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Pancreas development and the role of adult stem cells in regeneration

Rozwój trzustki i rola komórek macierzystych dojrzałego narządu w jego regeneracji

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S u m m a r y

Pancreatic morphogenesis is precisely regulated and coordinated by inductive signalling pathways; Hedgehog, Notch, Wnt as well as numerous transcription factors. Some of them e.g. Pdx1, p48 and Mist1 are responsible for progenitor cells differentiation into exocrine phenotype, others such as Hex, Ngn3 and Pax4 promote the formation of endocrine cells. Adult organ contains relatively small amount of stem cells which seem to play a significant role in regeneration following the pancreatectomy or pathological injury. These undifferentiated cells are present in the structure of intercalated ducts, exocrine acini and endocrine islets of Langerhans, their niches are generally located near a basement membranes and blood vessels. Pluripotential pancreatic Oct4- and Sox2-positive cells show the expression of the same surface antigens as hemopoietic stem cells and they are able to differentiate into both mesodermal and endodermal cells. A populations of multipotential nestin-positive cells probably derived from excretory ducts are also able to undergo formation of both endocrine and exocrine pancreatic components.

Key words: adult stem cells, pancreas, regeneration, organogenesis

S t r e s z c z e n i e

Morfogeneza trzustki jest procesem precyzyjnie regulowanym i koordynowanym zarówno przez indukcyjne szlaki sygnałowe (Hedgehog, Notch, Wnt), jak i przez liczne czynniki transkrypcyjne. Niektóre spośród nich, jak Pdx1, p48 i Mist1 są odpowiedzialne za różnicowanie trzustkowych komórek progenitorowych do fenotypu egzokrynowego, inne natomiast, takie jak Hex, Ngn3 i Pax4 promują wytwarzanie komórek endokrynowych. Dojrzała trzustka dysponuje względnie niewielką liczbą komórek macierzystych, które zdają się jednak odgrywać istotną rolę w regeneracji narządu w następstwie pankreatektomii lub jego patologicznych uszkodzeń. Owe niezróżnicowane komórki są obecne w strukturze wstawek, pęcherzyków wydzielniczych oraz wysp Langerhansa, ich nisze są generalnie zlokalizowane blisko błon podstawnych i naczyń krwionośnych. Pluripotencjalne trzustkowe komórki Oct-4 i Sox2-pozytywne charakteryzują się ekspresją tych samych antygenów powierzchniowych co hemopoetyczne komórki macierzyste i są zdolne do różnicowania do komórek mezodermalnych i endodermalnych. Populacja nestyno-pozytywnych komórek multipotencjalnych wywodzących się prawdopodobnie z przewodów wyprowadzających trzustki bierze również udział w formowaniu zarówno wewnątrz- jak i zewnątrzwydzielniczej części narządu.

Słowa kluczowe: komórki macierzyste organizmu dojrzałego, trzustka, regeneracja, organogeneza

INTRODUCTION

The liver and the pancreas are organs that develop from two different groups of endodermal epithelium of the primitive foregut. The liver is formed from the segment of the hepatic recess, and proliferating cells penetrate the mesoderm of the transverse septum. A hepatic bud comprises the head part, which develops into the liver, and the caudal part, responsible for creating the gallbladder and bile ducts. Furthermore, the pancreas develops from two buds, dorsal and ventral, which are bulges of the proximal part of the duodenum. The ventral bud develops into the uncinate process and the inferior part of the pancreatic head, whereas the rest of this organ derives from the dorsal bud endoderm. In humans, the dorsal bud of the pancreas is visible in the fifth week of the development, penetrating into the dorsal mesentery of the stomach. The ventral bud appears slightly later and it is located in the area of the common bile duct. During the turn of the intestines, also of the duodenum, the ventral bud moves

in the dorsal direction to finally merge with the dorsal bud and form the organ. In humans, pancreatic islets appear in the third month of the foetal development, whereas insulin secretion begins about the fifth month. The pancreatic and hepatic buds differentiate due to regulatory signals from the surrounding mesodermal cells.

