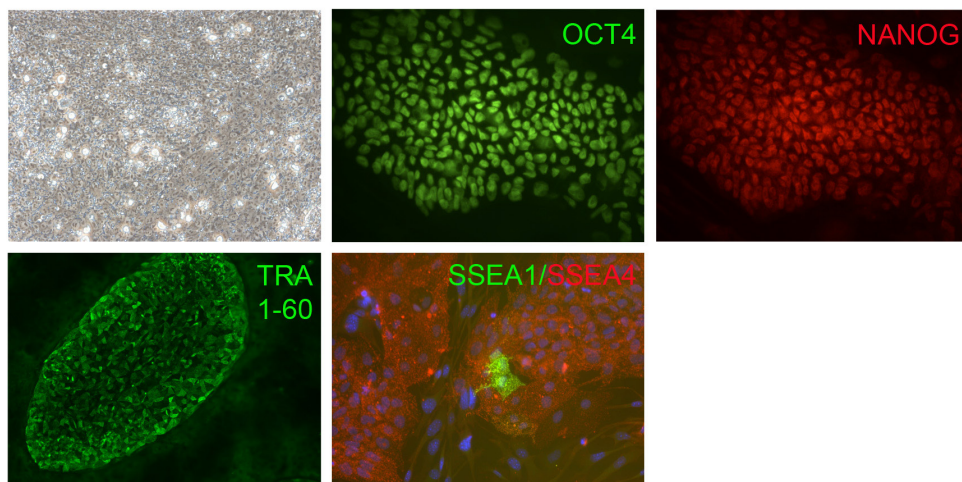
Figure 10. Characterization of hESCs. Undifferentiated hESCs are characterized by high ratio of nucleus to cytoplasm (seen to the left) and expression of specific markers such as OCT4, NANOG, TRA1-60 and SSEA-4. NANOG and OCT-4 are expressed in the nuclei of the hESCs whereas TRA 1-60 and SSEA 4 are membrane bound. Differentiated hESCs are stained by SSEA-1 (all stainings performed by A.K. Häger).

hESC propagation and availability

As mentioned above, hESCs are routinely cultured on mouse feeders that besides providing an attachment substrate also secrete unknown soluble factors that are important for the maintenance of undifferentiated hESCs. The most commonly used feeders are mouse embryonic fibroblasts, however, human feeders are also widely used [204]. As many researchers strive towards more defined systems feeder free conditions are also available such as matrigel [205]. However, as matrigel is neither defined nor xeno-free other more defined substrates such as recombinant fibronectin and laminin are frequently used. Furthermore, to grow hESCs under feeder-free conditions the media can either be supplemented by conditioned feeder medium [205] or a mixture of factors such as FGF2, Activin A, N2, and B27 supplements, known to promote the self-renewal [206].
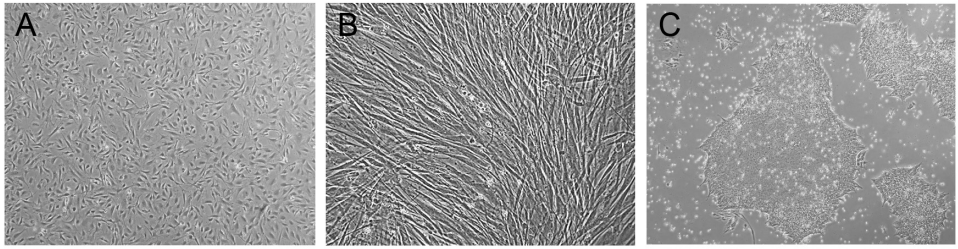
Figure 11. hESCs can be propagated on different substrates. A: mouse embryonic feeder cells, B: human feeder cells, and C: undifferentiated hES colony maintained on matrigel.

Since 1998, when the first hESC line was established by Thompson and colleagues [198], more than 400 new hESC lines are available worldwide. We have mainly used the hESC lines SA121 and SA181 (available from Cellartis AB, Gothenburg, Sweden) that have been derived and characterized in our laboratory [196] and the various HUES cell lines [207] available from the Melton laboratory, Harvard University, USA. The Gothenburg cell lines were originally passaged mechanically with a stem cell knife. However, as mechanically passaged cell lines grow more slowly and are more tedious to scale up these cell lines have been adapted to enzymatic dissociation with trypsin similar to the HUES cell lines. The enzymatic dissociated cell lines grow faster, and large amounts of cells can be obtained fairly quickly. Collagenase [208, 209], accutase [210] are other enzymes that are routinely used to passage the hESCs. The xeno-free alternatives are the old fashion mechanical way or with recombinant enzymes e.g. Trype Select [211] .
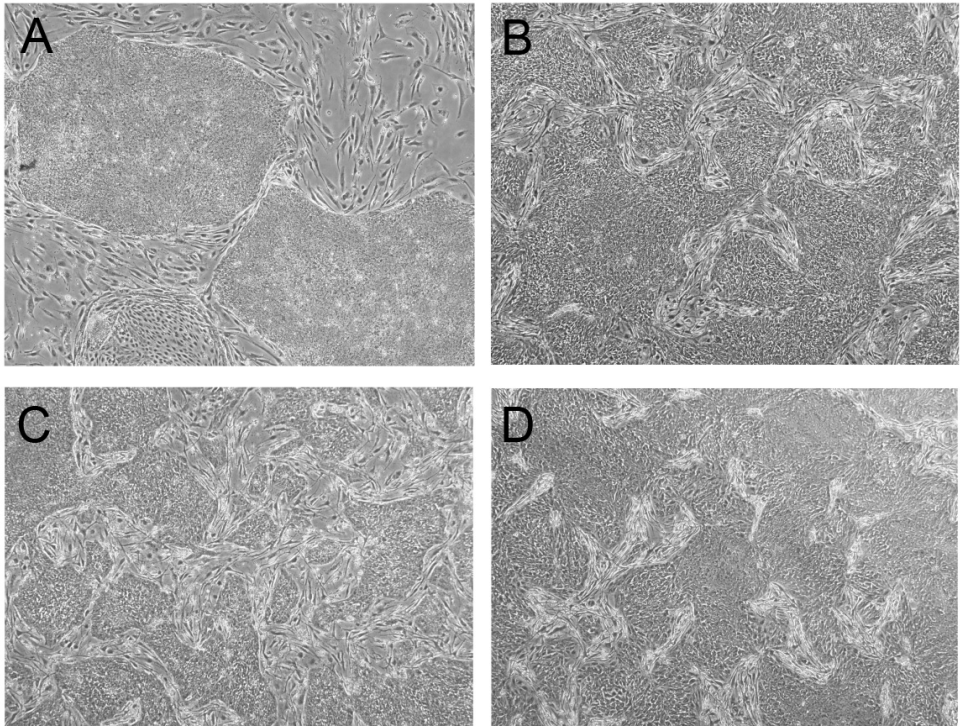
Figure 12. Morphology of different hES cell lines. A: mechanically passaged SA121, B: trypsin adapted SA181, C: HUES-4, D: trypsin adapted SA121.

However, hESC lines that have been passaged for extended periods tend to develop chromosomal aberrations [208, 212-214]. These karotypic changes often involve gain of chromosomes 12, 17q and 22 [208, 212, 213], which give a growth advantage. Importantly, the dissociation method seems to play a role in this. Specifically, passaging with trypsin appears to increase chromosomal aberrations in contrast to mechanical passaging [208, 213]. Hence, many researchers combine the mechanical and enzymatic dissociation methods for long-term maintenance. It is however important to keep in mind that the growth rate, karyotypic stability, and the propensity to differentiate into various germ layers differ between different hES cell lines [215].

hESC application

Because of their inherent capacity to self-renew and to form any cell types in the human body, hESCs present numerous possibilities for therapeutic applications. Transplantation of differentiated hESCs is not only of interest for curing Diabetes but also presents possibilities for treating other degenerative disorders such as Parkinson and Alzheimers. ES cells are also being investigated for treatment of various heart, liver, and blood cell diseases. In all these cases hESCs provide a replenishable source of transplantable cells. In addition, hESCs offer a great model for understanding different genetic disorders (as some hESC lines carry genetic disorders), but they also serve as model for cytotoxicity and drug screening [216, 217]. Last but not least, hESCs allow us to investigate and understand basic human development and biology.
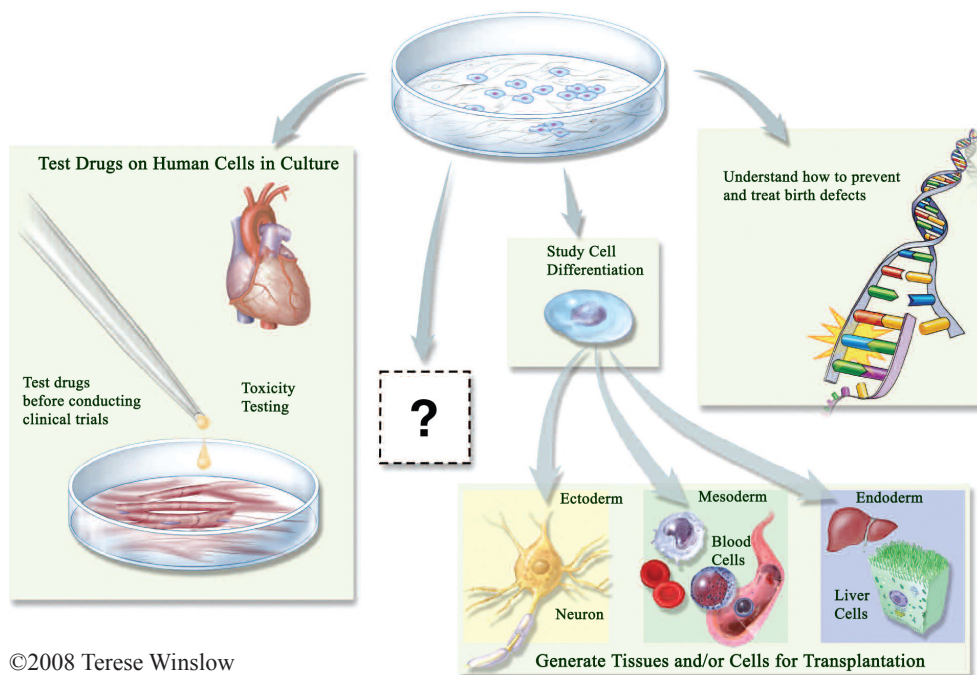

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Figure 13. hESCs can be used in various applications. The hESC system can be used as a model for understanding human genetic diseases, to study cell specification and to derive a variety of tissues or cell types for transplantation. In addition, they are also useful for drug development and toxicity screenings.

