

Activin A-Induced Differentiation of Embryonic Stem Cells into Endoderm and Pancreatic Progenitors—The Influence of Differentiation Factors and Culture Conditions

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Abstract The differentiation of murine and human embryonic stem (ES) cells into pancreatic cell types has been shown by several methods including spontaneous differentiation, formation of multi-lineage progenitors, lineage selection or transgene expression. However, these strategies led to a mixture of cells of all three primary germ layers and only a low percentage of definitive endoderm cells giving rise to pancreas, liver, lung and intestine. To reproducibly generate functional insulin-producing cells, ES cells have to be differentiated via definitive endoderm and pancreatic endocrine progenitors recapitulating the *in vivo* development. Activin A, a member of the transforming growth factor beta superfamily, has been shown to induce definitive endoderm cells dependent on concentration, culture conditions and time of application. Moreover, serum components or contamination by feeder cells as well as differentiation and proliferation factors are critical for successful generation of activin A-induced ES cells into endoderm and pancreatic cells. The review presents an

overview on those factors that influence activin A activity on endoderm and endocrine progenitor cells and determines the role of signaling factors in the differentiation process into the pancreatic lineage.

Keywords Embryonic stem cells · Human · Mouse · *In vitro* differentiation · Activin A · Definitive endoderm · Pancreatic endocrine lineage · Insulin-producing beta cells

Introduction

Embryonic stem (ES) cells established as permanent cell lines from pluripotent undifferentiated cells of mouse [1] and human [2] embryos are capable of almost unlimited proliferation and differentiation into diverse cell types of the organism [3]. During *in vitro* differentiation, both murine (m) and human (h) ES cells recapitulate early stages of embryonic development [4]. These properties qualify ES cells as unique model to study early embryonic development of mammalian cells *in vitro* and, as far as human ES cells are concerned, as potential donor cells for therapeutic application in regenerative medicine [5].

When ES cells are cultured in three-dimensional aggregates, called embryoid bodies (EBs), they spontaneously differentiate into cell types of all three primary germ layers, ectoderm, mesoderm and endoderm, the latter giving rise to liver, pancreas, lung and intestine [6]. Because after spontaneous differentiation the number of specifically differentiated cell types is relatively low, the application of defined differentiation factors and selection of lineage-specific progenitor cells seems to be necessary for directed differentiation of ES cells into the desired cell types. It is generally believed that the generation of lineage-specific progenitor cells will be crucial for future applications in

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basic research and applied medicine. In animal experiments, it has been shown that the transplantation of hES-derived pancreatic progenitors [7] rather than in vitro differentiated insulin-producing cells [8] results in the formation of glucose-responsive beta-like cells that are able to normalize glycemia in diabetic mice [7]. Therefore, the derivation of a suitable number of potential pancreatic progenitor cells will be extremely important for future developments and applications.

In this review, following a general introduction on pancreatic differentiation of ES cells, we summarize strategies for the generation of endoderm and pancreatic endocrine progenitor cells from mouse and human ES cells. In particular, we discuss the influence of crucial factors and cultivation conditions, which affect the activin A-induced differentiation of ES cells into endoderm and pancreatic endocrine progenitors, ultimate precursors of functional beta-cells. In this context it should be noted that particularly hES cell lines reveal heterogeneity within the pluripotent stem cell compartment [9] suggesting that different lines may represent different developmental stages and could react differently to signaling factors. For example, 17 hES cell lineages showed marked (>100) differences in their in vitro differentiation capacity [10]. Therefore, a detailed knowledge of the mechanisms and the activity of differentiation factors and signaling molecules and their cellular interactions are indispensable.

ES Cell Differentiation into Pancreatic Cell Types via Transgene Expression, Selection for Nestin-Positive Cells or Multi-lineage Progenitors

Spontaneous differentiation of murine ES cells into the pancreatic lineage resulted in a relatively low yield of endocrine beta-like cells [11]. Therefore, numerous strategies have been established to increase the number of insulin-producing cells, by (a) directed differentiation through lineage selection of pancreatic phenotypes using pancreas-specific promoters [12, 13], (b) transgene expression of pancreatic developmental control genes [14–21], (c) application of specific signaling molecules, such as PI3K-inhibitors [22] or retinoic acid [23–25], (d) use of conditioned medium from fetal pancreatic buds [26] and (e) selection and enrichment of a nestin-expressing cell population [27–29]. However, in the latter case, nestin-positive cells that may be also committed to neural fate lacked properties of functional beta cells after pancreatic differentiation, and eventually underwent apoptosis [28–31].

Although the sequence of expression of developmental control genes in EBs parallels the expression pattern of in vivo embryonal development, after in vitro differentiation lineage-specific progenitors develop in a stochastic manner.

Growth and extra-cellular matrix factors used to induce pancreatic differentiation included progesterone, putrescine, insulin, transferrin, sodium selenite, fibronectin (ITSFn), nicotinamide [26] and laminin [27], all of which have been shown to activate both neural and pancreatic differentiation. As a consequence, the protocols mentioned above promoted not only pancreatic, but also significant neuronal differentiation.

During embryogenesis, neuroectodermal and pancreatic differentiation are spatially and temporally regulated by the same transcription factors, such as Ngn3, Isl1 or Pax6 [32–36]. Likewise, nestin is transiently expressed in ES-derived neuronal [37], pancreatic [14, 38] and hepatic [39] differentiation. Therefore, nestin-positive cells have been regarded as a type of “multilineage” progenitor cells [40]. Because in EBs neuroectodermal and pancreatic progenitors are not temporally and spatially separated from each other (as during embryonic development), any induction and/or selection of cells expressing nestin will lead to parallel induction of neuronal and pancreatic cell types.

The application of a three-step differentiation protocol via EB formation and multilineage progenitors [without selection for nestin-positive cells [14, 38, 41]] resulted in functional insulin-producing cells. The rationale of this strategy was the development of a transient developmental state containing ectoderm and mesoderm progenitor cells secreting growth factor activity necessary to induce the development of endoderm cells. A comparative analysis of this differentiation system [38] with protocols selecting for nestin-positive cells [27] or applying a PI3-Kinase-inhibitor [22] showed the proof-of-principle of the three-step differentiation protocol via multilineage progenitors [38] in generating at least partially, glucose-responsive insulin-producing cells [42]. However, these experiments also demonstrated that the differentiation and maturation status of in vitro differentiated insulin-producing cells was insufficient and improvement of differentiation efficiency would be indispensable.

