Embryogenesis and neogenesis of the endocrine pancreas
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CHAPTER 6

Ex vivo differentiation of pancreas derived c-met and c-kit carrying cells to insulin producing cells
ABSTRACT

Objective: In the present study we investigated whether isolated c-met or c-kit positive cells from neonatal rats have the potential to differentiate into insulin producing cells after ex vivo manipulation.

Research Design and Methods: C-met and c-kit positive cells were first studied for expression of the following transcription factors associated with endocrine and exocrine differentiation: c-met, c-kit, nestin, Beta2, Islet-1, Pax6, Pax4, Pdx-1, ngn3, Glut-2, and insulin, glucagon, and amylase. Subsequently, freshly isolated c-met or c-kit positive cells were cultured in medium containing betacellulin, activinA, GLP-1, and HGF (for c-met positive cells) or SCF (for c-kit positive cells). After 10 days of culture the gene profile and protein level of insulin and c-met (on c-met positive cells) and c-kit (on c-kit positive cells) was studied.

Results: Freshly isolated c-met positive cells express c-met, c-kit, nestin, Beta2, Pax6, ngn3, Pdx-1, insulin, glucagon, and amylase. Upon culture these cells loose exocrine (amylase), alpha-cell specific markers (Pax6 and glucagon); upregulate or retain the expression of beta-cell specific markers (Islet-1, Pax4, and insulin). Increasing numbers of cells become insulin positive on a protein level. Freshly isolated c-kit positive cells express c-kit, Beta2, Islet-1, Pax4, insulin, and amylase. Upon culture these cells loose the exocrine marker (amylase), beta-cell specific transcription factors Pax4 and Beta2; and they upregulate beta-cell specific transcription factor ngn3; and remain insulin positive. On protein level, only a minor population of c-kit cultured cells produce insulin. Both c-met and c-kit cultured cells retain c-met or c-kit expression on mRNA and/or protein levels.

Conclusions: C-met carrying neonatal pancreatic cells can be manipulated ex vivo to differentiate into insulin producing cells.

INTRODUCTION

The neonatal endocrine pancreas contains a number of cell populations with the ability to form insulin producing cells [1-7]. We recently showed that a group of these cells express the extracellular receptor c-kit, i.e. the receptor for stem cell factor and c-met, i.e. the receptor for hepatocyte growth factor (chapter 4 and 5). The c-met positive cells in the pancreas are considered to be multipotent and able
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...to form cells of different pancreatic lineages. It was also shown that some c-met positive cells can be clonally expanded and therefore qualify as organ bound stem cell [5,7]. C-kit expressing cells in the pancreas are associated with neogenesis and reported to be expressed in the prenatal and postnatal rat pancreas, and during islet-cell development [2,6,7] and regeneration. Downregulation of c-kit results in decreased pdx-1 and insulin expression, suggesting downregulation of beta-cell differentiation [7,8].

We recently demonstrated that a portion of the c-met and c-kit positive cells contain beta-cell specific transcription factors such as ngn-3 and islet-1 (chapter 5). The cells can be found in every situation in which growth of the endocrine pancreas is required, i.e. from the embryonic status till adult suggesting a role for these cells in homeostasis of the endocrine pancreas. Both c-met and c-kit positive cells can be isolated from the pancreas by applying the receptor as a ligand for selection and purification. In the present study we applied the c-met and c-kit receptor for producing purified populations of these cells. The cells were subsequently cultured with beta-cell specific growth factors in order to determine whether the cells can be manipulated ex vivo to become insulin producing cells.

MATERIALS AND METHODS

Design of the study
C-met or c-kit positive cells were cultured in medium containing betacellulin, activinA, GLP-1, and HGF (for c-met positive cells) or SCF (for c-kit positive cells). The cells were cultured at a density of 50,000/cm². The cells were cultured for 10 days on this differentiation medium. During the 10 days of culture we examined the morphology of the cells. Thereafter, the cells were harvested and subjected to RT-PCR assessments to study the gene profiling of the cultured cells. We measured the mRNA levels of c-met, c-kit, nestin, Beta2 (transcription factor associated with beta-cell formation), Islet-1 (transcription factor specific for endocrine cell formation), Pax6 (transcription factor specific for alpha-cell formation), Pax4 (transcription factor specific for beta- and delta-cell formation), Pdx-1(transcription factor specific for beta-cell formation), ngn3 (transcription factor specific for endocrine cell formation), Glut-2 (glucose
transporter expressed by beta-cell), insulin (beta-cell secreted hormone), glucagon (alpha-cell secreted hormone), and amylase (acinar cell secreted enzyme).