Directed differentiation of hESCs -Induction of definitive and pancreatic endoderm

hESCs have the potential to offer an endless supply of functional cell types for regenerative medicine. In fact numerous multistage protocols have been reported where mature foregut-derived cells such as hepatocytes and pancreatic insulin producing cells have been derived from hESCs [10-12, 217-219]. Unfortunately, the number of insulin positive cells generated so far is very few and in most cases these cells either fail to become glucose responsive or secrete insufficient amount of insulin. This is most likely due to that the cells do not demonstrate a correct beta cell phenotype. However, it is believed that if we can determine exactly what signals are normally required for specifying endodermal cells into beta cells, we may be able to apply this knowledge in vitro and recapitulate these events to generate functional beta cells from hESCs. Hence, the most successful strategy for directing the differentiation of the hESCs is based on mimicking signals used during embryonic pancreatic development.
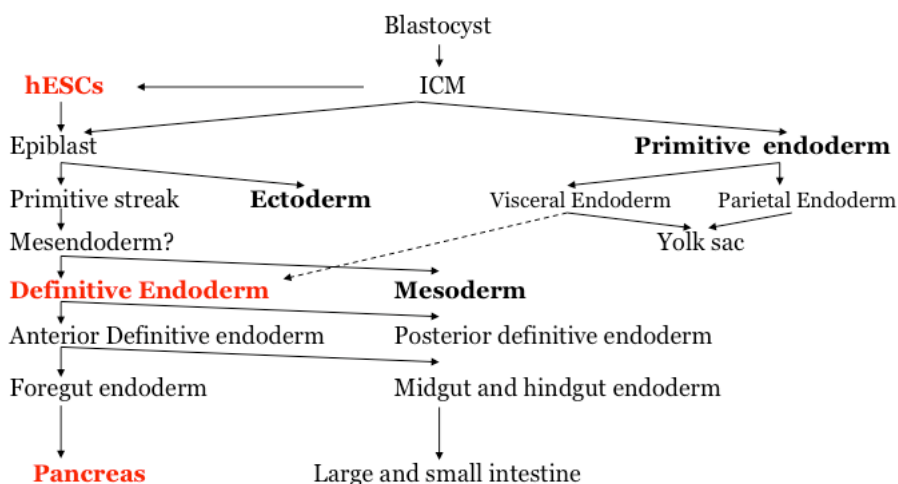

Figure 14. Simple overview of the developmental path from the blastocyst to pancreas.

The first step in this approach involves differentiation of hESCs into definitive endoderm. Signaling pathways controlling DE development are conserved between invertebrates and mammalians, including primates [20, 58]. Based on these studies it has been concluded that members of the TGF-beta family (Activin A and Nodal) are crucial for endoderm formation. Thus, mesendoderm/DE can be reliably generated

from both mouse and human ESCs through addition of Nodal or Activin A [22, 57, 92-94]. Other growth factor families that have been suggested to have a role in endoderm formation include Wnts [92] and BMPs [78, 220, 221]. Furthermore it has also been suggested that inhibition of the PI3-kinase pathway is required for differentiation of hESCs into definitive endoderm [13, 222]. Specifically, it has been shown that the presence of serum can activate the PI3-kinase pathway and in turn inhibit DE induction [222]. Thus, the most efficient DE induction protocol for hESCs today, involves treatment with high concentration of Activin A in combination with Wnt3a under low serum conditions [9]. Using this protocol we are able to generate more than 90% SOX17/FOXA2 double positive cells. Moreover, these cells are characterized by up-regulated levels of other endoderm specific markers such as GSC, CER and CXCR4. Through immunofluorescence stainings we have also been able to confirm co-expression of CXCR4 in the SOX17+ cells. As many of the available genetic markers are expressed in both definitive endoderm and visceral endoderm it is critical that DE formation is confirmed through characterization of multiple markers. Recently, two novel small chemical compounds (IDE1 and IDE2) have also been presented with the capacity to induce DE in both mouse and human ESCs. Part of their activity seems to also involve activation of the TGF-beta pathway similar to Activin A [223]. However, we have so far not been able to reproduce this data with our cell lines, it remains to be seen if this is achievable in other laboratories.
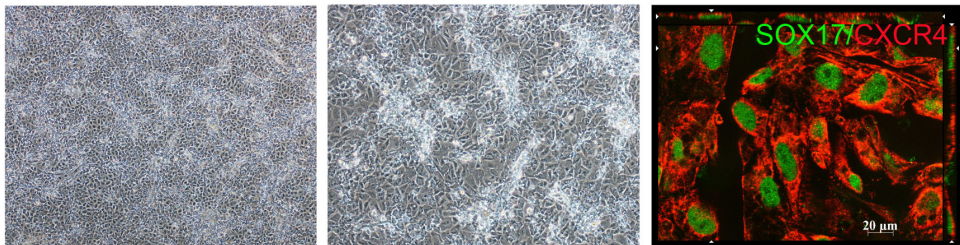

Figure 15. DE induction in hESCs. The two phase contrast pictures to the left show the morphology of the hESCs at day 3 after DE induction with Activin A and Wnt3a. The staining to the right shows a SOX17/CXCR4 co-staining. These double positive cells characterize the hESC-derived DE.

Next step is to direct hESC-derived DE into multipotent PDX1 expressing pancreatic progenitor cells via primitive gut endoderm. Removal of Activin A after the DE

induction has been shown to be necessary for formation of hESC-derived primitive gut endoderm, which is characterized by HNF1β and HNF4α expression [9]. The two of the most successful strategies reported recently [9, 12] include the addition of FGFs, RA and a SHH inhibitor after DE induction to obtain PDX1+ and NGN3+ endocrine progenitors, respectively. Although the addition of these factors was based on previous studies on vertebrate pancreas development [18, 82, 84, 192, 224, 225] only 7% insulin positive cells were obtained during in vitro differentiation. This suggests that either additional cues are required or that the timing/concentrations of the added factors are suboptimal. Furthermore, co-expression analysis revealed that the insulin+ cells resemble immature fetal β-cells as they also expressed multiple hormones such as glucagon and somatostatin and were only minimally responsive to glucose [9]. In contrast, if the PDX1+ pancreatic progenitor cells were transplanted into SCID mice, insulin-secreting cells that are glucose responsive were obtained [12]. Importantly, these hESC-derived insulin+ cells were able to rescue hyperglycemia induced by streptozotocin [12]. This demonstrates that these progenitor cells are capable of giving rise to insulin positive cells in the presence of the correct stimuli. Although, it remains to be seen which additional signals are required for efficient differentiation of the PDX1+ cells into true endocrine progenitors and insulin+ cells in vitro, the most recent publication by Zhang et al [14] shed some light on the matter. In this study DE induction was performed with Activin A and the PI3 kinase inhibitor Wortmannin, resulting in a culture comprised of >95% CXCR4/SOX17 double positive cells. Similar to the study by Kroon et al they also used FGF7 in combination with RA and NOGGIN (a BMP4 antagonist) to specify hESC-derived definitive endoderm into PDX1+ pancreatic endoderm. By applying EGF for additional five days they succeeded in expanding these progenitor cells and also obtaining significantly more proliferating PDX1+ cells characterized by Ki67 co-expression. Additional treatment with four other factors (Nicotinamide, bFGF, Extendin-4 and BMP4) resulted in maturation of these cells into 25% insulin producing cells with the same gene expression pattern as mature beta cells. This is the most successful strategy reported so far on obtaining hESC-derived insulin+ cells.

Interestingly, the same group has now also developed a strategy to differentiate hESCs into a 70% pure PDX1+ cell population [226]. This high amount of PDX1+

cells has previously not been reported. In this study, the hESC-derived definitive endoderm cell population was dissociated and replated at low cell density before additional treatment was performed. The authors hypothesized that the low percentage of PDX1+ cells obtained by previous protocols were due to the inhibitory effect of cell-cell interaction and that this could be avoided if the cells were replated at low density. Further treatment of the replated cells resulted in approximately 70% PDX1+ cells, whereas in the absence of RA signaling the cells differentiated into a hepatic fate, characterized by ALB and AFP expression. Moreover, by sorting the Activin A induced cells with CXCR4 prior to replating and treatment with RA they succeed in obtaining a homogenous cell population consisting of more than 90% PDX1+ cells! Interestingly, by testing the optimal time window for RA addition, they could conclude that RA only has an inhibitory effect on hepatic induction if it is added within 2 days after replating. This inhibitory effect on hepatic induction is believed to occur through inhibition of the BMP downstream targets Smad1/5/8. But since Noggin, which also inhibits Smad1/5/8 activity was not enough to induce pancreatic differentiation, this implies that RA activates/inhibits other signaling pathways necessary for inducing pancreatic endoderm. It is speculated that RA mediates pancreatic induction through PKC (protein kinase C) signaling. Although PKC signaling has not been reported in pancreatic embryogenesis, the following observations suggest a role for PKC during pancreas development: I) RA can directly bind to PKCalpha and activate PKC signaling [227, 228], II) the small chemical compound Indolactam V (ILV) induce pancreatic progenitor cells in hESCs by activating PKC signaling [229]. Briefly, ILV in combination with FGF10, RA and cyclopamine direct differentiation of hESCs into 45% PDX1+ cells. Furthermore, few insulin-expressing cells (less than 7%) were generated after transplantation in SCID mice. By using PKC inhibitors it was demonstrated that ILV induces pancreatic specification via PKC signaling [229]. However, additional studies are required to establish the precise role of PKC signaling during pancreatic specification.
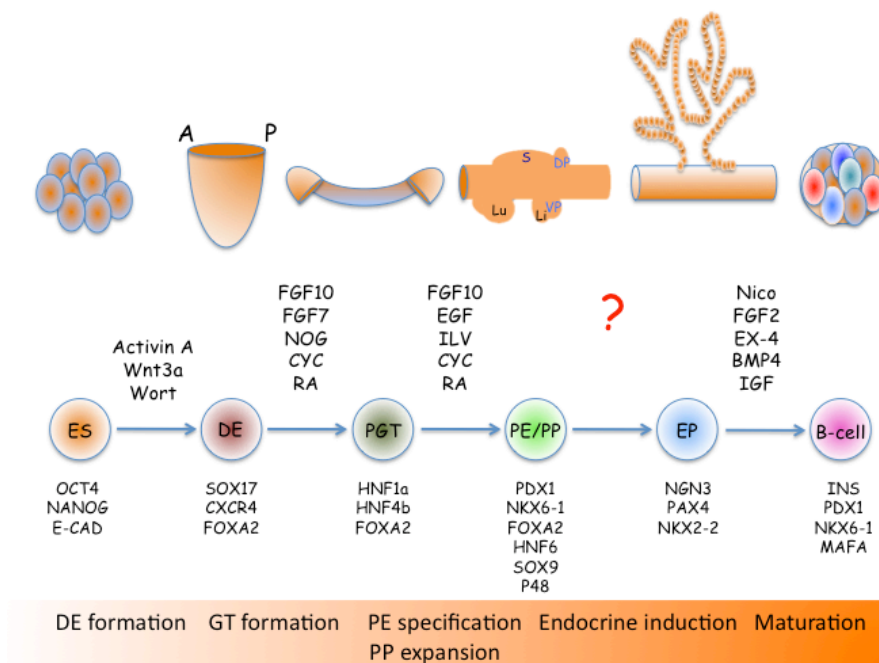
Figure 16. Step-wise differentiation of hESCs based on pancreatic development in mouse. hEScs are differentated into definitive endoderm (DE) by the TGF-beta member Activin A in combination with Wnt3a or the PI3-kinase inhibitor wortmannin. The small molecules IDE-1 and IDE-2 have also been reported to have the capacity to differentiate hESCs towards DE. DE is patterned into a primitive gut tube state by the removal of Activin A. However, many researchers add FGFs, BMP or SHH inhibitors such as Noggin (NOG) and Cyclopamine (CYC) in combination with RA to facilitate this. Pancreatic specification and expansion are achieved by basically the same factors used during the previous stage. The small molecule ILV can also be used at this stage. Currently there is no specific cocktail of growth factors used for differentiation of PDX1+ progenitor cells into NGN3+ endocrine progenitor cells. Based on mouse biology we are however aware that inhibition of the NOTCH pathway is important for accomplishing this. The finale step involves maturation of the endocrine progenitors into insulin+ cells. Some of the factors used in this step are Nicotinamide (Nico), FGF2, Extendin-4 (Ex-4), BMP4 and IGF.

