Culture of hESC-derived pancreatic progenitors in alginate-based scaffolds

Kjetil Formo[1], Candy H.-H. Cho[2], Ludovic Vallier[2] and Berit L. Strand[1, 3*]

[1]*Department of Biotechnology,* [2] *Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, Norway*

[2]*Wellcome Trust – Cambridge Stem Cell Institute, Anne McLaren Laboratory for Regenerative Medicine, Cambridge, United Kingdom*

[3] *Central Norwegian Regional Health Authority, St. Olav's Hospital, Trondheim University Hospital, Trondheim, Norway*

*Corresponding author.

Address for correspondence:

Berit Løkensgard Strand

NTNU, Department of Biotechnology,

Sem Saelandsvei 6/8

N-7491 Trondheim

Norway

e-mail: berit.l.strand@ntnu.no

Phone: +47 73594069

Culture of hESC-derived pancreatic progenitors in alginate-based scaffolds

Abstract

The effect of alginate-based scaffolds with added basement membrane proteins on the *in vitro* development of hESC-derived pancreatic progenitors was investigated. Cell clusters were encapsulated in scaffolds containing the basement membrane proteins collagen IV, laminin, fibronectin, or extracellular matrix-derived peptides, and maintained in culture for up to 46 days. The cells remained viable throughout the experiment with no signs of central necrosis. Whereas non-encapsulated cells aggregated into larger clusters, some of which showed signs of morphological changes and tissue organization, the alginate matrix stabilized the cluster size and displayed more homogeneous cell morphologies, allowing culture for long periods of time. For all conditions tested, a stable or declining expression of insulin and PDX1 and an increase in glucagon and somatostatin over time indicated a progressive reduction in beta cell-related gene expression. Alginate scaffolds can provide a chemically defined, xeno-free and easily scalable alternative for culture of pancreatic progenitors. Although no increase in insulin and PDX1 gene expression after alginate-immobilized cell culture was seen in this study, further optimization of the matrix physicochemical and biological properties and of the medium composition may still be a relevant strategy to promote the stabilization or maturation of stem cell-derived beta cells.

Keywords

Alginate, extracellular matrix, β-cells, stem cells, encapsulation

Introduction

Cell therapy for treatment of diabetes is today limited by a shortage of donor organs, and the development of an unlimited source of glucose-responsive, insulin-producing cells for this application is a major objective in regenerative medicine. Human β-cells have a very low

post-natal replication rate, and efforts to stimulate expansion (both *in vivo* and *ex vivo*) have largely been unsuccessful [1]. Alternative sources of β-cells are therefore being explored, and pluripotent stem cells (PSCs) such as human embryonic stem cells (hESCs) [2] or induced pluripotent stem cells (hIPSCs) [3] are particularly attractive candidates due to their capacity to proliferate indefinitely *in vitro* and their potential to differentiate into any of the more than 200 different cell types in the adult human body, including pancreatic cells [4-6].

A breakthrough for *in vitro* differentiation of PSCs to insulin secreting cells was recently reported by the Kieffer group [7] and by the Melton group [8]. Rezania and coworkers [7] used air-liquid interface and high glucose concentrations in their culture of pancreatic endocrine precursors (PE) in 1-2 mm cell clusters that together with tailored composition of the culture media resulted in cells with glucose response similar to human islets in static incubation assays. Pagliuca and coworkers [8] applied a new multistep differentiation protocol in suspension culture using soluble factors, and produced cells that were comparable to human primary beta cells in the expression of insulin and other relevant markers measured, as well as showing functional characteristics of β-cells such as packing of insulin in granules, and the flux of Ca^{2+} and secretion of insulin in the response to glucose. Prior to the recent publications, several groups have published differentiation protocols highly successful in producing pancreatic progenitors expressing PDX1 (pancreatic and duodenal homeobox domain 1), and to a lower degree also insulin-expressing cells, from PSCs [4-6, 9]. The $insulin^{+}$ cells generally did not show the expression levels or glucose responsiveness seen for β-cells in mature islets, and often co-express other hormones such as glucagon or somatostatin [4, 5], indicating that the cells were immature [10, 11]. Despite the limited expression *in vitro*, pancreatic cells derived from hESCs, differentiated by several different protocols, have been shown to mature and be capable of restoring euglycemia in diabetic mice *in vivo* [5, 9, 12-14].

The basement membrane (BM) is a thin layer of specialized extracellular matrix (ECM) that both separates cells from and connects them to the interstitial ECM, and it is this layer that is available for direct ECM-cell membrane receptor contact [15]. The composition of BM vary widely, but the major structural components are sheets of collagen IV connected to various laminin isoforms via nidogen, in addition to proteoglycans such as perlecan [15]. Laminins are heterotrimeric glycoproteins built up from α, β, and γ chains, and known chains can assemble at least 18 different laminin isoforms [16]. Cells interact with laminins through receptor-ligand interactions, mostly via various integrins [16]. The isoforms are recognized by different receptors, and will consequently induce different cellular responses. Virtanen *et al.* [17] found that the BM in and around adult human islets contained collagen IV, nidogen, and the laminin isoforms Lm-511 and -521. Lm-511 was co-localized with expression of the laminin receptor Lutheran blood group glycoprotein, and also the integrin subunits α3 and β1 were present. In developing human islets the composition of the BM was found to be similar to the adult islets, mostly containing the laminin-511 isoform, but also a smaller amount of laminin-111 [18]. Cirulli *et al.* [19] also identified vitronectin and fibronectin in developing islets.

The effect of various BM-cell interactions on β-cell development and function is still unclear, although some studies have been done. Laminin interaction with integrin α6β1 has been found to promote insulin transcription in mouse β-cells [20], but not in dissociated human islets [21]. The insulin secretion from human β-cells has been reported to be positively influenced by binding to collagen IV, an effect that was abolished by blocking the binding to integrin α1β1 [21, 22].

Alginate is a popular material for cell immobilization, particularly due to its ability to spontaneously form gels under close to physiological conditions in the presence of divalent cations, as well as its good biocompatibility [23]. Alginates are natural polymers of 1→4-

linked α-L-guluronate (G) and β-D-mannuronate (M). They are considered as block polymers; with homopolymeric blocks of M and G, as well as blocks with an alternating sequence. The polymer in its native form does not contain any sites for cell attachment or other specific receptor interaction. However, some alginates have been shown to influence keratinocyte morphology and gene expression in a structure- or calcium-dependent manner [24]. Cell receptor-BM interaction often occurs by binding to short peptide sequences in the BM proteins [25, 26]. In order to make the alginate matrix more bioactive, the polymer may be chemically functionalized with cell-interactive residues such as receptor-binding peptides [27].

Several groups have reported the use of alginate gels in the context of pluripotent stem cell maintenance and differentiation. Alginate scaffolds/gels have been shown to support long-term maintenance of pluripotency [28], proliferation [29, 30], endodermal differentiation [31], as well as neural [32] and hepatic [33, 34] specification. Pancreatic specification was achieved for murine stem cells encapsulated in alginate [29]. All of these studies applied natural (non-functionalized) alginate. In the study herein, we studied the development of hESC-derived pancreatic progenitors in alginate gels, both in alginate alone and in the presence of various ECM components, including three ECM-derived peptides; RGD, YIGSR, and IKVAV. The RGD peptide sequence was chosen due to its role in cellular adhesion and presence in fibronectin, laminin and several other ECM proteins [25]. The laminin-derived peptides IKVAV and YIGSR were included due to their presence in laminins found in islet BM [26]. Moreover, the peptides have been shown to positively influence the viability (all three peptides) and insulin secretion (IKVAV) in a mouse β-cell line [35] and in murine islets (IKVAV) [36].

