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In vitro-produced pancreas organogenesis models in three dimensions: self-organization from few stem cells or progenitors

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Abstract

Three dimensional models of organ biogenesis have recently flourished. They promote a balance between stem/progenitor cell expansion and differentiation without the constraints of flat tissue culture vessels, allowing for autonomous self-organization of cells. Such models allow the formation of miniature organs in a dish and are emerging for the pancreas, starting from embryonic progenitors and adult cells. This review focusses on the currently available systems and how these allow new types of questions to be addressed. We discuss the expected advancements including their potential to study human pancreas development and function as well as to develop diabetes models and therapeutic cells.

Keywords

stem cells; therapy; diabetes; modelling; mechanics; bioengineering

Introduction

Stem cells can be studied *in vivo* in their chemically and structurally complex biological niche, or, *in vitro* where their environment is synthetic and often 2-dimensional (2D) but can be tightly controlled. The artificial nature of *in vitro* culture systems is tempered with the advantages they convey in expansion, essential for experiments requiring large amounts of otherwise unobtainable stem cells. Hybrid systems where stem and progenitor cells are cultured in 3 dimensions (3D) have recently emerged combining the simplicity and

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controllability of *in vitro* culture with the possibility to reconstitute niches more similar to the natural niche. Pivotal examples are the generation of mini-organs *in vitro* from adult organ stem cells, embryonic progenitors or embryonic stem (ES) cells, with structural and functional similarities to parental organs such as intestine, stomach, liver, optic cup, and brain (1–7).

In contrast to organotypic culture, 3D hybrid systems offer the potential to initiate mini-organs from one or few stem or progenitor cells, which can be defined by cell sorting. The mini-organs are tools to decipher the potency of stem cells, the nature of their niches and the development of the organ structure in a self-organizing process (7,8). When starting from human stem cells, they can be powerful tools to generate 3D models of human organs. This review focuses on the most recent advancements in *in vitro* 3D culture systems of pancreatic cells and their potential use for understanding pancreas development, homeostasis and regeneration as well as for cell therapy and disease modeling.

In vivo pancreas organogenesis: a progressive loss of multipotency?

The pancreas is a compound gland that serves exocrine and endocrine functions which regulate two major processes: digestion and metabolism. The exocrine pancreas consists of acinar and ductal cells, while the endocrine pancreas consists of hormone-producing cells: alpha-, beta-, delta-, and pp-cells respectively producing glucagon, insulin, somatostatin and pancreatic polypeptide. The pancreatic primordia arise from the foregut endoderm, starting from a group of multipotent progenitors expressing the transcription factors PDX1, PTF1a, SOX9 and HNF1b (9). These cells undergo massive proliferation, sustained by active epithelial Notch signaling and initially by mesenchymal FGF10. Progenitors become progressively polarized and organized into a branched tubular system. This morphogenetic process is accompanied by a potency restriction: the $PTF1a^+$ cells at the “tips” of the tubes become irreversibly committed to an acinar fate, while cells in the “trunks” ($HNF1b^+$ and $SOX9^+$) remain bipotent approximately until birth and give rise to both endocrine and ductal cells (10–12).

Pancreas organogenesis follows a highly regulated tridimensional development and the correct specification of each cell type not only relies on the proper intra and inter-cellular signaling, but also requires architectural cues. Indeed apico-basal and planar polarity complex activities are required for appropriate endocrine cell differentiation (13,14).

Many lineage tracing systems have been elegantly used to investigate the latent differentiation potential of embryonic progenitors and adult pancreatic cells, under normal or regenerative conditions, in order to identify an elective adult stem cell reservoir. Under homeostatic conditions, alpha, beta, ductal, and acinar cells are maintained by self-duplication of pre-existing cells (15–17). Most of the lineage tracing studies have shown that ductal and acinar cells do not contribute to the endocrine compartment in the adult under homeostatic conditions (11,12,18,19), although controversies do exist (20) based on issues of specificity and biases of tracer lines. A different scenario has been described in the context of pancreas regeneration after severe damage. It has been demonstrated that *de novo* beta cells can be derived from the trans-differentiation of alpha cells (21), rarely from acinar

cells (22) and possibly from ductal cells (23), although this latter case is still questionable (11,24). Indeed, while in the model of pancreatic duct ligation, the induction of *Ngn3* expression has been univocally demonstrated, the effective maturation of newly generated beta cells has not been proved. The plasticity of pancreatic cell types appears to be highly dependent on the type of injury and can also be uncovered by transcription factor reprogramming (25).

