Short-reactivation of Neurogenin-3 and mesenchymal microenvironment is require for β-cells differentiation during fetal pancreas development and islet regeneration

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Abstract

**Purpose:** To investigate influencing factors of β-cells differentiation and microenvironment in embryonic development and regeneration, in order to conduct therapeutic efforts to broaden β-cells mass in diabetes. **Materials and Methods:** The expression of Ngn3 (Neurogenin-3) and microenvironment of β-cells differentiation during embryonic pancreas development at 4–12 weeks of gestation and regeneration after pancreatic islet injure observed by immunohistochemical staining. **Results:** The results showed that the expression of Ngn3 not only in pancreas development but also in β-cells regeneration in rat diabetic model (DM) by streptozotocin (STZ) treatment. Pancreatic mesenchymal tissue always accompanied by islet cells differentiation and there is a short expression of Ngn3 occurrence before all islet cells differentiated from pancreatic epithelium. The expression of Ngn3 including ectopic expression also appearance in β-cells injured rat pancreas. In addition, there are some Nestin-positive cells where located in pancreatic duct, islets and mesenchyme were detected in DM. Double immunostaining witness Brdu/Ngn3-positive cells was only in pancreatic mesenchyme after β-cells injure. **Conclusions:** Our data demonstrated the expression of transcription factor Ngn3 and pancreatic mesenchymal microenvironment are important and necessary to promote pancreatic progenitors differentiated to islet cells regardless of pancreatic development or islets regeneration.

**Keywords:** Neurogenin-3, pancreatic development, mesenchymal microenvironment, islet regeneration.

**Introduction**

It is fully affirmed that transcription factor Neurogenin-3 (Ngn3), one basic helix-loop-helix has a vital role in pancreatic endocrine cells differentiation. Previous study confirmed all endocrine islet cells derived from Ngn3+ precursors. However, regeneration of β-cells in pancreas stills poorly understood, yet stimulation of adult β-cells neogenesis could lead to therapies for type 1 and type 2 diabetes [1]. Thus, investigate the factors and mechanisms including β-cells differentiation in embryonic development and regeneration of β-cells in pancreatic islet will guide therapeutic endeavor to augment β-cells mass in patients with diabetes. However, the mechanism and influence related the differentiation of islet β-cells including microenvironment of pancreatic mesenchyme and the expression of Ngn3 is still not very clear. The basic helix-loop-helix transcription factor Ngn3 is essential and sufficient to induce endocrine islet cell differentiation for the differentiation of islet cells in pancreatic development [2–5]. Studies of ahead in mouse embryos development have reported transient expression of Ngn3 in scattered cells within the developing pancreatic epithelial cells during mid-gestation. Ngn3 controls islet cells fate specification in multipotent pancreatic progenitor cells and Ngn3 transcripts are obviously more widespread in the pancreatic epithelium than Ngn3 protein, indicating that post-transcriptional regulation may be play a critical role during endocrine differentiation [6–8]. However, all of these evidences are from the embryonic pancreas development, pancreatic β-cells regeneration so far remains controversy and understood it little about [9–12]. Especially, the observation of mesenchymal microenvironment for β-cells differentiation in pancreas development and pancreatic islet regeneration was involving less. Thus, we investigated the expression and distribution of Ngn3 during the fetal development of pancreas in human being and the regeneration of pancreas islet after β-cells injure in newborn rats. The purpose is to investigate the role about the expression of Ngn3 and mesenchymal microenvironment for islet cells differentiation. In addition, Nestin-positive cells, one marker was considered of pancreatic stem cell [13] also detected in pancreatic duct and its periphery islets after pancreas regeneration. The location and features of cells involve differentiation and proliferation observed in the pancreatic mesenchymal tissue. From the observation of pancreas development and islet regeneration, our studies demonstrate Ngn3, as an important transcripts regulator is indispensable and play a critical role to controls the expression of multiple genes that influence both endocrine differentiation and function. Meanwhile, pancreatic mesenchyme provides an essential microenvironment whether during endocrine cells differentiation in fetal pancreas development or the regeneration of pancreatic islets after β-cells injured.

**Materials and Methods**

**Observation of fetal pancreatic development**

**Embryo sample collection**

Embryo samples collection (4–12 weeks gestation, based on menolipsis and the size of the fetus) obtained from donors at the department of Dali Obstetrics Hospital,
were according to the manufacturers' instructions. Blood glucose measurements and normal value were measured in the USA). Blood glucose levels were measured from each pancreas. The reaction sections observed from each pancreas. The reaction (Model CX61, Olympus Optical Co., Ltd.). At least 10 sections cell nuclei counted per tissue block of pancreas. The rate of proliferation is the percentage of pancreatic cell nuclei that are also BrdU positive. Double staining was according to the details of Histostain™-DS kits (Beijing Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China). During the whole procedure, PBS applied as a negative control in place of a primary antibody.

