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Original article

Stem progenitor cells in the human pancreas

Giorgia Locci[1], Anna Paola Pinna[2], Angelica Dessì[3], Eleonora Obinu[1], Clara Gerosa[1], Maria Antonietta Marcialis[3], Maria Cristina Pintus[3], Marco Angiolucci[4], Vassilios Fanos[3], Rossano Ambu[1], Gavino Faa[1]

Department of Surgical Sciences, [1]Division of Pathology, [2]Section of Pediatric Clinic, [3]NICU, Neonatal Pathology and Neonatal Section, [4]Section of Obstetrics and Gynecology, University of Cagliari, Cagliari, Italy

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Stem cells: present and future
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Abstract

Early pancreas development, given its complexity, is generally considered as a paradigm for branching morphogenesis and for the development of two organs in one: the Langherans islets, programmed to secrete hormones into the bloodstream, and the exocrine pancreas compartment, composed of two major cell types, acinar and ductal cells, devoid to secrete digestive enzymes into the duodenum through a branched network of ducts. Exocrine and endocrine pancreas are generally presumed to originate from a common multi-lineage progenitor cell (MPC), emerging within the definitive endoderm surrounding the posterior foregut. Bipotential precursors committed to the pancreatic fate originate the MPC, that are considered the progenitors of all pancreatic cells operating in the mature pancreas, including acinar, ductal, endocrine and stromal cell types. Pluripotent stem cells (PSCs) are able to differentiate into several cell types, including acinary cells, duct cells and islet cells, depending on certain transcription factors, which function in a coordinated way during pancreas development. The epidemiological entity of pancreatic diseases such as diabetes mellitus and issues regarding the management of the diabetic patient have constantly stimulated the great current interest aimed at regenerative pancreatic medicine. Several studies in rats have demonstrated the existence of stem/progenitor cells in the adult pancreas and have clarified the mechanism by which pancreatic stem cells differentiate into acinar, ductal and endocrine cells. In this context, the cellular microenvironment called

"niche" plays a major role in inducing differentiation of stem/progenitor cells by adequate cellular signals. Within the niche, undifferentiated pluripotent cells give rise to asymmetrically dividing daughter cells. The main purpose of this work was to identify stem cells and progenitor cells in the human pancreas during intrauterine development in relation to what is already known in the adult pancreas and in experimental models.

Keywords

Human pancreas, pancreas development, molecular pathways, morphogenesis, stem progenitor cells, niche.

Corresponding author

Giorgia Locci, Department of Surgical Sciences, Division of Pathology, University of Cagliari, Cagliari, Italy; email: giorgettinaloc@gmail.com.

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Introduction

Early pancreas development, given its complexity, is generally considered as a paradigm for branching morphogenesis and for the development of two organs in one: the Langerhans islets, programmed to secrete hormones into the bloodstream, and the exocrine pancreas compartment, composed of two major cell types, acinar and ductal cells, devoid to secrete digestive enzymes into the duodenum through a branched network of ducts [1]. The development of the human pancreas is a complex process, the mature pancreas of mammals is the final product of two buds, one ventral and one dorsal, emerging from the definitive endoderm germ layer on both sides of the foregut, around the 3rd week of gestation in humans. In particular, the ventral foregut endoderm of the mammalian embryo gives rise to genetic programming of both liver and ventral pancreas stem/progenitor cells [2]. The ventral pancreas arises adjacent to the hepatic diverticulum and the cardiac mesenchyme, whereas the dorsal pancreatic bud is in strict contact with the notochord and dorsal aorta [3] and originates from a bipotential stem/precursor population for pancreas and liver within the embryonic endoderm [4]. Around the 7th week of gestation, when stomach and duodenum rotate, the ventral bud moves around, coming in contact and eventually fusing with the dorsal pancreatic bud [5]. If the ventral pancreatic bud forms the posterior part of the head (uncinate process), the dorsal bud forms the rest of the organ. During the bud fusion process, the ventral pancreatic duct fuses with the distal part of the dorsal duct, originating the duct of Wirsung, the main pancreatic excretory structure; at the same time, the proximal part of the dorsal duct gives rise to the duct of Santorini, the small accessory pancreatic duct [6]. The fusion between two buds necessitates molecular interactions between the pancreatic endoderm and the adjacent notochord, eventually leading to the coordinate development of multiple highly specialized stromal, vascular, acinar, ductal and endocrine cell types [7]. In particular, in ventral foregut, bone morphogenetic proteins (BMPs) and fibroblast growth factor (FGF) signaling activity promote liver and inhibit pancreas specification. The Notch signaling effector hairy and enhancer of split-1 (Hes1) is involved in pancreato-biliary segregation, acting to prevent ectopic pancreas development in adjacent tissues. In dorsal endoderm, retinoic acid (RA) is required to promote pancreas specification [4]. Overall, pancreas morphogenesis appears as a peculiar self-regulating process, whereby exocrine and endocrine cells, that are both presumed to derive from a common MPC, progressively differentiate and compartimentalize, giving rise to the final architecture of the "two in one" organ [8]. The entire pancreatic tree arises from an endodermally derived protodifferentiated epithelium [9]. The tubular epithelium originating within the acini progressively ramifies as small ducts, giving rise to ductal branches that make up the pancreatic tree. During organogenesis, the epithelial cells receive essential signals from the overlying mesenchyme. Previous studies have identified an important role for the mesenchyme in regulating the expansion of progenitor cells in the early pancreas epithelium [10]. Precursors located at branch tips give rise to the vast majority of pancreatic cells, including acinar, ductal and endocrine lineages [11]: acinar cells differentiate and mature at branch tips, whereas ductal and endocrine progenitors emerge in branch stalks. Endocrine cells escape from their epithelial neighbors via delamination and progressively coalesce into islets. Recent studies

have highlighted multiple factors that play a role in the proliferation and differentiation of different cell types participating to pancreas organogenesis, evidencing that pancreas development is controlled by precisely timed signaling events [12]. Pancreas development requires a fine balance of many factors, including genetic and epigenetic ones, which can be disturbed by various prenatal events, including intrauterine growth restriction [13], and perinatal asphyxia [14]. Recently, another factor, represented by maternal diet during pregnancy, has been hypothesized to play a relevant role in pancreas development [15].