To summarize, multipotent pancreas progenitors have been exquisitely characterized during development, while the existence of adult multipotent stem cells remains debatable and their nature and markers elusive.

In vitro pancreas organogenesis

Acinar, alpha, beta and ductal cell lines do exist but there is no cell line that behaves like a pancreas progenitor or stem cell. *In vitro* culture of primary cells fills the gap between immortalized cell cultures and whole-animal systems in many fields. The culture of dissociated primary cells in 2D has proven to be challenging for the pancreas and isolated pancreatic progenitors were maintained at best for a few days in small numbers (18,26). However, innovative protocols for the 3D culture of *bona fide* embryonic pancreatic multipotent progenitors were recently published and fall into two categories that lead to cell expansion in hollow spheres or branched organoids (Fig. 1).

Pancreatospheres

Sugiyama at al. described an approach to isolate multipotent embryonic progenitors based on the positive selection for SOX9 presence and a negative selection for NEUROG3 (27) (Fig. 1b). They demonstrated that these E11.5 mouse pancreatic progenitors diluted to clonal density proliferate *in vitro* in solidified Matrigel. They form epithelial polarized $SOX9^+$ spheres with a subpopulation of $SOX9^-$ cells expressing C-peptide, glucagon and NEUROG3. This expansion protocol required the presence of mesenchymal cells in co-culture and a defined culture medium, containing B27, FGF10, RA, IGF1, insulin and several other components. The cells in contact with the inner lumen of the spheres expressed Mucin1, as observed during pancreatic tubular network formation. Genetic manipulations of these cells in culture confirmed the conservation of differentiation pathways that had been first described *in vivo*. The spheres could be passaged: however, the expansion could be achieved only from partially dissociated clusters of $SOX9^+$ cells and, after 3 passages, a decrease in multipotency was documented.

Of note, culturing progenitors at physiological oxygen levels (5%) induced an increase of several endocrine markers, at the expense of cell proliferation. As a result, this culture system allowed the generation of glucose-responsive $Insulin^+$ cells, which accounted for 20% of the total epithelial cells in culture. In *ex vivo* pancreatic explants vascularization and oxygenation have been shown to promote endocrine differentiation (28,29): thus, it is unclear whether low oxygen has an effect on differentiation or rather on selection in the pancreatosphere model.

Multiple culture conditions are likely to promote the expansion of pancreas progenitors in spheres. We independently produced similar results from E10.5 progenitors cultured in Matrigel with B27, FGF2 and the ROCK-inhibitor Y-27632 (30) (Fig. 1a). Detailed time-lapse imaging with a nuclear progenitor marker revealed strong cooperative effect in pancreatic progenitors. If a single progenitor had a 2% probability to form a sphere, groups of 2 or 4 cells had, respectively, 50 and 90% chances of generating spheres *in vitro*. The resulting spheres resembled the structures obtained by Sugiyama et al., with homogeneous expression of progenitor markers such as SOX9, PDX1 and HNF1b and rare endocrine and acinar cells.

In these culture systems cells expand for weeks and appear to be molecularly similar to the *in vivo* transient early progenitors with acinar, ductal and endocrine potential. Whether the different media expand exactly the same cells remains to be investigated.

Mini-pancreas/pancreas organoid

We have recently identified conditions that recapitulate normal pancreas development in 3D, so that dissociated progenitors expand, mature, differentiate and self-organize into a miniature organ in a dish (30) (Fig. 1a). Also in Matrigel, these organogenesis conditions are based on a medium whose important components are knockout serum replacement, high levels of FGF10 whose activity was potentiated by heparin, the ROCK-inhibitor Y-27632 and other factors that increase the efficiency of progenitor expansion (R-spondin1, EGF). Pancreatic organoids formed from clusters of at least 4 cells, leading to 100% organoid formation from clusters of more than 12 starting cells. Under these culture conditions, progenitors expanded massively, relying on FGF and Notch signaling similarly to *in vivo*. This proliferation was accompanied by impressive architectural rearrangements, which lead to the generation of pancreas-like organs *in vitro*. This includes polarization into a ductal network, a proper segregation of acinar cells to the tips of the *in vitro*-formed tubes, while endocrine cells and HNF1b/SOX9-double positive cells were confined to the central core. Again, it appears that different culture conditions may lead to the formation of similar organoids since Sugiyama et al. described organoids after the exposure of progenitors to high Wnt levels in the absence of retinoic acid.

