Molecular role of neonatal diabetes factor MNX1 in human beta-cell formation and function

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Abstract

Diabetes is one of the most prevalent conditions in the world, with almost half a billion people worldwide affected by one form of the disease. Current treatment strategies are often inefficient and entail a severe reduction of patient's quality of life.

Regenerative strategies by using *in vitro* derived pancreatic tissue offer great promise for the treatment of diabetes, but differentiation strategies developed so far are unable to fully recapitulate pancreatic development.

A comprehensive understanding of the molecular regulation of β-cell formation and function is a key requirement for the development of efficient replacement or regeneration therapies for patients with diabetes. This project aims to improve this understanding by providing a detailed characterization of the pancreas specific gene regulatory network (GRN) acting downstream of the homeobox factor MNX1, a transcription factor involved in pancreas development.

Studies in different model systems (Harrison *et al.*, 1999; Li *et al.*, 1999; Wendik, Maier and Meyer, 2004; Dalgin *et al.*, 2011; Arkhipova *et al.*, 2012; Pan *et al.*, 2015) and the association of MNX1-mutations with neonatal diabetes (Bonnefond *et al.*, 2013; Flanagan *et al.*, 2014) suggest Mnx1 as a conserved major regulator of pancreas morphogenesis, β-cell differentiation and β-cell fate-maintenance. MNX1 is also important for pancreatic progenitor cells to enter a β-cell developmental program resulting in bona fide β-cells instead of polyhormonal cells, which is crucial for a normal physiological function (Petersen *et al.*, 2017).

Despite these important activities, the molecular role of MNX1 is poorly understood and to date no direct pancreatic *in vivo* targets have been identified. In order to better understand the role MNX1 in the human pancreas, *in vitro* generation of β-cells from human stem cells with modifications of the MNX1 gene are an ideal tool to investigate the function of MNX1 during human pancreas development.

Here, an analysis of MNX1 expression in human development and differentiating stem cells along with first steps to differentiate genetically modified human stem cells towards pancreatic β-cells are presented.

Introduction

Pancreas organogenesis

The pancreas is an organ that arises from the endoderm. While species specific differences exist, there are conserved events shared among species. During development, endoderm and mesoderm are specified during gastrulation by Nodal signalling. Initially, definitive endoderm is not committed to a single fate, but able to differentiate to all endodermal tissues. Regionalization of the endoderm along the anterior-posterior (A-P) and dorsal-ventral (D-V) axes occurs after gastrulation. During early embryogenesis, the endoderm is a flat sheet of cells that then folds up to form the primitive gut tube. The gut tube is then patterned into foregut, midgut and hindgut. This A-P axis specification is mediated mainly by FGF, Wnt, retinoic acid and BMP signalling pathways, which all promote a posterior fate. Further in development, primordia of the endodermal organs (e. g. thymus, lung, liver, pancreas) are specified in different regions of the gut tube in response to signalling cues from the surrounding tissues (Zorn and Wells, 2009).

In the case of the pancreas, these primordia are the dorsal and ventral pancreatic buds that form at the foregut-midgut boundary, i. e. between prospective stomach and duodenum. The pancreatic buds first become apparent as thickenings of the gut tube (Jennings *et al.*, 2013). They then continue to grow and the ventral pancreas rotates until it contacts and fuses with the dorsal pancreas. The ventral bud contributes mainly to the pancreatic head adjacent to the duodenum, whereas the pancreatic tail is formed by cells originating from the dorsal pancreas (Shih, Wang and Sander, 2013).

The structure of the pancreas arises by branching morphogenesis, with the tips of the branches forming the acinar cells and the trunk cells forming the duct. Acini and ducts together form the exocrine pancreas that secretes digestive enzymes into the gut. The endocrine compartment of the pancreas forms from endocrine progenitors located in the duct domain. These precursors migrate to the pancreatic mesenchyme and cluster together to form the islets of Langerhans. In the islets, insulin, glucagon, somatostatin and other hormones are produced and released in the bloodstream, providing an exquisitely tight control over blood glucose levels (Pan and Wright, 2011).

Expression of MNX1 during pancreas organogenesis

In mouse, Mnx1 expression is found as early as E7.5 in the node. Expression then expands to the notochord and definitive endoderm by E8.0 and consolidates in the endoderm, tailbud and notochord at E8.5. Expression in the early endoderm is evident in the medial region adjacent to the notochord. After tubulogenesis of the endoderm at E8.5, Mnx1 expression follows a dorsal-ventral gradient with the highest expression levels in the dorsal part (Sherwood, Chen and Melton, 2009).

Following increasing regionalization of the gut endoderm in distinct organ progenitors beginning at E9.5, Mnx1 expression is maintained at high levels in the dorsal and ventral pancreatic buds, colocalizing with Pdx1 (Harrison *et al.*, 1999; Li *et al.*, 1999)

Uniform expression of Mnx1 in the pancreatic buds is maintained until E12.5 when the pancreatic lobes begin to form. However, cells expressing low levels of Mnx1 are scattered throughout the Ptf1a/Cpa1 positive domains, with an expression pattern reminiscent of Sox9+ multipotent progenitor cells (Pan *et al.*, 2015).

At E13.5, increased expression levels can be found in islet cells and parts of the exocrine pancreas (Harrison *et al.*, 1999). Interestingly, Mnx1 expression is excluded from Neurog3 positive endocrine progenitors, but it colocalizes with Pax6 endocrine precursors and is found in the first insulin expressing endocrine cells at E14.5 (Pan *et al.*, 2015).

Expression of Mnx1 is then maintained in the exocrine pancreas and insulin positive cells of the endocrine compartment, becoming gradually more restricted to β-cells, with almost all β-cells being positive for Mnx1 in the juvenile and adult pancreas (Harrison *et al.*, 1999; Li *et al.*, 1999; Pan *et al.*, 2015).

Information about the expression of human MNX1 during pancreas development is more sparse, but seems to be broadly conserved. At gestational week 7, corresponding to mouse E10.5, MNX1 was detected in the Sox9 multipotent progenitor cells. At G18w (E16.5), MNX1 expression is found predominantly in INS producing β-cells and Sox9+ trunk/duct cells, an expression pattern maintained in juvenile pancreatic tissue (Pan *et al.*, 2015)

The role of MNX1 in pancreas development

Homozygous deletion of Mnx1 in mice leads to complete agenesis of the dorsal pancreatic lobe (Harrison *et al.*, 1999; Li *et al.*, 1999). The phenotype becomes apparent at E9.5, when loss of Mnx1 leads to absence of Pdx1 expression in the pancreatic epithelium. Expression of a Mnx1:nlsLacZ reporter allele can be detected in the region of the gut tube where the dorsal bud forms, but these cells fail to evaginate in absence of Mnx1 and remain part of the duodenum (Li *et al.*, 1999). The ventral pancreas does not seem to be affected by Mnx1 loss of function. Although all pancreatic cell types form during later development, the number of β-cells is strongly reduced, with a concomitant increase in delta cells. Mutant endocrine cells further fail to organize in the typical structure of the mature murine islet and β-cells do not express mature β-cell markers like Glut-2. Detailed studies of the postnatal phenotype of Mnx1 mutant mice were precluded by their death at birth due to defects in innervation of the diaphragm.

Conditional disruption of Mnx1 by a Cre-lox system under the control of either a Ngn3 promoter or a rat insulin promoter sequence revealed more details of the function of Mnx1 in pancreas organogenesis (Pan *et al.*, 2015).

Deletion of Mnx1 in Ngn3+ endocrine precursors (after formation of the dorsal bud, before formation of endocrine progenitors) led to severe hyperglycemia and death by P10. This was caused by a threefold reduction in β-cells and a correlated increase in delta and to a lesser extent alpha cells (a phenotype that strongly resembles Nkx6-1 KO). Lineage tracing indicated that β-cells failed to differentiate and maintain their fate in the absence of Mnx1. The overall number of endocrine cells was also reduced, while Pax6+/hormone neg. progenitor numbers were increased, pointing to a delayed or stalled differentiation program.

Further, several observations support the hypothesis that β-cells fail to mature without Mnx1: although Pdx1 and Nkx6-1 were still detected and localized to the nucleus, MafA, a transcription factor that is essential for the maturation of mouse β-cells – was restricted to the cytoplasm (Pan *et al.*, 2015). These results indicate a role for Mnx1 in the formation of the dorsal pancreas, β-cell specification and maturation. Conditionally deleting Mnx1 in insulin expressing cells (RIP2:Cre) led to a hyperglycemic phenotype caused again by a fate shift from beta towards delta cells. Some cells failed to express Cre and showed an increased proliferative potential, being able to repopulate the pancreas with sufficient numbers of β-cells to reestablish euglycemia after a few months. The highly proliferative nature of

these escaper cells was demonstrated by a remarkable recovery of pancreas function after β-cell ablation with streptozotocin.

On the other hand, constant overexpression of Mnx1 under the control of the Pdx1 promoter in mouse embryos impairs pancreatic development and pancreatic tissue fails to develop normally after embryonic day 12.5, when Mnx1 expression becomes downregulated in wild type conditions (Li and Edlund, 2001). This shows that the biphasic expression of Mnx1 is biologically relevant and downregulation of Mnx1 after early pancreatic induction is important for pancreatic organogenesis to proceed normally.

Previous studies in zebrafish revealed *mnx1* gene function and regulation during pancreas formation (Wendik, Maier and Meyer, 2004; Kimmel *et al.*, 2011; Arkhipova *et al.*, 2012). Using morpholino knock-down approaches a conserved requirement for *mnx1* in β-cell differentiation was revealed. Later studies suggested that loss of *mnx1* in zebrafish causes a conversion of β-cell progenitors into α-like cells (Dalgin *et al.*, 2011).

Detailed molecular mechanisms of the way Mnx1 operates have so far been only established in the specification of neuronal fates. The V2-interneuron specific gene Vsx1/Chx10 is the only Mnx1 target that has been analyzed in detail in the context of vertebrate development (Lee *et al.*, 2008). Mnx1 is required to prevent Vsx1 activation in motorneurons by Lim-transcription factors expressed in motorneurons and interneuron subtypes. As Vsx1 also represses Mnx1, cross-repressive interactions between Mnx1 and Vsx1 have been suggested as a major driving force for mutual exclusion of neuronal subtype choices. The data hint at an evolutionary conserved role for Mnx genes in cross-repressive networks that mediate specific fate decisions leading to cell fate bifurcations, a mechanism that could also be involved in cell fate specification in the endocrine pancreas.

The function of Mnx1 in human pancreas development is not well understood. Mutations in the MNX1 locus have been associated with permanent neonatal diabetes, confirming its relevance in human development (Bonnefond *et al.*, 2013; Flanagan *et al.*, 2014). However, the molecular mechanisms regulated by MNX1 in humans are not known, which is surprising considering the wealth of data available for other key factors in pancreas development. One of the few recent studies about the function of MNX1 in the developing human pancreas (Zhu *et al.*, 2016) employed gene editing in human stem cells to investigate the function of key lineage determinants in pancreas development. The authors did not find any significant differences during in vitro differentiation of wild type versus

MNX1 knock out cell lines. However, the differentiation protocol employed did not allow to generate functionally mature beta cells, where the late effect of MNX1 loss of function is to be expected. The lack of an apparent phenotype during early development likely reflects differences between *in vivo* and *in vitro* differentiation rather than dispensability of MNX1 in this process.

