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Human-induced pluripotent stem cell technology for the establishment of a versatile pancreatic ductal disease platform

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Abstract

Nowadays, a biotechnological game changer in regenerative medicine and regenerative pharmacology is indisputably induced pluripotent stem cell technology. Particularly, human-induced pluripotent stem cells (hiPSCs) construct the basis of various functional models in both health and disease states. Undifferentiated, like their natural embryonic counterparts' human embryonic stem cells (hESCs), hiPSCs represent a model for pluripotency and self-renewal. Once differentiated, hiPSCs give rise to endoderm, mesoderm and ectoderm, and produce numerous cell lineages derived from these three germ layers. Thereby, hiPSCs provide a robust system to recapitulate the development that naturally occurs in the human embryo. These cells provide an effective platform to produce disease-specific target cells. These remarks have far-reaching implications to study the congenital dysfunction in monogenic and multifactorial diseases to reproduce *in vitro* pathophysiology of such diseases. The advent of three-dimensional (3D) biofabrication strategies combined with human pluripotent stem cells has also opened the door to a better understanding of mechanisms of pancreas development and pathophysiology of pancreatic congenital abnormalities and both endocrine and exocrine pancreas diseases. The possibility of obtaining pancreatic ductal epithelial cells from patient-derived hiPSCs improved our ability to recapitulate *in vitro* the complex pathophysiology of several pancreatic diseases, such as the life-threatening pancreatic ductal adenocarcinoma, chronic pancreatitis leading to permanent pancreas damage, pancreatic cystic fibrosis and cystic fibrosis-related diabetes. Herein, it was discussed how integration of human-induced pluripotent stem cell technology and 3D bioprinting employed "organ-on-a-chip" devices emerged as a guiding tool precisely offering clues of human ductal epithelial pancreas diseases with their potentials establishing rich pipelines for biopharmaceutical innovations, large-scale toxicology testing and next frontier of precision medical therapies.

Introduction

hiPSCs as an emerging tool that revolutionizes disease modeling and biopharmaceutical industry

The pluripotent nature of human embryonic stem cells (hESCs) raised the idea of their utilization in regenerative medicine in the 1990s following their first discovery. In previous research attempts, *in vitro* human embryonic stem cell cultures showed significant heterogeneity in the cell state-pluripotency and spontaneous commitment to extraembryonic cell lineages or different embryonic germ layer-derived cells, resulting in tumorigenesis after transplantation of hESCs. The need for prevention of the spontaneous differentiation of hESCs, along with the need for a synchronized temporal and spatial differentiation under *in vitro* culture conditions, limited the reliable application of such cells in both pre-clinical and clinical settings. Today, a vast amount of reprogramming strategies based on

epigenetic instructions and specific signaling pathways operating during different developmental stages - such as Notch signaling pathway - allow for the epigenetic regulation of the embryonic stem cell fate[1-7]. Groundbreaking research also provided deeper insights into the role of different extrinsic microenvironment factors - such as oxygen supply - on embryonic stem cell identity[8-10]. These scientific advances improved our abilities of mimicking the *in vivo* conditions and controlling pluripotent cell cultures and differentiation. Despite the remarkable progress that has been made in hESC culture and differentiation strategies over the last years, the tumorigenic potential of hESCs, the immune-mediated rejection of hESC allografts and the ongoing ethical debate about the embryonic origin of hESCs are issues that still remain unaddressed. Cell differentiaton has long been considered as an irreversible process. However, discoveries on the reversal of histone methylation - which is responsible for permanent heterochromatin condensation and gene silencing - bring a new perspective to the epigenomic plasticity during embryonic development. This was a unique exception limited to primordial germ cells, although genome-wide reorganization of epigenetic modifications has recently overcome the barriers to epigenomic plasticity[11-13].

Importantly, human-induced pluripotent stem cells (hiPSCs) represent the cornerstone of scientific approaches lying at the root of this perspective. In their pioneering work, Takahashi et al[14] demonstrated the generation of iPS cells from terminally differentiated somatic cells by transduction of four defined transcription factors, namely Oct3/4, Sox2, Klf4, and c-Myc, among 24 pluripotency-related genes[5,15-19]. Both hESCs and hiPSCs provide an unlimited source to obtain functional tissue cells that can be used for modeling a wide spectrum of human diseases and for investigating unique pharmacological agents. Yet, only hiPSCs provide a convenient pluripotent stem cell source since their use prevents the immune-mediated rejection (as these cells derive from a patient's own somatic cells) and avoids the employment of oocytes and/or embryos that may not be in compliance with the ethical rules in force across different countries.