An alternative strategy has been recently established [7, 43] based on activin A-mediated and step-wise differentiation induction of ES cells into definitive endoderm (DE) and pancreatic endocrine cells by factors specifically involved in the regulation of endoderm and pancreas development.

Selective Induction of Definitive Endoderm (DE) Progenitor Cells by Activin A

Studies using the *Xenopus* animal cap assay revealed the induction of endoderm including pancreatic cells by activin A [44, 45]. *Xenopus*, zebrafish and chicken experiments demonstrated the central role of nodal signaling for the

development of endoderm [46, 47]. Nodal is known as a member of the transforming growth factor- β (TGF- β) superfamily. During gastrulation in mice, the definitive endoderm lineage originates from a common bipotential progenitor cell type called mesendoderm. The mesendoderm population has been shown to be induced by nodal signaling from the most anterior region of the primitive streak epiblast layer [48].

Activin A, a member of the TGF- β superfamily, has been shown to mimicking nodal [47]. Activins interact with two types of cell surface transmembrane receptors, which have intrinsic serine/threonine kinase activities in their cytoplasmic domains. Activin A binds to the type II receptor and initiates a cascade reaction that leads to the recruitment, phosphorylation, and activation of type I activin receptors. This activation is followed by phosphorylation of SMADs2/3, two of the cytoplasmic SMAD proteins. SMAD3 then translocates to the nucleus and interacts with SMAD4 through multimerization, resulting in the activation of transcription factor complexes responsible for specific gene expression [49].

Using the embryoid body (EB) differentiation model of mES cells, first studies showed that endoderm cells could be induced by treatment with activin A [50, 51]. It has to be mentioned that activin A also induced the formation of neuronal extensions and neurofilament proteins in PC12 cells [52] pointing towards the involvement of activin A in neural differentiation. The potential of activin A to induce ES cells into both pancreatic and neural lineages [on the basis of data published by Shi et al. [24]] has been demonstrated [53].

It became evident that the induction of hES cells into DE cells required a sophisticated schedule including the sequential application of activin A and other endoderm- and pancreas-specific growth factors. D'Amour et al. [8] showed that hES-derived DE cells (Sox17+/Cxcr4+) could be differentiated into foregut endoderm (Hnf4a+/Hnf1b+), pancreatic endoderm (Nkx6.1+/Pdx1+) and endocrine precursors (Ngn3+/Nkx2.2+) and finally, hormone expressing endocrine cells [see [43]]. Because in the 2006 study many of the ES cell terminal derivatives showed expression of more than one hormone, i.e., insulin and glucagon co-expression, the lack of glucose-dependent insulin release in such cells pointed to an immature stage of in vitro differentiated cells [8]. After advancement of the differentiation protocol and transplantation of hES-derived pancreatic precursor cells into diabetic mice, in vivo differentiation and maturation facilitated the development of glucose-sensing beta-like cells that normalized high blood glucose levels [7]. These data suggest that the in vivo maturation of appropriate activin A- and growth factor-induced pancreatic progenitors (rather than transplantation of fully differentiated cells) is advantageous to generate functional hES-derived beta-like

cells. However, it also means that entire in vitro methods to direct ES cells into terminal stages of islet-like clusters are not yet available.

The directed differentiation of ES cells into DE progenitors includes selective induction and enrichment of DE cells by activin A. However, numerous studies and our own experiments provided evidence that successful differentiation of ES cells into DE and pancreatic progenitors are significantly affected by parameters, such as contaminating constituents in culture media during activin A application, activin A concentration, time and duration of treatment, and differentiation models used. Such parameters and factors will be discussed in the following chapters.

Critical Parameters Affecting the Differentiation of Definitive Endoderm Progenitor Cells by Activin A

Serum- and Feeder-Free Culture Conditions

In previous experiments, the differentiation process into pancreatic cell types was initiated by complex culture media, and the addition of several growth factors, such as insulin, laminin, niacinamide and fetal calf serum [FCS; [12, 13, 18, 20, 22, 27, 28, 30, 54–57]].

However, these culture conditions evidently induced not only endoderm, but also a mixture of cells of other lineages, including neuroectoderm, resulting in inefficient differentiation of endoderm.

It is generally known that growth of mammalian cells in vitro is dependent on animal serum supporting proliferation, adhesion and cell survival. However, the source of FCS varies from batch to batch and the level of protein constituents is mainly undefined. This may lead to unspecific interactions between activin A with growth factors present in FCS-supplemented media during in vitro differentiation. Kubo et al. [50] for the first time showed that FCS specifically influenced activin A-mediated ES cell differentiation into DE cells, resulting in lower level of endoderm formation at high serum levels. Consequently, serum levels were reduced during activin A treatment [7, 8, 58], or alternatively, serum substitutes were applied instead of FCS. Specifically, ES cells were differentiated in the presence of activin A in media supplemented either by low FCS concentration [7, 8, 58, 59], knockout serum replacement [KSR [50, 60]; note that KSR contains serum components and cannot be defined as “serum-free” [61]], bovine serum albumin (BSA) or polyvinyl alcohol [PVA [62, 63]], or by polytetrafluoroethylene [PTFE [64], in the case of mES cells]. Our own studies with mES cells showed that BSA of defined quality (i.e. Sigma) was more suitable for DE generation as polyvinyl alcohol (PVA), where ES cells frequently failed to form proper EBs (Fig. 1).