These systems recapitulate many aspects of pancreas development but the degree of maturity and functionality of differentiated exocrine and endocrine cells remain to be evaluated as well as the extent of the similarities between culture methods.

In vitro expansion of adult ductal cells with endocrine differentiation potential

The primary culture of adult pancreatic cells has been reported for the three main adult populations (acini, ducts and islets). The differentiated exocrine and endocrine cells plated in 2D rapidly lose their differentiated characteristics while suspension culture, particularly for islets, enables maintenance for a few weeks but without expansion (31–33).

Clonal suspension culture from non-ductal sources

Although the existence of multipotent stem cell populations in the pancreas is still debated, tissue culture may uncover rare populations or latent properties. Starting from dispersed adult islet or ductal populations in suspension cultures, Seaberg et al. identified cells that could form colonies leading to differentiation of insulin-expressing cells and neurons, both in mouse and human (34), in the presence of FGF2 and EGF. Subsequent work showed that insulin-expressing cells reduce several differentiation markers as they expand: however, it is still unclear whether a specific insulin-expressing population can expand under these conditions or all beta cells do so at low frequency (35).

An alternative source for adult pancreatic stem cells could be the population of centroacinar cells, first described as unique domains of active Notch/HES1 signaling in the adult pancreas rapidly proliferating following pancreatic injury (36,37). Rovira et al. demonstrated that this population is characterized by the expression of ALDH1 and E-cadherin, a molecular signature used to isolate them (38). These cells could clonally expand to form pancreatospheres in suspension in the presence of EGF, FGF2, LIF and serum and could be passaged. The resulting spheres were composed of acinar and endocrine cells as well as a small $SOX9^+$ population, which would deserve a deeper characterization. These culture conditions may uncover the ability of exocrine or centroacinar cells to become endocrine under exceptional circumstances not found in homeostatic conditions *in vivo* (15,19,39–41).

Clonal culture from adult pancreatic ducts

More recently at least three independent groups published reports on the long-term culture of adult ductal cells. Albeit different, they all rely on the use of Matrigel as a 3D scaffold for the generation of spheres. Some common traits are also present at the level of soluble factors, such as the use of R-spondin1 in all the protocols, even with slightly different effects.

Jin et al. first reported a method to clonally expand ductal $CD133/SOX9^+$ cells from adult mice (42) (Fig. 2a). Those cells were cultured in a methylcellulose/5% Matrigel-based semisolid medium, ESC-derived pancreatic-like cell conditioned medium and FCS supplemented with Nicotinamide, activin B, exendin-4 and VEGF-A. The spheres in culture were mainly composed of ductal-like cells, but cells expressing very low levels of acinar and/or endocrine markers were detected. The addition of R-spondin1 promoted the growth of these colonies over 11 weeks. Interestingly, R-spondin1 also induced the generation of compact high-cellularity colonies with higher endocrine progenitor markers. PolyEthyleneGlycol(PEG)-laminin increased endocrine and acinar differentiation although it is unclear whether this promotes differentiation or selection.

Along the same lines, the groups of H. Clevers and H. Heimberg reported on the unlimited expansion of adult ductal cells (43) (Fig. 2b). Ductal cells could be clonally expanded in Matrigel in the presence of EGF, R-spondin1 and FGF10. Additional factors such as Noggin and Nicotinamide were proven to be essential for cultures longer than 2 months. In particular, it was shown that *in vitro* culture promoted the enrichment of a small $LGR5^+$ cell population, a marker that is not expressed in the pancreas unless it is injured by partial duct

ligation. The spheres grown *in vitro* maintained an adult duct signature. A latent endocrine differentiation potential was revealed after transplantation *in vivo* under the kidney capsule following aggregation with embryonic pancreatic cells. How similar these cells are to endogenous endocrine cells remains to be addressed.

