

Induction and Selection of Sox17-Expressing Endoderm Cells Generated from Murine Embryonic Stem Cells

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Key Words

Mouse embryonic stem cells • In vitro differentiation • Activin A • Definitive endoderm • Pancreatic endocrine lineage

Abstract

Embryonic stem (ES) cells offer a valuable source for generating insulin-producing cells. However, current differentiation protocols often result in heterogeneous cell populations of various developmental stages. Here we show the activin A-induced differentiation of mouse ES cells carrying a homologous dsRed-IRES-puromycin knock-in within the *Sox17* locus into the endoderm lineage. Sox17-expressing cells were selected by fluorescence-assisted cell sorting (FACS) and characterized at the transcript and protein level. Treatment of ES cells with high concentrations of activin A for 10 days resulted in up to 19% Sox17-positive cells selected by FACS. Isolated Sox17-positive cells were characterized by definitive endoderm-specific Sox17/Cxcr4/Foxa2 transcripts, but lacked pluripotency-associated Oct4 mRNA and protein. The Sox17-expressing cells showed downregulation of extra-

embryonic endoderm (Sox7, Afp, Sdf1)-, mesoderm (Foxf1, Meox1)- and ectoderm (Pax6, NeuroD6)-specific transcripts. The presence of Hnf4 α , Hes1 and Pdx1 mRNA demonstrated the expression of primitive gut/foregut cell-specific markers. Ngn3, Nkx6.1 and Nkx2.2 transcripts in Sox17-positive cells were determined as properties of pancreatic endocrine progenitors. Immunocytochemistry of activin A-induced Sox17-positive embryoid bodies revealed coexpression of Cxcr4 and Foxa2. Moreover, the histochemical demonstration of E-cadherin-, Cxcr4-, Sox9-, Hnf1 β - and Ngn3-positive epithelial-like structures underlined the potential of Sox17-positive cells to further differentiate into the pancreatic lineage. By reducing the heterogeneity of the ES cell progeny, Sox17-expressing cells are a suitable model to evaluate the effects of growth and differentiation factors and of culture conditions to delineate the differentiation process for the generation of pancreatic cells in vitro.

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Abbreviations used in this paper

ALK	activin receptor-like kinase
BAC	bacterial artificial chromosome
BSA	bovine serum albumin
CDM	chemically defined medium
CLSM	confocal laser scanning microscope
DE	definitive endoderm
EB	embryoid body
EDTA	ethylenediaminetetraacetic acid
ES	embryonic stem
ESC	embryonic stem cells
ETOH	ethanol
F12	Ham's F12 medium
FACS	fluorescence-assisted cell sorting
FCS	fetal calf serum
FITC	fluorescein-isothiocyanate
G418	geneticin
GMEM	Glasgow modified minimum essential medium
H&E	hematoxylin and eosin
IMDM	Iscove's modified Dulbecco's medium
IRES	internal ribosomal entry site
LIF	leukemia inhibitory factor
MTG	monothioglycerol (3-Mercapto-1,2-propanediol)
NOD/SCID	non-obese diabetes/severe combined immuno-deficiency
PFA	paraformaldehyde
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
TGFβ1	transforming growth factor β1

Introduction

The increasing incidence of diabetes mellitus and the shortage of transplantable pancreatic islets to treat patients suffering from the disease require alternative methods to generate insulin-producing cells of a sufficient amount and quality [Guo and Hebrok, 2009]. Due to their almost unlimited replication potential and ability to form all differentiated cell types of the body [Wobus and Boheler, 2005], embryonic stem (ES) cells have been used to generate insulin-producing cells in vitro. Initially, the following strategies were applied: (1) the directed differentiation through lineage selection of pancreatic phenotypes using pancreas-specific promoters [Leon-Quinto et al., 2004; Soria et al., 2000], (2) the constitutive expression of pancreatic developmental control genes [Blyszczuk et al., 2003; Ku et al., 2004; Miyazaki et al., 2004; Shiroy et al., 2005; Treff et al., 2006; Boretto and Gooch, 2007; Serafimi-dis et al., 2008], (3) the application of specific signaling molecules, such as phosphoinositide 3-kinase inhibitors [Hori et al., 2002] or retinoic acid [Micallef et al., 2005; Shi

et al., 2005; McKiernan et al., 2007], (4) the differentiation via multilineage progenitors [Blyszczuk et al., 2004; Schroeder et al., 2006], (5) the use of conditioned medium from fetal pancreatic buds [Vaca et al., 2006] and (6) the selection and enrichment of a nestin-expressing cell population [Lumelsky et al., 2001]. However, in these earlier studies using mouse ES cells, differentiation was insufficient with respect to the number of insulin-positive cells and their maturation status, and in many cases, insulin-producing neural progeny rather than pancreatic cells were generated [Rajagopal et al., 2003; Hansson et al., 2004; Sipione et al., 2004; Hori et al., 2005; Roche et al., 2005].

A prerequisite for pancreatic differentiation is the formation of definitive endoderm (DE), which in turn gives rise to the pancreatic lineage. A crucial transcription factor governing DE development is Sox17, as underlined by a lack/depletion of gut formation in Sox17-null mutant mice [Kanai-Azuma et al., 2009]. In vivo, the generation of DE is induced via nodal signaling [Tam et al., 2003; Grapin-Botton and Constam, 2007; Tam and Loebel, 2007], a member of the transforming growth factor-β superfamily. Activin A mimicking nodal signaling [Tam et al., 2003] has been shown to specifically induce differentiation of ES cells into DE [Kubo et al., 2004; D'Amour et al., 2005; Shi et al., 2005; Cai et al., 2007]. However, several critical culture parameters have to be considered that vary between human and murine ES cells, i.e. serum- and feeder-free cell culture and the concentration, time and duration of activin A application [Sulzbacher et al., 2009].

In vitro, activin A [D'Amour et al., 2006; Jiang et al., 2007; Kroon et al., 2008] was successfully applied to human and murine ES cells to generate DE, and by consecutive application of differentiation factors, to derive pancreatic cells. However, the current differentiation protocols resulted in a heterogeneous population containing cells of various developmental stages including undifferentiated pluripotent, potentially tumorigenic stem cells and a remarkable number of nonpancreatic cells. The selection of a homogeneous population of DE progenitors will be one prerequisite for efficient pancreatic differentiation [McKnight et al., 2010]. Such selection was attempted by Tada et al. [2005], who used *goosecoid* (*Gsc*) expression as a marker for mesendodermal cells. They identified *Gsc*⁺/*E-cadherin*⁺/*PDGFRα*[−] cells as intermediates that would eventually differentiate into DE and could be distinguished from *Gsc*⁺/*E-cadherin*[−]/*PDGFRα*⁺ mesoderm. Similarly, Cai et al. [2010] sorted activin A-induced cells using *Cxcr4* as a marker to obtain

a homogeneous Pdx1⁺ population upon further differentiation with retinoic acid. However, like Gsc, Cxcr4 is a marker of mesoderm, endoderm and their mesendodermal precursors.

Here, we present the activin A-induced differentiation of murine ES cells that carry a homologous dsRed-internal ribosomal entry site (IRES)-puromycin knock-in within the *Sox17* locus, followed by fluorescence-assisted cell sorting (FACS) selection of Sox17-positive DE cells and analysis of their transcript levels, protein abundance and cellular localization.

Materials and Methods

Generation of Sox17-dsRed Knock-In ES Cells and Culture of Undifferentiated ES Cells

Feeder-free CGR8 ES cells carrying a homologous dsRed-IRES-puromycin knock-in within the *Sox17* gene locus were used. The strategy for generating the dsRed-IRES-puromycin knock-in cell line involved the generation of the targeting construct, the modification of a mouse bacterial artificial chromosome clone encompassing the *Sox17* locus by recombineering in *Escherichia coli* [Zhang et al., 2000] and subcloning of the modified *Sox17* locus, followed by homologous recombination in ES cells. Correctly targeted ES cells were identified by Southern blotting [unpubl. data]. dsRed expression allowed real-time identification, selection and characterization of Sox17-positive DE progenitors by FACS. In further studies, resistance to puromycin would permit the selective isolation and cultivation of DE cells.

The Sox17-dsRed ES cells were maintained on gelatine (0.1%) coated tissue culture dishes in Glasgow minimum essential medium ES cell culture medium (Invitrogen) supplemented with 10% fetal calf serum (FCS), nonessential amino acids (1:100), 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin/streptomycin (all Invitrogen), 100 μ M β -mercaptoethanol (Merck), 10 ng/ml leukemia inhibitory factor (Millipore) and 200 μ g/ml G418 (Calbiochem). Cultures were passaged every day at a 1:3 split ratio [Wobus et al., 2002; Blyszczuk et al., 2006].

Differentiation of ES Cells

For differentiation, ES cells were washed 2 times with serum-free chemically defined medium (CDM) [Johansson and Wiles, 1995]. Basal CDM was prepared from Iscove's modified Dulbecco's medium (IMDM) with Glutamax-I and Ham's F12 medium (F12) with Glutamax-I (both Invitrogen, 1:1) supplemented with 450 μ M monothioglycerol, 2 U/ml leukemia inhibitory factor, 1% chemically defined lipid concentrate (Invitrogen), 5 mg/ml bovine serum albumin (BSA, Sigma), 150 μ g/ml transferrin (Sigma) and 7 μ g/ml insulin (Sigma).