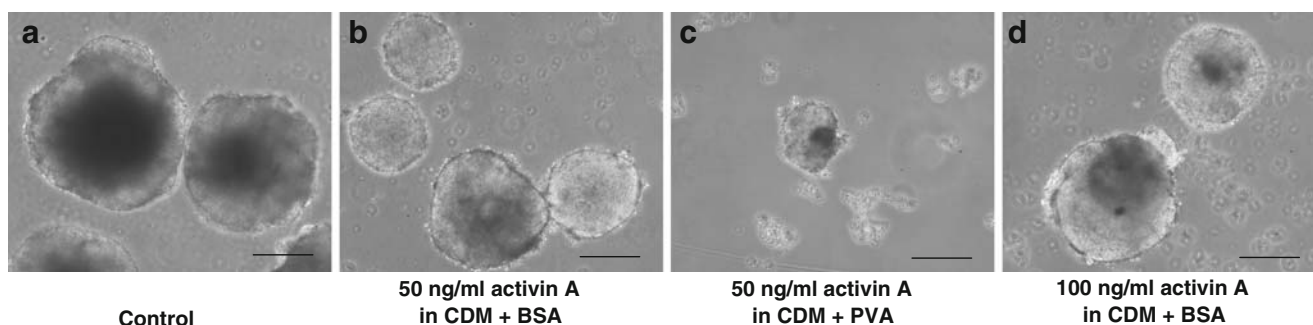


Fig. 1 Morphology of murine ES cell (line R1)-derived embryoid bodies (EBs) cultivated in Iscove's modified Dulbecco's medium (IMDM) in the presence of fetal calf serum (FCS) used as control (**a**) and in chemically defined medium (CDM) supplemented with activin A and bovine serum albumin (BSA) or polyvinyl alcohol (PVA) (**b–d**). Culture of ES cells in CDM without any supplements for more than

5 days was not successful, therefore, IMDM/FCS-supplemented medium variants served as control. Shown are EBs cultured for 9 days in **a** IMDM containing 20% FCS (control), in **b** CDM with BSA (5 mg/ml) + 50 ng/ml activin A, **c** CDM with PVA (0.1%) + 50 ng/ml activin A and **d** CDM with BSA (5 mg/ml) + 100 ng/ml activin A. Scale bar=50 μ m

Differentiation of hES cells in FCS (20%)-containing medium followed by activin A treatment in serum-free culture resulted in up-regulation of *Pdx1*, a marker of pancreatic progenitors [65], whereas serum-free medium without activin A induced ectoderm-specific *Pax6* and *Sox1* expression. Further reduction of the FCS concentration up to 0.2% to 0.5% during activin A treatment was successful for DE induction in hES cells suggesting that the inductive effect of activin A was most effective at very low serum level [58]. In subsequent studies with hES cells the authors used protocols with serum-free cultivation of cells during activin A treatment for the first 1–2 days, whereas FCS concentration was increased to 0.2% during the following 1–2 days to support the viability of early differentiated endoderm progenitors [7, 8].

There is evidence that specific growth factors and hormones present in FCS influence the activin A-dependent induction of endoderm. Using hES cells, McLean et al. [60] demonstrated an inhibitory effect of insulin/insulin-like growth factor (IGF) signaling on activin A-dependent DE differentiation. The authors showed that phosphatidylinositol 3-kinase (PI3K) antagonized the differentiation into mesendoderm and DE. However, inhibition of PI3K by LY 294002 led to up-regulation of mesendoderm (*Brachyury*, *Mixl1*) and endoderm (*Gsc*, *Sox17*) marker genes, while ectoderm (*Sox1*) and extra-embryonic endoderm (*Sox7*) genes were down-regulated. From the observation that activin A promotes DE differentiation only in low serum/serum replacement conditions they concluded that factors in the FCS/KSR activate PI3K signaling and identified insulin and IGF as the main agonists of PI3K-dependent signaling in FCS- or KSR-containing media [60].

In our own experiments with mES cells, the use of a chemically defined medium [CDM [62]] substituted by high activin A concentration (50 to 100 ng/ml) and BSA (5 mg/ml) under conditions of EB formation by the hanging

drop technique was effective to up-regulate DE-specific *Sox17* and to down-regulate neuroectoderm-specific *Pax6* transcript levels (see Fig. 2).

In conclusion, all experiments using both human and murine ES cells presented evidence for effective DE induction by activin A in the absence of FCS, or at very low FCS or KSR levels. Alternatively, FCS may be substituted by other components known to support growth and survival of ES cells, such as BSA.

Concentration of Activin A

One of the most important parameter for efficient endoderm differentiation is the activin A concentration. Previous *Xenopus* studies clearly demonstrated the concentration-dependent influence of activin A on endoderm differentiation [for review, see [47]].

Concentrations higher than 30 ng/ml (up to 100 ng/ml) were found to be sufficient for induction of DE cells and activation of *Pdx1* expression in murine [50] and human [65] ES cells. Treatment with 30 ng/ml led to a decrease in (neural-specific) *Sox1* and increase in endoderm-specific *Foxa2*, *Sox17*, *Mixl1* and *Pdx1* transcript levels [65]. However, studies by Phillips et al. [66] demonstrated that not only the specific activin A level, but in parallel, low BMP4 levels were necessary to induce DE formation in hES cells (see “BMPs/Noggin” section).

In most of the recent studies, consequently, for the differentiation of ES cells into definitive endoderm, high concentrations of activin A (100 ng/ml) were used [7, 8, 24, 58, 60, 64, 67, 68]. However, lower activin A levels (10 ng/ml) have been also applied to differentiate mES cells in monolayer culture [51, 69] suggesting that ES cells differentiating in monolayer (instead of EBs) may require lower doses of activin A for DE induction (see “Differentiation Models: EB Versus Monolayer Differentiation”).

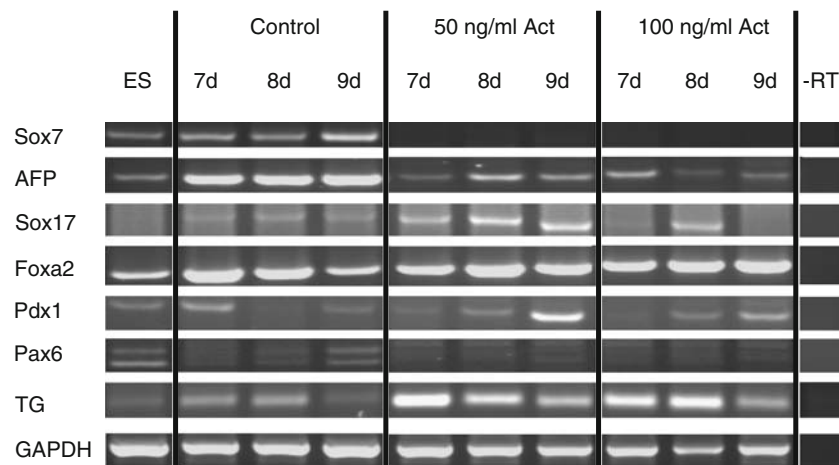


Fig. 2 Transcript levels of lineage-specific genes of EBs cultured in FCS-containing medium (control) and in CDM in the presence of activin A for 7, 8 and 9 days (*d*). Total mRNA was analyzed by RT-PCR for mRNA expression of Sox7 and AFP (extra embryonic endoderm), Sox17 and Foxa2 (definitive endoderm), the pancreatic progenitor marker Pdx1, Pax6 (ectoderm) and Brachyury (TG,

mesendoderm) in ES cells (ES) and in 7, 8 and 9 day old EBs cultivated in Iscove's modified Dulbecco's medium containing 20% FCS (control, *left*) and BSA containing CDM + 50 (*middle panel*) and 100 ng/ml activin A (*right panel*), respectively. GAPDH expression was used as a control