Materials and methods

hESC culture conditions. hESCs (H9 from WiCell, Madison, WI, USA) were maintained and

differentiated according to Cho *et al.* with the use of defined culture media [5]. The differentiation protocol to pancreatic progenitors avoids the use of feeder, stroma cells or serum that can interfere with experimental outcomes and that is relevant for future clinical application [5]. For the maturation stage the cells, either in capsules or non-encapsulated, were maintained in Advanced DMEM/F12 supplemented with 1% B27, 1% Pen/Strep, 1% L-glutamine (all from Life Technologies), 0.5% BSA, and 0.9 µg/ml all-trans retinoic acid (Sigma). A schematic presentation of the hESC culture is given in figure 1.

Encapsulation materials. Ultra-pure high-G (67% G, $N_{G>1}$=12, Mw=187 kDa) sodium alginate was purchased from Novamatrix (UPLVG, FMC Biopolymer, Norway). Recombinant laminins (Lm-511, -411, -111) were purchased from Biolamina AB (Stockholm, Sweden), collagen IV (CollIV) was purchased from BD (New Jersey, NJ, USA), and fibronectin (Fn) was purchased from Sigma Aldrich (St. Louis, MO, USA). All ECM proteins were of human origin.

Peptide-functionalized alginate. Alginate was functionalized with GRGDSP [25], GIKVAV [37] or GYIGSR [38] peptides (CASLO laboratories, Denmark) using carbodiimide chemistry [27]. The alginate was dissolved in 2-(N-morpholino)ethanesulfonic acid **(**MES) buffer (1 % alginate, 0.1 M MES, 0.3 M NaCl, pH 5.5). Sulfo-N-hydroxysuccinimide (sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were added at 1:10 molar ratio to the uronic acid monomers of the alginate. The solution was stirred for 30 minutes at room temperature before adding peptide at a corresponding 1:20 molar ratio. The coupling reaction proceeded at RT for 16 hours before the solution was transferred to a dialysis membrane (Spectra/Por® dialysis membrane, MWCO 12-14,000, Spectrum Labs Inc.) and dialyzed against three changes of distilled water, three changes of 100 mM NaCl, and finally against distilled water until conductivity measurements read < 0.1 µS. Finally, the peptide-functionalized alginates were purified by the addition of active carbon, followed by filtration

and lyophilisation. Degree of peptide coupling was estimated using ^{1}H-NMR spectroscopy (400MHz, Bruker, Germany) to be 0.4%, 1.0% and 0.7%, for Alg-GYIGSR, Alg-GIKVAV and Alg-GRGDSP, respectively, of monomers in the alginate substituted with peptide. ^{1}H-NMR spectrum of the respective samples and assigned peaks are given in supplementary materials. The biological activity of the peptide-coupled alginates was confirmed in a cell attachment study, also described in supplementary material.

Encapsulation of pancreatic progenitors. At day 18-19 of differentiation, collagenase (Sigma) was added to the adherent cells. After 10 minutes exposure to the enzyme, the cells were removed with cell scraper, resulting in cell clusters of varying size, along with a smaller fraction of dissociated single cells. The collagenase was inactivated by addition of BSA-containing media, and the cells were subsequently washed in Advanced DMEM/F12 without BSA. The cell suspension was added to a 5 ml syringe containing the alginate/ECM encapsulation materials and carefully mixed. The final concentration of alginate in the formulation was 1.5 % for all conditions. Gel beads were made by dripping the alginate solution into a gelling bath containing 50mM $CaCl_2$, 1 mM $BaCl_2$, 0.15M mannitol and 10mM MOPS buffer (pH 7.2-7.4) using an electrostatic bead generator with electrostatic potential difference 7kV, flow 10ml/h, needle with an outer diameter of 0.35mm, and a distance of 2 cm between needle and gelling solution surface, producing beads with a diameter of about 450-500 μm [39]. The concentration of ECM additions to the alginate beads was roughly based on Weber and Anseth [36], and is given in table 1 for all conditions.

Cell viability. The viability of the cells was qualitatively assessed using Live/Dead® viability/cytotoxicity kit (Life Technologies), visualizing viable and dead cells by staining with the fluorescent dyes calcein and ethidium homodimer-1 (EthD-1), respectively. Cells cultured in alginate beads were stained with 2 mM EthD-1 and 2 mM Calcein AM for 60 min before the viability was assessed using fluorescence microscopy (Olympus IX71).

RealTime-PCR. The expression of several pancreatic markers was assessed using qPCR at days 14 and 46 after encapsulation for all samples (encapsulated and non-encapsulated samples). Total RNA was isolated using High Pure RNA isolation kit (Roche, Indianapolis, IN, USA). Reverse transcription was performed using First-Strand cDNA Synthesis kit (Amersham Biosciences), following the manufacturer's instructions. PCR primers were purchased from Sigma Aldrich (St. Louis, MO, USA) (see supplementary table S1 for a list of primer sequences). All the primers used in the manuscript were validated by analyzing the dissociation curve and tested on multiple samples. Importantly, they were also used on time course where the expression of the corresponding gene gradually increased as shown by Cho *et al.* [5] and Hannan *et al*. [40]. RT-PCR was performed on Applied Biosystems® 7500 Real-Time PCR System, with Power SYBR Green Master Mix (Life Technologies), and gene expression relative to undifferentiated hESCs was calculated using the $\Delta\Delta C_t$ method [41]. The expression of PBGD was used as endogenous control.

Immunohistochemistry. All samples were embedded in a "coagel" by first adding human plasma and then gelling with thrombin. Next, the samples were fixed in 10% formalin overnight, dehydrated, and embedded in paraffin before sectioning and staining with hematoxylin and eosin (H&E).

Statistical analyses. Results were expressed as mean ± standard error. Comparisons of independent groups were analyzed with unpaired t-test.

Results and Discussion

Screening of encapsulation materials for efficiency as facilitator for the maturation of beta-cells

In order to identify encapsulation materials warranting a more thorough investigation, a

screening experiment was performed in which clusters of hESC-derived pancreatic progenitors were encapsulated in alginate gels containing a variety of ECM proteins or peptides, and cultured for two weeks. An overview over the conditions tested is given in Table 1. Cell viability was qualitatively assessed using the Live/Dead fluorescence assay, and viability appeared to be high (>80%), constant throughout the 14-day experiment, and similar for all conditions (data not shown). Assessment of the expression of selected pancreatic markers (figure 2) at day 14 post encapsulation revealed no increase in the β-cell markers PDX1 (pancreatic and duodenal homeobox domain 1) and insulin for any of the encapsulated samples relative to the non-encapsulated control, with the exception of Alg-GRGDSP for which a small increase in insulin expression was measured ($P<0.05$) Rather, the general trend was insulin and PDX1 expression levels similar to or lower than the non-encapsulated cells of both of these markers for all conditions. PDX1 is also a common marker of pancreatic progenitor cells [42], whose expression is down-regulated upon differentiation to endocrine cells other than β-cells. A decline in expression of this gene could therefore indicate that the progenitor cells either assume a non-β-cell endocrine fate, or revert to an earlier stage of differentiation. Interestingly, all encapsulated samples showed a higher expression of the endocrine hormones glucagon (GCG) (normally expressed in α-cells) and somatostatin (SST) (normally expressed in δ-cells) than the non-encapsulated control, indicating that, compared to the cells in suspension culture, the encapsulated cells had a higher likelihood of assuming endocrine specificities other than β-cells. Also the expression of neurogenin3 is higher in most of the encapsulated samples. During normal development, NGN3 is transiently expressed in endocrine progenitors and downregulated as the progenitors start to express hormones [43]. A higher expression in the encapsulated cells may therefore indicate a lower degree of progenitor maturation under these conditions.

In order to explore synergistic effects of combinations of ECM proteins, combinations of collIV and various laminins, as well as a mixture of Lm-411/-511 were included in the screening. Overall, there was little difference between gene expression for cells encapsulated with the combination of proteins and their respective single components. A possible exception is the combination of Lm-411 and collIV, for which the expression of neurogenin3, PDX1 and somatostatin is marginally higher than for Lm-411 or collIV alone. The opposite effect is seen for the combination of collIV and Lm-111, where the expression for all genes is lower compared to alginate mixed with Lm-111 alone.