To detect pancreatic progenitor proliferation and differentiation, number of BrdU+ cells in pancreatic tissue sections counted and double staining used for BrdU with NBT (Nitro blue tetrazolium chloride), DAB with Ngn3 as substrate. Imaging performed using Olympus CX-61 microscope.

Western blot analysis

To assist evaluate the expression of Ngn3, Western blot analysis taking from normal and DM group. Samples of pancreas performed with the supernatant from total protein of contents as described elsewhere [15]. Samples boilded for 10 minutes before loaded onto 10% Sodium Dodecyl Sulfate (SDS)-polyacrylamide electrophoresis gel. After electrophoresis, proteins transferred onto nitrocellulose membrane (Millipore Corp.) in 25-mmol Tris, 192-mmol Glycine, and 15% Methanol. The membranes blocked in 5% dried skim milk in PBS and then probed with antibodies.

The primary antibody used was a polyclonal antibody against Ngn3, and horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:2000) was used as a secondary antibody (all antibodies from Santa Cruz).

Immunoreactive proteins were observed by using the ECL Western blotting detection reagents (Amersham Corp.).

Statistical analysis

Two-tailed Student’s t-tests were performed, with p<0.05 or p<0.01 considered to be significant. Values give as mean ± SD or ± SEM.

Animals and Streptozotocin (STZ) treatment

All rats’ experiments were performed in accordance with our institutional Ethical Committee for Animal Experiments and national guidelines and regulations. Male Sprague Dawley rats (Dali, China), aged 3–4 weeks, were used for all experiments. A single intraperitoneal injection of STZ (Sigma, St. Louis, MO, USA) was intraperitoneally injected.

The basic experimental regimen consisted of a single 300-μL intraperitoneal injection of STZ within 15 minutes of dissolution in freshly prepared 20-mmol cold citrate buffer (pH 4.5); Non-STZ treated rats received 300-μL citrate buffer alone. Twenty-four hours after STZ treatment is DM 1d, 48 hours is DM 2d, and 72 hours is DM 3d. At 24 hours intervals, to confirm the diabetic status of the animals, rat showing blood glucose >16.5 mmol/dL consistently considered diabetic.

Blood glucose of all experimental rats was measured at 1, 2, 3, 4 and 5 days after STZ. Six hours before rats sacrifice, BrdU (Bromo-deoxyuridine, BrdU) 100 mg/kg (Sigma, St. Louis, MO, USA) was intraperitoneally injected.

Cells proliferation analysis

For cells differentiation and proliferation measurements, Nestin, BrdU incorporation assessed using a BrdU-specific mouse monoclonal antisera and rabbit antiserum (BrdU 1:200, Nestin 1:500) (Sigma, St. Louis, MO).

Immunohistochemical staining method is same as above descriptive. At least 10 sections cell nuclei counted per tissue block of pancreas. The rate of proliferation is the percentage of pancreatic cell nuclei that are also BrdU positive. Double staining was according to the details of Histostain™-DS kits (Beijing Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China). During the whole procedure, PBS applied as a negative control in place of a primary antibody.

To detect pancreatic progenitor proliferation and differentiation, number of BrdU+ cells in pancreatic tissue sections counted and double staining used for BrdU with NBT (Nitro blue tetrazolium chloride), DAB with Ngn3 as substrate. Imaging performed using Olympus CX-61 microscope.

Microscopy and imaging of the immunohistochemical stained sections done with an Olympus microscope (Model CX61, Olympus Optical Co., Ltd.). At least 10 sections observed from each pancreas. The reaction result for the SABC method was yellow-brown in cytoplasm of cell in pancreatic tissues of human embryo. The tissues of the control were all negative.

Animals and Streptozotocin (STZ) treatment

All rats’ experiments were performed in accordance with our institutional Ethical Committee for Animal Experiments and national guidelines and regulations. Male Sprague Dawley rats (Dali, China), aged 3–4 weeks, were used for all experiments. A single intraperitoneal injection of STZ (Sigma, St. Louis, MO, USA), at a dose of 240 mg/kg, was used to induce β-cell damage and overt hyperglycemia. Blood glucose levels were measured daily in blood collected from the tail vein of non-fasted animals during the time (between 8 to 10 a.m.), using a glucometer (One Touch Ultra, Life Scan, Milpitas, CA, USA). Blood glucose measurements and normal value were according to the manufacturers’ instructions.
Results

Histology and immunochemical staining for pancreas development and Streptozotocin (STZ) treated pancreas

During human pancreatic development, the pancreatic bud, namely the pancreatic primordia, emerged in primitive gut and began to grow and branch at 5th week of gestation. The growing epithelia cells with polarity aggregated and surrounded by bushy mesenchymal cells (Figure 1).