The possibility to expand adult ductal populations in spheres *in vitro* was recently confirmed in human (44) (Fig. 2c). The culture system was very similar to the reports from other groups: it required Matrigel and the soluble factors EGF, R-spondin1, FGF10, Noggin and Nicotinamide. These cells could be passaged up to 3 months. Although no endocrine cells were found to spontaneously differentiate *in vitro*, adenoviral overexpression of NEUROG3, MAFA, PDX1 and PAX6 converted the ductal cells into endocrine progeny with many hallmarks of beta cells (synthesis, process, storage and secretion of insulin in response to glucose or depolarization stimuli).

These extensive investigations have shown that adult pancreatic populations can be expanded *in vitro* and are amenable to endocrine differentiation, especially after overexpression of transcription factors and *in vivo* maturation. The degree of differentiation without transcriptional reprogramming needs to be further explored.

How has 3D *in vitro* culture advanced our understanding of pancreas biology?

The 3D culture systems of embryonic progenitors and adult ductal cells begin to fill the gap between animal models and immortalized cell lines for the pancreas. These systems increase our toolset, providing different angles to study pancreas development and adult cell potential at the single cell level and have already uncovered new aspects of pancreas biology.

The pluripotency of embryonic progenitors has been well studied by *in vivo* lineage tracing however the evidence that adult ductal cells can give rise to endocrine cells is under debate and probably restricted to injury conditions. The culture of ductal cells may simulate injury conditions, leading to the activation of LGR5 and Wnt signaling (43). The three adult studies suggest that the potential to give rise to endocrine cells does exist in the ducts but requires very strong reprogramming with transcription factors, along with an appropriate milieu for maturation. However, the large number of ductal cells that can be cultured *in vitro* for a long period opens the way to multiplexed screening for conditions that promote endocrine differentiation or other aspects of development as pioneered by Sugiyama et al. (27).

These methods also offer a setting to decipher the components of the niche of pancreas progenitors, as well as normal and regenerating adult ductal cells. It is notable that a key component of all culture systems is Matrigel. Matrigel has been replaced by PEG-laminin (30,42), although with less efficient outcomes. This suggests that Laminin is a key component of Matrigel for embryonic and adult duct culture and their endocrine differentiation. Laminin is naturally abundant in the basal lamina lining these cell types *in vivo* although the type of Laminin may matter. Mesenchymal cells were excluded from most culture systems but one (27). While the mesenchyme has an important role during

development as component of the niche, it can be replaced by FGF10, which it naturally secreted, and possibly other components in the Matrigel and B27 or KOSR culture supplements. The mesenchyme is also likely to provide mechanical support, which is replaced by the gel structure of Matrigel, and preliminary evidence suggests that the stiffness of the gel matters (30).

Another component of multiple culture systems is R-spondin1/Wnt. Several groups have already demonstrated the importance of Wnt activity for pancreatic progenitors and their commitment towards the endocrine and acinar compartments *in vivo* (45–50). The *in vitro* data suggest that R-spondin1, although dispensable, sustains longer expandability of ductal cells and possibly promotes embryonic endocrine differentiation (27,42). The epithelial or mesenchymal *in vivo* source of these Wnts remains to be identified.

Single cells can give rise to expanding spheres: nevertheless there is evidence that other epithelial cells contribute to the niche. Indeed, spheres and organoids formed more efficiently from multiple cells (30). It remains to be determined whether this is due to different cell types interactions, possibly via the Notch pathway, as shown for intestinal organoids that expand upon NOTCH ligand stimulation from Paneth cells (51). Although Notch signaling promotes sphere and organoid expansion from embryonic progenitors, endocrine cells are not the sole source of NOTCH ligands since their absence does not preclude expansion (30). The role of this pathway in adult duct expansion remains to be investigated.

