Chapter

Ontogeny of the Human Pancreas

Alexandra E. Proshchina, Yuliya S. Krivova, Larisa E. Gurevich, Valeriy M. Barabanov, Dmitriy A. Otlyga, Iya A. Voronkova and Sergey V. Saveliev

Abstract

Pancreatic disorders are the most common pathologies in humans worldwide. Detailed information on pancreatic cytoarchitecture, vascularisation, innervation, morphogenesis, and cell differentiation is required for the development of new approaches to the treatment of these diseases. Currently, the majority of studies on pancreas development are performed on experimental animals (mainly rodents). Studies on human pancreatic prenatal development are restricted in number by ethical constraints and some technical difficulties. However, interspecies differences in pancreatic structure and development are considerable. Therefore, data obtained in experiments on animals and cell cultures must be supplemented with information obtained directly from human pancreatic autopsies. In this chapter, we summarise our previous results and the literature data on human pancreatic ontogeny. Special attention has been paid to the endocrine pancreas, which undergoes morphogenetic restructuring during human development. Several forms of structural organisation of the endocrine pancreas (single endocrine cells, small clusters of endocrine cells, mantle, bipolar, and mosaic islets) gradually appear during development. It is important that this restructuring is accompanied by changes in the ratio of pancreatic endocrine cells. The mechanisms of these changes are still unclear. The difficulties in identifying progenitor cells and tracking cell differentiation are the main problems associated with this issue.

Keywords: pancreas, islets of Langerhans, human development, transcription factors, Nkx6.1, Neurod1

1. Introduction

The pancreas is a unique organ that combines both endocrine and exocrine functions, which determines the diversity of its pathology. Pancreatic disorders (such as malignant neoplasms, pancreatitis, and diabetes mellitus) are the most common pathologies in humans worldwide. For example, according to the International Diabetes Federation, worldwide, 10% of adults will have type 1 or 2 diabetes by 2030 [1]. Currently, there are not enough effective methods of diabetes treatment aimed at replenishing and maintaining the population of insulin-producing cells. New techniques for the recovery of islet cells based on the natural mechanisms of the endocrine cell pool renewal are required.

For a long time, it was believed that the ability of the pancreas to respond to insulin demands is very limited during life. Recently, evidence of pancreatic

endocrine part plasticity has been found, indicating that the number of β-cells varies under the influence of some physiological parameters. For example, it increases in cases of altered metabolic demand such as pregnancy and obesity, as well as during normal physiological growth [2, 3]. According to the literature, mechanisms of pancreatic β-cell plasticity are associated with the processes of cell proliferation, cell death, neogenesis, and changes in cell volume [4–6]. Recent work in rodents has indicated a previously unappreciated proliferative capacity of β-cells. However, the age of the animal appears to play a central role in determining the proliferative capacity of β-cells, as older animals display a significant decline in cellular expansion [7]. At the same time, the endocrine pancreas consists not only of β-cells but also contains several types of endocrinocytes. The endocrine cells of the pancreas are grouped into formations called pancreatic islets, but the details of the mechanisms and stages of human islet morphogenesis are still not fully resolved [8, 9].

All basic cell types of the pancreas originate from a single pluripotent cell with ductal phenotype [10–14]. It has been suggested that, in addition to the proliferation of existing β-cells, new cells can be obtained from differentiated adult pancreatic cells by interconverting between endocrine and exocrine parts. There are also suggestions regarding ontogenesis from duct cells or from other progenitor cells [6, 15]. Acinar cells are another potential source of new pancreatic endocrine cells. So-called acino-insular cells have been identified in many vertebrate species [16]. These cells simultaneously contain granules with digestive enzymes and granules containing hormones.

Recently, along with hypotheses about the replication of β-cells and the neogenesis of islets from progenitor stem cells, the hypothesis of transdifferentiation of some insulin-containing cells into glucagon-containing cells and the reverse transformation have been suggested [17, 18]. Most of these studies have been conducted on rodents, and there is still not enough data to confirm the existence of such mechanism in humans. However, in the endocrine cells of the developing human pancreas, co-expression of several hormones has been observed (see below). Some researchers believe that, in the human pancreas, these cells may be the precursors of endocrine cells [8].

It is also necessary to emphasise the importance of research directly on human pancreas samples. Currently, most studies into pancreas development are performed on experimental animals (mainly rodents) and cell cultures. However, adult β-cells have a limited lifespan in culture and undergo dedifferentiation, with the loss of insulin expression [7]. Interspecies differences in pancreatic structure and development are also considerable. For example, the formation of pancreatic islets in rodents is observed only in the first 2 weeks after birth, whereas, in the developing human pancreas, islets can be observed starting from 12 weeks of gestational development. Differences in the structure of the endocrine part of the pancreas in rodents and humans are also considered. In rodents, islets have a mantle-type structure, in which glucagon (α)- and somatostatin (δ)-containing cells are located at the islet periphery, and β-cells occupy the central part of the islet. In humans, islets of a mixed type are characteristic, in which α- and δ-cells can be not only at the periphery but also in the centre of the islet. In addition, bipolar islets (β-cells located at one pole of the islet and α- and δ-cells at another) are present during prenatal human development. In rodents, this stage of islet development has not been identified. Therefore, data obtained in experiments on animals and cell cultures must be supplemented with information obtained directly from the human pancreas. Although the basic developmental programme may be similar, the identity and/or timing of activity of some factors may be critically different.

In this chapter, we summarise our previous results and the literature data on the ontogeny of the human pancreas. We examined pancreatic autopsies derived from human foetuses from the 10th to the 40th week of development and from adults, using histological, IHC, and morphometric methods.

2. Pancreas structure in adults

The human pancreas is a well-defined unpaired elongated organ, in which three parts are usually distinguished: the head, body, and tail.