Embryoid bodies (EBs) were formed as hanging drops (600 cells/20 μ l) for 3 days and subsequently cultured in bacteriological dishes for 4–7 days.

For the induction of endoderm differentiation, 50 and 100 ng/ml activin A (R&D Systems, PeproTech), respectively, were added. Control EBs were cultured in CDM alone, CDM supplemented with 20% FCS with or without activin A and in IMDM/F12 sup-

plemented with 2 mM glutamine, nonessential amino acids (1:100), penicillin/streptomycin, 450 μ M monothioglycerol and 20% FCS. To analyze the specificity of the activin A treatment, a subset of cells was additionally treated with 5 μ M SB431542, an inhibitor of transforming growth factor- β ₁ superfamily activin receptor-like kinase (ALK) receptors, in CDM + 50 ng/ml activin A. The morphology of the cells was analyzed by the Eclipse T300 microscope (Nikon). The data presented here are based on 7 independent experiments.

Flow Cytometry and Cell Sorting

Cells of EBs were dissociated with 0.08% EDTA/0.5% trypsin (1:1) at room temperature (RT) for 3 min. The reaction was stopped with serum-containing medium. After washing with PBS, the cell suspension was collected in 0.75 \times PBS at 4°C, filtered through a 50- μ m mesh, stained with propidium iodide (PI) (1 μ g/ml) and analyzed within 1 h by a FACS Aria flow sorter (BD Biosciences) equipped with a 488-nm laser. For detection of dsRed, PI and FITC the following filters were used: 575/26, 610/20 and 539/30, respectively. Sorting was performed in 0.75 \times PBS and the data processed using the Diva Software (BD Biosciences).

Reverse Transcription PCR

Total RNA was extracted with RNeasy Mini Kit (Qiagen) followed by cDNA synthesis using reverse transcription system (Fermentas) according to the manufacturer's recommendations.

Quantitative Real-Time PCR

For real-time PCR of cells at defined time points during differentiation and of FACS-sorted Sox17-positive and Sox17-negative cells, the mRNA was isolated with DynaBeads mRNA direct Micro Kit (Invitrogen) followed by DNase treatment (Abcam) and cDNA synthesis using a reverse transcription system (Fermentas) according to the manufacturer's recommendations. Changes in mRNA levels of specific genes were quantified using the ABI PRISM 7900HT Sequence Detection System and the Step One Plus (Applied Biosystems). A set of specific oligonucleotide primers was designed using Primer Express software 2.0 (Applied Biosystems). Amplification of the target genes was monitored via SYBR green fluorescence signals generated during each PCR cycle. Fluorescence signals were regarded as positive, if the fluorescence intensity reached 10 \times the standard deviation of the baseline fluorescence (threshold). The amplification mix was prepared following the SYBR green PCR Master Mix (Applied Biosystems) protocol. Each reaction was performed in triplicate in 96-well optical reaction plates under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing/extension). Relative mRNA levels were calculated using the standard curve method (see ABI PRISM 7700 Sequence Detection System User Bulletin #2; Applied Biosystems).

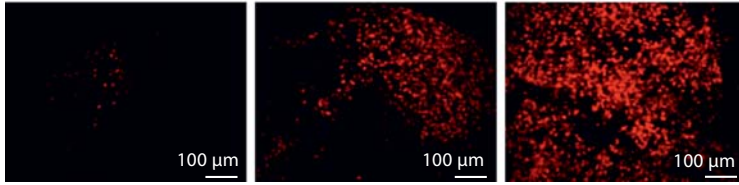
Immunocytochemical Analyses of EBs

EBs were fixed in 4% paraformaldehyde at RT for 40 min. Afterwards, EBs were treated with 0.2% Triton X-100 (Merck) for 15 min for nuclear staining and then blocked with 1% BSA at RT for 1 h. Whole EBs were stained with the primary antibody at 4°C overnight. The following primary antibodies were used: rat anti-Oct3/4 (1:50, R&D), rabbit anti-T (1:50, Abcam), rat anti-Cxcr4 (1:50, R&D) and rabbit anti-Foxa2 (1:50, Chemicon). The EBs

Control (IMDM/F12 + 20% FCS)



Activin A (50 ng/ml)

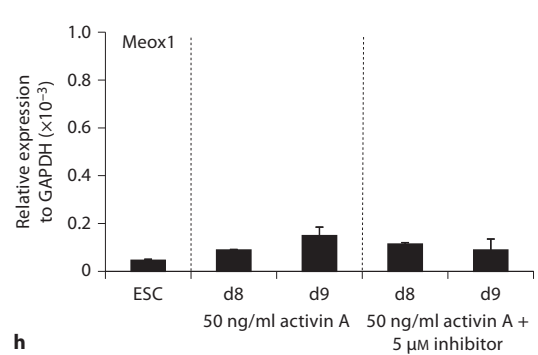
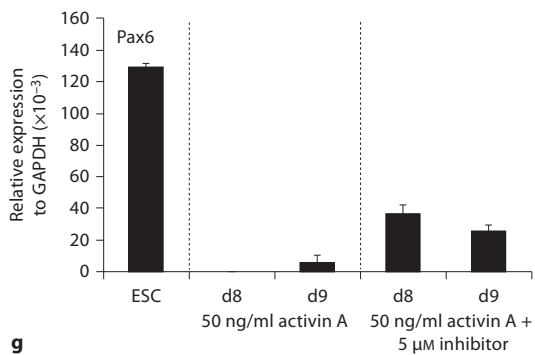
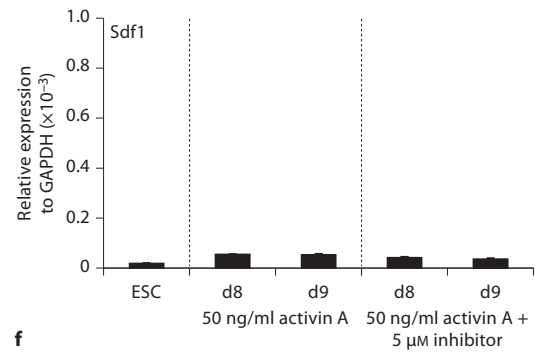
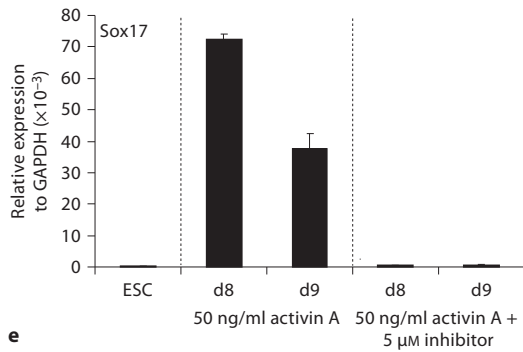
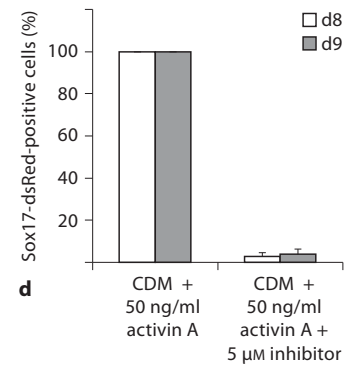
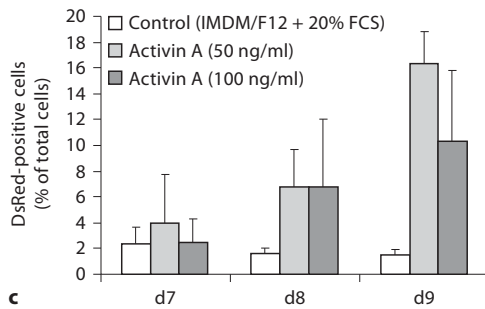
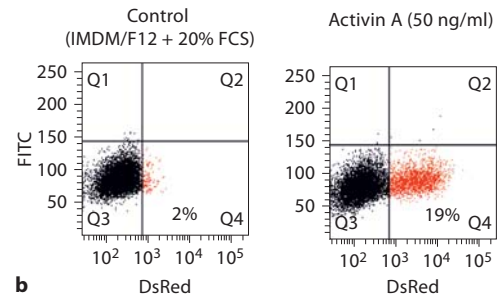


a

d7

d8

d9



were incubated with the secondary antibodies (diluted in 0.5% BSA) Alexa 488-conjugated rabbit anti-rat IgG or Alexa 488-conjugated chicken anti-rabbit (both 1:100, Molecular Probes) at 37°C for 45 min.

Paraffin sections of EBs were dried at 56°C overnight. Deparaffination was carried out twice in xylol for 10 min, once in 100, 98 and 70% in ETOH, each for 5 min. After washing in water, the sections were boiled in Retrieval solution (BD) for 10 min. The cooled sections were blocked with 1% BSA at RT for 30 min. The sections were incubated with the antibodies rabbit anti-E-cadherin (1:50, Santa Cruz) and goat anti-N-cadherin (1:50, Santa Cruz), respectively, at RT for 2 h, or with rat anti-Cxcr4 (1:50, R&D), rabbit anti-T (1:50, Abcam) and rabbit anti-Ngn3 (1:50, Abcam), respectively, at 37°C for 30 min. Staining with anti-Hnf1 β (1:100, Santa Cruz) and anti-Sox9 (1:250, Santa Cruz) was performed at 4°C overnight. The secondary antibodies Alexa 488-conjugated chicken anti-rabbit, Alexa 488-conjugated donkey anti-goat, Alexa 488-conjugated rabbit anti-rat and Alexa 488-conjugated chicken anti-rabbit (all 1:200, Molecular Probes, diluted in 0.5% BSA) were used at 37°C for 30 min.