In the context of community signals, an additional layer of regulation could be offered by mechanobiological forces. From this perspective, the interaction among cells providing the community effect that maintains progenitor properties (30) and the interaction between cells and the matrix can be both studied as geometric constraints. Indeed, both interactions result in the generation of traction or compression forces that impact the stiffness of cellular cytoskeletons and reciprocally, the surrounding structures across adhesion sites. We have provided evidences that adherent culture of pancreatic progenitors as well as the encapsulation in stiff matrices is not permissive for pancreatic progenitor maintenance and growth. In addition, we have shown that the tensional state of pancreas progenitors dictated by cytoskeletal dynamics tightly controls cell proliferation and identity, as shown by their potentiation by inhibition of ROCK or downstream actin dynamics to initially sustain embryonic pancreatic progenitors (30).

An important challenge in the generation of 3D pancreas models *in vitro* is now to combine engineering and biology in order to provide better understanding of these cellular forces and exploit this knowledge (52,53). The development of new matrices that are more controllable than Matrigel, with a wide range of stiffness and with the ability to pattern precisely anchoring peptides, may answer these still unsolved questions and finally help to decipher the complexity of pancreas development, function and disease.

How can 3D *in vitro* culture advance our approach to disease and therapy?

A stemness assay

The ability to grow cells that retain the potential to generate appropriate differentiated progeny from single adult cells has been used as a surrogate marker for stemness in many organs and is now available for the pancreas. This will enable the characterization of populations sorted for different markers and the assessment of the signaling pathways required for stem cells/progenitors under homeostatic, regenerative or diseased conditions. In particular, these assays could also be used to identify the tumor-initiating cancer stem cells in pancreatic ductal adenocarcinoma, whose identity has only recently been tackled (54). Spheres and organoids are amenable to live-monitoring of single cell dynamics with live reporters/genetic tracers for visualization of individual cell behaviors in the context of a developing surrogate organ.

Many cells for biochemistry and screening

The expansion of low abundance cell populations will enable to perform experiments to assess protein amounts, modifications and molecular interactions between proteins, DNA or RNA. In addition to this, the *in vitro* culture systems opened the path to ShRNA (27), small molecule or matrix screens (30). Although still at a small scale, further developments are expected to enable scaled-up screenings to test the effect of individual or combined compounds on differentiation, stemness and morphogenesis.

Genetic manipulations were also demonstrated to be possible in culture, as for example the deletion of floxed alleles (using CreERT2 in conjunction with tamoxifen) (27) and are expected to improve owing to the recent development of more powerful genetic manipulations.

Models of human development and disease

An exciting perspective is the production of 3D model systems of human pancreas development and possibly function and disease. In principle, embryonic human pancreatic cells could be used for the purpose but considering their scarcity and ethical considerations, the breakthrough is more likely to come from the conversion of human ES and induced pluripotent stem cells (iPSCs) to 3D pancreas, as done for other organs such as the intestine, eye or brain (4–6). The aforementioned genetic tools may be applied to introduce disease-alleles and develop disease models, particularly for genetic diseases such as Maturity Onset Diabetes of the Young. Patient-derived disease models could also potentially be developed via iPSCs in order to better understand the disease itself and to screen for compound and/or metabolites that could alleviate/treat the syndrome.

Production systems for replacement cells

Type 1 diabetes is caused by an almost complete loss of beta cells due to an auto-immune attack. Theoretically, the missing cells could be generated *in vitro* and grafted in an immune-suppressed patient thus curing the disease. The generation of iPSCs from adult somatic cells has rendered regenerative medicine even more appealing (55).

However, the generation of functional beta cells *in vitro* starting from ESCs/iPSCs has proven to be more challenging than initially anticipated, since the Insulin$^+$ cells produced exclusively *in vitro* do not properly respond to glucose (56). Since 3D cues such as apico-basal and planar polarity complex activities are needed for appropriate endocrine cell differentiation (13,14), it is possible that 3D culture systems improve the production of functional beta cells from ESCs/iPSCs *in vitro*. However, better differentiation protocols will be required to render this approach a valuable alternative: in particular, limitations due to the signals exchanged by cells in 3D culture, leading to self-organization and differentiation will complicate the control that can be exerted over it.