Using immunofluorescence, on the protein level, we investigated the expression of insulin in both c-met and c-kit positive cells (before and after culture). We also studied the expression of c-met (on c-met positive cells) and c-kit (on c-kit positive cells) before and after culture of the cells.

**Animals**

All experiments were conducted in accordance with NIH-guidelines for the care and use of laboratory animals. Female Wistar rats (Harlan; age 3-4 months and weighing ~200 g) were kept in a temperature- and light-controlled room (lights on from 6 AM to 6 PM).

Animals to obtain pregnancy were subjected to daily vaginal smears. Pregnancy was achieved by housing the female rats on the night of pro-oestrus with a fertile male for one night. Neonatal rats were used 1-2 days after natural delivery.

**Surgery**

The neonatal rat pancreata were obtained from 1-2 days old rat pups. The pups were decapitated and the pancreas was surgically removed. This was done by laparotomy, replacing the stomach aside, and taking the pancreas out by cutting it loose from the spleen, duodenum, and stomach wall. The neonatal pancreata were processed for cell-isolation. For cell-isolation, the pancreata were stored in 5 ml of 0.20 μm filtered Krebs-Ringer-Hepes buffer (KRH: 133 mM NaCl, 4.69 mM KCl, 1.18 mM KH₂PO₄, 1.18 mM MgSO₄·7H₂O, 10 mM HEPES and 2.52 mM CaCl₂·2H₂O (pH 7.4)) containing 5% bovine serum albumin (BSA) on ice.

**Cell-isolation**

Neonatal pancreata were digested with collagenase to transform them into single cells. The pancreata were first cut in small fragments of approximately 1 mm. The fragments were then washed once with KRH containing 5% BSA for 5 minutes at 4°C. The pancreas fragments were digested with 5.5 mg/ml Collagenase P (Boehringer Mannheim, Germany) in KRH containing 1% BSA for 12 minutes at 37°C under continuous agitation in a water bath. For the separation of the single
cells from the debris, the mixture was centrifuged at 500 rpm for 30 seconds at 20ºC. The supernatant (containing the single cells) was aspirated. The single cell suspension was washed 3 times with KRH containing 1% BSA for 5 minutes at 4ºC. After washing, the cells were resuspended in KRH containing 1% BSA. Cell viability was determined with Trypan blue dye exclusion, viability was always 90%. After this procedure the single cells were divided in a portion for c-met and a portion for c-kit cell-sorting.

According to standard methods the following procedure was performed for c-met and c-kit cell-sorting. First the single cells were centrifuged at 1500 rpm for 5 minutes at 4ºC. The supernatant was discarded and the pellet was pre-incubated with undiluted swine serum for 30 minutes. After incubation the cells were washed with PBS containing 0,5% BSA and 0,1% sodium azide. Subsequently, the mixture was centrifuged at 1500 rpm for 5 minutes at 4ºC. Next the cells were incubated with the primary antibody anti-c-met (1:100) \((H-190, Santa Cruz Biotechnology)\) or anti-c-kit (1:100) \((C-19, Santa Cruz Biotechnology)\) for 60 minutes. Then the cells were incubated with secondary antibody swine-anti-rabbit FITC (1:50) (DakoCytomation, Denmark) for 30 minutes in the dark. The whole procedure was performed on ice. Positive selection for c-met and c-kit was performed using a Fluorescence Activated Cell Sorter (FACS, MoFlo flow cytometer) (Cytomation, USA). The sorted cells were collected in a tube with sterile RPMI 1640 containing 10% FCS, and 10 mg/ml Gentamycine (GIBCO).

**Culture of isolated c-met and c-kit cells**

Freshly isolated c-met or c-kit cells from neonatal rat pancreata were cultured for 10 days in medium supplemented with growth factors that have been reported to induce differentiation of precursor cells into pancreatic-endocrine cells. The medium contained RPMI 1640, 10% FCS, and 10 mg/ml Gentamycin, 25 ng/ml Hepatocyte Growth Factor (HGF, only for culturing c-met positive cells) (R&D systems), 25 ng/ml Stem Cell Factor (SCF, only for culturing c-kit positive cells) (Invitrogen), 40 ng/ml Glucagon-Like Peptide (GLP-1), 10 ng/ml Activin-A (R&D systems), and 10 ng/ml Betacellulin (R&D systems). The cells were cultured in tissue culture flat bottom wells-plates at a cell density of 50,000 cells per cm². The medium was changed twice a week. During the 10 days of culture the cells were not passaged. Cells were incubated at 37ºC in a humidified atmosphere of 5% CO₂.
**RNA isolation and cDNA synthesis**