In conclusion, as evidenced from *Xenopus* studies, ES cells of both human and mouse origin need a high activin A level to differentiate into DE. But culture conditions (monolayer vs. EB culture, *see* “[Differentiation Models: EB Versus Monolayer Differentiation](#)”) or the application time (early vs. late stage, *see* “[Time and Duration of Activin A Application](#)”) could further influence the activin A effects on DE induction.

Time and Duration of Activin A Application

Also the time of activin A application during *in vitro* differentiation was found to be critical for induction of ES cells into DE, with particular differences between human and mouse ES cells. The application of activin A starting from the beginning of *in vitro* differentiation in monolayer was successful for hES cells to differentiate into DE cells [7, 8, 58, 60, 67, 68]. In contrast, activin A application to mES cells followed different schedules in different culture systems. For example, mES cells were precultured as EB mass culture without activin A for 2 [59, 70] or 4 to 5 [71] days followed by treatment with 100 [24] or 50 [70, 71] ng/ml activin A. In the case of monolayer culture of mES cells, also low concentration of 10 ng/ml activin A without precultivation was used for DE differentiation [51, 69]. In mES cells, the duration of activin A treatment (with high concentration) varied between 1 [24], 2 [70] and 3 [64] days (in EB culture), and between 4–6 days for low activin A level [in monolayer culture [51, 69]].

In conclusion, the different periods of activin A application for the induction of DE from ES cells are dependent on the culture system (EB vs. monolayer), which

are significantly different between mouse and human ES cells.

Differentiation Models: EB Versus Monolayer Differentiation

Whereas in early differentiation studies in general, ES cells were differentiated via EBs [in mass or hanging drop culture, *see* [3]], at present, differentiation in monolayer is commonly applied. The differentiation of ES cells in monolayer culture permits a more selective formation of DE cells, if signaling molecules that regulate lineage development are applied under stage-specific conditions. Again, differences between mouse and human ES cells were found.

In general, human ES cells were successfully differentiated into DE cells by activin A in monolayer [7, 8, 58, 60, 67, 68], and only exceptionally, in mass EB culture [65]. In contrast, murine ES cells were mainly differentiated into DE cells in EBs via mass culture [24, 64, 70, 71], but only occasionally in monolayer culture [51, 69].

A comparison of activin A concentrations used for differentiation of mES cells under EB or monolayer condition revealed that DE differentiation from EBs required higher activin A levels. Whereas concentrations of 10 ng/ml activin A were applied to differentiate mES cells into DE in monolayer [51, 69], 50 and 100 ng/ml were required in EB culture systems [24, 50, 64, 70].

In conclusion, hES cells have been successfully differentiated into DE cells by activin A application to monolayer cultures, whereas mES cells show also DE formation from EBs, but need higher activin A levels as in monolayer

culture. In our own experiments using mES cells, EBs differentiating in hanging drops for 3 days followed by suspension culture for up to 6 days and application of 50 to 100 ng/ml activin A resulted in up-regulation of DE-specific Sox17 transcript levels (*see* Fig. 2).

As potential reasons of these differences (reflected also by the different requirements for activin A in EB and monolayer culture) different developmental stages of human and murine ES cells have to be considered.

Role of Activin A Signaling in Maintenance of Pluripotency in hES Cells and mEpiSC

Whereas activin A in murine ES cells clearly induced differentiation into mesendoderm, endoderm and specifically DE cells [i.e. [50, 51, 64, 69, 71]], in hES cells activin A at low concentration is also able to maintain the undifferentiated pluripotent state. Specifically, concentrations of activin A between 5 and 20 ng/ml in serum-free media have been used to keep hES cells undifferentiated [72–75], and also long-term culture of hES cells could be achieved by 50 ng/ml activin A [with additional supplementation by KSR, KGF and NIA [76]].

In the mouse system, activin/nodal signaling is not required to maintain pluripotency of ES cells [73], however, it was shown recently that another pluripotent murine embryonic stem cell type derived from the epiblast tissue of the post-implantation embryo, called EpiSC (epiblast-derived stem cells), is dependent on activin/nodal signaling [77, 78]. Murine EpiSC share also other properties (besides activin/nodal signaling) with human ES cells, i.e. flat colony growth, absence of LIF and BMP dependency and FGF5 expression [78]. This would suggest that mEpiSC and hES cells resemble a similar developmental stage (of post-implantation late primitive ectoderm), which is different to mES cells originating from pre-implantation blastocysts.

These data called into question the interaction of activin A with pluripotency-associated genes, such as Oct4. Previous studies with mES cells showed that the Oct4 transcript level determined lineage decisions: low Oct4 level stimulated trophoblast, high Oct4 level induced endoderm and mesoderm differentiation, whereas an intermediate Oct4 level was required for maintenance of ES cells [79]. High Oct4 levels were also found to up-regulate endoderm markers in hES cells [80].

Obviously, the application of activin A to ES cells critically affects the decision between proliferation (pluripotency) and differentiation (i.e., endoderm induction) in a concentration- and stage-dependent manner. For example, sustained *nodal* expression in mES cells maintained Oct4 levels and prevented the differentiation of DE cells [81], and activin treatment of ES cells for a long period resulted in up-regulation of both,

Oct4 and Sox17 expression in a dose-dependent manner [82].

Consequently, to avoid activin A and Oct4 interaction that would result in uncontrolled Oct4 activation and inhibition of endoderm differentiation, the activin A concentration (*see* “Concentration of Activin A”), time and duration of activin A treatment (*see* “Time and Duration of Activin A Application”) have to be carefully controlled and standardized in ES cell differentiation experiments.