Lately, substantial progress has been made in directing the hESC-derived definitive endoderm into PDX1+ pancreatic progenitors and even insulin+ cells. Notably, all these protocols involve recapitulation of the key events that normally occur during embryogenesis. The majority of the published protocols result in heterogeneous populations of PDX1+ cells except for the most recent report from Deng and colleagues [226]. The absence of correct inductive signals required for human pancreas specification and the presence of inhibiting signals from adjacent cells

could explain the low number of PDX1+ cells obtained in the previously published protocols. The latter is strengthened by the recent findings by Cai et al that suggest proper cell-cell interactions are crucial during/after DE induction to achieve a prominent source of PDX1+ pancreatic progenitor cells. For example, repressive signals could explain the difficulty to reproducibly generate β-cells from ES cells. Duville and co-authours recently demonstrated that the mesenchyme have a repressive effect both upstream and downstream of Ngn3 [115]. Specifically, they showed that in the absence of mesenchyme Ngn3 is induced followed by β-cell differentiation whereas in the presence of mesenchyme Ngn3 induction is delayed and few β-cells develop. In addition to its repressive effect upstream of Ngn3, the mesenchyme acted downstream of Ngn3 by repressing the differentiation of Ngn3+ cells into β-cells. Notably, whereas a direct cell-cell contact between the mesenchyme and epithelium was necessary for repressed Ngn3 expression, the β-cell differentiation downstream of Ngn3 was inhibited by soluble factors from the mesenchyme. If we can identify these repressive signals from adjacent cells and override them a more efficient in vitro differentiation protocol should be achievable. In conclusion, it remains to be seen if the recent exciting findings described above can be reproduced. Although, the two protocols by Baetge et al are major breakthroughs, others have so far not been able to reproduce these protocols with other cell lines. Hence, it is important to establish new differentiation strategies to obtain PDX1+ pancreatic progenitor cells in a more reliable manner.

AIM OF THIS THESIS

The overall objective of this thesis was to develop robust strategies for differentiating hESCs into pancreatic endoderm.

The specific aims were:

Paper I.   To characterize the role of FGF2 in A-P patterning and specification of hESC-derived foregut endoderm.

Paper II.   To investigate the role of FGF4 and retinoic acid (RA) in specification of the hESC-derived definitive endoderm. In particular, to assess whether FGF4, similar to what has been observed in chick and mouse, exhibits a broad A-P patterning activity during human endoderm development

Paper III.   To establish a method for gene targeting by homologous recombination to generate hESC reporter cell lines.

PAPERS IN SUMMARY

PAPER I

Rationale

While an inductive role of FGF2 during dorsal pancreas formation has been demonstrated in mouse and chick, ventral pancreas induction proceeds in the absence of cardiac mesoderm and FGFs. Furthermore, although FGF2 lacks a broad anterior-posterior patterning activity in vivo, a more restricted patterning activity within the ventral foregut endoderm has been established. FGF2 secreted from the cardiac mesoderm, patterns the adjacent multipotent ventral foregut endoderm in a concentration-dependent manner into liver and lung. The aim of the present study was to investigate whether FGF2 controls anterior-posterior patterning and specification of hESC-derived foregut endoderm.

Experimental design

The differentiation protocol was divided in two parts: first hESCs were induced into definitive endoderm according to the "D'Amour protocol" [92]. RNA samples were taken at day 3 and analysed for various anterior definitive endoderm markers with QPCR. Immunofluorescence analysis with SOX17 and FOXA2 were also performed. Finally, the hESC-derived definitive endoderm was exposed to different concentrations of FGF2 (0, 4, 16, 32, 64 and 256 ng/ml) during eight additional days. As cell survival has been an issue in the previously tested protocols a different medium composition (KO-DMEM + 12% KoSr) than the one used in the D'Amour protocol was used for the second step. The effect of FGF2 was determined by collection of RNA samples at different time points (days 9-11). These samples were then analyzed for different markers expressed along the gut tube. Immunofluorescence analysis was also performed on the differentiated hESCs at day 11.

Results

Anterior definitive endoderm associated markers were up-regulated at day 3
SOX17, CXCR4, CER1, GSC, HHEX and FOXA2 expression was up-regulated in the Activin A/Wnt3a treated samples confirming efficient DE induction.

Increasing FGF2 concentrations inhibits hepatic cell fate

Hepatic associated markers Albumin, HNF4a, ONECUT1 were up-regulated at day 11 in the control samples treated with AA/Wnt3a and to a lesser extent in the samples treated with 4 ng/ml FGF2. Albumin stainings in the samples treated with different FGF2 concentrations confirmed that hepatic fate is inhibited with increasing FGF2 concentrations. Moreover, ALB/AFP double stainings of the control samples and the samples treated with 4 ng/ml FGF2 showed that only a fraction of the AFP+ cells express ALB, suggesting that these are immature hepatocytes.

Intermediate FGF2 concentration (64 ng/ml) induces PDX1+/NKX6-1+ pancreatic progenitor cells

Since Pdx1+ cells are also found in the posterior stomach and the duodenum, the expression level of several pancreatic markers (PDX1, SOX9, NKX6-1 and FOXA2) was assessed both at RNA and protein level. PDX1 expression was significantly up-regulated at 64 ng/ml and this was also the case for NKX6-1. Moreover, SOX9 expression was up-regulated with increasing FGF2 concentration while FOXA2 expression remained unchanged. In addition, PDX1 expression was confirmed at protein level and the highest number of PDX1+ cells (17%) was obtained at 64 ng/ml consistent with the mRNA data. Further characterization of the PDX1+ cell population at 64 ng/ml showed that while the majority of the PDX1+/FOXA2+ cells also co-expressed SOX9 and ONECUT1, a fraction (9%) of these cells co-expressed NKX6-1 reminiscent of pancreatic endoderm. However, the majority of the PDX1+ cells were non- proliferating. The PDX1+ cell population at 64 ng/ml resembled the PDX1+ cells obtained at 256 ng/ml with the exception that no NKX6-1 expression could be detected in the latter.

High FGF2 concentrations promote pulmonary and intestinal cell fates

Although, PDX1+ cells were also observed at 256 ng/ml, the lack of NKX6-1 expression suggests that they are of non-pancreatic origin. Furthermore, the expression of hepatic markers was also significantly decreased at this concentration. Instead, the expression of the anterior foregut associated marker SOX-2 was up-regulated at 256ng/ml. The expression pattern of markers associated with various anterior foregut derivatives (e.g. lung and thyroid) was hence assessed at the mRNA level. Concomitant up-regulation of NKX2-1 (earliest marker of lung and

thyroid specification) together with down-regulation of the thyroid specific marker thyroglobulin (TG) indicated induction of pulmonary cell types. The more mature lung markers CC10 and SP-C were undetectable, whereas other markers associated with pulmonary induction such as FGF10, SHH, and PTCH1 were all up-regulated.

Interestingly, expression of the midgut/hindgut markers CDX2 and MNX1 were also significantly increased at 256 ng/ml FGF2, suggesting a simultaneous induction of intestinal cell types. Consistent with the mRNA measurements the highest number of CDX2+ cells was obtained at 256 ng/ml. Moreover, co-stainings with FOXA2 and MKI67 showed that all of the CDX2+ cells co-expressed FOXA2 whereas 50% were MKI67+. These results suggest that the CDX2+ cells are endodermal and that they are formed by proliferation and differentiation.

Further characterization of the PDX1+/NKX6-1 cell population showed that whereas none of the PDX1+ cells co-expressed SOX2 (excluding cells in the stomach), a few PDX1+/CDX2+ duodenal cells were seen. Furthermore, there was no overlap between the CDX2+ and the SOX2+ cells.

FGF2 induced PDX1 induction occurs via the MAPK pathway
By monitoring FGFR expression at the mRNA level we concluded that FGFR1 and 3 were up-regulated, whereas FGFR2 and 4 were down-regulated, with increasing FGF2 concentration. To determine whether FGFR-mediated signaling is required for the induction of PDX1 transcription, the effect of the FGFR tyrosine kinase inhibitor SU5402, MAPK inhibitor, U1026, and PI3K inhibitor, LY294002, was investigated. Simultaneous treatment with FGF2 and the different inhibitors showed that PDX1 expression was significantly inhibited in the presence of U1026 and SU5402 while LY294002 had no effect. This demonstrates that the FGFRs and the MAPK pathway are crucial for the FGF2-mediated PDX1 induction.

Reproducibility
Robust induction of PDX1 and NKX6-1 mRNA expression at 64 ng/ml FGF2 was confirmed in multiple experiments using five different hESC lines (HUES-3, HUES-4, HUES-15, SA121tryp and SA181tryp) to avoid cell line specificity.

Conclusions

FGF2 posses a broader patterning activity in hESCs than what has previously been suggested based on studies on animal studies. Specifically, FGF2 has the capacity to specify the hESC-derived foregut endoderm into various gut endodermal derivatives in a concentration dependent manner. Low FGF2 levels promote hepatic cell fate, whereas intermediate and high FGF2 levels induce pancreatic and pulmonary cell fates, respectively. Importantly, high FGF2 concentrations also promote differentiation into intestinal progentiors, suggesting a posteriorizing activity. Finally, it is shown that the induction of PDX1+ pancreatic progenitors relies in part on FGF2-mediated activation of the MAPK signaling pathway.