The formation of embryonic stem cell (ES) culture requires their isolation from a blastocyst before implantation. A growth medium for culturing such types of cells should contain, beside standard foetal calf serum, nourishing cells (fibroblasts) and leukemia inhibitory factor (LIF). Under such conditions, ES cells form colonies composed of both undifferentiated and spontaneously differentiating cells. Self-renewable ES require binding LIF to a binary system of receptors, including a receptor specific for LIF and the gp130 receptor. The receptor activation initiates the JAK/STAT3 pathway, that is, one of pathways promoting cell proliferation. The activation of ERK by gp130 and other membranous receptors suppresses the effect of the LIF-STAT3 pathway. Thus, the maintenance of ES ability to divide results from the balance between the actions of different signalling pathways. The expression of the transcription factor Oct-3/4, a member of the POU family identified in embryonic neoplastic cells, and Nanog that plays a key role in blastocyst formation, is necessary for ES to maintain the ability to differentiate. The knowledge of the final formation of B cells from pluripotential precursors is hindered by the fact that they appear at relatively late stages of organogenesis. This brings up a question what molecular events regulate a sequential activation and inhibition of the adequate number of homeotic genes controlling the formation of endodermal cell line, and then, the generation of precursor exocrine cells, endocrine cells and finally, mature B cells (1).

MOLECULAR MECHANISMS OF PANCREATIC DEVELOPMENT

The suppression of mesodermal signalling pathways, Wnt and FGF4, which is predominant in the early phase of the foregut development, enables the induction of morphogenesis of both the liver and the pancreas, whereas the activation of the Wnt pathway in the hindgut prevents the initiation of this process (2). FGF released from the cardiac mesoderm of the ventral part of the foregut and BMP from mesenchymal cells of the transverse septum in a coordinated way, induce the molecular liver formation programme and at the same time block the pancreatic development programme (3, 4). Interestingly enough, the activation of the MAPK pathway as a reaction to FGF occurs earlier in cells located laterally inside the bud than in medially situated cells (5). At the time of foregut closing, ventrolateral endodermal cells, which move in the caudal direction in relation to the cardiac domain, cease to be affected by FGF and initiate the development of the ventral pancreatic bud (6) he dorsal part of the foregut, signals from the dorsal chord, activin and FGF, inhibit the Shh (Sonic hedgehog) pathway in endodermal cells, which activates the pancreatic development programme. It is worth emphasizing that all of the phenomena previously described occur in vertebrate embryos within a few hours only (7, 8).

Bipotential hepatic stem cells called hepatoblasts show the expression of genes characteristic for hepatocytes. They code serum proteins: albumin (alb1) and transthyretin (ttr). They undergo a differentiation into both hepatocytes and cholangiocytes (8). Tbx3 gene promotes the expansion of the hepatoblast population via $p19^{ARF}$suppression. At early stages, the pancreatic endoderm is characterised by the expression of genes for transcription factors, Pdx1 and Ptf1a (9). They play a key role in the pancreatic development (10, 11). Following the formation of hepatoblasts and pancreatic progenitor cells, they change their shape from cuboidal into columnar, and then they form a pseudostratified structure. This process is similar to the morphogenesis of the nervous epithelium, and in the foregut it is controlled by the transcription factor Hhex (6). The pancreatic epithelium spreads and penetrates into the stroma, where it forms the pancreatic bud, whereas, in the case of the hepatic epithelium, the basement membrane is disrupted and cells proliferate into the immediate surroundings of the stroma. Later morphological changes are controlled by the genes for transcription factors, Prox1, Hnf6/OC-1, Hnf6 and OC-1 regulating the synthesis of E-cadherin, thrombospondin-4, and Spp1, which participate in the process of the cell adhesion and migration (8). Neighbouring progenitor cells of both organs also receive regulatory signals from surrounding endothelial cells (12, 13). Interestingly enough, endothelial cells may also promote liver regeneration after its parenchymal damage, mainly via the HGF signalling pathway (14). Signalling factors produced by endothelial cells have not been identified yet. However, it has been established that sphingosine phosphate (present in blood) that is a substance of endothelial origin, promotes the development of the dorsal pancreatic bud (15). The cells of neural crests migrating into the developing pancreas are transformed into neurons and thus influence the number of B cells (16). The stimulating role of endothelial cells and neural crest cells illustrates the importance of cooperation of the mesenchymal stroma in the development of progenitor hepatic and pancreatic cells. In the liver and in pancreatic buds, the components of the Notch signalling pathway ensure the balance between hepatocytes and cholangiocytes formed from hepatoblasts as well as endo- and exocrine cells derived from pancreatic progenitor cells. The blockade of the Notch system initiates a preferential development of endocrine cells. The main role in this process is played by the Ngn3 transcription factor which is essential for the development of pancreatic islets and which has the features of a classic marker of pancreatic precursor endocrine cells. Ngn3 expression is considerably reduced at the moment of birth, and in the mature organ it is almost