Influence of Exogenous Signaling Molecules that Affect Activin A-Induced Endoderm Differentiation

WNTs

At gastrulation, the epiblast cell layer at the posterior end of the embryo forms the primitive streak (PS), a structure from which cells migrate to form both mesoderm and endoderm layers. Among the most important regulators of cell fate decisions during formation and patterning of the anterior–posterior (A–P) axis are members of the Wnt/Wg family of secreted factors. Wnts make up a large family of cysteine-rich glycoproteins that activate signaling cascades that induce cytoplasmic responses and/or transcription of target genes [83–85]. The activation of target genes is dependent on cytoplasmic accumulation of β -catenin and its translocation to the nucleus, where it associates with TCF/LEF family members.

Earlier studies demonstrated the direct involvement of Wnt family members in regulating endoderm formation. The finding that Wnt3a mutants lack *nodal* expression [86] suggested that canonical Wnt3 signaling is involved in PS and endoderm specification. In *Xenopus*, β -catenin drives the expression of endoderm-specific target genes, like *Hnf1 β* , *Edd*, *Foxa1* and *Foxa2* [87], whereas blocking of the Wnt/ β -catenin pathway induced the expression of cardiac-specific genes [88]. In mice, disruption of β -catenin signaling resulted in cell fate changes from endoderm to the pericardiac mesoderm [89]. Loss of Tcf4 and Tcf1/Tcf4 disruption led to loss of epithelial stem cells in the endoderm-derived small intestine [90] and severe defects in the formation of the hindgut [91], respectively.

These and other data suggested the involvement of (canonical) Wnt signaling for endoderm development also during ES cell differentiation. Recently, mES cells supplemented with Wnt3a showed induction of PS-specific *brachyury*, *gooseoid*, *Foxa2* in m/hES cells, and of the endoderm-specific *Sox17* in mES cells [71]. Addition of inhibitors of β -catenin signaling (i.e. by DKK1) blocked the formation of PS gene expression in mES cells, and incubation with Wnt3a and SB-431542 (inhibitor of TGF β /

nodal/activin pathway) resulted in a loss of PS (Foxa2+/Bry+) cell induction. The data underlined that the simultaneous activation of Wnt and activin signaling pathways is essential for PS formation, a requirement for DE development [59].

To support endoderm differentiation of hES cells, Wnt3a has been applied during the first two days of activin A-induced differentiation leading to increased efficiency of mesendoderm specification and synchrony of DE formation [8].

BMPs/Noggin

Bone morphogenetic proteins (BMPs), members of the transforming growth factor- β (TGF β) superfamily, are important signaling molecules in early pattern formation. At least, 20 BMPs have been described, which play an important role in mesoderm formation. BMP signaling is mediated by activated SMADs 1, 5 and 8, which interact with the common partner SMAD 4 [92].

In contrast to Wnts, BMPs inhibit endoderm induction as shown in zebrafish [93] and *Xenopus* [94]. Inhibition of BMP signaling via the BMP antagonist noggin resulted in increased number of endoderm precursors [93]. Noggin binds to BMPs thus preventing the interaction with their respective receptors [95]. Over-expression of the BMP antagonists chordin and noggin in animal caps of *Xenopus* induced the endoderm markers endodermin and *XIHbox8*. This activity could be counteracted by co-injection of BMP4 [94].

In the murine system, the role of BMPs was studied in knock out mice. BMP4-deficient mice failed to gastrulate and to form mesoderm [96], and BMP-receptor-1 and -2b-deficient embryos showed reduced epiblast proliferation and no mesoderm development [97].

As a consequence, BMPs function as activin A antagonists. For example, treatment of differentiating murine EBs with BMP7 caused a reduction of E-cadherin+/CXCR4+ endoderm cells, whereas addition of activin A and noggin increased the proportion of E-cadherin+/CXCR4+ cells. The antagonistic effects of BMP7 and activin A seemed to be mediated at the level of SMADs. Phosphorylated SMAD 1 was localized in FOXA2-/Brachyury+ mesoderm cells, whereas phosphorylated SMAD 2/3 (transducing activin A signaling) was observed in FOXA2+ endoderm cells [98].

The interaction of BMP-inhibition and the β -catenin/Wnt-signaling pathway to support endoderm development was shown by treatment of β -catenin-activated hES cells with noggin. Noggin induced PS/endoderm markers (TG, gooseoid, Foxa1, Foxa2, Cer1, Shh and Sox17) and rapidly reduced PS/mesoderm markers (Mixl1, KDR, FoxF1, Ventx). About 70% of the differentiated cells were FOXA2 positive and co-expressed SOX17 or TG. The

expression profile also showed that the treatment did not lead to differentiation into visceral endoderm, ectoderm or trophoderm. But the activin/nodal signaling is also important for noggin/ β -catenin-interaction: Inhibition of the activin/nodal signaling pathway by SB431542 completely suppressed FOXA2 induced by β -catenin and noggin. These results demonstrate that during ES cell differentiation canonical Wnt/ β -catenin, activin/nodal and BMP signaling pathways cooperatively interact for the induction and specification of PS, mesoderm and endoderm [99].

Recently, in a detailed study, Phillips et al. [66] applied different concentrations of activin A alone and in combination with BMP4 to hES cells and analyzed the effects on Pdx1 expression. BMP4 treatment of EBs resulted in reduced Pdx1 transcript levels, which underlined the inhibitory effect of BMP4 on endoderm and early pancreatic differentiation. However, when BMP4 was applied together with activin A at equal concentrations (50 ng/ml) in the presence of Matrigel, the transcript level of Pdx1 was 10 times higher as with activin A alone. The synergistic effect of activin A and BMP4 disappeared, when EBs formed in the absence of Matrigel resulting in suppression of visceral endoderm [100]. Phillips and coworkers speculated that the loss of visceral endoderm formation might provide an environment necessary to realize BMP4 effects on PS patterning and endoderm specification [66].