The gene expression from the screening experiment, with a focus on elevated levels of PDX1 and insulin, aligned with known composition of the BM in human islets, formed the basis for selecting a set of five conditions; Alg-GRGDSP, Alg+Lm111, Alg+Lm511, and non-encapsulated cells and simple alginate bead controls, that were tested in two additional biological replicates and subjected to further analysis described in the following.

Encapsulated cells are viable and maintain a stable morphology

Collagenase treatment and cell scraping at day 18 of differentiation resulted in cell clusters of a wide range of sizes up to approximately 400 μm in diameter. The cell clusters were encapsulated or cultured without capsules, and analyzed at 14 and 46 days after encapsulation. Live/Dead® staining (figure 3) revealed that the alginate beads contained larger cell clusters (50 – 400 μm) with very high viability for all conditions tested, with no apparent change over the timeframe of the experiment. Dead cells were mostly single cells and smaller clusters (figure 3). Most dead cells were present early in after encapsulation and indicate that cell death is mostly due to the collagenase/cell scraping procedure or the encapsulation process. As no single cells and small clusters were seen for the non-encapsulated sample, these have most likely been washed off or aggregated to larger clusters. The stable and high degree of viability for the larger clusters in the longer term indicates that the alginate bead environment

is compatible with cell survival. The alginate bead environment has previously been shown to be compatible with high cell viability for several cell types, including pluripotent stem cells [28] and human islets [44].

The non-encapsulated cells showed a marked increase in cluster size over time, due to aggregation of clusters as seen by an increase in cluster sizes to more than 1000 μm. As expected due to the limitations on cell mobility imposed by the alginate network, no such aggregation was seen for the encapsulated cell clusters. This is in line with previous work where alginate beads were shown to be efficient in preventing aggregation of porcine neonatal pancreatic cell clusters [45]. The encapsulated clusters were found to be of similar size at both day 14 and 46 after encapsulation, ranging from 50 to 400 μm. However, some cells were able to escape the beads and proliferate to form clusters in suspension (these cells were removed prior to RNA extraction). The escape of cells from alginate-based microcapsules has previously been reported for dividing cells [46], both from the surface of apparently intact capsules and from broken capsules upon mechanical destabilization caused by the growing cells. Hence this indicates that in this study, the alginate network prevents cell proliferation, but that the cells are capable of proliferation given no mechanical restrictions. In turn this also implies that increase in cluster size for the non-encapsulated clusters may also be an effect of cell proliferation, in addition to aggregation.

Interestingly, the non-encapsulated cell clusters showed tissue organization, while no such development was seen in the encapsulated samples, where the cell morphology was more homogenous. Teratoma formation is a critical safety concern in PSC differentiation. From morphological assessment of H&E-stained sections of the tissues formed in suspension culture, no ecto- or mesodermal cells were identified, leading to the conclusion that the tissue organization did not represent teratoma formation. This is in accordance with previously published results following the same differentiation protocol [5], however, the observation

was not verified by immunostaining. Another concern when encapsulating cell clusters is central necrosis due to limited oxygenation caused by the lack of vascularization and increased diffusion distance through the bead [47, 48]. Sectioning and H&E staining (figure 3) revealed no evidence of central necrosis in any of the samples.

Alginate matrices do not stimulate progenitor maturation into insulin-producing cells.
Expression of selected pancreatic genes relative to non-encapsulated cells is shown for one representative biological replicate in figure 4 (see supplementary data figure S1 for another biological replicate).

Normalization of the qPCR data for non-encapsulated cells at day 14 against nondifferentiated hESCs revealed relative expression values for beta cell markers in the three biological replicates ranging between 50-160 for PDX1 and between 1600-4500 for insulin. These values are in the low range of what has previously been reported for fetal beta cells with relative expression of about 200 and 5000 for PDX1 and insulin, respectively [40]

Overall, there was little difference in gene expression between the different encapsulation materials, and alginate without any ECM additions showed similar or higher expression of pancreatic markers than the conditions including ECM components, indicating that the additions did not stimulate pancreatic differentiation. The non-encapsulated cells had a higher expression of PDX1 than the encapsulated cells, but no difference was seen in the expression of the other pancreatic markers. The difference between the different time-points was more prominent. For all conditions, including the non-encapsulated control, there was an increase in the expression of somatostatin and glucagon, while the expression of insulin generally declined with extended time in culture or was kept at a constant level (figure S1). The expression of PDX1 declined over time for all encapsulated samples, while it remained stable and was significantly higher ($P<0.05$) for the non-encapsulated EBs. PDX1 is initially

expressed in common pancreatic precursor cells, and over the course of pancreatic development it is expressed in multihormonal precursor cells before being further restricted to expression exclusively in β-cells as the cells mature [42]. The loss of PDX1 expression and simultaneous increase in SST and GCG in pancreatic progenitors may therefore indicate maturation from the progenitor stage into α- or δ-like cells. Neurogenin3 (NGN3) expression was slightly reduced in long-term culture for the cells encapsulated in alginate, as well as the non-encapsulated cells ($P<0.05$), while the trend for the other conditions is unclear. As previously discussed, the expression of neurogenin3 is normally downregulated as the expression of hormones is turned on [43], and the reduction in NGN3 expression may therefore also be related to the general increase in GCG and SST expression.

One could speculate if limited oxygen supply could have affected the differentiation of the cell clusters, as recently suggested by Rezania *et al.* [7] that used air interface culture to promote beta cell maturation in clusters from the stage of pancreatic endocrine precursors. Too low oxygen tension may be a reason that the clusters fail to mature over time in culture. Higher oxygen tension for the non-encapsulated clusters could be the reason for a higher PDX1 expression relative to the encapsulated clusters. On the other hand, our recent experience with culture of human islets in capsules of similar size (450-500 μm) points towards a protective role of the alginate hydrogel for encapsulated tissue when cultured under limited oxygen supply [49].

Limited access to the molecules in the culture media for the clusters in the capsules due to restrictions in the capsule permeability could also be a reason for failure maturation of the cells in the capsules. Calcium-alginate gels with 1.8% alginate are permeable to IgG (150 kDa) [50], hence most culture media additions will freely diffuse into the alginate beads, including BSA (66 kDa). Indeed, pancreatic islets have been shown to be viable and functioning in alginate gels over the lifespan of mice [51], indicating that diffusion

restrictions of the material is not limiting to the viability and function of the encapsulated tissue. In this study, the similarities of expression of pancreatic markers in encapsulated and non-encapsulated samples indicate that there is no significant reduction in diffusion of relevant molecules to the encapsulated tissue.

Interestingly, the two conditions that stood out as particularly promising in the screening; RGD-alginate and alginate mixed with Lm-111 ($p = 0.003$ and $p = 0.018$, respectively, relative to alginate encapsulated sample for insulin expression), did not show the same trend in the follow-up experiments, indicating that the relatively high expression of pancreatic genes in the screening experiment may have been due to stochastic differentiation, or other unexplained factors.

The addition of ECM proteins did not seem to influence the viability or pancreatic gene expression of the encapsulated cells. The cells were encapsulated as clusters, and any cell-matrix interactions will therefore be limited to cells on the surface of the clusters. Moreover, in order for receptor binding to occur, the substrate must be correctly presented to the cells. For example, affinity towards RGD peptides as well as the type of signaling and cellular response induced by the interaction has been shown to depend on peptide conformation and immobilization [52, 53]. Unlike fibrous proteins such as collagen, laminin does not participate in matrix formation. In normal BM, laminin is tethered to the collagen network, and as the laminin added to the system in this study was not immobilized by conjugation to the matrix, it may not have been appropriately presented for proper receptor binding and signaling to occur. Weber and Anseth [36] reported significant improvements in mouse islet function when encapsulated in the presence of a similar set of ECM components physically entrapped in a poly(ethylene glycol) hydrogel. These results indicate that detectable effects of pancreatic cells interacting with the ECM components should be possible to determine also in the present alginate encapsulation system. In the present study, cell-matrix interactions were only

assessed indirectly based on a potential downstream effect on pancreatic gene expression, and direct investigation e.g. of integrin expression or inhibition studies, may show interactions previously undetected.