To label the nuclei, the EBs were incubated at RT with 5 μ g/ml Hoechst 33342 for 5 min. EBs were analyzed with the confocal laser scanning microscope LSM-510 META (Carl Zeiss) or the BZ-8000 fluorescence microscope (Keyence).

Tissue taken from whole mouse embryos, embryonic liver and brain on embryonic day 13 (E13) and adult pancreases served as controls (see online supplementary figure 1, www.karger.com/doi/10.1159/000329864). Hematoxylin/eosin (H&E) staining was performed according to conventional methods.

Results

Time-Dependent Sox17 Expression Monitored via dsRed Immunofluorescence

CGR8 cells carrying a homologous dsRed-IRES-puro-mycin knock-in within the *Sox17* locus allowed the analysis of DE-specific Sox17 expression on the basis of the dsRed reporter. After differentiating ES cells in EBs in the presence of 50 ng/ml activin A, clearly visible dsRed/Sox17-positive cells appeared at day 7, whereas control cells (cultured in IMDM/F12 + 20% FCS) differentiating without activin A showed only very few Sox17-positive cells (fig. 1a). At day 8, the number of Sox17-positive cells clearly increased forming large clusters (fig. 1a), reached

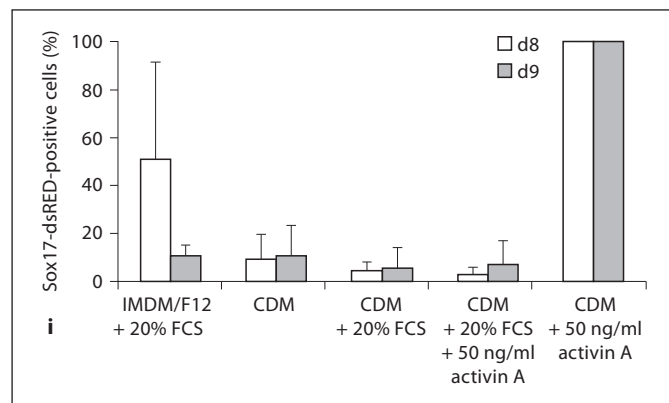
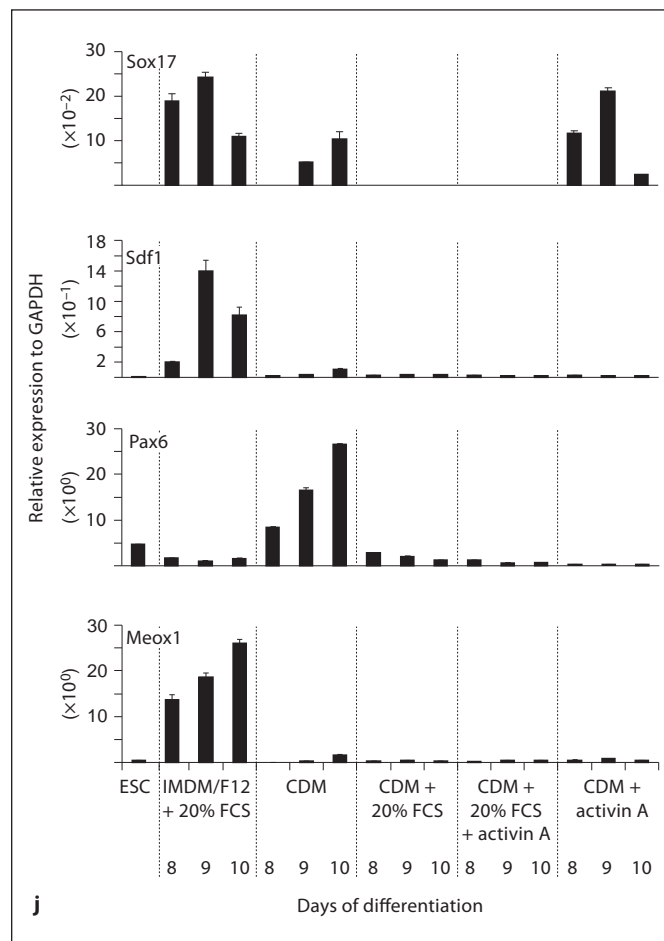


Fig. 1. Differentiation analysis of Sox17-expressing cells from murine ES cells in the absence (control) or presence of activin A. **a** Sox17-dsRed labeling in control and activin A (50 and 100 ng/ml)-treated EBs at days 7, 8 and 9. **b** Gating parameters for FACS selection of control (left) and activin A-treated samples (right) at day 9. **c** Percentage of FACS-selected dsRed/Sox17-positive cells in the entire cell population of control and activin A-treated samples at days 7, 8 and 9, $n = 3$. **d** Percentage of Sox17-dsRed-positive cells after treatment with activin A and the ALK inhibitor SB431542. **e–h** Transcript levels of Sox17, Sdf1, Pax6 and Meox1 in cells after treatment with activin A, with or without supplementation of the ALK inhibitor SB431542. **i** Percentage of Sox17-dsRed-positive cells differentiated in IMDM/F12 + 20% FCS, CDM alone, or CDM supplemented with 20% FCS with or without activin A (compared to cells treated with activin A in CDM without FCS), $n = 3$. **j** Transcript levels of Sox17, Sdf1, Pax6 and Meox1 after differentiation in IMDM/F12 + 20% FCS, CDM alone, CDM supplemented with 20% FCS with or without activin A and CMD with activin A. d = Day.



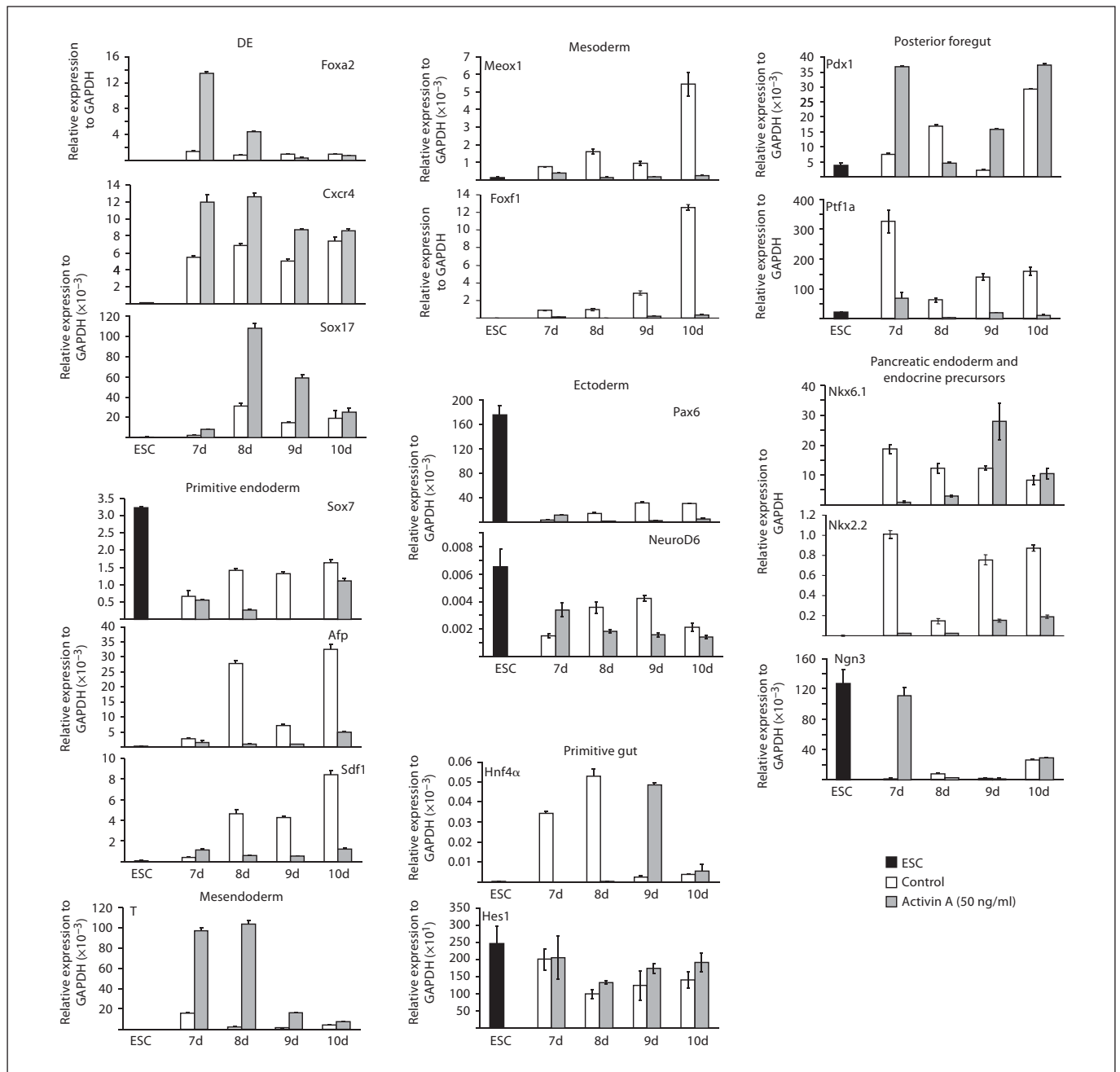


Fig. 2. Time-dependent relative transcript levels of various lineage-specific and pancreatic control genes of control and activin A (50 ng/ml)-treated ES cells (ESC) cultured as EBs at days 7, 8, 9 and 10 analyzed by quantitative RT-PCR. Data (mean \pm SEM) were normalized to GAPDH (internal standard) and compared to undifferentiated ES cells. d = Day.

a maximum at days 9–10 and declined within the following 2–3 days (data not shown). Comparable results were obtained with 100 ng/ml activin A.