From genesis of pancreatic buds to the branches of pancreatic original catheter, the microenvironment formed by pancreatic mesenchyme always accompanied with pancreatic endothelium differentiation from beginning to end. There is a lot of micrangium located in mesenchyme wrapped around the pancreatic primordia (Figure 2a). From the 6th week of gestation, Ngn3 expression was detected only on pancreatic epithelia cells, which it continued to the 8th week (Figure 2b). After this, Insulin-positive cells, Glucagons and Somatostatin-positive cells (data not show) emerge until to the 9th week of gestation. These endocrine positive cells have show the character of differentiation but still locate in primary ductal epithelium and no migration occurs (Figure 2c). Furthermore, we also found, in the whole differentiation period of endocrine cells from pancreatic epithelial cells, IGF-1 express all the time in pancreatic mesenchyme and lasting to pancreatic islet formation at gestation of 14 weeks (Figure 2d).

Figure 2 – (a–d) Expression of CD34, Ngn3, Insulin, IGF-1 detected by IHC staining in developmental pancreas of embryo: Primitive pancreatic ductal epithelium surrounded by dense mesenchymal cells, which express CD34 in pancreas development (a); Ngn3 express in primary pancreatic ductal epithelium at six weeks of gestation (b); Insulin-positive cells had differentiated and migrated from ductal epithelium at nine weeks of gestation (c); IGF-1 protein expression showing in peripheral mesenchyme of ductal epithelium at eight weeks of gestation and last to islets formed (d). IHC, ×400 (a–d).
With STZ-treated pancreas (DM), HE staining showed that pancreatic islets atrophied and cytoplasm of β-cells reduced compared with normal morphology. The positive reaction of insulin in normal islet cells had a visible difference when compared with those in the pancreatic islet of DM (data not show).

**Blood glucose levels of STZ-treated mice (DM)**

The DM with STZ-treated groups showed frank hyperglycemia at 24 hours (blood glucose level of 21.3±1.6 mmol/L). The hyperglycemia by STZ-treated rats, continue to observe for five days (Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Blood glucose [mmol/L]</th>
</tr>
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<tbody>
<tr>
<td>DM 1d</td>
<td>10</td>
<td>21.3±1.6*</td>
</tr>
<tr>
<td>DM 2d</td>
<td>10</td>
<td>28.6±2.4*</td>
</tr>
<tr>
<td>DM 3d</td>
<td>10</td>
<td>25.2±2.1*</td>
</tr>
<tr>
<td>DM 4d</td>
<td>10</td>
<td>26.8±1.7*</td>
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*P<0.05, compared with normal control group.

Average blood glucose level of DM was significantly different from that of the normal group (4.9±0.8 mmol/L) (p<0.05).

**Samples for immunohistochemistry of diabetic model (DM)**

To determine whether Ngn3 has ever taken part in the islet regeneration after islet injury, we detected cell proliferation and differentiation in pancreatic tissue after STZ-treated rats (DM). The data showed there were a few BrdU-positive cells locating in the pancreatic islet, duct, mesenchyme and acini. Cell proliferation mainly located in the periphery mesenchyme of pancreatic duct and between acini (Figure 3, a and b).

While a strong activation of Ngn3 expression, a well-established marker for embryonic islet progenitors, observed specifically in pancreatic tissue taken from STZ-treated rat. Ngn3-positive cells at 24 hours were detected dispersedly located between alveolus, especially in the pancreatic mesenchyme where around pancreatic duct, which Ngn3-positive cells gathered in small groups (Figure 4a). At DM 2d, all Ngn3-positive cells aggregated and formed cell group in variant size (Figure 4b). Interestingly, we also found when pancreatic islets regeneration after β-cells injury, there are some Nestin-positive cells simultaneously appearing, which located in pancreatic ductal epithelium, periphery islets near to duct and mesenchyme between gland alveolus (Figure 4c). In addition, BrdU-positive cell reaction after β-cells injure was mainly present in the pancreatic mesenchyme. Two kinds of BrdU-positive cells detected at the same time in pancreatic mesenchymal tissue, which one is small, oval-shaped and another is large, round. Double immunostaining displayed that the large and round cells not only BrdU-positive, but also Ngn3-positive (Figure 4d). This phenomenon of Ngn3-positive reaction continues to performance until at DM 4d. Maximal levels of Ngn3 transcript reached at DM 2d and subsequently decreased up to the DM 5d (Figure 4e).