Pancreas development

Exocrine and endocrine pancreas are generally presumed to originate from a common MPC emerging within the definitive endoderm surrounding the posterior foregut [16]. Differentiation of the primitive endoderm originates multiple organs, including thymus, thyroid, lungs and gut, whereas the ventral endoderm gives origin to bipotential precursors able to differentiate towards the hepatic or the pancreatic fate. Bipotential precursors committed to the pancreatic fate originate the MPCs, that are considered the progenitors of all pancreatic cells operating in the mature pancreas, including acinar, ductal, endocrine and stromal cell types. PSCs are able to differentiate into several cell types, including acinary cells, duct cells and islet cells depending on certain transcription factors, which function in a coordinated way during pancreas development. PSCs, including embryonic stem cells (ESCs) and induced PSC (iPSCs), present the ability to differentiate into all cell types of the body [17]. PSCs differentiation into pancreatic lineage is organized in several steps that start with the differentiation into definitive endoderm (DE), which is recognized by the expression of specific markers. The initiation of DE differentiation is properly induced in human ESCs (hESCs) and human iPSCs (hiPSCs) by NODAL and WNT signals [18]. NODAL signals have been previously indicated as the main inducer of endogenous endoderm [19], and are activated by one of the members of TGF-beta family, activin A. Activin A is crucial for NODAL signaling activation and in turn for DE differentiation [20]. In the dorsal endoderm, BMP signaling inhibition is required for specific differentiation into pancreatic lineage, whereas its presence after the formation of pancreatic cells is essential to maintain pancreatic and duodenal Homebox 1 gene (PDX1) expression [21]. PDX1 is expressed by all pancreatic precursor cells and has been shown to be essential for early pancreatic development [22]. The concerted action of both PDX1 and pancreas-specific transcription factor (Ptf1α) is generally considered to be necessary for the initiation of pancreatic development from endodermal pluripotent/stem cells. Two other signaling pathways, NOTCH and HEDGEHOG, are involved in the differentiation into pancreatic endocrine cells [23]. The inhibition of HEDGEHOG-signaling by cyclopamine or KAAD cyclopamine induces the generation of PDX1-expressing cells, whereas fibroblast growth factor 10 (FGF-10) activates NOTCH signaling, which is involved in the proliferation of PDX1-expressing pancreatic progenitors [24]. Another transcription factor considered a marker for late pancreatic cell development is neurogenin 3 (NGN3). NGN3 expression peaks during endocrine differentiation stage, which is subsequent to the generation of PDX1-positive pancreatic progenitors. In contrast to PDX1, NGN3 expression is stimulated by a reduction in NOTCH signaling. A recent study on a xeno-free culture system showed that a high NOGGIN concentration is crucial for inducing the differentiation of iPSCs into pancreatic progenitors (PDX1-positive) and then pancreatic endocrine progenitors (NGN3-positive cells) [25] (**Fig. 1**). The generation of mature pancreatic beta cells from hESCs and hiPSCs is characterized by the expression of a panel of different factors, including PDX1, MAFA, NKX6.1, NEUROD, ISL-1, GLUT2, C-peptide, and INS (insulin) [26]. Among these factors, NKX6.1 expression has been shown to be essential for the production of functional mature beta cells.

Pancreatic exocrine cell differentiation

The exocrine pancreas is composed of three types of cells: acinar cells, duct cells and centroacinar cells. The acinar cells are polarized pyramidal-shaped cells with high secretory capacity that derive from multipotent progenitors. Acinar cells express, initially and during development, Ptf1α paralleled by repression of NKx6, thereby suppressing alternative duct and endocrine cell fates. Absence of Ptf1α results in failure of acinar cell formation [27]. The proliferative activity of pancreatic acinar cells progressively decreases after birth whereas adult acinar cells show a low basal proliferative index and appear to have poor regenerative capacity