The pancreas combines both endocrine and exocrine functions. The exocrine compartments are identified as acinar and ductal cells. Acinar cells synthesise digestive enzymes that aid in nutrient metabolism, and ductal cells line the channels that transport these secretions to the gastrointestinal tract. The human exocrine pancreas (together with the mesenchyme) occupies 96–99% of the total pancreas volume [19, 20]. The endocrine pancreas occupies 1–4% of the total human pancreas volume. The main function of the endocrine pancreas is the regulation of carbohydrate metabolism. The mammalian (and human) endocrine pancreas contains four main types of endocrine cells: β-cells that produce, store, and secrete insulin (51 amino acid residues); α-cells that produce, store, and secrete glucagon (29 amino acid residues); δ-cells that produce, store, and secrete somatostatin (14 amino acid residues); and PP-cells that produce, store, and secrete pancreatic polypeptide (36 amino acid residues). The fifth type of pancreatic endocrine cells is the ghrelin-containing cells (ε), which are present in small numbers in the developing pancreas but disappear after birth [21, 22]. In the endocrine pancreas of some mammals, two other cell types have been described: EC-cells, which synthesise serotonin, and G-cells, which contain gastrin. These cells are extremely rare and can be found only at some stages of prenatal development.

However, this scheme is probably oversimplified. Recent studies have spurred an intense debate about whether β-cells represent a single homogeneous population or consist of subpopulations with functional and molecular variations to facilitate specialised tasks and responses to changes in the physiological environment [23].

In addition, several basic forms of endocrine pancreas structural organisation have been identified in the human pancreas (**Figure 1**): single endocrine cells, small clusters of cells, small islets of the mantle type, and large mosaic (mixed) islets [24–26]. The adult human pancreas contains from 100,000 to 2,000,000 islets [27], each of which contains from several hundreds to several thousands of endocrine cells. Large islets have a developed capillary network and are surrounded by a capsule. In humans, mice, and some other studied animals, the maximum islet size is about 500 microns [28, 29]. In addition, as mentioned above, acino-insular single cells have been identified in the pancreas of many vertebrates [16].

In mantle-type islets (**Figure 1c**), β-cells predominate and occupy the central islet part, while other cell types (α-, δ- and PP-cells) are located on the islet periphery. In our previous study, it was shown that the diameter of such islets in humans is less than 100 microns. In rodents (e.g. mice and rats), pancreatic islets of the mantle type are characteristic [30, 31], and mosaic islets (**Figure 1d**) are characteristic in the human pancreas [25, 31, 32]. However, in our study, it was shown that, in adults not suffering from disorders of carbohydrate metabolism, the contribution of the mantle type islets to the islet number was up to 65% of all insulin-containing cells. Islets with diameter more than 100 microns (i.e. mixed islets, since bipolar islets have not been identified in adults) represent only 10–15% of all islets, although they contain up to 35–40% of all the β-cells in the human endocrine pancreas. Similar data were obtained by Kilimnik et al. [26]. Moreover, it is believed that, in mosaic

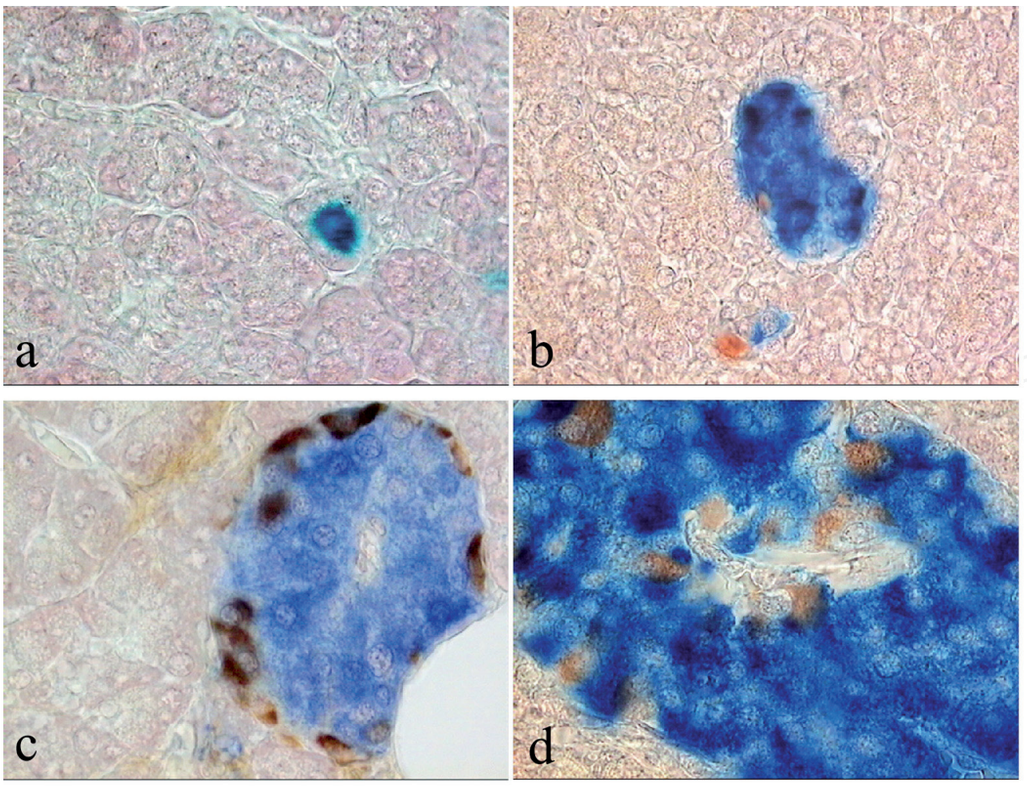

Figure 1.
Forms of endocrine pancreas structural organisation in adults (women, 75 years of age): (a) single endocrine cell, (b) small cluster of endocrine cells, (c) islet of mantle type, and (d) fragment of mosaic islet. Double immunohistochemical labelling with antibodies to glucagon (red) and insulin (blue), objective x100.

islets, the cells are located without clear ordering. In some studies [25, 33, 34], it was indicated that mixed (mosaic) islets of Langerhans have a clear, ordered structure. Within these islets, segments restricted by blood vessels have been identified. β-cells are located in the central part of the lobules, while α- and δ-cells are located at the periphery and around blood capillaries.

In humans, the main mechanism by which the endocrine pancreas grows during the neonatal period is an increase in the islet size rather than their number [35, 36], i.e. a 10% increase in the β-cell mass occurs in a small proportion (10%) due to the formation of new islets and to a much greater proportion (90%) due to an increase in islet size. It is important to note that, during adolescence in humans, no significant changes in islet number or cellular architecture have been detected.