To quantify the number of Sox17-positive progenitors, cells were analyzed by FACS. To discriminate viable from

nonviable cells, dissociated cells of EBs were additionally stained with PI. Figure 1b shows the gating parameters used to determine Sox17-positive cells. The control cell population (fig. 1b, left) was mostly composed of dsRed-negative cells. Only a small fraction of Sox17-positive/PI-

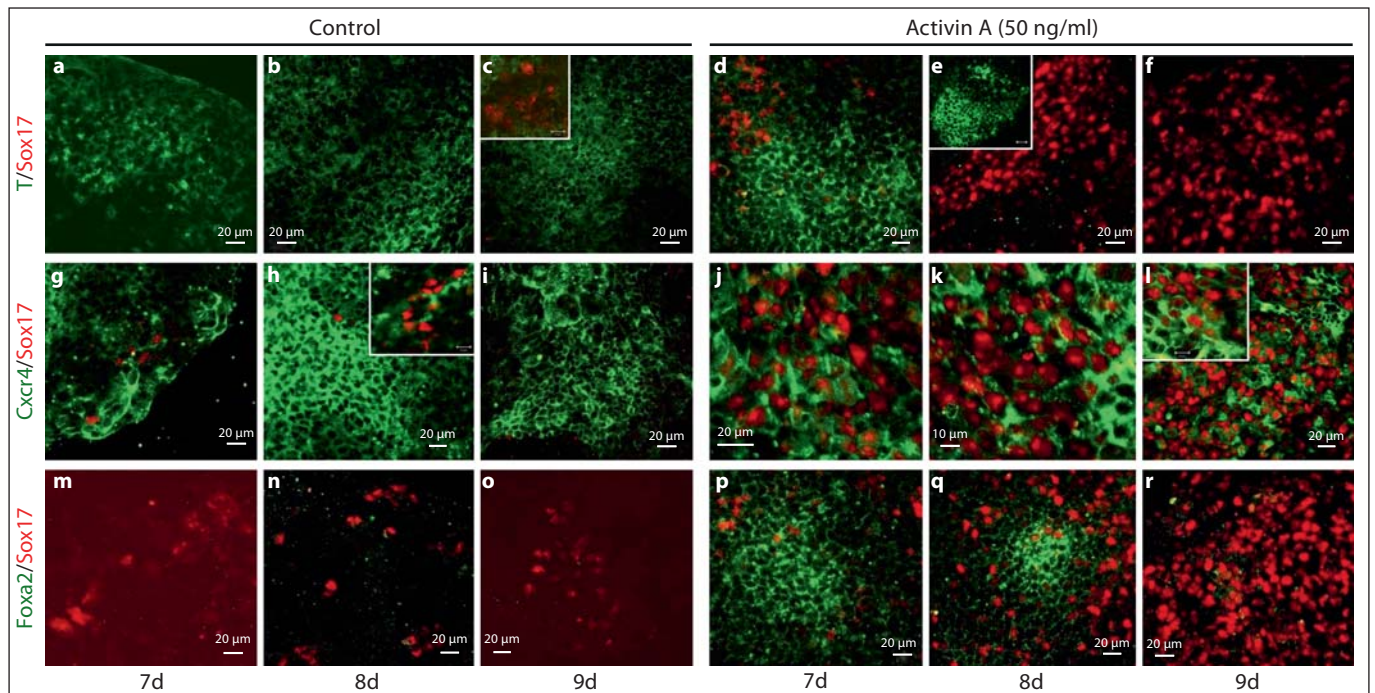


Fig. 3. Protein abundance and localization at days 7, 8 and 9 of selected developmental markers in Sox17-positive EBs analyzed by confocal laser scanning microscope. Brachyury (T)-positive cells in controls (**a–c**) and activin A (50 ng/ml)-treated samples (**d–f**), Cxcr4-positive cells in controls (**g–i**) and activin A-treated samples (**j–l**), and Foxa2-positive cells in controls (**m–o**) and activin A-treated samples (**p–r**). Bar = 20 μ m, except for **k, m** (10 μ m). **Insets** Different areas of the EBs (**c, e, h**) or magnification of the area shown (**l**). d = Day.

negative cells was detectable. In contrast, activin A-treated cells displayed a high percentage of dsRed/Sox17-positive cells (fig. 1b, right).

Whereas control cells after day 7 did not reach amounts beyond those of 2% dsRed/Sox17-positive cells, activin A-treated cells attained levels up to 19% after treatment with 50 ng/ml activin A, and up to 16% with 100 ng/ml activin A (fig. 1c). Treatment of EBs with activin A in the presence of SB431542, an inhibitor of transforming growth factor- β 1 superfamily ALK receptors, almost completely abolished the number of dsRed/Sox17-positive cells (fig. 1d), and the Sox17 mRNA level was downregulated (fig. 1e). However, Pax6 (fig. 1g) transcripts were slightly elevated and Sdf1 and Meox1 (fig. 1f, h) levels were not changed.

Because comparable Sox17 transcript levels in cells treated with 50 and 100 ng/ml activin A were detected, the following experiments were mainly performed with 50 ng/ml activin A. Culturing cells in CDM alone or CDM supplemented with 20% FCS, with or without activin A, resulted in very low numbers of dsRed/Sox17-positive cells (fig. 1i). While control EBs showed high

Sdf1 and Meox1 levels, cells cultured in CDM revealed high Pax6 transcript levels. Cells differentiated in CDM supplemented with 20% FCS, with and without activin A, displayed no Sox17, Sdf1, Pax6 or Meox1 mRNA (fig. 1j).

Analyses of Lineage-Specific Transcripts during in vitro Differentiation

To determine the stages of maximal expression, transcript levels of endo-, ecto-, and mesoderm-specific genes as well as markers of the early pancreatic lineage were analyzed by quantitative RT-PCR during in vitro differentiation. Endoderm- and mesendoderm-specific transcripts showed maximum levels at days 7 and 8, whereas most of the pancreas-specific markers revealed highest levels at days 9 and 10 (fig. 2).

Analyses of Lineage-Specific Proteins during in vitro Differentiation

ES cells differentiating as EBs in the absence or presence of activin A showed different protein abundance over time. Brachyury (T) protein was detected throughout differentiation in control cells (fig. 3a–c) with only