**Discussion**

Diabetes has become one of the major threats for human health in the twenty-first century [16]. The diabetes prevalence of varies of world significantly from developed countries to developing countries [17]. Until now, β-cells replacement still is an useful treatment for type 1 diabetes, but it’s apply was restricted because of short of donor tissue. The growing needs ask us to look for alternative method or tissue sources to cure diabetes mellitus whether type 1 or type 2. These years, human embryonic stem cells (hESCs) got more attention because of their prospect as a renewable resource of tissue regeneration for β-cells differentiation [18].

Yet, ethical and moral issues restricting the use of hESCs for cell replacement therapy involve in clinical application. On the other hand, stem cells resident in pancreatic tissue have certified that possess self-renewal and multi-differentiated proficiency, perhaps it is a great value for diabetes cellular therapies and is filled successful progress.
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Figure 4 – (a–e) Ngn3 express in DM 1d, arrowhead show gathered Ngn3-positive cells. IHC, ×400 (a). Ngn3 express in DM 2d, Ngn3-positive cells gathered to form groups. IHC, ×400 (b). Nestin express in DM 2d, Nestin-positive cells located in rudimental islets, mesenchyme between acini (blue arrowhead) and ductal lining (red arrowhead). IHC, ×400 (c). Ngn3 express and BrdU-positive cells in DM 2d, arrows showed Ngn3+/BrdU+ cells in mesenchyme between acini, double immuno-staining, ×400 (d). Ngn3 protein detected in pancreatic tissue of normal (N) and DM 1–5 d was performed by using Western blot analysis. Tissue lysates separated on a 10% SDS-polyacrylamide gel and electroblotted onto Trans-Blot membranes. The blot was probed with anti-Ngn3 antibody and 27 kD indicates a detected signal (e).

Previous studies have known about the cause in both type 1 and type 2 diabetes were related to the failure of β-cell mass. However, β-cells regeneration of pancreatic islets nowadays remains poorly understood. Therefore, found an effective method to stimulate adult β-cells or newborn cells would favorite to deal with type 1 and type 2 of diabetes. In clinical trials, although donor islets transplantation partly has achieved success, an incomparable approach still is the stimulation of endogenous β-cells regeneration in diabetic patients [19]. Although there are a lot of violent controversy relating the origin about pancreatic stem cells, the expression of Ngn3 as a transcript factor in islet differentiation and regeneration reach a consensus. Thus, study the influencing factors and regulation mechanisms involved in β-cells differentiation and regeneration consist of microenvironment of stem cell niche [20, 21] would strengthen β-cells mass formed in patients that reached the purpose to cure diabetes. Current evidence in this field was suggested when β-cells injure, both β-cells reproducing and neogenesis from stem cells in the adult pancreas are actually participate in the process [10, 22–24].

During pancreatic development, all of islet endocrine cells in pancreas were arising from precursors that express Ngn3. These endocrine cells in pancreatic islets differentiates respectively into four major endocrine cell types, including α, β, δ, and PP-cells, and which respectively express Insulin, Glucagons, Somatostatin, and Pancreatic Polypeptide. Cell’s fate of islet is determined by unavoidably regulated by transcription factors, of which Ngn3 is one of earliest and vital factor that specifically regulates β-cells differentiation and development until pancreatic
islet formation. Thus, the important role of Ngn3 gives the direction to find therapeutic approaches to raise Ngn3 expression in diabetes as an effective mean to increase β-cells amount and functions [25–28]. However, β-cells regeneration in adult pancreas, whether has Ngn3 expression or not is required now still existing controversy [9, 29, 30]. Recently reports display that adult β-cells regeneration arise from epithelium of pancreatic duct undergo it of fetal β-cells development. The directed and clear evidence of Ngn3 express to be a key transcript play a vital role recently has demonstrated [10], which it is also an essential protein for all fetal islet cell development [27] and in the adult pancreas [31]. In our study, we found the occurrence of β-cells differentiation from pancreatic ductal epithelium was later to the Ngn3 expression in pancreatic primary ductal epithelium. Ngn3 protein is detected in the pancreatic primary ductal epithelium at embryonic 6th week and gradually declined until to 8th week in human fetal development, which it not similar to the development of the pancreas in mouse showing the gap and occur in two distinct temporal waves of Ngn3 expression in development [7, 32].