Discussion

It is generally accepted that mesenddoerm/DE can be induced in both mouse and human ESCs by the addition of high concentrations of Activin A. However, there are still dissimilarities between the induced DE in mouse and human ESCs that needs to be addressed. One of these differences is lack of E-cadherin expression in hESC-induced DE. This could either imply a difference between these species or suggest that the Activin A induced hESCs might not actually represent bona fide DE and that additional signals are required. Furthermore, it still remains unclear whether this Activin A induced cell population represents a naïve or a pre-patterned endoderm. Based on this study we propose that the hESC-induced DE most likely represents a heterogeneous cell population, consisting of distinct foregut and midgut/hindgut progenitors. This is based on the observation that foregut and midgut/hindgut endodermal cell types characterized by FOXA2+/AFP+/ALB+ and FOXA2/AFP+/CDX2+ expression, respectively spontaneously appear in the samples treated with Activin A. Furthermore, we speculate that these progenitors respond differently to specific FGF2 concentrations. Specifically, whereas induction of hepatic fate occurs by default among the foregut progenitors and is sustained by low FGF2 levels, induction of pulmonary and pancreatic cell fates require specific concentrations of FGF2. This is also the case for the midgut/hindgut progenitors giving rise to intestinal cells, which are only significantly up-regulated at 256ng/ml.

Based on immunofluorescence stainings it could be concluded that the majority of the hepatic-like cells were immature as only a fraction of the AFP+ cells co-

expressed ALB. This was also the case for the pulmonary cells characterized by NKX2-1 expression as neither SP-C nor CC10 could be detected. To address whether the lack of mature lung markers was due to too low FGF2 concentration, 500 ng/ml FGF2 was also tested similar to studies in mouse [80, 180], however this resulted in no change. These observations suggest that additional inductive factors are required during human liver and lung development.

Studies involving mouse and chicks have established a role for FGF2 in dorsal pancreas induction, whereas induction of ventral pancreas in the ventral foregut endoderm occurs by default and in the absence of cardiac mesoderm secreted FGFs. This suggests that FGF2 induced PDX1+ cells in the hESCs most likely represent dorsal pancreatic endoderm but it cannot be excluded based on this study that the induction of ventral pancreas in humans also requires FGF2 signaling. Furthermore, we could conclude that the FGF2 induced PDX1 expression is mediated through the MAPK pathway.

In contrast to mouse studies, where high FGF2 concentrations induce pulmonary cell fate we could also observe a posteriorizing activity at this concentration. This was confirmed by CDX2 stainings, which also showed that while 52% of these cells are formed through proliferation the remaining cells arise through differentiation, confirming a FGF2 specific effect.

In summary, these findings suggest a broader patterning activity for FGF2 in human endoderm development in contrast to what has previously been advocated in mouse and chick models.

PAPER II

Rationale

Previous studies have established a role for FGF4 in anterior-posterior patterning of the endoderm. Furthermore, RA signaling has proven to be important for pancreas development and Pdx1 expression. Hence, the aim of this study was to investigate the role of FGF4 and RA in patterning of the hESC-derived definitive endoderm.

Experimental design

In the first step, hESCs were induced towards definitive endoderm according to D'Amour et al (2005). Activin A and Wnt3a were added to a confluent monolayer of undifferentiated hESCs in RPMI1640 medium containing low levels of serum. To confirm an efficient DE induction samples were taken 3 days later and analyzed by QPCR and immunofluorescence stainings

In the second step, the hESC-derived definitive endoderm was exposed to different concentrations of FGF4 to induce pancreatic endoderm. Low (0-10 ng/ml), intermediate (10, 25, 50 ng/ml) and high (0-100 ng/ml) concentrations of FGF4 were tested. The medium composition was mainly based on the D'Amour protocol from 2006 and involved DMEM supplemented with 2% FBS. The effect of FGF4 was determined by collection of RNA samples at different time points. These samples were then analyzed for pancreatic specific markers. Differentiated hESCs were also fixed with PFA for immunofluorescence analysis.

In the third step, the hESC-derived definitive endoderm was exposed to different concentrations of FGF4 in combination with RA. The same medium composition was used here as in step two and to assess the combinatorial effect of FGF4 and RA RNA samples were taken at different time points and analyzed with QPCR. Differentiated hESCs were also fixed with PFA for immunofluorescence analysis. Furthermore, cell viability was assessed by using the cell survival assay AlamarBlue.

Results

FGF4 alone has no patterning effect

FGF4 alone has no patterning effect on the hESC-derived definitive endoderm and no PDX1 expression could be detected at any concentration. This is in contrast to the in vivo studies done in mice and chick. Instead FGF4 increased cell viability in hESCs.

FGF4 in combination with RA induces PDX1 expression

As FGF4 alone did not have the capacity to induce pancreatic endoderm it was combined with RA due to its ability to induce pancreatic tissues. With this combination PDX1 expression was induced at day 12 and this was confirmed both at RNA and protein level. Since the RA receptor RARβ was up-regulated at day 4, RA was added immediately after the DE induction with Activin A and Wnt3a.

PDX1+ cells lack NKX6-1 expression but express other posterior foregut markers

By extending the original 12 days protocol to 16 days instead, a larger fraction of PDX1+ cells were obtained. The PDX1+ cell population was then characterized further as PDX1 is not only restricted to pancreatic foregut endoderm. This was primarily done by QPCR. The broad endodermal marker FOXA2 was expressed at all time points and unaffected by the FGF4/RA treatment. The expression of the midgut/hindgut marker CDX2 was however inconsistent between the different experiments. It was slightly up-regulated at days 12 and 16 in two experiments while it was down-regulated at day 16 in another experiment. SOX9 and HNF6 expression was also up-regulated at day 16 in all experiments, except for SOX9 that remained unchanged in one experiment. Notably, neither NKX6-1 nor PTF1α could be detected in the PDX1+ cells. Furthermore, based on AFP, Albumin and PROX1 expression it was evident that the control cells treated with Activin A and Wnt3a had adopted a hepatic cell fate.

Immunofluorescence analysis of PDX1+ cells showed that the majority of these cells co-express SOX9, HNF6, and FOXA2 and that very few of the PDX1+ cells were proliferative, as they lacked expression of the proliferation marker PH3. Moreover, PDX1 expression was manually quantified and determined to represent

32% of all cells. The PDX1 expression level was also compared to human islets and corresponded to 50%.

FGF4 and RA receptor expression
The FGF4 receptors FGFR1c and FGFR2c were both up-regulated at day 8 in the FGF4/RA protocol. RA receptor RARβ was up-regulated at day 4 and expression was maintained until day 8 before it declined.

Inhibition with the RA antagonist AGN193109 inhibits PDX1 expression
Addition of the RA antagonist AGN193109 between days 4 and 15 completely blocked PDX1 expression suggesting that RA is required for PDX1 induction. Interestingly, when RA was combined with the FGFR inhibitor SU5402, PDX1 expression was slightly down-regulated.

Reproducibility
The FGF4/RA protocol was reproduced in three separate experiments and in two different cell lines (HUES-3 and HUES-15) to confirm that PDX1 induction with FGF4/RA is reproducible and cell line independent.

Conclusions
The hESC-derived DE can be efficiently converted into PDX1+ foregut endoderm in the presence of FGF4 and RA. Furthermore, in contrast to the chick and mouse studies, FGF4 neither patterns hESC-derived definitive endoderm, nor induces PDX1+ pancreatic progenitors from hESC-derived DE, suggesting that FGF4 is not responsible for anterior-posterior patterning of the primitive gut during human development. Instead, FGF4's main role is to promote cell viability.

Discussion
As many of the published in vitro differentiation protocols have been difficult to reproduce in other laboratories, it is important to establish new differentiation strategies to obtain PDX1+ cells in a more reliable manner. Although, both RA and FGF4 have been implicated during endoderm patterning and pancreas induction, the combinatorial role of these factors during in vitro differentiation of hESCs have previously not been assessed. By testing out the optimal concentration and timing

of these factors individually and in combination we show that RA and FGF4 in a synergistic manner induce PDX1+ cells. Additionally, we show that in contrast to the in vivo situation, FGF4 does not influence anterior-posterior patterning of the gut endoderm and is alone not capable in inducing PDX1 from hESC-derived DE but instead promotes cell survival. Moreover, we show that RA is required for PDX1 induction in the hESCs as addition of the RA antagonist AGN193109 completely blocked PDX1 expression. Interestingly, part of RA's inducing activity on PDX1 expression is exerted by FGF-signaling. Importantly, whereas a high fraction of PDX1+ cells were obtained none of these cells co-expressed NKX6-1. Characterization of the PDX1+ cells suggests that these cells could represent a multipotent cell population within the foregut endoderm with the potential to give rise to pancreatic, posterior stomach, or duodenal cell types. Additional optimization is required to increase the number PDX1+ cells and to convert these cells into pancreatic progenitors co-expressing PDX1 and NKX6-1. Following changes in the protocol resulted in an increased number of PDX1+ cells however, still without expression of NKX6-1:

- Replacing the RPMI medium to DMEM during DE induction
- Combination of 2μM RA together with higher FGF4 concentration (100 ng/ml instead of 1.1ng/ml)
- Addition of B27 instead of FBS

Moreover, both FGF10 and cyklopamine were tested in different concentrations and combinations but resulted in no change in PDX1 and NKX6-1 expression levels.

PAPER III

Rationale

Reporter cell lines are extremely valuable tools when generating in vitro differentiation protocols. As described in the above-mentioned papers, hESC differentiation and expression of various tissue specific genes is routinely monitored through time consuming methods such as immunofluorescence stainings and QPCR. Hence, the aim of this paper was to establish an efficient method for establishing reporter cell lines in hESCs through gene targeting.

Experimental design

Briefly, the bacterial artificial chromosomes (BAC) RP11-426J19 (SOX 1), CTD-2252P3 (PTF1a) and CTD-2317D19 (NANOG) were obtained from Invitrogen and verified by restriction analysis. Using conventional restriction cloning two vectors containing the GFP/Neo^R reporter cassette (pEGFP-N1/bGl/loxP-Neo^R) and the TK cassette (pBR322/TK), respectively, were created. The recombineering experiments were done according to previously published protocols [230-233]. The reporter cassettes was inserted into the 5´ untranslated region (UTR) of the target gene and for retrieval, recombineering target sites were chosen at least 12kb upstream and 3kb downstream of the reporter cassette, creating the required homologous arms for gene targeting in hESCs. After retrieval, the final targeting constructs were verified by PCR, restriction analysis and sequencing.

The hESC lines HUES-1, HUES-3 and HUES-15 were used for transfections. 2-3 days after electroporation, G418 selection was initiated and maintained for additional 7-10 days. GFP positive clones were picked as described previously [234]. Targeted clones were detected by PCR and southern blot analysis. In addition, hESCs were differentiated with Activin A according to D'Amour et al (2005) to verify the functionality of the established NANOG reporter cell line.

Results

Generation of gene targeting vectors
Using BAC recombineering gene targeting vectors were created for the pluripotency

marker NANOG, the pancreatic transcription factor PTF1A (expressed in pancreatic progenitors), and for the neural precursor-specific marker SOX1.