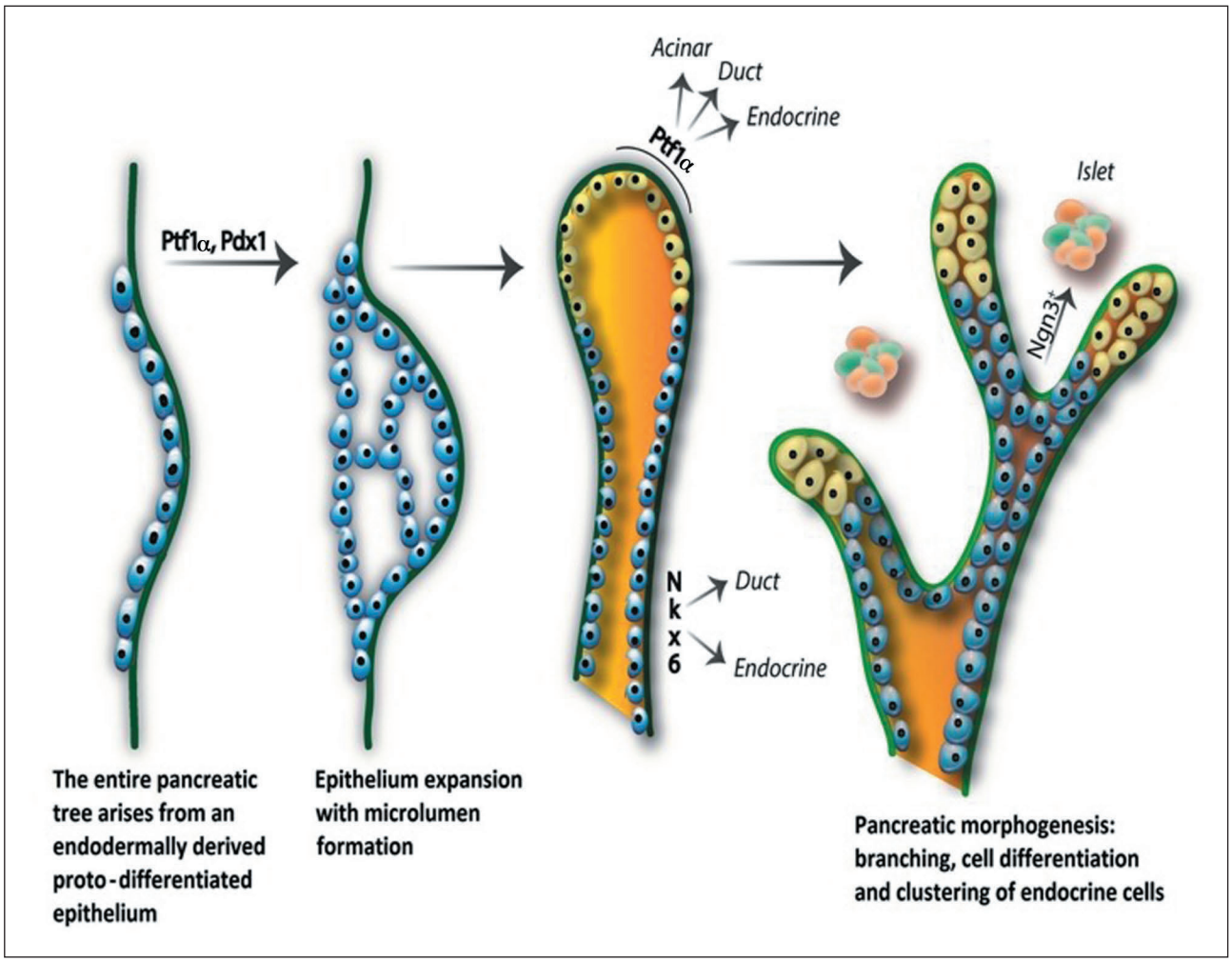

Figure 1. Pancreatic morphogenesis and developmental regulation.

[28]. Duct cells are ciliated, polarized epithelial cells that secrete bicarbonate, mucins, and form an extensive network of tubules originating from centroacinar cells. The morphological and functional heterogeneity, reflecting duct cell location, has likely complicated attempts to understand duct cell development [29]. It is commonly believed that columnar epithelia form the main ducts, whereas stratified squamous and simple squamous epithelia form the interlobular and intralobular ducts, respectively [30]. Duct cells derive from multipotent progenitors that become polarized through unknown mechanisms and form primary cilia, an organelle whose development requires the transcription factors Hnf6/Onecut1 and Hnf1β. The centroacinar cells are located at the junction of the secretory acinus and its associated terminal duct epithelium. These cells are variably depicted as an extension of the most terminal ductal epithelium as it invaginates into the secretory acinus [31], or alternatively as providing a fenestrated "cap" to the apical surface of acinar cells [32]. In addiction to ongoing NOTCH-pathway activation, at least some centroacinar cells and terminal ductal cells also express Sox9 [33].

The endocrine pancreas development

Mature pancreatic endocrine cells derive from a subset of epithelial cells that transiently express NGN3 (7th week) [5]. Lineage tracing studies suggest that NGN3 endocrine progenitors are unipotent, post-mitotic cells that engender separately five endocrine cell types: α, β, δ, PP, and ε cells (**Fig. 2**). The β-cells are mixed with other endocrine cells, and are distributed evenly throughout the islet structure. During differentiation, endocrine cells delaminate from the ductal epithelia (9th week), migrate toward the mesenchyme and aggregate into clusters called islets (11th-12th week); this is followed by

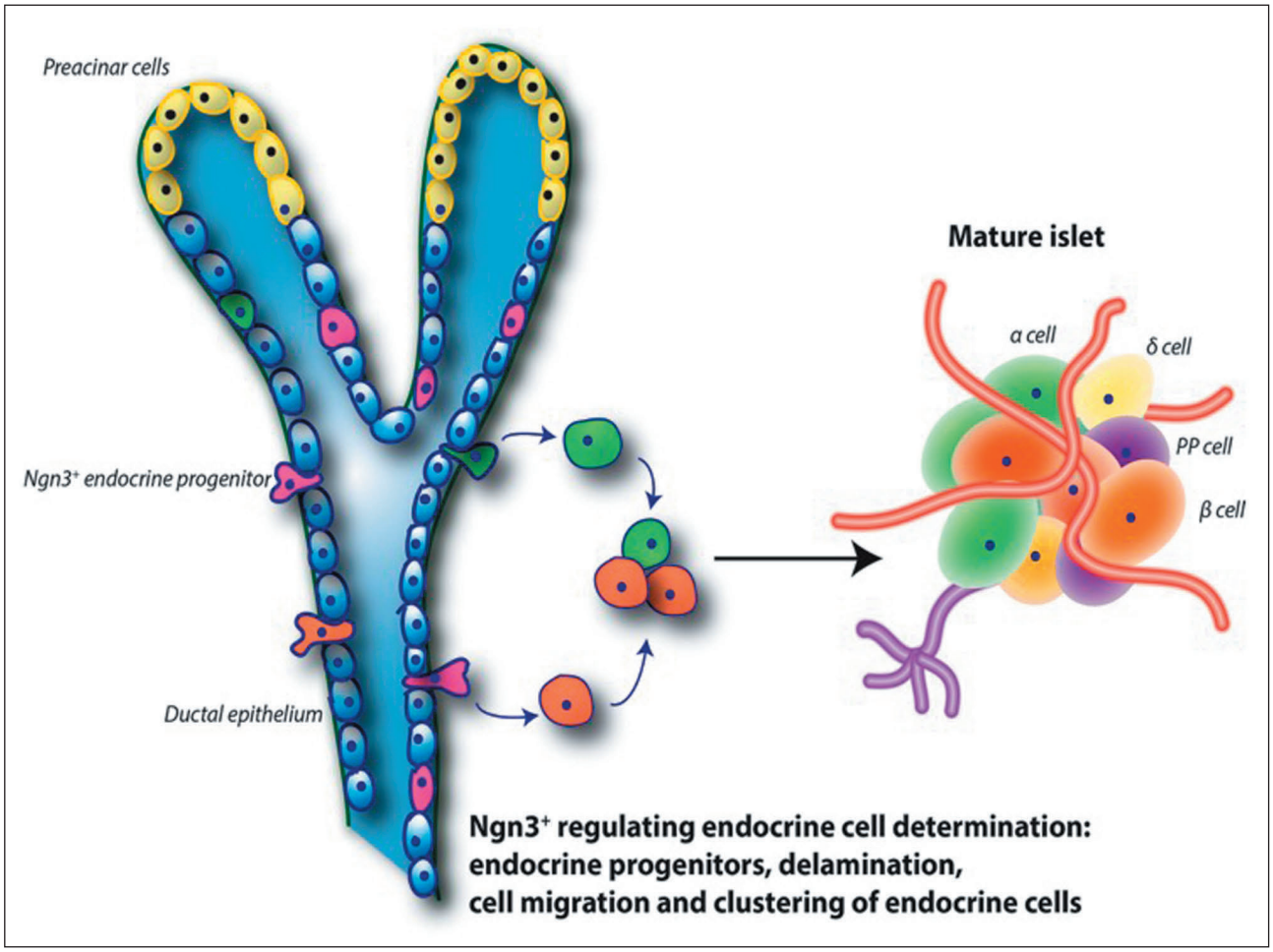

Figure 2. Endocrine pancreas development.