Therefore, it is important to study the function of MNX1 in a differentiation protocol closer to *in vivo* development like the one developed in the Hebrok group (Nair *et al.*, 2019) that we aimed to employ in this study. A huge advantage of this protocol is that it allows to generate functionally mature beta cells that are very close in function and genetic makeup to the corresponding population *in vivo*. This will allow to study the function of MNX1 in human development in detail.

When the established genetically modified cell lines will be successfully differentiated towards pancreatic fates, multiple methods can be used to disentangle the regulatory network in which MNX1 is embedded. First, the MNX1 knock in line can be used to study the protein levels of MNX1 throughout differentiation by immunofluorescence and Western blotting. While this is already possible using MNX1-specific antibodies, all these antibodies show low affinity for their target protein and endogenous levels of MNX1 are often not detected with them; for reference, compare the immunostaining results of (Pan *et al.*, 2015) to (Li *et al.*, 1999) and (Harrison *et al.*, 1999). Since the knock in construct allows to detect endogenous levels of MNX1 protein through the detection of the attached 2xTY1 epitope tag with higher sensitivity, additional insights about the dynamic expression profile of MNX1 protein can be gathered. Expression profiling by RNA sequencing of wild type and MNX1 knock out cell lines at the pancreatic progenitor and mature beta cell stages will allow to establish an overall phenotype of MNX1 loss of function. The epitope tag attached to MNX1 in the knock in line also enables genomic studies such as chromatin immunoprecipitation and protein interaction studies by co-immunoprecipitation of MNX1 and associated genomic loci or proteins, respectively.

Methods

Analysis of fetal pancreas scRNA-Seq data

Raw gene counts of scRNA-Seq of fetal human pancreas were downloaded from GEO (GSE110154, contributed by Martin Enge, Karolinska Institute).

Normalization, dimensionality reduction and clustering were performed using the self assembling manifold algorithm implemented in the 'sam-algorithm' Python package (Tarashansky *et al.*, 2019). Gene counts were standardized to the median cumulative read count per cell prior to normalization by Freeman-Tukey variance-stabilization transformation. Genes expressed in less than 5% and more than 95% of all cells were removed from subsequent analyses, with a count equal to 1 as minimum detection level.

The SAM algorithm was run on the preprocessed data with 50 nearest neighbours and using the average of the top 100 dispersed genes for calculating the weights. Stopping condition was an error of less than 5e-4 and the results were embedded in an UMAP graph for visualization. Clustering was performed using the Leiden algorithm with a resolution of 0.1 to separate endocrine, ductal and acinar cells from mesenchymal and epithelial populations. Clustering and marker gene expression largely agreed with the population labels deposited in the GEO database. Acinar, ductal and endocrine cells were subset from the entire dataset and reanalyzed with the settings above, except 40 nearest neighbours were used for SAM and a resolution of 0.5 for Leiden clustering.

For visualization of gene expression levels, the kNN averaged gene expression was used because it provides a way of visually smoothing noisy gene expression pattern, thereby improving qualitative assessment of gene expression clusters in dimensionality reduced plots.

Analysis of adult pancreas scRNA-Seq data

Raw UMI counts for healthy human donors from (Baron *et al.*, 2016) were downloaded from GEO (GSE84133). Normalization, dimensionality reduction and clustering were performed as above. Examination of clusters revealed clear separation of all major cell types (alpha, beta, gamma, delta,

acinar and ductal) and a heterogeneous cluster of endothelial and immune cells, which was excluded from subsequent analyses. Notably, alpha and β-cells were divided in two clusters, correlating with sample origin; sample number 2 segregated from the rest. As all cell types were clearly separated and the source of this separation could also be biological, no batch correction was performed.

Analysis of in vitro differentiation scRNA-Seq data

The single cell RNA sequencing dataset for stem cell derived definitive endoderm (Li *et al.*, 2019) was downloaded from GEO (GSE109524). Symbols for protein coding mRNAs (Ensembl genes 99, human genome assembly GRCh38.p13) were obtained from BioMart and used to remove non coding RNAs from the gene expression matrix. Further analysis was performed as above.

Single cell RNA expression data for the in vitro differentiation time course (Veres *et al.*, 2019) were obtained from GEO (GSE114412, 'x1' conditions). Due to limited computational resources, timepoints were analyzed separately using the same pipeline as for the other datasets.

Maintenance of hESCs

Maintenance and differentiation of hESCs followed the procedures outlined in Nair et al., 2019 with minor modifications outlined below and in the text where appropriate. The cell line used was MEL1$^{INS\text{-}GFP/W}$, human embryonic stem cells were obtained from S. J. Micallef and E. G. Stanley (Monash Immunology and Stem Cell Laboratories, Australia). Besides the wild type line, newly generated edited derivatives of it were used: two homozygous knockout lines lacking MNX1 and one line with a MNX1 C-terminal knock in allele that adds a 2xTY1 tag, followed by a T2A cleavage site and the red fluorescent protein mScarlet. These lines were generated at the University of Innsbruck using CRISPR/Cas9 based editing strategies.

Human embryonic stem cells were maintained on a feeder layer of irradiated mouse embryonic fibroblasts in DMEM/F12 with 20% Knockout Serum Replacement, 100 µM beta-mercaptoethanol, 1x GlutaMAX and 10ng/ml FGF2 (R&D Systems). Medium was changed daily, except for Monday, Thursday and Friday when it was changed twice daily.

Cells were passaged twice a week on Tuesdays and Fridays. For passaging, cells were washed with PBS, incubated with TrypLE Select at 37°C for 6 minutes and dissociated to single cells by trituration

with a serological pipette after addition of four volumes of maintenance medium without FGF2. Cell concentration in the suspension was determined by counting an aliquot on a Moxi Z automatic cell counter (Orflow). Cells were pelleted by centrifugation at 1000rpm and RT for three minutes and resuspended in maintenance medium at a concentration of 11x10e6 cells per ml. 4.3 million cells and 3.17 million cells per 10 cm dish were plated on Tuesdays and Fridays, respectively.

Feeder free maintenance of hESCs

For feeder free maintenance of hESCs in defined conditions, cells were cultured on recombinant Vitronectin-N in Essential 8 medium. The same passaging schedule and density as for feeder-dependent maintenance was used. Cells were passaged with TrypLE select and plated in Essential 8 medium with the addition of 10 µM Y-27632 to promote cell survival under feeder free conditions.

Sphere formation

For sphere formation, cells dissociated in a single cell suspension were seeded in untreated 6 well plates at a density of 1 million cells per ml in 5.5 ml sphere medium per well. Sphere medium consisted of maintenance medium without FGF2 and with 10 ng/ml Activin A and 10 ng/ml Heregulin beta-1. Sphere formation was allowed to proceed overnight on an orbital shaker set to a constant speed of 100 rpm. For some cell lines, the addition of 10µM Y-27632 was required to induce sphere formation.

Pancreatic β-cell differentiation

Differentiation of the cells towards the pancreatic lineage was started 24 hours after seeding cells for sphere formation. Formed spheres were collected in a 50 ml conical tube and allowed to settle by gravity for three minutes. The supernatant was carefully aspirated and the spheres were resuspended in 25 ml of RPMI 1640. Spheres were allowed again to settle by gravity for three minutes and the supernatant aspirated. The spheres were resuspended in 5.5 ml differentiation medium, plated in 6 well plates in a volume of 5.5 ml per well and incubated with orbital shaking at 100 rpm. Differentiation medium was changed daily by concentrating the aggregates in the center of the wells by swirling and carefully aspirating the medium, leaving ~0.5 ml behind to avoid the cells contacting air. The appropriate medium for each day of differentation was then added and the cells returned to the orbital shaker. Unless otherwise specified, the following differentiation strategy was used. Base medium for days 1-5 was RPMI 1640 with 1x Glutamax and 0.2% FBS. Day 1 medium contained 100 ng/ml

Activin A, 25 ng/ml recombinant mouse Wnt3a and ITS diluted 1:5000. Day 2 medium contained only 100 ng/ml Activin A and ITS at 1:2000. Some cell lines required an additional day in day 2 medium to succesfully differentiate towards endoderm. Day 3 medium included 25 ng/ml KGF and 2.5 µM TGFbi IV (also known as A-83-01). On days 4 and 5, 25 ng/ml KGF and additional 0.2% FBS were added.

From day 6 to day 12, base medium was switched to DMEM with 1x Glutamax and 1:100 B27. On day 6 and 7, 3 nM TTNPB was included in the medium. On day 8, in addition to 3 nM TTNPB, 50 ng/ml EGF was included in the medium. Medium for days 9 to 11 contained 50 ng/ml KGF and 50 ng/ml EGF. Medium for days 12-20 included 1x NEAA, 10 µM ALK inhibitor II, 500 nM LDN-193189, 1 µM gamma secretase inhibitor XXI, 1 µM Triiodo-L-thyronine, 0.5 mM ascorbic acid, 1 mM N-acetylcysteine, 10 µM zinc sulfate and 10 µg/ml heparin sulfate.

Flow cytometry

For flow cytometric analysis of differentiation, approximately 50 spheres were sampled, washed with PBS and dissociated with 250 µl Accumax for 6-15 minutes at 37°C with occasional mixing of the suspension by flicking the tube. Cells were fixed by the addition of 500 µl 4% paraformaldehyde in PBS and incubation for 15 minutes at room temperature. Cells were pelleted by centrifugation, the supernatant removed and the pellet resuspended in 1 ml PBS. The cell suspension was stored at 4°C until staining.

For staining, the cell suspension was filtered into FACS tubes through a 20 µm filter, permeabilized with 3 ml permeabilization buffer and pelleted by centrifugation at 1500 rpm for three minutes. The cell pellet was resuspended in 50 µl CAS block with 0.2% Triton, 5% donkey serum and 1% BSA and primary or directly labeled antibodies at the appropriate dilution. Samples were incubated overnight at 4°C in the dark and washed with 3 ml Perm buffer. Cells were pelleted by centrifugation as above. If necessary, 50 µl of secondary antibodies diluted in CAS block with 0.2% Triton, 5% donkey serum and 1% BSA were added to the cell pellet and the samples washed again with 3 ml Perm buffer and pelleted. Stained samples were were resupended in 100 µl FACS buffer (PBS, 1 mM EDTA, 1% BSA) and analyzed on a LSRFortessa X20 (BD Biosciences). Results were processed in FlowJo VX. Antibodies used were TRA-1-60-Alexa647 (1:200), SOX17-Alexa488 (1:100), FOXA2-PE (1:100), PDX1-PE (1:40), NKX6-1-Alexa647 (1:50), all from BD Biosciences.

