The number of nestin, c-met, and c-kit positive cells is associated with growth and differentiation of the embryonic, neonatal, and adult rat pancreas
ABSTRACT

Objective: Nestin, c-met, and c-kit positive cells are thought to be responsible for development of the primitive pancreas and have also been reported to be present in the adult pancreas. However, a lot of dispute about the exact localization of these cells in the pancreas exist.

Research Design and Methods: Histological analysis was performed to investigate the localization of these cells in the endocrine, i.e. the islets, and exocrine, i.e. ductules, exocrine tissue, and blood vessels, compartments in the embryonic, neonatal and physiological growing rat pancreas.

Results: In the embryonic pancreas nestin and c-met positive cells were abundantly present, whereas c-kit positive cells were absent. In the neonatal and adult pancreas during growth nestin, c-met, and c-kit positive cells are present in the islets, near the ductules and blood vessels.

Conclusions: Our results suggest that nestin, c-met and c-kit positive cells contribute directly or indirectly to pancreas neogenesis. Furthermore, these cells seem to be also involved in vessels formation.

INTRODUCTION

There is substantial effort by the endocrine pancreas to enhance the beta-cell mass during increased metabolic demand such as during for instance pregnancy, obesity, or insulin resistance [1-6]. To study the mechanisms of growth of the beta-cell mass. The advantage of using pregnancy for the study of beta-cells mass growth rather than models of beta-cell mass growth after damage of the pancreas is that pregnancy induced growth takes place in a small well defined time frame. Growth of the beta-cell mass is assumed to occur by various mechanisms, i.e. replication of already differentiated beta-cells, neogenesis from precursor cells or suppression of apoptosis [7]. Many have shown that these processes occur but solid insight in the processes guiding this homeostasis in beta-cell mass is still lacking. In the embryonic pancreas a number of cell types have been shown to contribute to the development and of growth of the endocrine pancreas [8-14].
During embryogenesis, the pancreas develops as an epithelial evagination of foregut endoderm into the surrounding splanchnic mesoderm. This epithelial-mesenchymal interaction is soon followed by acinar and islet cell differentiation [15]. During this early stage, a multipotent stem cell may differentiate into cells that possess either an endocrine or exocrine phenotype [16]. Endocrine precursor cells develop further by budding from embryonic duct-like cells [17]. This process leads to the formation of primitive islets in the mesenchyme adjacent to the ducts. Much new insight has been gained during recent years in the processes responsible for development of the pancreas in embryogenesis and a number of cell types that are being hold responsible for development of the primitive pancreas have been reported to be also present in the adult pancreas. These cells are so-called nestin, c-met, and c-kit, positive cells [8-10,18], which have been suggested to either guide the development of the endocrine pancreas or to directly differentiate in endocrine cells [8-10]. Unfortunately, only minor insight is available on possible similarities with embryogenesis and growth in the adult pancreas, and the involvement of these cells in the adult growth of the pancreas.

As none of the presently published studies conclusively show the presence of nestin, c-met, and c-kit positive cells in different stages of development (after embryo to adult pancreas) and also because there is still dispute whether these cell-types are present in the endocrine pancreas or in other compartments of the pancreas, i.e. the exocrine pancreas, the blood vessels, and ductal system, we decided to do a histological analysis of these cell types during development of the rat pancreas. We not only did that in the embryonic rat pancreas, but we also studied the presence and localization of these cells during physiological growth of the rat pancreas to demonstrate possible similarities with the embryonic development of the endocrine pancreas.

