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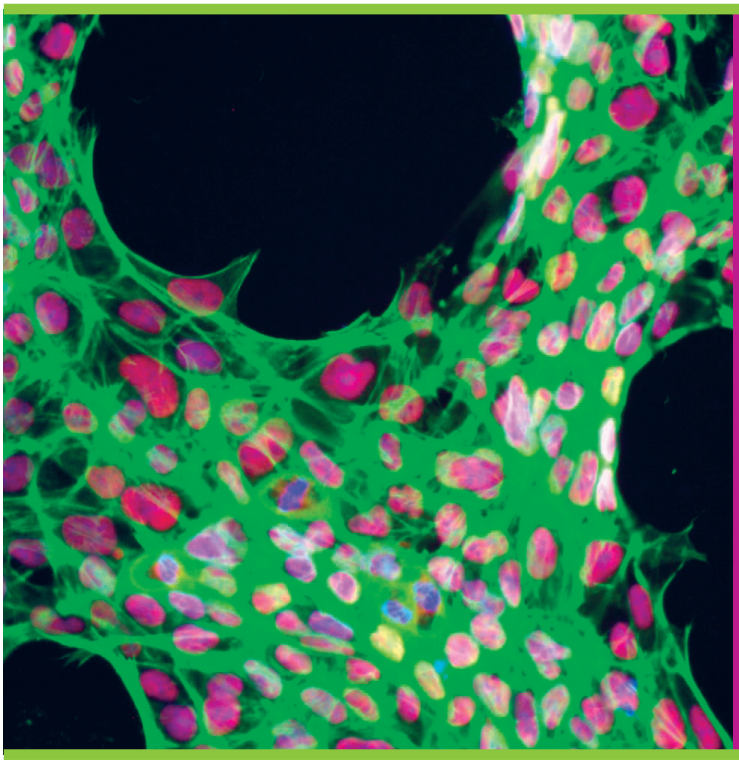
*Publication date:*
2016

*Document Version*
Publisher's PDF, also known as Version of record



# Manipulation of the extracellular microenvironment by micro- and nanotechnology approaches to improve the generation of pancreatic endocrine cells from human embryonic stem cells

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PhD Thesis April 2016

DTU Nanotech
Department of Micro- and Nanotechnology

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PhD Thesis

December 2015

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&

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**Title:** Manipulation of the extracellular microenvironment by micro- and nanotechnology approaches to improve the generation of pancreatic endocrine cells from human embryonic stem cells

# Preface

The project is a 3 year industrial PhD program conducted in collaboration between the company Novo Nordisk A/S and the university Technical University of Denmark (DTU). The project is funded by Innovation Fund Denmark and Novo Nordisk A/S. The majority of laboratory work was carried out at Novo Nordisk. At Technical University of Denmark the array screen was developed and produced. Further, a 3 months external research stay took place in the fall of 2013 at University of Glasgow in Dr. Nikolaj Gadegaards research group.

The PhD project resulted in 3 manuscripts; 1) Collagen type I improves the differentiation of human embryonic stem cells towards definitive endoderm, 2) Enhanced differentiation of human embryonic stem cells towards definitive endoderm on ultra-high aspect ratio nanopillars, and 3) The impact of cell seeding methods on density dependent differentiation.

In paper 1 the PhD candidate conducted all the experimental work and analysis, with the exception of statistical programming for the screen and the micro-array analysis. Paper 2 is a collaboration with University of Glasgow. Here, University of Glasgow produced the nanopillars, conducted the mechanical measurements of the nanopillars and did the image quantification. The PhD candidate did all the cellular experiments and subsequent analysis and imaging. For paper 3, which is also a collaboration with University of Glasgow, the PhD candidate performed all the experiments with human embryonic stem cells and the subsequent analysis and imaging.

The thesis consists of an introduction with a background covering the 3 publications, a discussion section, covering the most important findings in the publications, and finally a conclusion and perspectives section.

# Acknowledgement

One of the things I learned during me PhD is that a scientific research project cannot be executed by a single person and without support and sparring. I am happy that I here can have the chance to express my warm gratitude to the many people I have been lucky to collaborate with.

First I will thank my supervisor at Novo Nordisk Mattias Hansson for letting me continue my study from a master thesis in a PhD project. Mattias believed in my competences and allowed me to direct the project. Moreover, I will thank Dorthe Rønn Petersen; my daily supervisor at Novo Nordisk, for making me prioritize what is “need to know” and what is “nice to know”. I will also thank Dorthe for her honesty, scientific sparring and mostly for editing the papers and the thesis.

A very special thanks to Martin Dufva, my supervisor at Technical University of Denmark. Martin taught me how to bridge two academic fields and emphasised the importance of communication when doing an inter-disciplinary study. Further, Martin always made sure of my wellbeing and continuous development. Martin greatly helped with the paper and thesis writing.

Moreover, I will like to thank Nikolaj Gadegaard for giving me the fantastic opportunity for spending three months in his laboratory at University of Glasgow. The collaboration with University of Glasgow was an eye-opener and a great motivation for the rest of my PhD. Nikolaj and his group gave me a warm welcome and I quickly became part of the group in the basement office. Nikolaj spent hours on knowledge sharing and scientific discussions both in Glasgow and subsequently by many phone calls and emails. Also a special thanks to Paul from University of Glasgow on the scientific collaboration which turned into two paper manuscripts.

I want to give a special thanks to the other students at Novo Nordisk; Jesper Due Jensen, Jeannette Schlichting Kirkegaard, Maja Borup Kjær Petersen and Anna Kirstine Ringgaard. They kept me company during late work hours as well as supported and motivated me. Beside the support they provided me with laboratory instructions and scientific discussions.

Moreover, I want to thank the scientists in the Islet and stem cell Biology Department at Novo Nordisk for scientific discussions and knowledge sharing. Special thanks to Christian Honoré for fruitful challenging me scientifically and provided me with “must read” papers. Thanks for the technicians in the department for teaching various laboratory methods. Thanks to Dorthe, Tove, Heidi and Fanny who help me with my cell culture work when I was away.

# Publications included in the thesis

**Paper 1**

***Collagen type I improves the differentiation of human embryonic stem cells towards definitive endoderm***

Camilla Holzmann Rasmussen, Jonas B. Moeller, Dorthe Roenn Petersen, Mattias Hansson, and Martin Dufva

(Paper accepted in PlosOne, December 2015)

**Paper 2**

***Enhanced differentiation of human embryonic stem cells towards definitive endoderm on ultra-high aspect ratio nanopillars***

Camilla Holzmann Rasmussen, Paul M. Reynolds, Dorthe Roenn Petersen, Mattias Hansson, Robert M. McMeeking, Martin Dufva, and Nikolaj Gadegaard.

(Published in Advanced Functional Materials, December 2015)

**Paper 3**

***The impact of cell seeding methods on density dependent differentiation***

Paul M. Reynolds, Camilla Holzmann Rasmussen, Dorthe Roenn Petersen, Mattias Hansson, Martin Dufva, and Nikolaj Gadegaard.

(Manuscript draft)

# Summary

Human embryonic stem (hES) cells have the ability to generate all cell types in the body, which suggest that they can provide an unlimited source of cells for cell replacement therapy to treat degenerative diseases such as diabetes mellitus. To achieve a stem cell therapy treatment for diabetes mellitus, the hES cells must be differentiated into mature functional insulin producing beta-cells. Current differentiation protocols focus on the addition of soluble molecules whereas the impact of the physical microenvironment has been mainly unattended. However, the physical microenvironment plays an essential role in cellular behaviour during development and recent studies have demonstrated the effect of the physical environment in *in vitro* stem cell differentiation. Thus, understanding the role of the physical microenvironment is vital for the development of effective and consistent differentiation protocols. In this study we manipulated the physical environment during the first two differentiation steps towards beta-cells; definitive endoderm (DE) and pancreatic endoderm (PE). Three different approaches were used: 1) systematically screening of extracellular matrix (ECM) substrates (paper 1), 2) alteration of substrate topography and elasticity (paper 2) and 3) initial seeding and cell distribution (paper 3).

With the first strategy an array screen was performed to systematically identify ECM protein coatings, which induced or inhibited the differentiation of hES cells towards DE. Almost 500 different ECM protein combinations were screened and several candidates were found. The majority of these candidates could be validated in microtitre well plates and further studies demonstrated that certain ECM proteins regulate the differentiation of hES cells towards DE. Netrin 1, collagen 1 and collagen 2 induced DE differentiation to a higher degree than the control fibronectin. Especially, all the analyses pointed to collagen 1 having unique properties. Cultures on collagen 1 had distinct morphology, proliferated faster and most importantly resulted in purer DE cultures with very few undifferentiated cells. Currently, the underlying biological mechanism is not known and is a subject for further studies. However, to our knowledge collagen 1, collagen 2 and netrin 1 have previous not been linked to embryonic stem cell differentiation. Notable, this study demonstrated that the ECM proteins do have a functional role in stem cell differentiation and should be taken into consideration in order to obtain efficient and consistent differentiation protocols.

With the second strategy the physical microenvironment was manipulated with topographies in micro and nanoscale which have different elastics characters. The study consisted of an investigation of the differentiation of hES cells towards DE and PE on nanopillars. The nanopillars with soft character were not favoured by hES cells with regards to attachment and growth. However, DE cells appeared earlier during the DE differentiation on the soft nanopillars when compared to the flat control surface. Moreover, DE cells intensively repopulated the area with soft nanopillars, whereas the undifferentiated cells remained on the flat surface area. The very fast repopulation of DE cells indicated DE cells migrated onto the soft nanopillars, implying that the DE differentiation could be durotaxin driven, where the differentiated cells migrated from rigid substrates towards soft substrates. In contrast, the differentiation towards PE was to a large extent repressed on the soft nanopillars in comparison to the flat control surface. This indicated that the requirements of the phys-

ical environment changes during the different stages of differentiation. Our study demonstrated that the differentiation and cellular behaviour of embryonic stem cells are affected by altering the physical properties of the cell culture surface with nanopillars. Such observations have previously not been reported with embryonic stem cells, indicating that additional dimensions, such as the physical environment, should be taken into account when directing stem cell differentiation.

With the last strategy, the cell seeding density and cell distribution across a well was investigated. With a simple cell seeder device, an even and consistent distribution of cells across individual experiments was obtained for human fibroblast cells, hES cells and DE cells derived from hES cells. A uniform seeding of DE cells resulted in a more uniform differentiation towards PE across the entire well (12-well plate). Such uniformity is important in reproducibility and for assays which includes material from the entire well.

# Dansk resumé

Humane embryonale stamceller har potentialet til at blive til typer celler i kroppen, og derfor kan disse celler være en mulig ubegrænset kilde til celleterapi for at helbrede degenerative sygdomme, som fx diabetes mellitus. For at opnå en stamcelleterapi behandling for diabetes mellitus, skal de humane embryonale stamceller differentieres til insulin-producerende beta-celler. Nuværende differentierings protokoller fokuserer på at tilsætte opløselige vækstfaktorer, imens man har set bort fra det fysiske mikromiljø. Dog har flere studier påvist at det fysiske mikromiljø har en afgørende indvirkning på celledifferentiering, både i udviklingsbiologien og i *in vitro* stamcelle differentiering. Derfor vil en forståelse af det fysiske mikromiljø være vigtig for at udvikle effektive og konsistente differentierings protokoller. I dette studie har vi manipuleret det fysiske mikromiljø i de to første differentieringstrin til beta-celler; definitiv endoderm og pankreatisk endoderm. Vi brugte tre forskellige strategier: 1) systematisk screen af ekstracellulær matrix proteiner (artikel 1), modificering af topografien og elasticiteten af overflader (artikel 2) og udsåningdensitet af celler samt celledistribution (artikel 3).

Med den første strategi udførte vi et protein array screen for systematisk at identificere ekstracellulær matrix protein kombinationer som påvirkede differentiering af humane embryonale stamceller til definitiv endoderm. Der blev screenet for næste 500 kombinationer og flere kandidater blev fundet. Største delen af disse kandidater kunne valideres i mikrotiter plader og videre studier viste at specifikke ekstracellulære matrix proteiner regulerede differentiering af humane embryonale stramceller til definitiv endoderm. Netrin 1, collagen 1 og collagen 2 inducerede definitiv endoderm differentieringen i en signifikant højere grad end kontrollen fibronectin. Den højeste induktion af definitiv endoderm var opnået på collagen 1 substrat, og tidsinterval studier viste DE induktionen på collagen 1 startede en dag tidligere og i langt større grad sammenlignet med kontrollen fibronectin. Dog blev den underliggende biologiske mekanisme ikke kortlagt i disse studier og er derfor et emne for fremtidige studier. Vores studier viste at ekstracellulær matrix har en rolle i stamcelle differentiering og burde tages med i overvejelserne for at opnå effektive og stabile differentierings protokoller.

I den anden strategi blev det fysiske mikromiljø manipuleret med overflade topografier i mikro- og nano skala, som havde elastisk karakter. Studiet bestod af at undersøge differentieringen af humane embryonale stamceller til definitiv endoderm og pankreatisk endoderm på nanosøjler. Humane embryonale stamceller favoriserede ikke bløde nanosøjler med blød karakter i forhold til adhæsion og proliferation. Derimod startede induktionen af definitiv endoderm meget tidlig i differentieringsprotokollen på de bløde nanosøjler sammenlignet med flad overflade. Derudover skete der under differentieringen en intensive invadering aft definitiv endoderm celler på bløde nanosøjler, imens de uddifferentierede celler forblev på de flade område.. Den hurtige og intense invadering af DE celler indikerede at DE celler migrerede fra det flade område ind på de høje nanosøjler. Det indikerede at definitiv endoderm differentiering kan blive durotaxin drevet, hvor differentierede celler kan migrere fra et hårdt substrat (flad overflade) imod et mere blødt substrat (5nN/µm nanosøjler). I kontrast hertil, blev differentiering imod pankreatisk endoderm undertrykt på de høje nanosøjler sammenlignet med flad substrat. Det indikerede at kravene til det fysiske mikromiljø ændres i det forskellige

differentierings stadier. Sådanne observationer er tidligere ikke blevet publiceret med humane embryonale stamceller. Vores studier indikerede at yderligere dimensioner, som fx, the fysiske miljø, skal tages i betragtning ved stam celle differentiering.

I den sidste strategi, blev celleudsåningsdensitet og celledistribution over en hel brønd undersøgt. Med et simpelt celleudsånings-device, kunne en jævn og konsistent celleudsåning over hele brønden opnås med humane fibroblast celler, humane embryonale stamceller og definitive endoderm celler. En jævn udsåning af definitiv endoderm celler resulterede af en jævn differentiering til pankreatisk endoderm over hele brønden (12 brønds format). En sådan ensartethed er vigtig for reproducerbarhed og for assays, hvor materiale fra hele brønden skal bruges.

# Table of contents

# Selected Abbreviations

| | |
|---|---|
| AFM | Atomic force microscopy |
| bFGF/FGF2 | basic fibroblast growth factor |
| BMP | bone morphogenic protein |
| Col | Collagen |
| DE | Definitive endoderm |
| ECM | Extracellular matrix |
| ECMP | Extracellular matrix proteins |
| EMT | Epithelial to mesenchymal transition |
| ES | Embryonic stem |
| FGF | fibroblast growth fact |
| Fn | Fibronectin |
| FnAD | Fibronectin adhesion promoting peptide |
| FoxA2 | Forkhead box protein A2 |
| Gsc | Goosecoid |
| Hep | Heparan sulphate proteoglycan |
| hES | Human embryonic stem |
| Ln | Laminin |
| ME | Mesendoderm |
| mRNA | Messenger ribonucleic acid |
| Ne | Netrin 1 |
| Nid | Nidogen/entactin |
| Hep | Heparan sulphate proteoglycan |
| NKX6.1 | NK6 homeobox 1 |
| Oct3/4 / Oct4 | octamer-binding transcription factor 3/4 |
| PDMS | Polydimethylsiloxane |
| Pdx1 | Pancreatic and duodenal homeobox 1 |
| PE | Pancreatic endoderm |
| qPCR | Quantitative polymerase chain reaction |
| SEM | Scanning electron microscopy |
| Sox | SRY (sex determining region Y)-box |
| Vn | Vitronectin |
| Wnt | Wingless-type |

# 1. Introduction

Within this section an introduction to the background for the project will be given. This PhD project is an interdisciplinary project combining stem cell research, extra cellular matrix biology, screening engineering and nanotechnology. This section will also provide an understanding in how these fit together in one project.

## 1.1 Stem cell therapy

The establishment of hES cells (Thomson et al. 1998) and later human induced pluripotent stem cells (Takahashi et al. 2007) opened the perspective of studying human development, which previously had been restricted to other species. Further, this also opened the perspective of using an unlimited cell source in regenerative medicine: stem cell therapy. The idea behind stem cell therapy is to differentiate the stem cells into the somatic cells of interest and subsequently transplant these cells into the diseased or injured human body (Reimann et al. 2009) (Figure 1). At the moment, several stem cell therapies using adult stem cells are clinically approved, including hematopoietic stem cells and epidermal stem cells, but no therapy with pluripotent stem cells has been fully established. Up till now, a few clinical trials with embryonic stem cells have been initiated (EuroStemCell 2012). The latest clinical trial was initiated in the second half of 2014 by ViaCyte Inc. using pancreatic precursors derived from hES cells to treat diabetes (ViaCyte 2014).

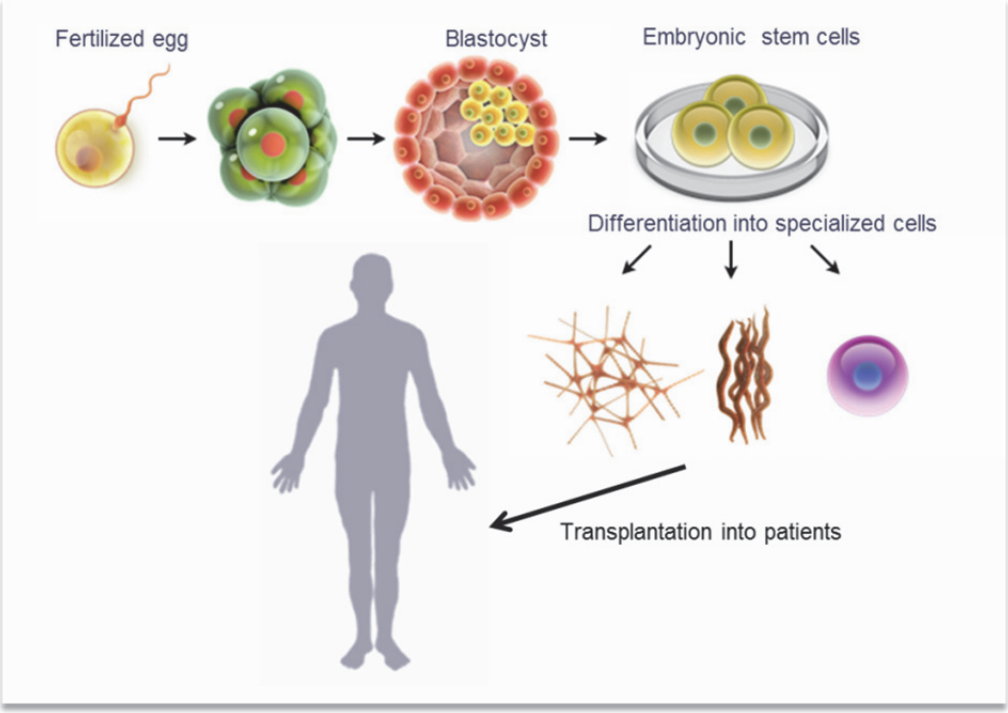


***Figure 1.*** *A schematic overview of the concept of stem cell therapy. Embryonic stem cells are isolated from a fertilized egg at the blastocyst stage. These cells can be cultured in vitro where they potentially can be differentiated into the somatic cells of interest and then transplanted cells into the diseased or injured human body. Modified from Biotech Academy* (Biotech Academy 2010)

A lot of effort has been put into the research of stem cell therapy to treat type 1 diabetes mellitus. Type 1 diabetes mellitus is characterized by a complete lack of insulin due to an autoimmune destruction of the insulin-producing beta-cells in the islets of Langerhans of the pancreas. The disease requires daily injections with insulin and causes diabetes related complications due to lack of complete metabolic homeostasis (Daneman 2006). Improved treatment, which can prevent such complications, will be beneficial. One potential treatment could be stem cell therapy. The idea for using stem cell therapy to treat diabetes, is to differentiate hES cells into insulin producing beta-cells *in vitro*, transplant these cells into the patients and hereby restore normal blood sugar regulation (Reimann et al. 2009; Schiesser & Wells 2014). Proof of principle already exits from transplanting healthy human islets from cadaveric donors to diabetic patients, which resulted in long term insulin-independence (Keymeulen et al. 1998; Ryan et al. 2001). However, the lack of an adequate source of donor islets is an important limitation, highlighting the need for an alternative source of insulin producing beta-cells. An alternative source could be hES cell derived beta-cells (Madsen & Serup 2006). To reach this goal, an *in vitro* protocol for differentiating embryonic stem cells into mature insulin producing beta-cells must be developed.

## 1.2 Human embryonic stem cells

hES cells are derived from the inner cell mass of a fertilized egg at the blastocyst stage. After fertilization of an egg, a diploid zygote is formed and multiple cell divisions generate the blastocyst. The blastocyst is composed of an outer cell layer, the trophoblast and an inner cell mass. The trophoblast will give rise to extra embryonic tissue and the inner cell mass will give rise to the embryo (Gilbert 2006). Embryonic stem (ES) cells have the capacity to divide indefinitely, which means that they can self-renew while maintaining their stem cell identity. Further, ES cells are pluripotent, and hereby they can potentially give rise to all cell types found in the human body. The pluripotent state is defined by the cells' ability to differentiate into derivatives of all 3 germ layers; ectoderm, mesoderm and endoderm either *in vitro* or by teratoma formation *in vivo* (Smith 2001). Pluripotent cells are only found in a short window of time during the embryos commitment. Further, it has been suggested that two phases of pluripotency exist; naïve and primed (Nichols & Smith 2009), primed , which will be further described in the section below. Pluripotent cells progress from a developmentally earlier state, the naïve state, to a primed state before lineage commitment. Primed state cells are molecular and epigenetically distinct from naïve state cells, but the details remains to be discovered. Most obvious differences are cell culture conditions as well as the fact that primed state female cells exhibit signs of inactivation of one of their X chromosomes (Wu & Izpisua Belmonte 2015).

The first ES cell lines was generated from the inner cell mass of a mouse blastocyst in 1981 (Evans & Kaufman 1981) and in 1997 human ES cells were isolated from human blastocyst (Thomson et al. 1998). Subsequently, it became clear that mouse and human embryonic stem cells differ in both morphology and their requirement to maintain pluripotency. Mouse ES cells form tall multi-layered clusters and depend on leukaemia inhibitory factory (LIF) to maintain pluripotency (Smith et al. 1988). Human ES form more flat clus-

ters and can be grown in homogenous monolayer (Anon 2015). Human ES cells do not require LIF but basic fibroblast growth factor (bFGF) to maintain their pluripotency (Amit et al. 2000). The differences between mouse and human ES were previously attributed to the differences in developmental programs between rodents and humans. However, this explanation was challenged by the generation of epiblast stem cells, which are derived from the post-implantation mouse epiblast (Brons et al. 2007; Tesar et al. 2007). Epiblast stem cells resembles human ES stem cells in morphology, in signals to maintain pluripotency and in controlling the early fate decision (Vallier et al. 2009). It has later been demonstrated that epiblast stem cells and human ES cells can be converted back to a more naïve state resembling mouse ES cells with regards to genetics, epigenetic and morphology (Guo et al. 2009; Valamehr et al. 2014). This indicates that the human ES cells used to day might already be primed for differentiation and thus are not as naïve as mouse ES cells.