On the other hand, hiPSCs have been shown to carry the risk of teratoma formation, as their pluripotent character counterparts hESCs[20,21]. Gutierrez-Aranda et al[22] showed that hiPSCs can lead to tumorigenesis more efficiently and faster than hESCs, regardless of the injection site. The same potential risk has been recognized in many early phase trials[23]. Safety is, therefore, the key concern for usage of hiPSCs as medicinal products. A challenge during the *in vitro* expansion of hiPSCs relies on the fact that cell culture systems are prone to produce abnormal rapidly-dividing cells. These cells may exhibit certain epigenetic modifications (e.g., aberrant histone methylations) that are similar to those found in cancer cells. Many cell culture approaches have been investigated to address this issue. One strategy consists of the introduction of inducible suicide genes into hiPSCs in order to prevent the tumorigenicity of such cells[24-26]. This strategy is effective even after cell transplantation and teratoma formation[27], although its major limitation relies on the fact that suicide gene expression needs to be precisely regulated. The transgenic forms can be shut down through epigenetic changes leading to gene silencing, even if they are inserted in permissive locations. Long-term gene expression can also be altered through gene mutations resulting in loss of function (loss-of-function mutations). Recently, Liang et al[28] developed a smart genome-engineering strategy aimed to define and improve cell therapy safety by effectively eliminating tumor-initiating cells. In order to obtain a reliable expression of suicide genes, authors selected a suitable location for inserting these genes to predict their behaviour and control their expression. In their newly developed strategy, authors created a transcriptional link between the suicide gene herpes simplex virus thymidine kinase (HSV-TK) and the cell-division gene cyclin-dependent kinase 1 (CDK1)[28]. HSV-TK expression enables cells to phosphorylate and convert ganciclovir (GCV) into a toxic monophosphate derivative. Cellular enzymes catalyze the conversion of the monophosphate derivative into GCV triphosphate, which is incorporated into DNA and promotes apoptosis[25,29]. In the study conducted by Liang et al[28], TK was inserted into the 3' untranslated region of CDK1 in murine and human ESCs, resulting in the synthesis of a bicistronic mRNA that is translated into two proteins. Authors claimed that this strategy has proven *in vitro* and *in vivo* efficacy in protecting a suicide system from inactivation in dividing cells, reducing the risk of (tumor) cell escape from immunosurveillance without interfering with the differentiation potential of hiPSCs[28].

While these advances will certainly expand hiPSC-based cell therapy platforms, the risk-benefit assessment of clinical applications of hiPSCs depends on the future development of novel three-dimensional (3D) biofabrication technologies using such cells to create complex tissue structures and even organs[30]. Further studies are needed to gain more insight into novel strategies aimed at engineering hiPSCs for their safe and rational clinical use, relying on the knowledge obtained from the aforementioned pioneering research.