indeterminable. In the nascent endocrine cells, the expression of Notch ligands (delta, serrate, jagged) is considerably increased. These ligands react with the Notch receptor on the neighbouring cells and prevent them from differentiating in a similar direction. Multipotential Cpa-1-positive cells located in distal endings of the branched epithelium give rise to both pancreatic ducts and endocrine cells located along their branches. In mice, this process takes place about the 14th day of the embryonic development. At further stages, cells showing Cpa-1 expression are also differentiated into acinar cells of the exocrine part of the pancreas. This process occurs at the time when mature B cells are formed, which is mediated by the MafaA factor.

Endodermal cells of the foregut show the expression of a wide range of genes responsible for creating transcription factors such as Hnf6, Hnf1β, Foxa2, Hnf4α, Hex, Gata4 and Gata 6. Hnf6 regulates the early phase of the pancreatic differentiation into endocrine cells. The presence of the Hnf1β factor determines the process of specialisation and differentiation of the visceral endoderm, whereas Hnf4α is a factor that participates in primitive and intestinal endodermal cells, in the poorly differentiated pancreatic epithelium and in mature endo- and exocrine pancreatic cells (17). A member of the Forkhead family of transcription factors is Foxa2 (Hnf3β) which shows an early expression in foregut endodermal cells, even before pancreatic buds are formed (18). Mice with a blocked Foxa2 gene die on the 11th day of the development due to several structural defects (19, 20). The Hex factor is responsible for the formation of the ventral pancreatic bud. The expression of the Gata4 factor at the early stages of the organ development seems to be limited to exocrine cells and it is maintained in the postnatal period; Gata 6 is present in developing ductal, precursor, endocrine and mature cells. In the later period, it is only limited to the islets of Langerhans. Despite the presence of a group of regulatory substances, essential for the pancreatic development, the Shh signal path inhibits the endodermal specialisation towards this organ phenotype via blocking Pdx1 factor expression. Shh repression by mesodermal signals, activin BB and ligands from TGFβ family, reveals Pdx-1 expression and determines the differentiation of the primitive duodenal endoderm into the pancreas. The inhibitors of the pancreatic development are FGF and BMP, mesodermals factors involved in the early stages of hepatic morphogenesis.

Potential pancreatic endodermal cells are characterized by the presence of all transcription factors, also occurring in primitive, undifferentiated foregut cells. However, new factors appear, such as Pdx1, Ptf1a and Hlx9b (fig. 1). Pdx1 is a homeodomain showing an early (the 8th day of the embryonic development) expres-