The endocrine pancreas is also affected by the exocrine pancreas, as well as the vascular, lymphatic, and nervous systems. For example, according to some authors, nervous system structures are the first target of an autoimmune attack in type 1 diabetes [37]. At the same time, relatively little is known about these interactions, especially in humans [38].

Thus, information on the cellular composition of pancreatic islets in humans and the contribution of different types of hormone-secreting cells to the plasticity of the endocrine pancreas is contradictory. The main focus of studies devoted to this issue has been on insulin-secreting cells. However, the intimate arrangement of heterogeneous cells in islets has been implicated in the functional regulation of islets [39]. The mechanisms responsible for changes in the number and ratio of endocrine cells are still unclear [4–6]. Therefore, it seems logical to examine these issues during prenatal human development.

3. Prenatal development of the human pancreas

All parenchymal cell types of the pancreas (acinar, ductal, and endocrine cells) are derived from primitive endodermal cells of the foregut. After gastrulation, the thickened endodermal epithelium along the dorsal and ventral surfaces of the posterior foregut gives rise to the pancreas. In humans, the pancreas originates from the two primary diverticula of the primitive gut at 26 days postconception (d.p.c.; ≈6 weeks of gestational development) [40]. The epithelia of the gut evaginate into the surrounding mesoderm-derived mesenchymal tissue and form dorsal and ventral pancreatic buds. In some studies, two ventral buds are described. However, the left ventral bud gradually regresses, whereas the right ventral bud starts to migrate posteriorly. According to several reports, failure of the left ventral bud to regress could lead to the pathological condition known as annular pancreas (reviewed in [41]).

These buds continue to expand, branch, and fuse as a result of the gut rotation that brings the buds together [7]. The parenchyma of the two buds merges during the 7th week of gestation. The ventral pancreas gives rise to the ventral or lower part of the head of the pancreas, whereas the dorsal pancreas gives rise to the dorsal or upper part of the head, the body, and the tail. Together with the parenchyma, the ducts of the primitive pancreas also merge (for more details, see [42]). Many factors are involved in these processes, including interactions with the mesenchyme and expression of pancreas-specific transcription factors (see below) [43].

The branching of the human pancreatic epithelium to form the duct system starts at the end of the embryonic period (56 d.p.c.) [8, 40, 44]. At 7 weeks of gestation, a network of tubes of undifferentiated epithelial cells forms, from which the endocrine and exocrine components of the pancreas derive. Thus, in the prenatal period, both endocrine and exocrine cells originate from cells located in the primitive pancreatic ducts.

The endocrine pancreas starts to develop earlier than the exocrine acini. Typical pancreatic lobes form only at 14–16 weeks of gestation. This process becomes more accentuated and dense at the tissue periphery and then throughout the gland between gestational week 25 and gestational week 41 [41].

Data on the timing of appearance of the major types of hormone-containing cells are contradictory; some authors suggest that the glucagon- [45, 46] or somatostatin-containing cells [47] are detected first during human pancreas morphogenesis. However, most authors have identified β-cells first. So, in the studies of Polak et al. [48] and Piper et al. [40], hormone expression, evident as rare epithelial cells immunoreactive for insulin, was first apparent at 10 weeks of gestational development (at 52 d.p.c). One week later (immediately after the embryonic period) at 8.5 weeks postconception (w.p.c.; ≈ 11 weeks of gestational development), glucagon and somatostatin were detected separately in isolated pancreatic epithelial cells. PP-containing cells were identified only at 12 weeks. Data obtained in a study by Jeon et al. [8] are similar: the first insulin-positive cells were detected at the 9th week of gestational development, glucagon-positive cells at the 10th week of development, and somatostatin-positive cells at the 11th week of development. However, the cells containing pancreatic polypeptide were identified by these authors only at the 17th week of development. Differences in the timing of endocrine cell differentiation can be explained by the variability in the antibody clones used in these studies or individual variability [9].

It should be noted that the co-expression of several hormones occurs in the endocrine cells of the developing human pancreas. Some researchers believe that these cells may be the precursor cells of endocrine cells in the human pancreas. The localisation of cells expressing several hormones in single cells and small clusters

indicates that they are present in forming islets [8]. Jensen et al. [49] and Kaligin et al. [50] found common multihormonal progenitor cells of α- and β-cells, which later differentiate into α- and β-cells, synthesising the corresponding hormones. Some of these cells remain within the duct epithelium, but most of them are located in islets in the early postnatal period. However, most authors note that there are very few of these cells [8, 40].

Data on the formation of human pancreatic islets are also controversial. In human studies on prenatal ontogenesis [8, 51–56], various forms of the cytoarchitectural organisation of the endocrine pancreas were identified: single endocrine cells, small endocrine cell clusters, as well as mantle, bipolar, and mixed islets (see above). On this basis, numerous schemes of islet morphogenesis during prenatal human development have been presented. However, there is still no consensus regarding either the number of endocrine pancreas structure forms or the time of their formation in ontogenesis.

In the study by Robb [53], the following stages of islet morphogenesis were identified: (1) formation of islet buds (10–14 weeks), (2) endocrine cell clusters with a capillary in the centre (10–16 weeks), (3) bipolar islets (after 16 weeks), (4) islets of the mantle type (after 20 weeks), and finally (5) mature islets (after 30 weeks). According to van Assche and Aerts [54], the first stage starts only at 16 weeks. In a study by von Dorsche et al. [55], islet morphogenesis was divided into three stages during human prenatal ontogeny. The first phase (14–16 weeks) is characterised by the formation of islet buds. During the second phase (17–20 weeks), islet buds separate from the ducts with the formation of mantle islets. During the third phase (21–26 weeks), mosaic islets are formed.

In the work by Jeon et al. [8], clusters containing several types of endocrine cells were found in the pancreas at 12 weeks of development. Between weeks 8 and 11, the distribution of endocrine cells was scattered, but by week 12, insulin- and/or glucagon-producing cells started to form small clusters; by week 14, these clusters were more prominent, with a core of insulin-positive cells surrounded by glucagon-positive cells, i.e. mantle islets. By weeks 16–17, the ring of glucagon-positive cells surrounding the core of insulin-positive cells appeared to 'open up', and, later, insulin- and glucagon-positive cells had expanded and formed homogeneous insulin-secreting and glucagon-secreting cell clusters.