In this thesis human embryonic stem cells are used, due to their clinical potential, and mouse ES cells and epiblast stem cells will not further be described. However, it is important to recognise that research using mouse ES cells has generated important knowledge used in the study of human ES cell differentiation.

hES cells keep their pluripotent state and can self-renew continuously under certain cell culture conditions. Traditionally, hES cells were cultured with feeder cells and serum to keep them in an undifferentiated state (Thomson et al. 1998). However, progress in elucidating the biological mechanism behind pluripotency has made it possible to culture hES cells in xeno free and chemically defined conditions. Xeno free and chemically defined culture conditions are important for therapeutic applications and for reproducibility (Hoffman & Carpenter 2005). The addition of basic fibroblast growth factor (bFGF) to the medium will help sustain hES cells in a pluripotent state. Further, repression of bone morphogenic protein (BMP) signalling by Noggin assists in maintaining the undifferentiated state (Xu et al. 2005). Besides culturing hES cells on feeder cells, hES cells can be maintained on extracellular matrices such as the undefined fibroblast derived Matrigel or ECM proteins, including fibronectin or laminin (Vazin & Freed 2010).

hES cells can be identified by their expression of certain cell surface markers and transcription factors, including stage specific embryonic antigen-4 (SSEA-4), SSEA-3, octamer-binding transcription factor 3/4 (Oct3/4), SRY-box containing gene2 (Sox2) and Nanog (Vazin & Freed 2010). The POU family transcription factor Oct3/4, which is expressed in early embryos and ES cells and down regulated upon differentiation, plays an essential role in maintaining self-renewal ability (Nichols et al. 1998; Looijenga & Stoop 2003; Zaehres et al. 2005). Thus, Oct3/4 is an ideal marker for undifferentiated hES cells. Another ES cell marker used in this project is the transcription factor Nanog, which is expressed in undifferentiated ES cells and is involved in the self-renewal process as a potential downstream target of Oct3/4 (Chambers et al. 2003; Silva et al. 2009; Boyer et al. 2005). The next sections will focus on the development of beta-cells in the embryo and the *in vitro* differentiation of hES cells towards the pancreatic lineage.

## 1.3 Pancreas development

Differentiation protocols of ES cells are built on the knowledge of the development of the cells or organ of interest. Development of vertebrates is highly conserved and a majority of the knowledge is gained from studying animal models such as mouse, chicken and zebra fish (Madsen & Serup 2006; Gilbert 2006). In this section an introduction to the pancreas development will be given with focus on the first part of the development process; formation of definitive endoderm (DE) and pancreatic endoderm (PE). For this overview mouse will be used as a model since specific knowledge about the human pancreas is limited, due to ethical and practical reasons. The section focuses on the morphology of the development and the major signalling pathways involved. Limited information about ECM and the mechanical properties in pancreatic development exists in the literature and will be briefly introduced in section 1.5 and 1.6.

### 1.3.1 Overview of the pancreatic development

Following fertilization, the egg undergoes a series of cleavages and develops into the blastocyst about 4 days after fertilization (E4.0). The blastocyst is a spherical structure composed of the outer cell layer, called trophectoderm giving rise to extra embryonic membranes and the placenta, and the Oct3/4 and Nanog expressing inner cell mass. The inner cell mass segregates further into two distinct cell types; the primitive endoderm and epiblast cells. The epiblast cells will give rise to the embryo (Arnold & Robertson 2009).

Around day E.6.5 the mouse embryo undergoes gastrulation, a series of cell movements which giving rise to the 3 germ layers: DE, mesoderm and ectoderm. The ectoderm (outer layer) will later form the skin, central nervous system and neural crest cells. The mesoderm (middle layer) will give rise to bones, muscle, connective tissue, urogenital system, heart, vasculature and blood cells. The DE (inner layer) will later form the lining of the respiratory tract and the gastrointestinal tract as well as several organs, including the liver and the pancreas (Gilbert 2006).

Gastrulation is completed around E.7.5 and at this point DE is formed into a cup-shaped sheet of cells covering mesoderm and ectoderm. Subsequently, DE will fold up to form the primitive gut tube. The gut tube becomes patterned along the anterior-posterior axis (head-toe body axis) with distinct gene expression in the different regions, giving rise to the different organs and gastrointestinal parts. Pdx1 positive cells from the posterior foregut will give rise to the pancreas. At E9.0 the dorsal (dorsal-ventral axis = back–belly body axis) pancreatic bud starts to appear and the next day the ventral pancreatic bud will emerge. These two buds are temporarily separated and are results of two independent regulatory signal networks. The budding is initiated as a thickening of the epithelial layer, which further proliferates and invades the surrounding mesenchyme where it branches out. Gut rotation brings the pancreatic buds in close proximity to each other resulting in fusion around E12.5 (Wells & Melton 1999; Collombat et al. 2006).

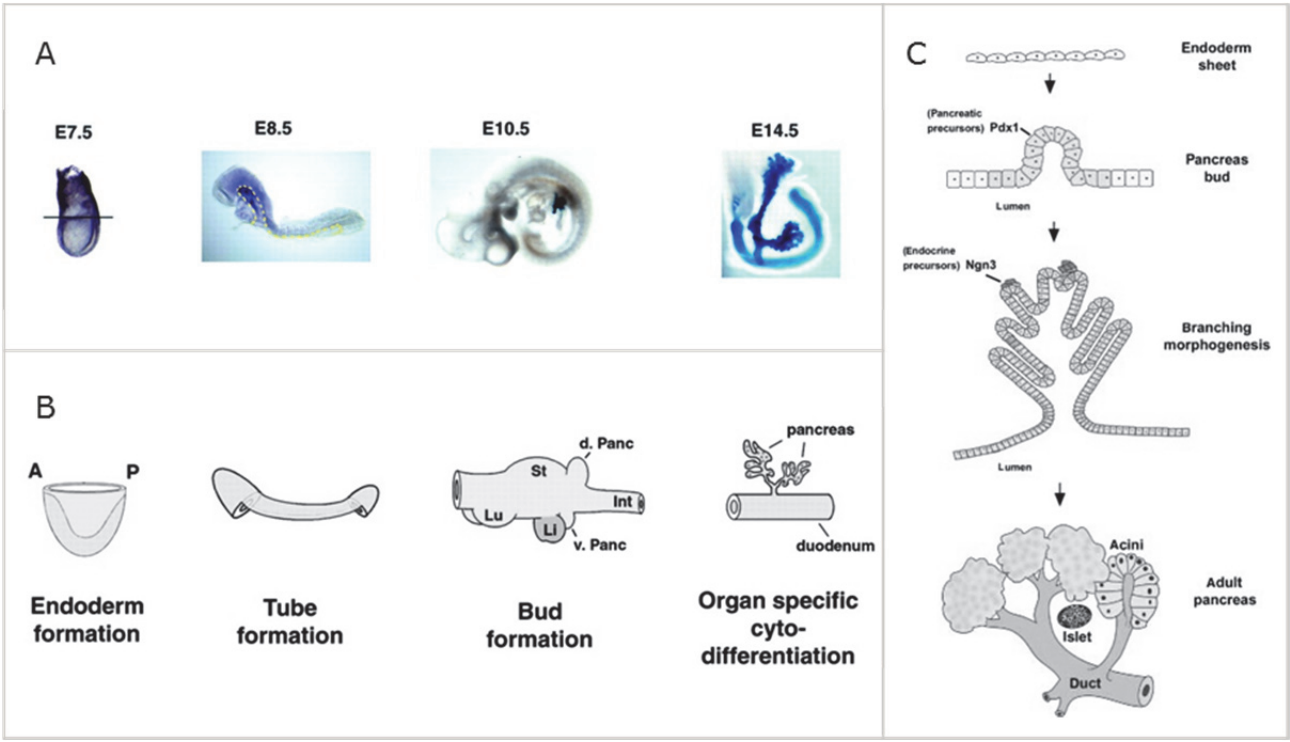


***Figure 2.*** *Overview of pancreas development in a mouse embryo. Panel A) are images of early mouse embryos at stages corresponding to the stages illustrated in panel B). At E.7.5 DE is formed during gastrulation along with the mesoderm and ectoderm. At this stage the embryo is cup-shaped. At E8.5 the primitive gut tube is formed and at E.10.5 the dorsal and ventral pancreatic buds appear in the posterior foregut. The buds grow and branch and eventually merge together to form the pancreas. Panel C) illustrates the close up of the development of an adult pancreas from the primitive gut tube. All pancreatic cells are Pdx1 expressing precursors arising from the primitive gut tube. Ngn3 expressing cells will delaminate from the branching pancreatic epithelium and migrate into the surrounding mesenchyme. The Ngn3 expressing cells will give rise to the endocrine cells in the pancreas, which are formed into islest of Langerhans. The figure is modified from (Wells 2003; Wells & Melton 1999).*

Regulated by transcription factors, individual cells of the branching pancreas differentiate into the different compartments of the pancreas; acinar tissue, the ductal tree as well as the Islets of Langerhans (Wells 2003). The acinar cells produce digestive enzymes which are led by the pancreatic duct into the intestine. The Islets of Langerhans consist of 5 types of endocrine cells, including the insulin producing beta-cells. The endocrine cells are formed by Ngn3 expressing endocrine precursors which exit from the pancreatic epithelium and migrate into the pancreatic mesenchyme where they aggregate into islet-like clusters (Murtaugh 2007).

In the next two sections, a more detailed description will be given on the DE and PE development, since this is the main focus of the PhD project.

### 1.3.2 Definitive endoderm development

DE is established together with the 2 other germ layers, ectoderm and mesoderm, during gastrulation, where cells proliferate, differentiate and rearrange. Gastrulation can be divided into 3 parts: 1) axis patterning of the embryo, 2) primitive streak formation and 3) germ layer formation (Figure 3). Gastrulation differs to some extent among the mammalian species. In mouse and rat embryos the epiblast forms a cup shape whereas the human epiblast is a disc shaped structure, resembling the chicken (Gilbert 2006). The following section is based on the mouse embryo.

1) Axis patterning of the embryo

At E6.5 the mouse embryo consists of a cup-shaped epiblast, surrounded by a layer of visceral endoderm with the extra-embryonic ectoderm contacting the proximal epiblast. At this stage the embryo has become asymmetric across proximal-distal axis and the anterior-posterior axis (Gilbert 2006; Wells & Melton 1999). Before the initiation of the gastrulation, the axis pattering of the epiblast is generated by a complex interplay between extra-embryonic ectoderm, distal visceral endoderm and epiblast. Following signalling pathways are involved: Wnt, Fibroblast Growth Factors (FGFs), bone morphogenetic proteins (BMPs) and transcription factors from the Transforming Growth Factor β (TGF-β) family, including Nodal (Reviewed in (Arnold & Robertson 2009).

2) Primitive streak formation

The first morphological sign of gastrulation appears by the formation of the primitive streak (PS), a shallow groove in the posterior epiblast at the border of the extra-embryonic ectoderm. The PS will elongate towards the distal tip of the embryo, while epiblast cells delaminate and ingress through the PS (Murry & Keller 2008). The delaminating epiblast cells undergo epihethial to mesenchymal transition (EMT), where cells loose cell-cell adhesion and polarity, breaks through the embryonic basement membrane and delaminate and ingress through the streak (Thiery et al. 2009). The high Nodal and Wnt concentration in the proximal posterior region and BMP4 signalling stimulates the PS formation and migration of the epiblast cells (Lu & Robertson 2004; Thiery et al. 2009). Cells migrating through the PS transiently express the markers Brachyury and Mix1 homeobox-like protein 1 (Mixl1) (Wilkinson et al. 1990; Hart et al. 2002). In the anterior region of the PS, cells also express Forkhead box A2 (FoxA2) and Goosecoid (Gsc) (Kinder et al. 2001; Sasaki & Hogan 1993).

3) DE formation

The cells ingressing through the PS will either form DE, which displaces the underlying visceral endoderm, or spread out between DE and the epiblast to form mesoderm. Epiblast cells which do not travel through the PS will form the ectoderm (Gilbert 2006). The fate of the cells migrating through the primitive streak is highly dependent on time and place. The majority of DE is formed from cells migrating through the anterior part of the PS at the mid to late streak stage (Lawson et al. 1991). Studies have demonstrated that DE and mesoderm both derive from a bipotent progenitor population, mesendoderm, which express Brachyury, Gsc, and FoxA2 (Kubo et al. 2004; Tada et al. 2005). The concentration of nodal decides the fate of mesendoderm.

High nodal concentration induces DE differentiation by activation of a transcription factor network, including Mixl1, Foxa2, Sox17, Eomes and GATA4-6 (Zorn & Wells 2009).

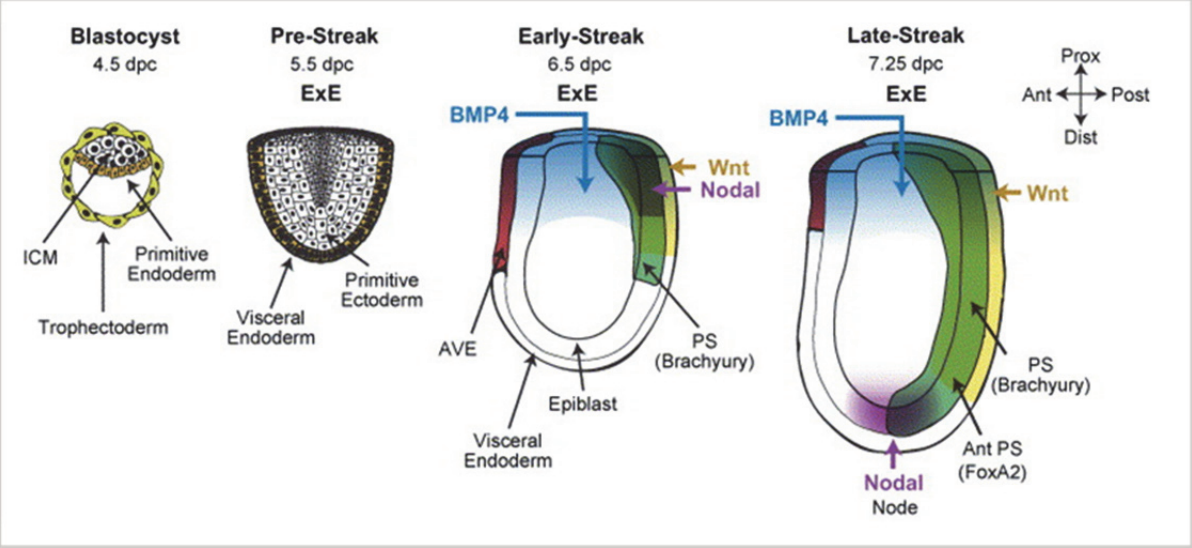


***Figure 3.*** *Gastrulation of a mouse embryo. Before initiating the embryo become patterned along the proximal-distal and anterior posterior axis, with Nodal, Wnt and BMP4 signalling. Gastrulation is initiated by the formation of the primitive streak. Cells in the primitive streak express Brachyury. The fate of the cells, that ingress through the primitive, is determined by time and place. The anterior part of the PS express Foxa2 and Gsc, which will give rise to DE cells. Reproduced from (Gadue et al. 2005).*

### 1.3.3 Pancreatic endoderm development

After gastrulation at E7.5, the DE consists of a cup-shaped sheet of about 500 cells overlaying the mesoderm and ectoderm. The sheet is patterned along the anterior-posterior axis, which results from the time and place of which a given cell ingress though the PS. The first DE to exit the PS migrates in an anterior direction and will later form the foregut, from where the pancreas will bud. Posterior DE will exit the PS later and give rise to the midgut and hindgut, which later will form the small and large intestine (Wells & Melton 1999). Anterior DE is specified by a network of downstream transcription factors of Wnt, high levels of Nodal and FGF signalling (Zorn & Wells 2009).

By a series of morphogenetic movements and growth, the DE is transformed into a tube like structure, the primitive gut tube, at around E9.0. The primitive gut tube becomes patterned along the anterior-posterior and dorsal-ventral axis and can be divided into four regions; dorsal foregut, ventral foregut, midgut and hindgut. Subsequently, the pattering becomes progressively refined into domains from where organs later will be formed. The pattering of the gut tube depends on signals from the adjacent mesoderm and ectoderm (Zorn & Wells 2009). FGF4 from the mesoderm has an essential role in the pattering. High concentrations of FGF4

induce hindgut and inhibits foregut and midgut gene expression. Intermediate levels of FGF4 induce the anterior pattering of the gut tube (Dessimoz et al. 2006).

The PE is specified from two distinct regions, the dorsal and ventral foregut, and by two distinct signalling networks. Specification of the ventral PE is induced by cardiac FGF signalling from the mesoderm. The dorsal PE is specified by Retinoic Acid from the mesoderm as well as signals from the notochord and dorsal aorta, such as bFGF and Activin A (Zorn & Wells 2009). Expression of the transcription factor *Pancreatic and duodenal homeobox 1* (*Pdx1*) is up regulated in the early PE. *Pdx1* is not restricted to the pancreas and is also found in the intestine and posterior stomach but is essential for the development of the pancreas. Several other transcription factors are essential for pancreatic development. One of these is NK6 transcription factor related, locus 1 (Nkx6.1*)*, which is only expressed in the Pdx1 positive endoderm which will form the pancreas. Further, NKX6.1 has an important role in endocrine specification of the pancreas (Murtaugh 2007).

### 1.3.4 Differences between mouse and human pancreatic development

It seems obvious that a detailed understanding of human pancreas development could be highly beneficial for finding the best protocols for *in vitro* differentiation of hES cells towards functional beta-cells. Due to practical and ethical reasons, knowledge about human development is limited. The knowledge we have about human development comes from studying genetic diseases and limited access to human embryonic and fetal tissues (Murtaugh 2007; Gilbert 2006). Thus, our understanding about developmental biology relies on the knowledge from the development biology in mouse and other model organism.

It is important to keep in mind that despite conservation across species of most transcription factors, differences between the mouse and human pancreas development exist (Domínguez-Bendala 2009). One evidence is the architecture of mature islets of Langerhans (islets). Islets in the mouse have a well-defined structure with a central core of beta-cells, which represent 60-80 % of the islet cells. Surrounding the beta-cells a mantle of other endocrine cells exists, including alpha cells (15-20% of the islet cells), delta cells and Pancreatic Polypeptide (PP) cells. In contrast human islets tend to contain fewer beta-cells and more alpha cells compared to mouse islets. Further, the endocrine cells in the human islet do not form the distinct distribution as seen in mouse islet (Steiner et al. 2010). These differences in the mature islets across species, implies that differences during the development also exist.

One example is that the expression of the definitive endoderm marker Sox17 is lost in the mouse but remains the presumptive pancreatic endoderm in humans (Jennings et al. 2013). Another example is later in the development, during the formation of the pancreatic epithelium. Here, Nkx2.2 is expressed in the mouse but will first be expressed in human after cells have committed towards the endocrine lineage (Jennings et al. 2013). Moreover, a study implies that the expression of Ngn3, which is required for endocrine cell specification, are expressed in two waves in the mouse (Villasenor et al. 2008), but only a single transition of Ngn3

is observed in humans (Salisbury et al. 2014). However, the study by Salisbury et al., needs to be further confirmed, since the specificity of the used Ngn3 antibody is currently being discussed (unpublished results, Novo Nordisk).

In the next section it will be described how the knowledge from the *in vivo* development of the pancreas is translated into the *in vitro* differentiation of ES cells towards beta-cells. A lot of the presented research had relied heavily on the information obtained from pancreas development in animal models.

## 1.4 Directed differentiation of human embryonic stem cells towards beta-cells

The hypothesis behind the differentiation of pluripotent stem cells towards beta-cells is to recapitulate the steps in the development of the pancreas (Figure 4). This requires removal of components that support pluripotency and the addition of correct components in the correct concentrations and at the right time (Vazin & Freed 2010). The goal is to differentiate the human pluripotent stem cells towards DE, PE, endocrine progenitors and finally into beta-cells. At each stage cells are characterized by the expression of certain markers, which is also found during development (Schiesser & Wells 2014) (Figure 4).

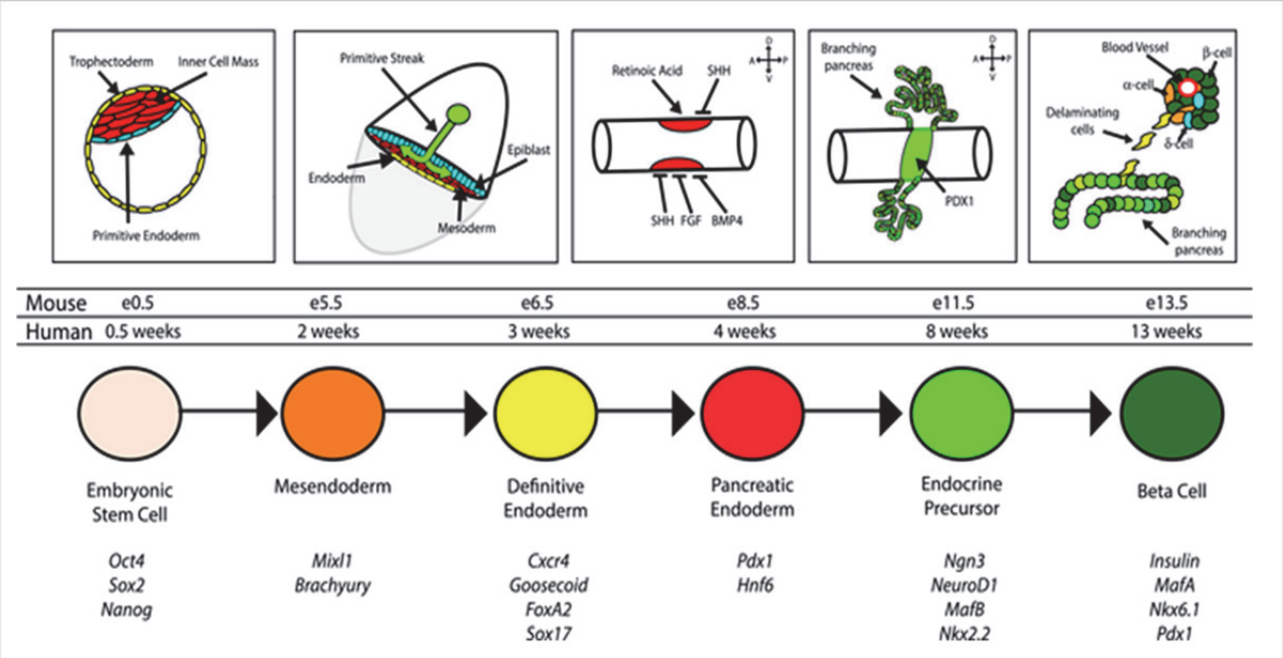


***Figure 4.*** *Schematic overview of the directed differentiation of hES cells towards beta-cells. The differentiation is a stepwise protocol mimicking the in vivo development of the pancreas. hES cells are differentiated into DE, PE, endocrine precursors and at last into beta-cells. The steps are characterized by the expression of certain markers which also is present in vivo development. Reproduced from* (Schiesser & Wells 2014).

The first insulin producing cells derived from human pluripotent stem cells were observed more than a decade ago in spontaneous differentiation of embryoid bodies (Assady et al. 2001). Since then, a lot of effort has been put into to generating protocols which can direct hES cells towards beta-cells. However, the production of fully mature beta-cells has proved to be difficult. Several published protocols resulted in polyhormonal cells, expressing both insulin and glucagon, or in cells that do not secrete insulin in a glucose-dependent manner (Kroon et al. 2008; D'Amour et al. 2006; Rezania et al. 2012). However, some of these immature cells have proven to be able to mature *in vivo* in mice (Rezania et al. 2012; Kroon et al. 2008; ViaCyte 2014). Recently, two independent studies achieved to yield insulin secreting cells with potential therapeutic value (Pagliuca et al. 2014; Rezania et al. 2014). These cells are glucose-responsive *in vitro* and are capable of rescuing diabetic mice, but it remains unclear whether these cells represent fully functionally mature beta-cells (Kushner et al. 2014). All attempts to differentiate hES into functional beta-cells focuses on growth factors, chemokines and small molecules, targeting downstream signalling pathways of growth factors and chemokines. However, the difficulty to develop a reproducible protocol to differentiate hES into functional beta-cells, questions whether stem cell differentiation is guided by more parameters than the ones used in the mentioned studies. Such additional factors will be described in section 1.5-1.7.

Since this project focuses on the first two differentiation steps towards beta-cells (DE and PE) a more detailed description of protocols towards these two populations will be given below.

### 1.4.1 Definitive endoderm differentiation

Several efficient DE protocols have been published describing targeting of the signalling pathways involved in gastrulation and DE formation. They all target the TGFβ-pathway, via Nodal or Activin A. The TGFβ-family member Activin A is most often used as a replacement to Nodal, since it can mimic Nodal, by activating the same pathway, and it is more stable and potent than Nodal. The addition of Activin A will drive ES cells through the transient mesendoderm stage, expressing Brachyury and Mixl1 and subsequently to DE, expressing Sox17, Goosecoid and Foxa2 (Tada et al. 2005; Kubo et al. 2004; D'Amour et al. 2005). Even though Activin A is widely used in DE differentiation as a replacement for Nodal, recent studies with mouse ES cells showed that Nodal induced DE had greater developmental potential than Activin A (Chen et al. 2013).