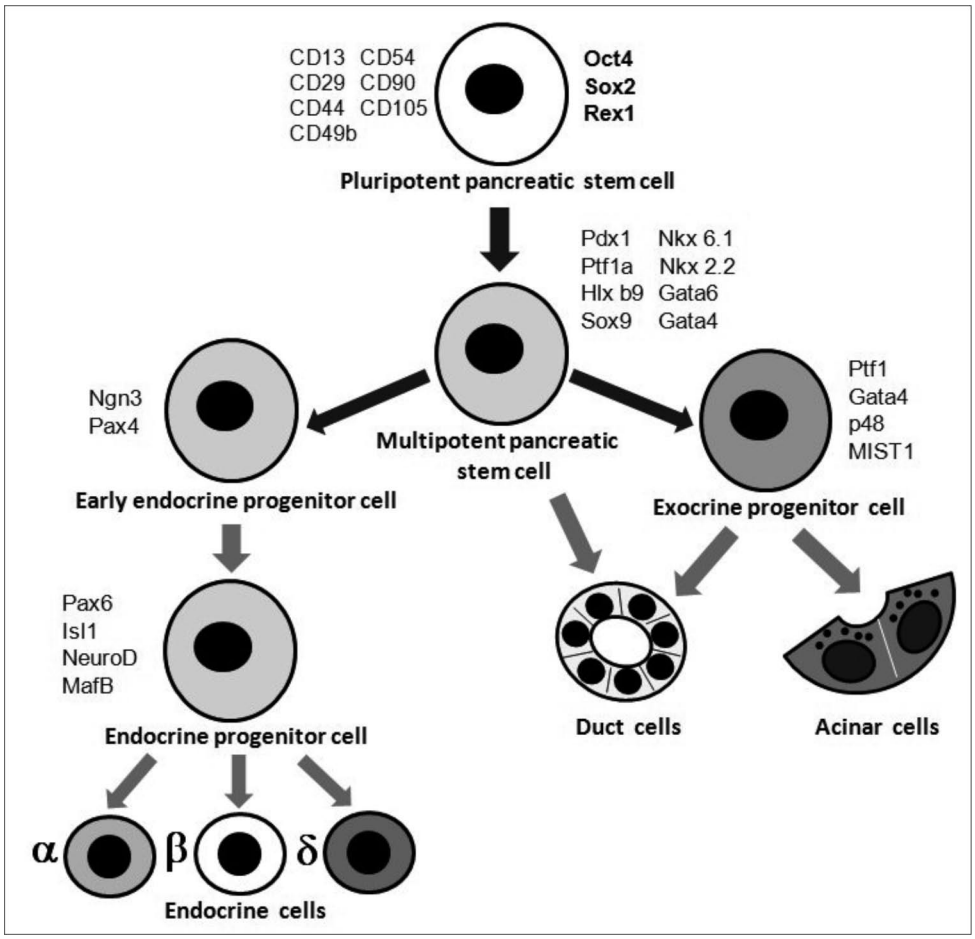

Fig. 1. Transcription factors expressed during formation of pancreatic exocrine and endocrine progenitor cells from pluripotential stem cell.

sion in endodermal cells of the dorsal and ventral parts of the foregut, so in the areas that give rise to pancreatic buds (21). It has also been identified in the common bile duct, the distal part of the stomach, Brunner's glands and duodenal epithelium. Pdx1 expression is continued in epithelial cells; it increases in the course of their differentiation and finally it becomes almost completely limited to B cells and a small subpopulation of D and PP cells. However, it is worth noting that a slight level of Pdx1 expression has also been identified in the nuclei of exocrine acinar pyramidal cells (17). Ptf1 is a heterooligomeric transcription factor composed of 4 subunits (bHLH, p48, p64 and p75), which jointly control the expression of exocrine cell genes, including the gene for elastase 1 (Ela1) (22). It determines the formation of the exocrine part of the pancreas. The crucial role in this process is played by the p48 subunit. Another factor should be also mentioned, Mist1 transcription factor, type bHlH protein, which is expressed in cells differentiating into exocrine cells and which is considered a key regulator of the functional stability of the pancreatic acinar pyramidal cells (23, 24, 25). Hlxb9 takes part in the early phase of ventral pancreatic bud formation, and at later stages it influences the development and differentiation of B cells.

In pancreatic progenitor cells that maintain the presence of all transcription factors from previous stages, next factors also appear, Nkx6.1, Nkx2.2 and Sox9. Nkx2.2 is a factor that functions as both an activator and a repressor of transcription (26). The factor is suggested to inhibit A and B cell formation in the course of the embryonic development, but it stimulates the maturation of B cells and determines their function in the postnatal period. It is also detectable in PP cells. Nkx6.1 factor is an NK type homeodomain with features similar to Nkx2.2. During the development, its expression is gradually limited to B cells (27). Sox 9 is a member of the HMG family of transcription factors. It plays a crucial role in maintaining the pool of pancreatic progenitor cells and it participates in the formation of a signalling net that regulates the early stages of organ development (28).

Maintaining pancreatic progenitor cells in an undifferentiated state is achieved via the Notch signalling pathway stimulated by Fgf10 released by mesenchymal cells. Differentiation towards exo- and endocrine cells is blocked via Ptf1 inhibition and Ngn3 expression. Suppressing the expression of early factors, Hex and Gata4, is characteristic for early, progenitor endocrine cells. In these cells, the synthesis of further factors specific for endocrine cells is initiated; these factors are Ngn3 and Pax4 (8, 29). However, Ngn3-null mice also show the abnormal development of pancreatic acini (30, 31). The transformation of these early cells into the proper endocrine progenitor cells is also determined by a progressing extinction of Hnf6 and Hnf1 expression. On the other hand, a new factors appear, including Pax6, Isl1, NeuroD and MafB. The extinction of Ngn3 expression in progenitor endocrine cells leads directly to their transformation into immature B cells, which, at the final stage of differentiation into fully formed cells, lose MafB expression in favour of a new transcription factor MafA (32, 33).