Characterization of the peptide coupling to alginate by ^{1}H-NMR spectroscopy showed that 0.4-1.0 % of the monomers were substituted with peptide. An attachment study indicated that the peptide-coupled alginates produced for the study herein are sufficiently modified to induce a cellular response in C2C12 cells (supplementary material). This result is in accordance with what is expected from the literature where several groups, including our own, have reported induction of various cellular responses using materials with a similar or lower degree of substitution [54-56]. It is still possible that this degree of coupling results in a peptide density too low to induce significant cellular response for the system studied herein [57], and further studies may include alginates with a higher peptide density produced with methods other than carbodiimid chemistry. Combinatorial approaches where the alginate is functionalized with combinations of different biofunctional ligands have also shown promise in several recent studies using other cell types [58, 59], and such strategies should also be explored. Finally, in light of the recent reports of *in vitro* differentiation of pluripotent stem cells to functional insulin producing cells by Melton and Kieffers labs [7, 8], the encapsulation of pancreatic progenitors at an earlier stage of differentiation, e.g. stage 4 of pancreatic endoderm, is interesting. Also the inclusion of heparin, or sulfated alginate as a heparin analogue [60] is intriguing, together with other modifications of the cell culture as described in the recent papers [7, 8].

Conclusion

Although no induction of β-cell specification was recognized due to encapsulation of pancreatic progenitors in alginate including extracellular components in this study, the progenitors were viable and expressed endocrine markers at similar levels as cell clusters in

suspension culture. Insulin expression was relatively stable for all conditions throughout the 46-day timeframe of the experiment, but a decrease in PDX1 and an increase in glucagon and somatostatin over time indicate a dynamic expression profile of the cell clusters in the alginate beads as well as in culture of non-encapsulated clusters. The alginate matrix stabilized the cluster size and cell morphology even for long-term culture, in contrast to the non-encapsulated clusters. Encapsulation in alginate can provide a chemically defined, xeno-free and easily scalable alternative for culture of pancreatic progenitors. Although no effect was seen in this study, further tuning of the microenvironment by manipulation of mechanical and chemical properties, including increased functionalization of the matrix along with other changes in culture conditions may still be a relevant strategy for stimulating maturation of stem cell-derived pancreatic cells.

Acknowledgements

We thank the cellular and molecular imaging core facilities at NTNU (CMIC) for performing immunohistochemistry, Dr. Rahmi Lale for assisting with qPCR, Wenche I. Strand for running NMR and Dr. Finn Aachmann for peak assignments. We are grateful for the financial support from NTNU, strategic funds (KF), Liaison Committee between the Central Norway Regional Health Authority and NTNU (BLS) and the Norwegian Research Council and the Marpol project 217708/O10 (BLS, KF).

References

1. Efrat, S. and H.A. Russ, *Making beta cells from adult tissues.* Trends Endocrinol Metab, 2012. **23**(6): p. 278-285.
2. Thomson, J.A., J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, and J.M. Jones, *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**(5391): p. 1145-7.
3. Takahashi, K., K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, and S. Yamanaka, *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-872.

4. D'Amour, K.A., A.G. Bang, S. Eliazer, O.G. Kelly, A.D. Agulnick, N.G. Smart, M.A. Moorman, E. Kroon, M.K. Carpenter, and E.E. Baetge, *Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells.* Nat Biotechnol, 2006. **24**(11): p. 1392-401.
5. Cho, C.H., N.R. Hannan, F.M. Docherty, H.M. Docherty, M. Joao Lima, M.W. Trotter, K. Docherty, and L. Vallier, *Inhibition of activin/nodal signalling is necessary for pancreatic differentiation of human pluripotent stem cells.* Diabetologia, 2012. **55**(12): p. 3284-95.
6. Zhang, D., W. Jiang, M. Liu, X. Sui, X. Yin, S. Chen, Y. Shi, and H. Deng, *Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells.* Cell Res, 2009. **19**(4): p. 429-38.
7. Rezania, A., J.E. Bruin, P. Arora, A. Rubin, I. Batushansky, A. Asadi, S. O'Dwyer, N. Quiskamp, M. Mojibian, T. Albrecht, Y.H. Yang, J.D. Johnson, and T.J. Kieffer, *Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells.* Nat Biotechnol, 2014. **32**(11): p. 1121-33.
8. Pagliuca, F.W., J.R. Millman, M. Gurtler, M. Segel, A. Van Dervort, J.H. Ryu, Q.P. Peterson, D. Greiner, and D.A. Melton, *Generation of functional human pancreatic beta cells in vitro.* Cell, 2014. **159**(2): p. 428-39.
9. Rezania, A., J.E. Bruin, M.J. Riedel, M. Mojibian, A. Asadi, J. Xu, R. Gauvin, K. Narayan, F. Karanu, J.J. O'Neil, Z. Ao, G.L. Warnock, and T.J. Kieffer, *Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice.* Diabetes, 2012. **61**(8): p. 2016-29.
10. De Krijger, R.R., H.J. Aanstoot, G. Kranenburg, M. Reinhard, W.J. Visser, and G.J. Bruining, *The midgestational human fetal pancreas contains cells coexpressing islet hormones.* Dev Biol, 1992. **153**(2): p. 368-75.
11. Polak, M., L. Bouchareb-Banaei, R. Scharfmann, and P. Czernichow, *Early pattern of differentiation in the human pancreas.* Diabetes, 2000. **49**(2): p. 225-32.
12. Kroon, E., L.A. Martinson, K. Kadoya, A.G. Bang, O.G. Kelly, S. Eliazer, H. Young, M. Richardson, N.G. Smart, J. Cunningham, A.D. Agulnick, K.A. D'Amour, M.K. Carpenter, and E.E. Baetge, *Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo.* Nat Biotechnol, 2008. **26**(4): p. 443-52.
13. Jiang, W., Y. Shi, D. Zhao, S. Chen, J. Yong, J. Zhang, T. Qing, X. Sun, P. Zhang, M. Ding, D. Li, and H. Deng, *In vitro derivation of functional insulin-producing cells from human embryonic stem cells.* Cell Res, 2007. **17**(4): p. 333-44.
14. Shim, J.H., S.E. Kim, D.H. Woo, S.K. Kim, C.H. Oh, R. McKay, and J.H. Kim, *Directed differentiation of human embryonic stem cells towards a pancreatic cell fate.* Diabetologia, 2007. **50**(6): p. 1228-38.
15. Kruegel, J. and N. Miosge, *Basement membrane components are key players in specialized extracellular matrices.* Cell Mol Life Sci, 2010. **67**(17): p. 2879-95.
16. Durbeej, M., *Laminins.* Cell Tissue Res, 2010. **339**(1): p. 259-68.
17. Virtanen, I., M. Banerjee, J. Palgi, O. Korsgren, A. Lukinius, L.E. Thornell, Y. Kikkawa, K. Sekiguchi, M. Hukkanen, Y.T. Konttinen, and T. Otonkoski, *Blood vessels of human islets of Langerhans are surrounded by a double basement membrane.* Diabetologia, 2008. **51**(7): p. 1181-91.
18. Otonkoski, T., M. Banerjee, O. Korsgren, L.E. Thornell, and I. Virtanen, *Unique basement membrane structure of human pancreatic islets: implications for beta-cell growth and differentiation.* Diabetes Obes Metab, 2008. **10 Suppl 4**: p. 119-27.
19. Cirulli, V., G.M. Beattie, G. Klier, M. Ellisman, C. Ricordi, V. Quaranta, F. Frasier, J.K. Ishii, A. Hayek, and D.R. Salomon, *Expression and function of alpha(v)beta(3) and alpha(v)beta(5) integrins in the developing pancreas: roles in the adhesion and migration of putative endocrine progenitor cells.* J Cell Biol, 2000. **150**(6): p. 1445-60.
20. Nikolova, G., N. Jabs, I. Konstantinova, A. Domogatskaya, K. Tryggvason, L. Sorokin, R. Fassler, G. Gu, H.P. Gerber, N. Ferrara, D.A. Melton, and E. Lammert, *The vascular basement*