Freshly isolated and trypsinized cultured cells were washed with PBS. Total RNA was isolated using an Absolutely RNA® Miniprep kit (Stratagene, Netherlands) according to the manufacturer’s protocol. Briefly, the cells were lysed in the lysis buffer provided in the kit. Ethanol (70%) was added and the solution was placed in a RNA-Binding Spin Cup (containing a silica-based fiber matrix). The RNA binds to the silica-based fiber matrix. Possible genomic DNA contamination was removed by incubation with RNase-Free DNase I and DNase Digestion Buffer (Stratagene). Several washing steps removed contaminants and the RNA was collected using an elution buffer. RNA concentration was measured using The NanoDrop® ND-1000 Spectrophotometer.

The cDNA was synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen), RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), 0.1M DTT (Invitrogen), 5x First Strand Buffer (Invitrogen), dNTPs and random hexamer primers (Invitrogen) according to manufacturer’s protocol.

**Polymerase Chain Reaction analysis**

PCR reactions were performed in a MJ Research PTC-100 Thermal Cycler. The PCR reaction (total 30 µl) was performed as follows: 100 ng cDNA, 3 µl 10x PCR buffer (Fermentas), 3 µl dNTP’s (2mM, Fermentas), 1.25 µl MgCl₂ (50mM, Fermentas), 0.2 µl Taq DNA Polymerase (Invitrogen), 1 µl forward primer (40 mM), 1 µl reverse primer (40mM) and MilliQ water add up to 30 µl.

PCR cycles were as follows: denaturation of the double-stranded DNA at 94°C for 5 minutes, followed by 94°C for 30 seconds, primer annealing temperature for 30 seconds, 72°C for 30 seconds (40 cycles), and final extension at 72°C for 5 minutes. The PCR products were separated on a 2% agarose gel in 0.5x TBE running buffer (150V, 60 minutes), and visualized by ethidium bromide staining. The primer sequences, annealing temperatures, and the expected fragment sizes are shown in Table 1. β-Actin was used as a housekeeping gene for the PCR reaction.
**Table 1:** The primer sequences, annealing temperatures, and the expected fragment sizes of the used primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Annealing temp.</th>
<th>Fragment size</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Met</td>
<td>ATGCCCTTCGAAAGCAACCAT</td>
<td>ATGAATAAGTCGACGCGCTGC</td>
<td>52.5°C</td>
<td>540 bp</td>
</tr>
<tr>
<td>c-Kit</td>
<td>AGCAAGAGTTAAAGGATCCGAG</td>
<td>CCAGAAAGGTGTAAAGGCTCCT</td>
<td>54.2°C</td>
<td>344 bp</td>
</tr>
<tr>
<td>Nestin</td>
<td>GCGGGCGCGTGGCTGACTAC</td>
<td>AGGCAAGGGGAAGGAAAGATGT</td>
<td>63.9°C</td>
<td>550 bp</td>
</tr>
<tr>
<td>Beta-2</td>
<td>AAGAGCAAGCAGATTCGAG</td>
<td>AATGGAGACGAGGTCTGGCTT</td>
<td>63.9°C</td>
<td>201 bp</td>
</tr>
<tr>
<td>Islet-1</td>
<td>GCAAGTGCAGCATAAGGCTT</td>
<td>AGGCTCTTCAGCTCTAGGC</td>
<td>62.7°C</td>
<td>201 bp</td>
</tr>
<tr>
<td>Pax-6</td>
<td>AGGGCAATCGGAGGCAAGTA</td>
<td>TTGCTTTTCGCTAGCCAGGT</td>
<td>52.5°C</td>
<td>201 bp</td>
</tr>
<tr>
<td>Pax-4</td>
<td>GGACTCTTTGTGAAATGGCC</td>
<td>GGCCAGACGTGGTTGCTT</td>
<td>61.0°C</td>
<td>201 bp</td>
</tr>
<tr>
<td>Pdx-1</td>
<td>ATCACTTGAGCAGGGAGGT</td>
<td>GCTACTACGGTTTCTATCT</td>
<td>60.0°C</td>
<td>247 bp</td>
</tr>
<tr>
<td>Ngn-3</td>
<td>TCACGCGTGCTATGCTGCTG</td>
<td>CAGTCAACCACTTCTGCTGCG</td>
<td>52.5°C</td>
<td>160 bp</td>
</tr>
<tr>
<td>Glat-2</td>
<td>GTAGCATGACGCCGCACTTT</td>
<td>CAGTGACGCGGCCGAGGAAG</td>
<td>52.5°C</td>
<td>201 bp</td>
</tr>
<tr>
<td>Insulin</td>
<td>TGCCCAGGCCTTTTGCAACACGACCTT</td>
<td>CTCCAGTGCCAAAGGTCTGAA</td>
<td>60.0°C</td>
<td>187 bp</td>
</tr>
<tr>
<td>Glucagon</td>
<td>CAGAGGAAGAACCCAGCATCA</td>
<td>TCATGACGTTTGGCAAGTTC</td>
<td>52.5°C</td>
<td>202 bp</td>
</tr>
<tr>
<td>Amylase</td>
<td>TCAGCTGCTGTCCGCTTGCT</td>
<td>CTCGTTCCCTGGGAGACCAT</td>
<td>54.2°C</td>
<td>201 bp</td>
</tr>
<tr>
<td>B-Actin</td>
<td>GATCGAACACTGTGCGTGTACCTACAGTGGTGAAGATGGGACCAGAT</td>
<td>TGTCGACGCAGGACTAGTACAG</td>
<td>56.4°C</td>
<td>100 bp</td>
</tr>
</tbody>
</table>
**Immunofluorescence**