islet expansion (12th-13th week) and adult islet conformation (26th week) (**Fig. 3**). Coincident with islet morphogenesis, vascularization, and innervation of islets by the autonomic nervous system occurs [34]. NGN3 expression is strictly associated with the differentiation and maturation of endocrine progenitors into functional endocrine cells, NGN3-regulating endocrine cell determination. Key α-cell transcription factors include Foxa2, NKx2.2, Pax6, and Arx, whereas β-cell differentiation requires expression of MafB, PDX1, Hlxb9, Pax4, Pax6, Isl1, Nkx2.2, Nkx6.1. δ-cells require Pax6 and Pax4, whereas PP-cells require Nkx2.2. ε-cell-positive Ghrelin cells are rare (< 1%), and disappear after birth. From the 13th week of gestation, the progressive expansion of the endocrine component occurs in the context of the pancreatic parenchyma, until the conformation of the adult islets of Langerhans that occurs around the 26th week of gestation. The final architecture of islets is not formed until 2-3 weeks after birth [35]. During the postnatal period, β-cells undergo two critical events that enable the establishment of a normal, functional β-cell mass. First, β-cells undergo functional maturation by increasing insulin production and enhancing glucose-stimulated insulin secretion (GSIS). Known transcriptional regulators of the maturation process include Neurogenic differentiation 1 (NeuroD1), MafA, MafB, Isl1, PDX1, NGN3, and Von Hippel–Lindau (Vhl). Second, β-cells undergo a transient burst of β-cell proliferation that coincides with a significant increase in β-cell mass expansion [36].

Preliminary personal data

In our study we used 20 pancreatic samples. 10 pancreatic samples were obtained from legal interruption of pregnancy and 10 from autopsies. Gestational age ranged from 11 up to 36 weeks. All living newborns were admitted to the Neonatal Intensive Care Unit (NICU) of Cagliari University Hospital. Pancreatic samples were formalin-fixed,

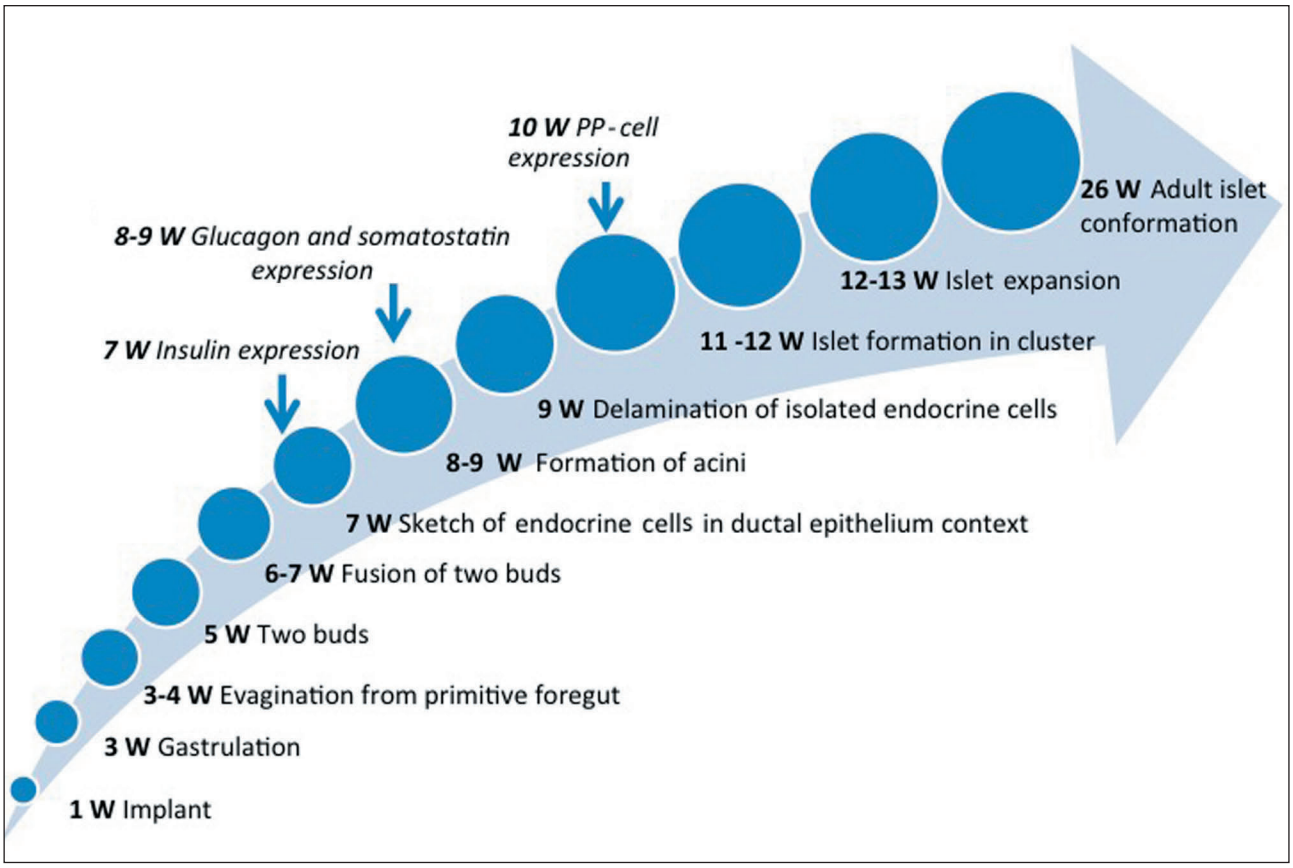

Figure 3. Timing of human pancreas development.

paraffin-embedded and routinely processed in 4 µ-thick sections for histological evalutation. The immunohistochemical study carried out on 4 fetuses, ranging from 12 to 27 weeks of intrauterine life, allowed us to detect the expression of 3 immunohistochemical markers including insulin, glucagon and somatostatin.