Transfection efficiency in different hESC-lines
Three different hES cell lines (HUES-1, HUES-3 and HUES-15) were transfected with the Nanog targeting vector pNANOG$^{eGFP/NeoR}$. Out of 5 million transfected hESCs, 146 stable clones were obtained for HUES-1, and 91 for HUES-3. The number of stable transfectants was ten times less when HUES-15 was transfected with the same protocol as for HUES-1 and HUES-3. However, addition of the Rock-inhibitor Y-27632 before and after transfection increased the transfection efficiency five times. The presence of Rock-inhibitor had no effect on the transfection efficiency in HUES-1 and HUES-3.

NANOG gene targeting efficiency
The relative targeting frequency in the different hES cell lines were as follows: 18% in HUES-3, 0.3-1.2% in HUES-1 and 1.1-2.4% in HUES-15. Importantly, targeted clones were only obtained in HUES-15 when the Rock-inhibitor was used.

NANOG/eGFP co-expression in targeted clones
Immunofluorescence staining of targeted undifferentiated hESCs confirmed co-expression of NANOG and eGFP. Furthermore, upon differentiation both NANOG and eGFP expression was downregulated in NANOG targeted hESCs.

Karyotyping of targeted clones
Karyotype analysis was performed on three clones from HUES-15 and six clones from HUES-3. A normal diploid karyotype was observed in four of the clones, whereas a gain of material from chromosomes 12, 17 or 21 was observed in five clones.

Conclusions
An efficient method for obtaining gene targeting in hESCs using BAC recombineering technology is presented. Using this technology we created targeting vectors for three different transcription factors, namely NANOG, PTF1a and SOX1. Provided that suitable reporter cassettes are available, this method enables researchers to create

gene-targeting constructs within just few weeks and to establish hESC reporter lines within 10-14 weeks.

Discussion

Gene targeting is a powerful method to create reporter cell lines, which allow the monitoring of gene expression in living cells. Compared to traditional transgenic approaches BAC recombineering strategy allows the expression of the reporter gene from the intact and complete regulatory elements that control the expression of the target gene. Although homologous recombination has been used extensively in the mouse ES field, there are only a few reports of this in hESCs [235-238]. Hence, gene targeting is still technically challenging and far from being a routine-application in the hESCs. This is partly due to the difficulties encountered when transfecting hESCs [239]. Unlike mESCs, chemical reagents and ordinary electroporation based transfection strategies have not been as successful in hESCs, and the survival rate of stable transfectants is much lower for hESCs. In this study higher stable transfection efficiency was obtained in hESCs by optimizing transfection conditions and adding a Rho-associated kinase (ROCK)-inhibitor both prior and after transfections. Using this optimized transfection protocol three targeting vectors were created for the human genes NANOG, PTF1a and SOX1. In addition, gene targeting of the NANOG locus was accomplished in three different hESC lines. The relative targeting frequency for the NANOG locus ranged between 0,6 and 18 percent. This is comparable to previous reports on gene targeting in hESCs, using both isogenic and non-isogenic DNA [235, 237, 240-242]. However, one should keep in mind that targeting frequencies vary significantly between different loci and cell lines. The relative targeting frequency was approximately 10-fold higher in HUES-3 (probably due to a higher recombination efficiency) compared to HUES-1 and HUES-15. Interestingly, the addition of the ROCK-inhibitor Y-27632 increased the stable transfection efficiency in HUES-15 and yielded targeted clones whereas it had no effect on HUES-3 and HUES-1. This suggests that HUES-15 in comparison to the other cell lines is more sensitive to single cell dissociation and the following transfection induced cells death.

Furthermore, since homologous recombination is a very rare event and finding the correctly targeted clones could involve tedious screening a retrieval vector

containing the thymidine kinase (tk) gene was also created. As the TK gene will be inserted outside the homologous regions of the targeting construct, only true-targeted clones will survive treatment with the drug gancyclovir.

Reporter cell lines not only allow for real-time monitoring of gene expression in hESCs but they are also valuable tools to establish directed differentiation protocols. In addition, they can also be used for screening purposes that could allow the identification of differentiation-inducing molecules. Furthermore, the reporter gene-expressing hESCs can be isolated and hence a purified cell population can then be cultured further, used for additional gene expression analysis or transplanted.

CONCLUDING REMARKS

Here, we show for the first time to our knowledge that FGF4 alone neither patterns hESC-derived definitive/foregut endoderm, nor induces PDX1+ pancreatic progenitors. This observation is in contrast to studies in chick and mouse models where it posses a high A-P patterning activity. Instead, we found a broad patterning activity for FGF2, which has previously only been suggested to have a restricted activity within the ventral foregut endoderm. Specifically, FGF2 specifies hESC-derived DE into foregut/midgut organ-specific lineages, such as hepatic, pancreatic, pulmonary, and intestinal progenitors, in a concentration-dependent manner, whereas FGF4 only promotes cell viability. These findings show that whereas FGF2's function in foregut endoderm patterning is conserved between humans and mice, FGF2 also possess a posteriorizing activity within the human primitive gut endoderm. Altogether, these results suggest that FGF4 is not responsible for anterior-posterior patterning of the primitive gut during human development and that FGF4's role in foregut-midgut patterning may have been replaced by FGF2 during evolution. Additionally, this discrepancy may be explained by the fact that previous studies in mouse and chick only examined the effects of low FGF2 concentrations (1-10 ng/ml) [62, 70, 72]. Finally, we demonstrate that both RA and FGF2 are required for the induction of PDX1+ cells as PDX1 expression is completely blocked when RA and FGFR antagonists, respectively, are added. Furthermore, as the induction of the FGF2 induced PDX1+ pancreatic progenitors relies on activation of the MAPK signaling pathway it would be interesting to test if an active MAPK pathway is also necessary for PDX1 induction by RA.

Although homologous recombination has been used extensively in the mouse ES field, there are only a few reports of this in hESCs [235, 236]. Using BAC technology we have established an efficient method for gene targeting in hESCs. This knowledge will be used to create additional reporter cell lines for various pancreatic markers to facilitate our efforts in establishing a reproducible in vitro differentiation protocol for pancreatic progenitors and finally insulin producing cells.

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REFERENCES

1. Limbert, C., et al., Beta-cell replacement and regeneration: Strategies of cell-based therapy for type 1 diabetes mellitus. Diabetes Res Clin Pract, 2008. 79(3): p. 389-99.

2. Shapiro, A.M., et al., Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med, 2000. 343(4): p. 230-8.

3. Ryan, E.A., et al., Five-year follow-up after clinical islet transplantation. Diabetes, 2005. 54(7): p. 2060-9.

4. Dor, Y., et al., Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature, 2004. 429(6987): p. 41-6.

5. Xu, X., et al., Beta cells can be generated from endogenous progenitors in injured adult mouse pancreas. Cell, 2008. 132(2): p. 197-207.

6. Inada, A., et al., Carbonic anhydrase II-positive pancreatic cells are progenitors for both endocrine and exocrine pancreas after birth. Proc Natl Acad Sci U S A, 2008. 105(50): p. 19915-9.

7. Butler, A.E., et al., Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes, 2003. 52(1): p. 102-10.

8. Bonner-Weir, S., et al., In vitro cultivation of human islets from expanded ductal tissue. Proc Natl Acad Sci U S A, 2000. 97(14): p. 7999-8004.

9. D'Amour, K.A., et al., Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol, 2006. 24(11): p. 1392-401.

10. Jiang, J., et al., Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells, 2007. 25(8): p. 1940-53.

11. Jiang, W., et al., In vitro derivation of functional insulin-producing cells from human embryonic stem cells. Cell Res, 2007. 17(4): p. 333-44.

12. Kroon, E., et al., Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol, 2008. 26(4): p. 443-52.

13. Zhang, D., et al., Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells. Cell Res, 2009. 19(4): p. 429-38.

14.   Zhang, D., et al., Generation of pancreatic islet cells from human embryonic stem cells. Sci China C Life Sci, 2009. 52(7): p. 615-21.

15.   Takahashi, K. and S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 2006. 126(4): p. 663-76.

16.   Zhou, H., et al., Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell, 2009. 4(5): p. 381-4.

17.   Rossant, J. and P.P. Tam, Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. Development, 2009. 136(5): p. 701-13.

18.   Wells, J.M. and D.A. Melton, Vertebrate endoderm development. Annu Rev Cell Dev Biol, 1999. 15: p. 393-410.

19.   Lu, C.C., J. Brennan, and E.J. Robertson, From fertilization to gastrulation: axis formation in the mouse embryo. Curr Opin Genet Dev, 2001. 11(4): p. 384-92.

20.   Grapin-Botton, A. and D. Constam, Evolution of the mechanisms and molecular control of endoderm formation. Mech Dev, 2007. 124(4): p. 253-78.

21.   Tam, P.P. and D.A. Loebel, Gene function in mouse embryogenesis: get set for gastrulation. Nat Rev Genet, 2007. 8(5): p. 368-81.

22.   Gadue, P., et al., Germ layer induction from embryonic stem cells. Exp Hematol, 2005. 33(9): p. 955-64.

23.   Gardner, R.L., Investigation of cell lineage and differentiation in the extraembryonic endoderm of the mouse embryo. J Embryol Exp Morphol, 1982. 68: p. 175-98.

24.   Fujikura, J., et al., Differentiation of embryonic stem cells is induced by GATA factors. Genes Dev, 2002. 16(7): p. 784-9.

25.   Nichols, J., et al., Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell, 1998. 95(3): p. 379-91.

26.   Feldman, B., et al., Requirement of FGF-4 for postimplantation mouse development. Science, 1995. 267(5195): p. 246-9.

27.   Niswander, L. and G.R. Martin, Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. Development,

1992. 114(3): p. 755-68.

28.   Rappolee, D.A., et al., Expression and function of FGF-4 in peri-implantation development in mouse embryos. Development, 1994. 120(8): p. 2259-69.

29.   Arman, E., et al., Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc Natl Acad Sci U S A, 1998. 95(9): p. 5082-7.

30.   Wilder, P.J., et al., Inactivation of the FGF-4 gene in embryonic stem cells alters the growth and/or the survival of their early differentiated progeny. Dev Biol, 1997. 192(2): p. 614-29.

31.   Arnold, S.J. and E.J. Robertson, Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. Nat Rev Mol Cell Biol, 2009. 10(2): p. 91-103.

32.   Duncan, S.A., et al., Expression of transcription factor HNF-4 in the extraembryonic endoderm, gut, and nephrogenic tissue of the developing mouse embryo: HNF-4 is a marker for primary endoderm in the implanting blastocyst. Proc Natl Acad Sci U S A, 1994. 91(16): p. 7598-602.

33.   Chen, W.S., et al., Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. Genes Dev, 1994. 8(20): p. 2466-77.

34.   Barbacci, E., et al., Variant hepatocyte nuclear factor 1 is required for visceral endoderm specification. Development, 1999. 126(21): p. 4795-805.

35.   Coffinier, C., et al., Essential role for the homeoprotein vHNF1/HNF1beta in visceral endoderm differentiation. Development, 1999. 126(21): p. 4785-94.