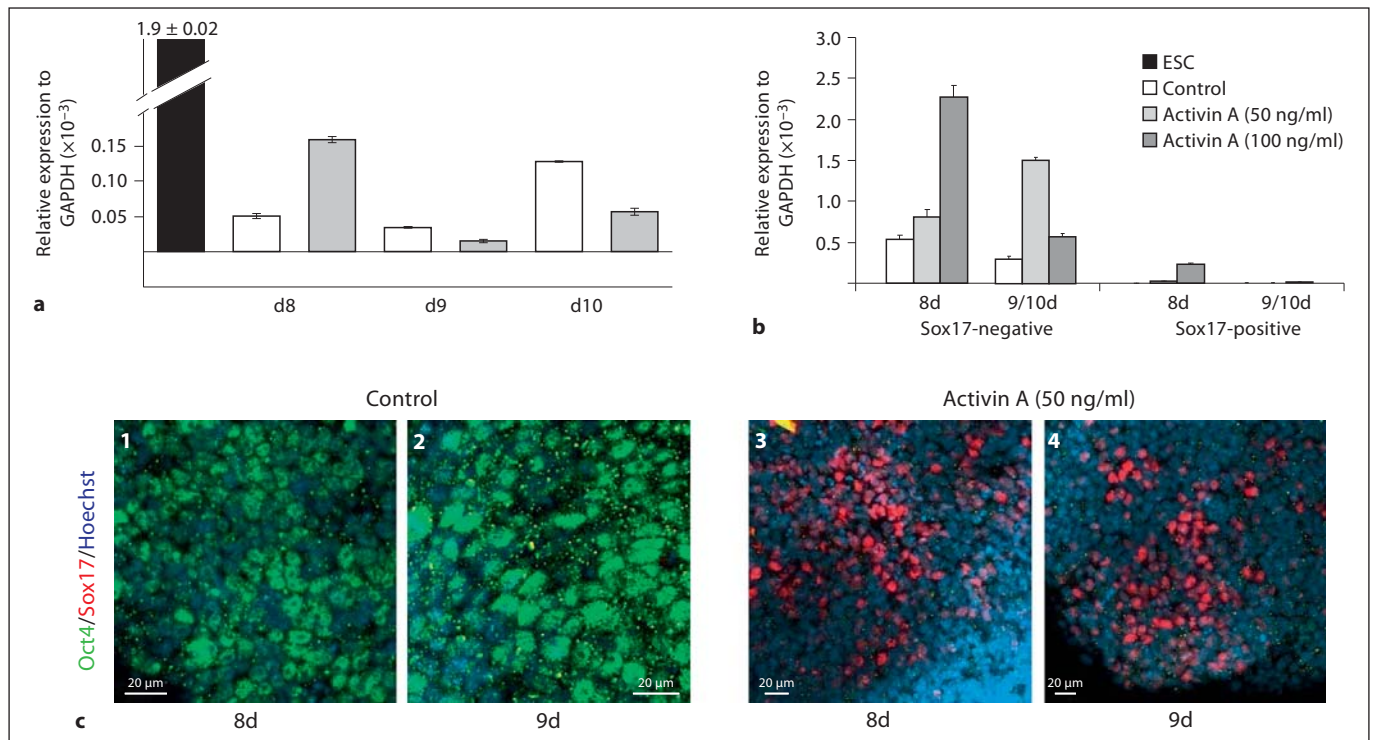


Fig. 4. Analysis of the pluripotency-associated marker Oct4. Oct4 mRNA levels during differentiation (days 8–10) (a) and in FACS-selected Sox17-negative and Sox17-positive fractions at day 8 and day 9/10 (b) analyzed by quantitative RT-PCR. Data (mean \pm SEM) were normalized to GAPDH (internal standard). c Oct4 protein abundance in control EBs (panel 1, 2) and EBs treated with activin A (50 ng/ml, panel 3, 4) at days 8 and 9 by confocal microscopy analysis. Bar = 20 μ m. d = Day.

occasional colocalization with Sox17 (fig. 3c, inset). In activin A-treated cells, T was coexpressed with Sox17 only in some cells at day 7, while others stained positive exclusively for T (fig. 3d). At day 8, activin A-treated cells showed either T-labeling (fig. 3e, inset) or Sox17-labeling (fig. 3e), whereas at day 9, T protein was absent (fig. 3f).

Cxcr4 protein was present in control and activin A-treated cells throughout differentiation (fig. 3g–i). In controls, Cxcr4 labeling was rarely accompanied by Sox17 staining (inset, fig. 3h), while in activin A-treated cells, most of the Cxcr4-positive cells were also Sox17-positive (fig. 3j–l). Foxa2 protein could not be detected in control cells (fig. 3m–o), but was clearly visible in activin A-induced cells at day 7, diminished at day 8 and was virtually absent at day 9 (fig. 3p–r).

Transcript and Protein Levels of the Pluripotency-Associated Marker Oct4

Oct4 (*Pou5f1*) transcripts, highly abundant in undifferentiated ES cells, showed decreased levels in both control and activin A-treated cells during differentiation

(fig. 4a). However, some Oct4 mRNA was still detected in activin A-induced cells, albeit lower than in the controls at days 9 and 10. FACS-isolated fractions of Sox17-positive cells isolated on day 8 and day 9/10 revealed only marginal levels or an absence of Oct4 mRNA, whereas Oct4 was present in the Sox17-negative population in control and activin A-treated cells (fig. 4b). Immunofluorescence analyses revealed Oct4-positive cells in the control population at days 8 and 9 (fig. 4c, panel 1, 2), whereas in activin A-treated EBs positively labeled by Sox17, Oct4 was not detectable (fig. 4c, panel 3, 4). In very rare cases, Oct4-positive EBs could be found after treatment with activin A. However, these EBs were never Sox17-positive.

Characterization of the FACS-Selected Sox17-Positive Cell Population at Day 8 and Day 9/10

FACS analysis allowed the characterization of transcript profiles of Sox17-positive and Sox17-negative cell populations by quantitative RT-PCR at day 8 and day 9/10 (fig. 5).

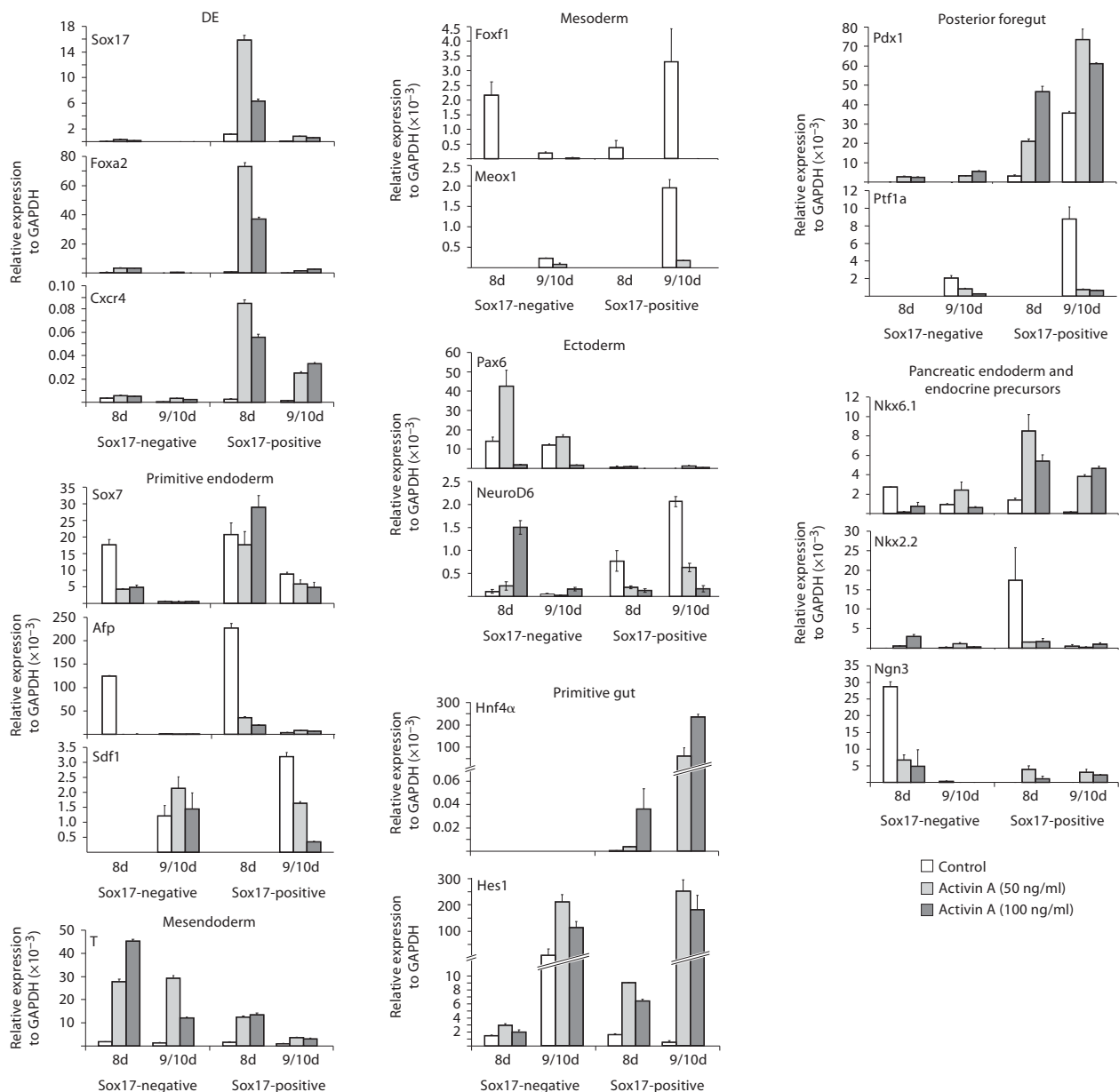


Fig. 5. Relative mRNA levels determined by quantitative RT-PCR of lineage-specific genes of FACS-selected Sox17-negative and Sox17-positive cells differentiated in the absence (control) or presence of activin A (50 and 100 ng/ml) at day 8 and day 9/10. Data (mean \pm SEM) were normalized to GAPDH (internal standard). d = Day.

First, endoderm-specific Sox17, Foxa2 and Cxcr4 mRNA levels were analyzed. As expected, Sox17, Foxa2 and Cxcr4 levels were low or undetectable in the Sox17-negative fractions at day 8 and day 9/10. The highest transcript levels of all three genes were found in Sox17-positive

cells treated with 50 ng/ml activin A at day 8, whereas 100 ng/ml resulted in lower levels.