In our studies, we examined pancreatic autopsies derived from human foetuses from the 10th to the 40th weeks of gestational development, using histological, IHC, and morphometric methods. To analyse the dynamics of pancreatic endocrine cell quantity, we used the classification in which the foetal period of human development can be divided into four subperiods (pre-foetal, 10–12 gestational weeks (g.w.); early foetal, 13–20 g.w.; mid-foetal, 21–28 g.w.; and late foetal, 29–40 g.w.) [44].

The spatio-temporal distribution of various structural forms of endocrine pancreas in general coincides with what has been described in the literature [8, 40, 41, 44, 48, 51, 57]. However, the data obtained in our study allowed us to clarify the main stages of the morphogenesis of the endocrine part of the pancreas during prenatal human development.

In the prefoetal period, only single pancreatic endocrine cells and their small clusters were detected. Starting from 10 g.w., insulin-, glucagon-, and somatostatin-containing cells were identified within primary ducts of the human pancreas. Mantle islets were detected starting from 12 weeks. The diameter of the mantle islets was approximately 70 microns. In prenatal human development, so-called bipolar islets were also identified (β-cells located at one islet pole and α- and δ-cells at the other pole). These bipolar islets were identified starting from 14 weeks. The average diameter of bipolar islets was more than 80 microns. The largest bipolar islets were detected during the early foetal period; their diameter gradually decreased after this point. In adults, bipolar islets are not detected. Currently, the role of bipolar islets in the formation of the endocrine pancreas is not clear. According to some literature

data, homogeneous clusters consisting of only β- or α-cells are present in the human foetal pancreas [8, 51]. In our study, large homogeneous clusters of endocrine cells in close contact with each other were also found. However, during the analyses of serial sections, it was shown that these clusters are parts of bipolar islets. Thus, islets consisting of only one type of endocrine cell are artefacts of two-dimensional images. The typical adult mixed islets appear in the mid-foetal period (after 25 weeks); their number gradually increases until birth. The diameter of these islets exceeds 100 microns. This 'mosaic' (mixed) islet formation seems to be related to the migration processes of different types of endocrine cells within islets.

Thus, various morphological forms of organisation of the endocrine pancreas appear consistently during human pancreas development (**Figure 2**). The first endocrine cells are detected in the central ducts of the developing human pancreas. At later stages of development, the largest pancreatic islets are also located in the central region, around the main pancreatic duct, which corresponds to literature data [8, 48]. During endocrine pancreas development, heterochronous maturation occurs, which may represent a physiological reserve for adaptation to increased metabolic load during this time.

It is important to note that all detected forms of endocrine pancreas organisation are described in pancreas phylogenesis. The chronology of the appearance of various forms of the human endocrine pancreas during ontogenesis repeats the phylogenetic stages of islet formation [58].

In normal physiological development, the ratio of various structural forms of the endocrine pancreas changes: the number of small islets and clusters of endocrine cells with a diameter of 40 to 55 microns gradually decreases, while the number of medium-sized (with a diameter of 55 to 100 microns) and large-sized (with a diameter of over 100 microns) islets increases.

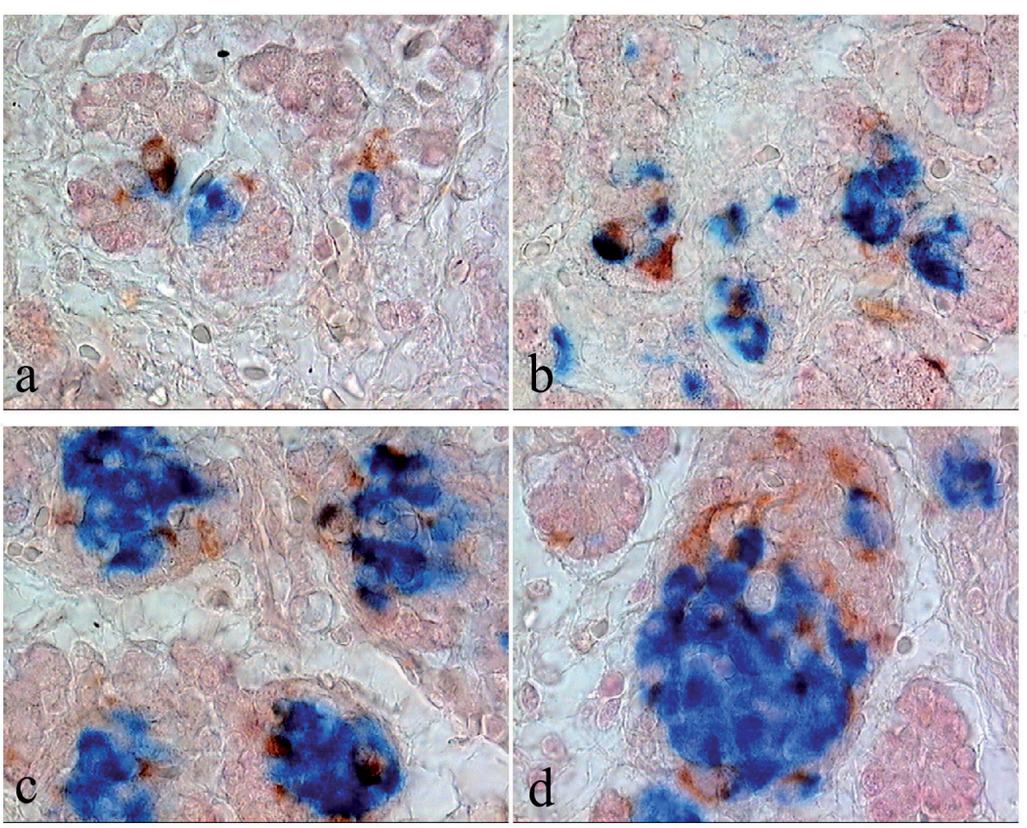

Figure 2.
Forms of endocrine pancreas structural organisation in the human foetus at 22 w.p.c.: (a) single endocrine cell, (b) small cluster of endocrine cells, (c) islet of mantle type, and (d) bipolar islet. Double immunohistochemical labelling with antibodies to glucagon (red) and insulin (blue), objective x100.

