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Silk matrices promote formation of insulin-secreting islet-like clusters

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ABSTRACT

Ex vivo expansion of endocrine cells constitutes an interesting alternative to be able to match the unmet need of transplantable pancreatic islets. However, endocrine cells become fragile once removed from their extracellular matrix (ECM) and typically become senescent and loose insulin expression during conventional 2D culture. Herein we develop a protocol where 3D silk matrices functionalized with ECM-derived motifs are used for generation of insulin-secreting islet-like clusters from mouse and human primary cells. The obtained clusters were shown to attain an islet-like spheroid shape and to maintain functional insulin release upon glucose stimulation *in vitro*. Furthermore, *in vivo* imaging of transplanted murine clusters showed engraftment with increasing vessel formation during time. There was no sign of cell death and the clusters maintained or increased in size throughout the period, thus suggesting a suitable cluster size for transplantation.

1. Introduction

Type I diabetes is currently representing around 10% of all diabetic patients, with serious implications for both quality of life and healthcare costs [1]. Transplantation of islets isolated *in vitro* from a donor pancreas has the potential to become a widely applicable treatment for insulin-dependent diabetes [2], but unfortunately currently available procedures suffer from both low efficacy and limited reproducibility [3,4]. Moreover, side-effects of immunosuppressive therapy [5] and the scarcity of donor islets [3] are limiting the therapeutic usage of islet transplantation. For these reasons, a search for alternative methods for transplantation of islet cells is very much needed.

Because of limited availability of organs, that have to be taken from deceased donors, *ex vivo* expansion of islet cells is often considered as an alternative source for islet transplantation. However, endocrine cells are subjected to senescence and loss of insulin expression during conventional culture by monolayer formation on a two-dimensional (2D) surface [6]. Moreover, the monolayer-cultured cells are fragile, with consequent cell death due to both necrosis and apoptosis once they are removed from their natural extracellular matrix (ECM) [7]. *In vitro* culture on an artificial support, designed to provide both a three-dimensional (3D) configuration and ECM-like biochemical interactions, could potentially grant better conditions and thus offer a way to both preserve function and viability, and possibly even allow expansion of endocrine cells.

The viability of isolated islets is adversely affected by hypoxia since the cells of the inner islet core do not receive an adequate supply of oxygen and nutrients [8,9] but depend on intra-islet capillary-mediated flow of blood [10]. A beneficial outcome of generated islet-like clusters in comparison to isolated native islets would thus be that the size can be optimized, in order to assure access to oxygen and nutrients also to the cells of the inner core. A suitable size and functional arrangement of the endocrine cells with tight connection to the vasculature is of key importance for a functional outcome *in vivo*.

In the last decade considerable effort has been paid to develop functional islet cells from a variety of stem cell sources. As cell engineering technologies are expected to continue evolving in the coming years, it will be of great value to have effective protocols for formation of islet-like clusters at hand.

We have recently described [11] a new strategy for *in vitro*

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maintenance of islets for prolonged times, accomplished by providing the islets with a temporary support made of a 3D silk matrix functionalized with cell binding motifs to imitate the natural cell environment [12]. Interestingly, the supporting silk matrix did not just contribute to higher islet viability, but also resulted in another unexpected novel finding; after one month in culture, newly formed islet-like clusters could be found on the silk matrices. These islet-like clusters were attached to the matrices and showed positive staining for insulin. When comparing cluster formation with age of the islet donor, we found that islets from younger donors (<35 years) had the highest ability to form islet-like clusters, and that this occurred particularly on silk with the RGD motif, where the amount of newly formed islet-like clusters found were almost double compared to the initial number of islets added. This prompted us to consider the silk matrices for generation of islet-like clusters suitable for transplantation. In the present study, we investigated the process of cluster formation starting from isolated and dispersed endocrine cells, and also evaluated how the formed clusters behave both *in vitro* and *in vivo*. The long-term goal of our work is to achieve pre-requisites for transplantable, functional islet-like clusters as an alternative to pancreatic islets for both research use and clinical application.

2. Materials and methods

2.1. Preparation of silk matrices

Conventional molecular cloning techniques were used to insert short peptides (Fig. 1c) at the N-terminus (RGD, RGE, FN and VN) or in the repetitive part (2R and 3R) of the recombinant spider silk protein 4RepCT (herein denoted WT) [13]. Plasmids with the different 4RepCT constructs were transformed to *Escherichia coli* BL21(DE3) cells (Merck Biosciences). Corresponding proteins were expressed and recovered essentially as previously described [13]. A procedure for depletion of Lipopolysaccharides (LPS) using EndoTrap columns (Profos AG) was also included [14]. After purification, the protein solution was sterile filtered (0.2 μm) and concentrated to 3 mg/mL by centrifugal filtration (Amicon Ultra, Millipore). The silk proteins were formulated to 3D foam matrices in either plates for suspension cultures (Sarstedt) or chambers slides (Labtek) as described in Widhe et al. [15]. During optimization of the cluster formation protocol, matrices of the 2R silk protein were used. All foams used for obtaining results presented in Figs. 2–7 were made according to the optimized protocol (20 μl protein deposited as pyramidal foam and kept dry before usage).