Differentiation of cells to definitive endoderm in adherent culture

To differentiate stem cells to DE, cells were seeded on vitronectin coated coverslips at a density of $1x10^5$ cells per cm^2 in hESC-A medium. After 24 hours, cells were washed with RPMI-1640 and medium changed to differentiation medium (RPMI-1640 supplemented with 1x GlutaMAX, 0.2 % FBS, 1 µM JNK-IN-8, 3 µM CHIR99021 and 100 ng/ml Activin A). The following two days, medium was switched to RPMI-1640 supplemented with 1x GlutaMAX, 0.2 % FBS, and 100 ng/ml Activin A and changed daily.

Immunostaining of adherent cells

Cells grown on coverslips were rinsed with PBS and fixed in 4% PFA in 1x PBS for 15 minutes at room temperature. Following fixation, PFA was aspirated and cells briefly washed three times in 0.1 M ammonium chloride in PBS to quench free aldehyde groups. Cells were then washed three times in PBS with 0.3% Triton X-100 for 5 minutes and blocked with CAS block with 0.3% Triton X-100. Primary antibodies diluted in CAS block with 0.3% Triton X-100 were applied for one hour at room temperature, followed by five washes in PBS with 0.3% Triton X-100. Incubation with secondary antibodies was performed following the same procedure. After incubation with secondary antibodies, cells were washed three times for 5 minutes each in PBS with 0.5 M NaCl, incubated for 5 minutes with Hoechst 33342 diluted 1:10000 in PBS and washed an additional three times with plain PBS. Coverslips were mounted in Mowiol 4-88 and cured overnight at 4°C. Representative images were acquired on a Zeiss Cell Observer Spinning Disk microscope. Antibodies used were rabbit FOXA2 (CST) 1:400, TY1 (clone BB1) hybridoma supernatant 1:20, Alexa488 goat anti-rabbit (Thermo) 1:500, Alexa647 goat anti-mouse (Thermo) 1:500.

Results

Analysis of MNX1 expression in the fetal pancreas

To complement the sparse data available about MNX1 expression during human pancreatic development, publicly available single cell RNA sequencing datasets were analyzed with a focus on MNX1 expression during different stages in human pancreatic development and in vitro differentiation of human embryonic stem cells or human induced pluripotent stem cells.

A scRNA-Seq dataset obtained from human fetal pancreata from gestational week 12 to 22, which was recently made available (GEO Series GSE110154, contributed by Martin Enge, Karolinska Institute), was analyzed using the self-assembling manifold algorithm. To improve resolution, single cells with a signature indicating an origin in blood or mesenchymal and epithelial tissues were removed prior to reclustering. Single cells separated into clearly defined populations. Marker gene expression indicated that these clusters corresponded to alpha cells (GCG+), beta cells (INS+), delta cells (SST+), duct cells (CFTR+) and acinar cells (CPA1+). A small population with high expression levels of NEUROG3, likely corresponding to endocrine progenitors, was also detected. In the latter population, a few cells with high levels of GHRL were evident; these were probably epsilon cells, but they could not be clearly separated from the endocrine progenitors. No PPY expressing PP/gamma cells were detected at these stages. CHGA was expressed throughout the endocrine compartment and absent from the putative exocrine clusters (Fig. 1).

The expression of MNX1 and other lineage determining transcription factors corroborated this classification and revealed a more nuanced substructure of the populations. In the exocrine compartment, an opposing gradient of CPA1 and SOX9 was visible, likely representative of cells in the process of differentiating from a trunk/duct fate towards a tip/acinar fate (Fig. 2).

Correlating these patterns with MNX1 expression, reveals that the expression data largely agree with the broad picture from immunostainings of human tissue. MNX1 expression is high in a CFTR/SOX9 positive cluster, presumably duct cells where also multipotent progenitor cells reside. Some expression is also detected in the SOX9-high subpopulation of the acinar cluster.

Another cell population expressing high levels of MNX1 is found in the INS expressing beta cell cluster. As in mouse, there is an overlap with PAX6 expression, although the PAX6 expression domain

is much broader and encompasses GCG expressing alpha cells. MNX1 expression is very low in the

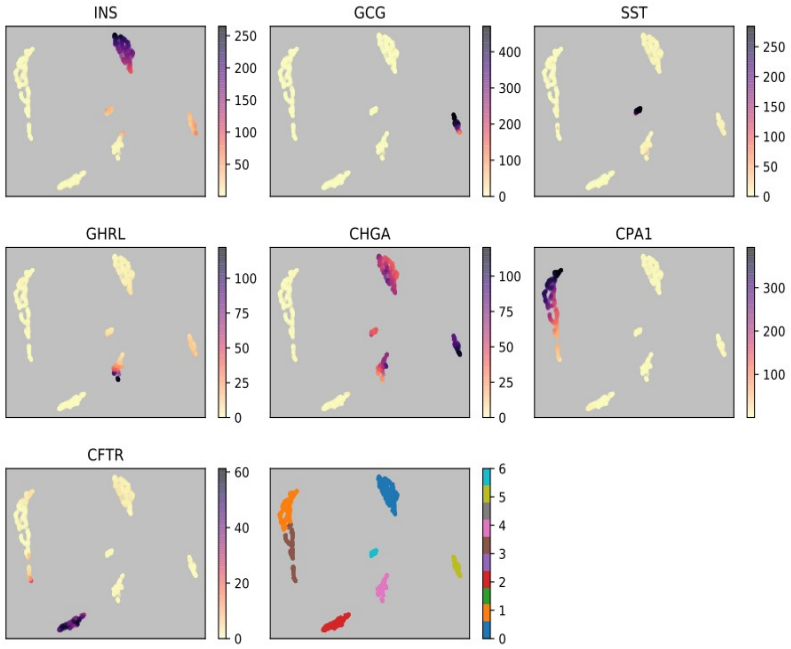

Figure 1 UMAP plot colored by kNN-averaged scRNA-Seq expression levels of endocrine and exocrine markers in fetal pancreata (gw12-22) and cells colored according to clusters obtained by the Leiden algorithm.

NEUROG3 progenitor cluster, as has been described in mouse development (Pan *et al.*, 2015).

In the beta cell cluster it is interesting to note that MNX1 was found mostly at one edge of the cluster, representing cells expressing high levels of insulin. This suggests that MNX1 expression is highest in terminally differentiated beta cells.

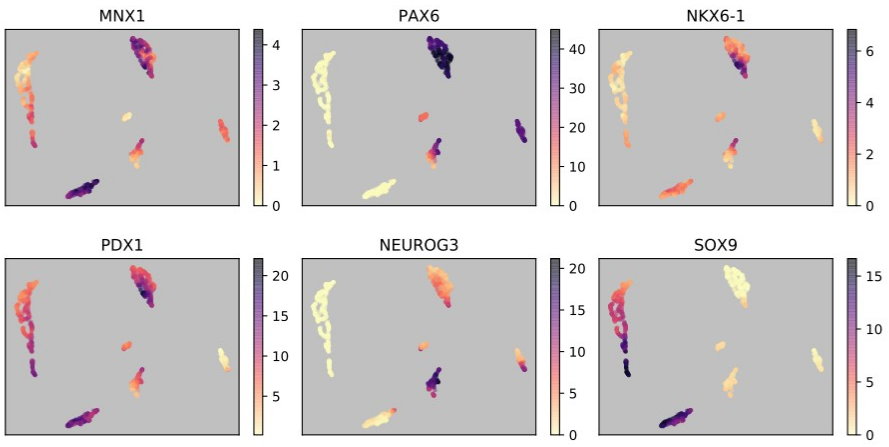

Figure 2: UMAP plot colored by kNN-averaged scRNA-Seq expression levels of MNX1 and other lineage determinants in the fetal pancreas.

MNX1 expression in the adult human pancreas

To examine expression of MNX1 in the adult human pancreas, data from healthy donors in the well known dataset from (Baron *et al.*, 2016) was analyzed for MNX1 expression. To get comparable results and take advantage of the higher resolution of the SAM algorithm, analysis was performed with a similar pipeline as for the fetal scRNAseq dataset. Mesenchymal, epithelial and immune cells were excluded from the original dataset prior to analysis. The clusters obtained represented cell populations and showed little structuring attributable to the tissue source, i. e. donor. Large populations of alpha, beta, and exocrine cells were found, along with smaller populations of delta and PP cells (Fig. 3). As expected, NEUROG3 expression was not detected in adults. Like in the original publication, no epsilon cells were found in the dataset. As expected, MNX1 expression was found in INS+ beta cells and the cells which highly expressed MNX1 were also highly expressing NKX6-1, PDX1 and MAFA, indicating that MNX1 expression is maintained in beta cells and likely to fulfill a function in beta cell maintenance in adults, as has been described in mouse. The heterogeneity of the expression of these genes however could also be caused by noise of technical nature, as expression levels for all genes are generally rather low, around forty to eighty times lower than those of endocrine hormones and exocrine enzymes. Notably, a small subset of the ductal population expressed high levels of SOX9 and weak expression of PDX1 and MNX1.