36.   Morrisey, E.E., et al., GATA6 regulates HNF4 and is required for differentiation of visceral endoderm in the mouse embryo. Genes Dev, 1998. 12(22): p. 3579-90.

37.   Coucouvanis, E. and G.R. Martin, BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. Development, 1999. 126(3): p. 535-46.

38.   Sirard, C., et al., The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. Genes Dev, 1998. 12(1): p. 107-19.

39.   Tam, P.P. and R.R. Behringer, Mouse gastrulation: the formation of a mammalian body plan. Mech Dev, 1997. 68(1-2): p. 3-25.

40.   Solnica-Krezel, L., Conserved patterns of cell movements during vertebrate gastrulation. Curr Biol, 2005. 15(6): p. R213-28.

41.   Barrow, J.R., et al., Wnt3 signaling in the epiblast is required for proper orientation of the anteroposterior axis. Dev Biol, 2007. 312(1): p. 312-20.

42.   Kispert, A. and B.G. Herrmann, Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. Dev Biol, 1994. 161(1): p. 179-93.

43.   Hart, A.H., et al., Mixl1 is required for axial mesendoderm morphogenesis and patterning in the murine embryo. Development, 2002. 129(15): p. 3597-608.

44.   Crossley, P.H. and G.R. Martin, The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development, 1995. 121(2): p. 439-51.

45.   Shamim, H. and I. Mason, Expression of Fgf4 during early development of the chick embryo. Mech Dev, 1999. 85(1-2): p. 189-92.

46.   Rivera-Perez, J.A. and T. Magnuson, Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. Dev Biol, 2005. 288(2): p. 363-71.

47.   Wang, Y. and H. Steinbeisser, Molecular basis of morphogenesis during vertebrate gastrulation. Cell Mol Life Sci, 2009. 66(14): p. 2263-73.

48.   Kosaka, N., et al., Pleiotropic function of FGF-4: its role in development and stem cells. Dev Dyn, 2009. 238(2): p. 265-76.

49.   Ciruna, B. and J. Rossant, FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell, 2001. 1(1): p. 37-49.

50.   Batlle, E., et al., The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol, 2000. 2(2): p. 84-9.

51.   Cano, A., et al., The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol, 2000. 2(2): p. 76-83.

52.   Tam, P.P., et al., The allocation of epiblast cells to the embryonic heart and

other mesodermal lineages: the role of ingression and tissue movement during gastrulation. Development, 1997. 124(9): p. 1631-42.

53.   Gilbert, S.F., Developmental Biology, Seventh Edition. 2003.

54.   Conlon, F.L., et al., A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. Development, 1994. 120(7): p. 1919-28.

55.   Sun, X., et al., Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. Genes Dev, 1999. 13(14): p. 1834-46.

56.   Winnier, G., et al., Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev, 1995. 9(17): p. 2105-16.

57.   Tada, S., et al., Characterization of mesendoderm: a diverging point of the definitive endoderm and mesoderm in embryonic stem cell differentiation culture. Development, 2005. 132(19): p. 4363-74.

58.   Lewis, S.L. and P.P. Tam, Definitive endoderm of the mouse embryo: formation, cell fates, and morphogenetic function. Dev Dyn, 2006. 235(9): p. 2315-29.

59.   Morrison, G.M., et al., Anterior definitive endoderm from ESCs reveals a role for FGF signaling. Cell Stem Cell, 2008. 3(4): p. 402-15.

60.   Kwon, G.S., M. Viotti, and A.K. Hadjantonakis, The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. Dev Cell, 2008. 15(4): p. 509-20.

61.   Deutsch, G., et al., A bipotential precursor population for pancreas and liver within the embryonic endoderm. Development, 2001. 128(6): p. 871-81.

62.   Wells, J.M. and D.A. Melton, Early mouse endoderm is patterned by soluble factors from adjacent germ layers. Development, 2000. 127(8): p. 1563-72.

63.   Shawlot, W., J.M. Deng, and R.R. Behringer, Expression of the mouse cerberus-related gene, Cerr1, suggests a role in anterior neural induction and somitogenesis. Proc Natl Acad Sci U S A, 1998. 95(11): p. 6198-203.

64.   Yasunaga, M., et al., Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat Biotechnol, 2005. 23(12): p. 1542-50.

65.   Dufort, D., et al., The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. Development, 1998. 125(16): p. 3015-25.

66.   Kanai-Azuma, M., et al., Depletion of definitive gut endoderm in Sox17-null mutant mice. Development, 2002. 129(10): p. 2367-79.

67.   McGrath, K.E., et al., Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol, 1999. 213(2): p. 442-56.

68.   Beck, F., et al., Expression of Cdx-2 in the mouse embryo and placenta: possible role in patterning of the extra-embryonic membranes. Dev Dyn, 1995. 204(3): p. 219-27.

69.   Grapin-Botton, A. and D.A. Melton, Endoderm development: from patterning to organogenesis. Trends Genet, 2000. 16(3): p. 124-30.

70.   Dessimoz, J., et al., FGF signaling is necessary for establishing gut tube domains along the anterior-posterior axis in vivo. Mech Dev, 2006. 123(1): p. 42-55.

71.   Ang, S.L. and J. Rossant, Anterior mesendoderm induces mouse Engrailed genes in explant cultures. Development, 1993. 118(1): p. 139-49.

72.   Kumar, M., et al., Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. Dev Biol, 2003. 259(1): p. 109-22.

73.   Kim, S.K., M. Hebrok, and D.A. Melton, Notochord to endoderm signaling is required for pancreas development. Development, 1997. 124(21): p. 4243-52.

74.   Soria, B., In-vitro differentiation of pancreatic beta-cells. Differentiation, 2001. 68(4-5): p. 205-19.

75.   Lammert, E., O. Cleaver, and D. Melton, Induction of pancreatic differentiation by signals from blood vessels. Science, 2001. 294(5542): p. 564-7.

76.   Fukuda, K. and S. Yasugi, Versatile roles for sonic hedgehog in gut development. J Gastroenterol, 2002. 37(4): p. 239-46.

77.   Shin, D., et al., Bmp and Fgf signaling are essential for liver specification in zebrafish. Development, 2007. 134(11): p. 2041-50.

78.   Tiso, N., et al., BMP signalling regulates anteroposterior endoderm patterning in zebrafish. Mech Dev, 2002. 118(1-2): p. 29-37.

79.   Jung, J., et al., Initiation of mammalian liver development from endoderm by fibroblast growth factors. Science, 1999. 284(5422): p. 1998-2003.

80.   Serls, A.E., et al., Different thresholds of fibroblast growth factors pattern the ventral foregut into liver and lung. Development, 2005. 132(1): p. 35-

47.

81.   McLin, V.A., S.A. Rankin, and A.M. Zorn, Repression of Wnt/beta-catenin signaling in the anterior endoderm is essential for liver and pancreas development. Development, 2007. 134(12): p. 2207-17.

82.   Molotkov, A., N. Molotkova, and G. Duester, Retinoic acid generated by Raldh2 in mesoderm is required for mouse dorsal endodermal pancreas development. Dev Dyn, 2005. 232(4): p. 950-7.

83.   Stafford, D. and V.E. Prince, Retinoic acid signaling is required for a critical early step in zebrafish pancreatic development. Curr Biol, 2002. 12(14): p. 1215-20.

84.   Hebrok, M., S.K. Kim, and D.A. Melton, Notochord repression of endodermal Sonic hedgehog permits pancreas development. Genes Dev, 1998. 12(11): p. 1705-13.

85.   Kim, S.K., et al., Activin receptor patterning of foregut organogenesis. Genes Dev, 2000. 14(15): p. 1866-71.

86.   Reed, R.A., et al., Morphogenesis of the primitive gut tube is generated by Rho/ROCK/myosin II-mediated endoderm rearrangements. Dev Dyn, 2009. 238(12): p. 3111-3125.

87.   Keng, V.W., et al., Homeobox gene Hex is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. Biochem Biophys Res Commun, 2000. 276(3): p. 1155-61.

88.   Martinez Barbera, J.P., et al., The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. Development, 2000. 127(11): p. 2433-45.

89.   Hallonet, M., et al., Maintenance of the specification of the anterior definitive endoderm and forebrain depends on the axial mesendoderm: a study using HNF3beta/Foxa2 conditional mutants. Dev Biol, 2002. 243(1): p. 20-33.

90.   Levinson-Dushnik, M. and N. Benvenisty, Involvement of hepatocyte nuclear factor 3 in endoderm differentiation of embryonic stem cells. Mol Cell Biol, 1997. 17(7): p. 3817-22.

91.   Ang, S.L., et al., The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. Development, 1993. 119(4): p. 1301-15.

92.   D'Amour, K.A., et al., Efficient differentiation of human embryonic stem

cells to definitive endoderm. Nat Biotechnol, 2005. 23(12): p. 1534-41.

93.   Yasunaga, M., et al., Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat Biotechnol, 2005. 23(12): p. 1542-50.

94.   Kubo, A., et al., Development of definitive endoderm from embryonic stem cells in culture. Development, 2004. 131(7): p. 1651-62.

95.   Ciruna, B.G., et al., Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for FGFR1 in morphogenetic movement through the primitive streak. Development, 1997. 124(14): p. 2829-41.

96.   Herrera, P.L., et al., Embryogenesis of the murine endocrine pancreas; early expression of pancreatic polypeptide gene. Development, 1991. 113(4): p. 1257-65.

97.   Kordowich, S., A. Mansouri, and P. Collombat, Reprogramming into pancreatic endocrine cells based on developmental cues. Mol Cell Endocrinol, 2009.

98.   Prado, C.L., et al., Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. Proc Natl Acad Sci U S A, 2004. 101(9): p. 2924-9.

99.   Wierup, N., et al., The ghrelin cell: a novel developmentally regulated islet cell in the human pancreas. Regul Pept, 2002. 107(1-3): p. 63-9.

100.   Habener, J.F., D.M. Kemp, and M.K. Thomas, Minireview: transcriptional regulation in pancreatic development. Endocrinology, 2005. 146(3): p. 1025-34.

101.   Teitelman, G., et al., Precursor cells of mouse endocrine pancreas coexpress insulin, glucagon and the neuronal proteins tyrosine hydroxylase and neuropeptide Y, but not pancreatic polypeptide. Development, 1993. 118(4): p. 1031-9.

102.   Ahlgren, U., et al., Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. Nature, 1997. 385(6613): p. 257-60.

103.   Harrison, K.A., et al., Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice. Nat Genet, 1999. 23(1): p. 71-5.

104.   Li, H., et al., Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9. Nat Genet, 1999. 23(1): p. 67-70.

105.   Ahlgren, U., J. Jonsson, and H. Edlund, The morphogenesis of the pancreatic

mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/ PDX1-deficient mice. Development, 1996. 122(5): p. 1409-16.

106. Ohlsson, H., K. Karlsson, and T. Edlund, IPF1, a homeodomain-containing transactivator of the insulin gene. Embo J, 1993. 12(11): p. 4251-9.