Next, the primitive/extraembryonic endoderm-specific markers Sox7, Afp and Sdf1 were analyzed. Sox7 transcripts were generally higher in Sox17-positive than

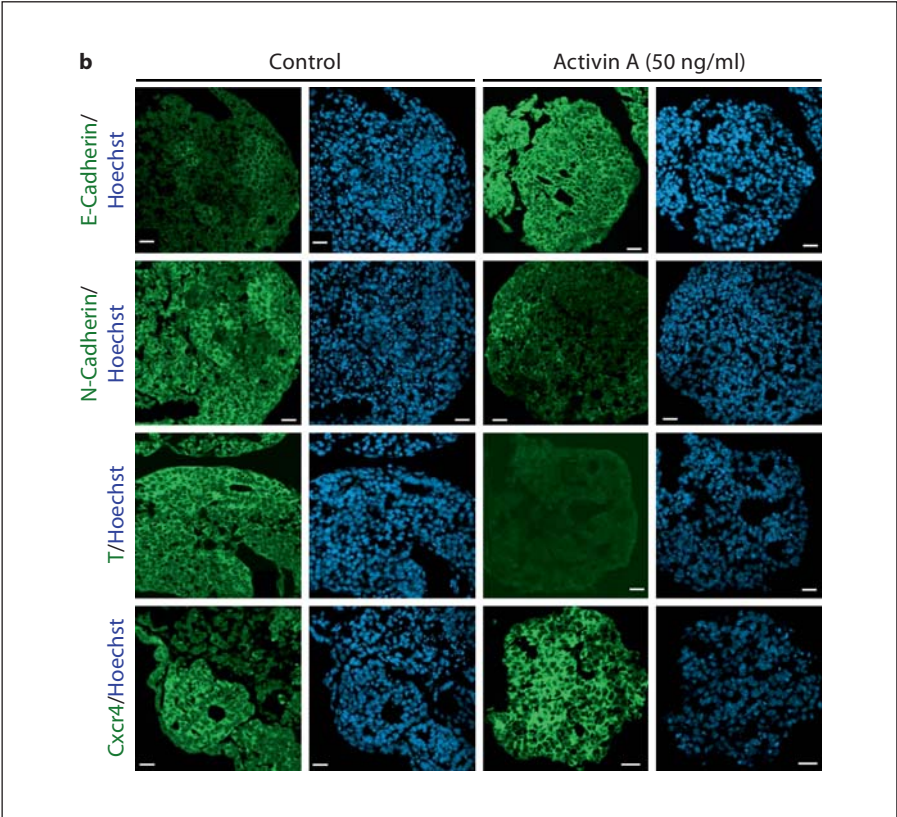
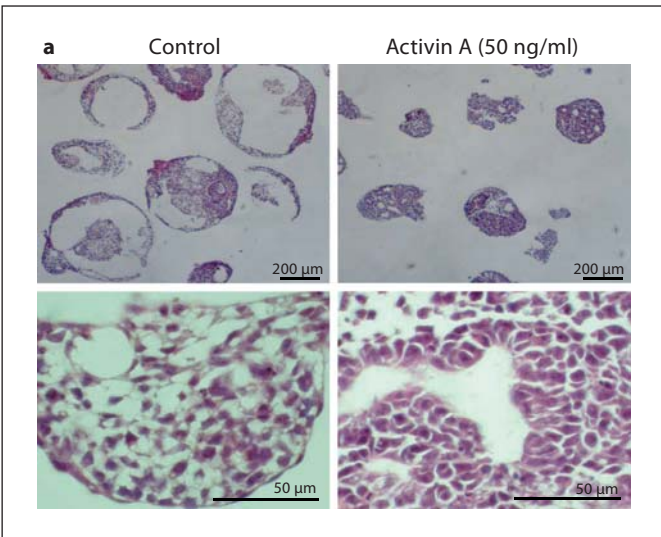


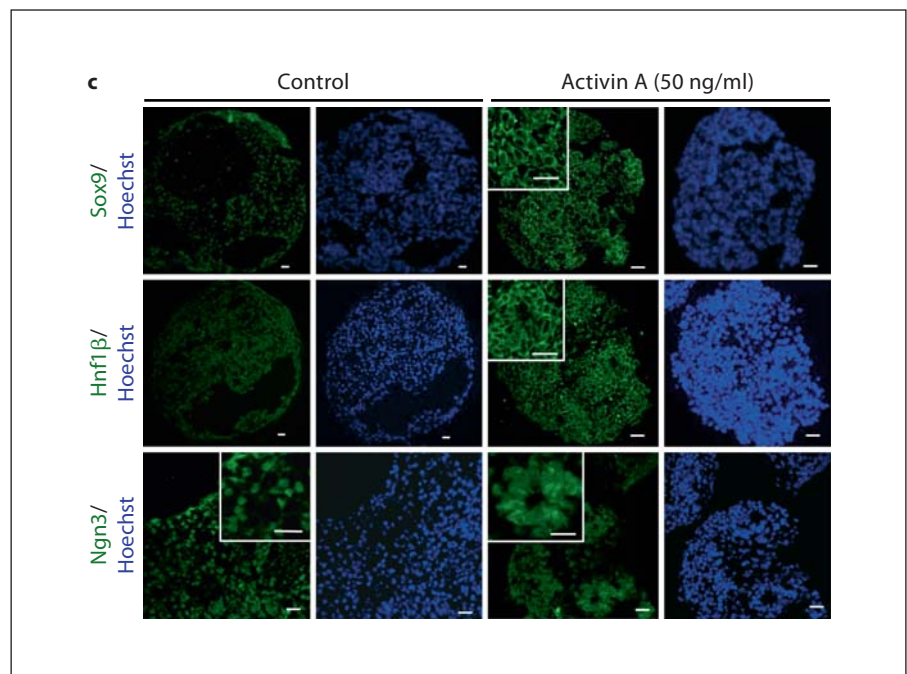
Fig. 6. Analysis of epithelial/duct-like structures in EBs at day 9 of culture. **a** H&E staining of control and activin A (50 ng/ml)-treated cells. **b** Confocal images of E-cadherin-, N-cadherin-, Brachyury (T)- and Cxcr4-labeled cells (green) in control and activin A-treated cells in addition to nuclear staining with Hoechst 33342 (blue). Bar = 20 μ m.

in Sox17-negative fractions, but Sox7 was lower at day 9/10 compared to day 8, and activin A-treated cells showed lower levels than the controls. Afp mRNA was highly detectable in Sox17-negative and Sox17-positive control cells at day 8, but almost absent in the activin A-induced population, and in both fractions at day 9/10. In contrast, Sdf1 transcripts were not detectable at day 8, but

at day 9/10. Whereas at day 9/10, the Sox17-negative fraction showed similar Sdf1 mRNA levels in control and activin A-treated cells, Sox17-positive cells showed lower levels in activin A-treated samples.

T, Foxf1 and Meox1 were analyzed as characteristic markers of mesendoderm and mesoderm, respectively. T was generally expressed at higher levels in activin A-

Fig. 6. (continued) Analysis of epithelial/duct-like structures in EBs at day 9 of culture. **c** Fluorescence images of Sox9, Hnf1 β and Ngn3 labeling (green) in control and activin A-treated cells. Nuclear staining with Hoechst 33342 (blue). Bar = 20 μ m.



treated than in control cells at both stages. Transcript levels were higher in cells at day 8 in comparison to day 9/10. Foxf1 mRNA was not detected in activin A-treated cells, but was clearly measured in control cells of the Sox17-negative (day 8) and Sox17-positive fractions (day 9/10). Meox1 transcripts were almost undetectable in activin A-treated cells, but were measured in Sox17-positive control cells at day 9/10.

Pax6 and NeuroD6 were analyzed as ectoderm-specific markers. Pax6 transcripts were almost absent in the Sox17-positive fraction, but were present in the Sox17-negative population with higher levels in activin A-treated cells than in controls. NeuroD6 mRNA was highly abundant in control cells of the Sox17-positive fraction, but downregulated in activin A-treated cells, whereas in Sox17-negative samples, a maximum level was detected in cells treated with 100 ng/ml activin A at day 8.

Hnf4 α , a marker of the primitive gut, showed remarkable transcript levels in activin A-treated cells of the Sox17-positive cell fraction, but was undetectable in all Sox17-negative fractions at day 8 and day 9/10. Hes1, another marker of early pancreatic and exocrine progenitors [Kopinke et al., 2011], was highly expressed in both the positive and negative fractions, at day 9/10.

Progenitor cells of the posterior foregut are characterized by *Pdx1* and *Ptf1a* expression [Van Hoof et al., 2009]. Activin A-treated cells of the Sox17-positive fraction revealed increased *Pdx1* mRNA levels compared to control

cells with a maximum at day 9/10, whereas *Pdx1* was almost absent in Sox17-negative cells at both stages. *Ptf1a* mRNA was almost absent in activin A-treated cells, and showed higher transcript levels in control cells at day 9/10.

Nkx6.1, Nkx2.2, and Ngn3 transcripts were analyzed as characteristic markers of pancreatic endoderm and endocrine progenitors [Kroon et al., 2008; Van Hoof et al., 2009]. Nkx6.1 showed maximum mRNA levels in activin A-treated cells of the Sox17-positive fractions, but low levels were detected in Sox17-negative samples. Nkx2.2 mRNA levels were rather low in activin A-treated cells of both fractions, with higher levels being detected in control cells of the Sox17-positive fraction at day 8. Ngn3 showed low levels in activin A-treated cells of the Sox17-positive cell fraction, but high transcript levels in the Sox17-negative control population at day 8.