In human pancreas development, we found that islet cells differentiation of pancreatic buds cannot do without microenvironment of mesenchyme. The emergence of pancreatic bud, primitive duct branch and epithelium differentiation are complete all the time at the microenvironment of pancreatic mesenchyme. In addition, immunostaining results demonstrate Insulin-, Glucagons- and Somatostatin-produce cells occur on ductal epithelium at 9th weeks of gestation, which take place later to Ngn3 expression, and has differentiated islet cells still located in primitive pancreatic ductal epithelium. These data further prove the evidence that Ngn3 expression is required for the differentiation of endocrine cells and indicate occurrence of islet cells differentiation is precede to migration. Ngn3 protein are undetectable in Insulin- and Glucagons-producing cells, suggesting that Ngn3 protein is vanish before the ultimate differentiation of the hormone-producing cells [8, 13, 33]. This brief activation of Ngn3 may be result from its ability to negative regulate its own expression spontaneously [34]. Therefore, the results indicated that Ngn3 expression is the premise of endocrine cells differentiation. We support the recently study results that the requirement of pancreas development in embryonic microenvironment can produce indispensable growth and differentiation factors [10]. When endocrine cells begin differentiating from pancreatic duct epithelial cells, IGF-1 factor constantly shows high reactivity in peripheral pancreatic mesenchymal cells until pancreatic islets had formed at 14 weeks of gestation [25, 35]. It indicated that paracrine of environmental mesenchymal cells where surrounded the pancreatic ductal epithelium plays an essential role, which may be as a stem cell niche to regulating the differentiation of endocrine cells [20, 21, 36].

On the other hand, our study demonstrates that the offspring rat pancreas have a short reactivation of endogenous Ngn3 expression in islets regeneration after β-cells injured by STZ. This ectopic expression of Ngn3 perhaps as an endocrine transcriptional program in adult and can promote pancreatic progenitors has gone in for endogenous islet regeneration. Meanwhile, this ectopic expression of Ngn3 may thus have therapeutic potential looking for alternative β-cells source for transplantation in the development [9]. The detection of Brdu/Ngn3-positive cells indicate simultaneously that an ectopic reactivation of endogenous Ngn3 expression happen perhaps promotes definitive islet cell progenitors differentiation and proliferation after β-cells mass injury by STZ treatment. These differentiated microenvironments no matter pancreas development in embryo or islets regeneration in adult are similar but also need Ngn3 expression and pancreatic mesenchymal microenvironment. As far as Nestin, which is generally consider as pancreatic stem cell marker [13], express in lining cells of pancreatic duct, islet cells and mesenchymal cells between gland alveolus. Only one explains is these adult stem cells locate in this position in pancreas, which can also express Nestin. Meanwhile, a few cells showed Brdu and Ngn3 positive reaction simultaneously in pancreatic mesenchyme with double staining detected suggesting pancreatic stem cells are located in pancreatic duct, pancreatic islet and even mesenchyme among exocrine alveolus. The Ngn3/Brdu+ cells observed maybe originate from pancreatic stem cells among the lining of ducts or elsewhere and must migrate to the islet to become hormone-positive cells in newborn rats. These Ngn3 positive cells only appeared in pancreatic mesenchyme, which suggest pancreatic mesenchyme maybe as an essential microenvironment of endocrine cells differentiation. Furthermore, the microenvironment of pancreatic mesenchyme perhaps is the niche of pancreatic stem cells differentiation and is necessary to the endocrine cells differentiation. They did not adequately exclude the substitute for possibility that endocrine cells dedifferentiated to Ngn3+ cells [10]. Not all Ngn3+ cells are pancreatic progenitor, but the ectopic expression of Ngn3 perhaps is the ensuring for pancreatic stem cells differentiation to endogenous progenitor. Those cells that showed Brdu+/Ngn3+ characteristic maybe are genuine endogenous progenitor and real could differentiate to endogenous β-cells. Further studies include Brdu+/Ngn3+ cells isolation culture and transplantation to promote endogenous β-cells regeneration with a microenvironment, which is good for islet cells regeneration, will likely be required to dispose of the questions in future for diabetes mellitus cellular therapy.

Conclusions

Our study demonstrated that pancreatic duct, islet and mesenchyme have the capability to act as stem cells. Ngn3 activation has a vital role for ductal epithelium differentiated to endocrine cells, including pancreatic development and islets regeneration after β-cells injure. Appearance of Brdu+/Ngn3+ cells in the pancreatic mesenchyme, combined with the role of stroma in pancreatic ductal epithelium differentiation, indicate mesenchymal microenvironment is require for β-cells differentiation during fetal pancreas development and islet regeneration.

Acknowledgments

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References