107. Slack, J.M., Developmental biology of the pancreas. Development, 1995. 121(6): p. 1569-80.

108. Edlund, H., Transcribing pancreas. Diabetes, 1998. 47(12): p. 1817-23.

109. Hebrok, M., S.K. Kim, and D.A. Melton, Screening for novel pancreatic genes expressed during embryogenesis. Diabetes, 1999. 48(8): p. 1550-6.

110. Rossi, J.M., et al., Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. Genes Dev, 2001. 15(15): p. 1998-2009.

111. Nikolova, G., et al., The vascular basement membrane: a niche for insulin gene expression and Beta cell proliferation. Dev Cell, 2006. 10(3): p. 397-405.

112. Edlund, H., Pancreatic organogenesis--developmental mechanisms and implications for therapy. Nat Rev Genet, 2002. 3(7): p. 524-32.

113. Scharfmann, R., Control of early development of the pancreas in rodents and humans: implications of signals from the mesenchyme. Diabetologia, 2000. 43(9): p. 1083-92.

114. Edsbagge, J., et al., Vascular function and sphingosine-1-phosphate regulate development of the dorsal pancreatic mesenchyme. Development, 2005. 132(5): p. 1085-92.

115. Duvillie, B., et al., The mesenchyme controls the timing of pancreatic beta-cell differentiation. Diabetes, 2006. 55(3): p. 582-9.

116. Miralles, F., et al., TGF-beta plays a key role in morphogenesis of the pancreatic islets of Langerhans by controlling the activity of the matrix metalloproteinase MMP-2. J Cell Biol, 1998. 143(3): p. 827-36.

117. Jensen, J., et al., Control of endodermal endocrine development by Hes-1. Nat Genet, 2000. 24(1): p. 36-44.

118. Gradwohl, G., et al., neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A, 2000. 97(4): p. 1607-11.

119. Gu, G., J. Dubauskaite, and D.A. Melton, Direct evidence for the pancreatic

lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development, 2002. 129(10): p. 2447-57.

120. Ye, F., B. Duvillie, and R. Scharfmann, Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. Diabetologia, 2005. 48(2): p. 277-81.

121. Fujimoto, K., et al., Autophagy regulates pancreatic beta cell death in response to Pdx1 deficiency and nutrient deprivation. J Biol Chem, 2009. 284(40): p. 27664-73.

122. Deltour, L., et al., Polyclonal origin of pancreatic islets in aggregation mouse chimaeras. Development, 1991. 112(4): p. 1115-21.

123. Herrera, P.L., Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development, 2000. 127(11): p. 2317-22.

124. Jonsson, J., et al., Insulin-promoter-factor 1 is required for pancreas development in mice. Nature, 1994. 371(6498): p. 606-9.

125. Offield, M.F., et al., PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. Development, 1996. 122(3): p. 983-95.

126. Stoffers, D.A., et al., Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet, 1997. 15(1): p. 106-10.

127. Stoffers DA, Z.N., Stanojevic V, Clarke WL, Habener JF, Pancreatic agenesis attributable to a single nucleotide deletion in the human IPF1 gene coding sequence. Nat Genet, 1997. 15(1): p. 106-10.

128. Ahlgren, U., et al., beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev, 1998. 12(12): p. 1763-8.

129. Gannon, M., P.L. Herrera, and C.V. Wright, Mosaic Cre-mediated recombination in pancreas using the pdx-1 enhancer/promoter. Genesis, 2000. 26(2): p. 143-4.

130. Gerrish, K., et al., Pancreatic beta cell-specific transcription of the pdx-1 gene. The role of conserved upstream control regions and their hepatic nuclear factor 3beta sites. J Biol Chem, 2000. 275(5): p. 3485-92.

131. Samaras, S.E., et al., Conserved sequences in a tissue-specific regulatory region of the pdx-1 gene mediate transcription in Pancreatic beta cells: role for hepatocyte nuclear factor 3 beta and Pax6. Mol Cell Biol, 2002. 22(13): p. 4702-13.

132. Wu KL, G.M., Peshavaria M, Offield MF, Henderson E, Ray M, Marks A, Gamer LW, Wright CV, Stein R, Hepatocyte nuclear factor 3beta is involved in pancreatic beta-cell-specific transcription of the pdx-1 gene. Mol Cell Biol, 1997. 17(10): p. 6002-13.

133. Gerrish, K., J.C. Van Velkinburgh, and R. Stein, Conserved transcriptional regulatory domains of the pdx-1 gene. Mol Endocrinol, 2004. 18(3): p. 533-48.

134. Marshak, S., et al., Functional conservation of regulatory elements in the pdx-1 gene: PDX-1 and hepatocyte nuclear factor 3beta transcription factors mediate beta-cell-specific expression. Mol Cell Biol, 2000. 20(20): p. 7583-90.

135. Weinstein, D.C., et al., The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. Cell, 1994. 78(4): p. 575-88.

136. Ben-Shushan, E., et al., A pancreatic beta -cell-specific enhancer in the human PDX-1 gene is regulated by hepatocyte nuclear factor 3beta (HNF-3beta), HNF-1alpha, and SPs transcription factors. J Biol Chem, 2001. 276(20): p. 17533-40.

137. Haumaitre, C., et al., Lack of TCF2/vHNF1 in mice leads to pancreas agenesis. Proc Natl Acad Sci U S A, 2005. 102(5): p. 1490-5.

138. Jacquemin, P., et al., Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Mol Cell Biol, 2000. 20(12): p. 4445-54.

139. Landry, C., et al., HNF-6 is expressed in endoderm derivatives and nervous system of the mouse embryo and participates to the cross-regulatory network of liver-enriched transcription factors. Dev Biol, 1997. 192(2): p. 247-57.

140. Rausa F, S.U., Ye H, Lim L, Fletcher CF, Jenkins NA, Copeland NG, Costa RH, The cut-homeodomain transcriptional activator HNF-6 is coexpressed with its target gene HNF-3 beta in the developing murine liver and pancreas.

Dev Biol, 1997. 192(2): p. 228-46.

141. Pedersen, J.K., et al., Endodermal expression of Nkx6 genes depends differentially on Pdx1. Dev Biol, 2005. 288(2): p. 487-501.

142. Oster, A., et al., Homeobox gene product Nkx 6.1 immunoreactivity in nuclei of endocrine cells of rat and mouse stomach. J Histochem Cytochem, 1998. 46(6): p. 717-21.

143. Sander, M., et al., Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development, 2000. 127(24): p. 5533-40.

144. Gittes, G.K., Developmental biology of the pancreas: a comprehensive review. Dev Biol, 2009. 326(1): p. 4-35.

145. Kawaguchi, Y., et al., The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet, 2002. 32(1): p. 128-34.

146. Hald, J., et al., Generation and characterization of Ptf1a antiserum and localization of Ptf1a in relation to Nkx6.1 and Pdx1 during the earliest stages of mouse pancreas development. J Histochem Cytochem, 2008. 56(6): p. 587-95.

147. Krapp, A., et al., The bHLH protein PTF1-p48 is essential for the formation of the exocrine and the correct spatial organization of the endocrine pancreas. Genes Dev, 1998. 12(23): p. 3752-63.

148. Sellick, G.S., et al., Mutations in PTF1A cause pancreatic and cerebellar agenesis. Nat Genet, 2004. 36(12): p. 1301-5.

149. Roux, E., et al., The cell-specific transcription factor PTF1 contains two different subunits that interact with the DNA. Genes Dev, 1989. 3(10): p. 1613-24.

150. Afelik, S., Y. Chen, and T. Pieler, Combined ectopic expression of Pdx1 and Ptf1a/p48 results in the stable conversion of posterior endoderm into endocrine and exocrine pancreatic tissue. Genes Dev, 2006. 20(11): p. 1441-6.

151. Lioubinski, O., et al., Expression of Sox transcription factors in the developing mouse pancreas. Dev Dyn, 2003. 227(3): p. 402-8.

152. Seymour, P.A., et al., SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci U S A, 2007. 104(6): p. 1865-70.

153. Lynn, F.C., et al., Sox9 coordinates a transcriptional network in pancreatic progenitor cells. Proc Natl Acad Sci U S A, 2007. 104(25): p. 10500-5.

154. Seymour, P.A., et al., A dosage-dependent requirement for Sox9 in pancreatic endocrine cell formation. Dev Biol, 2008. 323(1): p. 19-30.

155. Jensen, J., et al., Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes, 2000. 49(2): p. 163-76.

156. Apelqvist, A., et al., Notch signalling controls pancreatic cell differentiation. Nature, 1999. 400(6747): p. 877-81.

157. Schwitzgebel, V.M., et al., Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. Development, 2000. 127(16): p. 3533-42.

158. Huang, H.P., et al., Regulation of the pancreatic islet-specific gene BETA2 (neuroD) by neurogenin 3. Mol Cell Biol, 2000. 20(9): p. 3292-307.

159. Wang, J., et al., Mutant neurogenin-3 in congenital malabsorptive diarrhea. N Engl J Med, 2006. 355(3): p. 270-80.

160. Sander, M. and M.S. German, The beta cell transcription factors and development of the pancreas. J Mol Med, 1997. 75(5): p. 327-40.

161. Sosa-Pineda, B., et al., The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature, 1997. 386(6623): p. 399-402.

162. Wang, J., et al., The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic beta-cell differentiation. Dev Biol, 2004. 266(1): p. 178-89.

163. Greenwood, A.L., et al., Notch signaling reveals developmental plasticity of Pax4(+) pancreatic endocrine progenitors and shunts them to a duct fate. Mech Dev, 2007. 124(2): p. 97-107.

164. St-Onge, L., et al., Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. Nature, 1997. 387(6631): p. 406-9.

165. Matsuoka, T.A., et al., The MafA transcription factor appears to be responsible for tissue-specific expression of insulin. Proc Natl Acad Sci U S A, 2004. 101(9): p. 2930-3.

166. Olbrot, M., et al., Identification of beta-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. Proc Natl Acad Sci U S

A, 2002. 99(10): p. 6737-42.

167. Zhang, C., et al., MafA is a key regulator of glucose-stimulated insulin secretion. Mol Cell Biol, 2005. 25(12): p. 4969-76.

168. Artner, I., et al., MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. Diabetes, 2006. 55(2): p. 297-304.

169. Artner, I., et al., MafB is required for islet beta cell maturation. Proc Natl Acad Sci U S A, 2007. 104(10): p. 3853-8.

170. Beenken, A. and M. Mohammadi, The FGF family: biology, pathophysiology and therapy. Nat Rev Drug Discov, 2009. 8(3): p. 235-53.

171. Ornitz, D.M. and N. Itoh, Fibroblast growth factors. Genome Biol, 2001. 2(3): p. REVIEWS3005.

172. Ornitz, D.M., et al., Receptor specificity of the fibroblast growth factor family. J Biol Chem, 1996. 271(25): p. 15292-7.