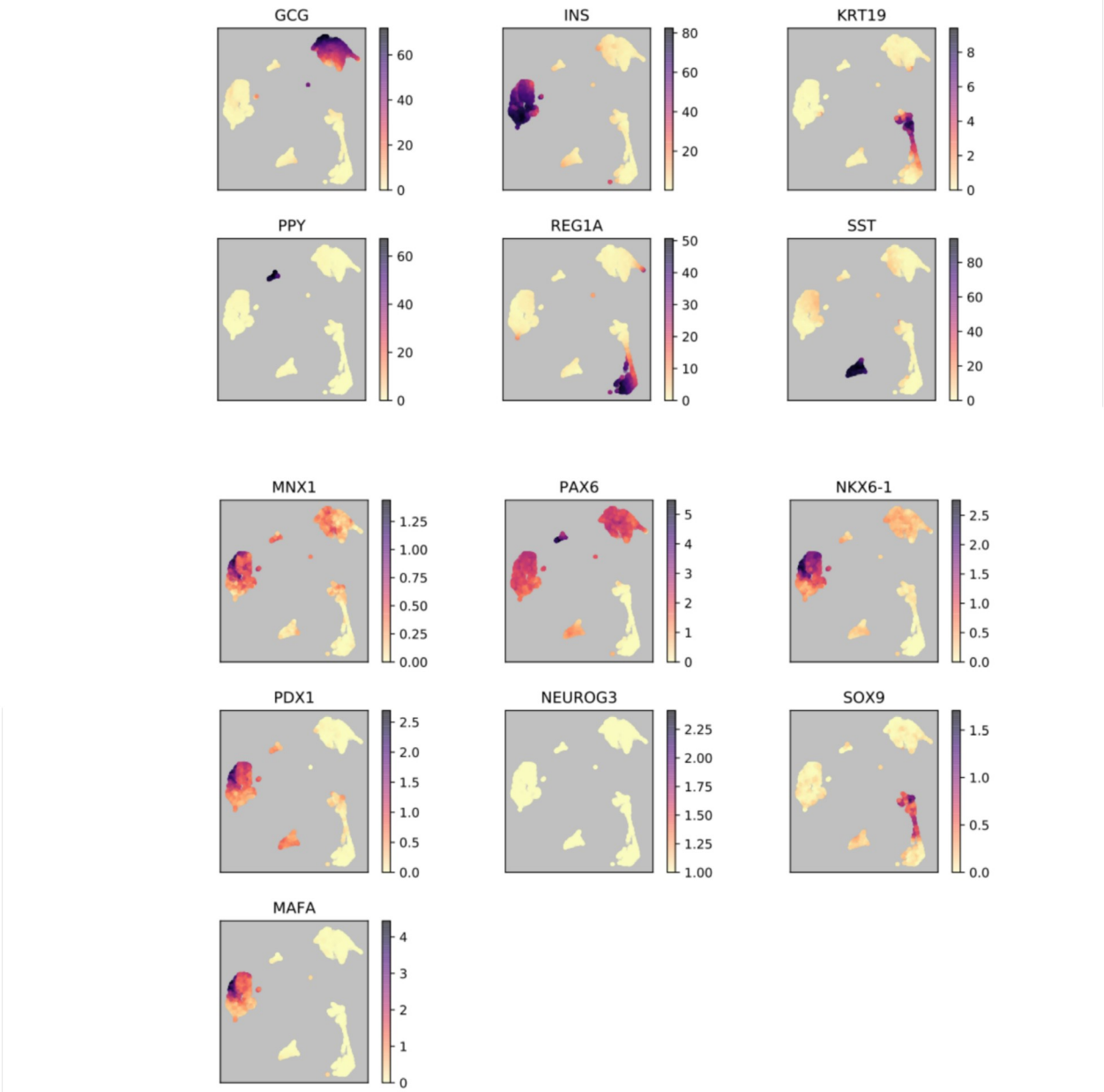

Figure 3: UMAP plots of adult pancreatic scRNA-Seq data. Shown is the expression of top ranked marker genes for each cluster with duplicates removed (top) and the expression of lineage determinants including MNX1 (bottom)

MNX1 expression during in vitro differentiation

As RNA sequencing data from human development is sparse for obvious reasons, scRNA-Seq datasets from in vitro differentiation experiments were analyzed. One such dataset is from (Li *et al.*, 2019), who looked at differences in definitive endoderm differentiation of wild type versus MKK7 knock out cells. Although MNX1 was expressed at low levels in relatively few cells, it was clearly coexpressed with definitive endoderm markers like SOX17, FOXA2 and GATA6 (Fig. 4). This is in line with previous data (Meyer lab, unpublished), where MNX1 expression is clearly detected in definitive endoderm cells (Fig. 5). The low expression level of MNX1 at this stage makes detection of the transcripts by single cell RNA sequencing methods challenging.

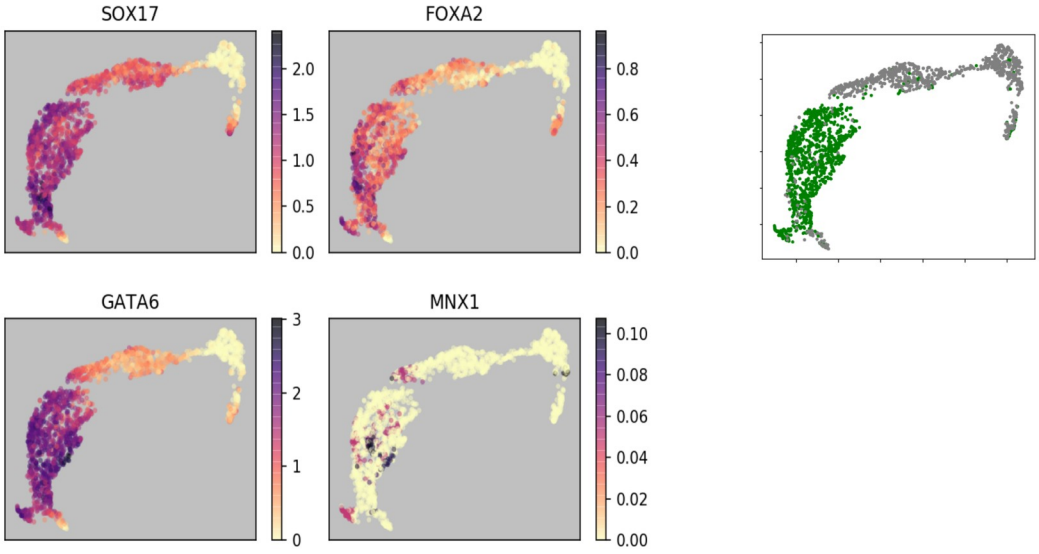

Figure 4: mRNA expression of MNX1 and definitive endoderm genes from in vitro differentiated stem cells. On the right, the same UMAP scatter plot with cells colored by genotype (grey, WT; green, MKK7KO).

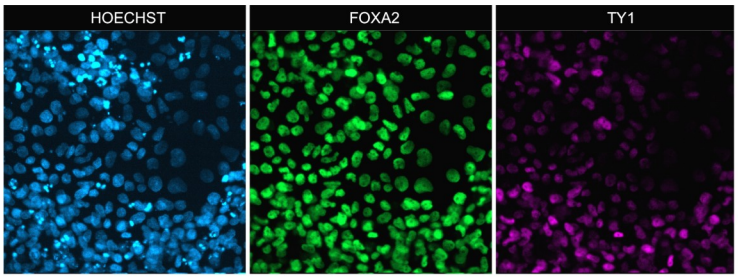

Figure 5: Immunostaining of a MNX1 knock in line differentiated to definitive endoderm in adherent culture. DNA was stained with Hoechst dye, FOXA2 was used as definitive endoderm marker and the MNX1-2xTY1 fusion protein was detected using a TY1-antibody.

One of the most detailed studies of stem cells differentiating towards pancreatic fates has been performed by (Veres *et al.*, 2019). The authors performed scRNA-Seq at multiple timepoints, starting at stage 3 of their protocol, corresponding to pancreatic endoderm. At this stage, almost all cells express PDX1, very similar to data from in vivo studies. As expected, at this stage MNX1 is relatively highly expressed in most cells and the expression profile is overall quite homogeneous (Fig. 6).

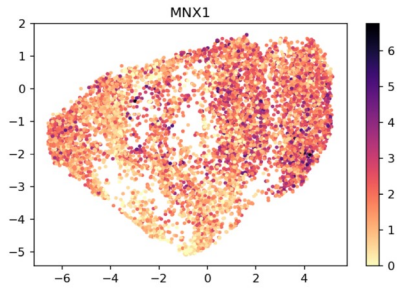

Figure 6: MNX1 expression in a scRNA-Seq dataset derived from in vitro differentiated human stem cells at stage 3 of differentiation.

At stage 4 of the dataset, corresponding to pancreatic progenitor stage, the picture was slightly more complex. Two clusters were evident, one larger population of cells that was positive for SOX9, NKX6-1 and PDX1 while not expressing transcripts of endocrine hormones and a smaller cluster expressing INS, SST and GCG along with NEUROD1. Notably, hormone expressing cells were mostly bihormonal, expressing INS and either GCG or SST. These cells are likely similar to “first wave” endocrine cells or prematurely differentiating endocrine cells, which are prevalent in protocols employing BMP inhibition at early stages (Russ *et al.*, 2015). Intriguingly, MNX1 was expressed weakly in most cells, but reduced in bihormonal cells compared to NEUROD1 positive, hormone negative cells (Fig. 7).

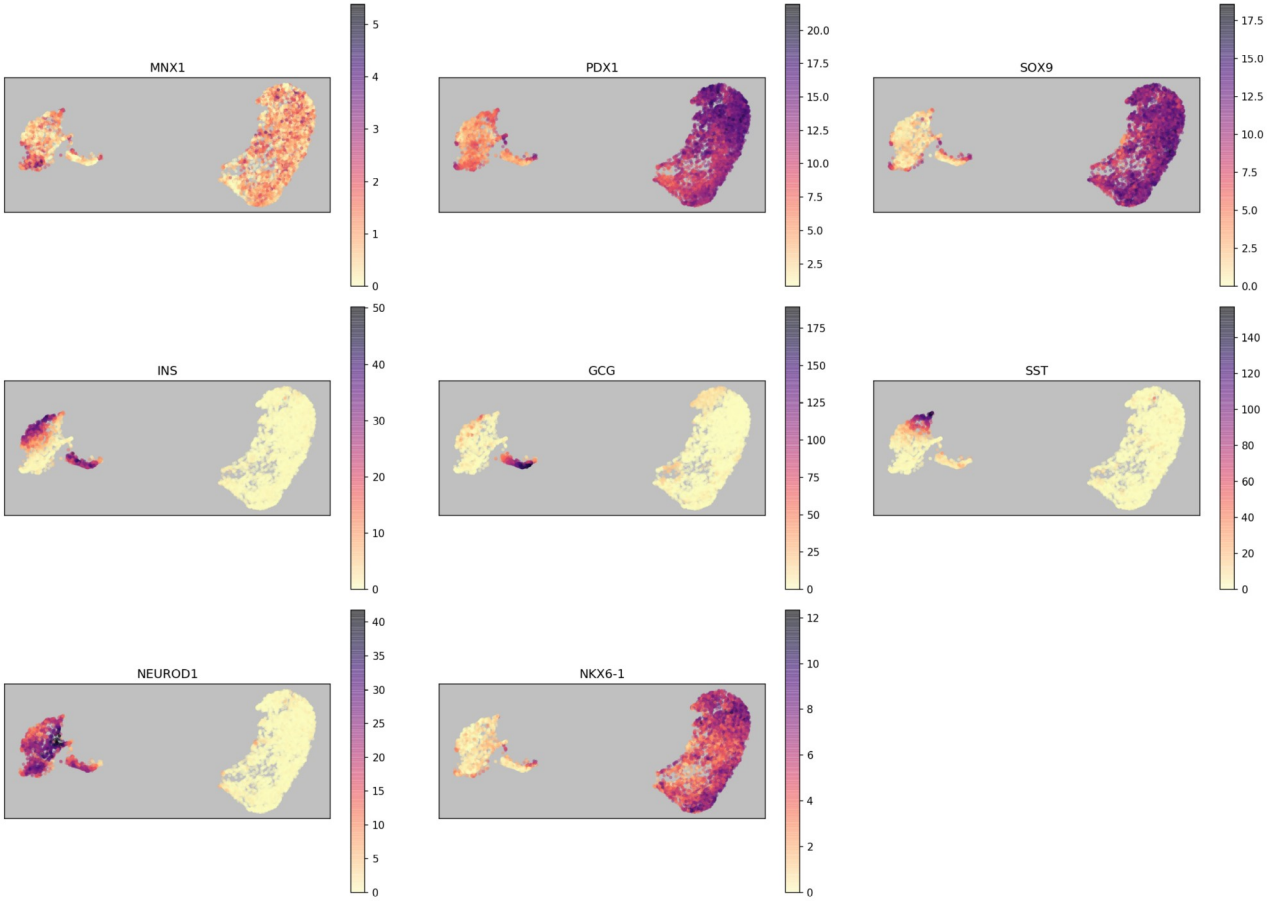

Figure 7: Expression of MNX1 and other relevant genes at stage 4 of differentiation.