Targeting the Wnt signalling pathway together with the addition of Activin A can further induce the DE differentiation. Wnt signalling plays in important role during gastrulation. Targeting Wnt signalling allows ES cells to escape pluripotency and differentiate into the transient mesendoderm stage, similar to the embryonic development (D'Amour et al. 2005). Wnt signalling has been targeted either by using Wnt3a (D'Amour et al. 2005) or using a the small molecules such as the GSK3β inhibitor CHIR99021 (Kunisada et al. 2012).

### 1.4.2 Pancreatic endoderm differentiation

In the *in vitro* directed differentiation towards PE, the two factors FGF and retinoic acid have been shown to be of key importance in specifying PE. This fits with the developmental biology, where FGF and retinoic acid from the notochord and cardiac mesoderm specify the ventral and dorsal PE domains respectively (Zorn & Wells 2009). Almost all published PE differentiation protocols are based on these two factors (D'Amour et al. 2006; Rezania et al. 2012; Ameri et al. 2010; Nostro et al. 2011). bFGF affects PE differentiation in a concentration dependent manner, where intermediate levels of bFGF seem to favour PE. Furthermore, in the absence of bFGF, no Pdx1 expressing cells appear (Ameri et al. 2010). In contrast, retinoic acid is not an essential requirement for obtaining Pdx1 expressing cells, but the addition of retinoic acid can significantly improve the efficiency of the differentiation (Johannesson et al. 2009). One study suggests that retinoic acid reduces the differentiation of hepatic endoderm, by inhibiting BMP signalling (Cai et al. 2010). Several other factors which inhibits the BMP signalling pathway have likewise been shown to reduce hepatic differentiation (Rezania et al. 2014; Zhang et al. 2009).

## 1.5 The extracellular matrix

As described above, much effort has been put into differentiating pluripotent stem cells towards beta-cells during the last decade, recently with great success. Previous studies have been focusing on the use of soluble molecules and their downstream signalling pathways, whereas the physical environment has been left unattended. However, accumulating results have shown the importance of the physical environment, including the extra cellular matrix (ECM) (Rozario & DeSimone 2010). In this section an introduction to the ECM and its role in stem cell biology will be given.

The ECM is a three-dimensional, non-cellular structure that consists of fibers in a gel like substance (Zagris 2001). Every cell in the body is exposed to ECM (Hynes 2009). ECM is secreted by cells into the extracellular environment and self-assemble in macromolecular structures such as fibers and fibrils. The ECM varies in composition within and between tissues, and it is constantly remodelled as cells build and reshape the ECM by degradation and reassembly (Bonnans et al. 2014; Tsang et al. 2010).

Traditionally it was believed that the ECM just provided structural support and filled the space between the cells. However, since the discovery of integrins, which are cell membrane proteins that binds to the ECM, the view of the ECM as passive was changed. Today the ECM is recognized as having a multifunctional role which is vital for normal development and physiology (Figure 5) (Tsang et al. 2010; Rozario & DeSimone 2010).

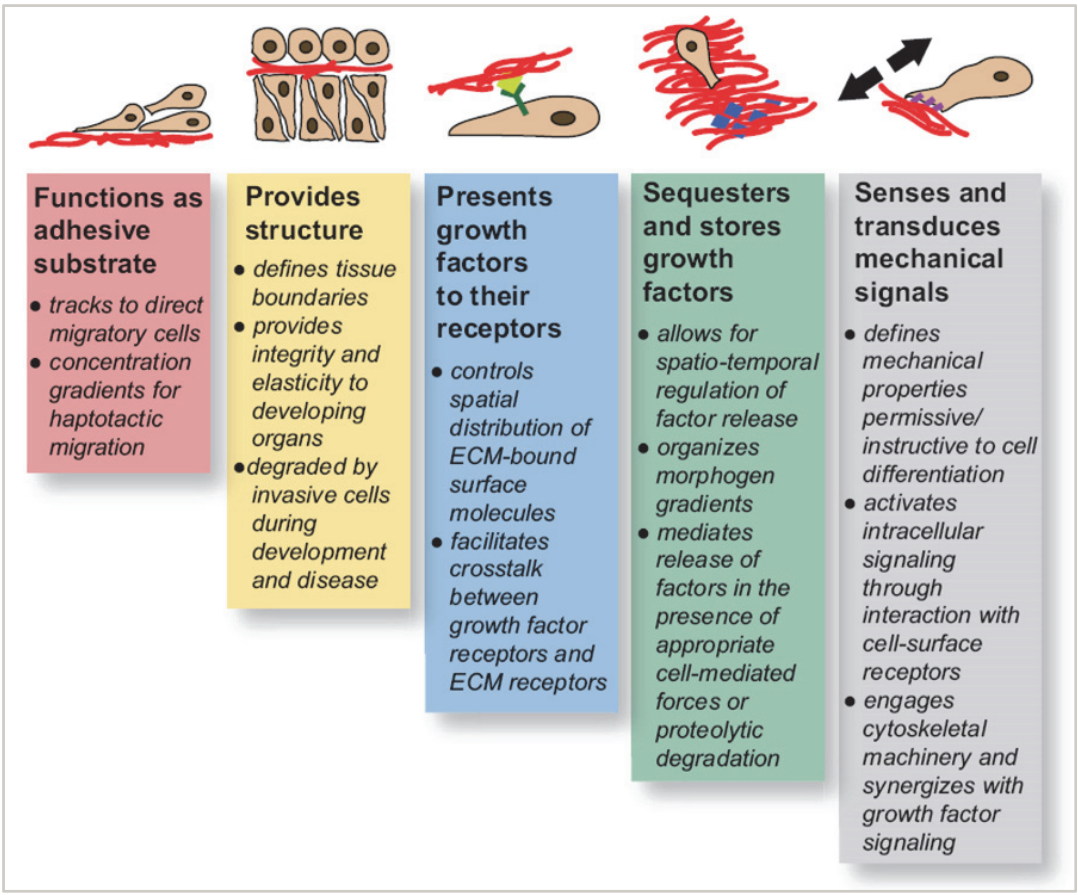


***Figure 5.*** *Schematic overview of the functions of the ECM. Reproduced from (Rozario & DeSimone 2010).*

First, the ECM functions as an adhesive substrate for cells and a platform for cell migration. Cell locomotion is facilitated by cycles of cell adhesion and adhesion to the ECM, together with contraction of the cytoskeleton (Rozario & DeSimone 2010). The ECM provides structural support to tissues and organs, and the basement membrane defines the boundaries between the different tissues (Hynes 2009).

Furthermore, the ECM plays an essential role in growth factor signalling. Many ECM proteins have binding sites for both growth factors and cell adhesion which allow growth factors to be released locally and bind to their cell surface receptors. Some ECM bound growth factors function as a solid phase ligand to an ECM component. Thus, the ECM functions as a cofactor and presents the growth factor for cell surface receptors. Further, localisation of growth factors by ECM binding, contributes to the establishment of gradients of soluble chemokines and growth factor morphogens, which play an essential role in developmental processes. Growth factors can also be sequestered to the ECM, which hereby function as a localized reservoir. Degradation of ECM will then release the growth factors from solid inactive growth factors to active soluble ligands (Kim et al. 2011; Hynes 2009).

The ECM itself also functions as signals to the cells by binding to specific cell surface receptors. This interaction results in a bidirectional interaction between the extracellular compartments and the cells with outside-in

and inside-out signalling (Harburger & Calderwood 2009). The physical and mechanical characteristics of the ECM provide signals to the cells, via mechanosensors or receptors which are connected to the intracellular cytoskeleton. The mechanical properties of the environment greatly affect cellular behaviour (Hynes 2009).

### 1.5.1 Components of the extracellular matrix

Almost 300 ECM proteins are encoded in the mammalian genome, and comprises around 1-1.5% of the mammalian proteosome. These proteins are highly conserved among different taxa and some of them are evolutionary ancient. ECM proteins are large and complex with repeats of characteristic set of domains and can be divided into different families, including collagens, non-collagenous glycoproteins and proteoglycans (Hynes & Naba 2012). In this section an overview of each family will be given.

**Collagens**

Collagens are triple helical proteins and are the main component of the ECM, existing in all tissues. It provides tensile strength and structural support to all kind of matrixes, including to the strong fibers of the tendons, in the organic matrices of the bone and cartilage or viscous matrices in the eye. Collagens also provide binding sites for other ECM proteins. To date, 44 human genes encoding collagen chains are identified and the product of these genes form at least 28 distinct trimeric proteins. Collagens contain repeats of the triplet Gly-X-Y, where X is often proline and Y is frequently 4-hydroxyprolin (Hynes & Naba 2012; Rozario & DeSimone 2010). Collagen type 1 is the most abundant ECM protein in the human body and is mainly expressed in the intrinsic part of the ECM. In general Col1 provides structural support and binds other ECM proteins (Rozario & DeSimone 2010; Zagris 2001).

**Non-collagenous glycoproteins**

About 195 glycoproteins have been identified in humans. They have multiple functions, including ECM assembly, cell binding, signalling and binding of growth factors (Hynes & Naba 2012). Glycoproteins contain oligosaccharide chains which are covalently attached to the side-chains of the polypeptide. Within the glycoproteins several distinct families exist and some of the glycoproteins are composed of multiple chains encoded by different genes whereas others differ due to alternative splicing (Rozario & DeSimone 2010). Two of the major families are laminin and fibronectins. Laminin are large trimeric proteins, consisting of an α-chain, a β-chain and a γ-chain. Laminin binds to cell surface receptors, including integrin and trigger several intracellular signalling pathways. Fibronectins are large and homodimeric proteins and are the product of a single gene, where alternative splicing results in several isoforms (Zagris 2001). Fibronectin contains the cell binding motif RGD (Arg-Gly-Asp) which also appears in vitronectin and other ECM proteins. The RGD sequences reside within a hydrophilic loop of the proteins and are critical for the binding to several integrins (Rozario & DeSimone 2010).

**Proteoglycans**

Proteoglycans are linear unbranched glycoproteins with attached glycosaminoglycans (GAGs), which are repeating polymers of disaccharide. The GAGs have a negative charge, which draws water to form a hydrous gel as well as attachment of divalent cations such as calcium (Hynes & Naba 2012). Proteoglycans bind growth factors and thus play a vital role in modulating growth factor activity and morphogenic patterns (Zagris 2001; Rozario & DeSimone 2010). One example is heparan sulphate proteoglycans which are able to bind members of the Wnt, TGFβ and FGF families (Gilbert 2006). 35 human genes encode proteoglycans and they can be divided into several families depending on their repeats. One of the biggest families has LRR repeats where many of these bind to various collagens and growth factors. Another large family has LINK domains, which often binds to glycoproteins. Many proteoglycans fall out of these families and have distinct domains. Moreover, some ECM proteins from the other categories contain GAGs and the boundary between proteoglycans and glycoproteins is not always clear (Hynes & Naba 2012).

**Basement membrane and interstitial extracellular matrix**

ECM in the body can be divided into two subgroups; the basement membrane and the interstitial ECM (Figure 6). The basement membrane separates the epithelium from the surrounding tissue and confers polarity for the epithelial cells, which interact only with the basement membrane on the basal site of their cell membrane. The basement membrane is compact and consists mainly of collagen IV, laminins and heparan sulphate proteoglycan (Bonnans et al. 2014). During development, the basement membrane creates barriers for cells and macromolecules, as well as segregates and differentiates cells into specific tissues (Zagris 2001). The interstitial ECM is more loosely arranged and tends to completely surrounds cells. The interstitial ECM mainly consists of collagen I and fibronectin (Bonnans et al. 2014).

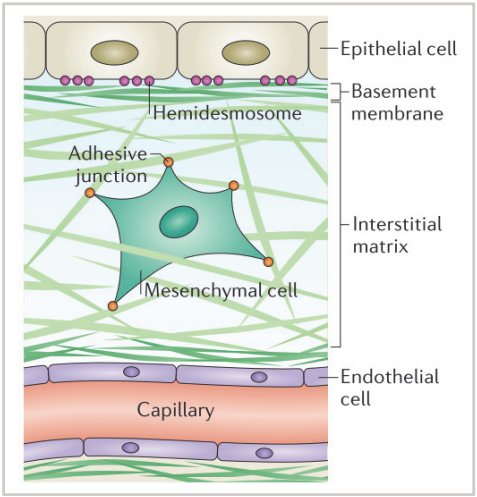


***Figure 6.*** *The different cell types which interact with different ECM. One side of the epithelial and endothelial cells interact with the basement membrane; a dense sheet of specialized ECMs. Mesenchymal cells are surrounded by interstitial matrix. Reproduced from (Watt & Huck 2013).*

### 1.5.2 Extracellular matrix receptors and downstream signalling pathways

For the ECM to affect cellular behaviour, it is obvious that there must be receptors and downstream signalling pathways for ECM proteins. In this section an introduction to ECM receptors and downstream signalling pathways will be given.

**Integrins**

The major ECM receptors are integrins, which are expressed in all metazoans. Integrins are a large family of 24 heterodimeric receptors. They are composed of non-covalently associated α- and β-subunits, each of which is a single-pass transmembrane protein. Each heterodimer have specific ECM binding partners, including collagen, fibronectin, laminin and vitronectin. Some integrins also bind to membrane-receptors on adjacent cells (Harburger & Calderwood 2009; Hynes & Naba 2012). Some integrin subunits, like β-subunits, are ubiquitously expressed whereas others are only expressed in specific cells (Prowse et al. 2011). *In vitro* the profile of integrin changes when hES cells differentiate (Wong et al. 2010). Integrins support cell adhesions and play a vital role in the communication between the cytoplasm and extracellular space. This communication is bidirectional, inside-out and outside-in signalling, and control cell motility, survival, differentiation and proliferation, ECM remodelling and embryonic development (Prowse et al. 2011).

With outside-in signalling, integrins in response to ECM binding generate clusters of integrin heterodimers, called focal adhesions sites. Integrin clustering occurs after ECM association with other cell surface receptors and the recruitment of many cytoplasmic signalling proteins (Prowse et al. 2011) and involves more than 150 components (Harburger & Calderwood 2009). The recruited cytoplasmic proteins can be divided into three categories: 1) integrin binding proteins, such as Talin and focal adhesions kinases, 2) scaffold and adaptors proteins, which interact with the cytoskeleton and 3) enzymes which affect the downstream signalling, thereby influencing tyrosine kinases and serine/threonine kinases. However, many of the focal adhesion proteins can be part of more than one category (Prowse et al. 2011). With such complex networks of proteins, integrins are involved in many signalling pathways. Activation of integrin signalling is dependent on the organisation and mechanical properties of the ECM, the cell type and the expression and subcellular location of integrin and co-signalling receptors (Harburger & Calderwood 2009). Downstream integrin signalling pathways include MEK/ERK, RhoA/Rock, PI3K, β-catenin (Harburger & Calderwood 2009; Prowse et al. 2011; Bonnans et al. 2014) (Figure 7).

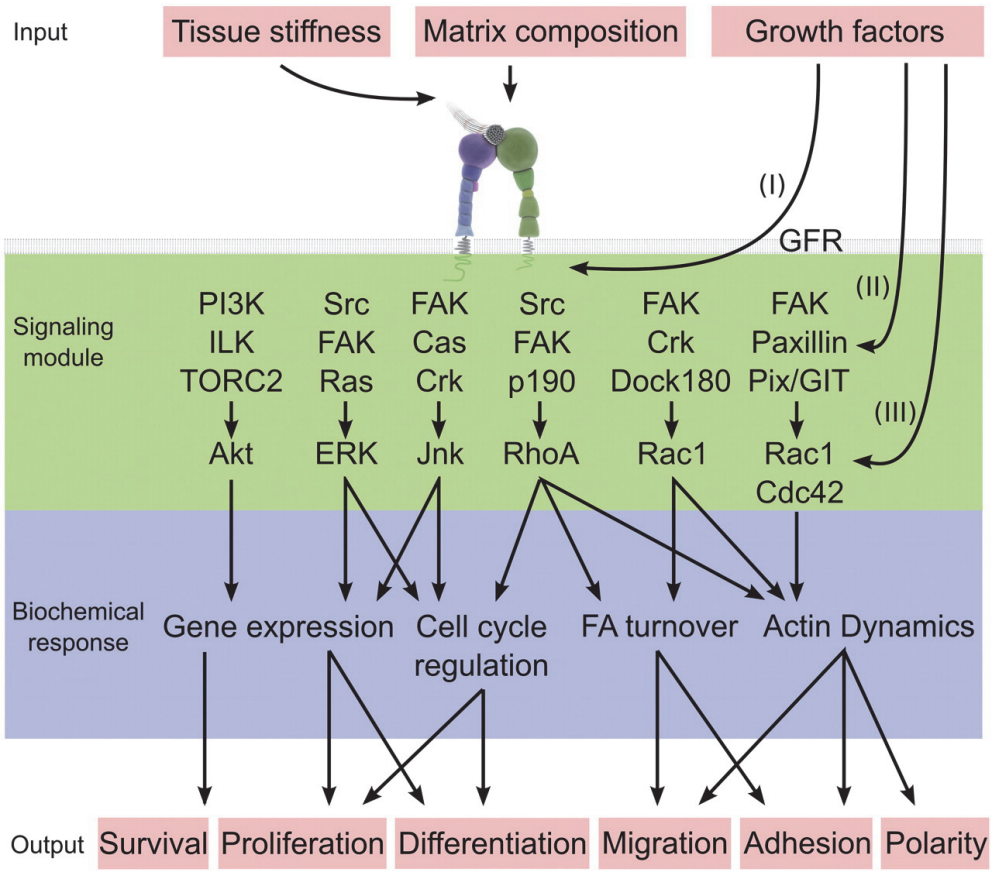


***Figure 7.*** *Schematic overview of integrin signalling pathways and downstream response (simplified). Integrin signalling is activated in response to ECM binding and stiffness as well as growth factors. Activation of downstream signalling pathways, such as PI3K, MEK/ERK, RhoA/Rock affects cellular behaviour, including survival, proliferation, differentiation and migration. Reproduced from (Legate et al. 2009).*

In addition to outside-in, integrins can regulate their affinity for their ligands by undergoing conformational changes in their extracellular domains in response to cytoplasmic signalling, a process called inside-out signalling (Calderwood 2004). During inside-out signalling an intracellular activator binds to the β-integrin cytoplasmic tail, leading to conformational changes that result in an increased affinity for specific ECM proteins. Inside-out signalling controls the strength of the adhesion, which allows integrins to transmit the forces required for cell migration, as well as ECM assembling and remodelling (Shattil et al. 2010). Moreover, integrins and its downstream signalling crosstalk intensively with growth factor signalling, adding further complexity (Prowse et al. 2011).

**Non-integrin Extracellular matrix cell receptors**

Besides integrins, multiple ECM receptors exist. However, only relatively few studies have focused on non-integrin receptors. Two of these receptors are the D receptor Tyr kinases discoidin domain-containing receptor (DDR) 1 and DDR2, which both bind to collagens (Hynes & Naba 2012). These receptors have mainly

been described in relation to cancer, they are, however, also involved in cell migration (Bonnans et al. 2014). A study has demonstrated that DDR2 is involved in EMT, during gastrulation and DE differentiation. DDR2 activation stabilises the EMT transcription factor SNAIL 1, which induces the expression of collagen I and collagen I metalloproteases, and promotes EMT (Shintani et al. 2008).

Dystroglycan is another receptor for ECM proteins, which binds to laminin, perlecan and agrin in/on the basement membrane. The dystroglycans contain LamG domains, which bind to dystroglycan in a glycosylation-dependent manner (Hynes & Naba 2012). Besides binding the cells to the basement membrane, dystroglycans can act as signal receptors. One study demonstrated that laminin111 binding to Schwann cells activated downstream Scr kinase family members in a dystroglycan dependent manner (Li et al. 2005).

CD44 binds to the ECM protein hyaluronan and is expressed in several cells. Binding to hyaluronan can stimulate cell adhesion, proliferation, migration as well as matrix assembly. CD44 interacts downstream with the cytoskeleton, various tyrosine kinases and RhoA/Rock signalling pathways (Turley et al. 2002). Netrins binds to Unc5-related tyrosine- kinase receptors and to Deleted in colorectal cancer (DCC). Both receptors are involved in cancer, however, it has been suggested that netrin is involved in the neural and pancreatic development and is expressed throughout the nervous system as well as the pancreas (Lai Wing Sun et al. 2011; Yebra et al. 2003).

In the above sections an introduction to the individually receptors has been given. However, ECM proteins have multiple domains, allowing them to interact with several receptors and growth factors at the same time. This multiple crosstalk process is called multi domain interaction and adds additional complexity to the cell-ECM interactions (Hynes 2009).

**Mechanotransduction**

In addition to direct signalling and its involvement in cytokine and growth factor signalling, the ECM also provides mechanical signalling to the cells. Mechanical load comes either from the ECM itself, from other cells or from outside the organism. Cells respond to the mechanical properties of the extracellular surroundings by membrane receptors, which are linked to the cytoskeleton and to signal transduction pathways. This process is called mechanotransduction (Hynes & Naba 2012; Humphrey et al. 2014). Mechanical cues are known to play an important role in early embryo development, including gastrulation (Krieg et al. 2008). Also *in vitro* the mechanical character of the microenvironment has significant implication for cell survival, proliferation and differentiation (Earls et al. 2013).

Integrins and the focal adhesions complex play an important role in mechanotransduction since they link to the cells cytoskeleton, where actin filaments, myosin and associated proteins transmit the mechanical signal into biochemical signalling. The exact process of how mechanical signals are transformed into intracellular biochemical signals is not known (Hynes & Naba 2012; Humphrey et al. 2014). Besides integrins, cadherins are suggested to be involved in mechanical signals by transducing mechanical forces between the cyto-

skeleton and the plasma membrane (Borghi et al. 2012). Further, mechanically gated ion channels, can concert mechanical force into a biochemical intracellular response. An example of such includes the tension driven modulation of protein phosphorylation at the kinetochore within the mitotic anaphase during mitosis (Geiger et al. 2001).

Mechanical properties can also function as homing signals for cells, where cells migrate towards an area with specific mechanical properties. This process is called durotaxis (Discher et al. 2009). Such processes are seen for mesenchymal stem cells which home to sites of injury and fibrosis (Pittenger & Martin 2004). The process is also observed *in vitro* where cells migrate towards a more rigid substrate (Lo et al. 2000). The underlying biological mechanism is not elucidated for durotaxis (Discher et al. 2009).

### 1.5.3 Extracellular matrix in the early development of an embryo

During development the ECM is dynamic, spatially distributed, and tightly regulated, indicating that the ECM has an important function in the developmental processes. However, knowledge about ECM in developmental biology is very limited and the downstream mechanism is not known. Most of this limited knowledge is gained by loss of function studies of ECM genes and their receptors, which frequently results in lethality or interruption in embryonic development (Bonnans et al. 2014). In this section some examples of the ECM functions in the early development towards pancreas will be given to emphasise the important functions of the ECM in developmental biology.

The ECM appears in the very early stage of development and can be both of maternal and embryonic origin (Rozario & DeSimone 2010). In the mouse, laminin 111 and laminin 511 are the first ECMP to be expressed and laminin 111 mRNA can be detected already at the 2-4 cell stage. Nidogen is detected at the 8-16 cell stage (Dziadek & Timpl 1985). However, the laminins and nidogen are first deposited and assembled into ECM around the blastocyst stage, during the formation of the first basement membranes. At this time collagen IV and fibronectin also appear. The first two basement membranes to form in the mouse embryo is the embryonic basement membrane, localized between the visceral endoderm and the developing epiblast, and in the Reichert's membrane, which surrounds the embryo (Leivo et al. 1980).