Another important issue is the timing of the appearance of mature endocrine cells, similar to those observed in adults. von Dorsche et al. [47, 55] suggested that islet β-cells start to function at 10–14 weeks. In a study by Piper et al. [40], it was shown that endocrine cells are in contact with CD34-positive cells of developing capillaries from the 10th week of development and a thin capillary islet network forms at the 14th week. According to Piper et al. [40], this is evidence of the similarity of pancreatic islets at 12–14 weeks of development with adult islets. Islet innervation starts at 12 weeks. Moreover, so-called neuro-insular complexes may be detected in the developing human pancreas starting at this time [59]. However, Jeon et al. [8] have shown that large pancreatic islets of the mixed type, typical in adults, are formed only after 21 weeks of development. In studies by Meier et al. [35] and Gregg et al. [36], the islets of Langerhans, typical in adults, were formed only at 2 years of age. This was accompanied by an increase in the number of β-cells. Thus, there is an increase in the ratio of β-cells to α-cells, as well as an increase in the ratio of β-cells to δ-cells, both due to an increase in β-cell proliferation and a decrease in the number of δ-cells [36]. The β-cell proliferation index is highest during the first 2 years after birth and then gradually decreases [35, 36].

In general, since the appearance of the first endocrine cells at 9–10 weeks of gestational development and up to 2 years of age, there is a gradual shift towards the predominance of mixed islets, but this prevalence is not complete [8, 35, 36, 53].

Modern ideas about the formation of the human endocrine pancreas are mostly limited to the primary stages of differentiation of endocrine cells and the formation of mantle islets, similar in structure to rodent islets (i.e. the period from 9 to 14 gestational weeks). Although the formation of mantle-type islets in rodents is well described, the formation of mixed islets in the developing human pancreas has been insufficiently studied [8, 9].

4. Transcription control of gene specification during pancreas ontogeny

A good model for studying the differentiation of pancreatic cells is the study of transcription factors, which play an important role in development. As described above, pancreatic endocrine cells are of endodermal origin and differentiate during prenatal development from the epithelial cells of the primary ducts. A specific programme of transcription factor gene expression is subsequently activated and defines the fate of pancreatic progenitors [60]. Through intense research over the last several years, a hierarchy of transcription factors regulating pancreas development has emerged. The expression of the same transcription factors as in mice and rats was found in human pancreatic samples using IHC methods, in situ hybridisation, and molecular biology methods (RT-qPCR, microarray). For some transcription factors, the dynamics of changes in expression levels were revealed during pancreas development [8, 40, 61, 62].

Early markers of emerging pancreatic cells include two transcription factors: pancreatic and duodenal homeobox 1 (Pdx 1) and pancreas-specific transcription factor 1a (Ptf1a). In mice, the initial expression of Pdx1 (E8.5–E9.0) marks the pre-pancreatic endoderm before it has visibly thickened. Early Pdx1 expression is therefore a useful marker of pancreatic identity, although it expands over the next several days of development to encompass the posterior stomach, duodenum, and bile duct. Although early pancreas buds still form, Pdx1 knockout mice show completely arrested pancreas organogenesis after the initial stages. Lineage tracing analysis has revealed the contribution of Pdx1-positive cells to all adult pancreatic fates. In the adult pancreas, Pdx1 is most highly expressed in β-cells and δ-cells, with lower expression in exocrine cells [7]. In humans, Pdx1 has been detected in

cell nuclei at the inception of pancreatic bud outgrowth from the duodenum at 26 d.p.c. [40]. Pdx1 detection was initially accompanied by very weak detection of SOX9 in the presumptive pancreatic endoderm [62]. This expression was much more robustly detected in all pancreatic epithelial cells at 41 d.p.c. and during the early foetal period. Following islet formation, Pdx1 remained in the nuclei of non-endocrine epithelial cells. However, a more diffuse pattern of staining was observed in islet cells, consisting of both cytoplasmic and nuclear detections. By dual immunofluorescence at 14 w.p.c., this expression colocalised strongly with insulin. Later, during human foetal development and in adult pancreatic sections, Pdx1 expression remained in duct cells but was most strongly detected in islets, in keeping with its established role in glucose-regulated insulin production [40]. According to Jeon et al. [8], IHC analyses showed that IPF1, the human homologue of Pdx1, is expressed in epithelial progenitor cells throughout the period from 7 to 21 weeks, as well as in insulin-expressing cells as they appear. However, IPF1 expression was also observed in occasional glucagon-expressing cells at early but not at later stages of development. The expression of IPF1 in glucagon-expressing cells was weaker than that observed in pancreatic progenitor cells or in insulin-expressing cells. In the absence of Pdx1 expression, pancreas aplasia has been observed, with impaired growth of the dorsal diverticulum [63, 64]. Haploinsufficiency of IPF1 leads to maturity onset diabetes of the young (MODY4), an autosomal dominant form of diabetes caused by monogenic mutations [64]. Recent reports further emphasise the important role of Pdx1 in β-cell formation and function. Thus, Pdx1 plays essential roles in both pancreas development and adult islet function [7].

Another transcription factor, Ptf1a, is also expressed in the early pancreas, and its endodermal expression remains pancreas-specific throughout development. Ptf1a expression is necessary for exocrine differentiation [65], as well as endocrine cells [66]. In the absence of Ptf1a expression, the differentiation of exocrine cell precursors into duodenal cells has been observed, as well as the localisation of a small number of endocrine cells in the spleen. Consistent with its later phase of expression, Ptf1a has been identified as an acinar gene activator, and Ptf1a-deficient pancreata entirely lack acinar cells [67]. Thus, the expression of these factors plays crucial roles in pancreas specification and is necessary for normal pancreas development.