REGENERATION FROM ADULT PANCREATIC STEM CELLS

Recent studies have demonstrated that the adult pancreatic structure contains a few percent of stem cells that may differentiate in a determined manner, towards the pancreatic phenotype. Data suggest that pancreatic ducts contain precursor cells that can differentiate into B cells; such cells were also identified in their immediate vicinity (34, 35). Up to the present, the group of potential candidates for the role of pancreatic stem cells participating in organ regeneration have included:

1. some of the epithelial cells of excretory ducts,
2. some cells of exocrine acini,
3. nestin-positive progenitor cells of pancreatic islets,
4. cells showing neurogenin-3 (ngn-3) expression,
5. multipotential cells formed in the process of dedifferentiation of finally mature cells (1, 36, 37, 38).

Several scenarios of endocrine cell neogenesis in a mature organ are considered. According to the first of them, the population of epithelial cells located in a small area of excretory ducts (intercalated ducts) may be transformed into endocrine cells, when influenced by specific morphogenetic stimuli. Another option assumes that stem cells are located between the epithelial cells of intercalated ducts and in the area surrounding their basement membranes (1, 39, 40). However, a more reasonable hypothesis suggests that stem cells form a separate population in the epithelium of intercalated ducts and the pancreatic islet structure. It has been observed that the cells have a considerable mobility. These cells may move quickly in the tissue environment, which makes them similar to macrophages (41). They probably migrate inside the islets and ducts, in search of a specific niche, to enable them a contact with adequate paracrine and endocrine signals that are essential for activating processes of their proliferation and differentiation (fig. 2).

Stem cells isolated from human pancreatic ducts show the expression of identical surface antigens as stem cells in bone marrow and umbilical cord blood. The presence of CD13, CD29, CD44, CD49b, CD54, CD90 and CD105 was detected, whereas CD34, CD45 and CD117 were not identified. In vitro, these cells differentiate into both mesodermal (osteocytes, chondrocytes and adipocytes) and endodermal cells (hepatocytes and B cells), so they are pluripotential cells (42, 43, 44). Mesenchymal stem cells of pancreatic islets also show Oct-4, Sox2 and Rex1 expression and they are characterised by a considerable proliferative dynamics determined by a high activity of telomerase (45).

During pancreatic morphogenesis, endocrine cells are formed from the cells of future excretory ducts; intercalated ducts (46). However, it is not commonly