173. Orr-Urtreger, A., et al., Developmental localization of the splicing alternatives of fibroblast growth factor receptor-2 (FGFR2). Dev Biol, 1993. 158(2): p. 475-86.

174. Tsang, M. and I.B. Dawid, Promotion and attenuation of FGF signaling through the Ras-MAPK pathway. Sci STKE, 2004. 2004(228): p. pe17.

175. Bhushan, A., et al., Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. Development, 2001. 128(24): p. 5109-17.

176. Dichmann, D.S., et al., Expression and misexpression of members of the FGF and TGFbeta families of growth factors in the developing mouse pancreas. Dev Dyn, 2003. 226(4): p. 663-74.

177. Hart, A., S. Papadopoulou, and H. Edlund, Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells. Dev Dyn, 2003. 228(2): p. 185-93.

178. Hart, A.W., et al., Attenuation of FGF signalling in mouse beta-cells leads to diabetes. Nature, 2000. 408(6814): p. 864-8.

179. Le Bras, S., et al., Fibroblast growth factor 2 promotes pancreatic epithelial cell proliferation via functional fibroblast growth factor receptors during embryonic life. Diabetes, 1998. 47(8): p. 1236-42.

180. Roszell, B.R., et al., Efficient Derivation of Alveolar Type II Cells from Embryonic Stem Cells for In Vivo Application. Tissue Eng Part A, 2009.

181. Kim, S.K. and R.J. MacDonald, Signaling and transcriptional control of pancreatic organogenesis. Curr Opin Genet Dev, 2002. 12(5): p. 540-7.

182. Itoh, N. and D.M. Ornitz, Evolution of the Fgf and Fgfr gene families. Trends Genet, 2004. 20(11): p. 563-9.

183. Yamauchi, H., et al., Fgf4 is required for left-right patterning of visceral organs in zebrafish. Dev Biol, 2009. 332(1): p. 177-85.

184. Igarashi, M., P.W. Finch, and S.A. Aaronson, Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). J Biol Chem, 1998. 273(21): p. 13230-5.

185. Miki, T., et al., Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene. Proc Natl Acad Sci U S A, 1992. 89(1): p. 246-50.

186. Pulkkinen, M.A., et al., The IIIb isoform of fibroblast growth factor receptor 2 is required for proper growth and branching of pancreatic ductal epithelium but not for differentiation of exocrine or endocrine cells. Mech Dev, 2003. 120(2): p. 167-75.

187. Jacquemin, P., et al., An endothelial-mesenchymal relay pathway regulates early phases of pancreas development. Dev Biol, 2006. 290(1): p. 189-99.

188. Norgaard, G.A., J.N. Jensen, and J. Jensen, FGF10 signaling maintains the pancreatic progenitor cell state revealing a novel role of Notch in organ development. Dev Biol, 2003. 264(2): p. 323-38.

189. Duester, G., Retinoic acid synthesis and signaling during early organogenesis. Cell, 2008. 134(6): p. 921-31.

190. Duester, G., F.A. Mic, and A. Molotkov, Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. Chem Biol Interact, 2003. 143-144: p. 201-10.

191. Simon, D.M. and T.J. Mariani, Role of PPARs and Retinoid X Receptors in the Regulation of Lung Maturation and Development. PPAR Res, 2007. 2007: p. 91240.

192. Martin, M., et al., Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice. Dev Biol, 2005. 284(2): p. 399-411.

193. Lee, J.C., et al., Regulation of the pancreatic pro-endocrine gene

neurogenin3. Diabetes, 2001. 50(5): p. 928-36.

194.   Jensen J, et al., Control of endodermal endocrine development by Hes-1. Nature Genetics, 2000. 24(1): p. 36-44.

195.   Thomson, J.A., et al., Embryonic stem cell lines derived from human blastocysts. Science, 1998. 282(5391): p. 1145-7.

196.   Heins, N., et al., Derivation, Characterization, and Differentiation of Human Embryonic Stem Cells. Stem Cells, 2004. 22(3): p. 367-376.

197.   Chen, A.E. and D.A. Melton, Derivation of human embryonic stem cells by immunosurgery. J Vis Exp, 2007(10): p. 574.

198.   Thompson, D.L., et al., The derivation and characterization of stromal cell lines from the bone marrow of p53-/- mice: new insights into osteoblast and adipocyte differentiation. J Bone Miner Res, 1998. 13(2): p. 195-204.

199.   Vemuri, M.C., et al., Derivation of human embryonic stem cells in xeno-free conditions. Methods Mol Biol, 2007. 407: p. 1-10.

200.   Ellerstrom, C., et al., Derivation of a xeno-free human embryonic stem cell line. Stem Cells, 2006. 24(10): p. 2170-6.

201.   Stojkovic, M., et al., Derivation, growth and applications of human embryonic stem cells. Reproduction, 2004. 128(3): p. 259-67.

202.   Amit, M., et al., Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Dev Biol, 2000. 227(2): p. 271-8.

203.   Heins, N., et al., Derivation, characterization, and differentiation of human embryonic stem cells. Stem Cells, 2004. 22(3): p. 367-76.

204.   Eiselleova, L., et al., Comparative study of mouse and human feeder cells for human embryonic stem cells. Int J Dev Biol, 2008. 52(4): p. 353-63.

205.   Carpenter, M.K., et al., Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn, 2004. 229(2): p. 243-58.

206.   Baxter, M.A., et al., Analysis of the distinct functions of growth factors and tissue culture substrates necessary for the long-term self-renewal of human embryonic stem cell lines. Stem Cell Res, 2009.

207.   Cowan, C.A., et al., Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med, 2004. 350(13): p. 1353-6.

208.   Brimble, S.N., et al., Karyotypic stability, genotyping, differentiation,

feeder-free maintenance, and gene expression sampling in three human embryonic stem cell lines derived prior to August 9, 2001. Stem Cells Dev, 2004. 13(6): p. 585-97.

209. Schulz, T.C., et al., Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. Stem Cells, 2004. 22(7): p. 1218-38.

210. Bajpai, R., et al., Efficient propagation of single cells Accutase-dissociated human embryonic stem cells. Mol Reprod Dev, 2008. 75(5): p. 818-27.

211. Ellerstrom, C., et al., Facilitated expansion of human embryonic stem cells by single-cell enzymatic dissociation. Stem Cells, 2007. 25(7): p. 1690-6.

212. Baker, D.E., et al., Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotechnol, 2007. 25(2): p. 207-15.

213. Draper, J.S., et al., Recurrent gain of chromosomes 17q and 12 in cultured human embryonic stem cells. Nat Biotechnol, 2004. 22(1): p. 53-4.

214. Mitalipova, M.M., et al., Preserving the genetic integrity of human embryonic stem cells. Nat Biotechnol, 2005. 23(1): p. 19-20.

215. Osafune, K., et al., Marked differences in differentiation propensity among human embryonic stem cell lines. Nat Biotechnol, 2008. 26(3): p. 313-5.

216. Cao, T., et al., Differentiated fibroblastic progenies of human embryonic stem cells for toxicology screening. Cloning Stem Cells, 2008. 10(1): p. 1-10.

217. Hay, D.C., et al., Highly efficient differentiation of hESCs to functional hepatic endoderm requires ActivinA and Wnt3a signaling. Proc Natl Acad Sci U S A, 2008. 105(34): p. 12301-6.

218. Pei, H., et al., Lineage restriction and differentiation of human embryonic stem cells into hepatic progenitors and zone 1 hepatocytes. Tissue Eng Part C Methods, 2009. 15(1): p. 95-104.

219. Shim, J.H., et al., Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. Diabetologia, 2007. 50(6): p. 1228-38.

220. Zorn, A.M. and J.M. Wells, Vertebrate endoderm development and organ formation. Annu Rev Cell Dev Biol, 2009. 25: p. 221-51.

221. Poulain, M., et al., Zebrafish endoderm formation is regulated by combinatorial Nodal, FGF and BMP signalling. Development, 2006. 133(11): p. 2189-200.

222. McLean, A.B., et al., Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed. Stem Cells, 2007. 25(1): p. 29-38.

223. Borowiak, M., et al., Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. Cell Stem Cell, 2009. 4(4): p. 348-58.

224. Hebrok, M., et al., Regulation of pancreas development by hedgehog signaling. Development, 2000. 127(22): p. 4905-13.

225. Stafford, D., et al., A conserved role for retinoid signaling in vertebrate pancreas development. Dev Genes Evol, 2004. 214(9): p. 432-41.

226. Cai, J., et al., Generation of Homogeneous PDX1+ Pancreatic Progenitors from Human ES Cell-derived Endoderm Cells. J Mol Cell Biol, 2009.

227. Ochoa, W.F., et al., Retinoic acid binds to the C2-domain of protein kinase C(alpha). Biochemistry, 2003. 42(29): p. 8774-9.

228. Radominska-Pandya, A., et al., Direct interaction of all-trans-retinoic acid with protein kinase C (PKC). Implications for PKC signaling and cancer therapy. J Biol Chem, 2000. 275(29): p. 22324-30.

229. Chen, S., et al., A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat Chem Biol, 2009. 5(4): p. 258-65.

230. Yu, D., et al., An efficient recombination system for chromosome engineering in Escherichia coli. Proc Natl Acad Sci U S A, 2000. 97(11): p. 5978-83.

231. Warming, S., et al., Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res, 2005. 33(4): p. e36.

232. Lee, E.C., et al., A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics, 2001. 73(1): p. 56-65.

233. Liu, P., N.A. Jenkins, and N.G. Copeland, A highly efficient recombineering-based method for generating conditional knockout mutations. Genome Res, 2003. 13(3): p. 476-84.

234. Tompers, D.M. and P.A. Labosky, Electroporation of murine embryonic stem cells: a step-by-step guide. Stem Cells, 2004. 22(3): p. 243-9.

235. Urbach, A., M. Schuldiner, and N. Benvenisty, Modeling for Lesch-Nyhan disease by gene targeting in human embryonic stem cells. Stem Cells, 2004. 22(4): p. 635-41.

236.   Zwaka TP and T. JA., Homologous recombination in human embryonic stem cells. Nature Biotechnology, 2003. 21(3): p. 319-21.

237.   Davis, R.P., et al., Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. Blood, 2008. 111(4): p. 1876-84.

238.   Irion, S., et al., Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat Biotechnol, 2007. 25(12): p. 1477-82.

239.   Eiges, R., et al., Establishment of human embryonic stem cell-transfected clones carrying a marker for undifferentiated cells. Curr Biol, 2001. 11(7): p. 514-8.

240.   Irion, S., et al., Identification and targeting of the ROSA26 locus in human embryonic stem cells. Nat Biotechnol, 2007.

241.   Costa, M., et al., A method for genetic modification of human embryonic stem cells using electroporation. Nat Protoc, 2007. 2(4): p. 792-6.

242.   Zwaka, T.P. and J.A. Thomson, Homologous recombination in human embryonic stem cells. Nat Biotechnol, 2003. 21(3): p. 319-21.