The cell population at stage 6, where fully differentiated, but immature endocrine cells were found, presented itself as very heterogeneous. MNX1 was expressed at low levels in a population enriched for SOX9 and mostly negative for endocrine hormones. Higher levels of MNX1 were found in cells expressing INS, but not GCG. MNX1 expression was high in cells expressing INS, PDX1 and NKX6-1 and not expressing GCG or SST, representing real beta cells. Interestingly, low levels of MNX1 persisted in INS/SST double positive cells. As discussed in (Veres *et al.*, 2019), one subset of cells expressed the gene TPH1, encoding a tryptophane hydroxylase involved in the biochemical synthesis of serotonin and usually found in neuronal cells. The authors postulated that this cluster shows similarity to serotonin producing enterochromaffin cells from the gut. In this population, the highest levels of MNX1 were found, along with NKX6-1 and NEUROG3. This was puzzling, as MNX1 should be excluded from pancreatic cells expressing NEUROG3. Therefore, these cells likely were not of pancreatic character, but assumed a more neuronal fate. Since NKX6-1, MNX1 and NEUROG3 are transcription factors also used in neuronal gene regulatory networks, these cells upregulated neuronal

genes in response to high levels of these factors and probably lacking another signal counteracting this fate (Fig. 8).

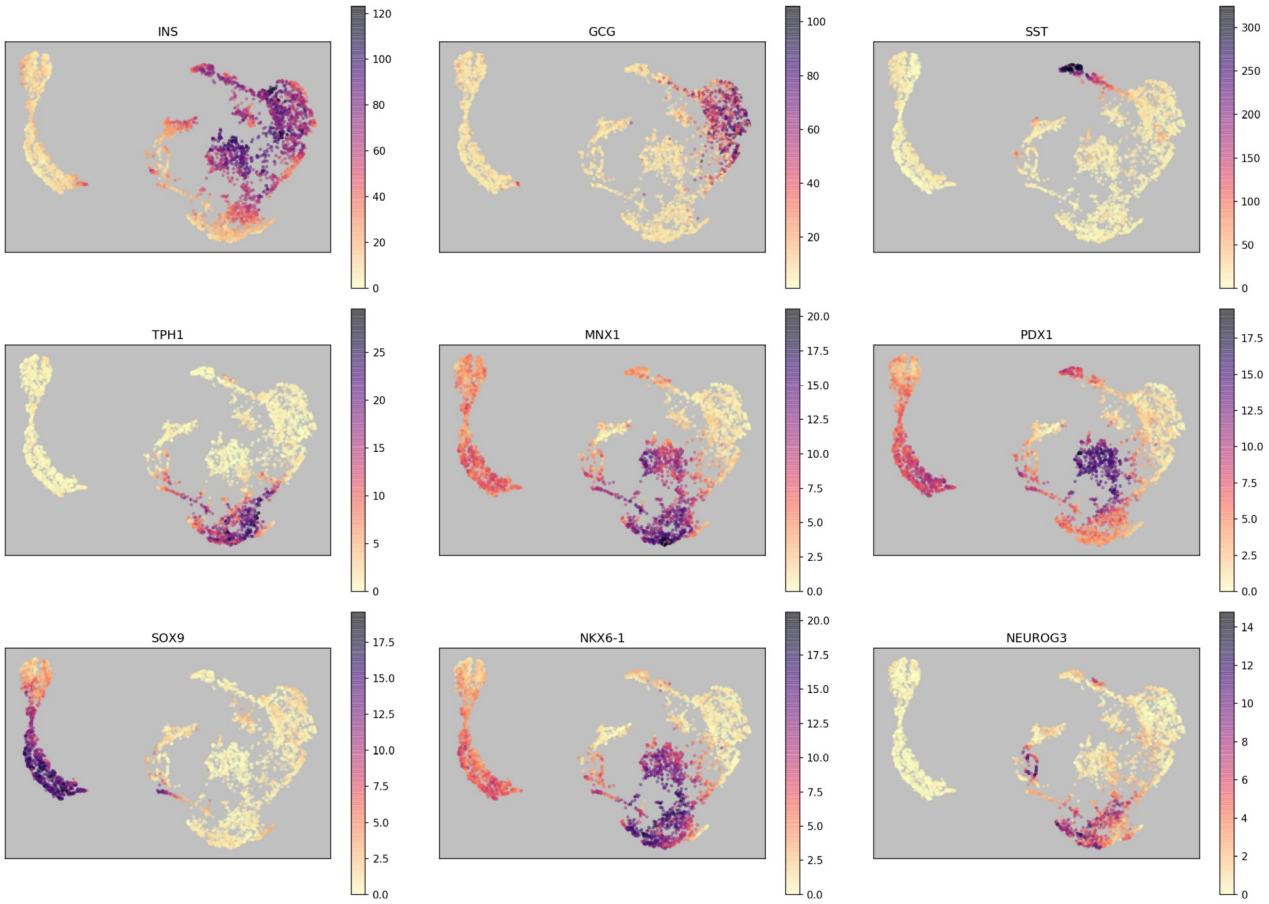

Figure 8: Expression of MNX1 and other relevant genes in differentiated stem cells at stage 5 of differentiation.

Differentiation of modified hESC lines to pancreatic beta cells

To examine the function of MNX1 in human beta cells, cell lines with MNX1 knocked out through CRISPR/Cas9 mediated genome editing were created previously. In addition, lines with a knock in construct adding a double TY1 epitope tag and the red fluorescent protein mScarlet preceded by a T2A cleavage site at the C-terminus of MNX1 were created. The knockout lines should allow to shed light on the function of MNX1 by analysis of the loss of function phenotype. The knock in lines on the other hand provide an epitope tag which allows to detect MNX1 by western blot and immunostaining, as well as enabling the pulldown of MNX1 in chromatin immunoprecipitation and co-immunoprecipitation. These methods can be employed to investigate direct transcriptional targets regulated by MNX1 and protein cofactors with which MNX1 interacts. By combining these methods with the highly efficient beta cell differentiation and maturation protocol recently established by the Hebrok group (Nair *et al.*, 2019), the dynamic role of MNX1 in pancreas differentiation and its role in the maintenance of beta cell function can be elucidated. Because the differentiation protocol is optimized for the MEL1$^{INS\text{-}GFP/W}$ line, the MNX1 knock out and knock in cell lines were derived from that line. One peculiarity of this specific differentiation protocol is that it is performed entirely in a high density suspension culture under constant agitation by an orbital shaker. Under these conditions, the embryonic stem cells should form clusters with a homogeneous size between 150 and 200 µm. The first step to establish this protocol for the modified lines was to investigate the proper conditions for spheroid formation. After several passages on a mouse embryonic fibroblast feeder layer, a single cell suspension was seeded in sphere formation medium. The original INS-GFP line forms aggregates without supplementation of the Rho kinase (ROCK) inhibitor Y-27632.

The modified lines however failed to form aggregates under these conditions (Fig. 9). While a few small and irregular clusters did form, most cells failed to aggregate. Supplementation of Y-27632 at a concentration of 10 µM, frequently employed in passaging of stem cells under feeder free conditions, improved aggregate formation and yielded aggregates of the expected size. The protocol calls for a density of 1 x 10^6 cells per ml of medium and 5.5 ml medium per well of a six well plate. As cell density is an important factor in aggregate formation, the influence of two different densities on sphere formation was tested: the standard density of 1 x 10^6 cells per ml and 1 x 10^5 cells per ml, both with the addition of 10 µM Y-27632 to promote sphere formation. Although even at the lower cell density

aggregates formed, those were of a much smaller size and unsuitable for successful differentiation. Therefore, sphere formation was carried out with 10 µM Y-27632 during all following differentiation experiments.

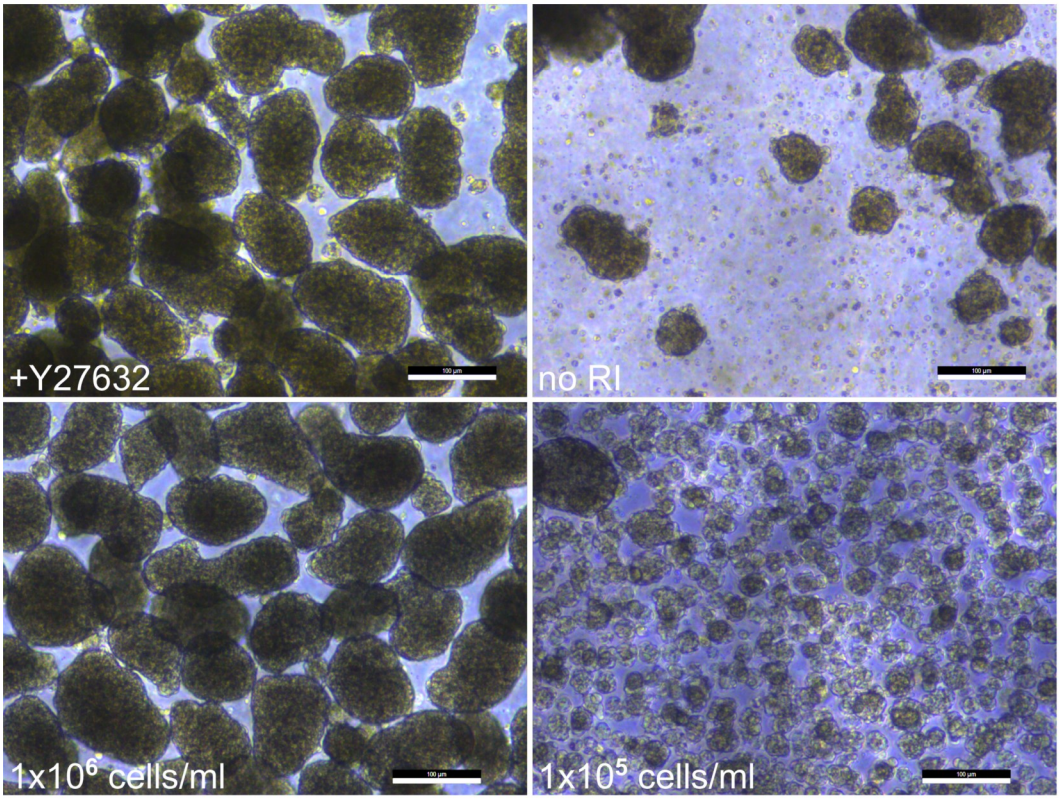

Figure 9: Bright field pictures of aggregate formation of human embryonic stem cells under different conditions. Top left, aggregates formed in the presence of the ROCK inhibitor Y-27632; Top right, aggregates formed without ROCK inhibition; Bottom left, aggregates formed with a seeding density of $1x10^6$ cells/ml; bottom right, aggregates formed with a seeding density of $1x10^5$ cells/ml. Scale bars: 100 µm.