MATERIALS AND METHODS

Design of the study
We studied the presence and localization of c-met-, c-kit- and nestin positive cells in the pancreas at different stages of development, i.e. in the embryonic, the neonatal, as well as in the (growing) adult rat pancreas. This was done by
immunohistology. Embryonic pancreata (n=5) were obtained from embryos of 14 days (E14) pregnant female rats. Neonatal rat pancreata (n=5) were isolated from 1-2 days old (P1-2) newborn pups. Non-pregnant female rat pancreata (n=5) served as controls. Growth under metabolic challenge was studied in pancreata from fourteen- (n=4) and seventeen-days (n=4) pregnant rats. Pregnancy-associated pancreas growth occurs in a defined time frame of 14-17 days after conception. In the rat the expansion of the beta-cell mass is reaching a peak level at day 14 of pregnancy [6,19]. This is the main rationally to concentrate on nestin, c-met, and c-kit expression on days 14. After excision the pancreata were snap frozen and processed for immunohistological assessment of nestin, c-kit, and c-met expression and localisation in the islets of Langerhans, the blood vessels, and ductal system. In case we observed by histological examination integration of nestin, c-met, or c-kit cells into blood vessels or the ductal system we performed double staining with CD31 (endothelial cells) or CK20 (ductal epithelial 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).

All animals were subjected to daily vaginal smears. Rats with regular 4-day oestrus cycles (*i.e.* rats in the follicular phase of the ovarian cycle) were selected for the experiments. Pregnancy was achieved by housing the female rats on the night of pro-oestrus with a fertile male for one night. The next day, when spermatozoa were detected in the smear, was designated as day 0 of pregnancy. In experiments in which neonatal pancreata were used natural delivery of the newborn pups was allowed.

**Surgery**

Embryonic rat pancreata were surgically dissected from the embryos by using a dissection microscope. The pregnant female rats were anaesthetised with isoflurane, oxygen, and NO₂. Laparotomy was performed after which the embryos were removed. The embryos were placed under the dissection microscope and fixed with needles. First the stomach and spleen were dissected. Removal of these two
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organs uncovered the pancreas with its dorsal and ventral lobs. These two lobs were removed and snap frozen in liquid nitrogen and stored at -80ºC until sectioning.

The neonatal rat pancreata were obtained from 1-2 days old newborn 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 either snap frozen in liquid nitrogen or processed for cell-isolation. The adult non-pregnant and pregnant pancreata were obtained from adult female non-pregnant and pregnant rats. These rats were anaesthetised with a combination of isoflurane, oxygen, and NO₂ and the pancreas was removed and subsequently snap-frozen in liquid nitrogen and stored at -80ºC until sectioning.

**Immunohistochemistry and immunofluorescence**

Embryonic and neonatal pancreata were sectioned at 6 μm. Adult and pregnant pancreata were sectioned at 4 μm and stored at -80ºC. Tissue cryosections were air dried and then fixed in acetone for 10 minutes and followed by air drying for 30 minutes. Next, the sections were incubated with normal goat serum for 30 minutes and subsequently blocked with a biotin blocking system kit (DakoCytomation, Carpinteria, CA) for 15 minutes. Subsequently, the sections were incubated with primary antibodies for 60 minutes. The whole procedure was performed at room temperature. The following first antibodies have been applied; mouse-anti-rat-nestin (1:500), rabbit-anti-rat-c-kit (1:100), rabbit-anti-rat-c-met (1:150). Then the sections were incubated with secondary antibodies for 30 minutes at room temperature. We applied the following secondary antibodies; goat-anti-mouse IgG-biotin (1:50) (Southern Biotechnology Associates, Inc, Birmingham, USA) or goat-anti-rabbit IgG-biotin (1:50) (DakoCytomation, Denmark). Staining was performed with the StreptABComplex-HRP (DakoCytomation, Denmark). Peroxidase activity was visualised by applying 3-amino-9-ethyl-carbazole (Sigma, Steinheim, Germany). The sections were analysed using Leica DMLB light microscope (including Leica DC 300 camera). For sections of neonatal, pregnant and non-pregnant rats, we counted the number of nestin positive cells in at least 1000 cells in the islets and expressed the results as a percentage of the total number of counted cells. The quantified sections were selected with an interval of 5 sections.
In some experiments we applied double immunofluorescent staining in which nestin, c-met, or c-kit staining was combined with either staining for CK20 or CD31. The sections were treated according to standard methods. In brief, tissue cryosections were air dried and then fixed in acetone for 10 minutes and subsequently again air dried for 30 minutes. Next the sections were incubated with normal goat or swine serum for 30 minutes and subsequently blocked with a biotin blocking system (DakoCytomation, Carpinteria, CA) for 15 minutes. Subsequently, the sections were incubated with primary antibody anti-nestin (1:500) or anti-c-met (1:150) or anti-c-kit (1:100) for 60 minutes. Next the sections were incubated with secondary antibody goat-anti-mouse IgG fluorescein isothiocyanate (FITC)(1:50) (for nestin) or goat-anti-rabbit IgG-biotin (1:50) (for c-met) or swine-anti-rabbit IgG FITC (1:50) (for c-kit) for 30 minutes followed by tertiary step with incubation with streptavidin-Cy3 (1:200) (Invitrogen, USA) or goat-anti-swine IgG FITC (1:50) (Jackson ImmunoResearch Laboratories, Inc) incubation for 30 minutes. Then the sections were incubated with primary antibody anti-CK20 (1:100) (DakoCytomation, Denmark) or anti-CD31-biotinylated (1:50) for 60 minutes. Subsequently, the sections were incubated with secondary antibody goat-anti-mouse FITC (1:50) or streptavidin-Cy3 (1:200) 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). All procedures were performed at room temperature. Analysis was performed using the Leica DMRXA fluorescent microscope and Leica Qwin Pro software.