The transitions of Pdx1 and Ptf1a expression coincide with the overall conversion of progenitors into mature endocrine and exocrine cells. This conversion is also reflected in the dynamic expression of the transcription factor neurogenin 3 (Neurog3, also known as Ngn3), which specifically marks precursors of all endocrine pancreas cells [49, 67–69]. Ngn3 is a basic helix-loop-helix protein and marks the progenitor population of cells fated to form the endocrine lineage. Animals lacking Ngn3 are devoid of islets and die shortly after birth due to hyperglycaemia [68]. Within the embryonic pancreas, Ngn3 expression peaks at embryonic day 15.5, shortly after the secondary transition that indicates the burst of endocrine specification, and subsequently declines. Ngn3+ cells appear in small numbers in the early organ [7]. Expression of Ngn3 peaks by the end of the first trimester and disappears at about the 35th week of gestation [70]. Probably, similar to neurons, islet cells are ordinarily generated during a restricted developmental window [67]. In general, ectopic expression of Ngn3 causes cells to exit from the cell cycle and to express some endocrine markers [71]. Inactivation of Ngn3 leads to the failure of pancreatic islet development [68]. However, it is clear that mere overexpression of Ngn3 does not guarantee expansion into the β-cell lineage. In fact, in the majority of studies, increased glucagon- or somatostatin-expressing cells are observed [69].

In contrast to published data from mouse embryos, during human pancreas development, Jennings et al. [62] detected only a single phase of Ngn3 expression and endocrine differentiation from approximately 8 weeks, before which Nirenberg

and Kim homeobox 2.2 (NKX2.2) was not observed in the pancreatic progenitor cell population. The expression of Ngn3 has been detected in the human pancreas at the 7th week of development. By the 9th week of development, the expression level of this transcription factor gradually increases and remains high until the 17th week, after which it gradually decreases [8]. Most authors have paid special attention to cells that co-express transcription factors and hormones of endocrine cells of the pancreas [61], considering these cells to be a population of progenitor cells. In the early (8–11) weeks of gestation of the human foetal pancreas, cells co-expressing Ngn3 and Pdx1, Ngn3 and insulin, and Ngn3 and glucagon have been observed.

Ngn3 controls the expression of a number of other islet-specific transcription factors, i.e. NeuroD1, Pax4, and Pax6. During pancreas development, NeuroD1 also takes part in the differentiation of endocrine cells and the morphogenesis of pancreatic islets. A reduction in the number of endocrine cells, especially β-cells, and the inability to form islets were shown in neonatal mice missing a functionally active NeuroD1 gene [72]. Unlike Ngn3+ precursors, cells expressing NeuroD1 do not proliferate (they leave the cell cycle before the activation of NeuroD1 expression) [49]. Pax4 is necessary for the differentiation of β- and δ-cells from Ngn3+ precursors, and Arx is required for the differentiation of α- and PP-cells from Ngn3+ precursors. These two transcription factors have the opposite effect (they inhibit each other) and regulate the ratio of various types of endocrine cells. In the absence of Pax4 expression (Pax4−/−), β- and δ- cells are not detected in islets [73]. In the absence of Arx expression (Arx−/−), islets are composed only of β- and δ-cells [74]. Pax6 is expressed in all types of endocrine cells; cells expressing Pax6 differentiate into α-cells, and cells simultaneously expressing Pax6 and Pax4 differentiate into β-, δ-, and PP-cells [74]. Inactivation of Pax6 leads to a decrease in the number of endocrine cells, especially α-cells, and the disorganisation of pancreatic islets [75]. Inactivation of Pax6 and Pax4 simultaneously leads to the absence of endocrine cells.

The expression of Pax4 (a marker of β-cell precursors) begins at the 9th week of human development, and the expression of Arx (a marker of α-cell precursors) begins at the 11th week [8] . Transcription factors (Nkx2–2 and Nkx6–1) play a regulatory role in the development of the endocrine part of the pancreas. The absence of Nkx6–1 expression in deficient mice leads to impaired β-cell differentiation [76]. In mice lacking Nkx2–2, there is a lack of β-cells and a decrease in the number of α-cells and PP-cells [77]. A number of transcription factors that are expressed during the differentiation of endocrine cells of the pancreas are also characteristic of the differentiation of nervous system cells [49, 68, 69, 71, 72, 78, 79]. Expression Nkx2.2 and Nkx6.1 has been detected in the human pancreas from week 7 of development, gradually increasing from week 7 to week 21 [8].

Thus, the use of transcription factors allows us to estimate the direction and features of differentiation of cellular populations in the pancreas. Data on the localisation of cells expressing various transcription factors in the developing human pancreas are few. It is important to note that, in the adult human pancreas, the maintenance of β-cell identity is associated with the presence of key transcription factors (in particular PDX1 and NKX6.1) and changes in their expression and/or localisation have been described in the islets of type 2 diabetic individuals, possibly contributing to β-cell dedifferentiation (i.e. the regression to a progenitor-like state) in this disease [70].

In our studies we have analysed the distribution of two transcription factors—NeuroD1 and Nkx6.1—in the developing human pancreas. The study was performed on pancreatic samples from 22 foetuses (gestational age 10–36-37 weeks) using double and triple immunohistochemistry with antibodies to NeuroD1 (mouse monoclonal; Abcam), Nkx6.1 (rabbit polyclonal; Thermo Fisher Scientific, Inc.), insulin (mouse monoclonal, Sigma), and glucagon (mouse monoclonal, Sigma, or

rabbit polyclonal; Thermo Fisher Scientific, Inc.). Reactions of double immunohistochemical labelling were visualised using the MultiVision Polymer Detection System Anti-Rabbit HRP + Anti-Mouse AP, LV blue and LV Red (Thermo Fisher Scientific, Inc.) according to specification. In the reactions of triple immunohistochemical labelling, at the first phase, NeuroD1 was visualised using UltraVision ONE Detection System, DAB Plus Chromogen (Thermo Fisher Scientific, Inc.). At the second phase, insulin and glucagon were visualised using MultiVision Polymer Detection System Anti-Rabbit HRP + Anti-Mouse AP, LV blue and LV Red (Thermo Fisher Scientific, Inc.).