The differentiation protocol was then continued with spheres of the appropriate size and density to examine how the cell lines performed during the following steps. Beginning at day 1 of differentiation, a zonation of the spheres became apparent, a phenomenon which was not observed in lines adapted to the protocol. The spheres developed a dense core and a less dense outer layer. This layering became more pronounced as the differentiation progressed and at day 4, the spheres began to become cystic, developing cavities of increasing size and some spheres seemed to lose adhese and began to disintegrate. This indicated a deviation from the desired differentiation and as the differentiation progressed, the cystic structures became larger and eventually the spheres fell apart (Fig. 10).

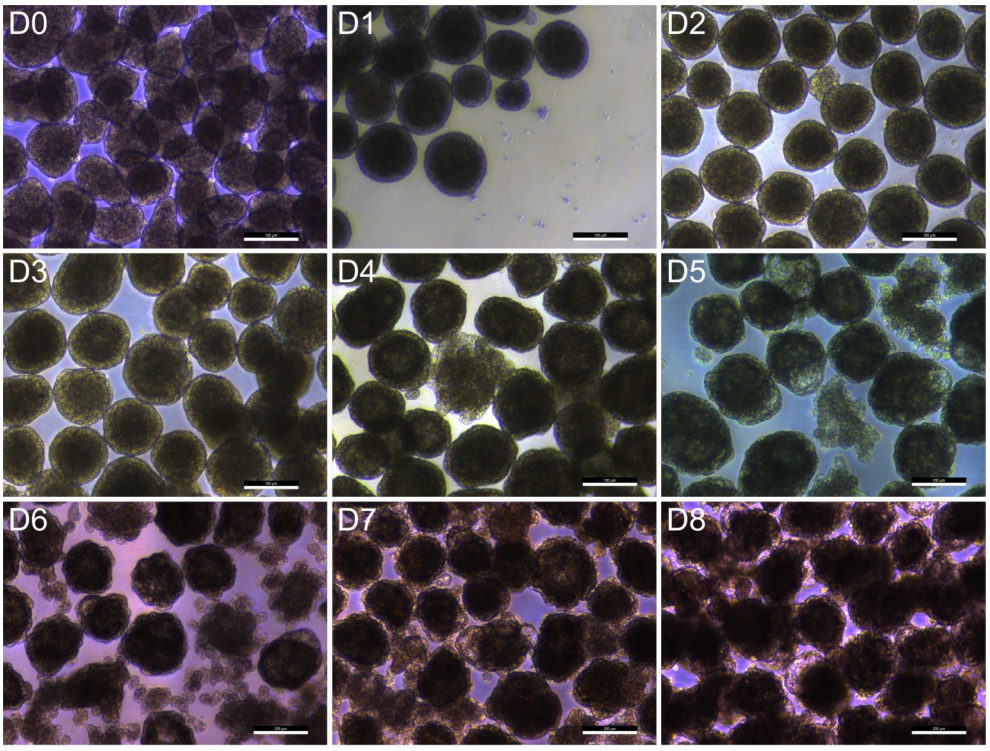

Figure 10: Brightfield pictures of a MNX1 knockout line during differentiation.

As the modified cell lines were previously maintained under feeder free conditions, they were maintained on feeder cells for three more passages in an effort to adapt them further to the new maintenance conditions. However, the differentiation capability did not change even with further adaptation. Flow cytometry of spheres stained with FOXA2 and SOX17 as endoderm markers revealed that less than 20% of all cells had differentiated towards definitive endoderm (Fig. 11), while the majority stained positive for the pluripotency marker TRA-1-60. This was independent of genotype, as both knock out and knock in lines behaved the same. The failure to differentiate further could therefore be explained by inefficient induction of definitive endoderm.

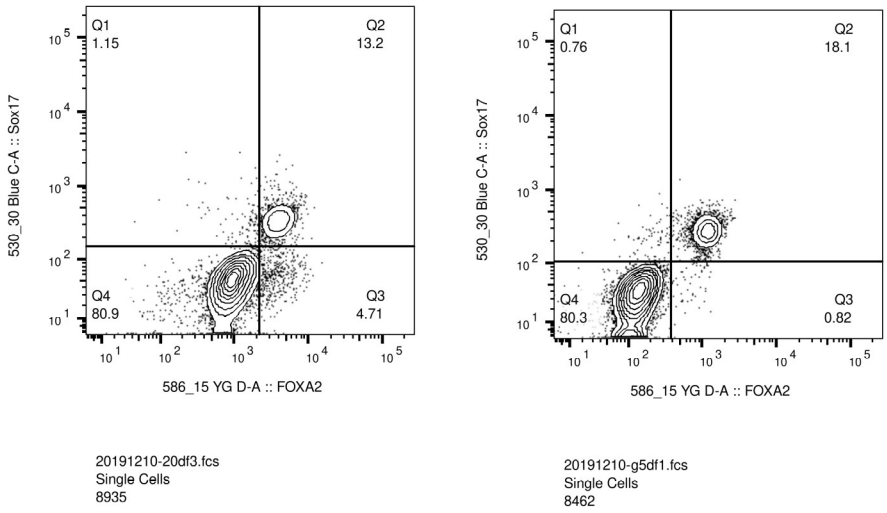

Figure 11: Flow cytometric analysis of definitive endoderm induction according to the standard protocol in a MNX1 knock out line (left) and a MNX1 knock in line (right).

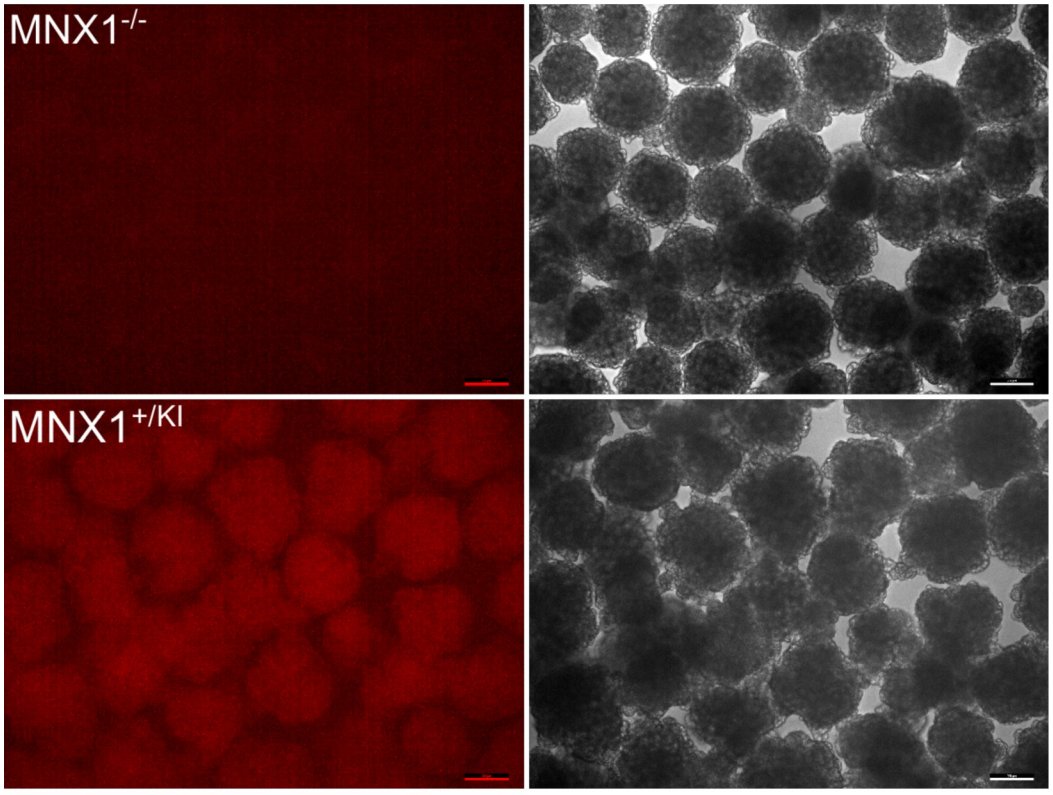

Figure 12: Live bright field and red fluorescence images of differentiated spheres at day 7. The MNX1 knock in line shows red fluorescence from transgenic mScarlet expression, while no red fluorescence is visible in the MNX1 knock out line at the same time.

To increase the proportion of definitive endoderm, the protocol was modified and the period of definitive endoderm induction was prolonged by one day. Cells analyzed for the expression of TRA-1-60, FOXA2 and SOX17 by flow cytometry after this additional day of endoderm induction showed a strong increase in definitive endoderm with a concomitant reduction of TRA-1-60 positive cells (Fig. 12).

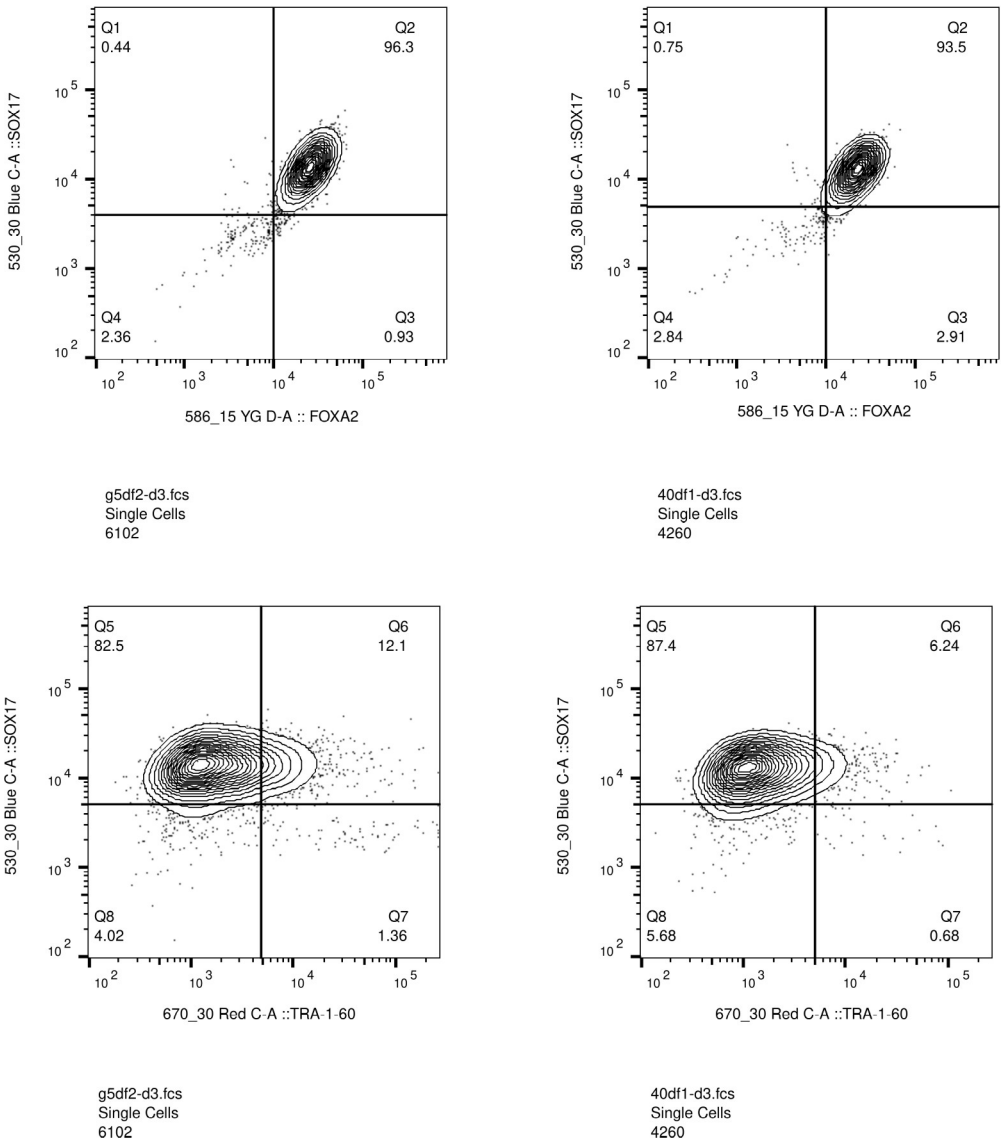

Figure 13: Flow cytometry of of a MNX1 knock in line (left) and a MNX1 knock out line (right) differentiated towards definitive endoderm for three days instead of two days.