Fibroblast Growth Factors (FGFs)

Gene targeting experiments and transgene over-expression revealed that fibroblast growth factors (FGFs) play an essential role in A-P patterning and endoderm formation. FGF-receptor (FGFR)-depletion or FGFR over-expression in *Xenopus*, zebrafish and mouse embryos resulted in phenotypic changes mostly in the posterior region. Inactivation of the FGFR-1 signalling pathway in mice led to embryonic lethality between E7.5 and E9.5 [101]. *FGFR-1* and -8 mutants showed defects in endoderm development [101–105]. *Fgfi2* knock out mice revealed defective ICM and visceral endoderm formation [106] and loss of *Fgf4* function caused defects in trophoderm and primitive endoderm development [107, 108].

Treatment of isolated endoderm of E7.5 mouse embryos with FGF4 induced dose-dependent expression of *Somato-statin* (expressed in the posterior endoderm that develop into duodenum and small intestine) and of *NeuroD* (expressed in the more anterior endoderm fated to become pancreas und duodenum). FGF4 at lower concentration induced, whereas higher concentrations repressed *NeuroD* suggesting threshold effects of FGF4-dependent signaling. These results in combination with the demonstration of FGF4 expression in the PS/mesoderm (closer to the

posterior than to the more anterior endoderm) indicated that high concentrations of FGF4 promote a posterior/intestinal endoderm cell fate, whereas lower FGF4 levels induce a more anterior/pancreas–duodenal cell fate [109].

An inductive effect by FGF2 on mouse ES cell-derived endoderm development was demonstrated by increased numbers of E-cadherin+/Cxcr4+ and Pdx1+ cells. Addition of activin A alone caused a higher up-regulation of E-cadherin+/Cxcr4+ and Pdx1+ cells, but the combination of FGF2 and activin A resulted in additive effects: ES-derived cells treated with activin A and FGF2 expressed higher Sox17 and Pdx1 levels than control cells. FACS sorted DE cells showed increased mRNA levels of the endocrine markers insulin 2, glucagon, pancreatic polypeptide, somatostatin, Nkx6.1 (but low neurogenin 3 [98]).

However, the activation of the FGF/ERK pathway may also negatively affect endoderm differentiation. In zebrafish, over-expression of *Fgf8* or of Ras and ERK2 signaling led to a reduction in the number of Sox17-expressing cells, whereas triple-knockdown of *Fgf8*, *Fgf17b* and *Fgf24* strongly increased the number of endoderm cells. In these cases, FGF seemed to cooperate with BMP to repress endoderm formation [93].

Exogenous Factors Regulating the Differentiation of Pancreatic Progenitors from Activin A-Induced DE Cells

Cyclopamine

Ten years ago, Kim and Melton showed that the teratogenic *Veratrum* alkaloid cyclopamine promoted the differentiation of pancreatic cells [110]. This effect was due to specific inhibition of sonic hedgehog (Shh) signaling by cyclopamine [111], which promoted pancreatic differentiation by expanding the endoderm *Pdx1*-expressing region [110]. The positive effect on pancreas development was further substantiated by the finding of pancreatic overgrowth/annular pancreas in *Shh*^{-/-} mutants [112].

In early development, Shh is highly expressed in stomach and duodenal endoderm, but not in pancreatic endoderm, indicating that hedgehog signaling is involved in the formation of distinct organ boundaries along the gastrointestinal tract [110, 113]. Accordingly, D'Amour et al. [8] used cyclopamine in combination with FGF10 and RA to direct activin A-induced hES-derived DE cells into the pancreatic lineage. Combined addition of cyclopamine and FGF10 resulted in a 160-fold increase in insulin mRNA in comparison to differentiation without cyclopamine/FGF10. Likewise, treatment with RA alone led to lower insulin mRNA levels compared to cyclopamine/RA-treated variants [7, 8].

In the adult pancreas however, also other members of the hedgehog (Hh) family, Indian hedgehog (Ihh) and desert hedgehog (Dhh) and their receptors patched (Ptc) and smoothened (Smo), are expressed in β -cells making them direct targets of active Hh signaling. As a result, Thomas et al. [114] demonstrated that the inhibition of Hh signaling in INS-1 cells by cyclopamine resulted in a decrease of insulin mRNA and insulin secretion. Therefore, time and duration of cyclopamine application during late stages of ES cell differentiation have to be carefully controlled.

Retinoic Acid (RA)

Retinoids, including all-trans retinoic acid (RA), are known differentiation inducers and morphogens during embryonic development [for review, see [115–117]] and during in vitro differentiation of ES cells [118]. Specifically, RA signaling has been shown to affect pancreas specification in *Xenopus* [119], chicken [120], zebrafish [121, 122] and mice [123].

In *Xenopus*, RA concentration-dependently modulated the effects of activin by influencing expression levels of lineage-dependent genes [i.e., *goosecoid*, *Xlim-1*, *XIhbox-6* [124]]. Activin A at low concentration induced mesodermal and ectodermal (notochord) lineages in the animal cap assay, but the combined application of activin A (at intermediate concentration) and RA (10^{-6} M) resulted in the formation of muscle cells, while a further increase of the RA concentration resulted in the formation of pronephric tubules. Only combined application of high activin (100 ng/ml) and high RA (10^{-4} M) levels induced the development of pancreas instead of mesodermal tissues [125].

Due to its morphogenetic effects on lineage development, RA was frequently used in combination with activins for ES cell-derived pancreatic differentiation in vitro. Shi et al. applied activin A and all-trans-RA to differentiate mES cells into pancreatic cells [24]. Although insulin-positive cells were detected, the authors did not analyze the potential formation of neural cells. However, differentiation induction by activin A and RA is crucially dependent on the specific cell types or developmental stage of cells. For example, in the case of neural PC12 cells, activin A was found to induce the formation of neuronal extensions and neurofilament proteins [52] pointing towards the involvement of activin A in neural differentiation. By repeating the differentiation protocol of Shi et al. [24] using activin A in combination with RA (10^{-6} M), we showed that mES cells differentiated into both, pancreatic and neuronal (β -III-tubulin+) insulin-positive cells [53].

Concentration-dependent effects on ES cell differentiation were also observed by combined application of activin A and RA with sodium butyrate (see also “Sodium Butyrate” section). Whereas RA and sodium butyrate application led to the formation of pancreatic endocrine cells, RA with

activin A and RA with betacellulin, respectively, induced neuronal- and glial-like cell types from mES cells [25].