**Statistics**

Data are expressed as the mean percentage (± SEM). Data was analyzed using nonparametric Mann Withney U tests. P < 0.05 were considered statistically significant.

**RESULTS**

We studied nestin, c-met, and c-kit positive cells in and around the different pancreas compartments *i.e.* the islets of Langerhans (the endocrine compartment), the exocrine tissue, the ductules, and the blood vessels. We did this in three phases
of pancreatic development, i.e. the embryonic, the neonatal pancreas and during the pregnancy induced growth of the adult pancreas.

**The embryonic pancreas**

We studied the localisation of nestin, c-met, and c-kit positive cells in rat embryonic pancreata of embryos from day 14 of gestation (E14). Nestin positive cells and c-met positive cells were abundantly present in the embryonic rat pancreas, whereas c-kit positive cells were absent (Figure 1). At this stage of development the endocrine and exocrine pancreas are still underdeveloped and do not allow reliable, separate histological study of the different cells types in the endocrine and exocrine pancreas.

![Figure 1: Photomicrograph of the embryonic rat pancreas after immunostaining for nestin (A), c-met (B), and c-kit (C). The pattern of expression was different for nestin and c-met. Nestin positive cells were found as single cells or small clusters throughout the whole embryonic pancreas with exception of the primitive pancreatic ducts (A). C-met was expressed in clusters in the embryonic pancreas (B). Furthermore, c-met positive cells were found as layers around the primitive pancreatic ducts (B). Staining for c-kit was negative in the embryonic pancreas (C). Original magnification 100X](image)

**The neonatal pancreas**

In the neonatal pancreas we found nestin positive, c-met positive and c-kit positive cells throughout the whole neonatal pancreas (Figures 2 and 3).

Nestin positive cells were clearly found in the islets of Langerhans of neonatal pancreata (Figure 2A). In total 34.1 ± 2.4% of the islet cells in the neonatal
pancreas were nestin positive (Figure 2D, Mann Whitney U test, \( P < 0.01 \)). In the exocrine compartment, nestin positive cells were found in cell-clusters near the neonatal ductules (Figure 3A, arrow); around the ductule columnar epithelial cells (Figure 3A); close to neonatal blood vessels (Figure 3B) and around the endothelial cells of the large blood vessels (Figure 3B). Since some nestin positive cells were localized near neonatal blood vessels we applied double staining for CD31 with nestin to decide whether these are endothelial cells. As shown in Figure 4A, a portion of the small blood vessels stain double positive for nestin and CD31 (Figure 4A, arrow). In the neonatal exocrine tissue, nestin positive cells were found as small clusters of cells. Also, some cells in the developing acini stain positive for nestin (Figure 3C, arrow).

We also examined the c-met expression pattern in the neonatal pancreas. C-met positive cells were found in the neonatal islets of Langerhans (Figure 2B). The expression of c-met was not restricted to the islets, but it was also found around ductules (Figure 3D, arrow) and near blood vessels (Figure 3E, arrow). To study the nature of these c-met positive cells around the blood vessels and ductule columnar epithelial cells we performed double immunofluorescence stainings for c-met and CD31 (Figure 4B) and c-met and for CK20 (Figure 4C). These results clearly show that c-met positive cells are neither CD31-positive endothelial cells nor CK20-positive ductal epithelial cells. Furthermore, c-met expression was also found in the developing acini (Figure 3F, arrow).