membrane: a niche for insulin gene expression and Beta cell proliferation. Dev Cell, 2006. **10**(3): p. 397-405.
21. Kaido, T., M. Yebra, V. Cirulli, C. Rhodes, G. Diaferia, and A.M. Montgomery, *Impact of defined matrix interactions on insulin production by cultured human beta-cells: effect on insulin content, secretion, and gene transcription.* Diabetes, 2006. **55**(10): p. 2723-9.
22. Kaido, T., M. Yebra, V. Cirulli, and A.M. Montgomery, *Regulation of human beta-cell adhesion, motility, and insulin secretion by collagen IV and its receptor alpha1beta1.* J Biol Chem, 2004. **279**(51): p. 53762-9.
23. Smidsrød, O. and G. Skjåk-Bræk, *Alginate as immobilization matrix for cells.* Trends in Biotechnology, 1990. **8**(3): p. 71-78.
24. Stenvik, J., H. Sletta, O. Grimstad, B. Pukstad, L. Ryan, R. Aune, W. Strand, A. Tondervik, S.H. Torp, G. Skjak-Braek, and T. Espevik, *Alginates induce differentiation and expression of CXCR7 and CXCL12/SDF-1 in human keratinocytes--the role of calcium.* J Biomed Mater Res A, 2012. **100**(10): p. 2803-12.
25. Ruoslahti, E., *RGD and other recognition sequences for integrins.* Annu Rev Cell Dev Biol, 1996. **12**: p. 697-715.
26. Yamada, K.M., *Adhesive recognition sequences.* J Biol Chem, 1991. **266**(20): p. 12809-12.
27. Rowley, J.A., G. Madlambayan, and D.J. Mooney, *Alginate hydrogels as synthetic extracellular matrix materials.* Biomaterials, 1999. **20**(1): p. 45-53.
28. Siti-Ismail, N., A.E. Bishop, J.M. Polak, and A. Mantalaris, *The benefit of human embryonic stem cell encapsulation for prolonged feeder-free maintenance.* Biomaterials, 2008. **29**(29): p. 3946-52.
29. Wang, N., G. Adams, L. Buttery, F.H. Falcone, and S. Stolnik, *Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells.* J Biotechnol, 2009. **144**(4): p. 304-12.
30. Serra, M., C. Correia, R. Malpique, C. Brito, J. Jensen, P. Bjorquist, M.J. Carrondo, and P.M. Alves, *Microencapsulation technology: a powerful tool for integrating expansion and cryopreservation of human embryonic stem cells.* PLoS One, 2011. **6**(8): p. e23212.
31. Chayosumrit, M., B. Tuch, and K. Sidhu, *Alginate microcapsule for propagation and directed differentiation of hESCs to definitive endoderm.* Biomaterials, 2010. **31**(3): p. 505-514.
32. Li, L., A.E. Davidovich, J.M. Schloss, U. Chippada, R.R. Schloss, N.A. Langrana, and M.L. Yarmush, *Neural lineage differentiation of embryonic stem cells within alginate microbeads.* Biomaterials, 2011. **32**(20): p. 4489-97.
33. Maguire, T., A.E. Davidovich, E.J. Wallenstein, E. Novik, N. Sharma, H. Pedersen, I.P. Androulakis, R. Schloss, and M. Yarmush, *Control of hepatic differentiation via cellular aggregation in an alginate microenvironment.* Biotechnol Bioeng, 2007. **98**(3): p. 631-44.
34. Ramasamy, T.S., J.S. Yu, C. Selden, H. Hodgson, and W. Cui, *Application of three-dimensional culture conditions to human embryonic stem cell-derived definitive endoderm cells enhances hepatocyte differentiation and functionality.* Tissue Eng Part A, 2013. **19**(3-4): p. 360-7.
35. Weber, L.M., K.N. Hayda, K. Haskins, and K.S. Anseth, *The effects of cell-matrix interactions on encapsulated beta-cell function within hydrogels functionalized with matrix-derived adhesive peptides.* Biomaterials, 2007. **28**(19): p. 3004-11.
36. Weber, L.M. and K.S. Anseth, *Hydrogel encapsulation environments functionalized with extracellular matrix interactions increase islet insulin secretion.* Matrix Biol, 2008. **27**(8): p. 667-73.
37. Tashiro, K., G.C. Sephel, B. Weeks, M. Sasaki, G.R. Martin, H.K. Kleinman, and Y. Yamada, *A synthetic peptide containing the IKVAV sequence from the A chain of laminin mediates cell attachment, migration, and neurite outgrowth.* J Biol Chem, 1989. **264**(27): p. 16174-82.
38. Graf, J., Y. Iwamoto, M. Sasaki, G.R. Martin, H.K. Kleinman, F.A. Robey, and Y. Yamada, *Identification of an amino acid sequence in laminin mediating cell attachment, chemotaxis, and receptor binding.* Cell, 1987. **48**(6): p. 989-96.