Formation of Epithelial/Duct-Like Structures in EBs after Activin A Treatment

H&E staining of control and activin A-treated EBs performed at day 9 revealed the presence of epithelial/duct-like structures almost exclusively in the activin A-treated samples (fig. 6a). Immunocytochemical analyses of EBs showed that the activin A-treated cells were positive for E-cadherin, but almost negative for N-cadherin (fig. 6b), whereas control cells showed the opposite pattern. Likewise, T was abundant in control cells, but absent

in activin A-treated EBs. Activin A-treated cells showed Cxcr4 labeling throughout the whole EB, but in control EBs only occasionally Cxcr4-positive duct-like structures were found. Additionally, Sox9, Hnf1 β and Ngn3 were analyzed (fig. 6c). While Sox9 and Hnf1 β were not detectable in control EBs, both markers were abundant in activin A-treated EBs. Ngn3 protein was detected in control and activin A-treated cells, but the morphology of Ngn3-positive cells varied between both variants. Whereas control cells showed short prolongations resembling a neural-like morphology, activin A-treated cells showed Ngn3-positive cells within the epithelial-/duct-like structures.

Discussion

ES cells offer an almost unlimited source for the generation of pancreatic cells usable for cell replacement strategies and/or in vitro test systems. This requires the effective formation of DE, which in turn gives rise to all pancreatic lineages. However, current differentiation protocols suffer from their inefficiency to produce homogeneous DE populations. We used ES cells carrying a homologous dsRed-IRES-puromycin knock-in within the SOX17 gene locus to generate DE cells. This experimental model allowed the rapid selection of DE cells by FACS and the characterization of Sox17-positive cells at the transcript and protein level. Our data suggest the potential of Sox17-positive cells to develop into the pancreatic lineage by further differentiation induction.

Activin A-Induced Differentiation of Murine ES Cells into DE

Treatment of ES-derived EBs with 50 ng/ml activin A under serum-free conditions resulted in an increased number of dsRed/Sox17-expressing DE cells, whereas control cells, differentiated in IMDM/F12 + 20% FCS, CDM alone or CDM supplemented with 20% FCS with or without activin A, revealed only marginal differentiation into DE. A comparison to wild-type ES cells showed a slightly delayed differentiation into DE of the transgenic ES cells and reduced levels of Sox17 expression due to the loss of one Sox17 allele. However, in contrast to Sox17 heterozygosity in vivo [Kanai-Azuma et al., 2009], which leads to neonatal lethality, the loss of one Sox17 allele in the transgenic cells does not change the differentiation modality in general. Treatment with higher activin A concentrations (100 ng/ml) did not further enhance the number of Sox17-positive cells. These findings confirm

earlier data of DE induction in ES cells by activin A [Kubo et al., 2004; D'Amour et al., 2006; Cai et al., 2007; Kroon et al., 2008] and are in accordance with previous results showing that concentrations of at least 30 (and below 100) ng/ml activin A are required for DE induction in murine [Kubo et al., 2004] and human [Shim et al., 2007] ES cells, respectively. Abolishment of Sox17 expression upon addition of the ALK inhibitor SB431542 underlined that the effect seen in this study can be unequivocally attributed to the activin A induction. We further showed that activin A-induced Sox17-positive cells are Oct4-negative.

Time-Dependent Expression of Developmental Control Genes during Activin A Treatment

Sox17 is essential for the formation of DE [Tam et al., 2003], which is severely impaired in Sox17 mutant mice [Kanai-Azuma et al., 2002]. In activin A-treated cells Sox17 expression peaked at day 8 of differentiation. Sustained treatment with activin A for more than 10 days did not perpetuate Sox17 expression. Sox17 expression was preceded by the expression of Foxa2, necessary for the development of gut [Weinstein et al., 1994] and early pancreatic epithelium [Gittes, 2009]. Early expression of Foxa2 is a prerequisite for successful differentiation into the endocrine lineage, plays a major role in the maturation of endocrine cells [Lee et al., 2002, 2005] and is necessary for Pdx1 expression in β -cells [Gerrish et al., 2000; Sund et al., 2001]. In contrast to Foxa2 and Sox17, Cxcr4 (specifically expressed in DE and mesoderm, but not extraembryonic endoderm [McGrath et al., 1999]) was expressed throughout differentiation. Although Cxcr4 transcripts were present in both control and activin A-treated cells, immunocytochemical analyses revealed clear differences between the two populations. In control cells, Cxcr4 was not colocalized with Sox17 labeling indicating the mesodermal nature of Cxcr4-positive cells (because Sox17 is not expressed in the mesoderm [Kanai-Azuma et al., 2002]). In activin A-treated cells, Cxcr4 was localized in Sox17-positive cells confirming their derivation from DE.

The derivation of DE was further substantiated by downregulated transcript levels of primitive/visceral endoderm-specific Sox7, Afp and Sdf1. At the stage of maximal Sox17 expression, all three markers were only weakly expressed in activin A-treated cells, but strongly abundant in the controls. Specifically, the increased Sdf1 level in control cells from days 8 to 10 could point towards mesoderm differentiation, because Sdf1 also serves as a marker for mesoderm [D'Amour et al., 2005]. The expression pattern of Brachyury (T) suggests that these cells

Table 1. Primer sets employed for real-time RT-PCR analysis

Gene	Primer sequence (5'–3')
Sox17 (NM_011441)	AGA AAC TGC AGA CCA GAA GCT ATC A (s) GCT CAT TGT ATC CAT GAG GTG ACA (as)
Cxcr4 (NM_009911)	ACC TCT ACA GCA GCG TTC TCA TC (s) TGT TGG TGG CGT GGA CAA T (as)
Foxa2 (NM_010446)	GAA CTC CAT CCG CCA CTC TCT (s) AGC CCT TGC CAG GCT TGT (as)
Sox7 (NM_011446)	GCC AAG GAT GAG AGG AAA CGT (s) ATG ACT TTC CCA GCA TCT TGC T (as)
Afp (NM_007423)	TCG ACC TCA CCG GGA AGA T (s) TCA CCA CAG CCG GAC CAT (as)
Sdf1 (NM_001012477)	GCA CCA CGG TCT CCA TCC T (s) AAT ATT GCC AGG TGA GGG TAG TG (as)
Oct4 (NM_013633)	GCA GCT CAG CCT TAA GAA CAT GT (s) TCT CAT TGT TGT CGG CTT CCT (as)
Brachyury (T) (NM_009309)	TGC TGC AGT CCC ATG ATA ACT G (s) TAT GAC TCA CAG GCA GCA TGC T (as)
Pax6 (NM_013627)	GCA ACA CTC CTA GTC ACA TTC CTA TC (s) TTG GTG AGG GCG GTG TCT (as)
NeuroD6 (NM_009717.2)	CAG GGT GCC ATG TTC AGG TT (s) GAG ATT GGC TGC GCA GAT GT (as)
Pdx1 (NM_010491)	GTT GGG TAT AGC CGG AGA GAT G (s) TTG GAG CCC AGG TTG TCT AAA (as)
Ngn3 (NM_009719)	TGA CTG ACC TGC TGC TCT CTA TTC T (s) TCC GGG AAA AGG TTG TTG TG (as)
Hnf4a (NM_008261)	ACG TGC TGC TCC TAG GCA AT (s) GAT GGA CAC ACG GCT CAT CTC (as)
Nkx6.1 (NM_144955)	TTC GGA GAA TGA GGA GGA TGA (s) GCT GCC ACC GCT CGA TT (as)
Nkx2.2 (NM_010919)	GCC GAC ACA GGT CAA GAT CTG (s) GCG TCA CCT CCA TAC CTT TCT C (as)
Ptf1a (NM_018809)	AGG CCC AGA AGG TTA TCA TCT G (s) GAA AGA GAG TGC CCT GCA AGA (as)
Meox1 (NM_010791)	CCT CAG GGC TGT CCC ATT ATC (s) GCT CGT TGA AGA TTC GCT CAG T (as)
Foxf1 (L35949)	CGG AGA AGC CGC CCT ACT (s) GCG CGC CTG AAG AAA CTG (as)
Hes1 (NM_008235)	CGG CTT CAG CGA GTG CAT (s) CGG TGT TAA CGC CCT CAC A (as)

transit through a mesendoderm progenitor stage and acquire DE characteristics between days 8 and 9. The activin A-specific endoderm induction is further substantiated by transcript downregulation of the mesoderm-specific marker genes *Meox1* and *Foxf1*.

Because activin A/nodal signaling can also induce neural differentiation [Iwasaki et al., 1996; Rolletschek et al., 2006], we determined neural-specific Pax6 and NeuroD6 levels. They were almost completely downregulated in activin A-treated cells, but were detectable in the con-

trols. Our data underline that the activin A concentration used was able to inhibit ectoderm differentiation, confirming the results of Shim et al. [2007].

Hnf4 α , a marker of primitive gut and extraembryonic endoderm [Duncan et al., 1994], showed distinct patterns in control and activin A-treated samples. The early Hnf4 α transcript appearance in control cells (days 7–8), parallel to high levels of Sox7, Afp and Sdf1, underlined the extraembryonal nature of Hnf4 α -positive cells, whereas in activin A-treated cells, Hnf4 α transcripts were not present before day 9, when extraembryonic markers were low. We may conclude that Hnf4 α -positive cells of the activin A-treated population do not belong to extraembryonic lineages. The Foxa2 transcript maximum preceding Hnf4 α expression is in line with the induction of *Hnf4 α* expression by Foxa2 [Ryffel, 2001].

Hes1, a marker of both pancreatic and exocrine progenitors [Kopinke et al., 2011], may be attributed to multipotent pancreatic progenitors. We could speculate that Hes1 transcripts in ES and control cells are correlated to neuronal commitment and differentiation, because Hes1 is also expressed in neural stem cells [Qu and Shi, 2009].