Freshly isolated and trypsinized cultured cells were spotted on glass slides at a density of 50,000 cells per slide. This was done with a Cytospin centrifuge at 500 rpm for 5 min. Cytospots were air dried and then fixed in acetone for 10 minutes and followed by air drying for 30 minutes. The cytospots of freshly isolated c-met or c-kit cells had already been labelled with first antibody and secondary antibody for the isolation procedure. Because fluorescence intensity of the secondary antibody decreased during the sorting procedure we had to increase the fluorescence of the cells for microscopic detection. This was done by completing the staining by incubation with tertiary antibody goat-anti-swine fluorescein isothiocyanate FITC labelled (1:50) (Jackson Immunoresearch Laboratories, Inc USA) for 30 minutes. Cytospots of cultured cells were incubated with normal goat serum for 30 minutes and subsequently blocked with a biotin blocking system kit (DakoCytomation, Carpinteria, CA) for 15 minutes in the case of staining for c-met or c-kit. Subsequently, the cytospots were incubated with primary antibodies for 60 minutes. The following antibodies have been applied; rabbit-anti-rat-c-kit (1:100), rabbit-anti-rat-c-met (1:150), and mouse-anti-rat-insulin (1:750) (clone K36AC10; Sigma). Next the sections were incubated with secondary antibody goat-anti-mouse IgG1 fluorescein isothiocyanate FITC labelled (1:50) (for insulin) (Southern Biotechnology Associates, Inc, Birmingham, USA) or goat-anti-rabbit biotin labelled (1:50) (for c-met or c-kit) (DakoCytomation, Denmark) for 30 minutes. For c-met and c-kit this was followed by tertiary antibody streptavidin-Cy3 (1:200) (Invitrogen, USA) incubation for 30 minutes. Finally the sections were incubated with 4’, 6-diamidino-2-phenylindole (DAPI) (1:2500) (Roche) for 10 minutes and mounted with Citifluor (Agar Scientific). The whole procedure was performed at room temperature. Analysis was performed using the Leica DMRXA fluorescent microscope and Leica Qwin Pro software.

**RESULTS**

After transforming neonatal pancreata into single cells, we found that 1.8 ± 0.9% are c-met positive cells, and 4.7 ± 0.8% are c-kit positive cells (Figure 1). After cell sorting the population was always composed of more than 90% of c-met or
c-kit positive cells. These pure populations were used for culturing the cells in the differentiation medium as described in the materials and methods section.

**Gene expression profile of c-met and c-kit positive cultured in differentiation medium containing beta-cell specific growth factors**

When compared with freshly isolated c-met positive cells we found that c-met cells lose the expression of c-kit, Beta2, ngn3, Pdx-1, glucagon, and amylase after culture for 10 days in differentiation medium. They gain the expression of Islet-1 and Pax4 (Figure 2A). Moreover, they lose amylase expression and glucagon expression, but remained insulin positive.