In agreement with the literature [8, 61], we showed that NeuroD1 and Nkx6.1 are present in the foetal pancreas already at 8 w.p.c. (10th g.w.). In the prefoetal period (10–12 g.w.), immunopositive reaction to NeuroD1 and Nkx6.1 was detected in the nuclei of single epithelial cells of primitive ducts (**Figures 3a** and **4a**). At these stages, the intensity of the reaction to both NeuroD1 and Nkx6.1 was very low. The reaction to these transcription factors became more intensive by the beginning of the early foetal period (14 g.w.) (**Figures 3b** and **4b**) and remains high in all subsequent stages of development. The same results demonstrating that the expression level of NeuroD1 was initially low and increases starting from week 15 onwards and the expression of Nkx6.1 gradually increases from the 7th to the 21st weeks of development were shown by other authors [8].

As it was previously shown, in the pancreas of foetuses from 8 to 21 weeks of development, NeuroD1 is colocalised with insulin, glucagon, somatostatin, and pancreatic polypeptide indicating the involvement of this transcription factor in the differentiation of all four types of endocrine cells [61]. At the same time, positive reaction to Nkx6.1 was detected only in the insulin-containing cells, suggesting that Nkx6.1 is β-cell-specific transcription factor.

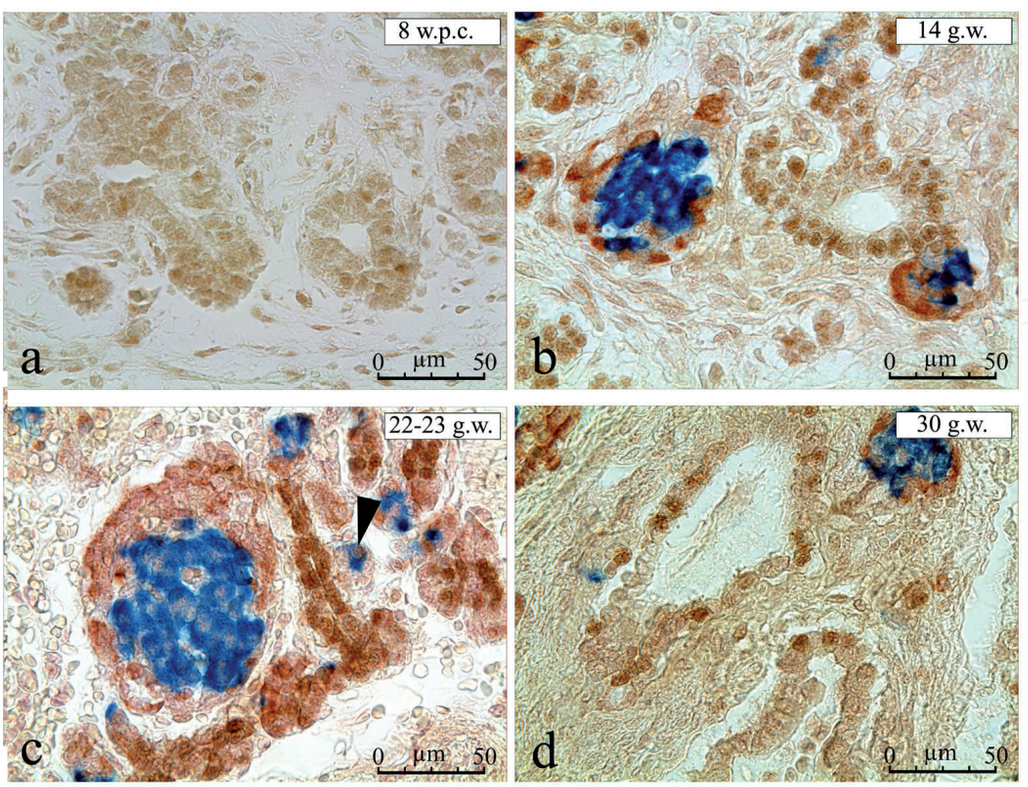

Figure 3.
Distribution of the transcription factor NeuroD1 in the human foetal pancreas. (a) Immunohistochemical staining to NeuroD1 in the foetal pancreas at 8 w.p.c. (b–d): triple immunohistochemical staining to NeuroD1 (brown nuclei), insulin (blue), and glucagon (red) in the foetal pancreas at 14 g.w. (b), 22–23 g.w. (c), and 30 g.w. (d). Arrowhead indicates β-cell with NeuroD1-positive nucleus.