Results

The most relevant data regarding the pancreatic stem cells niche were obtained with the study of pancreas in a 12-week old fetus (**Fig. 4**). In this case, stem cell niche were identified at the periphery of the developing pancreas, in the sub-capsular zone. Pancreatic stem/progenitor cells appeared as large cells, with oval clear nuclei, enveloped by a loose mesenchyme. In some fields, stem cells were arranged around a vessel with prominent endothelial cells (**Fig. 4**). Stem cells frequently surrounded developing acinar ductal structure, giving rise to a complex stem/vascular/mesenchymal and epithelial niche (**Fig. 4**). Stem/progenitor cells observed in developing pancreas had a very different morphology when compared to acinar cells. It was also possible to distinguish groups of pancreatic stem cells arranged in multiple rows in the sub-capsular region. These cells were, in the majority of fields, disorganized and, when located in the vicinity of a vessel, showed the tendency to surround the vascular wall. Stem/progenitor cells were polymorphous, showed a scant cytoplasm, prominent nucleolus, and occasionally had a plasmacytoid appearance. We found cells not yet differentiated in the capsule, which differed from the cells observed in the sub-capsular region and in the interstitial space. We focused our attention on the presence of undifferentiated stem/progenitor cells located in a sub-capsular band disposed below the capsule, showing a greater cell density so that it was possible to identify a gradient going from the capsule toward the deep interstitial spaces (**Fig. 5**). Pancreatic samples under 20 weeks of gestation didn't show any organized Langerhans islet. We only observed scattered endocrine cells forming small clusters distributed through the whole parenchyma, indistinguishable at H&E from other surrounding cell types. Islet burden was strictly related to gestational age, as demonstrated by the increasing number of islets

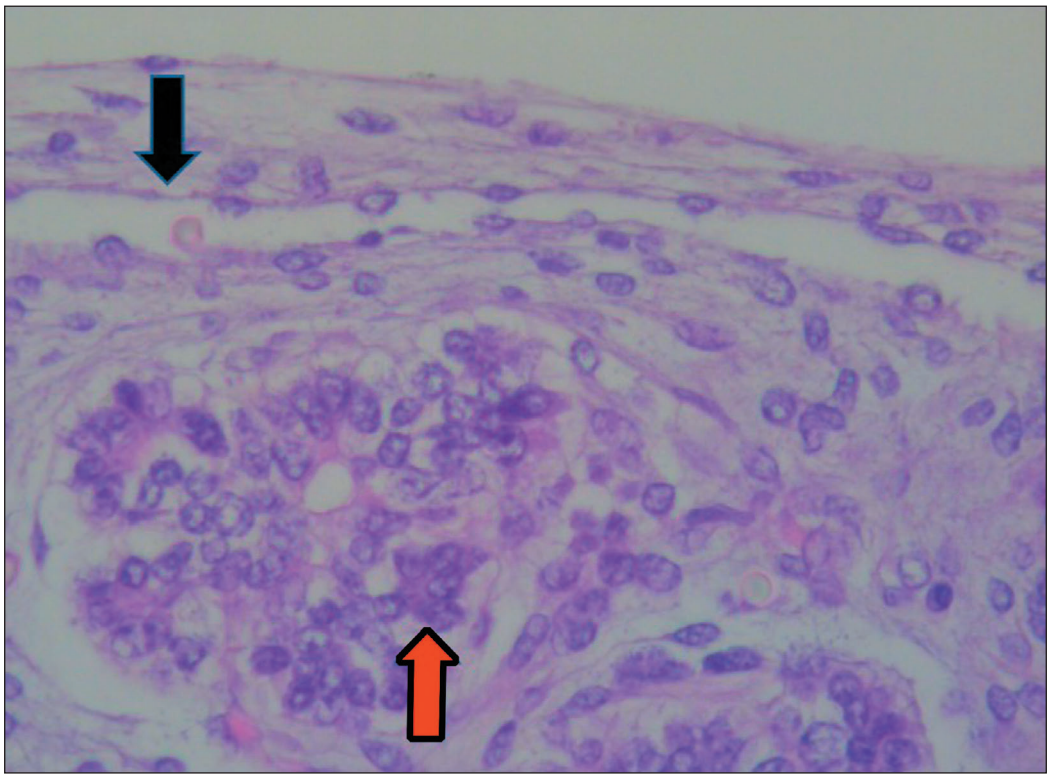

Figure 4. 12-week human pancreas H&E (40X): a stem cell niche was identified at the periphery of the developing pancreas, in the sub-capsular zone. Pancreatic stem/progenitor cells appeared as large cells, with oval clear nuclei, enveloped by a loose mesenchyme. In some fields, stem cells were arranged around a vessel with prominent endothelial cells (black arrow). Stem cells frequently surrounded developing acinar ductal structures, giving rise to a complex stem/vascular/ mesenchymal and epithelial niche (red arrow).