Formation of the embryonic basement membrane is essential for the embryo to develop. However, some of the components seem to have a limited effect. One example is collagen IV and mice lacking collagen IV appears to synthesize and assemble the embryonic basement membrane and survive until E9.5, which is after the primary gut tube formation (Pöschl et al. 2004). In contrast, laminin is important in early embryonic development. Mice lacking laminin 511 form an embryonic basement membrane and the Reichard's membrane normally, probably due to the compensation by laminin 111 (Miner et al. 1998). In contrast, mice lacking laminin 111 develop a normal basement membrane but lack the Reichard's membrane, leading to embryonic lethally at E6.5 (Miner et al. 2004). When mice lack both laminin 111 and laminin 511, the embry-

onic basement membrane as well as the Reichard's membrane fail to form and development is terminated at e5.5 (Smyth et al. 1999).

Fibronectin is expressed in early development and is assembled into a fibrillar structure prior to gastrulation. During gastrulation fibronectin appears along the blastocoel roof in amphibians (Lee et al. 1984). Studies indicate that fibronectin-integrins interaction is required for mesendoderm migration (Davidson et al. 2006). However, in mouse fibronectin null mutants, gastrulation occurs and embryos die around E10.5, due to cardiovascular and neural tube malfunctions (George et al. 1993).

Expression of basement membrane proteins at the early stage of development appears to be regulated by key signalling pathways, which indicates interplay between the ECM and biochemical signalling pathways in the early development. One example is an *in vitro* study with embryoid bodies performed by Li and colleagues, which demonstrated that FGFs and the downstream Gata6 induce endoderm differentiation by regulating laminin expression (Li et al. 2004). Another study demonstrated that loss of Smad4, a downstream TGFβ signalling molecule, results in increased expression of laminin and decreased expression of ECM metalloproteinase in ES cells. This resulted in thickening of the basement membrane, which disrupts signalling between the visceral endoderm and the epiblast as required for gastrulation (Costello et al. 2009). Fibronectin expression appears to be regulated by PI3K/Akt1 within the developing embryo, and the graded expression of fibronectin appears to regulate endoderm patterning and differentiation (Villegas et al. 2013).

As previously described, the ECM plays a vital role in generating morphogenic gradients and local reservoirs for growth factors, which is important in the developmental process. One example is that heperan sulfate proteoglycan, which plays an essential role in FGF signalling by stabilizing the FGF-receptor stability (Ornitz 2000).

Additionally, TGFβ ligands, such as Nodal and Activin A first bind to proteoglycans, which present the ligands to their receptors. The gradient of the morphogen BMP in a developing embryo appears to be regulated by collagen IV. Collagen IV binds to the Drosophila BMP4 and chordins homologs, sequestering them in the ECM (Wang et al. 2008). FGFs are important in both DE and PE development, whereas TGFβ ligands and BMP play a vital role in gastrulation and DE formation.

### 1.5.4 Extracellular matrix in culturing and differentiation of stem cells

The limited knowledge about ECM in embryonic development is only to some degree transferred to the *in vitro* culturing of ES cells where the main focus to date has been on soluble molecules. However, several observations and the recent evidence that ECM directs the differentiation of stem cells have highlighted the importance of ECM in the *in vitro* culturing of stem cells. Considering ECM in directed stem cell differentiation, adds another dimension which may solve some of the difficulties in the field. In this section, an overview of ECM in stem cell research will be presented and a comparison to embryonic development is made.

The discovery of Matrigel as a feeder free alternative for culturing pluripotent ES cells demonstrated that ECM together with growth factors can maintain pluripotency. Matrigel is basement membrane substrates secreted by Engelbreth-Holm-Swarm mouse sarcoma cells. ES cells maintain pluripotency when cultured on Matrigel, whereas they undergo differentiation on substrates like fibronectin (Xu et al. 2001; Greenlee et al. 2005). The basement membrane in mouse embryoid bodies appears to also have an inhibitory effect on the differentiation (Fujiwara et al. 2007). Moreover, hES cells express their own ECM proteins, such as nidogen, laminin 511 and laminin 111, indicating that these ECM proteins have a function in the microenvironment (Evseenko et al. 2009; Vuoristo et al. 2009).

Laminins are one of the ECM proteins which have been investigated in stem cell biology, due to its expression in the early embryo and its cell adhesive properties. Some of the first evidence that specific ECMPs can maintains pluripotency was demonstrated by Rodin and colleagues. They showed that laminin 511 induce long term self-renewal of mouse ES cells (Domogatskaya et al. 2008) and later hES cells (Rodin et al. 2010). This fits with the embryonic development, where laminin 511 is present early in the embryonic basement membrane, localized between the visceral endoderm and the developing epiblast (Leivo et al. 1980). In contrast, two other studies demonstrated that a synthesized basement membrane induced the differentiation of mouse ES cells towards pancreatic linages or hepatic lineage, which were mainly due to laminin 511 in the basement membrane (Higuchi et al. 2010; Shiraki et al. 2011). However, other ECM proteins and trapped soluble molecules in the synthesised basement membrane could have induced the pancreatic differentiation. Another study demonstrated that fragments of laminin 111, derived from metalloproteases cleavage, modulated cellular behaviour and EMT signals of mouse ES cells (Horejs et al. 2014). This fits with the *in vivo* initiation of gastrulation, where the epiblast cells undergo EMT by delaminating from the epithelial epiblast layer and breaks down the underlying basement membrane, where laminin 111 is present (Thiery et al. 2009).

Fibronectin is also linked to influencing stem cell differentiation. In 2005 Flaim and colleagues investigated how combinations of ECM proteins will affect the differentiation of mouse ES cells towards hepatic linage. In the array screen they found that fibronectin and collagen I substrates had a positive influence on the differentiation (Flaim et al. 2005). Further studies have demonstrated that fibronectin induce of DE differentiation of ES cells (Brafman et al. 2013a; Taylor-Weiner et al. 2013) and that the concentration of fibronectin patterns the DE (Villegas et al. 2013). Moreover, integrin expression is changed throughout the *in vitro* differentiation of stem cells, indicating that the ECM composition also change throughout the differentiation. Studies have demonstrated that laminin specific integrins are down regulated and integrins specific for fibronectin and vitronectin are up regulated when ES cells are differentiated to DE (Wong et al. 2010; Brafman et al. 2013b).

Morphogenic gradients and local reservoirs of growth factors and chemokines are essential for proper development of an embryo. *In vitro* interaction between ECM and growth factors has been shown to have a function. One study demonstrated that the ECM protein perlecan, a heperan sulphate proteoglycan, medi-

ates the binding and delivery of bFGF to FGF receptors (Smith et al. 2007). However, only limited studies exist on how growth factor-ECM binding affects the differentiation of embryonic stem cells.

To sum up, several studies have indicated that ECM plays a role in stem cell differentiation and few studies have indicated that fibronectin, laminins and vitronectin affect stem cell differentiation. However, identification of other ECM proteins that direct stem cell differentiation and the underlying biology mechanism remain unsolved.

## 1.6 Surfaces in stem cell culture and differentiation

Some of the physical and mechanical cues which the ECM's provide *in vivo* can be mimicked *in vitro* by changing the physical parameters of the cell culture surfaces with engineering methods. Currently, the majority of stem cell research is carried out in plastic culturing dishes, which is a rigid 2-dimensional system significantly different from the *in vivo* environment (Lutolf et al. 2009). However, various studies have demonstrated that changing the surface composition influences stem cell behaviour. Most of these studies focuses on mesenchymal stem cells, possibly due to the ease of culture, yet one study demonstrated that the same is the case for ES cells (Marklein & Burdick 2010). In this section some examples will be given of how surface composition can influence stem cell behaviour.

The mechanical properties of the ECM can be mimicked by changing the mechanical properties of the cell culture surface. One example is the stiffness of the surface. *In vivo* the stiffness of tissues varies depending on function, location and age. Natural and synthetic matrices can be produced to create cell culture substrates with known stiffness (Lutolf et al. 2009). Pelham and Wang showed the importance of substrate stiffness to control cell migration and spreading by using hydrogels (Pelham & Wang 1999). The stiffness can also influence stem cell behaviour. Engler *et al*. demonstrated how substrate stiffness can direct the differentiation of mesenchymal stem cells into different linages. Rigid substrates favoured bone differentiation and soft substrates promoted adipocyte differentiation (Engler et al. 2006). Other studies have followed, using the same stiffness of the desired tissue to direct stem cell differentiation (Saha et al. 2008; Gilbert et al. 2010). However, it has been suggested that it is not the stiffness but other parameters of these gel substrates, such as ECM tethering and organization that influence the cellular behaviour (Trappmann et al. 2012). Matrix elasticity can be regulated by changing the topography of the surface in micro and nanoscale. Yang and colleagues showed that elasticity can be regulated in Polydimethylsiloxane (PDMS) substrate with micropillars, by changing the height of the pillars (Yang et al. 2011). A study has demonstrated that less elastic micropillars promoted osteogenic differentiation whereas micro pillars with high elasticity induce adipogenic differentiation of mesenchymal stem cells (Fu et al. 2010). Thus, a comparable effect can be obtained by changing the height of the micropillars or stiffness of a hydrogel.

Mechanical properties and cell behaviour are even more complicated than described above. One study has shown that mesenchymal stem cells cultured on hydrogels can sense underlying substrates at a depth of about 20μm (Buxboim et al. 2010). Moreover, cells can most likely remember past physical signals, which will affect their present behaviour. A recent study demonstrated that human mesenchymal stem cells possess mechanical memory through the cytoskeleton signalling molecules YAP/TAZ that stores information from past physical environment and influence cellular behaviour (Yang et al. 2014).

The topography and architecture of the surface can also influence stem cell differentiation. One study showed that different surface wrinkles in micrometer scale, lamellar or hexagonal shaped, direct the differentiation of human mesenchymal stem cells to either osteogenic or adipogenic lineages (Guvendiren & Burdick 2010). However, the underlying biological mechanism remains unsolved. Topography on nanoscale also greatly influence stem cells behaviour, as well as change the mechanical properties of the substrate (Yang et al. 2011) (Figure 8). One study demonstrated that mesenchymal stem cell differentiation towards the osteogenic lineage can be affected by nanopatterns with 330nm deep grooves with different widths. Cells differentiated on nanogrooves which were 100nm wide showed a significant up regulation of skeletal developmental genes compared to the other nanogrooves (Biggs et al. 2008). Furthermore, disordered nanopits can stimulate human mesenchymal stem cells to produce bone mineral *in vitro* in the absence of osteogenic supplements (Dalby et al. 2007). Another study with the same nano topographies as described above, showed that hES differentiation on substrates displaying a near square arrangement of nanopits provided an enhanced mesodermal differentiation with a greater efficiency than planar substrates (Kingham et al. 2013).

The biological mechanism behind the directed differentiation by micro and nanotopographies remains unsolved. However, some characteristics have been reported. Cells can respond to patterns by aligning and elongating in the direction of the topography, and hereby change cell shape and cytoskeleton arrangement. It has been reported that nanotopographies can cause increased filopodia formation and change in focal adhesion complex formation (Figure 8). Furthermore, nanotopographies can alter the gene expression profiles of cells (Bettinger et al. 2009). The cellular response to nanotopographies can also be due to indirect mechanisms, such as changes in the protein absorption at the surface (Kriparamanan et al. 2006).

Moreover, stem cell behaviour can be controlled by the size of the substrate area. One study demonstrated that the size of circular Matrigel spots dictated the direction of hES cell differentiation. Cells cultured on large Matrigel spots (1200μm in diameter) gave rise to the mesoderm lineage while cell cultured on small sport gave rise to the DE lineage (Lee et al. 2009).

Other engineering strategies can be used to alter the microenvironment. This includes immobilization of growth factors to the surface, which can improve the stability and potency of the growth factor (Nur-E-Kamal et al. 2008; Alberti et al. 2008). Further, 3-dimensional culture system is another approach which mimics the *in vivo* microenvironment to a much larger extent than 2D culture systems. The potential of these engineer-

ing strategies should not be ignored but is out of scope for this thesis and is reviewed elsewhere (Lutolf et al. 2009).

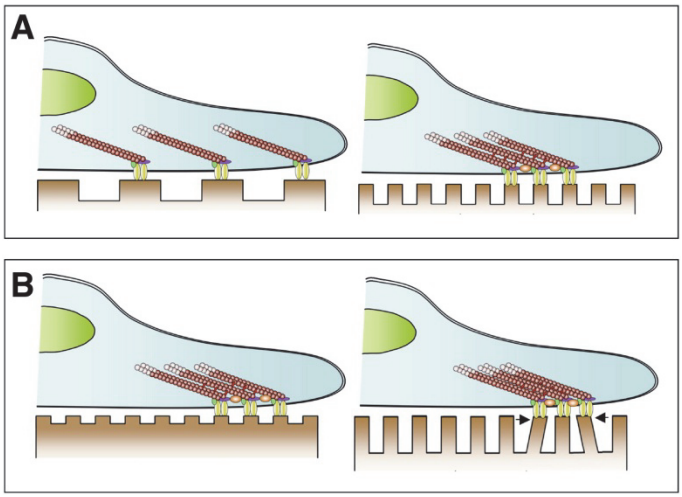


***Figure 8**. Nanoscale geometry and size of the topography of the surface/ECM can affect cellular behaviour, by altering the focal adhesions and cytoskeleton. A) The size of the feature can have impact on cell adhesions by altering the degree of cell spreading. B) Nanotopographies with different height can cause alteration of the surface stiffness, which affects the cellular behaviour by influencing the cytoskeleton tension as well as molecular and biochemical signals. Reproduced from (Guilak et al. 2009).*

The studies described above, demonstrate that cellular behaviour, including stem cell differentiation, can be regulated by changing the composition and properties of the cell culture surfaces. Most studies are carried out on mesenchymal stem cells and established cell lines, whereas knowledge of the behaviour of ES cells on surfaces is very limited. Furthermore, the downstream biological mechanisms are not well understood and elucidating such mechanisms will require extensive collaboration between engineers, biophysics and biologists.

## 1.7 Other parameters influencing stem cell differentiation

Even though no references exist, it is common knowledge in the stem cell research field that stem cell differentiation protocols can sometimes yield inconsistent results and be difficult to reproduce. This indicates that we still have a lot to learn to fully control the differentiation process and there are uncontrolled parameters possible in the microenvironment which greatly influence the differentiation.

One example is the timing and concentration of growth factors, which is highly controlled in the development of an embryo (Kumar et al. 2014; Chung et al. 2005). Neighbouring cells also play an essential role in the *in vivo* development, and co-culturing has shown to affect the directed differentiation of ES cells (Talavera-

Adame et al. 2011). Moreover, chemical parameters, such as pH and $O_2$, largely effect stem cell differentiation and cell maturation (Chaudhry et al. 2009; Heinis et al. 2010; Ezashi et al. 2005).

Cell density and number of cells within the environment is also an important parameter for cellular behaviour. One interesting study demonstrates that the initial cell seeding density influences the differentiation of hES towards pancreatic endocrine cells (Gage et al. 2013). Similar studies show that the seeding density is important for stem cell differentiation (Chetty et al. 2013; Ghosh et al. 2010).

The above sections emphasize that the microenvironment of the stem cells is complex and highly dynamic with crosstalk between many factors, including soluble, insoluble, physical and mechanical factors. To obtain fully controllable differentiation protocols of ES cells, all the various factors must be considered.

# 2. Objectives

The overall objective for this thesis is to improve the differentiation protocols of hES cells towards definitive endoderm and pancreatic endoderm by manipulating the physical microenvironment with micro- and nano-technology approaches. This is achieved by combining different disciplines: stem cell biology, engineering and micro- and nanotechnologies. The thesis is composed of 3 different strategies for manipulating the microenvironment of hES cell differentiation.

**1) Using extracellular matrix proteins to improve the differentiation protocol of hES cells towards definitive endoderm**

The study will systemically screen for ECM combinations which induce DE differentiation. This is achieved by performing an array screen with about 500 different extracellular matrix protein combinations. Potential hits will further be investigated and elucidating the downstream biological mechanisms will be attempted.

**2) Investigation of the influence of nanotopographies on hES cell differentiation towards definitive endoderm and pancreatic endoderm**

The aim is to study if and how different nanotopographies influence the cellular behaviour during hES cell differentiation towards definitive endoderm and pancreatic endoderm. If any difference in cellular behaviour is observed, a further investigation will be attempted.

**3) Investigation of the effect of initial cell seeding in the differentiation towards pancreatic endoderm**

The aim is to investigate if a uniform seeding of hES cells and definitive endoderm cells can be achieved by using a cell seeder device. Furthermore, it is investigated whether uniform cell seeding across the well and seeding density affect the differentiation towards pancreatic endoderm.

# 3. Strategy and scientific rationale

In this section the strategy and scientific rationale of my studies for the first two objectives (papers) will be given.

The purpose for this PhD project is to investigate how the physical environment affects differentiation of hES cells towards the pancreatic lineage. Only limited knowledge and literature about this subject exist. On one hand, extensive research has been conducted about the role of soluble molecules and their downstream signalling pathways in embryonic stem cell research. On the other hand biophysicist and engineers have investigated how the physical environment affects cellular behaviour and cell morphology. However, the link between the two research fields appears to be lacking. Thus, to focus on the physical environment in a stem cell differentiation setting, we decided to study the first two differentiation steps towards beta-cells, where the soluble factors and their downstream signalling pathways are well known.

As described in the introduction, differentiation of stem cells is a result of multiple factors, which interacts with highly complexity and interdependencies. We acknowledge this complexity, but simplification, is essential to study the effect of the physical environment on stem cell differentiation. This simplification is similar to the early research about soluble molecules in stem cell differentiation, where only one factor was investigated at the time. Accordingly, we decide to have the physical environment as the only variable factor in the differentiation of hES cells.

Endless opportunities exist within engineering of the physical environment, including 2D/3D strategies (Lutolf & Hubbell 2005), altering the basic material/surface (Ying Mei et al. 2007), addition of coatings such natural ECM proteins (Rodin et al. 2010) or engineered molecules (Haque et al. 2010) as well as altering the architecture (Guvendiren & Burdick 2010) and mechanical parameters (Fu et al. 2010). However, artificial physical environments must mimic the function of the natural ECM in which the underlying mechanisms are not fully understood. Therefore, we decided to start by looking into the effect of ECM protein coatings on hES cell differentiation.

## 3.1 Strategy for the extracellular matrix protein study

Few studies have proved that ECM influence stem cell differentiation (Higuchi et al. 2010; Shiraki et al. 2011; Domogatskaya et al. 2008; Horejs et al. 2014; Thiery et al. 2009), but the biology behind these observations is not known. That is why it is not possible to predict the cellular behaviour on the different ECM coatings and a trial-and-error strategy is requisite. Further, ECM has a multifunctional role and the different ECM proteins greatly interacts with each other (Tsang et al. 2010; Rozario & DeSimone 2010). One ECM protein may bind the cells to the surface, another ECM protein may signal to the cells, while another ECM protein binds solu-

ble growth factors. With a trial-and-error strategy and a large number of ECM protein combinations, a systematic screen will be useful.

Several screening platforms have been developed to screen for biomaterials. One possible screening method, which have been used in stem cells research, is the microarray platform (Y Mei et al. 2007; Mei et al. 2010; Brafman et al. 2013a; Flaim et al. 2005). Here it is possible to screen thousands of different ECM composition by robotically spotting ECM components to a basic surface or within a micro well (Y Mei et al. 2007). We decided to use such a screen, since it has previous been used in stem cell research and it fits well with DTU Nanotech's knowledge and equipment. The screening method was developed and optimized, including selection of basic materials, spot size, distance between spots, sterilisation technique, differentiation protocol, quantification, data analysis and NanoPlotter settings. A semi-optimal but stable DE differentiation protocol was chosen, to allow a large enough signal window for potential positive or negative effects by the ECM coating. Stability and a large enough signal window are essential aspects in designing a high quality screening assay (Iversen et al. 2012). Based on literature studies (S2 table in the manuscript) and own laboratory experience, an ECM library was built by combining 14 ECM proteins giving total of 500 different combinations.

The screen allowed us to test many ECM combinations at once in a systemic way. With a screen, a huge and complex dataset was generated, which requires a clear and simple readout. Immunocytochemistry was used for quantification, and we chose to stain for expression of the well-established markers Oct3/4 (pluripotent marker) and Sox17 (DE marker). The readout was number of cells and percentage of Sox17 positive cells at the end of the differentiation (day 8). It will be interesting to add additional markers or do analysis at different time point, but such addition will multiple the size and complexity of the dataset. As described above a screen gives the opportunity to test many ECM combinations at once, but it is important to acknowledge that a screen has some limitations. A screen simplifies the biology and assumptions are made. Further, in a screen, false-negative and false-positive results can occur due to various reasons, including error in the ECM library and high variability between the biological replicas. This means that results from a screen cannot stand alone, and the results need to be validated and further investigated.

Further investigation was performed in microtitre plate format to 1) validate hit combinations from the screen, 2) elucidate the effect of individual ECM protein on DE differentiation and 3) evaluate the quality of the screen. To obtain this, the hits and new combinations based on the hits were tested in microtitre plates.

After this investigation, four ECMPs (extracellular matrix proteins) stood out and along with the reference ECM, they were further investigated. The majority of previous studies on altering cellular behaviour by the physical environment have focused on the cell morphology (Guvendiren & Burdick 2010). However, the link between morphology and cellular behaviour has not yet been solved (Hynes & Naba 2012; Humphrey et al. 2014). Furthermore, studying morphology on tight packed confluent cell layer will be difficult. Thus, we choose a biochemical strategy, which also links to the stem cell research field methodology. In our previous

studies, only the expression of Sox17 and Oct4 was investigated on the last day of differentiation (day 8). To characterize the differentiated DE cells on the different ECM coatings, 9 different markers for pluripotency and DE differentiation was investigated by qPCR at days throughout differentiation protocol.

Knowledge about how the ECM affects intracellular pathways and hereby cellular behaviour is limited. Due to the novelty of our results and the lack of literature within the field, we did not have any obvious mechanism to further investigate. Again, a trial-and error strategy seemed to be the only option. We performed a global gene expression analysis (DNA micro-arrays) on cells differentiated on the different ECM coating at the last day of the differentiation, to see which genes had been regulated. Results from the global DNA microarray screen gave us an idea where to look and what to further investigate. This is discussed in the paper and the thesis discussion and perspective.

## 3.2 Strategy for studying nanostructured surfaces

The SMALL group at University of Glasgow, headed by Nikolaj Gadegaard, has specialized in injection moulding nanotopography plastic surfaces for biological and clinical studies (Pedersen et al. 2015; Stormonth-Darling & Gadegaard 2012). A collaboration with Nikolaj Gadegaards laboratory was established by a three months external research stay.

Studies with nanostructured surfaces were performed to see if physical and mechanical properties of a surface affected hES cell differentiation. DE and PE differentiation on various nanotopographies were investigated with stable semi-effective differentiation protocols. The semi-effective protocols gave a large enough signal window to see the potential effect of the nanostructured surfaces. For testing the PE differentiation, cells were differentiated towards DE in cell culture flasks, seeded on the nanostructured surfaces and the differentiated to PE. This allowed the same starting point for the PE differentiation on the different surfaces.

The nanotopgraphy which have been shown in previous studies to influence mesenchymal stem cell differentiation (Dalby et al. 2007), did not affect the differentiation of hES towards DE and PE (results not shown). However, surfaces with soft nanopillars, developed by Nikolaj Gadegaards laboratory (Stormonth-Darling & Gadegaard 2012), showed a very interesting effect on DE differentiation. Daily bright field microscopy observations made it clear that cellular attachment and movements were distinct on the nanopillars when compared to the flat surface. Next, time point experiments were set up as well as an attachment assay was set up to investigate for cell specific attachment.

The layout of the samples (Figure 13), made it impossible to harvest cells from individually nanopillared areas for biochemical assays, e.g. qPCR. Therefore, immunocytochemistry seemed to be the only option. Furthermore, studies of the cell morphology were difficult with a dense cell layer. For example preliminary

electron microscopy results demonstrated it was not possible to distinguish individual cells in the dense cell layer. Only individually cells or small cell clumps could be observed using electron microscopy, but these did not represent the majority of the cells.

If time and current surface layout had not been a limitation, it would have been interesting to further study this novel observation. Potential further studies are described in the thesis perspective.

# 4. Summary of results

In this section a summary of the results presented in the 3 manuscripts will be given. The results in this section are presented in chronological order whereas the manuscripts focus on the major findings.