The morphology of spheres was also improved and showed the expected homogeneous structure without obvious zonation. As differentiation progressed, the compact spheres began to show the less dense morphology of pancreatic endoderm with rougher edges. Beginning at day 6 of differentiation, the knock in line also began to show red fluorescence, indicating expression of mScarlet from the modified MNX1 locus (Fig. 13). This is in line with MNX1 expression data from mouse development, where high level expression of MNX1 was detected in pancreatic endoderm. Flow cytometry of spheres at day 7 of differentiation showed that 50% to 70% of cells stained positive for PDX1. At day 8, the percentage of PDX1 positive cells was increased to 60% to 80% (Fig. 14). The knock in line consistently showed a higher of PDX1 positive cells compared to the knock out line, but this could have been caused by different behavior of the cell lines rather than MNX1 loss of function. Unfortunately the spheres were not able to survive past day 11 and could therefore not be differentiated into beta cells.

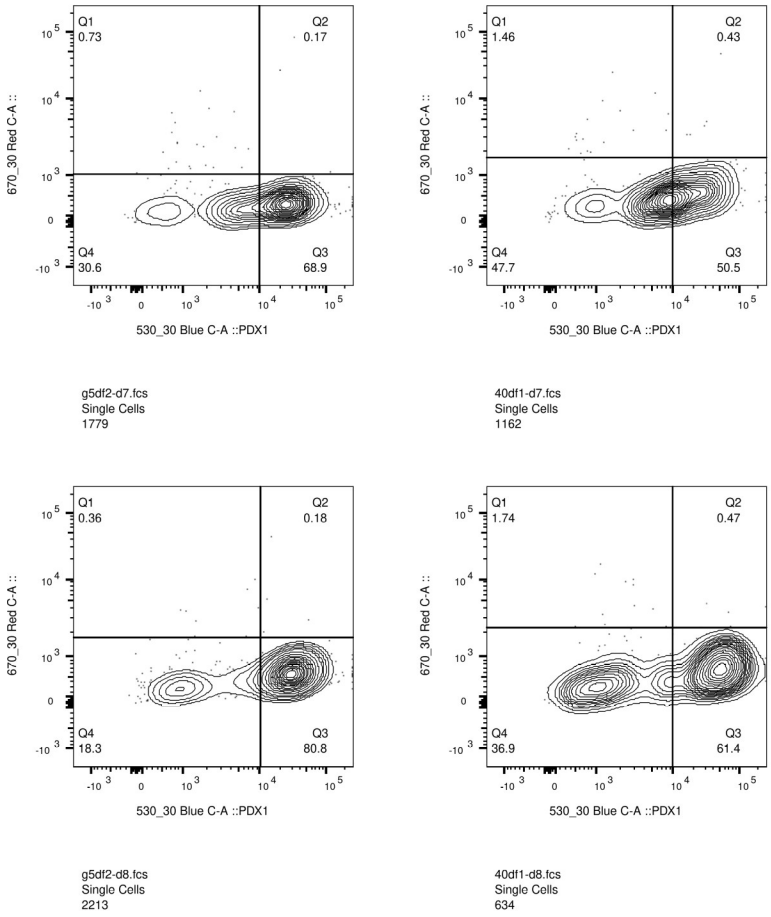

Figure 14: FACS analysis of PDX1 expression at day 7 (top) and day 8 (bottom) of differentiation of a MNX1 knock in cell line (left) and a MNX1 knock out line (right). PDX1 is on the X-axis, the Y-axis shows background fluorescence in the far red channel.

As the modified cell lines were cultured under feeder free conditions prior to adaptation to MEF feeder culture, the differentiation potential of these cell lines when cultured on truncated human Vitronectin in chemically defined Essential 8 medium was assessed. Cells continously cultured in these conditions for a similar number of passages than those adapted to MEF feeder culture were differentiated with the modified protocol and stained for FOXA2 and SOX17 after definitive endoderm induction. Surprisingly, even with prolonged exposure to definitive endoderm inducing medium containing Activin A, these cells failed to progress towards definitive endoderm as shown by flow cytometry (Fig. 15).

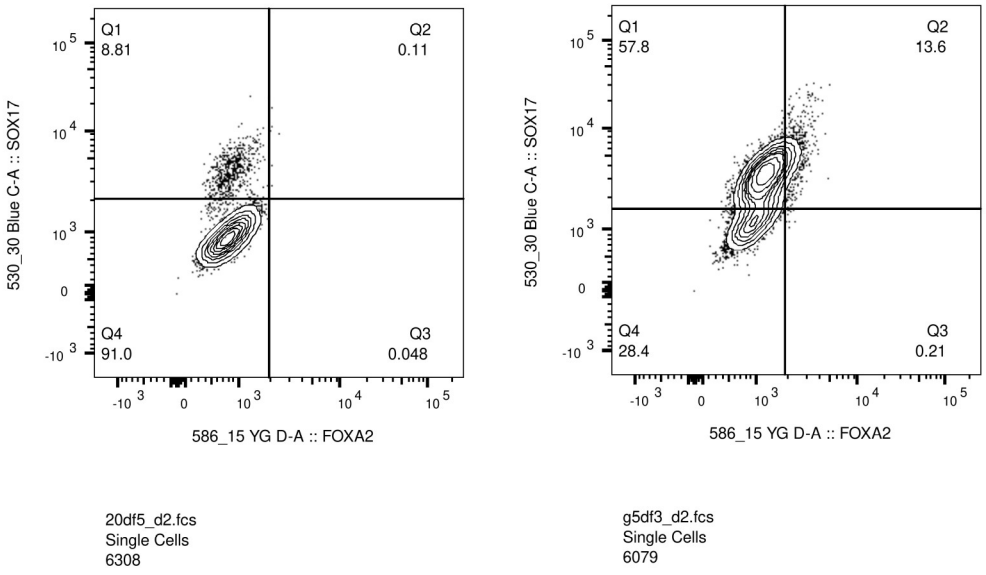

Figure 15: Flow cytometric analysis of definitive endoderm induction of modified cell lines cultured under feeder free conditions.

Since stem cells can be efficiently differentiated towards definitive endoderm in adherent culture on Vitronectin, a possible cause is the high concentration of FGF2 necessary to maintain pluripotency in the simplified E8 medium. The concentration of FGF2 in Essential 8 is tenfold higher (100 ng/ml) compared to the FGF2 concentration in hESC-A maintenance medium. Essential 8 also includes Insulin as a cell survival and proliferation promoting factor (Chen *et al.*, 2011).

Another factor influencing differentiation capability towards endoderm might be the dependence on ROCK inhibition for sphere formation. Besides their roles in the regulation of the cytoskeleton and cell contractility, ROCKs have also been shown to downregulate Insulin receptor mediated signalling by inhibiting the phosphorylation of Insulin receptor substrate-1 (IRS-1) (Farah *et al.*, 1998). Insulin receptor signalling inhibits endoderm differentiation (Xu, Browning and Odorico, 2011), therefore inhibition of ROCK could lead to higher IGF signalling and thereby reduce endoderm differentiation capability.

Even though many groups routinely maintain their stem cells in E8 on Vitronectin and succesfully differentiate them towards multiple endodermal lineages (e. g. (Li *et al.*, 2019)), they employ differentiation strategies based on adherent culture, which might be less sensitive to slight differences in the ground state of stem cells prior to differentiation. Insulin is included in the differentiation media as part of the Insulin-Transferrin-Selenium (ITS) supplement, albeit at an initially very low concentration. To test whether omission of ITS changes the differentiation dynamics of the modified cell lines, cells were seeded for sphere formation as usual and differentiation towards definitive endoderm was carried out in the presence of ITS according to the standard protocol or without ITS. Cell survival was not compromised by the omission of ITS and no obvious morphological differences between spheres differentiated without ITS and with ITS was visible, except a slightly larger size of the spheres differentiated without ITS (Fig. 16). Cells were assayed for the expression of the definitive endoderm marker SOX17 and the pluripotency marker TRA-1-60 by flow cytometry after three days. Spheres differentiated without ITS showed a higher percentage of SOX17 positive cells and a lower percentage of cells positive for TRA-1-60 compared to spheres differentiated according to the standard protocol (Fig. 17). While this suggests that ITS negatively impacts endoderm differentiation, the result might be attributable to technical variability.

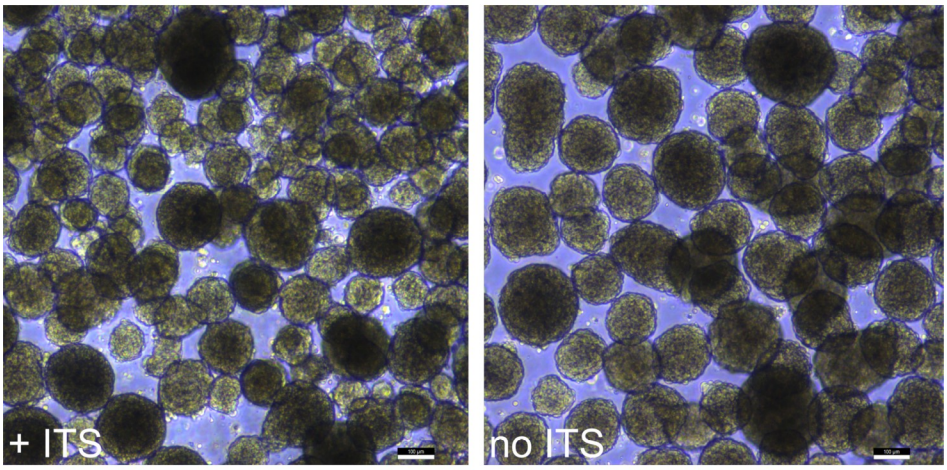

Figure 16: Bright field images of spheres differentiated for three days with (left) or without (right) the addition of ITS.