In hES cells, sequential application of FCS, activin A and RA resulted in the induction of endoderm-specific Sox17, Foxa2, Pdx1 and Hb9 transcript levels, while ectoderm-specific Pax6, and Sox1 levels were decreased [65]. Because of significant effects on endoderm and pancreatic endocrine cell formation, RA application was also component of the differentiation protocols established by the Baetge group [7, 8]: Activin A-induced hES cells were treated by sequential application of cyclopamine (0.25 μ M) for 2–4 days and of RA (2 μ M in combination with cyclopamine, 0.25 μ M) for another 2–4 days into pancreatic progenitors.

Noggin

Noggin was also applied to promote pancreatic differentiation at later stages of development. Application of noggin to VegT/ β -catenin-injected animal caps of *Xenopus*, which formed clusters of exocrine and endocrine pancreatic cells upon treatment with RA, further enhanced the expression of endocrine (insulin) and exocrine (XPDip) markers, whereas simultaneously, liver specific Ttr and XHex were reduced [119]. A similar observation was described for the murine system. In murine tissue explants containing ventral foregut endoderm, septum transversum mesenchyme and cardiac mesoderm, noggin treatment blocked hepatic differentiation and induced the pancreatic progenitor marker Pdx1 [126]. Consequently, noggin was used to differentiate hES cells by combined application with EGF and bFGF, which induced the differentiation of DE into pancreatic exocrine and endocrine cells [127]. Also, Kroon et al. substituted FGF10 [see [8]] by noggin together with RA and cyclopamine to differentiate primitive gut-like cells (Hnf1b, Hnf4) into posterior foregut-like (Pdx1, Hnf6, Prox1, Sox9) derivatives [7].

These results demonstrate that the inhibition of BMP signaling is not only important for early endoderm induction, but also for the continuous differentiation into pancreatic progenitors.

Sodium Butyrate

Previous studies revealed inductive effects of sodium butyrate on pancreatic differentiation of ES cells [128]. Since liver and pancreas arise from common endoderm progenitors, and hepatic differentiation has been achieved by long-term exposure to sodium butyrate, Goicoa et al. [129] hypothesized that short exposure of mES cells to sodium butyrate (2 mM) could induce differentiation into early stages of pancreatic development. This regime led to down-regulation of hepatic markers, but significantly

induced alpha-fetoprotein (AFP). In contrast, genes expressed in pancreatic precursors, like *Pdx1* and *Isl-1*, were up-regulated, while endocrine-specific genes, such as *Ngn3*, *NeuroD* and *Pax4*, were slightly down-regulated in comparison to undifferentiated cells. Interestingly, *Pdx1* mRNA levels sharply decreased after butyrate removal. Butyrate further decreased the expression of neuroectoderm, but increased expression of mesendoderm genes. The data were substantiated by immunocytochemistry showing abundance of Foxa2-positive clusters, similar to those found in early pancreatic epithelium. The authors concluded that sodium butyrate “stimulates early events of pancreatic specification, prior to the onset of endocrine differentiation” [129].

Moreover, McKiernan et al. [25] analyzed the effects of sodium butyrate in combination with betacellulin (BTC) and activin A on mES cell differentiation. EBs cultured in the presence of RA for 5 days were treated with sodium butyrate (1 mM), BTC (1 nM) or activin A (2 nM) for another 4 days (see also “Betacellulin (BTC)”). Sodium butyrate induced expression of pancreatic transcription factors and the extra-embryonic endoderm marker AFP, whereas neuronal genes were repressed. In activin A- and BTC-treated cultures, insulin I/II and *glut2* mRNA levels were down-regulated, while GFAP, *Shh* and somatostatin were up-regulated. From these results the authors concluded that sodium butyrate preferentially induced the differentiation of pancreatic endocrine insulin-producing cells, while (under the conditions used) activin A and betacellulin gave rise to neuronal- and glial-like cell types [25].

Another study using hES cells analyzed the effects of sodium butyrate, activin A and the combination of both on the formation of DE cells. Human ES cells were cultured in 1 mM sodium butyrate and 4 nM activin A for one day and 0.5 mM sodium butyrate and 4 nM activin A for another 6 days. Sodium butyrate alone was ineffective in inducing DE cells, but the combination of sodium butyrate and activin A resulted in higher Foxa2, Hnf4 α and similar Sox17 levels when compared to activin A treatment alone. Flow cytometric analysis revealed only 2–3% of the total cell population as Sox17-positive/CXCR4-negative cells indicative of extra-embryonic endoderm. Further differentiation of sodium butyrate and activin A-treated cells in B27 medium containing EGF, bFGF and noggin produced maximal levels of Pdx1-positive cells [127] suggesting that also the formation of extra-embryonic endoderm is necessary for pancreatic differentiation.

Growth Factors

Protocols for the differentiation of ES-derived endoderm progenitors into pancreatic cell types applied various growth factors, including members of the fibroblast growth

factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor (IGF) families. In addition, medium supplements, such as B27 containing growth factors, are routinely used for differentiation, expansion, and terminal maturation of pancreatic endocrine cells [see [130]]. However, the relevance of these factors on terminal pancreatic differentiation has been questioned. For example, D'Amour et al. [8] routinely used N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine-t-butylester (DAPT, LY374973), exendin-4, IGF-1 and HGF during terminal differentiation stages, but observed only minor effects on differentiation, when these factors were omitted. Likewise, Phillips et al. [66] applied B27, FGF18, EGF, TGF α , IGF1 and 2, VEGF, forskolin, HGF, exendin 4, heparin and pancreatic polypeptide Y. While the authors found that eliminating either FGF18 or B27 significantly reduced insulin expression and C-peptide release, the effect of the other growth factors on differentiation of Pdx1-/C-peptide+ pancreatic cells could not be unequivocally demonstrated.

However, several growth factors have been found to act in conjunction with activin A also to stimulate the proliferation of pancreatic progenitors.

Betacellulin (BTC)

A mitogenic role for β -cells was found for betacellulin (BTC), member of the epidermal growth factor (EGF) family, in combination with activin A on the formation of insulin-producing cells from exocrine AR42J cells [131]. Specifically, Demeterco et al. [132] analyzed the effects of activin A and/or BTC on islet development and growth from primary cultures of human fetal pancreatic tissue. BTC acts as a mitogen for undifferentiated pancreatic epithelial cells accompanied by an increase in the number of islet-like cell clusters, whereas activin A promoted increased insulin contents. Interestingly, the combination of activin A and BTC led to slightly weaker effects when compared to those variants using each factor alone. The authors conclude, that “activin A may stimulate the cells towards differentiation and BTC to proliferation, one occurring at the expense of the other” [132].