In contrast to the embryonic pancreas, c-kit expression was observed in the neonatal pancreas. We found that c-kit positive cells are localised in the islets of Langerhans (Figure 2C). Similar to c-met positive cells, we also found that c-kit positive cells formed a cell layer around the ductule columnar epithelial cells (Figure 3G, arrow). Near blood vessels, c-kit positive cells were localised in small clusters (Figure 3H, arrow). In the exocrine tissue, c-kit positive cells were also organised as small clusters of cells (Figure 3I, arrow).
Figure 2: Photomicrograph of the neonatal rat pancreas after immunostaining for nestin, c-met, and c-kit for the islets of Langerhans. Nestin (A), c-met (B), and c-kit (C) positive cells were found throughout the neonatal islets of Langerhans. These nestin positive cells were observed in both the periphery and centre of the islets. C-met positive cells were scattered throughout the whole neonatal islet. Mean percentage (± SEM) of nestin positive cells in the islets of Langerhans of neonatal rat pancreas (open bar), adult non-pregnant rat pancreas (black bar), 14-days pregnant rat pancreas (check bar), and 17-days pregnant rat pancreas (striped bar) (D). Statistical comparisons were made with the Mann Whitney U test. For all data differences were considered significant. ** P-value < 0.01; * P-value < 0.05. Original magnification 200X.
Figure 3: Photomicrograph of the neonatal rat pancreas after immunostaining for nestin, c-met, and c-kit for the specific structures, i.e., the ductules and blood vessels in the exocrine pancreas. Nestin positive cells were found around the ductules (A), and around blood vessels (B). The developing acini stained positive for nestin (C). C-met and c-kit positive cells were found around and near the ductules in small clusters (D and E), and near blood vessels in small clusters (G and H). In the exocrine pancreas the developing acini stained positive for nestin (C), c-met (F), and c-kit (I). Original magnification 200X
Figure 4: The neonatal rat pancreas after immunostaining for nestin and CD31 (A), c-met and CD31 (B), and c-met and CK20 (C). In the neonatal pancreas the small blood vessels stain double positive for nestin and CD31 (A). C-met positive cells do not stain double positive with CK20, but they surround ductules (B, arrow). Also c-met positive cells do not stain double positive with CD31, however they are found in small clusters near blood vessels (C, arrow). Original magnification 400X
The adult pancreas in steady state

In the adult female rat pancreas we found nestin positive cells in the islets of Langerhans (Figure 5A). In total 3.9 ± 0.8 % of the islet cells in the adult pancreas were nestin positive (Figure 2D). No nestin positive cells were found near the ductules (Figure 5B). Nestin positive cells were also found in clusters of 2 or 3 cells near blood vessels (Figure 5C, arrow).

In the adult female rat pancreas, no c-met positive cells were found in any of the compartments, i.e., of islets (Figure 6A), ductules (Figure 6B), and blood vessels (Figure 6C).

C-kit positive cells were found in all compartments of the adult female rat pancreas (Figure 7). In the islets they were detected in both the periphery and in the centre of the islets (Figure 7A). Furthermore, the duct cells (columnar epithelium) also stained positive for c-kit (Figure 7B, arrow). Near the blood vessels c-kit positive cells were also detected (Figure 7C, arrow).