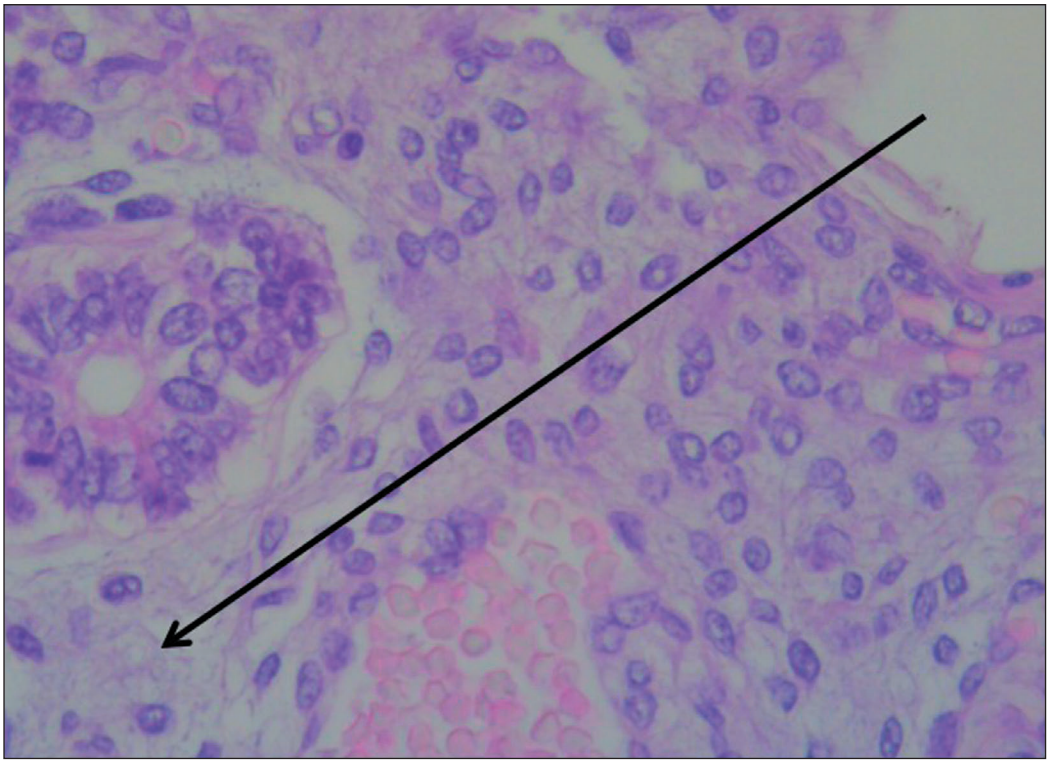

Figure 5. 14-week human pancreas H&E (40X): there was the presence of undifferentiated stem/progenitor cells located in a sub-capsular band disposed below the capsule, showing a greater cell density so that it was possible to identify a gradient going from the capsule toward the deep interstitial spaces (black arrow).

that reached an adult conformation around the 26th week (**Fig. 6**). Significant changes were observed in the cells of the pancreatic capsule at different gestational age. Whereas in younger fetuses the capsule showed a large number of cells, some of which could be interpreted as a stem cells (**Fig. 4**), in older fetuses the pancreatic capsule became more fibrotic, thinner and the number of stem/progenitor decreased progressively. The pancreas at 36 weeks of gestation showed the presence of oval isolated cells in the interstitial space, suggesting the persistence of a few number of progenitor cells in the deep pancreatic parenchyma. The immunohistochemical study carried out on 4 fetuses, ranging from 12 to 27 weeks of intrauterine life, allowed us to detect the expression of 3 immunohistochemical markers namely insulin, glucagon and somatostatin. With these markers we were able to detect the presence of isolated endocrine cells in the parenchyma, sometimes organized in nests. β-cells were marked with insulin (**Fig. 7**), α-cells were marked by means of the hormone glucagon and δ-cells were marked with the hormone somatostatin. At 27 weeks of gestation, islands of Langerhans cells with the adult shape and the typical insulin expression of β-cells (**Fig. 8**), α-cell and δ-cell were detectable by immunohistochemical staining of the respective hormones.

Discussion

The epidemiological entity of pancreatic diseases such as diabetes mellitus and issues regarding the management of the diabetic patient have constantly stimulated the great current interest aimed at regenerative pancreatic medicine. Several studies in rats have demonstrated the existence of stem/progenitor cells in the adult pancreas and have clarified the mechanism by which pancreatic stem cells differentiate into acinar, ductal and endocrine cells. In this context, the cellular microenvironment called "niche" plays a major role in inducing differentiation of