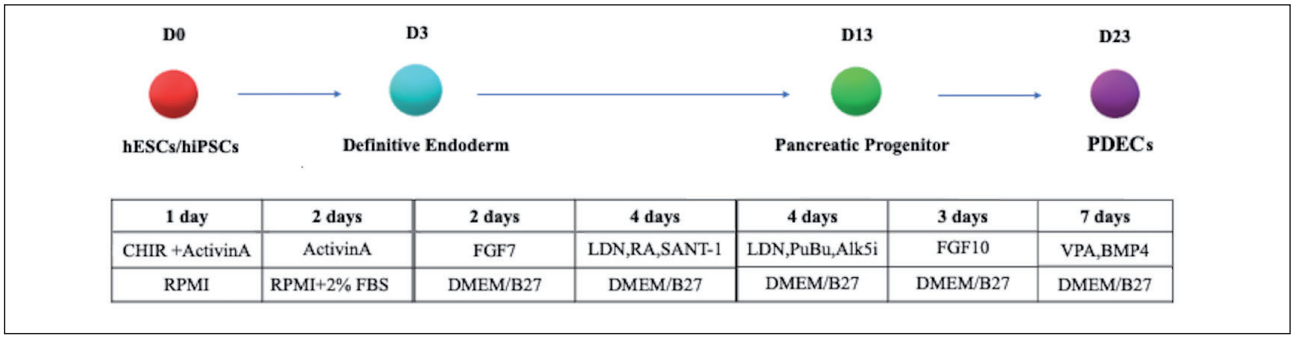
Induced pluripotent stem cell technology provides an unlimited source of starting materials to obtain distinct functional cell lines that can be used for disease modeling, pharmaceutical therapy and other novel clinical and therapeutic applications. Most of our current knowledge on disease-specific phenotypes derives from studies conducted in animal models, globally available disease-specific cell lines, disease-specific primary cells from affected individuals and patient-derived xenografts[31]. However, there are major impediments to the translation of this knowledge into the diagnosis and treatment of human diseases, and into the progress in drug discovery for such diseases. Results obtained from animal models often do not translate into clinical relevance. For example, Mus musculus (mouse) has long been served as a model of human and mammal diseases due to shared anatomical, physiological, metabolic and reproductive features with humans. At the same time, Mus musculus genome sequence shows a high degree of similarity with the human genome sequence. Additionally, certain mouse models recapitulate human diseases such as lissencephaly, a neurological disorder characterized by severe brain malformations resulting in developmental delays and mental disability[32]. Yet, murine models fail to accurately mimic the pathophysiology of most human diseases such as cystic fibrosis, as they exhibit distinct phenotypes. It seems that the severity of disease pathology at birth differs widely across a variety of animal species. Therefore, these limitations of murine models affect experimental tools and are associated with poor predictability of distinct human disease phenotypes. Genetically engineered mouse models may enlighten the processes behind the disease initiation and support the development of novel drugs. However, these experimental systems are highly expensive and time-consuming.

The use of primary cells from affected individuals has both ethical and technical hurdles. First, biopsy specimens as well as specific cell types directly obtained from living patients for research purposes raise universal ethical concerns. In addition to this, their culture systems are neither supportive for cell proliferation and growth, nor successful for the maintenance of long-term culture. A large number of disease-specific cell lines (available worldwide) offer easy, inexpensive and stable disease platforms. Nevertheless, their cellular behavior in a dish cannot fully recapitulate that occurring *in vivo* in human diseases. Finally, patient-derived xenografts have yielded insights into drug development, but their generation requires a large amount of tissue and time.

With respect to the aforementioned limitations, hiPSCs represent a valid tool able to provide a deeper insight into the pathophysiology of human diseases, with subsequent implications for their potential use for pharmacological and transplantation purposes[33,34]. Over the last years, most scientific efforts were directed to the modeling of human autoimmune diabetes arising from the immune-mediated destruction of insulin-secreting beta cells within the pancreatic islets (endocrine pancreas). On the other hand, recent investigations raised interest in the potential clinical application of hiPSC-based technology in devastating diseases involving the ductal epithelium of the exocrine pancreas. However, there is still a long road ahead to fully understand the physiology of pancreatic ductal epithelial cells, as well as their involvement in the pathophysiology of distinct diseases of the exocrine pancreas[35].

Recapitulating the physiology and pathophysiology of human exocrine pancreas in vitro

The pancreas is a complex organ divided into an exocrine portion (acinar and duct tissue) and an endocrine portion (islets of Langerhans). The exocrine portion accounts for 85% of the mass of the pancreas and is involved in the production of digestive enzymes, whereas the endocrine portion is responsible for the production of hormones, such as insulin and glucagon, that regulate glucose homeostasis[36,37]. The functional unit of the exocrine pancreas



1 day	2 days	2 days	4 days	4 days	3 days	7 days
CHIR +ActivinA	ActivinA	FGF7	LDN,RA,SANT-1	LDN,PuBu,Alk5i	FGF10	VPA,BMP4
RPMI	RPMI+2% FBS	DMEM/B27	DMEM/B27	DMEM/B27	DMEM/B27	DMEM/B27

