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

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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 varies 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]. Alginites 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 keratinoocyte 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 ΔΔCt 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 alginate 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.

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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+CollIV, 8: Alg+Lm411+CollIV, 9: Alg+Lm111+CollIV, 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.

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