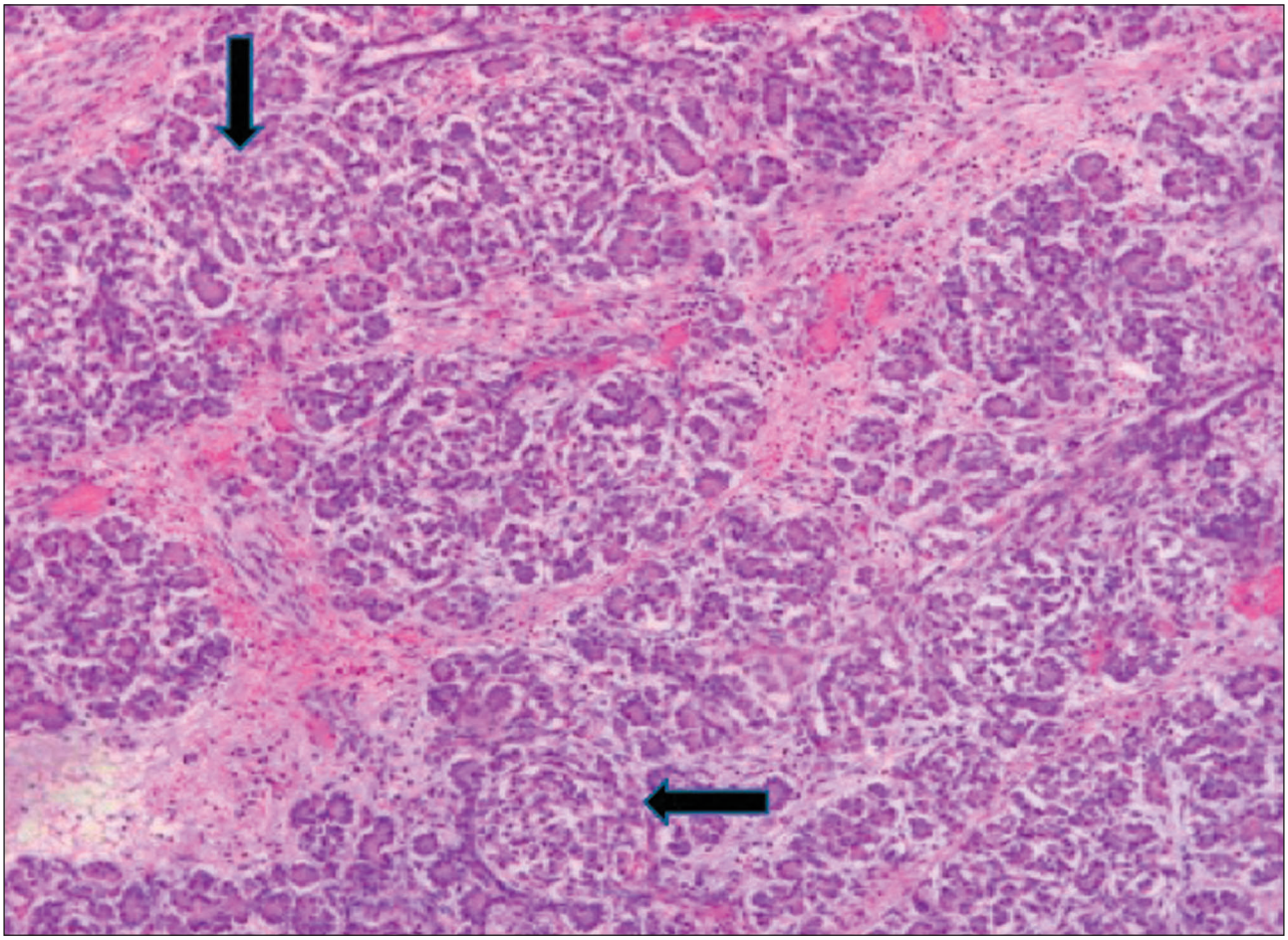

Figure 6. 26-week human pancreas H&E (20X): islet burden was strictly related to gestational age, as demonstrated by the increasing number of islets that reached an adult conformation around the 26th week (two black arrows).

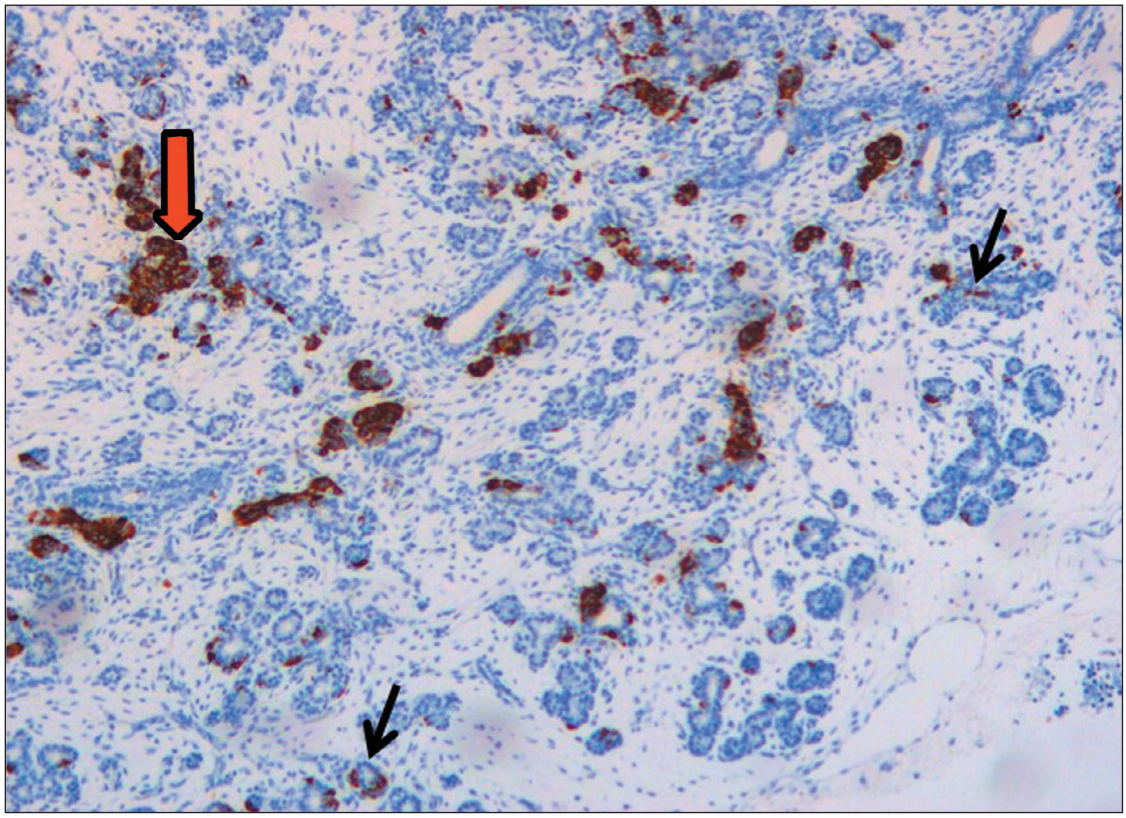

Figure 7. 13-week human pancreas insulin (10X): β-cells were marked with insulin. With insulin, glucagon and somatostatin we were able to detect the presence of isolated endocrine cells (two black arrows) in the parenchyma, sometimes organized in nests (red arrow).