Pdx1, significant for pancreatic development, is expressed in a biphasic pattern with highest expression at E9.5–10 and E18 in the mouse [Soria, 2001]. During early development, *Pdx1* correlates with Foxa2 expression. We also observed a biphasic pattern of *Pdx1* transcript levels with peaks at day 7 and day 10, respectively, and found that *Pdx1* coincided with maximal Foxa2 and *Ptf1a* levels. *Ptf1a* expression is induced by *Pdx1* and is a prerequisite for exocrine development of the pancreas [Bonafant-Hare and Herrera, 2008]. Stanger et al. [2007] found that the number of *Pdx1*-positive cells between E8.5 and E12.5 determines the final size of the pancreas. Given the maximum *Pdx1* levels at days 7 and 10, the in vitro differentiation pattern is thought to mimic the in vivo development and substantiates the role of *Pdx1* in the development of insulin-producing cells [Stoffers et al., 1997; Dutta et al., 1998]. Mimicking the biphasic pattern of *Pdx1* expression was a prerequisite of inducing endocrine pancreatic cell phenotypes [Bernardo et al., 2009].

The potential formation of pancreatic endoderm of activin A-treated cells is reflected by increased Nkx6.1 and Nkx2.2 transcript levels. Both Nkx2.2 and Nkx6.1 are crucial for pancreatic β -cell development. Nkx2.2-null mice failed to develop β -cells [Sussel et al., 1998], and loss of Nkx6.1 function led to a decrease in β -cell mass [Sandberg et al., 2000].

Ngn3, the key transcription factor of endocrine differentiation [Gu et al., 2002], showed a biphasic pattern sim-

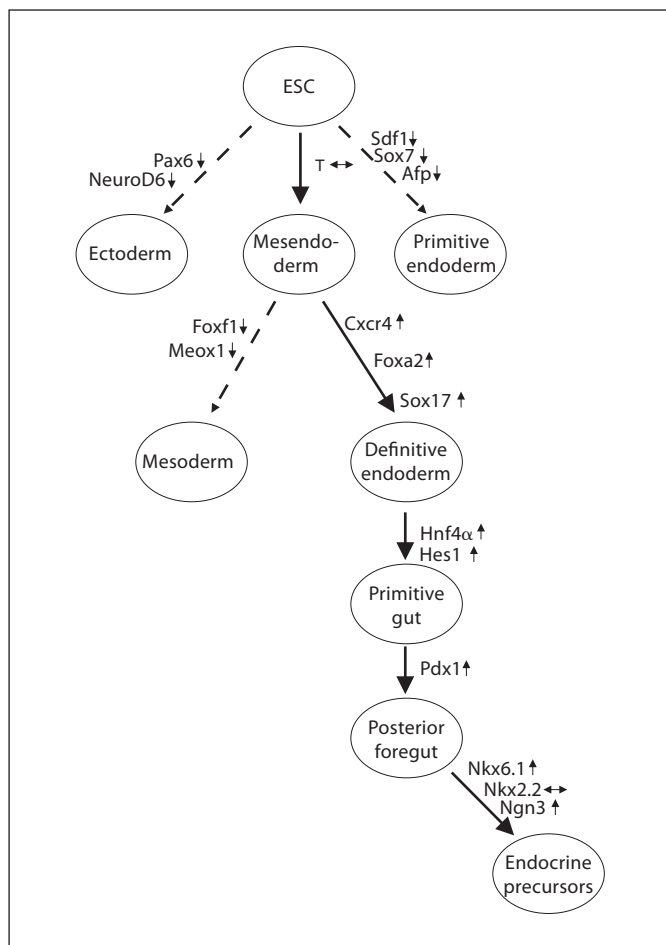


Fig. 7. Summary of the characteristics of Sox17-positive cell populations after activin A treatment showing differentiation of ES cells (ESC) into cells of the endoderm and early pancreatic lineage. Dashed lines represent differentiation pathways inhibited by activin A.

ilar to Pdx1 in activin A-treated cells. In contrast, in the controls, the high transcript levels of Ngn3, but also of Pax6, NeuroD6 and Hes1 may be due to neural commitment of feeder-free ES cells, because these genes are also expressed in developing neural cells [Lee et al., 2003; Rolletschek et al., 2006].

Characterization of Selected Sox17-Positive Cell Populations at Day 8 and Day 9/10

ES cells differentiating via EBs generally result in a mixed cell population. Sox17-positive cells were selected at 2 time points, with maximal Sox17 mRNA levels (day 8) and maximal Sox17 protein abundance (day 9/10). Cells at both stages (day 9/10) were collectively analyzed, because of almost no differences in transcript levels.

The concordant patterns of endoderm-, mesoderm- and mesoderm-specific transcripts of activin A-induced Sox17-positive cells at day 8 and day 9/10 provided evidence for a transient progression through a mesendoderm progenitor stage.

The transcript pattern of mesodermal genes in control cells was different in Sox17-positive and Sox17-negative cells at day 8 and day 9/10. While Foxf1 showed maximal levels in the Sox17-negative fraction at day 8, Meox1 and Foxf1 were upregulated in the Sox17-positive population at day 9/10. These data would be in line with findings that Sox17 is also associated with hematopoiesis and vascular development [Kim et al., 2007; Liao et al., 2009], although there is evidence that two different promoters drive Sox17 expression in the endoderm and vascular lineage. Because Foxf1 acts also as a vasculogenic factor by activating Bmp4 (which in turn promotes the assembly of vascular tubes from mesodermal progenitors [Astorga and Carlsson, 2007]), the Sox17/Foxf1-positive fraction of control cells at day 9/10 may represent cells determined to vasculogenesis.

The downregulation of mesoderm-specific transcripts paralleled by the lack and/or downregulation of neuroectoderm-specific Pax6 and NeuroD6 mRNA, respectively, confirmed the activin A-induced differentiation into the endoderm lineage.

DE differentiation characterized by Sox17, Foxa2 and Cxcr4 transcripts was generally higher in the activin A-treated Sox17-positive populations than in the controls. The same was true for the primitive gut marker Hnf4α and the posterior foregut marker Pdx1. In general, posterior foregut progenitors are characterized by coexpression of Pdx1 and Ptf1a [Oliver-Krasinski and Stoffers, 2008]. Therefore, it was surprising that compared to the relatively high Pdx1 levels in the activin A-treated Sox17-positive fraction at day 9/10, only low Ptf1a levels were detected. However, in the foregut, Pdx1 is expressed in a much broader region than Ptf1a, and Pdx1 expression is not dependent on Ptf1a expression [Gittes, 2009]. This interpretation would also be supported by the observation that in Ptf1a-null mice, although acini and ducts were lacking, endocrine cells developed [Krapp et al., 1998; Lin et al., 2004].

The high Ngn3 levels in the Sox17-negative control population at day 8 may be due to neuronal rather than endocrine pancreatic differentiation. This is substantiated by the lack of DE markers in this fraction, but Ngn3 expression in neuronal progenitors [Sommer et al., 1996]. Neural commitment of control cells was also demonstrated by Ngn3-positive neural-like cell types (fig. 6), where-

as in activin A-treated cells, Ngn3 labeling was almost exclusively restricted to epithelial/duct-like structures.

Nkx6.1, a marker of pancreatic progenitors [Gittes, 2009], is preceded by *Pdx1* expression during pancreas development. Based on relatively high *Pdx1* levels in activin A-treated cells at day 7, it is not surprising that Nkx6.1 showed highest levels in the Sox17-positive fraction at day 8 and day 9/10. Because *Nkx6.1* expression is later restricted to insulin-positive cells and supports β -cell function [Sander et al., 2000], this finding would further support our hypothesis that via selection of Sox17-positive progenitors, insulin-producing, but also exocrine and ductal cells could be generated (fig. 7).

The formation of epithelial/duct-like structures in activin A-treated EBs labeled by epithelial (E-cadherin) and not mesenchymal (N-cadherin) markers, but by Ngn3-labeled epithelial structures, excludes a neuronal origin. We may hypothesize that these cells represent early pancreatic progenitors. This would be further corroborated by the presence of Ngn3-positive ductal epithelial cell clusters in activin A-treated cells, which are also labeled by Sox9 and Hnf1 β , known as key mediators of Ngn3-positive pancreatic endocrine progenitors [Gittes, 2009].

In summary, activin A-treated Sox17-positive cells are committed to the pancreatic endocrine fate and potentially capable of further differentiation into the pancreatic lineage in vitro, whereas control cells are also committed to mesodermal and neuroectodermal differentiation.

Conclusion

Treatment of murine ES cells with activin A and subsequent selection of the Sox17-positive population enabled the generation of cells that are committed to establish a pancreatic endoderm and endocrine fate as summarized in figure 7. By reducing the heterogeneity of the ES cell progeny, Sox17-expressing cells are a suitable model to address fundamental issues in more detail, including the evaluation of growth and differentiation factors and culture conditions to delineate the pancreatic differentiation process and to generate endocrine and exocrine pancreatic cells in vitro. According to McKnight et al. [2010] and Wang et al. [2011], the system we present here fulfills one requirement for successful pancreatic differentiation of ES cells.

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