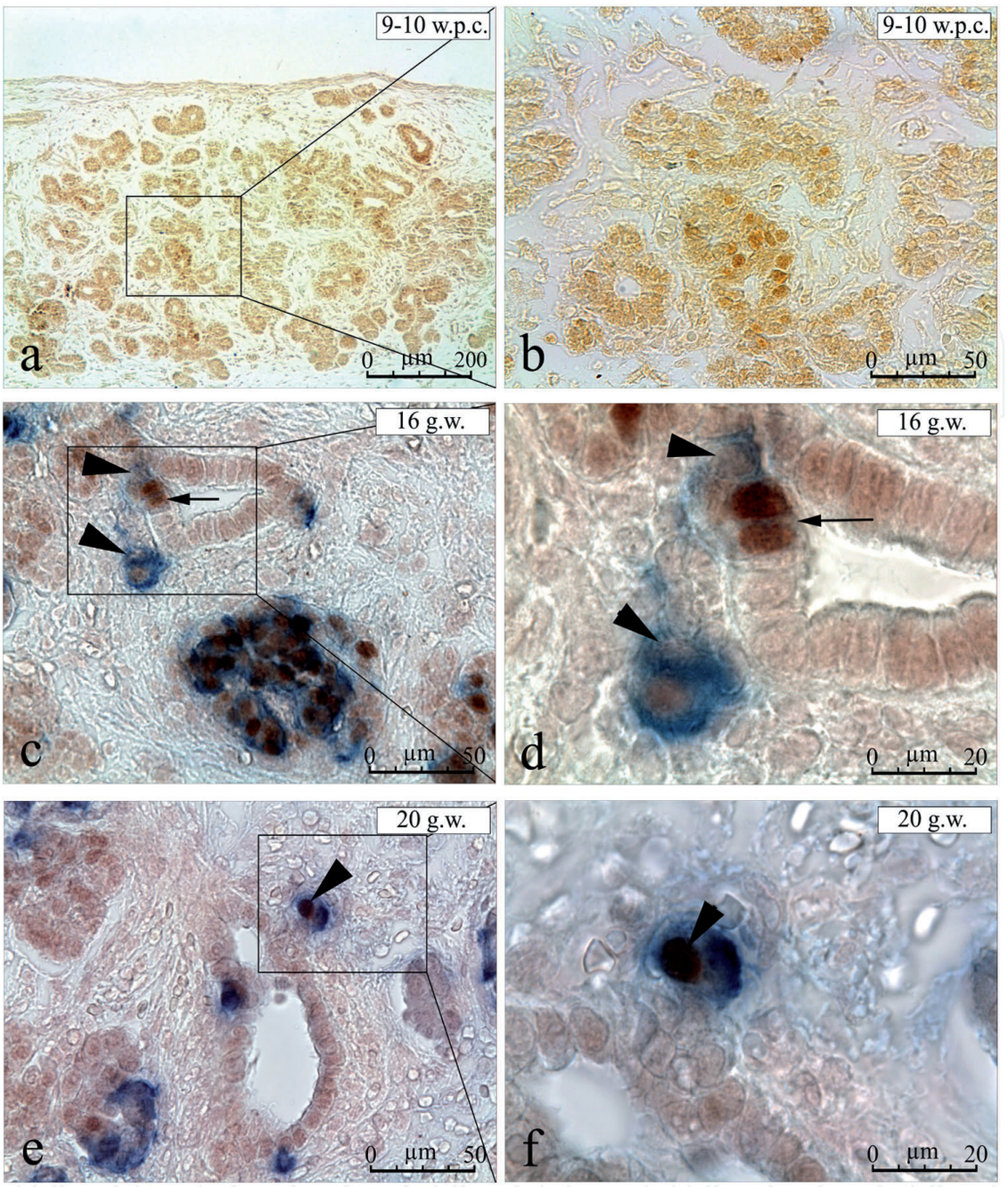

Figure 4.
Distribution of the transcription factor Nkx6.1 in the human foetal pancreas. (a, b) Immunohistochemical staining to Nkx6.1 in the foetal pancreas at 9–10 w.p.c. and (c, d) double immunohistochemical staining to Nkx6.1 (brown) and insulin (blue) in the fetal pancreas at 16 g.w.; arrowheads indicate β-cells with Nkx6.1-negative nuclei; arrows indicate hormone-negative cells with Nkx6.1-positive nuclei. e, f - double immunohistochemical staining to Nkx6.1 (brown) and glucagon (blue) in the fetal pancreas at 20 g.w.; arrowheads indicate α-cells with Nkx6.1-positive nucleus. b, d and f – marked fragments of a, c and e at higher magnification.

Our findings demonstrate that the distribution of NeuroD1 and Nkx6.1 in the developing human pancreas was different. The amount of NeuroD1-positive cells gradually increases during prefoetal period, and, in the early and middle foetal periods, such cells were more numerous. Starting from 14th g.w. onwards, positive reaction to NeuroD1 was observed predominantly in the epithelial ductal cells (**Figure 3b–d**). In the majority of endocrine islet cells, the reaction to NeuroD1 was immunonegative. However, in all investigated pancreatic samples, we found single β- and α-cells with NeuroD1-positive nuclei (**Figure 3c**), which is in agreement with the literature [61]. In the late foetal period, NeuroD1 is also expressed predominantly in the epithelial ductal cells, but the distribution of NeuroD1-positive cells became sparser (**Figure 3d**).

Starting from the beginning of early foetal period (14 g.w.) and in all subsequent stages of development, positive reaction to Nkx6.1 was observed predominantly in insulin-containing β-cells (**Figure 4c–f**) which indicates the involvement of Nkx6.1 in β-cell differentiation. In all investigated pancreatic samples, we observed two types of β-cells: β-cells with the Nkx6.1-positive nuclei which represent the majority of β-cells and rare population of β-cells with the Nkx6.1-immunonegative nuclei (**Figure 4c,d**). In addition, in the pancreas of all foetuses from 14th to 36–37th g.w., we identified hormone-negative cells with Nkx6.1-positive nuclei (**Figure 4c,d**). It can be suggested that Nkx6.1 is transiently expressed in the differentiating β-cells prior to hormone synthesis, and then its expression decreases and is further restored in the mature β-cells. On the other hand, differentiation of some β-cells may occur without Nkx6.1 expression. As it was shown in mice lacking Nkx6.1 (Nkx6.1−/−) expression, Nkx6.1 does not affect the early stages of pancreas development and β-cell differentiation [76]. The defect in Nkx6.1 expression appears after E12.5 and results in the significant reduction of the amount of β-cells (up to 85% of wild type) and in the absence of the expression of the mature β-cell markers MAFA and Clut2 [76, 78].

In contrast to other researchers [61], we found rare glucagon-containing cells with Nkx6.1-positive nuclei in the developing human pancreas (**Figure 4e, f**). Activation of Nkx6.1 expression in α-cells has been previously observed in a mouse model after extreme β-cell loss [17] and in humans with type 1 diabetes [80]. This activation of Nkx6.1 possibly indicates α- to β-cell conversion during restoration of β-cell mass [17] or a partial change of α-cells towards a β-cell phenotype [80].

5. Conclusions

This chapter has provided a brief analysis of the current state of research in the field of the origin of pancreatic endocrine cell populations and islet growth. However, further research must be performed to deepen our understanding of these processes, due to their fundamental importance in the restoration of normal glucose homeostasis in humans with impaired carbohydrate metabolism.

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Conflict of interest

The authors declare no competing interests.

Author details

Alexandra E. Proshchina[1*], Yuliya S. Krivova[1], Larisa E. Gurevich[2], Valeriy M. Barabanov[1], Dmitriy A. Otlyga[1], Iya A. Voronkova[3] and Sergey V. Saveliev[1]

1 Research Institute of Human Morphology, Moscow, Russia

2 Moscow Regional Clinical Research Institute n.a. M.F. Vladimirskiy, Moscow, Russia

3 Endocrinology Research Centre, Moscow, Russia

*Address all correspondence to: proshchina@yandex.ru

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