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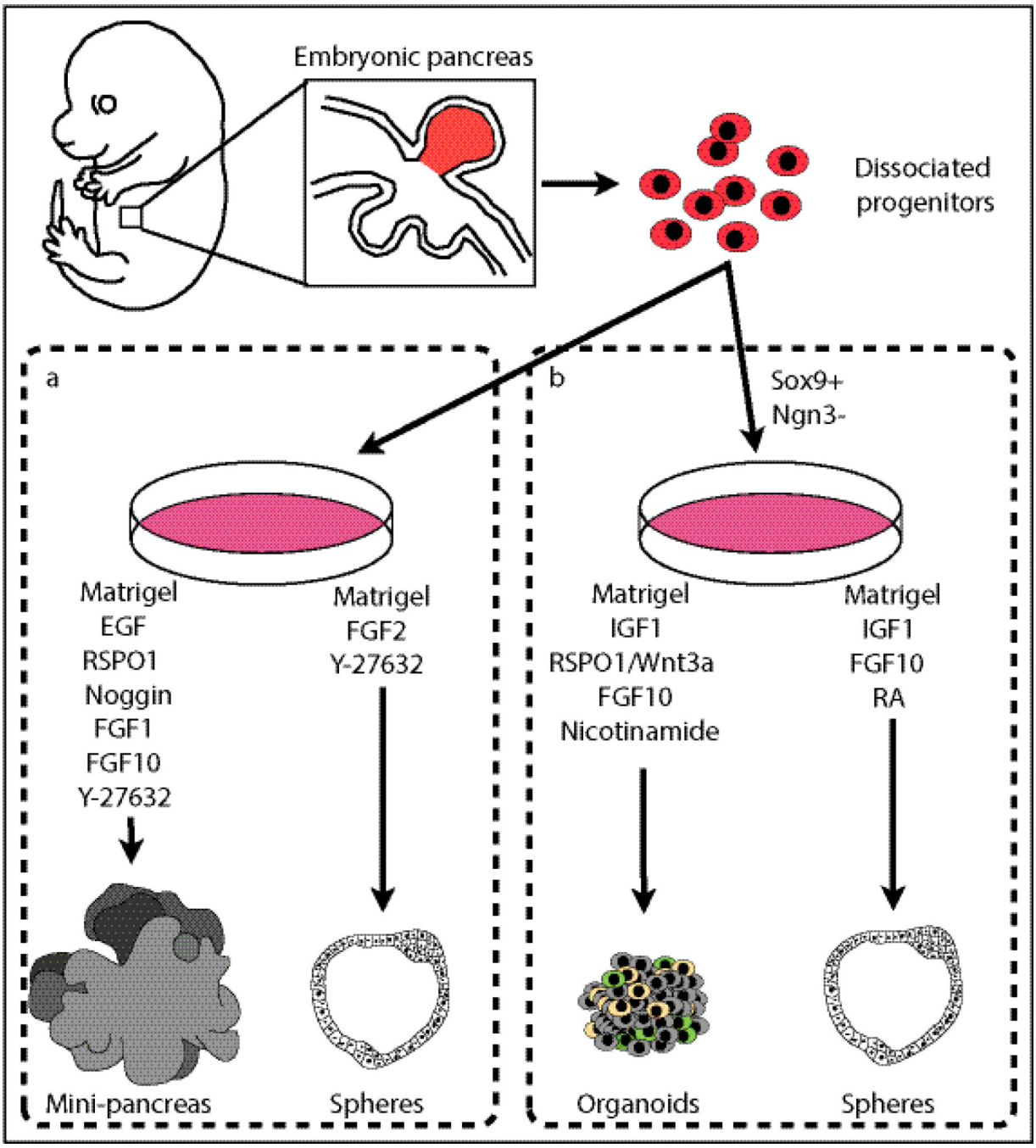

Figure 1. Spheres/cysts and organoids from embryonic tissues

Embryonic pancreatic progenitors are isolated from E10.5 (a (30)) or E11.5 (b (27)) mouse embryos. They are expanded in Matrigel and in presence of the chemicals/growth factors indicated. The expanding cells self-organize in different structures: mini-pancreas, hollow spheres and organoids composed of pancreatic progenitors (spheres) and differentiated cells (mini-pancreas, organoids).