## 4.1 Collagen type I improves the differentiation of human embryonic stem cells towards definitive endoderm

This study included a systematic array screen to identify ECM proteins that induce differentiation of hES cells towards DE using a semi-effective differentiation protocol (Figure 9). The protocol was based on Wnt3a and Activin A in order to observe potential effects of the ECM proteins. The screen was built on a protein array platform on microscope slides where almost 500 combinations of 14 different ECM proteins were tested. Prior to the onset, the screening method was developed and optimize, including selection of microscope slide material (polystyrene) as well as selection of size of the individual protein spots and distance between them.

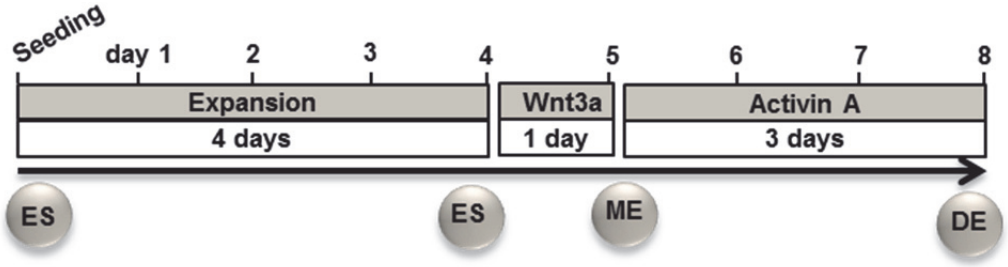


***Figure 9.*** *Schematic overview of the DE differentiation protocol used in this study. hES cells are seeded and cultured for 4 days to allow expansion. The DE differentiation is initiated by priming with Wnt3a which direct the cells towards mesendoderm fate. Subsequently 3 days exposure to Activin A will direct the cells towards DE.*

From the screen several ECM protein combinations were identified as effectors of differentiation and then divided into 2 groups: the ECM protein combinations resulting in high percentage of Sox17 positive cells and the ECM protein combinations resulting in a high number of cells but only intermediate percentages of Sox17 positive cells. The interesting ECM protein combinations were validated in microtitre well plate format. All ECM protein combinations resulting in high number of cells and 70% of the ECM protein combinations yielding a high percentage of Sox17 positive cells were confirmed in microtitre well plates.

Further studies in microtitre well plates identified individual ECM proteins as regulators of DE differentiation as compared to the control samples cultured on fibronectin (summarized in Table 1). Collagen I significantly improved the differentiation of hES cells towards DE compared to the fibronectin. When hES cells were differentiated on collagen 1, the output was 83 % Sox17 positive cells whereas differentiation on fibronectin gave 64 % Sox17 positive cells. Furthermore, collagen 2 improved DE differentiation, but resulted in a low

number of cells. However, the addition of fibronectin to collagen 2 significantly increased the number of cells while maintaining high DE induction. Netrin 1 improved the differentiation towards DE, but only when it was combined with fibronectin. In addition, human laminin had a positive effect on the differentiation to DE, where nidogen and vitronectin appeared to repress the differentiation towards DE. Differentiation on vitronectin had a positive effect on the total cell number.

***Table 1.*** *Summary of individual ECM proteins and their effect on the percentage of Sox17 positive cells (DE differentiation) or the total cell number when compared to the control fibronectin.*

| ECM protein | DE differentiation | Total cell number |
|---|---|---|
| Collagen 1 | ↑ | - |
| Collagen 2 | ↑ | ↓ |
| Netrin1+ fibronectin | ↑ | - |
| Laminin human (mix) | ↑ | - |
| Vitronectin | ↓ | ↑ |
| Nidogen | ↓ | - |

↑ Positive effect
↓ Negative effect
- No effect

The following four ECM proteins or combinations of ECM proteins were selected for further studies: 1) collagen 1, 2) collagen2 + fibronectin, 3) netrin 1 + fibronectin and 4) vitronectin. These ECM proteins were assessed in a time course experiment, which was analysed by immunocytochemistry and qPCR for selected genes. Gene expression analysis by qPCR showed similar kinetics for differentiation on the ECM protein substrates compared to previous published DE protocols. One day after Wnt3a priming, the cells on collagen 1 had a distinct morphology (Figure 10 and 11) and the mesendoderm marker *BRACHYURY* was upregulated. Furthermore, on day 6 a significantly higher percentage of Sox17 positive cells appeared on collagen 1 substrates ($P<0.05$) compared to cells cultured on fibronectin,indicating that collagen 1 influences the initiation of DE differentiation. Cells on the other ECM coatings showed similar kinetics with regards to gene expression and the appearance of Sox17 positive cells compared to cells on control surface (fibronectin).

To further look into the mechanism of our findings, a global gene expression analysis was performed at day 8 (at the end of the differentiation protocol). Data showed that cells cultured on collagen 1 showed significant changes in gene expression compared to cells cultured and differentiated on fibronectin. About 1200 genes were differentially expressed in cells cultured on collagen 1 compared to cells on fibronectin. The microarray data also confirmed the qPCR results.. The most down-regulated gene was *DACT1* which is an inhibitor of Wnt signaling (Cheyette et al. 2002; Zhang et al. 2006). Other down-regulated genes were *POU5F1* and *SOX2* which both are markers for pluripotent cells, indicating a better differentiation. One interesting gene that was up regulated was Nitric oxide synthase 2 (*NOS2*), which has been shown to activate β-catenin and EMT in breast cancer cells (Switzer et al. 2012). Pathway analysis showed that cells differentiated on colla-

gen 1 had differentially expressed genes involved in focal adhesion, neural crest differentiation, spinal cord injury and Notch signalling when compared to cells differentiated on fibronectin.

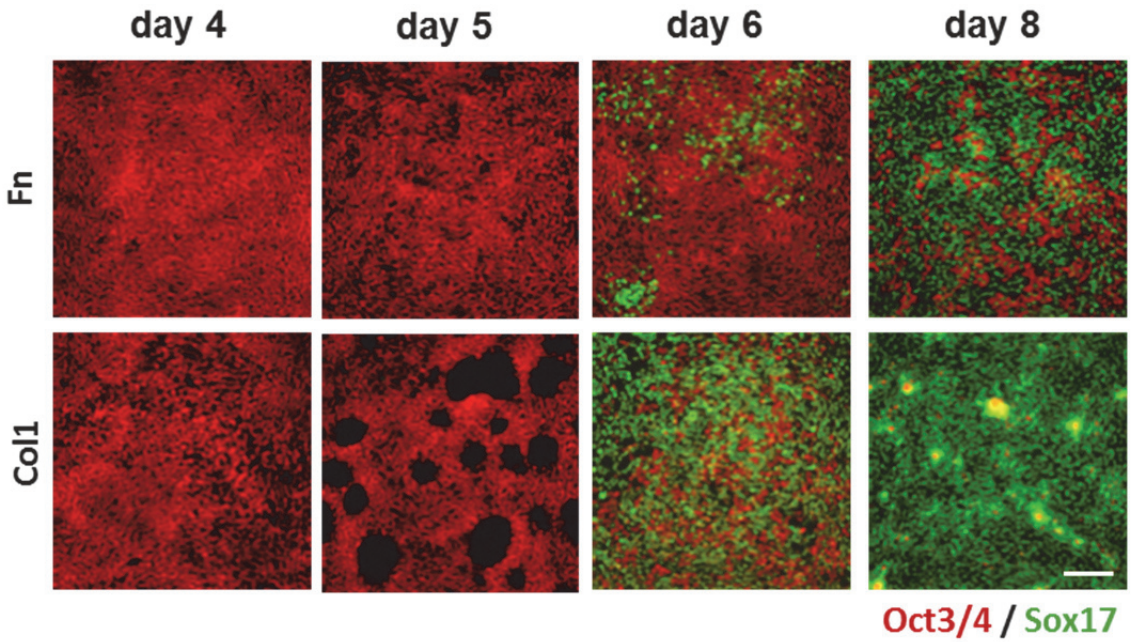


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***Figure 10.*** *Key findings in the time course experiment. DE Differentiation on collagen I (col1) showed a morphology difference on day 5, faster DE induction on day 6 and a significant higher percentage of Sox17 positive cells, compared to fibronectin (Fn). Fluorescence microscopy, 10x images, showing the expression of markers representing pluripotency (Oct3/4) and DE (Sox17). Scale bar 200µm.*

**Additional results not presented in the paper**

Figure 11 in the thesis is also displayed in the supplementary material for the manuscript (S3 Fig). The results will be described here, since they are not further described in the manuscript. The cell morphology on the difference ECMP substrates was analysed at different time points during DE differentiation (Figure 11). One day after seeding, fewer cells attached and the cell morphology was different on collagen 1 compared to fibronectin. Cells on collagen 1 were clustered together and some cells showed an elongated morphology (Figure 11, day 1). Whether these differences in cell morphology were due to lower cell number or ECMP substrate is not known. However, on day 4 the cell layer had grown to confluence on the different substrates. On day 4, it appeared that the cell morphology on fibronectin was more structured and showed similarities to an epithelial cell layer. The cell morphology on the other substrates appeared to be more unstructured and the actin filaments were more distinct and elongated. The day after Wnt3a treatment (Figure 11, day 5), holes in the otherwise confluent cell layer appeared on collagen 1 but not on the other substrates. The actin filament appeared to be more distinct on collagen 1 and collagen 2 + fibronectin substrates compared to the other substrates. One day after Activin A treatment, Sox17 positive cells started to appear and they had a different morphology different than the Oct4 positive cells. The DE cells had less distinct actin filaments in the middle and more distinct actin filaments in the membrane area where they are in contact to other cells.

Despite the difference in the distribution of Sox17 and Oct3/4 positive cells on the various ECMP substrates, not obvious cell morphology difference was observed on the various ECMP substrates.

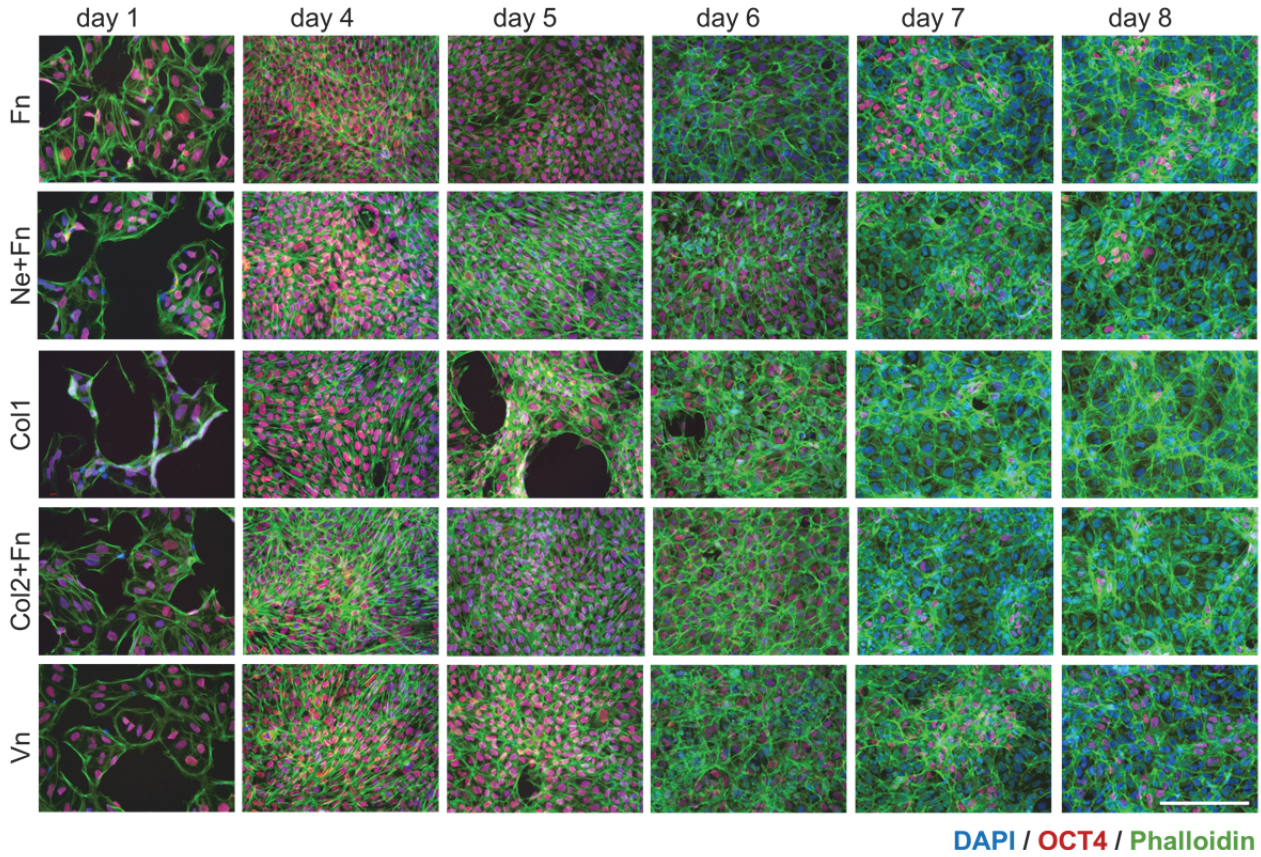


***Figure 11.*** *(copy from paper 1 supplementary) Cell morphology during DE differentiation on different ECMP substrates in microtitre plates at different time points. Representative immunofluorescence images from 3 independent experiments of cells cultured to different time points during the DE differentiation protocol. The cells were stained for Oct3/4, nucleus (DAPI) and F-actin (Alexa Fluor 488 Phalloidin). The tested ECMP combinations were fibronectin (Fn), collagen 2 plus fibronection (Col2+Fn), collagen 1 (Col1), netrin 1 plus fibronectin (Ne+Fn) and vitronectin (Vn) (scale bar=200µm).*

It is well known that the variability of differentiation of hES cells is high between individual experiments (biological replicas) and you might sometimes find differentiation unexpectedly low for unknown reasons. This also occurred in our hands and therefore made use of a positive control (fibronectin). Experiments, where the positive control gave below 50% of Sox17 positive cells in the DE differentiation, were discarded. However, even in experiments which gave below 50% of Sox17 positive cells on positive control, the differentiation on collagen 1 resulted in above 80% Sox17 positive cells. This indicated that collagen 1 appeared not only to improve the percentage of Sox17 positive cells during DE differentiation, but also rescued poor differentiation and decreased the variability between independent experiments.

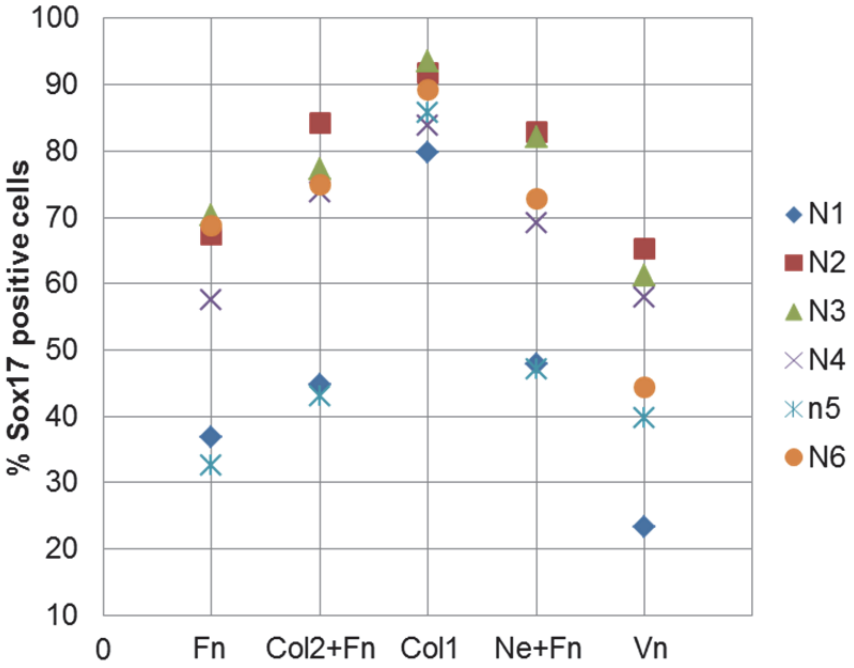


***Figure 12.*** *DE differentiation Variation among different biological replica (N1-N6) at different ECM substrates. Fibronectin (Fn), collagen 2 plus fibronectin (Col2+Fn), collagen 1 (Col1), netrin 1 plus fibronectin (Ne+Fn) and vitronectin (Vn).*

## 4.2 Enhanced differentiation of human embryonic stem cells towards definitive endoderm on ultra-high aspect ratio nanopillars

This study includes an investigation of the differentiation of hES cells towards DE and PE on various nanotopography substrates. The differentiation was affected by high nanopillars whereas the other tested nanotopographies did not show any differences compared to the flat control surface.. The polymer substrates with the nanopillar topography were produced by injection moulding (Figure 13) and characterized with atomic force microscopy (AFM) and scanning electron microscopy (SEM). The nanopillar topographies had an elastic character and the height and width of the pillars controlled the elasticity.. Initial studies showed that at the end of DE differentiation the soft nanopillars had a significantly higher percentage of Sox17 positive cells (94%) compared to the control (flat) surface (48%) (Figure 14).

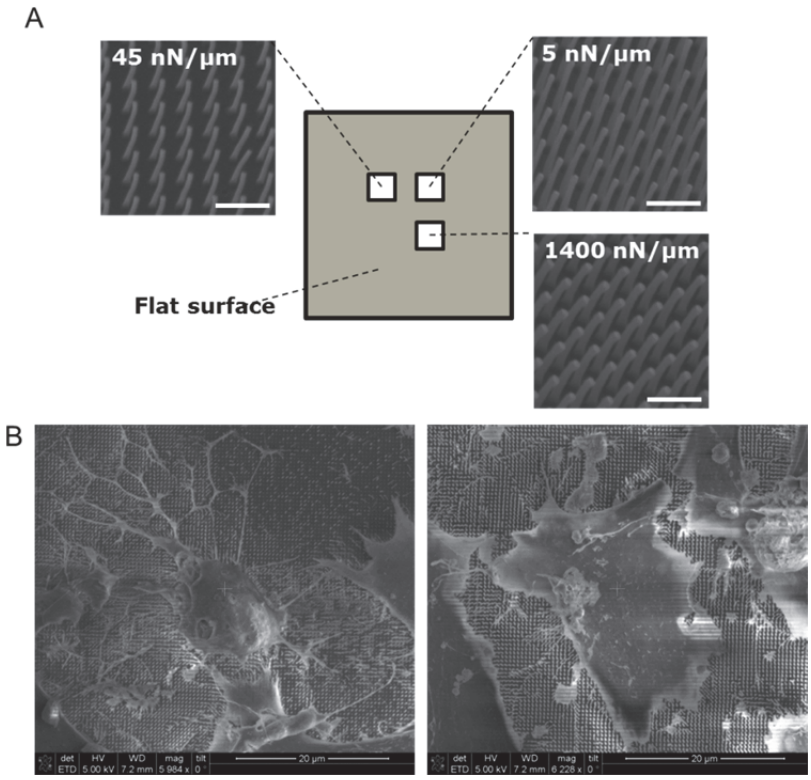


***Figure 13**. **A)** Overview of the nanopillar substrates. On a polycarbonate chip three different nanopillar structures are assembled. Outside the nanopillar areas the surface is flat. **B)** Examples on how cells interact with the nanopillars during DE differentiation, SEM images.*

To further investigate thisobservation, time course studies of the DE differentiation were performed using the protocol outlined in Figure 9. The initial attachment of hES cells was significantly lower on the nanopillars compared to the control surface. The day after seeding, cells on the soft nanopillars had a distinct cell morphology; they were elongated and aligned to the pillar pattern. After four days in culture (Figure 14, day 4) the hES cells had proliferated extensively, and the cell layer was confluent on the control surface. However, on the soft nanopillars, cells attached at the edge of the nanopillar area and with a few small tight clusters of cell in the middle of the area. This indicated that the soft nanopillar substrate did not favour hES cell proliferation and/or attachment. Subsequently, DE differentiation was initiated by Wnt3A priming to drive the cells towards mesendoderm. One day after Wnt3a priming (Figure 14, day 5) the first Sox17 positive cells appeared on the soft nanopillars, at the border of the confluent cell layer. Such early Sox17 induction was not observed in the control sample, indicating an early induction of DE cells on the soft nanopillars. The DE differentiation protocol was continued with the addition of Activin A for three days. After one day with Activin A treatment (day 6) the previous cell empty space on the soft nanopillars was filled with Sox17 positive cells. This could indicate that Sox17 positive cells migrate into the soft nanopillar area from the surrounding flat surface substrates.

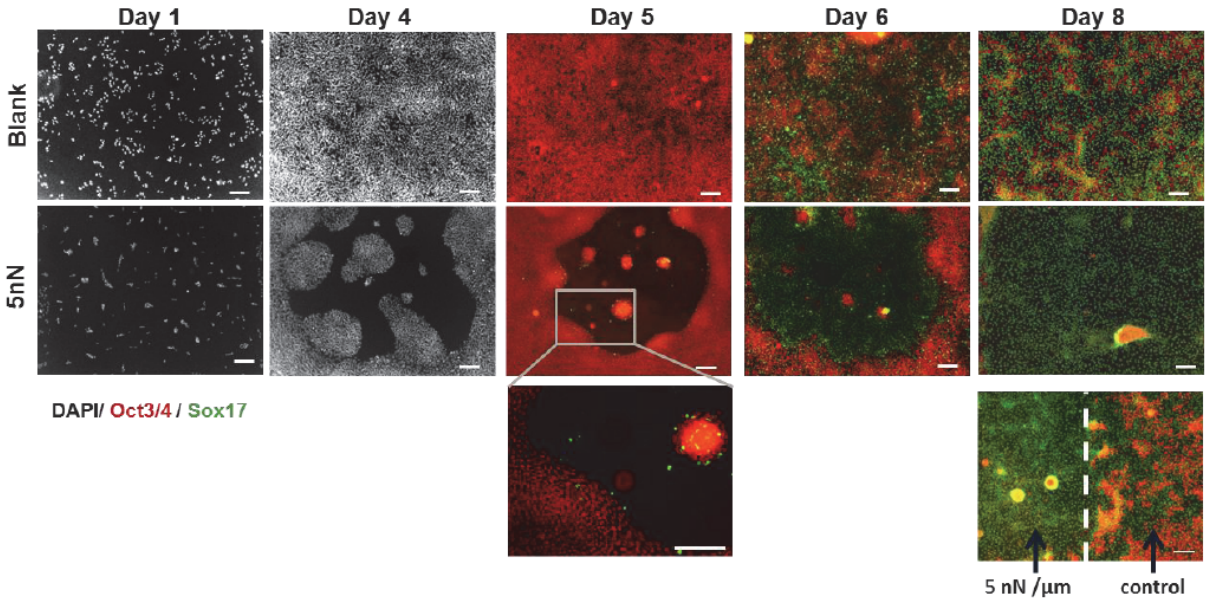


***Figure 14.*** *Overview of the DE differentiation on flat and soft nanopillar substrates. The initial attachment of hES cells was significantly lower (P<0.05) on the soft nanopillars (Day 1), and after 3 days on culture cells only attached at the border between the flat surface and the soft nanopillars (Day 4). Already on day 5, after Wnt3a priming, the first Sox17 positive cells appeared on the soft nanopillars (Day 5). During DE differentiation Sox17 positive cells migrated into the soft nanopillar area from the surrounding flat surface substrates (Day 6 and Day 8). Fluorescence microscopy, 4x and 10x images, showing the expression of markers representing pluripotency (Oct3/4) and DE (Sox17). Scale bars 200µm.*

To investigate if the DE favours attachment to the soft nanopillars, a heterogeneous cell population, composed of undifferentiated hES cells and DE cells, was seeded and fixed the following day. Results from this study showed that the attachment of DE cells was slightly favoured on the soft nanopillars compared to the control surface

The next differentiation step towards pancreatic cells, PE, was also assessed on the nanopillars. In contrast to DE differentiation, the softnanopillars had a repressive effect on PE differentiation. On the nanopillars, Pdx1 and Nkx6.1 positive cell clusters were significant smaller compared to the control substrate.

## 4.3 Comparison of cell seeding techniques

The third study presented a simple methodology by which the uniformity of cell seeding across large culture areas can be improved. Using a cell seeder device, the cell seeding suspension is limited to a defined space above the culture substrate, which will cause minimal turbulence disturbance of the cell suspension. Hereby, fluctuations in cell density across the culture surface will be minimised.