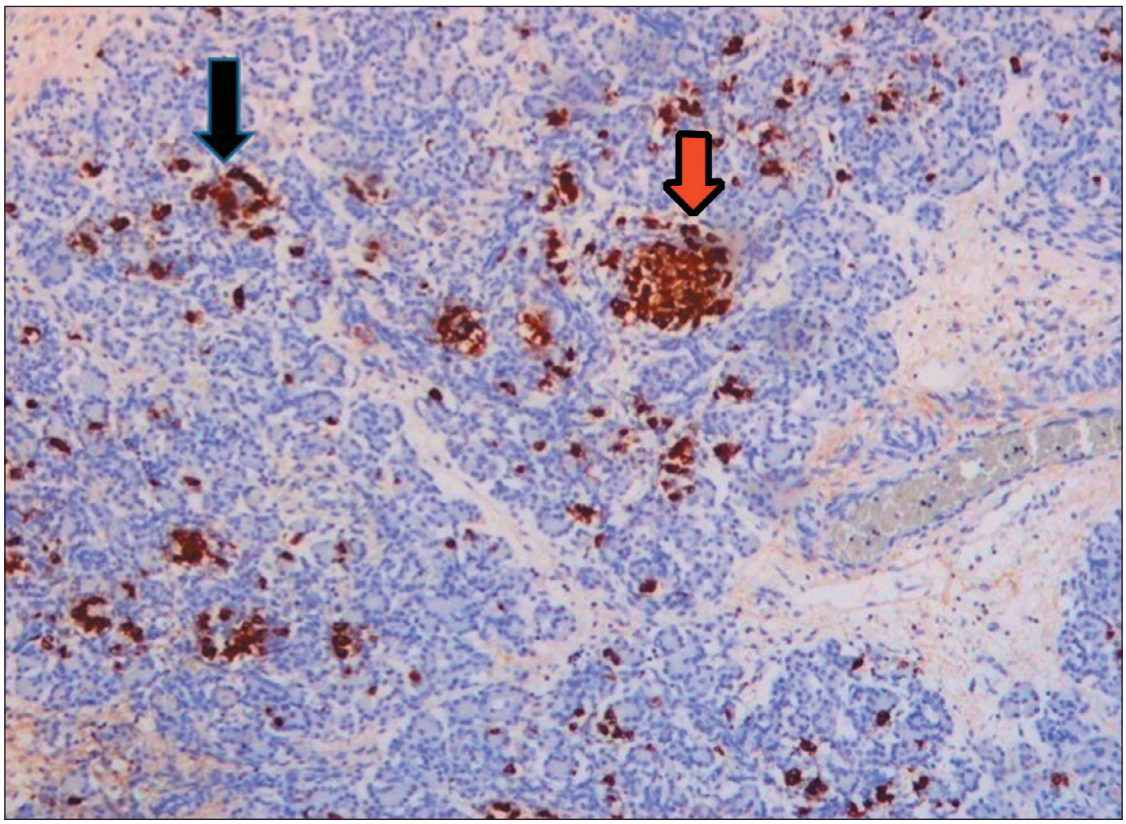

Figure 8. 27-week human pancreas insulin (10X): islands of Langerhans cell with adult shape, the typical insulin expression of β-cells (red arrow) and cluster of β-cells marked with insulin (black arrow).

stem/progenitor cells by adequate cellular signals [37]. Within the niche, undifferentiated pluripotent cells give rise to asymmetrically dividing daughter cells [38]. The main purpose of this work was to identify stem cells and progenitor cells in the human pancreas during intrauterine development in relation to what is already known about adult pancreas and in experimental models.

The morphological identification of pancreatic stem/progenitor cells was the first goal of this study. This identification was difficult because of conflicting indications and the lack of precise information from the international scientific literature, regarding the shape and location of stem/progenitor cells and stem cell niches in the developing pancreas. In order to detect stem cell niches, we operated the accurate scanning of all capsular, sub-capsular and interstitial areas of the pancreatic samples under study. This approach enabled us to achieve this first objective, i.e. the identification of niches of stem/progenitor cells and pancreatic stromal cells associated. The study of stem/progenitor cell morphology performed using H&E-stained sections allowed us to identify the presence of pancreatic stem cell niches. In fact, this study showed the presence of cells with morphology very different from the adult pancreatic acinar, ductal and endocrine cells. In cases concerning the first trimester of pregnancy until the 19th week of pregnancy, these cells were easily identifiable, in small clusters, or arranged to form a sub-capsular band. The niche was defined as a structure consisting of randomly oriented ellipsoidal structure, with cellular and extracellular components. These stem cell niches were observed disposed in the vicinity of a vessel, or in a region where there is a low hemodynamic stress and a low oxygen tension. The niche is usually located away from exogenous stimuli [39, 40]. In fact the scientific literature also reports that low oxygen tension is a condition that provides a selective advantage to stem cells, encouraging their primitive state of rest [41]. Even cases that relate to the second trimester of pregnancy highlighted cells with marked polymorphism, arranged in niches (although less in number) and isolated in the context of the pancreas. All these data taken together suggest a kind of maturational gradient that goes from the capsule into the interstitial space. Cases involving the last trimester of pregnancy revealed, in this study, further progressive reduction in the number of stem cell niches with persistence of individual cells in the context of the pancreas. The relevance of the microenvironment for the understanding of the behavior of stem cells is such that, in the absence of the niches, the stem cells cannot be stored [42, 43]. The pancreatic stem/progenitor cell goes through a finished number of divisions; after each division the replicative capacity decreases and the degree of maturation and differentiation gradually increases. The number of stem cells can be reduced owing to exogenous stimuli or because of physiological aging; the microenvironment observed in cases of internal abortion (cases concerning the 20th week of pregnancy onwards) may have resulted in a premature differentiation of what should have been a pool of quiescent cells. In particular, the 36-week case showed, in fact, a sub-capsular region, whose cells did not present significant differences with respect to the acinar cells present at the sub-capsular level and the absence of stem cells could be observed: a very different condition compared to that found in the earliest gestational ages. This would indicate the possibility of variability in karyogenesis linked to multiple external factors. It proved useful to break down the pancreas into 3 areas: capsular, sub-capsular and interstitial areas. This division has allowed us, after localizing niches of stem cells, to assess the number, the changes over the weeks of gestation, the inter-individual variability. Within the niches, the pancreatic stem cells are typically grouped together with the first cells in the process of differentiation. The number of pancreatic stem cells and their niches has been reported to be higher in the sub-capsular area, as this area is anatomically protected, characterized by a lower hemodynamic stress, low oxygen and low power consumption compared to other regions of the pancreas. The next phase of the work was to apply immunohistochemical staining as insulin, glucagon and somatostatin. The future will be to apply immunohistochemical stains that allow us to typify the immunophenotype characteristic of stem cells. With the characterization and identification of human pancreatic stem cells you can have important clinical implications in terms of regenerative medicine with the hope of a cure for patients with chronic pancreatic disease like diabetes. This study is part of a larger experimental context, that of fetal programming, in which our group is identifying the different cell types of stem/progenitor cells and specific niches of different organs and tissues, in order to better understand the human embryonic development and organogenesis. This is the context of regenerative

medicine, which uses the mechanisms of organogenesis and the cells responsible in order to repair the damage produced by harmful diseases and cell aging. However, further studies are needed to confirm our findings.

Declaration of interest

The Authors declare that no conflict of interest exist.

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