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Mesenchymal cells are required for epithelial duct cell-to-beta cell maturation and function in an injured adult pancreas in the rat

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ABSTRACT

The islet, the endocrine portion of the pancreas − develops from an invagination of the pancreatic duct epithelial cells (PDECs) into the surrounding tissue. The contact of the PDECs with mesenchymal cells (MSCs) may be an essential drive for endocrine cell fate. During pancreatic development, cells that express Neurogenin-3 (Ngn3) biomarker are precursors of insulin- producing beta cells. These precursors have been reported in the neogenesis of islets from adult tissues following the surgical ligation of the main pancreatic duct (PDL). But the capacity of these precursors to induce the appropriate signals to complete the entire neogenesis program has been questioned. We studied the fate of co-culture of PDECs and MSCs from the ligated adult pancreas and established the exact location of adult stem- or progenitor-like cells that give rise to beta cells. PDECs were cultured in direct contact with or without MSCs in serum-containing culture media. The cytomorphology of the cells in co-cultures was determined and the immunocytochemical study of the cells was carried out using anti-Ngn3, anti-insulin and anti-cytokeratin-7 (CK7) antibodies. Both the PDEC/MSC- and PDEC/MSC+ cultures showed out- pocketing from duct epithelium by the end of the second week, which are distinct as cell clusters only in PDEC/MSC+ cells later in week four, exhibiting numerous branching ducts. Co-expression of Ngn3 with insulin was observed in both cultures from the second week. However, characterizations of these Ngn3+ cells in the PDEC/MSC+ culture revealed that these cells also co-expressed a CK7 biomarker. This study provides new evidence of the ductal epithelial nature of beta cells in injured adult pancreata; and that the mesenchymal stromal cells are required to sustain Ngn3 expression for beta cell maturation and function.

1. Introduction

The process that culminates in the formation of the islets of Langerhans resides in the signaling pathways during pancreas organogenesis. The miR-21 and its targets are involved in the differentiation and proliferation of the insulin-producing cells (IPCs) from pancreatic progenitors (Bai et al., 2016). During embryogenesis, both exocrine and endocrine components of the pancreas develop by invagination of the epithelial cells into the connective tissue underlying an epithelial membrane. The connection with the epithelial membrane from the invagination persists as a duct and acinar, forming the exocrine pancreas. Whereas the loss of the connection results in the formation of the endocrine pancreas − the islet. Then, the beta cells in the islet produce insulin, a hormone that promotes the uptake and metabolism of glucose by the body's cells (Saltiel and Kahn, 2001).

Evidence from studies on fetal (Dudek et al., 1991) and neonatal (Popiela et al., 1986) pancreata has shown that there is a morphological similarity between an *in vitro* islet neogenesis from pancreatic duct epithelial cells (PDECs), and an *in vivo* islet neoformation. After birth, however, the insulin producing beta cells (IPCs) become terminally differentiated and the mitotic process remains quite low during endocrine remodeling, despite the presence of PDECs (Teta et al., 2005). Why islet cell neogenesis from PDECs is not significantly activated in an adult pancreas remains unknown.

In the adult pancreas, islet neogenesis from PDECs has been the subject of intense debate in the literature based on the nature of its origin. Dor et al. (Dor et al., 2004) clearly established that beta cells do not arise from PDECs in an adult pancreas, but rather from pre-existing beta cells. While using adult models of pancreatic injury, many studies (Inada et al., 2008; Murtaugh and Kopinke, 2008; Solar et al., 2009; Xu et al., 2008) demonstrated that neogenesis can be reactivated from PDECs. This suggests however, that PDECs contain precursor cells which, upon stimulus, have the capacity to differentiate into endocrine cells and give rise to functional beta cells (Bonner-Weir and Sharma, 2002; Inada et al., 2008).

The surgical ligation of the main pancreatic duct (PDL) has been

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reported to induce regeneration of small pancreatic ductal epithelial structures in adult rodents after a degeneration of exocrine tissues in the portion distal to the ligation point (Chintinne et al., 2012; Tchokonte-Nana et al., 2011; Xu et al., 2008). Indeed, in the tail portion of the ligated pancreas, Neurogenin-3 positive (Ngn3 +) cells are expressed within PDECs as well as in small cell clusters close to the metaplastic pancreatic ducts (Inada et al., 2008; Murtaugh and Kopinke, 2008; Solar et al., 2009; Wang et al., 1995; Xu et al., 2008). Lineage-tracing studies (Kopp et al., 2011; Solar et al., 2009) on PDL models of pancreatic injury demonstrated that Ngn3 + cells in metaplastic pancreatic ducts do not differentiate further into mature islet cells, including beta cells. These controversial opinions suggest that surgical injury by ligation of the main pancreatic duct does not induce the appropriate signals to complete the entire neogenesis program, even though factors regulating neogenesis from PDECs were not elucidated.

Knowledge of molecular events that promote the differentiation of endocrine progenitors into mature beta cells during pancreatic development is essential. During early pancreatic development, distinct signals secreted by adjacent MSCs and notochord (Kim et al., 1997), aortic endothelial cells and cardiac mesoderm (Lammert et al., 2001) are required for pancreatic endoderm patterning. The importance of epithelial–mesenchymal interactions in fetal epithelial cytodifferentiation into endocrine cells has been suggested (Gittes et al., 1996; Golosow and Grobstein, 1962). The implication of MSC replication in endocrine formation as a requirement for an early extracellular signaling pathway in the specification of beta cell fate has also been proven in many investigations (Rahavi et al., 2015; Seyedi et al., 2015). In addition, the effective role of MicroRNAs in the induction of IPCs from MSCs has been demonstrated (Bai et al., 2017). The fibroblastic MSC replication reported in injured adult pancreatic tissues in the early hours following PDL (Tchokonte-Nana, 2011) may be a clear demonstration that MSCs play an important role in the differentiation of endocrine cells.