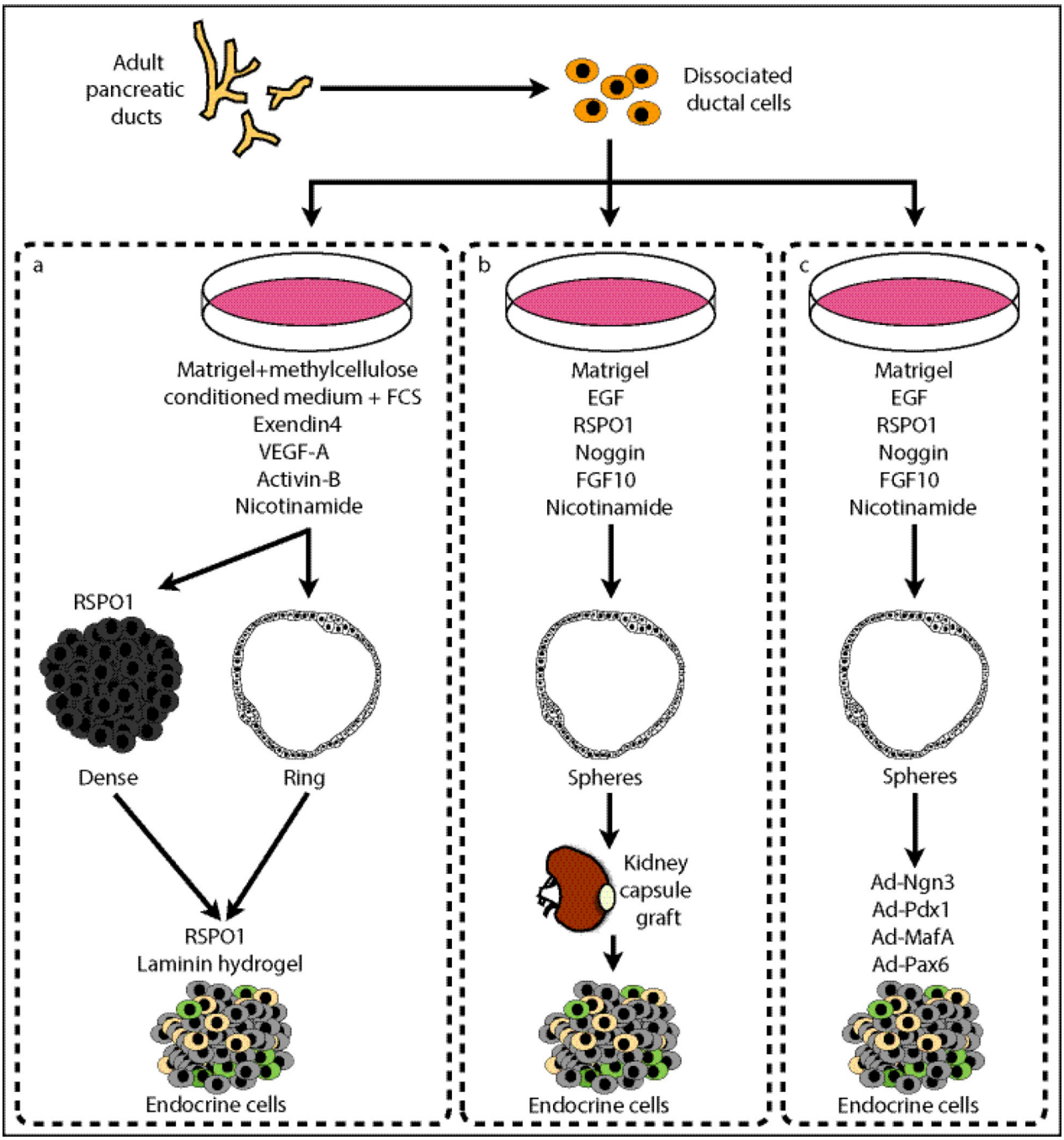

Figure 2. Adult ductal spheres/cysts and further endocrine differentiation

Ductal cells are dissociated from adult murine (a (42), b (43)) or human (c (44)) pancreatic ducts. They are expanded in semisolid matrixes (Matrigel) and generate dense (a) or hollow (a, b, c) spheres in presence of the soluble factors indicated. Different approaches have been used to generate endocrine cells from these cultures: exposure to R-Spondin1 and Laminin (a), grafting *in vivo* under the kidney capsule (b) and transfection with pro-endocrine transcription factors (c).