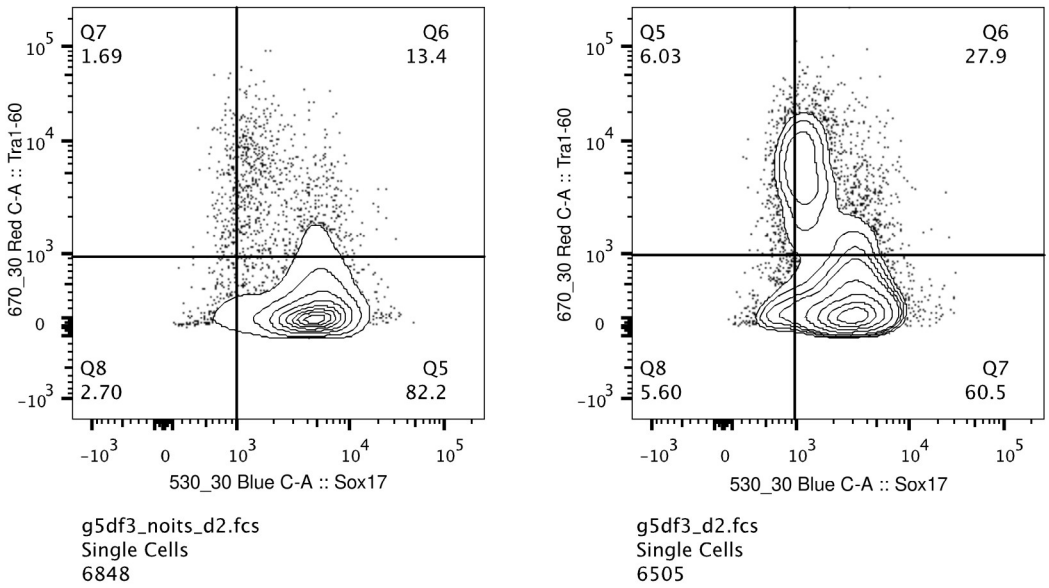

Figure 17: Flow cytometric analysis of spheres differentiated for three days without (left) or with ITS (right), showing the expression of TRA-1-60 and SOX17

Discussion

The importance of MNX1 in pancreatic development in vivo is well known. However, the role of MNX1 in pancreas development is not well understood. Since MNX1 is important for the formation of the dorsal pancreatic bud, from which most endocrine cells develop and its role in fate maintenance of pancreatic beta cells and thereby glucose homeostasis, understanding the molecular mechanisms of MNX1 in the mammalian pancreas will yield important insight for improving current strategies to derive pancreatic tissue from human stem cells for regenerative therapies and disease modelling from patient derived pluripotent stem cells.

Through the comparison of data available in the literature from mouse models with very recent single cell RNA sequencing datasets, strong arguments for the conserved function of MNX1 in mammalian pancreas development could be made. The complex role of this factor in human development is only beginning to be appreciated to its full extent.

In this project, first steps towards the establishment of an *in vitro* model for the study of MNX1 were performed. Fateful recapitulation of the multiple stages in development that lead to the generation of complex organs such as the pancreas is necessary to yield meaningful data about the factors involved. However, guiding embryonic or pluripotent stem cells towards the desired fate is a delicate procedure involving manipulation of a complex interconnected system of biological processes that even in this day and age are still incompletely understood. As evidenced by the results presented here, the ground state of cell lines, influenced by culture conditions and other poorly understood factors, can have profound effects on their behaviour in a differentiation protocol. Therefore, no protocol exists today that allows to efficiently generate desired differentiation outcomes for every cell lines. It is also well known that cell lines can differ enormously in their ability to differentiate to the three germ layers *in vitro.* All these pitfalls make first hand experience indispensable to successfully transferring a protocol from one laboratory to another. Even though I was not able to generate beta cells from the cell lines generated in our lab, good progress was made in determining the factors crucial for succeeding in this endeavor.

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References

Arkhipova, V. *et al.* (2012) ‘Characterization and regulation of the hb9/mnx1 beta-cell progenitor specific enhancer in zebrafish’, *Developmental Biology*, 365(1), pp. 290–302. doi: 10.1016/j.ydbio.2012.03.001.

Baron, M. *et al.* (2016) ‘A Single-Cell Transcriptomic Map of the Human and Mouse Pancreas Reveals Inter- and Intra-cell Population Structure’, *Cell Systems*. Elsevier Inc., 3(4), pp. 346-360.e4. doi: 10.1016/j.cels.2016.08.011.

Bonnefond, A. *et al.* (2013) ‘Transcription factor gene MNX1 is a novel cause of permanent neonatal diabetes in a consanguineous family’, *Diabetes and Metabolism*, 39(3), pp. 276–280. doi: 10.1016/j.diabet.2013.02.007.

Chen, G. *et al.* (2011) ‘Chemically defined conditions for human iPSC derivation and culture’, *Nature Methods*. doi: 10.1038/nmeth.1593.

Dalgin, G. *et al.* (2011) ‘Zebrafish mnx1 controls cell fate choice in the developing endocrine pancreas’, *Development*, 138(21), pp. 4597–4608. doi: 10.1242/dev.067736.

Farah, S. *et al.* (1998) ‘A Rho-associated protein kinase, ROKα, binds insulin receptor substrate-1 and modulates insulin signaling’, *Journal of Biological Chemistry*, 273(8), pp. 4740–4746. doi: 10.1074/jbc.273.8.4740.

Flanagan, S. E. *et al.* (2014) ‘Analysis of transcription factors key for mouse pancreatic development establishes NKX2-2 and MNX1 mutations as causes of neonatal diabetes in man’, *Cell Metabolism*. The Authors, 19(1), pp. 146–154. doi: 10.1016/j.cmet.2013.11.021.

Harrison, K. A. *et al.* (1999) ‘Pancreas dorsal lobe agenesis and abnormal islets of Langerhans in Hlxb9-deficient mice’, *Nature Genetics*, 23(1), pp. 71–75. doi: 10.1038/12674.

Jennings, R. E. *et al.* (2013) ‘Development of the human pancreas from foregut to endocrine commitment’, *Diabetes*, 62(10), pp. 3514–3522. doi: 10.2337/db12-1479.

Kimmel, R. A. *et al.* (2011) ‘Requirement for Pdx1 in specification of latent endocrine progenitors in zebrafish’, *BMC Biology*, 9(October). doi: 10.1186/1741-7007-9-75.

Lee, S. *et al.* (2008) ‘A Regulatory Network to Segregate the Identity of Neuronal Subtypes’, *Developmental Cell*. doi: 10.1016/j.devcel.2008.03.021.

Li, H. *et al.* (1999) ‘Selective agenesis of the dorsal pancreas in mice lacking homeobox gene Hlxb9’, *Nature Genetics*, 23(1), pp. 67–70. doi: 10.1038/12669.

Li, H. and Edlund, H. (2001) ‘Persistent expression of Hlxb9 in the pancreatic epithelium impairs pancreatic development’, *Developmental Biology*, 240(1), pp. 247–253. doi: 10.1006/dbio.2001.0440.

Li, Q. V. *et al.* (2019) ‘Genome-scale screens identify JNK–JUN signaling as a barrier for pluripotency exit and endoderm differentiation’, *Nature Genetics*. Springer US, 51(6), pp. 999–1010. doi: 10.1038/s41588-019-0408-9.

Nair, G. G. *et al.* (2019) ‘Recapitulating endocrine cell clustering in culture promotes maturation of human stem-cell-derived β cells’, *Nature Cell Biology*. Springer US, 21(2), pp. 263–274. doi: 10.1038/s41556-018-0271-4.

Pan, F. C. *et al.* (2015) ‘Inactivating the permanent neonatal diabetes gene Mnx1 switches insulin-producing -cells to a -like fate and reveals a facultative proliferative capacity in aged -cells’, *Development*, 142(21), pp. 3637–3648. doi: 10.1242/dev.126011.

Pan, F. C. and Wright, C. (2011) ‘Pancreas organogenesis: From bud to plexus to gland’, *Developmental Dynamics*, 240(3), pp. 530–565. doi: 10.1002/dvdy.22584.

Petersen, M. B. K. *et al.* (2017) ‘Single-Cell Gene Expression Analysis of a Human ESC Model of Pancreatic Endocrine Development Reveals Different Paths to β-Cell Differentiation’, *Stem Cell Reports*, 9(4), pp. 1246–1261. doi: 10.1016/j.stemcr.2017.08.009.

Russ, H. A. *et al.* (2015) ‘Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro’, *The EMBO Journal*, 34(13), pp. 1759–1772. doi: 10.15252/embj.201591058.

Sherwood, R. I., Chen, T. Y. A. and Melton, D. A. (2009) ‘Transcriptional dynamics of endodermal organ formation’, *Developmental Dynamics*, 238(1), pp. 29–42. doi: 10.1002/dvdy.21810.

Shih, H. P., Wang, A. and Sander, M. (2013) ‘Pancreas Organogenesis: From Lineage Determination to Morphogenesis’, *Annual Review of Cell and Developmental Biology*, 29(1), pp. 81–105. doi: 10.1146/annurev-cellbio-101512-122405.

Tarashansky, A. J. *et al.* (2019) ‘Self-assembling manifolds in single-cell RNA sequencing data’, pp. 1–29.

Veres, A. *et al.* (2019) ‘Charting cellular identity during human in vitro β-cell differentiation’, *Nature*. Springer US, 569(7756), pp. 368–373. doi: 10.1038/s41586-019-1168-5.

Wendik, B., Maier, E. and Meyer, D. (2004) ‘Zebrafish mnx genes in endocrine and exocrine pancreas formation’, *Developmental Biology*, 268(2), pp. 372–383. doi: 10.1016/j.ydbio.2003.12.026.

Xu, X., Browning, V. L. and Odorico, J. S. (2011) ‘Activin, BMP and FGF pathways cooperate to promote endoderm and pancreatic lineage cell differentiation from human embryonic stem cells’, *Mechanisms of Development*. doi: 10.1016/j.mod.2011.08.001.

Zhu, Z. *et al.* (2016) ‘Genome Editing of Lineage Determinants in Human Pluripotent Stem Cells Reveals Mechanisms of Pancreatic Development and Diabetes’, *Cell Stem Cell*, 18(6), pp. 755–768. doi: 10.1016/j.stem.2016.03.015.

Zorn, A. M. and Wells, J. M. (2009) ‘Vertebrate endoderm development and organ formation.’, *Annual review of cell and developmental biology*, 25, pp. 221–251. doi: 10.1146/annurev.cellbio.042308.113344.