The adult pancreas during pregnancy (i.e. during growth of the beta-cell mass)

In the 14-days pregnant rat pancreas we found relatively high numbers of nestin positive cells in the islets of Langerhans (Figure 5D). The number of nestin positive cells in the 14-days pregnant rat pancreas was 10.8 ± 1.3 % which was significantly increased when compared to the control adult pancreas (3.9 ± 0.8 %) (Figure 2D, Mann Whitney U test, P < 0.05). Occasionally nestin positive cells were found near the ductules (Figure 5E, arrow). Since we found nestin positive cells near ductules in the adult pregnant rat pancreas, we questioned whether nestin positive cells are ductal epithelial cells. To determine whether these nestin positive cells are ductal epithelial cells, we performed double immunofluorescence staining for nestin and CK20 (Figure 5G). As shown in Figure 5G no nestin and CK20 double positive cells were found illustrating that the nestin positive cells are not ductal epithelial cells. No nestin positive cells were found near blood vessels (Figure 5F, arrow).
Figure 5: The adult pancreas under basal conditions (A, B, and C) and in the growing adult pancreas at 14-days pregnancy (D, E, and F), after immunostaining for nestin in the pancreatic islets, ductules, and blood vessels. In the adult pancreas nestin positive cells were found in the islets. These cells were not grouped, but scattered in a diffuse manner throughout the islets of Langerhans (A), were not found near ductules (B), and were present near blood vessels (C, arrow). In the 14-days pregnant pancreas nestin positive cells were found in the islets (D), were spotted near the ductules (E, arrow) and near the blood vessels (F, arrow). In the adult pregnant rat pancreas the ductule columnar epithelial cells do not stain double positive for nestin and CK20 (G), nestin positive cells are found around the ductule (G, white arrow). Original magnification 200X.
We also studied c-met expression (Figure 6). In contrast to the pancreas under normal conditions we found quite some c-met positive cells in the pancreas under metabolic challenge. C-met positive cells were found in the islets of Langerhans (Figure 6D). Moreover, c-met expression was detected near ductules (Figure 6E, arrow) and near blood vessels (Figure 6F, arrow). In some animals columnar epithelial cells of the ductules stained positive for c-met (Figure 6G, arrow), however in general the ductules were c-met negative (Figure 6G).

C-kit positive cells were also detected in the islets of Langerhans of rats on day 14 of pregnancy (Figure 7D). The columnar epithelial cells of the ductules stained positive for c-kit (Figure 7E, arrow). The amount of c-kit positive cells near the ductules (Figure 7E) and blood vessels (Figure 7F) was increased when compared with the adult female rat pancreas.

The presence of nestin, c-met, and c-kit positive cells was also studied in the 17-days pregnant rat pancreas. In the 17-days pregnant pancreas, nestin positive cells were found in the islets (Figure 8A). The number of nestin positive cells in the islets, however, was decreased as compared with day 14. Nestin positive cells were up regulated around the ductules (Figure 8B, arrow), and near the blood vessels (Figure 8C, arrow); this was not different from day 14 of pregnancy.

In the 17-days pregnant pancreas c-met positive cells were still found in the islets (Figure 8D), were increased in number and surrounded the ductules (Figure 8E, arrow). Also, c-met was found in clusters near blood vessels (Figure 8F, arrow).

In the 17-days pregnant pancreas c-kit positive cells were also found in the islets (Figure 8G), increased near the ductules and the duct cells stained positive for c-kit (Figure 8H, arrow), and slightly increased near blood vessels (Figure 8I, arrow).
Figure 6: The adult pancreas under basal conditions (A, B, and C) and in the growing adult pancreas 14-days pregnancy (D, E, and F) after immunostaining for c-met for the specific compartments i.e., islets, ductules, and blood vessels. In the adult pancreas c-met was not expressed in the islets as small clusters of cells in the centre of the islets (A) or near the ductules (B) or blood vessels (C) in specific small clusters. In the 14-days pregnant pancreas c-met positive cells was found in the islets (D), in small clusters near ductules (E, arrow) and blood vessels (F, arrow). In general in the adult pregnant pancreas the ductule columnar epithelial cells do not stain double positive for c-met and CK20 (G), however some ductule columnar epithelial cells stained double positive for c-met and CK20 (G, white arrow). Original magnification 200X
Figure 7: The adult pancreas under basal conditions (panel A, B, and C) and in the growing adult pancreas at 14-days pregnancy (panel D, E, and F) after immunostaining for c-kit for the specific compartments i.e., islets, ductules, and blood vessels. C-kit was found more abundantly in the adult pancreas when compared to nestin and c-met. It was, however, less pronounced than in earlier stages of development. In the adult pancreas c-kit was expressed in the islets (A). The duct cells (columnar epithelium) also stained positive for c-kit (B, arrow). C-kit positive cells were found near the blood vessels (C, arrow). In the 14-days pregnant pancreas c-kit positive cells was found in the islets (D; insert shows a control pancreas which is negative for c-kit), were up regulated near the ductules and the duct cells (columnar epithelium) stained positive for c-kit (E, arrow), and did not increase near blood vessels (F). Original magnification 200X
Figure 8: Photomicrograph of the 17-days pregnant rat pancreas after immunostaining for nestin, c-met, and c-kit for the specific compartments i.e., islets, ductules, and blood vessels. In the 17-days pregnant pancreas nestin positive cells found in the islets (A), the ductules (B, arrow), and near the blood vessels (C, arrow). C-met positive cells were found in the islets (D), increased and surrounded the ductules (E, arrow), and were also present in clusters near blood vessels (F, arrow). C-kit positive cells were also found in the islets (G), increased near the ductules and the duct cells stained positive for c-kit (H, arrow), and slightly increased near blood vessels (I, arrow). Original magnification 200X