Figure 1. Directed differentiation of hESCs/hiPSCs into PDECs. Schematic of the directed differentiation protocol from hESCs/hiPSCs to PDECs. Abbreviations: D0, day zero; D3, day 3; D13, day 13; D23, day 23; hESCs, human embryonic stem cells; hiPSCs, human-induced pluripotent stem cells; PDECs, pancreatic ductal epithelial cells.

is represented by an acinus and its draining ductule. The acinar cells synthesize, store and secrete digestive enzymes, whereas the draining ductules drain into interlobular ducts, which in turn drain into the main pancreatic ductal system. The pancreatic duct cell epithelium consists of cells containing abundant mitochondria necessary for energy products required for ion transport. The pancreatic ductal system extends from the lumen of the acini to the duodenum[37]. Importantly, pancreatic ductal epithelial cells (PDECs) can be involved in the pathophysiology of several serious diseases of the exocrine pancreas, including pancreatic ductal adenocarcinoma (PDAC), pancreatic cystic fibrosis and cystic fibrosis-related diabetes, as well as chronic pancreatitis leading to permanent pancreas damage.

Because of the limited access to human embryonic and fetal pancreas tissues, the molecular mechanisms underlying the development of the exocrine pancreas as well as the formation of the exocrine pancreatic cell lines have remained elusive for years. More recently, thanks to the greater access to human embryonic and fetal pancreas tissues, many molecular and morphological events of the exocrine pancreas development have been elucidated and have emerged as conserved between humans and rodents. The development of human pancreas begins with the generation of the foregut endoderm, in a similar manner as occurs in rodent models. The first step in pancreas specification involves the formation of the dorsal and ventral pancreatic buds from the foregut endoderm. The trunk domain of the embryonic pancreas consists of bipotent epithelial cells expressing PDX1, SOX9, HNF1B and NKX6.1[38,39]. In 2003, Murtaugh et al[40] showed that expression of activated Notch1 throughout the pancreas prevents both exocrine and endocrine development, trapping early and late progenitors in an undifferentiated state.

Historically, the fundamental knowledge and insights gained into the pancreas development (mainly derived from rodent studies) facilitated the advent of technologies allowing for the differentiation of pancreatic progenitor cells and pancreatic endocrine and exocrine lineages. In 2015, Huang et al[41] established 3D cell culture conditions to induce the directed differentiation of human pluripotent stem cells into pancreatic ductal and acinar cells for *in vitro* modeling of pancreatic cancer initiation and for identification of targeted therapeutic strategies. In 2017, Hohwieler et al[42] improved 3D cell culture conditions and differentiation protocols, which yielded over 19% of pancreatic acinar cells and 42% of pancreatic ductal cells expressing specific markers, including carbonic anhydrase. Of note, authors aimed to recapitulate patient-specific clinically relevant physiological abnormalities through more robust differentiation protocols. Simsek et al[43] developed another efficient strategy to induce the differentiation, in a stepwise manner, of human pluripotent stem cells (both hESCs and hiPSCs) into PDECs and mimic functional defects observed at the cellular level in patients with pancreatic cystic fibrosis (Figure 1). Their scientific efforts provided a new methodology for deriving pure and functional PDECs from the heterogeneous mixture of cells generated by directed differentiation. At the same time, this methodology allows for disease modeling in a dish platform, thus representing a valid strategy to identify drug candidates to rescue the pancreatic defects occurring in cystic fibrosis, and to implement recent gene-editing tools aimed to correct mutations related to pancreatic cystic fibrosis phenotypes.