To assess whether using the seeding device impacted cell distribution and differentiation, it was compared to two traditional seeding techniques: droplet seeding (a concentrated droplet of cell suspension is placed in the centre of the well) and open seeding (the well was filled with medium containing a cell suspension)

(Figure 15). When seeding human fibroblast cells with these 3 methods, the seeding method with the cell seeder device resulted in a significant improvement in the uniformity of cell distribution within the well. The two other seeding methods resulted in high cell density in the centre of the well.

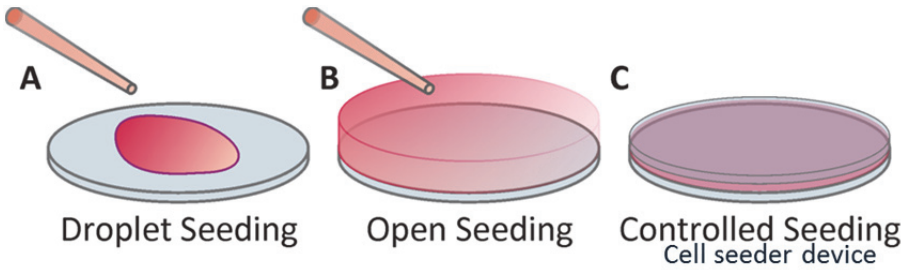


***Figure 15.*** *Different cell seeding techniques. A) droplet seeding, whereby a concentrated droplet of cell suspension is placed in the centre of the well. B) Open seeding whereby the well was filled with cell suspension medium C) Controlled seeding using the cell seeder device.*

Similar results were obtained with hES cells and DE cells in 12 well plates when the cell seeder device was used. The DE cells were seeded at three different densities with either open seeding or controlled seeding (using the seeding device) and subsequently differentiated towards PE. The outcome of PE differentiation depended on the seeding method and seeding density. With low seeding density (100K cells per $cm^2$), Pdx1 and Nkx6.1 double positive cells (mature PE cells) were only observed in the centre of the well, when using open seeding. This indicated that differentiation to PE was only possible in areas with high cell density. With intermediate seeding density (200K cells per $cm_2$) it was possible to obtain Pdx1 and Nkx6.1 double positive cells both with open seeding and controlled seeding. However, when using the controlled seeding technique, a more uniform distribution of clusters of Pdx1 and Nkx6.1 double positive cells across the entire well was obtained, compared to using open seeding. With high seeding density (300K cells per $cm^2$) the differences in the seeding was less pronounced using the two different seeding techniques.

# 5. Discussion

Detailed discussions are given in the manuscripts. In this section an overall discussion of the thesis results will be presented.

A significant effort has gone into developing protocols for the directed differentiation of hES cells to mature functional beta-cells, due to their potential use in cell replacement therapy to treat diabetes. Recently, two improved protocols were published (Pagliuca et al. 2014; Rezania et al. 2014), but it is still questioned whether these stem cell derived beta-cells are mature and fully functional. The majority of the protocols try to mimic the *in vivo* development by adding soluble factors to target signalling pathways involved in the *in vivo* development (Pagliuca et al. 2014; Rezania et al. 2014). Although several studies have highlighted the essential role of the physical microenvironment in stem cell biology (Taylor-Weiner et al. 2013; Brafman et al. 2013a; Kingham et al. 2013), current differentiation protocols do not take the physical environment into consideration. However, taking the physical environment into consideration might improve the current differentiation protocols and help to achieve fully functional mature cells. Furthermore, investigating the physical environment of stem cells will provide an understanding of how the physical environment affects cellular behaviour and how it interacts with the signals from the soluble molecules.

Here we have demonstrated that manipulating the physical microenvironment using three different strategies greatly influenced the differentiation of hES cells to DE and PE. With the first strategy, we identified ECM protein substrates that can direct the differentiation of hES cells towards DE. With the second strategy we demonstrated that nanopillar substrates highly influence the differentiation of hES cells to DE and PE. Finally, with the third strategy we showed that uniform seeding across the entire well can easily be obtained by using a cell seeder device and that such uniform seeding had a big influence on the differentiation to PE.

The three presented strategies focus on simple cell culture systems and are only representing a small fraction of engineering methods used to manipulate the physical environment (Vazin & Schaffer 2010; Edalat et al. 2012). Moreover, timing (Murry & Keller 2008), chemical (Chaudhry et al. 2009; Heinis et al. 2010) and biochemical (Pagliuca et al. 2014) parameters influence stem cell differentiation. And it is important to acknowledge that stem cell fate is regulated by a complex dynamic interplay between all parameters (Watt & Huck 2013). Using the three strategies provided in this thesis represent simple models to study the physical environment in stem cell differentiation and downstream biological mechanisms as well as providing improvements for directed stem cell differentiation protocols.

## 5.1 Biological interest of our findings

In this study we demonstrated that the physical environment, in the form of ECM proteins and nanopillars, influenced differentiation of hES cells towards DE. To our knowledge such observations have not previously

been reported and it provides another dimension to the traditional perception of stem cell biology. However, the downstream mechanism behind our observations was not fully elucidated. Differentiation of hES cells to DE and PE *in vitro* resembles events during embryonic development (Murry & Keller 2008). Whereas the majority of the biochemical cues and downstream signalling pathways are mapped (Kumar et al. 2014), the physical microenvironment is not well studied during the stem cell differentiation process (Rozario & DeSimone 2010). However, many of the signalling pathways involved in the differentiation to DE and PE, are activated through integrins and other cell receptors which are involved in ECM signalling and mechanotransduction. This includes MAPK/ERK (Prowse et al. 2011; Ameri et al. 2010), PI3K (McLean et al. 2007; Prowse et al. 2011; Villegas et al. 2013), TGFβ (Kloeker et al. 2004; Massagué & Chen 2000) and GSK3/β-catenin signalling (Crampton et al. 2009; Liu & Habener 2010; Rezania et al. 2014). This connection strongly indicates that interplay exists between soluble molecules and the physical microenvironment with stem cell differentiation, since they are targeting the same downstream signalling pathways. Such interplay might explain our observations regarding how the physical environment induced DE differentiation and could be a subject for further studies.

In our study we demonstrated that ECMP substrates influence the differentiation of hES cells towards the DE stage, which aligns with several recent studies that have highlighted the essential role of ECM in stem cell biology (Taylor-Weiner et al. 2013; Rodin et al. 2010; Higuchi et al. 2010), Our studies indicated that collagen 1, collagen 2 and netrin 1 improved the DE differentiation of hES cells, whereas vitronectin appeared to have a positive effect on the total cell number. Within all our analyses, cultures on collagen 1 was notable by having distinct morphology, proliferated faster and importantly givingg purer DE cultures with very few Oct3/4 positive cells.

Looking into existing literature, collagen 1 is found to be the most abundant ECM protein in the body, where it provides structural support and binds other ECM proteins (Rozario & DeSimone 2010; Zagris 2001). To our knowledge, collagen 1 has not been associated with differentiation of hES cells towards DE or with the development of DE previously. Although, a previously published screen demonstrated that collagen 1 had a negative effect on hES cells differentiation towards DE (Brafman et al. 2013a) and another array study reported that collagen 1 positively influenced the differentiation of mouse ES cells towards hepatic linage (Flaim et al. 2005). Collagen 1 appears not to be presence in early embryo, since collagen 1 is not expressed by mouse ES cells in various cell culture conditions (Taylor-Weiner et al. 2013) and *in vivo* disruptions of the collagen 1 is not lethal until a late stage of development, due to aortic rupture (Löhler et al. 1984). Further, collagen 1 appears not be expressed in the embryonic basement membrane through which epiblast cells ingress during gastrulation (Gersdorff et al. 2005). However, direct evidence whether collagen 1 is expressed in the early embryo or extra embryonic tissue is lacking. This limited literature about the role of collagen 1 in development biology and stem cell differentiation, indicates that our observations are novel and can be subject for further investigation in the stem cell and developmental biology fields.

Our qPCR results (paper 1, figure 4) suggested that collagen 1 induced a more rapid and consistent mes-endoderm differentiation (day 5). After Wnt3a treatment, we observed holes in the cell layer on collagen 1 substrates and these holes were covered with cells after one day of Activin A treatment. This relatively fast disappearance of the holes and appearance of cells suggests cell migration or movement of the whole cell layer by a contraction mechanism. Previously, It has been reported that Wnt3a increased fibroblast mediated contraction of collagen 1 lattices and increased the migration of myofibroblasts during differentiation (Carthy et al. 2011). One hypothesis of our observations is that collagen 1 provides anchorage sites for cell movement or contraction.

Further, the qPCR results indicated a stronger down-regulation of *POU5F1* and upregulation of e.g. *BRACHYURY in cells on* collagen 1 , . The microarray analysis showed that *DACT1* was down-regulated and *NOS2* was upregulated on collagen 1 substrates compared to fibronectin. Previously, it has been deomonstrated hat *NOS2* activates β-catenin (Switzer et al. 2012) and that *DACT1* inhibits Wnt signalling (Cheyette et al. 2002; Zhang et al. 2006), indicating that that the effects of Wnt3a could be enhanced by a reduction of *DACT1* and/or increased *NOS2* activity. Further, it has been reported that *BRACHUYRY* is a downstream target of Wnt signalling (Arnold et al. 2000), which may explain why *BRACHUYRY* showed a large induction the day after Wnt3a stimulation . A possible hypothesis is that collagen 1 increases *NOS2* expression and reduces *DACT1* expression at the undifferentiated stage, making the cells more susceptible to Wnt3a signalling, and thereby increasing the fraction of cells entering the differentiation program.

The microarray gene expression analysis demonstrated that in cultures on collagen 1 substrate the focal adhesion pathway was upregulated. The focal adhesion pathway affects diverse cellular functions, including cell migration (Prowse et al. 2011) and epithelial to mesenchymal transition (EMT) (Cicchini et al. 2008). The pathway is regulated by binding of ECM to the integrin cell receptors (Prowse et al. 2011). During development, DE is formed during gastrulation where cells migrate and undergo EMT (Thiery et al. 2009). Interestingly, when cancer cells invade tissues or metastasize, they use a mechanism similar to the EMT during gastrulation and several studies have reported that collagen 1 promote EMT in cancerous cells (Shintani et al. 2008; Shintani et al. 2006; Koenig et al. 2006). Another study demonstrated that collagen 1 is highly upregulated after EMT in fibrosis (Hosper et al. 2013). Similar mechanisms may be involved during DE differentiation of hES cells where collagen 1 substrates promote EMT and hereby induce the DE differentiation. Moreover, it is noteworthy that NOS2, which found to be upregulated on collagen 1 , increases EMT in breast cancer cells (Switzer et al. 2012).

We observed an increased number of DE cells after differentiation on collagen 1 substrates compared to fibronectin substrates. In principle, we are not aware whether this is due to induced DE differentiation, induced proliferation of DE cells and/or improved survival or attachment of DE cells compared to the hES cells. However, the qPCR data strongly point to an increased DE induction, as seen by the pattern of upregulation of mesenendoderm markers and DE markers in our cell cultures on collagen 1 substrate. The increased number of DE cells on collagen 1 substrate could be a result of increased proliferation of DE cells. However,

the total cell number on collagen 1 was similar to the total cell number on fibronectin at the end of the differentiation. The number of cells on collagen 1 was unchanged throughout the differentiation, indicating that increased proliferation attachment is not be the dominant factor for the increased fraction of DE cells on collagen 1 substrate. However, further proliferation assays along with Sox17 stainings are needed to fully confirm this.

Another potential explanation to the increase fraction of DE cells on collagen 1 substrates is that collagen 1 primed the cells to enter into DE differentiation. Our qPCR results demonstrated that the expression of markers for pluripotency, mesendoderm and definitive endoderm were similar in the cells cultured on the different substrates. However, such priming by collagen 1 can be due to differences within the focal adhesion, cytoskeleton, epigenetic level, protein level or the expression of other genes and so further studies are required to fully explore this.

In our study we demonstrated that nanopillars greatly influenced the differentiation of human embryonic stem cells to DE and PE. Previously, it has been demonstrated that mechanical properties of the environment affect cell differentiation of mesenchymal stem cells (Engler et al. 2006; Dalby et al. 2007; Kilian et al. 2010). To our knowledge it has not previously been reported that topographies or mechanical properties of the surface affects the differentiation of hES cells towards DE and PE. At the end of the DE differentiation protocol The soft nanopillars had significantly higher percentages of Sox17 positive cells (94%) compared to the flat control surface (48%). Our studies suggest that the high percentages of Sox17 positive cells is caused by a combination of factors, including , early and fast induction of Sox17 positive cells on the soft nanopillars, migration of DE cells towards the nanopillars and surface dependent selective attachment.

It has previously been reported that nanotopography provides selective cell attachment for different cell types (Chen et al. 2012; Reynolds et al. 2012), however it has not been observed for hES cells and hES cell derived DE cells. Studies have indicated that cells preferring a stiffer matrix have a more developed and distinct actin cytoskeleton, whereas cells on a softer substrate have a far less developed cytoskeleton (Engler et al. 2006). This is consistent with our results; staining for actin revealed that DE cells on flat surfaces had a far less defined cytoskeleton with only cortical actin organisation compared to the hES cells, which had clear visible cytoskeleton (Figure 11). Another explanation to the selective attachment could be due to differences in integrins and cadherins expression profiles in hES and DE cells. The studies of Chen and coworkers indicated that the topographical sensing and the selective attachment might include feedback regulation involving mechanosensory integrin-mediated cell-matrix adhesion, myosin II, and E-cadherin (Chen et al. 2012). The integrin profile (Wong et al. 2010) and the expression of E-cadherin (Thiery et al. 2009; D'Amour et al. 2005) changes when hES cells differentiate towards DE. Thus, the selective attachment we observed might be due to difference in the integrins and cadherins expression profile in hES and DE cells.

Our resulted demonstrated that the differentiation was initiated very early on the soft nanopillars, indicating that the pillars may directly and actively induce the differentiation. Already after the Wnt3a priming, Sox17

positive cells appeared on the soft nanopillars and such early induction was not seen on the flat control surface. It has been previously reported that targeting Wnt signalling, using Wnt3a, increase the efficiency of mesendoderm specification(D'Amour et al. 2006; D'Amour et al. 2005; Tada et al. 2005). However, Sox17 is first expressed in the later in the DE stage and not at the mesenedoderm stage (Tada et al. 2005; D'Amour et al. 2005), indicating that the soft nanopillars in combination with Wnt3a provided sufficient signals to differentiate the hES cells all the way to the DE stage. One possible explanation to this could involve the TGF-β pathway. Previously studies have demonstrated that the intracellular proteins YAP and TAZ are found to be involved in mechanotransduction (Dupont et al. 2011) and these proteins are situated at the centre of the TGF-β signalling pathway (Morgan et al. 2013). Interestingly, during DE formation the TGF-β signalling pathway is activated through Nodal/Activin A (Zorn & Wells 2009). Thus, the soft nanopillars may provide mechanical signals to activate the TGF-β signalling pathway without the need of Nodal/Activin A.

We observed that the area of soft nanopillars was repopulation with Sox17 positive cells within a day while the hES cells (Oct3/4 positive cells) were mostly restricted to the more rigid flat surface. Such fast repopulation of a rather large and almost empty area cannot be only caused by cell division alone, since cells do not divide as fast. Moreover, the total cell number decreased throughout the Activin A treatment on the flat surface, suggesting that the differentiation protocol did not support high proliferation of DE cells. The most likely explanation to such a fast repopulation is cell migration which is consistent with the literature describing the development of an embryo and *in vitro* DE differentiation. In the early development of an embryo the DE is generated during gastrulation where epiblast cells undergo EMT and delaminate from the epiblast epithelial layer. The cells break through the embryonic basement membrane and migrate through the primitive streak. During the migration the cells go through a mesendoderm transition state and subsequently become DE cells (Murry & Keller 2008; Thiery et al. 2009). The directed migration through the primitive streak is guided by cues from the extracellular matrix and soluble factors (Gilbert 2006). In the *in vitro* differentiation of hES cells towards DE, EMT and migration capabilities have also been reported (Tada et al. 2005). In our study we see directional migration in regards with mechanical cues, a process called durotaxis. Such process has previously been observed for mesenchymal stem cells which home to sites of injury and fibrosis (Discher et al. 2009), but to our knowledge, not in the development of the early embryo and neither in the *in vitro* differentiation of hES cells. This observation questions the current perception that directional cell migration in developmental biology is controlled by chemokines (Gilbert 2006). Perhaps the mechanical properties have an undiscovered role in the directional cell migration in development biology.

In our ECM study we found that cultures differentiated on collagen 1 had a distinct morphology, showed an earlier induction of DE cells and importantly gave purer DE cultures with very few Oct3/4 positive cells compared to the control cells cultured on fibronectin. Similarly, the increased number of DE cells on the soft nanopillars was due to a combination of factors, including surface dependent selective attachment, early and fast DE induction and migration of DE cells towards the nanopillars. Such similarity could raise the question whether the nanopillars and collagen 1 substrates target the same mechanism. In our studies we see migration capabilities of DE cells, both on the nanopillars and collagen 1 substrates. Further, both cultures gave an

early and fast induction of DE, indicating that both substrates may play an important role in escaping pluripotency and initiation of the DE formation. Further studies will be required to fully elucidate this phenomenon. Future studies could include a gene expression comparison of cultures differentiated on soft nanopillars and collagen 1 would clarify if the two substrates target the same mechanism.

Besides the cell culture media and the physical microenvironment, the cells themselves contribute to the microenvironment, in the form of cell-cell signalling, secretion of growth factors, chemokines and ECM proteins as well as assembly and remodelling of the ECM (Discher et al. 2009). In our study we found that the cell seeding density greatly affected the differentiation and that PE differentiation required high cell density. Despite the availability of soluble signalling molecules from the cell culture media, differentiation to PE was not possible with low cell seeding density. This indicated that the cells somehow contribute with essential factors to the microenvironment or that cell-cell contact is essential for the differentiation. With uneven seeding, PE could be generated in the centre of the well, where the initial seeding density was high. This suggests that the PE differentiation was affected by the local cell density, and hereby local environment, and similar studies have indeed demonstrated that the seeding density affects stem cell differentiation (Ghosh et al. 2010; Gage et al. 2013). It has been suggested that low seeding density arrests cells in the G2/M phase of the cell cycle, whereas cells in G1 phase allow cell to undergo differentiation (Gage et al. 2013). Investigation of cell cycle phases at different seeding densities was not performed in our study, but could be a subject for further investigation since the cell cycle is important for regulating differentiation. Another explanation could be that the cells create their own microenvironment with signals not provided in the cell culture medium and these signals might not present in high enough concentrations when the cell density is too low. A third possible explanation could be that signalling molecules from the cell culture medium only provide a starting point for cells to initiate differentiation when a critical density is reached. Such mechanism is observed in the embryonic development, where the embryo possesses a capacity to express a development potential only when a critical cell density of induced cells is present. This mechanism is called the community effect, previously described as mass effect or homotypic induction. This means that once a group of cells has been induced, autocrine and/or paracrine factors can sustain the induction and complete the differentiation (Gilbert 2006). Thus, cells density can provide biological information of the local niche and cell-cell contact.

Our results demonstrate that the physical environment significantly affects the stem cell differentiation and this provides another dimension to the traditional perception of stem cell biology. The ECM and mechanotransduction may play a larger role in stem cell differentiation and development biology than previous believed.

## 5.2 Development of stem cell differentiation protocols

The majority of published differentiation protocols focus on soluble molecules, including the two recently published protocols eluting to the differentiation of ES cells to beta-cells (Pagliuca et al. 2014; Rezania et al.

2014). However, it is still questioned if these protocols provide fully functional and mature beta-cells. Changing the physical environment might improve and further develop the directed differentiation protocols.

Our findings provided improvements to the DE differentiation protocols. By using collagen 1 as cell culture substrate, it was possible to obtain 84% Sox17 positive cells with a suboptimal DE differentiation protocol with 10ng/ml Activin A. Similar results could only be obtained with 100ng/ml Activin A on fibronectin substrates. Thus, by using collagen 1 substrate it is possible to reduce the use of the expensive growth factor Activin A by 10 fold. Such a reduction will be highly beneficial for many laboratories and in a large scale cell production to take the cells into the clinic. In this study, we used animal derived collagen 1 from rat tail, since it was the only collagen 1 available at the initiation of the study. Animal derived factors are not suitable for clinical application and GMP production. However, ECM proteins are highly conserved across species including collagen 1 (Hynes & Naba 2012) which indicates that results similar to ours can be obtained with recombinant rat or human collagen 1.

Our nanopillar studies demonstrated an early and fast DE induction on the soft nanopillars as well as migration of DE cells towards the soft nanopillars during the differentiation. These findings can potentially be used to develop a surface which can either purify DE cells or improve the DE differentiation. One suggestion is to have areas of flat surfaces, which allow hES cells to attach and proliferate, and surrounding surfaces with nanopillars, where the DE cells migrate onto (Figure 16). Such cell culture surfaces could easily be produced by injection moulding which is inexpensive, consistent and produce large quantities.

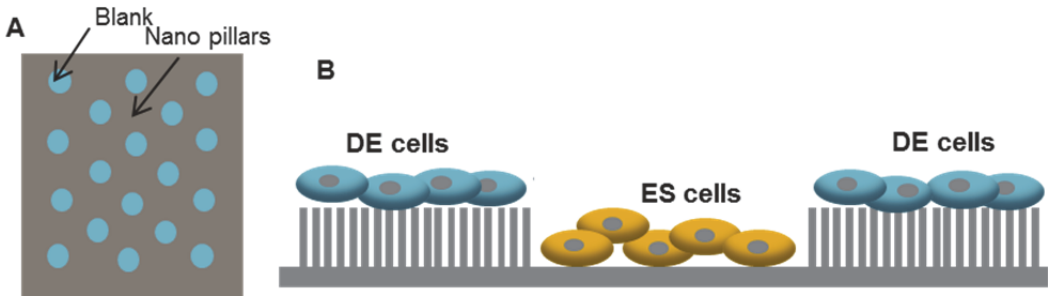


***Figure 16.*** *A potential substrate design to purify or induce DE differentiation. A) The surface showed from above. The substrate is filled with nanopillars expect for islets with flat surface. B) The surface showed from the side. hES cells will attached and proliferate on the area with a flat surface. When the DE differentiation is initiated, DE cells will migrate towards the pillared area of the surface.*

Further, our nanopillar study demonstrated that nanopillars with various spring constant could be used as model substrates with different stiffness. Previous studies have demonstrated that the stiffness of the cell culture substrate hydrogel greatly affected cellular behaviour, including stem cell differentiation (Engler et al. 2006). However, others have challenged Engler's study by suggesting that it was not the stiffness but other parameters which regulated the cell fate (Trappmann et al. 2012). We used a similar approach as Chen *et al.* with pillars to regulate the apparent stiffness of the cell culture substrate (Fu et al. 2010; Yang et al. 2011),

and were hereby able to regulate the apparent stiffness of the substrate. The nanopillar samples are manufactured with injection moulding, which is a highly reproducible and high content production method.

In published stem cell differentiation protocols, soluble molecules are added in different concentrations at different time points to mimic the stepwise embryonic development (Kumar et al. 2014). Likewise, the physical microenvironment changes during embryonic development (Bonnans et al. 2014), and hereby the different differentiation steps may require different physical environments. Our results with the nanopillars clearly demonstrate this as DE differentiated cells favoured the soft nanopillars, whereas the PE differentiation was repressed by the soft nanopillars. This may also account for ECM protein substrates, where previous studies have reported changes in integrin expression during the directed differentiation (Wong et al. 2010). Our results indicate that the ECM substrate/physical environment should be considered at the different step or differentiation direction.

The studies presented in this thesis focused on the first two differentiation steps, but they also provide a proof of principle that the physical microenvironment greatly influences stem cell differentiation. To obtain a successful differentiation towards beta-cells or other mature cells linages, manipulation of the physical microenvironment could be part of the solution. Moreover, culturing mature cells derived from hES cells as well as the subsequent transplant into patients requires the creation of specific microenvironments and here the physical environment may play an essential role.