DISCUSSION

In the present study we evaluated the presence and localisation of nestin, c-met and c-kit positive cells in the foetal, neonatal, and adult growing pancreas. We clearly show an association between enhanced numbers of nestin, c-met, and c-kit positive cells and the embryonic, foetal or adult growth and differentiation and growth
of the endocrine pancreas. This corroborates the findings of others [4,8-10,20]. Nestin was shown to be present in the foetal pancreas and was suggested to be associated with pancreatic development [13]. The interaction of c-met with HGF was shown to play an essential role in pancreatic and beta-cell development [11,21]. C-kit is the receptor for stem-cell factor (SCF) and its down regulation results in decreased pdx-1 and insulin expression, suggesting down regulation of \( \beta \)-cell differentiation [22].

The presence of nestin in a pancreas has been suggested to be associated with growth of the pancreas and it has even been suggested that nestin positive cells are beta-cell precursor-cells [8]. This latter suggestion that nestin positive cells might be beta-cell precursor cells was debated by Lardon et al. and Treutelaar et al. [23,24] who showed that nestin positive cells differentiate into endothelial cells \textit{in vivo} and not into beta-cells. Therefore, many have subsequently concluded that nestin positive cells are not precursors for islet-cells [23-26]. Recently, however, we have shown that nestin positive cells from the neonatal rat pancreas express \textit{ngn3}, while Wang et al. [27] have shown that nestin positive cells from the human foetal pancreas also express \textit{ngn3}. Since \textit{ngn3} is a transcription factor specific for beta-cell development, these finding may suggest that the nestin positive cells may be precursors cells for both endothelial cells [23-2] and beta-cells and, probably, even more pancreatic cell types. This is corroborated by our present findings which show that nestin positive cells are located near the blood vessel, the ductal system, and in the islets during development and growth.

Our findings in the foetal pancreas corroborate previous studies in mice [28,29] and in humans [27], where nestin expression was demonstrated in the foetal pancreas. We, however, did not only find nestin expressing cells in the foetal pancreas, but also in the adult pancreas. We found some nestin positive cells in the adult islets and near the blood vessels. This number was elevated in the growing pancreas, \textit{i.e.} during pregnancy. This elevation coincides with the peak proliferation of beta-cells at day 14 of pregnancy [6,19], which suggests that nestin positive cells play a role in growth of the beta-cell mass during pregnancy. The phenotype of the nestin positive cells remains to be determined. We also found some nestin positive cells near the ductules. These cells were not ductule epithelial cells. We expect that these cells have a supporting function for ductal development or growth. This
suggestion is however in conflict with the findings of Kim *et al.* [30]. In their model of beta-cell mass growth, *i.e.* partial pancreatectomy, they show that nestin positive cells are in fact the columnar epithelial cells of the ductules themselves. In another model for beta-cell mass growth, *i.e.* STZ treated rats, no nestin positive cells were observed in the pancreas following STZ treatment [31]. This difference in findings might be explained by the application of different animal models or antibodies.