Figure 1: Mean percentage (± SEM) of c-met (open bar) and c-kit (black bar) positive cells in neonatal rat pancreas (n=3).

Also the c-kit positive cells change their mRNA expression profile after 10 days of culture. When compared with freshly isolated c-kit positive cells we found that these cells lose the expression of c-kit, Beta2, Pax4, and amylase. They gain the expression of nestin, and ngn3 (Figure 2B). Similar to c-met positive cells, they lose the expression of the exocrine marker amylase. The cells are still producing insulin mRNA.
Figure 2: Agarose gel electrophoresis of RT-PCR products of c-met, and c-kit positive cells after culture in differentiation medium containing beta-cell specific growth factors. Freshly isolated and cultured c-met, and c-kit positive cells were harvested and following RNA isolation and cDNA synthesis subjected to RT-PCR using primers for c-met, c-kit, nestin, Beta2, Islet-1, Pax6, Pax4, Pdx-1, ngn3, Glut-2, insulin, glucagon, and amylase, respectively. B-actin was used as a housekeeping gene. Note that c-met cultured cells loose the expression of c-kit, Beta2, ngn3, Pdx-1, glucagon, and amylase. They gain the expression of Islet-1 and Pax4 and remained insulin positive (A). C-kit cultured cells loose the expression of c-kit, Beta2, Pax4, and amylase. They gain the expression of nestin, and ngn3 and remained insulin positive (B).

Protein profile of c-met and c-kit positive cultured in differentiation medium containing beta-cell specific growth factors

We applied immunofluorescence to study whether c-met and c-kit cultured cells express insulin on protein levels. We furthermore investigated whether c-met or c-kit positive cells retained their expression of the markers c-kit and c-met after culture. This was done by investigating the expression of c-met for c-met cultured cells or c-kit for c-kit cultured cells on protein levels.

Although only a few freshly isolated c-met cells were insulin positive (Figure 3A), we found that after culture all c-met cultured cells express insulin
Ex vivo differentiation c-met and c-kit cells to insulin producing cells

(Figure 3B), although at a low level. We furthermore found that after culture the cells lost their c-met expression on protein level (Figure 3D).

**Figure 3:** Photomicrograph of cytospots of c-met positive cells before and after culture in differentiation medium containing beta-cell specific growth factors. The cytospots were immunostained for insulin (A and B), and c-met (C and D). Freshly isolated c-met positive cells expressed insulin (A) after culture the number of insulin expressing cells increased (B). Freshly isolated c-met positive cells express c-met on their cell surface (C), after culture the cells loose their c-met expression (D). Original magnification 200X

Only a few of the freshly isolated c-kit cells expressed insulin (Figure 4A). After the 10 days culture period this number was not increased. It remained restricted to a minority of the cells (Figure 4B). We furthermore found that the majority of c-kit cultured cells still expressed c-kit on protein level (Figure 4D).
**Figure 4:** Photomicrograph of cytospots of c-kit positive cells before and after culture in differentiation medium containing beta-cell specific growth factors. The cytospots were immunostained for insulin (A and B), and c-kit (C and D). Freshly isolated c-kit positive cells expressed insulin (A). After culture the cells remained their insulin expression, however, insulin expressing cells did not increase in number (B). Freshly isolated c-kit positive cells express c-kit on their cell surface (C), after culture the cells did not loose their c-kit expression (D). Original magnification 200X

**DISCUSSION**

In this study we have isolated c-met and c-kit positive cells from neonatal pancreata and subjected them to *in vitro* culture with various factors which are known to be involved in differentiation of beta-cells. In our previous study we have shown that these cells express transcription factors specific for both exocrine and different endocrine (precursor) cell-types suggested that these cell populations are a heterogenous population with different functions and different differentiation potentials. After 10 days of culture in medium containing beta-cell specific growth factors, c-met and c-kit positive cells show a different mRNA expression profile. This new profile suggests that we may have differentiated the c-met and c-kit positive cells (or specific populations within these cell populations) towards beta-cells.
Ex vivo differentiation c-met and c-kit cells to insulin producing cells

C-met carrying cells from the neonatal pancreas can be transformed into a pure population of insulin producing cells after a ten day culture period in the presence of the beta-cell specific growth factors betacellulin, activinA, GLP-1 [5,9-12]. In the c-met carrying cell cultures we also added HGF. This was done not only because HGF is the ligand for the c-met receptor [13,14], but also because it was recently reported that HGF induces the differentiation of epithelial monolayers into insulin producing cells [7].