For clinical application of cells it is essential to obtain large scale industrial differentiation of hES stem cells. For this purpose it would be beneficial to carry out the differentiation in stirring bioreactor tanks, similar to the tanks used in the field of vaccines and recombinant protein production, where cultivation of mammalian cell lines in up to 1000 L dimensions has thoroughly been established (Kehoe et al. 2009). However, such a technique cannot be directly transferred to large scale stem cell production and differentiation, since stem cell proliferation and differentiation require cell level parameters such as heterogeneity, endogenously produced factors, and the local physicochemical microenvironment (Kirouac & Zandstra 2008). Both the literature (Placzek et al. 2009) and our observations pointed out that the physical environment and cell-cell contact are important factors during stem cell differentiation, and these parameters must also be taken into consideration in developing large scale production in bioreactors. This could include the consideration of the size of the cell aggregates in the stirring bioreactors, incorporation of ECM proteins in the cell aggregates, using micro carrier beads for cells to attach and the mechanical stimuli in the bioreactors, i.e. sheer stress. Further, stirring bioreactors do not reflect the interplay between the physical environment and the soluble molecules. This include ECM dependent stability and signalling activity of growth factors, which could be solved by adding ECM proteins to the culture media or fusing growth factors with ECM proteins (Assal et al. 2013). This is beyond the scope of this thesis, but it is important to emphasize that the physical environment also must be incorporated in large scale differentiation of stem cells.

The results presented in this thesis suggest that the physical microenvironment should be taken into account in addition to the biochemical cues, when differentiating hES cells to specific cell linages. Simple modifications such as changing the ECM substrates or using a cell seeder device can improve the efficiency and consistency of directed differentiation protocols.

# 6. Conclusion

In this thesis we used three different strategies to manipulate the physical microenvironment to induce the differentiation of hES cells towards definitive endoderm and pancreatic endoderm. With the first strategy we demonstrated that certain ECM protein substrates could regulate the differentiation of hES cells towards definitive endoderm. Collagen 1, collagen 2 and netrin 1 induced definitive endoderm differentiation, whereas vitronectin and nidogen inhibited definitive endoderm induction. All our analyses pointed to collagen 1 having unique properties, as seen been by cultures on collagen 1 having distinct morphology, displayed a faster induction of definitive endoderm and importantly gave purer definitive endoderm cultures with very few pluripotent stem cells compared to the control substrate (fibronectin). With the second strategy we demonstrated that topographies in the form of soft nanopillars influence the differentiation of hES cells towards definitive endoderm and pancreatic endoderm. hES cells favoured the rigid flat surface, whereas the number of DE differentiated cell were significant higher on the elastic nanopillars and pancreatic endoderm induction was inhibited on the elastic nanopillars. With the third strategy we obtained a consistent and uniform cell seeding using a cell seeder device. Initial cell seeding density and cell distribution across the well largely influenced the differentiation towards pancreatic endoderm. Uniform cell seeding resulted in uniform pancreatic endoderm differentiation across the entire well. Such uniformity is important in reproducibility and for assays which includes material from the entire well. Thus, the physical microenvironment greatly influenced the directed differentiation of hES cells and such findings contribute both to the biological aspect and the protocol design within stem cell differentiation.

# 7. **Perspective**

The greatest learning during this 3 year PhD, is that we only scratched the surface of the subject of interest and a lot remains to be investigated. In this section, future possible studies will be described.

Our discovery that collagen 1 enhanced DE differentiation, will be interesting to further investigate both in a developmental perspective and stem cells perspective. To our knowledge collagen 1 has not previously been connected to gastrulation or early development. Future developmental studies should include an investigation of the presence of collagen 1 during gastrulation and, if possible, its role during gastrulation. Within the field of stem cell biology, it could be interesting to further study the pathways and genes identified in the global gene expression analysis. First, the regulation of these genes must be confirmed by qPCR. Further, it could be interesting to investigate the expression on protein level as well as manipulation of the pathways/gene of interest by looking at phenotypes after upregulation, or repression of the expression of selected genes using knockdown or inhibitors. The global gene analysis was performed on the last day of differentiation. However we made some interesting observations the day after Wnt3a treatment and it could be exiting to do a global gene analysis at this time point and subsequent biological studies as described above. Moreover, a study of the cells prior to the onset of differentiation will provide information about similarities of the cells on the different substrates and if the cells on collagen 1 substrate may be primed for the differentiation on collagen 1 substrate.

Our gene expressions analysis showed that the focal adhesion pathway was significantly upregulated in cultures differentiated on collagen 1 substrate. One hypothesis could be that collagen 1 affects the focal adhesion and hereby EMT. It could be interesting to look further into EMT on collagen 1 substrates, including regulation of EMT proteins and cell migration capabilities. Further studies of focal adhesions are also a possible direction. Using morphology methods and immunocytochemistry staining for cytoskeleton fibers and focal adhesion might be challenging due to the tight cell clustering that hES requires. Another approach could be to look at the collagen1 cell interactions. Various cell receptors bind to collagen 1 including different integrin and DDRs (Hynes & Naba 2012). Studies could include an investigation of the expression of the different collagen 1 receptors and manipulation of the receptor-collagen 1interaction by blocking antibodies blocking (receptors (Ahmed et al. 2005) and up or downregulation of receptors.

Serval similar studies as described above could be performed on cells differentiated on nanopillars. Some studies may require a new design of the brick with the different nanopillar areas. Our results indicated that cells migrated onto the pillars after one day of Activin A treatment. Migration studies with time-lapse microscopy using a Sox17-GFP hES cell line, will provide direct evidence for migration of these cells. Further migration studies could also give knowledge about whether the migration is directed by mechanical cues, durotaxis. Moreover, the very early appearance of Sox17 positive cells on the tall nanopillars could be subject for further studies. One direction could be to investigate if the tall nanopillars induce the TGF-β pathway.

Beside further investigation of our observations, our findings could be used to develop high efficient differentiation protocols for industrial application or look into later differentiation steps towards beta-cells. Further levels of complexity can also be added, e.g. investigating the interactions between ECM and growth factors.

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# Paper 1

## Collagen type I improves the differentiation of human embryonic stem cells towards definitive endoderm

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Published: 29 December 2015, PLOS ONE

DOI:10.1371/journal.pone.0145389

Link: http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0145389

### Abstract

Human embryonic stem cells have the ability to generate all cell types in the body and can potentially provide an unlimited source of cells for cell replacement therapy to treat degenerative diseases such as diabetes. Current differentiation protocols of human embryonic stem cells towards insulin producing beta cells focus on soluble molecules whereas the impact of cell-matrix interactions has been mainly unattended. In this study almost 500 different extracellular matrix protein combinations were screened to systemically identify extracellular matrix proteins that influence differentiation of human embryonic stem cells to the definitive endoderm lineage. The percentage of definitive endoderm cells after differentiation on collagen I and fibronectin was >85% and 65%, respectively. The cells on collagen I substrates displayed different morphology and gene expression during differentiation as assessed by time lapse studies compared to cells on the other tested substrates. Global gene expression analysis showed that cells differentiated on collagen I were largely similar to cells on fibronectin after completed differentiation. Collectively, the data suggest that collagen I induces a more rapid and consistent differentiation of stem cells to definitive endoderm. The results shed light on the importance of extracellular matrix proteins for differentiation and also points to a cost effective and easy method to improve differentiation.

# Paper 2

## Enhanced differentiation of human embryonic stem cells toward definitive endoderm on ultrahigh aspect ratio nanopillars

Camilla Holzmann Rasmussen, Paul M. Reynolds, Dorthe Roenn Petersen, Mattias Hansson, Robert M. McMeeking, Martin Dufva, and Nikolaj Gadegaard*

First published: 15 December 2015
DOI: 10.1002/adfm.201504204
Link: http://onlinelibrary.wiley.com/doi/10.1002/adfm.201504204/full

### Abstract

Differentiation of human embryonic stem cells is widely studied as a potential unlimited source for cell replacement therapy to treat degenerative diseases such as diabetes. The directed differentiation of human embryonic stem cells relies mainly on soluble factors. Although, some studies have highlighted that the properties of the physical environment, such as substrate stiffness, affect cellular behavior. Here, mass-produced, injection molded polycarbonate nanopillars are presented, where the surface mechanical properties, i.e., stiffness, can be controlled by the geometric design of the ultrahigh aspect ratio nanopillars (stiffness can be reduced by 25.0003). It is found that tall nanopillars, yielding softer surfaces, significantly enhance the induction of definitive endoderm cells from pluripotent human embryonic stem cells, resulting in more consistent differentiation of a pure population compared to planar control. By contrast, further differentiation toward the pancreatic -endoderm is less successful on “soft” pillars when compared to “stiff” pillars or control, indicating differential cues during the different stages of differentiation. To accompany the mechanical properties of the nanopillars, the concept of surface shear modulus is introduced to describe the characteristics of engineered elastic surfaces through micro or nanopatterning. This provides a framework whereby comparisons can be drawn between such materials and bulk elastomeric materials.

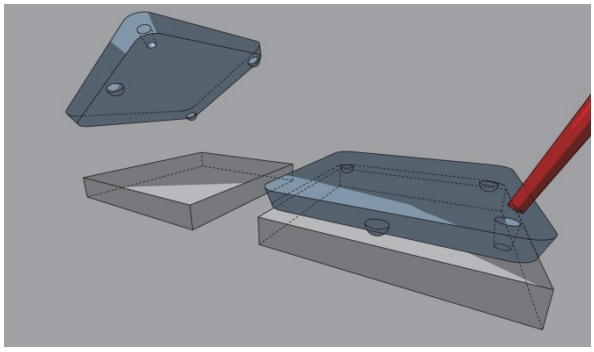
# Paper 3

## The impact of cell seeding methods on density dependent differentiation

Paul Reynolds[1], Camilla Holzmann Rasmussen[2,3], Dorthe Roenn Petersen[2], Mathias Hansson[2], Martin Dufva[3] and Nikolaj Gadegaard[1]*

### Abstract

Cell seeding density plays a critical role in the outcome of cell culture experiments. Standard seeding methods in large culture vessels (12 & 6 well plates, culture flasks etc) do not provide a homogeneous distribution of cells on the culture surface, as the macroscopic flow of culture media redistributed the cell population around the well. Commonly users find cells aggregated around the circumference, claustered in the centre, or some combination of the two. These local variations in cell density may affect the global outcome of an experiment – hampering accuracy and subsequent reproducibility. We present a simple methodology by which the uniformity of cell seeding across large culture areas can be drastically improved. By containing the cell seeding suspension in a defined space above the culture substrate, local fluctuations in cell density are minimised. Seeding efficiency, as defined by the homogeneity of intercellular spacing, was improved by a factor of 10. We go on to demonstrate the impact of local variation in cell density on the lineage commitment of human embryonic stem (hES) cells towards pancreatic endoderm (PE) – with variations in the differentiation profile of cells across a culture well closely mirroring variations in cell density introduced by seeding method.



### Introduction

Every biological experiment starts with cell being seeded. This is a process which to many seem trivial, yet there are procedures to ensure regularity/uniformity between experiments and experimenters. It has often been seen that there is a strong user dependence. Biological experiments involving cell culture, from biomaterial testing [1–4] to drug discovery[5,6] often begin with cells being seeded onto a flat substrate to form a two-dimensional culture. This is the foundation on which the experiment as a whole is built, and is therefore arguably one of the most critical steps. Common practice for this crucial step often varies between disciplines, laboratory's, individual researchers, and even from day to day. Suggested protocols exist, however these recommended methods by no means eliminate user dependence[7]. Such user dependence can in turn introduce huge variability to the results and mean others struggle to reproduce the work. There is a growing concern in the scientific community regarding the reproducibility of high profile work[8,9], and as such a method for the standardisation and deskilling of a key part of cell culture studies is of critical importance. This compliments published methods for statistical analysis and data presentation, towards a more consistent experimental process across cell biology studies. The manner in which almost all researchers seed cells

onto a flat surface can be roughly categorised into two methods (figure 1a and b). Either a droplet of fluid is placed on the substrate, and the vessel back filled with culture media after a period of attachment, or the substrate is immersed in culture media, with a concentrated cell suspension then pipetted over the substrate in as even a manner as possible. The literature also suffers from confusion stemming from interchangable use of seeding densities quoted in either $ml^{-1}$ or $cm^{-2}$.

Uneven seeding arises within these methods due to three factors – the macro scale turbulent flow of cell seeding suspension as it is added to the well, disturbing the cell suspension as plates are moved to the incubator, and also to a lesser extent the meniscus which forms around the wall of the culture plate. The problems associated with uneven cell seeding are aparent in the volume of forum posts (e.g. at researchgate.net) from students looking for help in improving their seeding, after struggling with variability. Studies investigating the ptimal means of cell seeding have sought to identify the source of uneven cell distribution[10].

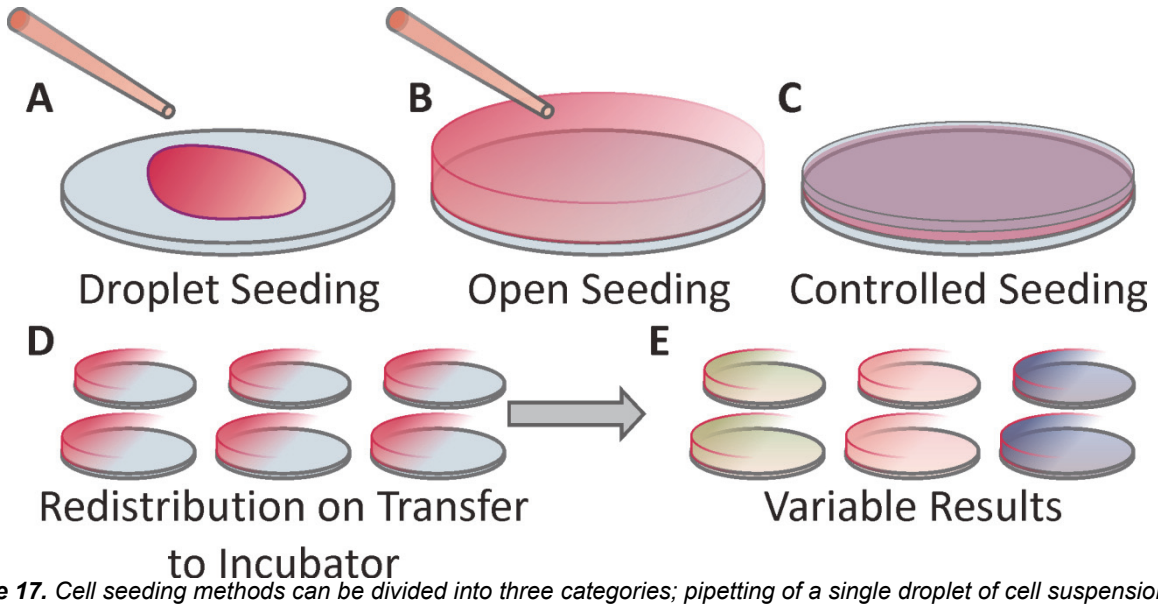



***Figure 17.*** *Cell seeding methods can be divided into three categories; pipetting of a single droplet of cell suspension onto the surface (A), filling the culture vessel with media and pipetting a concentrated cell suspension into the fluid (B), and the method presented here – a rigid surface placed 1mm above the surface which confines the cell seeding volume in a defined volume across the full sample (C). Movement to the incubator for culture (D) represents a critical step, whereby redistribution of unattached cells due to flow within the well causes uneven distribution, generally in the centre of the well due to centrifugal flow. Final analysis of results (E) is therefore affected by this uneven distribution in the plate – leading to increased experimental error.*

An uneven seeding will create local cell densities across a well or an assay, figure 2. Several studies have shown that the cell density effect cellular behaviour due to cell-cell communication, secretion of signilalling factors, cell shape and mechanically properties [11]. The commitment and differentiation of stem cells are highly regulated by the cell density. One interesting study demonstrates that the initial cell seeding density

influences the differentiation of hES towards pancreatic endocrine cells [12]. Similar studies show that the seeding density is important for stem cell differentiation [13,14].

Issues with uneven seeding arise in the case of array and screening platforms, whereby a disparity in cell density may introduce noise and variability – leaving the assays open to errors [16,17,12,18]. Whilst not reported in the literature, uneven seeding may skew results when conducting biomolecular assays across an entire well, including measures in supernantant, cell lysate and DN/RNA, *Figure 18.*[19]

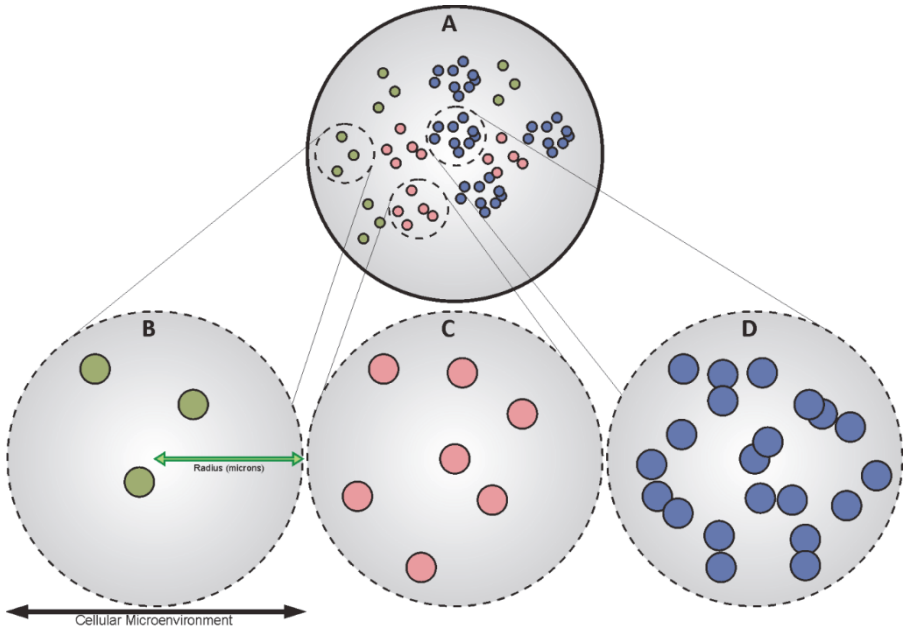



***Figure 18.*** *Local cell densities influences cell behaviour through cell-cell communication - both chemical and mechanical. Frequently, studies investigate the modulation of cell behaviour by modulating small microenvironments. We present a technique which allows the precise homogeneous control of the cell microenvironment across millimetre length scales – yielding improvements in attachment efficiency, differentiation potential and final experimental outcomes. Local density has been shown to influence the lineage commitment of various stem cell types, for example three distinct seeding densities may exist in a single well due to uneven seeding distribution.*

Here we present a device which improves the quality of cell seeding on a flat surface by an order of magnitude, spreading the cells in a consistent and uniform manner which is not easily reproducible with currently used techniques and reducing the likelihood that local variations in cell density yield local variations in cell response, and therefore poor results. With the device, an even and consistent distribution of cells across individually experiments was obtained for human fibroblast cells, human embryonic stem (hES) cells and definitive endoderm (DE) cells derived from hES cells. A uniform seeding of DE cells resulted in a more uniform differentiation towards PE across the entire well (12-well plate). Such uniformity is important in reproducibility and for assays which includes material from the entire well.

## Results

### Comparison of the three seeding tehniquies

To compare the seeding device against the current used seeding tehniquies we seeded human fibroblast cells with the three different seeding technuqes (Figure 1). Our results demonastrated a widespread variation in homogeneity, both between methods and also between users. Droplet seeding created a dome of fluid which is higher in the centre than at the edges – as cells settled out of suspension the greater volume in the centre resulted in a greater concentration in the centre (Figure 3). In the case of the three samples shown in Figure 3, a single sample had both sparsely populated and fully confluent regions within 6 h of seeding. Similarly, using the open seeding method did not distribute cells evenly. In this case, where the substrate was submerged in culture media and a concentrated seeding suspension was pipetted onto it, the location of droplets influences density across the substrate. The controlled seeding with the device used capillary force to hold the cell suspension 1 mm above the substrate across the whole substrate area. Using this techqnique resulted in even seeding across the substrate and with low expermital vatiation (Figure 3).

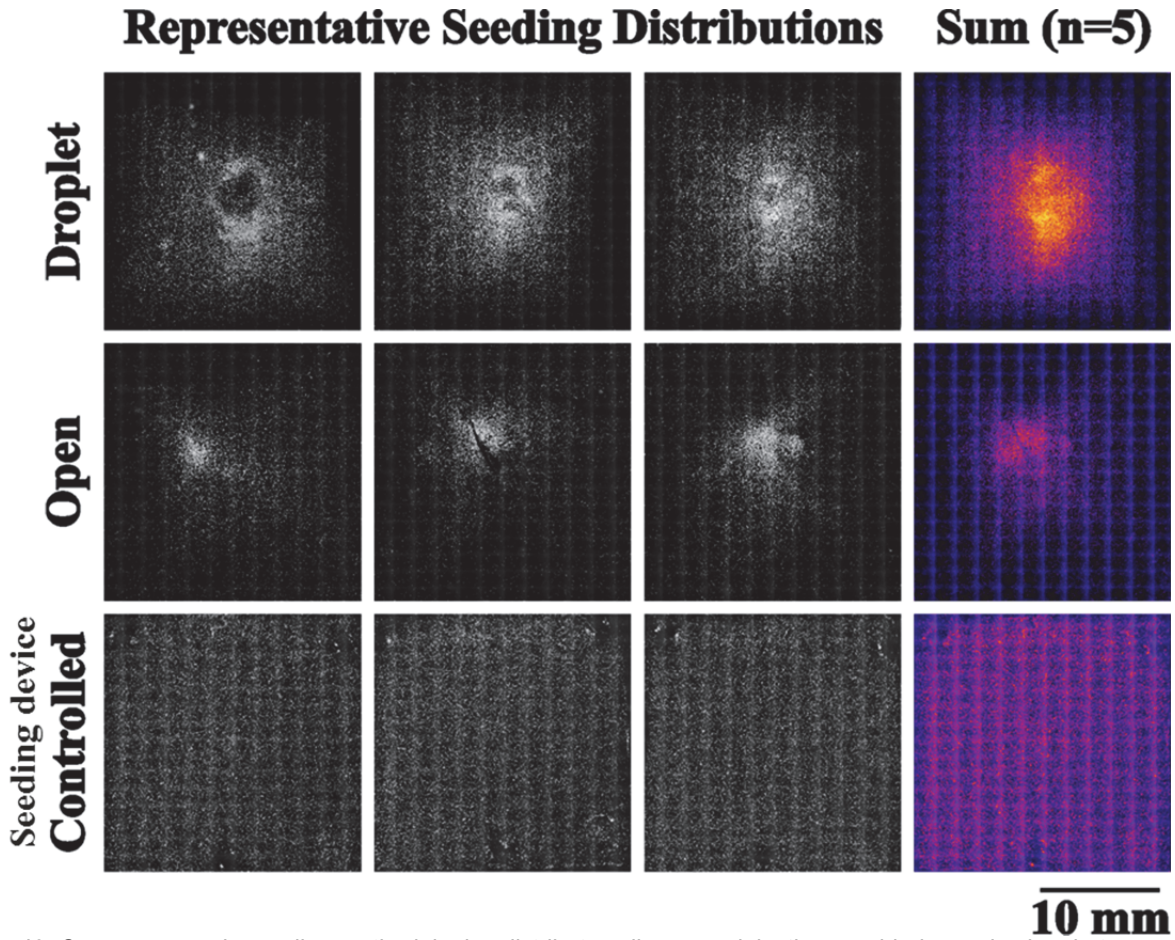



***Figure 19.*** *Common sample seeding methodologies distribute cells across injection moulded samples in a heterogeneous manner which is undesirable. Controlled seeding using the seeder device results in a drastic improvement in the uniformity of cell distribution on the substrate – as evidenced by the lack of cell density 'hot spots' in summed images of 10 samples (rightmost images).*

**Seeding of human embryonic stem cells**

To test whether the controlled seeding with the seeding device could be obtained with other cell lines, hES cells were seeded with seeder device or by open seeding at different cell seeding densities, We obtained similar results as with the fibroblast cells. Using the seeder device we were able to obtained much more uniform seeding compared to the open seeding at all tested seeding densities (10.000-40.000 cells/cm$^2$) (Figure 4).