Cho et al. [133] modified the protocol published by D'Amour et al. [8], by applying BTC and nicotinamide (NIA) to hES cells at late stages of pancreatic differentiation. BTC and NIA treatment resulted in sustained Pdx-1 expression and led to subsequent insulin-production. However, this effect could not be observed with BTC or NIA alone. McKiernan et al. [[25], see above] showed that directed differentiation of mouse ES cells in the presence of BTC alone led to the formation of glial-like cells suggesting the induction of ectodermal differentiation.

Fibroblast Growth Factors (FGFs)

FGFs are not only essential for early endoderm lineage determination (see above) but are also playing a role at later stages of pancreatic differentiation. Loss and gain of function experiments indicated that FGF10 is involved in the control of pancreas development [134–136]. In FGF10^{-/-} mouse embryos the initiation of dorsal and ventral pancreatic buds occurred normally, whereas PDX1+ progenitor cells within the pancreatic buds showed a marked decrease in proliferation. Addition of FGF10 rescued the PDX1-expressing epithelial cells in cultured pancreas derived from *Fgf10* mutants [134].

A proliferation-inducing effect of FGF7 and FGF10 on human pancreatic rudiments was demonstrated by Ye et al. [137] leading to induction of the early growth response gene *egr1*. A similar positive effect was found for FGF7 (= KGF) on proliferation of ductal cells, which after transplantation subsequently differentiated into β -cells [138]. In mES cells, treatment of EBs with FGF7 caused high up-regulation of exocrine markers p48 and amylase in addition to up-regulated Pdx1 expression [139, 140].

The stimulating effects of FGF7 and 10 on pancreatic development were used in the hES cell differentiation protocol by D'Amour et al. [8]. Activin A-induced endoderm progenitor cells were treated with FGF10 together with cyclopamine followed by RA treatment. FGF10 and cyclopamine application resulted in a 160-fold increase of insulin mRNA expression in comparison to activin A treatment alone [8]. Later, Kroon et al. [7] substituted FGF10 by FGF7 in the differentiation protocol, whereas Gouon-Evans et al. [70] stimulated activin A-induced endoderm progenitor proliferation by FGF2, however, these cells had the potential to differentiate into the hepatic and not the pancreatic lineage.

Hepatocyte Growth Factor (HGF)

Hepatocyte growth factor (HGF) known to stimulate hepatic differentiation was also found to exert beneficial effects on β -cell mass and function, potentially as a mitogen. Garcia-Ocana et al. [141] demonstrated in β -cells of a transgenic mouse model (in which HGF expression is regulated by the rat insulin promoter (RIP)-II) higher insulin levels, increased glucose-induced insulin secretion and up-regulated Glut2 and glucokinase mRNA levels.

In vitro studies showed that activin-treated AR42J cells differentiated into insulin-producing cells when further treated with HGF [131]. HGF was also found to increase the number of insulin-producing cells in cultured human islets [142].

In hES cells, HGF in combination with pancreatic polypeptide (PP) and forskolin applied during late stage of

pancreatic differentiation and at high glucose levels increased insulin transcription paralleled by C-peptide release. However, this up-regulation was attributed to the effects of forskolin and glucose, and it was questionable, whether the addition of HGF specifically potentiated this effect [66]. D'Amour et al. [8] used HGF within the last stage of pancreatic differentiation of hES cells (in combination with exendin-4 and IGF1), but were unable to demonstrate a specific beneficial effect of these three factors.

In summary, the present data on ES cell differentiation do not allow a final conclusion about potential mitogenic or differentiation-inducing effects of HGF and growth factors on pancreatic differentiation. As a consequence, terminal differentiation steps in vitro were omitted from the protocol introduced by D'Amour et al. [8]. Instead, the authors successfully used the hES-derived activin A-, cyclopamine- and RA-induced progenitor population (expressing Nkx2.2, Nkx6.1, Ngn3, and Ptf1a) for successful direct transplantation into diabetic animals [7].

Summary and Conclusion

The enrichment of endoderm progenitors is a prerequisite for the development of efficient strategies to generate functional pancreatic cells. The application of activin A to ES cells substantially induces DE cells in vitro. However, activin A-induced endoderm differentiation from murine and human ES cells depends on concentration, culture conditions, time of application, and interactions of activin A with various signaling pathways and differentiation factors:

1. The experimental design of activin A-induction strategies is dependent on the organism used, i.e. whether human or murine ES cells are applied. While hES cells are successfully differentiated in monolayer culture, mES cells are routinely cultured in EBs. These different culture systems may reflect different developmental stages of both ES cell types.
2. The concentrations of activin A required for endoderm induction range from 10–100 ng/ml with treatment durations of up to 10 days. EBs require higher activin A levels due to concentration gradients within EBs, while in monolayer culture lower concentrations may be used.
3. All studies show that FCS in the differentiation medium interferes with activin A activity. Therefore, alternatively, low concentrations of FCS or KSR, and BSA- or PVA-containing serum-free and chemically defined media are applied.
4. To enhance the inductive effect of activin A, factors like Wnt3a, noggin (as BMP inhibitor), and FGF2/4 are successfully used.

5. The induction of activin A-induced DE cells into pancreatic endocrine progenitor cells is facilitated by various growth and differentiation factors, such as the pancreas-specific inducers cyclopamine and RA, and by proliferation inducers including sodium butyrate, betacellulin, FGFs and HGF.

However, at present, all those factors mentioned above do not allow in vitro terminal differentiation of ES cells into functional beta cells. Additional factors and cultivation conditions (i.e. 3D-culture systems) will be required to induce complete differentiation and full maturation of ES-derived progenitors into glucose-responsive islet cells. The importance of endothelial cell-released growth factors, the role of the vascular endothelial system for pancreatic differentiation and specification [143, 144] and of cell-matrix interactions during pancreatic differentiation [145] demands more sophisticated in vitro systems to generate sufficient amounts of functionally competent insulin-secreting pancreatic cells.

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