2.2. Pancreatic islet donors and islet isolation

As rodent pancreatic islet donors, ob/ob, lean (+/+) (both of B6 origin) and MIP-GFP transgenic mice [16], all inbred in the animal core facility at Karolinska Institutet, were used. All animal work within this study was conducted in accordance with the Swedish Animal Research Committee's guidelines. The study was approved by the Swedish Animal Research Committees at Karolinska Institutet, Stockholm, Sweden (permit number N445/12). The mice were sacrificed by cervical dislocation. Islets were isolated from pancreas by injecting 1.2 mg/ml collagenase in Hank's Balanced Salt Solution (HBSS) buffer containing 25 mM HEPES, pH 7.4, with the addition of 0.25% BSA into the bile duct. The pancreas was taken out and put into a flask containing the same concentration of collagenase in HBSS buffer. The flask was then put into a 37 °C water bath and shaken very slowly for 15 min. Thereafter the islets were washed in HBSS and handpicked under a stereomicroscope into a Petri dish with complete RPMI 1640 medium (Gibco) supplemented with L-glutamine (2 mM), penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}) and 10% heat-inactivated fetal bovine serum (FBS).

Human islets from declared diseased donors (n = 10) were provided by the Nordic Islet Transplantation Program (www.nordicislets.org). Islets were released for research after approval by the ethics committee at Uppsala University Hospital. The human islets were obtained from the unavoidable excess of islets generated within the Nordic Network for Clinical Islet Transplantation. Only organ donors who explicitly had agreed to donate for scientific purposes were included. Informed written consent to donate organs for medical and research purposes was obtained from donors, or relatives of donors, by the National Board of Health and Welfare (Socialstyrelsen), Sweden. Experimental procedures were done according to the approved ethical permit from the Ethical Committee for Human Research (permit number 2011/14667-32).

2.3. Cell culture and dispersion of islets

A very mild enzymatic method was used to disperse the mouse or human islets into single cells. The islets were first washed two times in PBS without Ca^{2+} and Mg^{2+}, incubated in Accutase (Gibco) at 37 °C for 5 min and finally pipetted up and down to disrupt the islet architecture and obtain a single cell suspension.

The endocrine cells obtained from mouse islets were cultured in RPMI 1640 medium (as described above) and the human cells were cultured in CMRL-1066 (ICN Biomedicals) supplemented with HEPES (10 mM), L-glutamine (2 mM), Gentamycin (50 mg ml^{-1}), Fungizone (0.25 mg ml^{-1}, Gibco), Ciprofloxacin (20 mg ml^{-1}, Bayer Healthcare AG), nicotinamide (10 mM), and 10% heat inactivated FBS.

MIN6m9 cells [17] were cultured in DMEM (Gibco) supplemented with β-mercaptoethanol (50 μM), penicillin (100 U mL^{-1}), streptomycin (100 μg mL^{-1}), 10% heat-inactivated FBS and glucose (11 mM). Medium was changed every second day.

2.4. Cluster formation and analysis

Primary endocrine cells from dispersed islets were seeded with the desired amount of cells in 8 well chamber slides (12,500 cells/cm^2), 24-well plates (10,000 cells/cm^2) or 96-well plates (6730 cells/cm^2) containing silk foam and cultured in a humidified atmosphere with 5% CO_2 at 37 °C, in duplicates. An inverted light microscope (Nikon Eclipse Ti-S, 10x magnifications) was used to monitor the cells and cluster formation over time during *in vitro* culture, and the amount of formed clusters within each well was counted at day 2 and 7 or 14.

The morphology of primary cell clusters formed on WT, RGD and 2R silk foam was evaluated after 7 days in culture. Clusters were washed in PBS and fixed in 4% formaldehyde and thereafter permeabilized in PBS containing 0.3% Triton x100 for 15 min. Blocking was done with 6% fetal calf serum (FCS) in PBS containing 0.1% Triton for 1 h at room temperature (RT). The clusters were then incubated with antibody against insulin (guinea-pig anti-insulin, 1:1000, Dako) overnight at 4 °C. The next day the cell clusters were probed with a secondary antibody against guinea-pig coupled to Alexa488 (Molecular Probes, 1:1000).

In order to verify presence of α cells, the clusters were stained for glucagon using rabbit anti glucagon (1:500, Sigma Aldrich) followed by Alexa Flour 546-labeled goat anti rabbit (1:1000, Invitrogen).

MIN6m9 cells [17] at passage 27–35 were cultured in 24-well plates (2500 cells/cm^2) or 8 well chamber slides (6250 cells/cm^2) and cultured for 4 days to reach an approximate number of 20,000 cells/well. The MIN6m9 cells were fixed in 4% formaldehyde, permeabilized with 0.3% Triton x100 in PBS and blocked in 6%