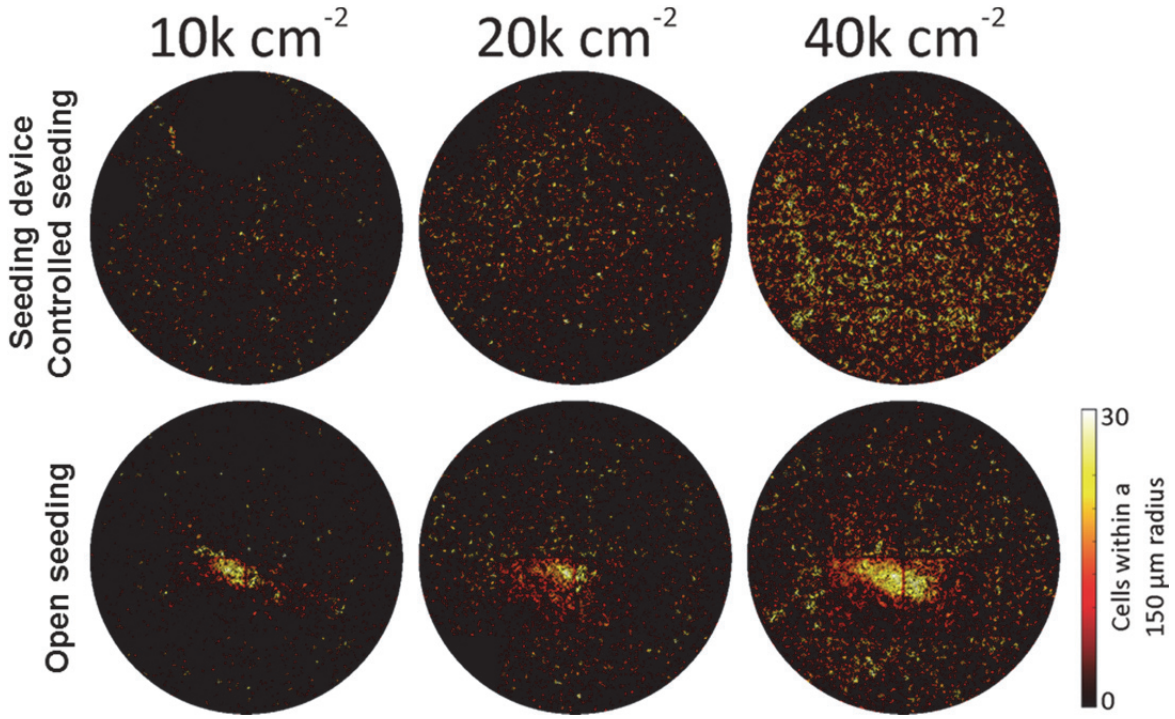



***Figure 20.*** *The Use of the seeding device leads to a marked improvement in the uniformity of cell seeding. Full culture wells were imaged, and images processed to calculate the number of nearby cells for any given cell. Cells were then colour coded to show the local density, where differences in colour indicate a difference in local density. The uniformity of local density is markedly better when the seeding device is used. Seeder = seeding with the seeding device. 10K, 20K and 40K represent the seeding densities; 10K, 20K and 40K cells per cm$^2$.*

To quantify our observations we defined the accuracy of a given cell seeding technique in terms of the consistency of the local microenvironment across a 12 well plate (20 mm diameter, 3.14cm$^2$). Defining this as the number of cells within a radius of 150 µm, large image arrays were captured across multiple wells and culture conditions to understand the seeding distribution resulting from each method. Controlled seeding carried out using the cell seeder device provided more consistent seeding in terms of the local microenvironment surrounding each cell – with a significant reduction in the standard deviation of local cell numbers (Figure 5).

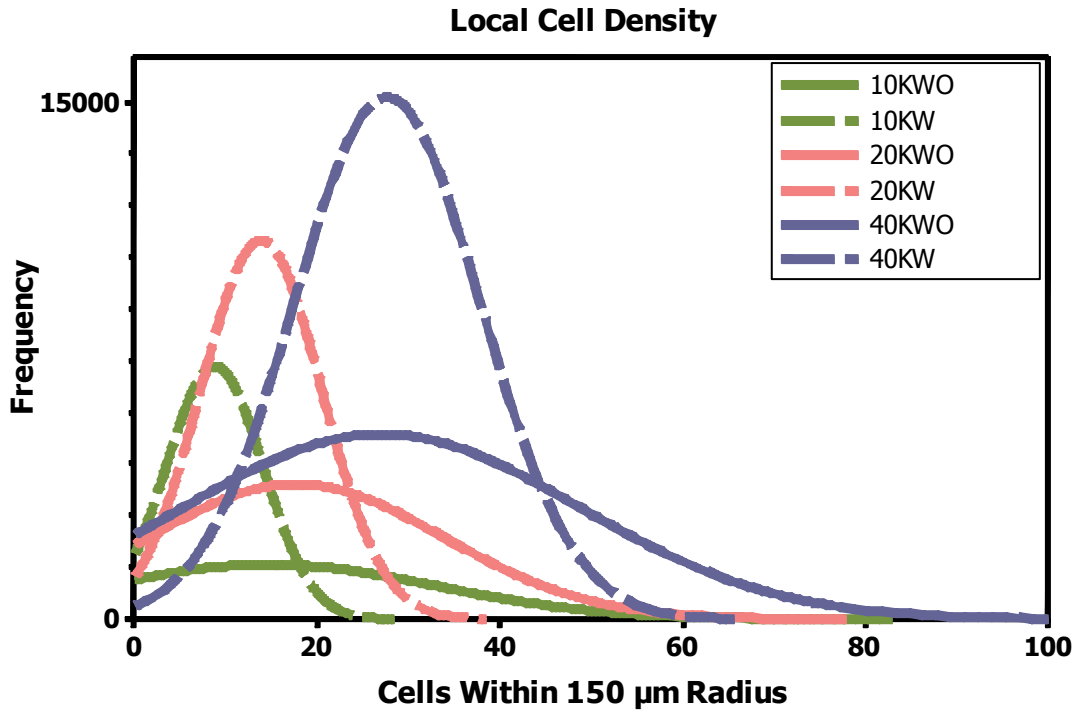



***Figure 21.*** *Cells can be more accurately seeded using the seeding device when compared to standard seeding in open wells. Sharper peaks (dotted lines) show a more consistent number of cells within a 150 µm across the full culture well as compared with standard seeding methods. 10K, 20K and 40K represent the seeding desinity (10K, 20K and 40K cells per cm$^2$). W = with the cell seeder (controlled seeding), WO= without the cell seeder (open seeding).*

**Pancreatic endoderm differentiation**

The resulted described above demonstrated a uniform and consistent seeding could be obtained using the seeding device. To test if a uniform cell seeding effect the differention, we seeded definitive endoderms with and without the cell seeder and with different seeding densities. The outcome of the PE differentiation depended on the seeding method and seeding density. With low seeding density (100K cells per cm$^2$), Pdx1 and Nkx6.1 double positive cells (mature PE cells) were only observed in the centre of the well, when using open seeding. Thus, differentiation into PE cells was only possible in areas with high cell density. With intermediate seeding density (200K cells per cm$^2$) it was possible to obtain Pdx1 and Nkx6.1 double positive cells both with open seeding and controlled seeding. However, when using the controlled seeding technique, a more uniform distribution of clusters of Pdx1 and Nkx6.1 double positive cells across the entire well was obtained, compared to using open seeding. With high seeding density (300K cells per cm$^2$) the differences in the seeding was lees pronounced with the two different seeding techniques.

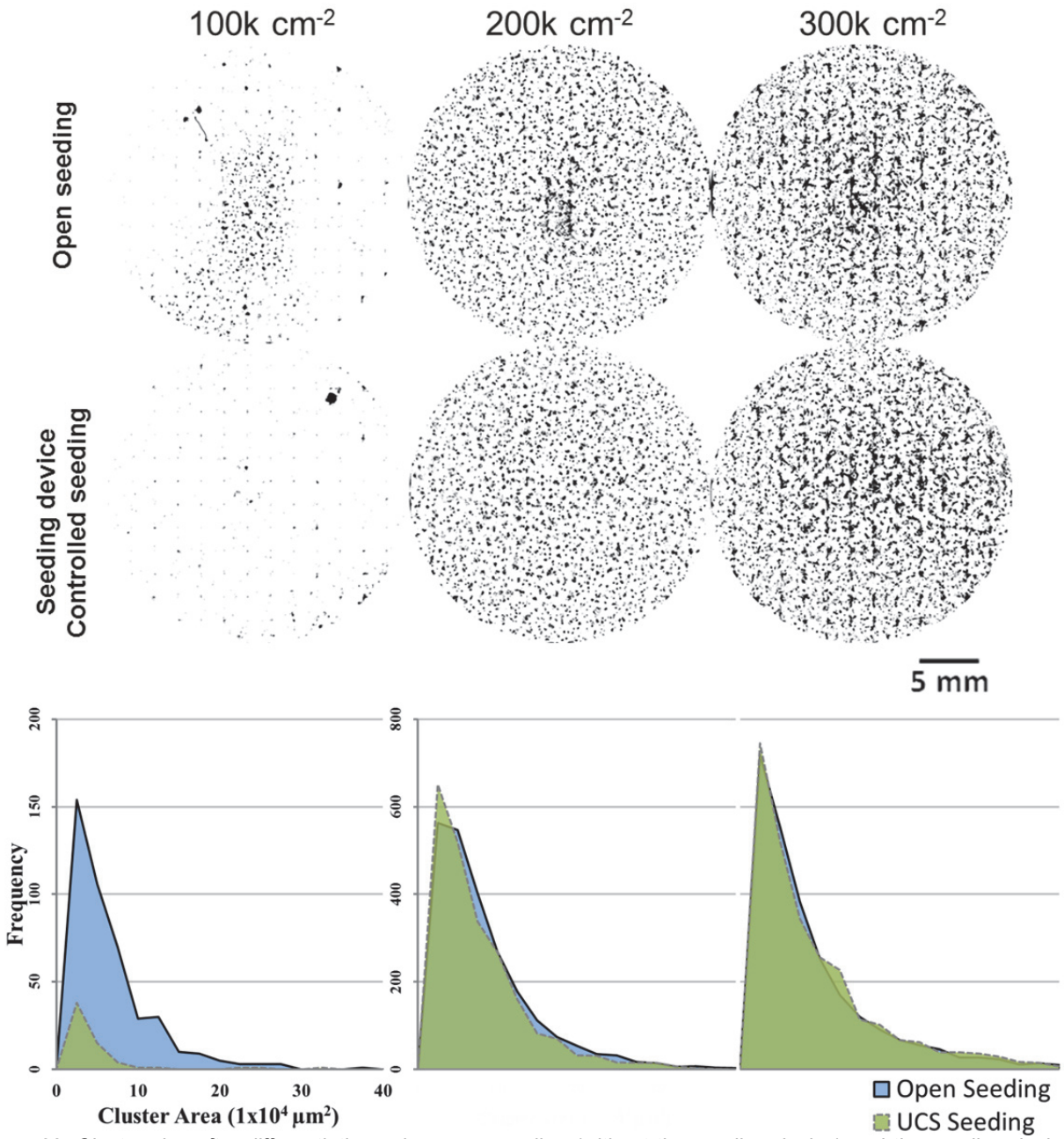



***Figure 22.*** *Cluster size after differentiation using open seeding (without the seeding device) and the seeding device to control cell seeding. Full well montages (top) show the inhomogeneous distribution of Nkx6.1 positive cells at low seeding densities, which is less pronounced at higher densities. Seeding artefacts are visible in the well centre for both seeding methods. Histograms of Nkx6.1 positive colony size (bottom) show a drastic reduction in differentiation efficiency using the UCS, whereas both seeding methods are comparable for higher seeding densities.*

## Conclusion

The UCS described here addresses a fundamental problem in the use of high-content screening studies (i.e. array and gradient platforms) and thereby ensuring that upon seeding, cells are distributed across the sample in a homogeneous manner. This allows us to 'trust' that changes in cell density and behaviour are the result of surface driven effects, rather than spurious seeding artefacts.

A quantifiable change in the quality of cell seeding has been demonstrated, potentially providing a means of homogenising seeding protocols across users, experiments and samples. The variation in seeded cell density on injection moulded array samples, and also in multiwell plates, has been demonstrated as flawed in multiple users work. Reducing the standard deviation of local cell density by a factor of almost 5 on injection moulded samples allows for increased confidence in experimental results. This has led to a direct improvement in the reliability of studies on gradient substrates fabricated in this thesis, and represents a potentially valuable tool for other high-content screening systems moving forward.

Furthermore, experiments were undertaken to elucidate the possible effect of cell seeding uniformity on the differentiation of human embryonic stem cells towards pancreatic endoderm. Initial results indicated that the differentiation profile within 12-well plates depends on the seeding method. Variation in density across the surface of the well led to a variation in positive differentiation of hES cells. Work is ongoing to confirm the quantitative change in differentiation yield using standard seeding approaches and the seeding device.

## Methods

### Production of the seeder device

UCS devices were injection moulded in polycarbonate, as a square of plastic with three feet which create a constant 1 mm gap between the surface and the UCS. An injection hole was included to allow cell suspension to be added. A custom injection moulding insert was milled from aluminium on a CNC machine, as well as individual UCS devices being machined from polycarbonate sheets.

### Fibroblast cells

Quantification and comparison of seeding methods was carried out using human fibroblast cells (hTERT-BJ1). Cells were suspended in DMEM media at a concentration of 500,000 cells per ml. Cells Cells were seeded onto planar tissue culture surfaces at three different seeding densities by three methods;

I. Droplet seeding, whereby a concentrated droplet of cell suspension is placed in the centre of the well. The cells were allowed to attach at room temperature for 1 – 2 hours before the well was backfilled with media. [Figure 1A]
II. Open seeding, whereby the well was filled with 2ml of culture media, and a concentrated cell suspension pipetted into the media. [Figure 1B]
III. Controlled seeding, by placing a UCS device in the well, and filling the fluid cavity with cell suspension through a filling hole. [Figure 1C]

Substrates were left stationary for 10 min, before being moved to an incubator for a further 50 min. They were then fixed using 4% paraformaldehyde at 37C for 10 min, and stained with Coomassie blue for 5 mins before washing 3 times in 1X PBS. Inspection revealed that in this timeframe, cells had been given enough time to attach to the surface without fully spreading or beginning to migrate. Fixing at this time point gives a realistic snapshot of cell distribution on the surface after seeding. Array images were acquired using an

Olympus CX41 upright microscope equipped with a Prior motorized stage and 10x objective. Automated scanning and acquisition across the surface was driven by ImageProPlus (Media Cybernetics, UK) to capture a contiguous array of at least 5 separate samples for all cell seeding methods outlined above. These images were analysed using the CellProfiler software suite (Broad Institute, Harvard, USA) to automatically detect and count cells in each frame.

**hES cells pancreatic endoderm differentiaon**

The human embryonic stem (hES) cells (SA121, Takara Bio Europe AB) were cultured in the feeder-free and defined DEF-CS™ 500 system according to instruction from the supplier (Cellartis AB). The hES cells were cultured and differentiated in a humidified incubator with 5% $CO_2$.

For the differentiation towards pancreatic endoderm the following protocol was used. The cells were first differentiated to definitive endoderm in fibronectin coated cell culture flask using a highly effective patented protocol (WO 2012175633 A1). Subsequently, the cells was rinsed with PBS and dissociated to single cell suspensions with TrypLE Select at room temperature. Seeding of the cells was performed with 100.000-300.000 cells/$cm^2$ in basal media with 100 ng/ml Activin A and 5μM RockI. The cells was differentiated towards pancreatic endoderm with a 13 days published protocol with RPMI medium containing 64ng/ml FGF2 (Peprotech), 12% knockout serum replacement (Gibco) and 0.1% pen strep [21]. Media was changed daily.

The cells were seeded in 12 well plates with differentiation medium with 2% B27 at 3 different seeding densities by the following 2 method II and III described aboved. With open seeding (method II), the wells were filed with total volume of 2ml. With the controlled seeding (III) the UCS device was removed 1.5-2 hours after seeding and media was added to a total volume of 2ml per well. The pancreatic endoderm differentiation were initiated the day after reseeding

The cells were fixed by washing once with phosphate buffered saline (PBS) (Invitrogen and adding 4% formaldehyde (Lilly's fixative, Mallinsckridt Baker) for 20 minutes. The cells were rinsed 2 times with PBS and permebilzed in 0.5% TritronX-100 (Sigma Aldrich??) in PBS. The cells was washed in PBS and blocked with TNB blocking buffer (0.1M tris-HCL pH 7.5, 0.15M NaCl and 0.5% Blocking reagent from Perkin Almer TSA kit) for 30 minutes. Primary antibodies were diluted in in 0.1% TritronX-100 in PBS and applied to the cells, followed by incubation overnight at 4°C. The following primary antibodies were used: goat polyclonal anti Sox17 (1:1000) (R&D Systems, AF1924), mouse polyclonal anti Oct3/4 (1:500) (Santa Cruz, sc, 5279), goat polyclonal anti Pdx1 (1:8000) (Abcam,, #ab47383) and mouse anti Nkx6.1 (1:500) (in house facility, F55A10). Cells were washed 3 times with PBS for 5 minutes. The secondary antibodies Alexa Fluor 594 conjugated donkey-anti-mouse IgG (Invitrogen) and Alexa Fluor 488 donkey-anti-goat IgG (Invitrogen) were added in a 1:1000 dilution together with DAPI (1:2000) (Sigma- Aldrich) in 1% TritronX-100 in PBS for 45 minutes at room temperature. The cells were rinsed 3 times with PBS for 5 minutes.

**Image acquisition and qantification**

Images were acquired using either an Olympus Olympus CX41 upright microscope equipped with a Prior motorized stage and 10× objective, operated by ImageProPlus (Media Cybernetics, UK), or using a GE InCell analyser, also equipped with a 10x objective. Contiguous arrays were captured across the full extent of each plate well, including the curved wall which was later cropped from the image to leave only the complete culture area for analysis. Images were analysed using CellProfiler (Broad Institute, Harvard) to detect individual cells using the nuclear stains. After detection of individual cells, their centroids were computed and the nearest neighbour and number of neighbour measurements computed. Processing of 1,000 2 megapixel images takes approximately 12h using CellProfiler 2.0 on an Intel Core i7 2600 CPU @ 2.4 GHz with 16Gb DDR2 RAM. The processing pipeline used in this work has been made available online at www.cellprofiler.org/published_pipelines.shtml. Representative images of cells coloured by metric data were generated by CellProfiler, and modified in ImageJ.

**Statistical analysis**

All values were presented as mean ±S.E.M. Data are presented for experiments performed at least as 3 independent experiments. For statistical analysis, paired–test was used and a P-value of 0.05 was considered statistically significant.

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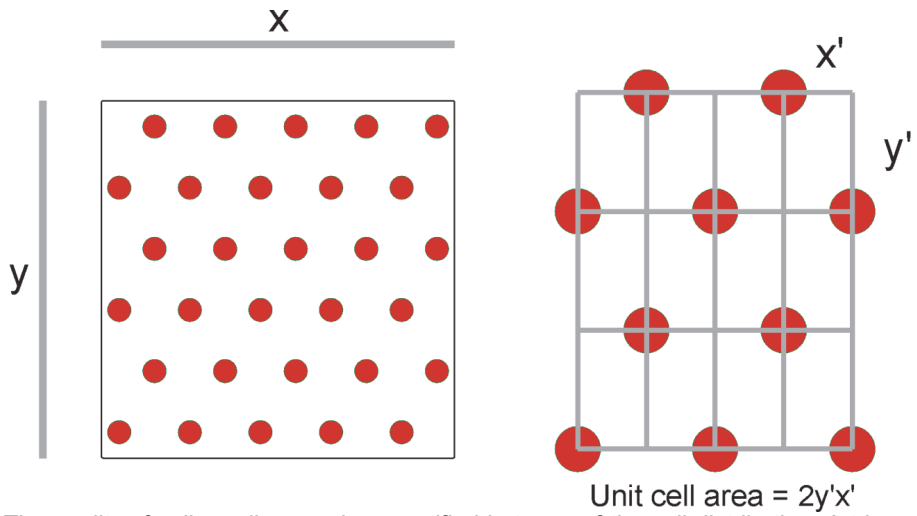
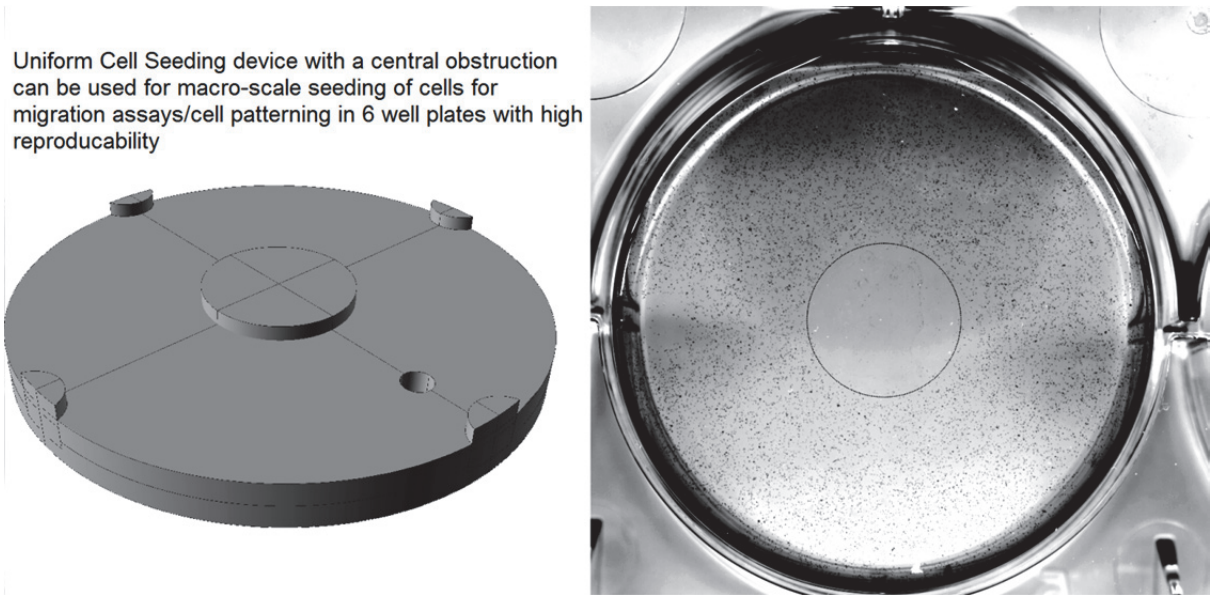
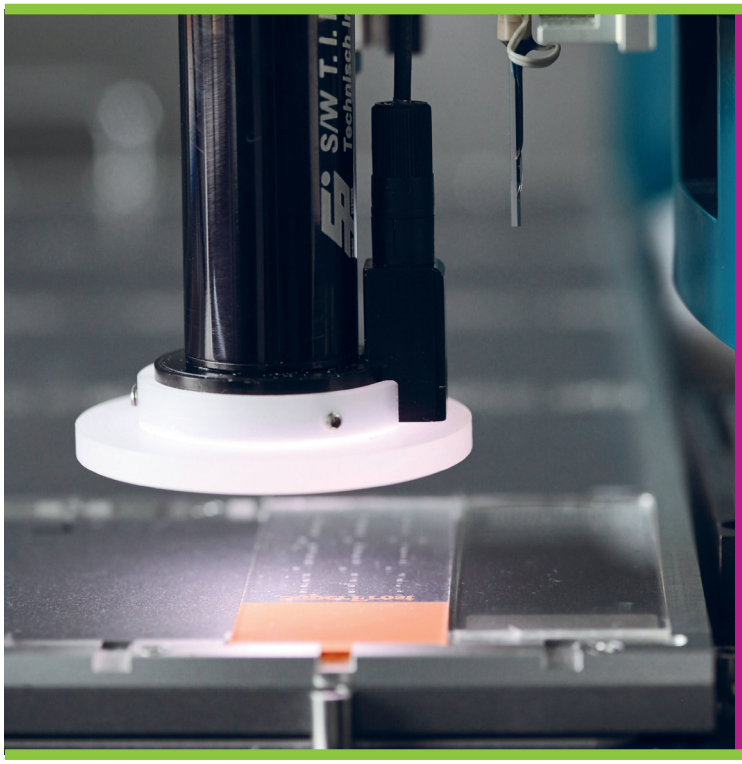
## Supplementary Section




***Suppl. figure 1**. The quality of cell seeding can be quantified in terms of the cell distribution. A triangular array is taken as the ideal seeding result, with a constant cell-cell spacing and therefore a deviation of 0.*




***Suppl. figure 2.** The seeding device can also include a barrier region to allow the shaped seeding of cells. This has applications in wound healing assays and other migration based assays*





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