39. Strand, B.L., O. Gåserød, B. Kulseng, T. Espevik, and G. Skjåk-Bræk, *Alginate-polylysine-alginate microcapsules: effect of size reduction on capsule properties.* Journal of Microencapsulation, 2002. **19**(5): p. 615-630.
40. Hannan, Nicholas R.F., Robert P. Fordham, Yasir A. Syed, V. Moignard, A. Berry, R. Bautista, Neil A. Hanley, Kim B. Jensen, and L. Vallier, *Generation of Multipotent Foregut Stem Cells from Human Pluripotent Stem Cells.* Stem Cell Reports, 2013. **1**(4): p. 293-306.
41. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
42. Kaneto, H., T. Miyatsuka, Y. Fujitani, H. Noguchi, K.-H. Song, K.-H. Yoon, and T.-a. Matsuoka, *Role of PDX-1 and MafA as a potential therapeutic target for diabetes.* Diabetes Research and Clinical Practice, 2007. **77**(3, Supplement): p. S127-S137.
43. Schwitzgebel, V.M., D.W. Scheel, J.R. Conners, J. Kalamaras, J.E. Lee, D.J. Anderson, L. Sussel, J.D. Johnson, and M.S. German, *Expression of neurogenin3 reveals an islet cell precursor population in the pancreas.* Development, 2000. **127**(16): p. 3533-42.
44. Qi, M., Y. Morch, I. Lacik, K. Formo, E. Marchese, Y. Wang, K.K. Danielson, K. Kinzer, S. Wang, B. Barbaro, G. Kollarikova, D. Chorvat, Jr., D. Hunkeler, G. Skjak-Braek, J. Oberholzer, and B.L. Strand, *Survival of human islets in microbeads containing high guluronic acid alginate crosslinked with Ca2+ and Ba2+.* Xenotransplantation, 2012. **19**(6): p. 355-64.
45. Tatarkiewicz, K., M. Garcia M Fau - Lopez-Avalos, S. Lopez-Avalos M Fau - Bonner-Weir, G.C. Bonner-Weir S Fau - Weir, and G.C. Weir, *Porcine neonatal pancreatic cell clusters in tissue culture: benefits of serum and immobilization in alginate hydrogel.* (0041-1337 (Print)).
46. Rokstad, A.M., B. Strand, K. Rian, B. Steinkjer, B. Kulseng, G. Skjak-Braek, and T. Espevik, *Evaluation of different types of alginate microcapsules as bioreactors for producing endostatin.* Cell Transplant, 2003. **12**(4): p. 351-64.
47. Mueller-Klieser, W.F. and R.M. Sutherland, *Influence of convection in the growth medium on oxygen tensions in multicellular tumor spheroids.* Cancer Res, 1982. **42**(1): p. 237-42.
48. O'Sullivan, E.S., A.S. Johnson, A. Omer, J. Hollister-Lock, S. Bonner-Weir, C.K. Colton, and G.C. Weir, *Rat islet cell aggregates are superior to islets for transplantation in microcapsules.* Diabetologia, 2010. **53**(5): p. 937-45.
49. Hals, I.K., A.M. Rokstad, B.L. Strand, J. Oberholzer, and V. Grill, *Alginate microencapsulation of human islets does not increase susceptibility to acute hypoxia.* 2013(2314-6745 (Print)).
50. Morch, Y.A., I. Donati, B.L. Strand, and G. Skjak-Braek, *Effect of Ca2+, Ba2+, and Sr2+ on alginate microbeads.* Biomacromolecules, 2006. **7**(5): p. 1471-80.
51. Cui, H., C. Tucker-Burden, S.M.D. Cauffiel, A.K. Barry, N.N. Iwakoshi, C.J. Weber, and S.A. Safley, *Long-Term Metabolic Control of Autoimmune Diabetes in Spontaneously Diabetic Nonobese Diabetic Mice by Nonvascularized Microencapsulated Adult Porcine Islets.* Transplantation, 2009. **88**(2): p. 160-169.
52. Aumailley, M., M. Gurrath, G. Muller, J. Calvete, R. Timpl, and H. Kessler, *Arg-Gly-Asp constrained within cyclic pentapeptides. Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1.* FEBS Lett, 1991. **291**(1): p. 50-4.
53. Verrier, S., S. Pallu, R. Bareille, A. Jonczyk, J. Meyer, M. Dard, and J. Amedee, *Function of linear and cyclic RGD-containing peptides in osteoprogenitor cells adhesion process.* Biomaterials, 2002. **23**(2): p. 585-96.
54. Re'em, T., O. Tsur-Gang, and S. Cohen, *The effect of immobilized RGD peptide in macroporous alginate scaffolds on TGFβ1-induced chondrogenesis of human mesenchymal stem cells.* Biomaterials, 2010. **31**(26): p. 6746-6755.
55. Duggal, S., K.B. Fronsdal, K. Szoke, A. Shahdadfar, J.E. Melvik, and J.E. Brinchmann, *Phenotype and gene expression of human mesenchymal stem cells in alginate scaffolds.* Tissue Eng Part A, 2009. **15**(7): p. 1763-73.
56. Sandvig, I., K. Karstensen, A.M. Rokstad, F.L. Aachmann, K. Formo, A. Sandvig, G. Skjak-Braek, and B.L. Strand, *RGD-peptide modified alginate by a chemoenzymatic strategy for tissue engineering applications.* J Biomed Mater Res A, 2014.

57. Rowley, J.A. and D.J. Mooney, *Alginate type and RGD density control myoblast phenotype.* J Biomed Mater Res, 2002. **60**(2): p. 217-23.
58. Fonseca, K.B., S.J. Bidarra, M.J. Oliveira, P.L. Granja, and C.C. Barrias, *Molecularly designed alginate hydrogels susceptible to local proteolysis as three-dimensional cellular microenvironments.* Acta Biomater, 2011. **7**(4): p. 1674-82.
59. Madl, C.M., M. Mehta, G.N. Duda, S.C. Heilshorn, and D.J. Mooney, *Presentation of BMP-2 Mimicking Peptides in 3D Hydrogels Directs Cell Fate Commitment in Osteoblasts and Mesenchymal Stem Cells.* Biomacromolecules, 2014.
60. Arlov, O., F.L. Aachmann, A. Sundan, T. Espevik, and G. Skjak-Braek, *Heparin-like properties of sulfated alginates with defined sequences and sulfation degrees.* Biomacromolecules, 2014. **15**(7): p. 2744-50.

Figure legends:

Figure 1: Schematic of the stepwise differentiation of pluripotent stem cells (PSC) to endocrine progenitors (EP) via definitive endoderm (DE), dorsal foregut (DF) and pancreatic endoderm (PE). Each stage represents different culture conditions and is given in detail by Cho *et al*. [5]. The EP was further cultured as clusters under encapsulated or non-encapsulated conditions in Advanced DMEM containing B27, BSA and retinoic acid for 14 – 46 days. The capsules contained various extracellular matrix components as outlined in Table 1.

Figure 2: Expression of pancreatic markers (results from qPCR) relative to non-encapsulated cells for pancreatic progenitors encapsulated at day 18 after start of differentiation. Samples were taken at day 14 after encapsulation (day 33 after start of differentiation). Keys: 1: hESC, Pancreatic progenitors in 2: Alginate, 3: Alg+CollV, 4: Alg+Fn, 5: Alg+Lm411, 6: Alg+Lm111, 7: Alg+Lm511+CollV, 8: Alg+Lm411+CollV, 9: Alg+Lm111+CollV, 10: Alg+Lm511+Lm411, 11: Alg-GIKVAV, 12: Alg-GRGDSP, 13: Alg-GYIGSR, 14: Non-encapsulated pancreatic progenitors. Error bars represent the standard error (SE).

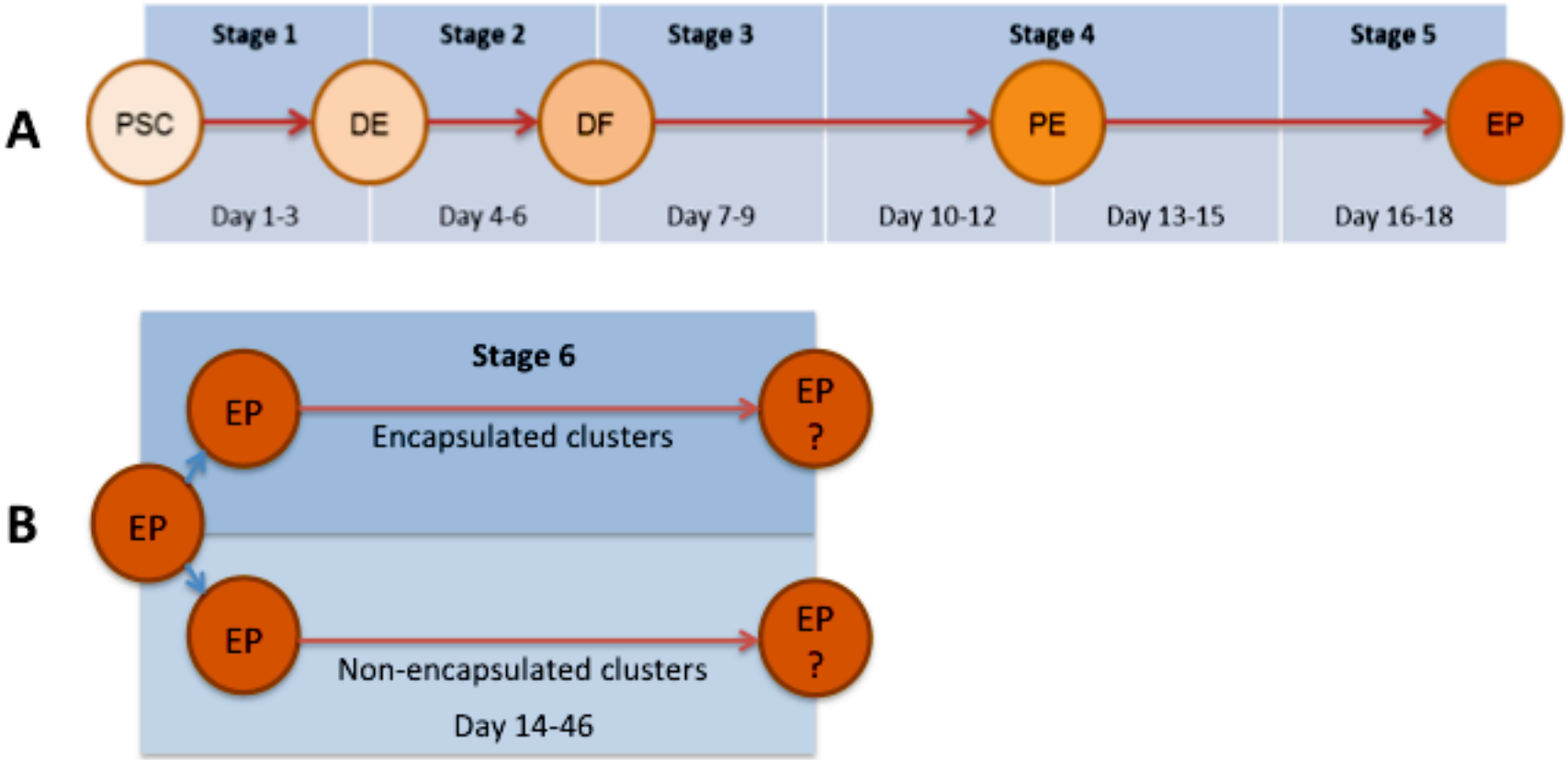
Figure 3: hESC-derived pancreatic progenitors encapsulated at day 18 of differentiation were analyzed at 14 (A) and 46 (B) days after encapsulation. i) Brightfield microscopy (scale bar A = 500, B = 250 μm). ii) Live/Dead® staining (live cells are green, dead cells are red) of the same area as in i). iii) Hematoxylin/eosin stain of fixed and paraffin-embedded samples. (scale bar = 100 μm, scale bar for EB = 200 μm).