Our report also suggests a role for c-met and c-kit positive cells in development and growth of the pancreas. C-met is involved in scattering of cells, invasion of cells into tissues, proliferation of cells, transformation, angiogenesis, and branching morphogenesis [32], while more importantly, it has been suggested that pancreatic c-met positive cells may be beta-cell precursors [14]. C-kit has also been shown to be important for pancreatic development [33]. Mice heterozygous for non-functional c-kit receptor show an early onset of diabetes and decreased beta-cell mass [33]. We did not focus on whether the c-met and c-kit positive cells might be precursor cells, but we show that in the foetal and neonatal pancreas high numbers of c-met positive cells can be found in various compartments, *i.e.* in the islets, around blood vessels en around the ductules in a pattern that suggests that c-met plays a role in proliferation, transformation, angiogenesis, and morphogenesis of the pancreas. Our data confirm findings in the rat and human foetal pancreas [34,35]. This observation also confirms the findings of Dai *et al.* [21] who recently demonstrated the essential role of c-met in islet-cell development by demonstrating a reduced islet size and decreased insulin content when c-met is absent during development.

In contrast to our study, Yaspal *et al.* [9] showed c-kit positive cells in the foetal rat pancreas, while Rachdi *et al.* [36] showed c-kit positive cells in the foetal mice pancreas. The difference between our study and the two other studies may be explained by the foetal age: we studied foetuses from day 14 pregnant rats, Yashpal *et al.* studied foetuses from pregnant rats on day 18 of pregnancy. The mice in the study of Rachdi *et al.* were studied on days 14.5 and 18.5, which is also later in foetal life. This may suggest that c-kit positive cells appear later in pancreatic development in the rat foetal pancreas. However, in the human foetal pancreas c-kit positive cells appear very early in foetal life, *i.e.* from week 8 of pregnancy [22,37].
As far as we know, we are the first to demonstrate that c-met and c-kit expression is associated with adult pancreatic growth during pregnancy. During pregnancy-induced growth, we found increased numbers of c-met and c-kit positive cells in the islets. However, these cells were also found in other compartments such as concentrated near ductuli and vasculature. The timing of the cells, i.e. maximal numbers at day 14 of pregnancy, during peak beta-cells growth, suggest that similar to nestin positive cells, also these cells have an important function in growth of the beta-cell mass during pregnancy. The location of these cells suggests that these cells may have multiple functions, i.e. in islet growth, vascular growth and neogenesis.

The c-met positive and c-kit positive cells near and in ductules need some further consideration with regard to neogenesis. Various studies have shown that ductule cells participate in the regeneration of the pancreas after damage [38] and in vitro studies have shown that cells from the ductule enriched part of the pancreas are responsible for the formation of insulin expressing cells [38]. Moreover, Suzuki et al. [14] demonstrated that c-met positive cells located around the ducts are able to form clonal colonies and may differentiate into multiple pancreatic lineage cells in vitro. It is tempting to speculate that the c-met positive cells around the ductules and the c-met positive ductule epithelial cells are similar to the c-met positive cells isolated by Suzuki et al. This may imply that the ‘budding’ islet-precursors are c-met positive. C-kit positive cells could have a similar function, since also in other models of pancreatic growth, i.e. STZ induced beta-cell damage or duct ligation, increased numbers of c-kit positive cells were found in the islets and in the ductal cells [31,39]. The role of the c-kit positive cells needs to be established.

After studying nestin, c-met, and c-kit positive cells at different time points of pancreatic development and during pancreatic growth, as well as in different compartments of the pancreas we suggest that these cells play an essential role in different processes during development and adult growth. Nestin, c-met, and c-kit might serve as a measure to assess whether growth-processes are occurring in the pancreas. The localisation in the islets and near ductules and its expression at different stages of differentiation of exocrine and endocrine cells may also suggest that these cells either directly or indirectly contribute to pancreas neogenesis. The localisation of nestin, c-met, and c-kit positive cells near blood vessels at different stages of the pancreas suggest that these cells are also involved in vessels formation.
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More cell-based studies and specific knock-out studies are required to predict the exact role of the nestin, c-met, and c-kit cells in growth of the pancreas.

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