Despite its role during endocrine pancreas development, the response of adult PDECs to MSC replication and their potential as stimulants of progenitors for islet neogenesis in the injured adult pancreas have not been explored. Hence, this study sought to determine the fate of a co-culture of PDECs and MSCs isolated from the ligated adult pancreas and establish the exact location of adult stem- or progenitor-like cells within the pancreas that give rise to beta cells.

2. Materials and methods

2.1. The animals

Healthy adult male Wistar rats (250–300 g) were obtained from the Animal Unit of the Faculty of Medicine and Health Sciences, Stellenbosch University. The animal ethics for this study was approved by the Stellenbosch University Animal Care and Use Committee (SU ACUC): ethics number (SU-ACUM13-00036).

2.2. Surgical procedure and tissue preparation

Animals (n = 10) were anesthetized by inhalation of Isofor (Safeline Pharmaceuticals (Pty) Ltd, Roodepoot, South Africa) and a midline laparotomy was performed as described by Tchokonte-Nana (Tchokonte-Nana, 2011). The splenic duct in the pancreas of the experimental animals (n = 5) was ligated, and the pancreas of the control animals (n = 5) was gently touched with a cotton swab for 30 s without ligation. A distal one third of these pancreata were excised 24 h following surgery, and placed on Hanks' balanced salt solution (HBSS) to clean out any blood tissue. These portions were sliced into 2 mm pieces 5 min later and placed in conical tubes containing 10 mL (1.5 mg/mL) Collagenase A (Sigma) ready for isolation procedures.

2.3. Isolation of PDECs

PDECs were isolated from pancreatic tissues using an adapted method from previous authors (Gotoh et al., 1985). Pancreatic tissues were first minced into 2 mm pieces in Hanks' balanced salt solution (HBSS) and then digested with collagenase A (SIGMA) for 30 min at 37 °C in a water bath. The resulting mixture contained PDECs, islets, acinar cell clusters, blood vessels and nerves. PDECs were isolated from the digest by first hand shaking and then the digest was vigorously pipetted and sieved on a 60 mesh stainless steel sieve (pore size 200 × 300 μm) (Millipore). Tissues in the supernatant were then left for 2 min to allow the lymph nodes and large blood vessels to sediment and these were later discarded. The supernatant was centrifuged in 1.06g/mL Ficoll density cushion at 800 × *g* for 15 min to sediment the remaining exocrine (acinar) tissue.

The floating tissue fragments were identified using the following specific morphological characteristic features: the main intra-pancreatic pancreatic duct presents large lumen with a thick columnar epithelial lining. These ducts have numerous distorted tubular evaginations (Githens et al., 1981), while intra-lobular pancreatic ducts are lined by simple low columnar to cuboidal epithelium underlined by a loose connective tissue. In addition, small-size pancreatic ducts are lined by simple cuboidal epithelium; and, intercalated pancreatic ducts − which are often attached to larger pancreatic ducts, contain a single layer of thick squamous epithelium with a straight unbranched duct of a smooth outer lining. These cells were hand-picked with a pipette under a Nikon Eclipse TE2000-S dissecting microscope (Nippon Kogaku KK, Japan). PDECs were cultured in DMEM in a T25 tissue flask for 24 h to recover from the effects of digestion and isolation. Pancreatic ducts were then transferred in a new T25 culture flask containing 15 mL of RPMI1640 serum free and supplemented by 1% (vol/vol) Penicillin-Streptomycin. The media was changed every 2 days for 14 days; following this, pancreatic duct cultures were then observed under a Nikon Eclipse light microscope (Nippon Kogaku KK, Japan).

2.4. MSCs separation

MSCs were isolated based on their ability to adhere to plastic dishes as described previously (Schauwer et al., 2011). The remaining collagenase digest obtained after PDECs isolation was cultured in T25 flasks. Supernatant was removed 48 h later and the resultant adherent comprising fibroblast-like cells (MSCs) were maintained as a monolayer at 37 °C in humidified air (95%) and CO_2 (5%) in Dulbecco's modified Eagle medium H-DMEM (GIBCO) supplemented by 1% (vol/vol) Penicillin-streptomycin (GIBCO) and 10% Fetal Bovine serum (FBS) for 3 passages. The monolayer was later detached from the plastic dish and was allowed to expand to 80–90% confluence, when they were co-cultured with isolated duct fragments (PDECs).

2.5. Co-culture of PDECs and MSCs

Approximately 150,000 MSCs of passage 3 were seeded, each on a Cell Star 15-mm Petri dish (Greiner Bio-One) and cultured for 24 h to form a confluent monolayer for the direct contact PDEC-MSC monolayer co-culture system. Five to six fresh duct fragments were seeded directly on the MSC monolayer. The co-culture system used serum free RPMI-1640 medium, supplemented by 1% (vol/vol) Penicillin-Strepotomycin (GIBCO).

Control PDECs were cultured alone, without MSCs, in groups of 5–6 PDECs per 15 mm Petri dish in RPMI-1640. The culture medium was changed every 2 days. Growth morphological changes of PDECs in the culture were investigated and images were captured every 2 days using a Nikon Eclipse light microscope (Nippon Kogaku KK, Japan). At this point, all PDECs were pipetted for an *in vitro* analysis.

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