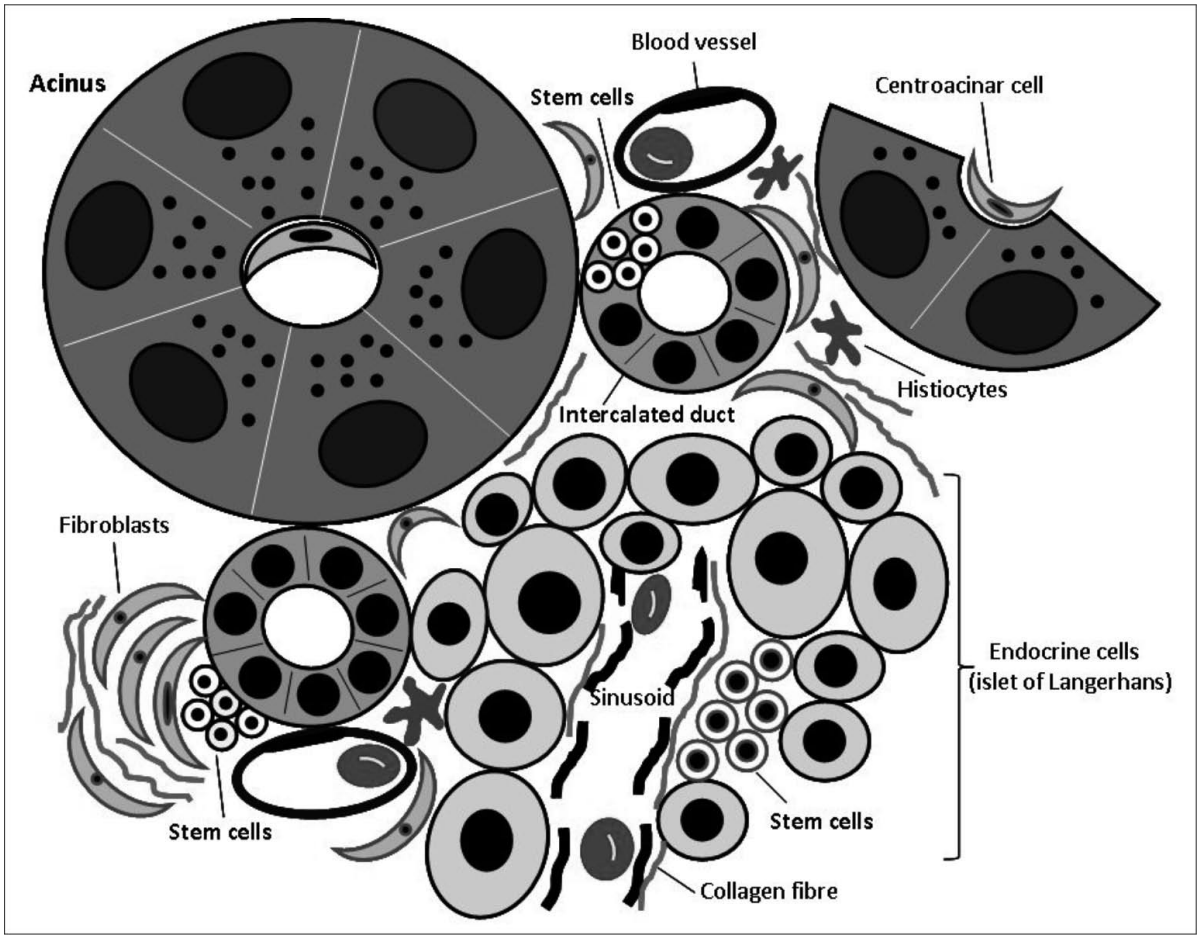

Fig. 2. Localization of adult stem cell niches in the exocrine and endocrine pancreas.

recognized that a number of stem cells may survive in the structure of a mature organ. It has been proven that neogenesis in the excretory ducts takes place in adult animals following the ligature of the Wirsung's duct or after a 90% pancreatectomy (46, 47, 48). A similar phenomenon is observed in the liver where the regeneration based on stem cells occurs after blocking hepatocyte mitosis, e.g. due to special diets or toxic damage. A mature pancreas contains cells showing mRNA expression of classic markers of stem cells, Oct4 and Sox2 (49). These markers play an essential role in maintaining the population of pluripotential stem cells. In mice, Oct4 is present in totipotential, germinal, undifferentiated and neoplastic cells (50). Oct4 and Sox2 jointly regulate the expression of the Nanog gene, a transcription factor critical for retaining a stem cell phenotype. The impairment of Oct4 and Nanog expression leads to the initiation of cellular differentiation (51, 52). Murine stem cells deprived of Oct4 die immediately after the implantation of an embryo (53). The extinction of Sox2 expression results in a similar effect in the early phase of embryogenesis (54). The expression of mRNA Oct4 and Sox2 was confirmed in the mature pancreatic cells in humans, without a clear segregation into the cells of the endo- and exocrine part of this organ. Immunohistochemical staining shows that Oct4 and Sox2 cells are scattered inside or near small excretory ducts. They do not show CK19, insulin and nestin coexpression. The absence of CK19 expression, a marker of epithelial cells, suggests that Oct4/Sox2 cells are precursors. Another considered hypothesis assumes that Oct4 and Sox2 expression in ductal cells occurs after the extinction of CK19 expression. Most Sox2-positive cells show Oct4 coexpression, whereas not all Oct4-positive cells are characterized by a detectable presence of Sox2, probably due to a low level of its expression- Sox2 protein. Moreover, it was observed that the level of Oct4 expression in mature pancreatic cells is higher than the level of Sox2 expression. No evidence was found to support the connection between the expression advancement of these markers and immunological criteria for an organ donor or individual age. Oct4 and/or Sox2-positive cells are present in a mature pancreas, being a source of new B cells (46). Most Oct4-positive cells show a cytoplasmic expression of this factor, only a small part of these cells (~1.6%) shows a nuclear expression. Probably, the presence of Oct4 and Sox2 proteins in cytoplasm is characteristic for stem cells that are mitotically inactive, while a nuclear location suggests that they are in the proliferation phase (55). A similar model of location was observed for Pdx-1 protein, which is found in cytoplasm in a dephosphorylated form, whereas, after penetrating into the cell nucleus and phosphorylation, it becomes an active transcription factor (56). In human pancreatic stem cells, the presence of mRNA and