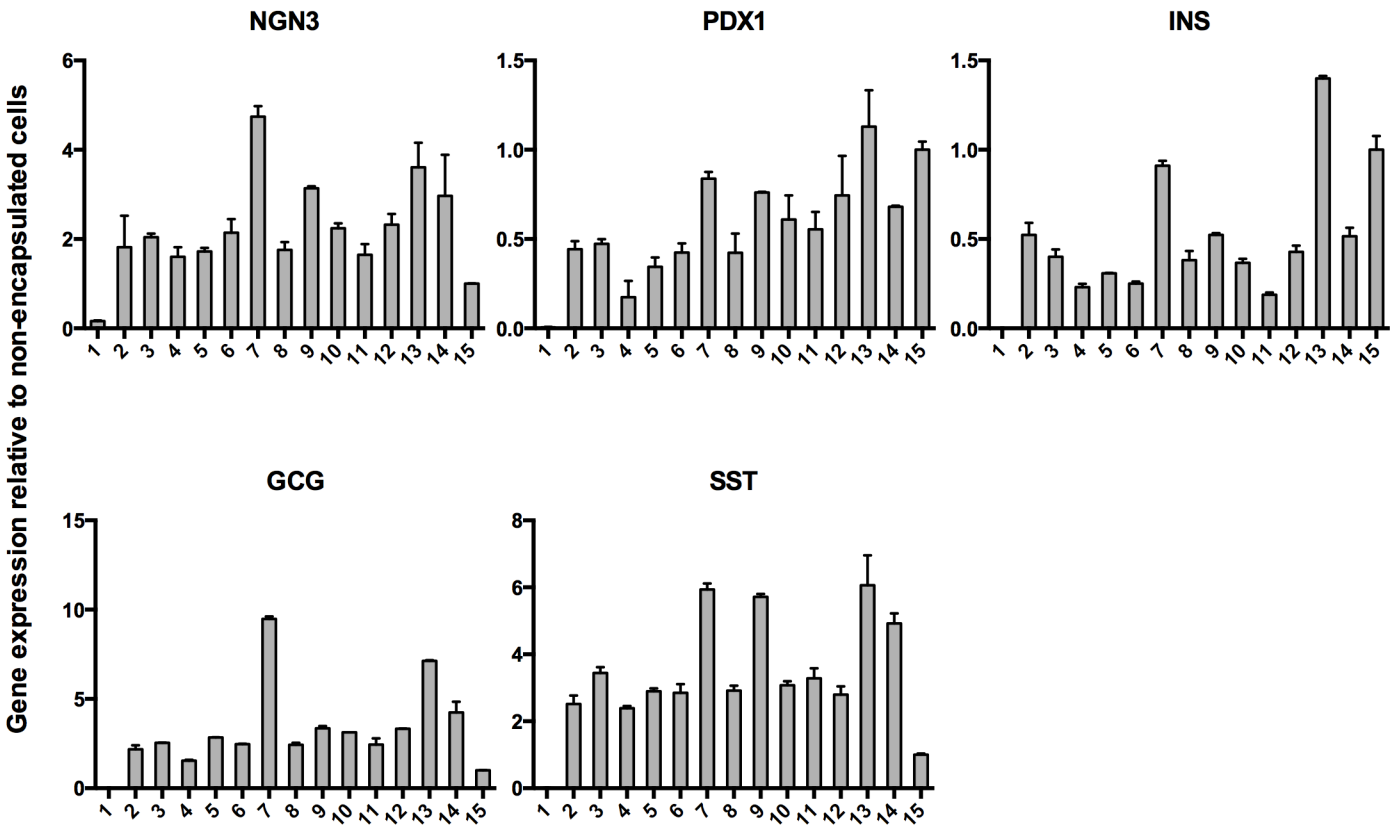
Figure 4: Expression of pancreatic markers (results from qPCR) relative to non-encapsulated cells (day 14) for pancreatic progenitors encapsulated at day 18 after start of differentiation. Data from one representative experiment. Samples were taken at day 14 (black bars) and day 46 (grey bars) after encapsulation. Error bars represent the standard error (SE).