The fact that cells produce insulin is not necessarily a suggestion that the cells are beta-cells [15]. However, we not only found that the c-met positive cells produce insulin, but also that they upregulate beta-cell specific transcription factors and lose transcription factors for other pancreatic cell types. Under the influence of the culture conditions the c-met carrying cells loose the transcripts for pancreatic cell types such as that of exocrine cells (amylase) [16] and alpha-cells (Pax6 and glucagon) [17]. The cells furthermore upregulate the expression of Islet-1, i.e. an important transcription factor for differentiation into endocrine cells [18], and more importantly the expression of Pax4, i.e. a beta-cell differentiation marker [19].

The fact that Pdx-1, ngn3, and Beta2 are absent in the cultured c-met expressing cells suggests that the cells are not yet in the end stage of beta-cell differentiation but are further in the process than in the starting cultures. Ngn 3 directs precursors towards an endocrine fate and is therefore expressed early in the differentiation process and disappears from the cells during the process of differentiation [20]. Beta2 and PDX-1 are expressed at the end of the process of differentiation. Beta2 is suggested to play a role in the maintenance of islets cell differentiation and in the process of islet morphology [21]. PDX-1 has an important function in adult beta-cells in insulin gene transcription [22-27]. The absence of Beta-2 and PDX-1 suggests that the cells are not yet entered the process of end stage differentiation.

C-kit positive cells could not be manipulated to increase the number of insulin positive cells in the 10 days culture period. This however does not mean that the cells did not differentiate towards an endocrine phenotype. The cells start expressing ngn3, which is associated with an endocrine cell development [20]. Furthermore, the c-kit positive cells loose their mRNA for amylase, which suggest that the cells loose the ability to differentiate into an exocrine phenotype. Interestingly, during
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culture, the cells also loose Pax4. It has been suggested that Pax4 is present in early development and disappears shortly after birth. In adult it is absent [19,28]. Again this suggests a progressive differentiation of the cells towards an endocrine phenotype.

Many groups focus on finding a source of insulin producing cells for the cure of diabetes. An approach that achieves much attention is generation of beta-cells from embryonic stem cell sources [29-33]. Although this approach has shown some success, it also contains the major threat of teratoma formation [34]. Therefore, many are the efforts to find alternative sources such as multipotent newborn or adult organ bound stem cells. These cells are not associated with teratoma formation and may be more easily differentiated than embryonic stem cells, since they already have multipotency towards the organ from which they are harvested. Also a portion of the c-met and c-kit expressing cells have many characteristics that qualify them as organ bound multipotent stem cell (chapter 5). The present study demonstrates the principle applicability of the cells as they do survive in culture for prolonged periods of time and can be manipulated with growth factors to differentiate into an endocrine phenotype.

One may argue that the insulin positivity of our c-met cultures may be achieved by selection of the insulin positive cells in the fresh cultures and not by differentiation of the population. Several arguments appose this suggestion. First we observed no significant changes in the cells density. Second the cells were not passaged, suggesting low replication rate in the cell-population. This implies that there was no significant loss of cells or of replication of pre-existing insulin producing c-met positive cells. Hence, the only explanation is that the insulin producing cells are formed by differentiation of the c-met positive cells in the cultures.

Thus, we show that both c-met and c-kit can be manipulated in vitro towards an endocrine phenotype. C-met producing cells can be forced to become insulin producing cells. The cells are not yet in the end-stage of differentiation and therefore can not be considered to be fully mature functional beta-cells. The cells do express beta-cell specific transcription factors, but still lack Glut-2 which implies the cells do not have developed the glucose-responsive machinery to respond on a glucose load [35]. Whether fully differentiated cells are required for application
Ex vivo differentiation c-met and c-kit cells to insulin producing cells of cells for therapeutic purposes is however questionable. It has been shown that precursor cells are able to further differentiate into beta-cells after in vivo transplantation [5,36].

Finally, our study demonstrates the principle applicability of organ bound c-met and c-kit precursor cells for obtaining cells with an endocrine insulin producing phenotype. Current efforts concentrate on determining the therapeutic potentials of these cells by studying the dose effect relationship for curing diabetes.

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REFERENCE LIST

14. Naldini, L. et al. (1991) Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. Oncogene. 6, 501-504
20. Gu, G. et al. (2002) Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 129, 2447-2457
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