CD34 protein was demonstrated as well. However, it is unlikely to ascribe mRNA Oct4, Sox2 and CD34 to hemopoietic cells that colonized the pancreas (46).

In pancreatic islets and in the epithelium of small excretory ducts (intercalated ducts), there are also cells showing the expression of nestin, a marker of neuronal stem cells. They are characterized by a prolonged proliferative activity in vitro. Among them, cells isolated from islets also show the expression of typical markers of endocrine cells: insulin, GLUT2, glucagon, IDX-1 transcription factor and other genes of both exocrine cells and hepatocytes. These multipotential cells seem to be genealogically close to progenitor cells (IPS cells) which occur in human cultures of islet buds (CHIBs). Probably, both these populations derive from small excretory ducts (39, 57). Nevertheless, they do not have a phenotype of epithelial cells with a characteristic CK-19 expression. Thus, it may be suggested that nestin-positive cells of pancreatic ducts are undifferentiated and different from epithelial cells. These cells may be regarded as multipotential stem cells which have not undergone the process of differentiation into endocrine or ductal cells.

A population of human and rat nestin-positive progenitor cells of islet origin (NIP) was cloned and managed to be maintained in a culture for 8 months (in the presence of βFGF, EGF and glucose). It was proven that a considerable number of these cells still maintained nestin expression. When the cells were enabled to form aggregates, by removing βFGF and EGF from the medium and adding HGF, activin-A, exendin-4 or nicotinamide, the expression of NCAM, CK19 and IDX-1 transcription factor was observed in these cells. It is not clear whether HGF, activin-A and nicotinamide are factors responsible for initiating the expression of pancreatic islet cell markers. In NIP cells exposed to dexamethasone, the expression of transthyretin of a hepatic acute phase protein was observed. In some of them, a low level of the synthesis of insulin, glucagon and GLP-1 was also detected. Although a combination of βFGF and EGF growth factors clearly stimulates the proliferation of NIP cells (also in nervous stem cells), it is still unclear which of them play a key role in the process of NIP differentiation into the endocrine phenotype. HGF, activin-A and exendin-4 were selected since they induce the differentiation of AR42J cells (a neoplastic line of rat ductal carcinoma) into cells producing glucagon and insulin (58). Yet, the exposure of AR42J cells to high doses of dexamethasone stimulates them to differentiate into hepatocytes. IDX-1 expression in islet-like cellular clusters suggests that they are able to differentiate into a pancreatic line. In the NIP cells mentioned above, a poor IDX-1 expression was detected in the cytoplasm and nucleus, whereas a strong expression was observed in stem cells (SC) gathered in spherical aggregates. This expression occurs at the early stages of the pancreatic development and during organ regeneration induced by pancreatectomy. IDX-1 is regarded as a key factor leading to the development of the pancreas and to the activation of genes specific for islet endocrine cells, including insulin in B cells. The ectopic expression of IDX-1 in A cells and in hepatocytes leads to their conversion into the phenotype of B cells in vivo (58). The introduction of IDX-1 gene into hepatocytes by means of an adenovirus vector results in their transformation into B cells. It cannot be excluded that pancreatic cells are able to undergo transdifferentiation into hepatocytes and cholangiocytes.

CONCLUSIONS

It has been established beyond doubt that the adult pancreas posseses a few percent population of pluripotential stem cells. In vitro, this self-renewing pool is able to differentiate into the various types of mesodermal and endodermal cells. However, under the influence of niche-derived signalling factors pluripotential stem cells undergo transformation into multipotential stem cells, a source of exocrine and endocrine pancreatic cells. Activation of stem cells-dependent organ regeneration takes place after surgical injuries.

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