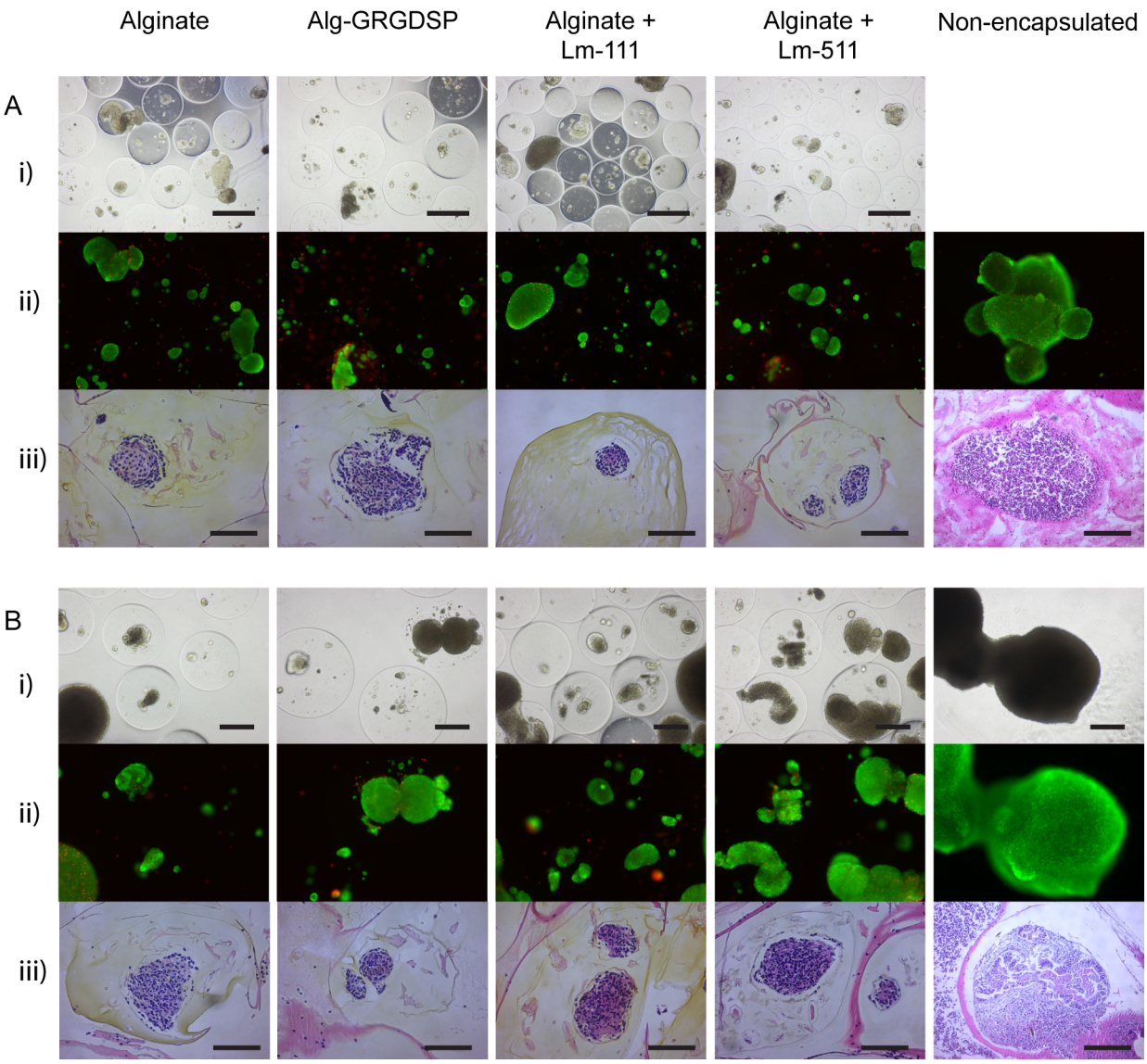
Table 1: Overview of the extracellular matrix components added to each condition. The concentration of alginate was 1.5% for all conditions.

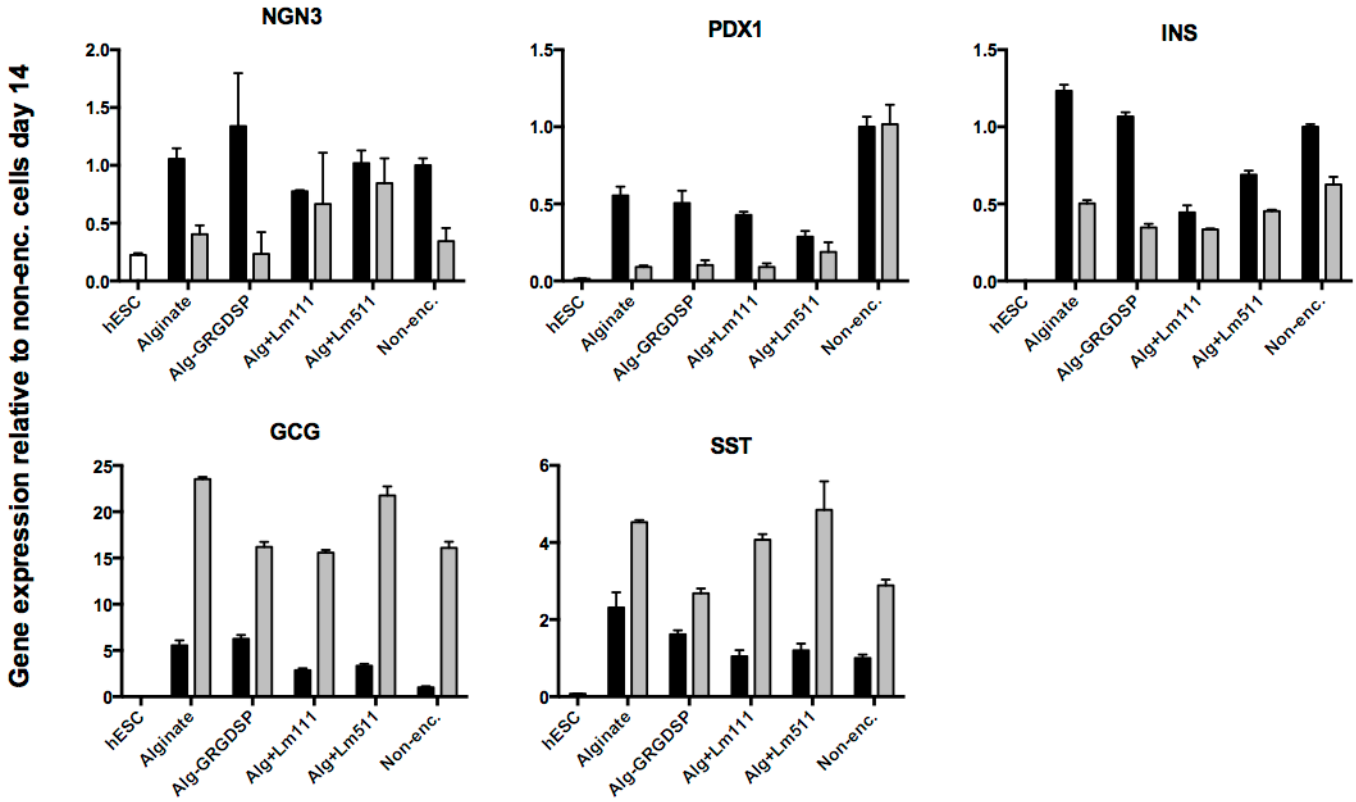
Abbreviation	Type of ECM addition (concentration)
Alginate	N/A
Alg+CollIV	Collagen IV (100 µg/mL)
Alg+Fn	Fibronectin (100 µg/mL)
Alg+Lm511	Laminin-511 (100 µg/mL)
Alg+Lm411	Laminin-411 (100 µg/mL)
Alg+Lm111	Laminin-111 (100 µg/mL)
Alg+Lm511+CollIV	Laminin-511 (75 µg/mL), collagen IV (25 µg/mL)
Alg+Lm411+CollIV	Laminin-411 (75 µg/mL), collagen IV (25 µg/mL)
Alg+Lm111+CollIV	Laminin-111 (75 µg/mL), collagen IV (25 µg/mL)
Alg+Lm511+Lm411	Laminin-511 (50 µg/mL), laminin-411 (50 µg/mL)
Alg-GIKVAV*	Gly-Ile-Lys-Val-Ala-Val peptide (1.0% monomers substituted)
Alg-GRGDSP*	Gly-Arg-Gly-Asp-Ser-Pro peptide (0.7% monomers substituted)
Alg-GYIGSR*	Gly-Tyr-Ile-Gly-Ser-Arg peptide (0.4% monomers substituted)
Non-encapsulated	N/A

*Peptide conjugated to the alginate


A
Stage 1
Stage 2
Stage 3
Stage 4
Stage 5
PSC
DE
DF
PE
EP
Day 1-3
Day 4-6
Day 7-9
Day 10-12
Day 13-15
Day 16-18
B
Stage 6
EP
EP
?
Encapsulated clusters
EP
EP
EP
?
Non-encapsulated clusters
Day 14-46


NGN3
PDX1
INS
GCG
SST
Gene expression relative to non-encapsulated cells


Alginate
Alg-GRGDSP
Alginate + Lm-111
Alginate + Lm-511
Non-encapsulated
A
i)
ii)
iii)
B
i)
ii)
iii)


Gene expression relative to non-enc. cells day 14
NGN3
PDX1
INS
GCG
SST
hESC
Alginate
Alg-GRGDSP
Alg+Lm111
Alg+Lm511
Non-enc.