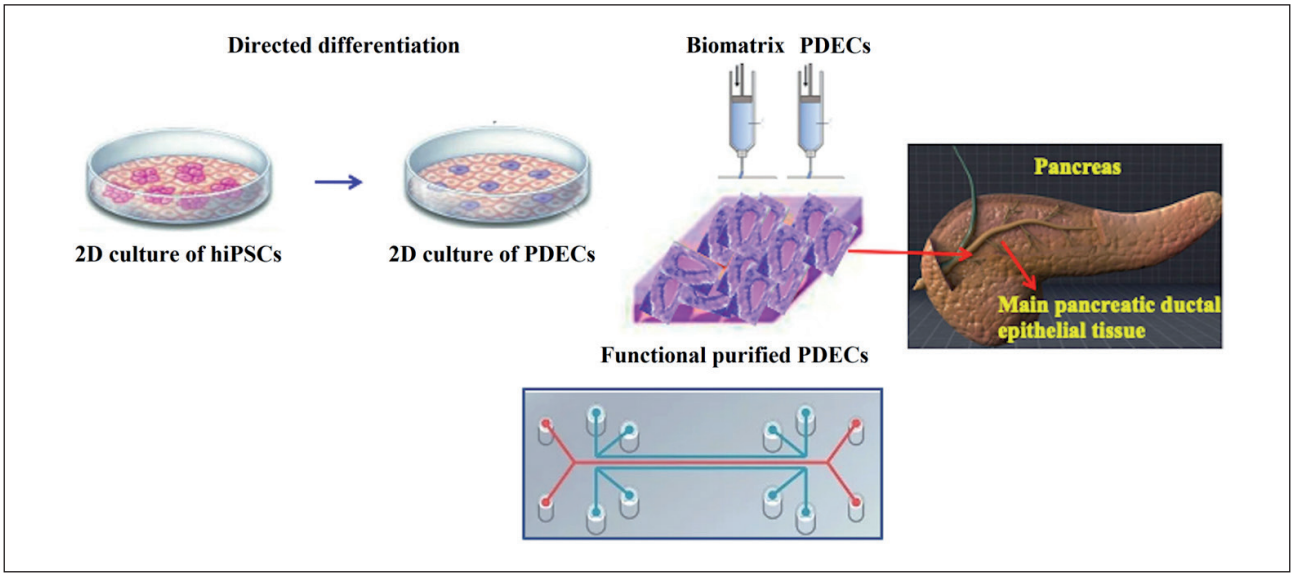


Figure 2. Schematic of an innovative biomimetic system used for the biofabrication of pancreatic ductal epithelium. Functional and impaired PDECs are generated through a stepwise directed differentiation of healthy and disease-specific hiPSCs. This strategy can be employed on a microfluidic "organ-on-a-chip" (OOC) platform. In this platform, a three-dimensional (3D) bioprinting system prints simultaneously biomatrices and cells bound to the chip. Abbreviations: 2D, two-dimensional; hiPSCs, human-induced pluripotent stem cells; PDECs, pancreatic ductal epithelial cells.

Conclusions

Generating a biomimetic model of pancreatic ductal diseases through the biofabrication of pancreatic ductal epithelium paves the way for the clinical applicability of precision medicine in the context of incurable disorders of the exocrine pancreas. Standard, static two-dimensional (2D) and 3D models of PDECs through hiPSC technology have proven to be successful. On the other hand, these models are still inadequate to explore the mechanisms that control the formation of pancreatic ductal epithelium, and to mimic PDAC pathophysiology, where cancer epithelial cells interact with stromal cells ("cancer-stromal interactions"). The first biomimetic model of PDAC was developed through a microfluidic model ("ductal tumor microenvironment-on-a-chip")[44]. In this system, pancreatic ductal cancer cell lines were embedded within the collagen matrix, thus mimicking the heterogeneity of PDACs. However, this system still warrants further improvement to mimic other aspects of PDACs. The collagen matrix within this system presents single components of the extracellular matrix (ECM). However, other components of the ECM, such as hyaluronic acid, fibrin and fibronectin are additional hallmarks of PDACs. It is envisioned that this system can be further improved by incorporating together patient-specific PDECs and stromal cells. The integration of robust hiPSC-based ductal differentiation protocols with the state-of-the-art advanced bioprinting methods, combined with microfluidic "organ-on-a-chip" (OOC) technologies, enable to print simultaneously multiple ECM components and cell types, such as ductal epithelial and stromal cells, and to locate them in a defined microenvironment without interfering with their growth, differentiation and response to internal and external stress stimuli. This strategy will certainly represent a landmark achievement towards the precise PDAC modeling, as well as the next frontier of pharmaceutical innovation platform, which targets different patient groups, shortens the time required for discovery and development of novel drugs, and allows for testing drugs through functional organ printing techniques (Figure 2). This strategy will also allow pharmaceutical companies to test their drug candidates more safely and in a cheaper manner by allocating the majority of their budget to the improvement of drug design and to the identification of the adequate dosage through a more accurate toxicology testing.

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Conflict of Interest:
The author declares that she has no conflict of interest to disclose.

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