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MARTIN POOK

Studies on artificial and extracellular matrix protein-rich surfaces as regulators of cell growth and differentiation

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Studies on artificial and extracellular matrix protein-rich surfaces as regulators of cell growth and differentiation

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Department of Cell Biology, Institute of Molecular and Cell Biology,
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LIST OF ORIGINAL PUBLICATIONS

This thesis includes the following publications, which will be referred to in the text by their Roman numerals:

I. Reemann, P., Kangur, T., Pook, M., Paalo, M., Nurmis, L., Kink, I., Porosaar, O., Kingo, K., Vasar, E., Kõks, S., Jaks, V., and Järvekülg, M. (2013). Fibroblast growth on micro- and nanopatterned surfaces prepared by a novel sol-gel phase separation method. J. Mater. Sci. Mater. Med. *24*, 783–792.

II. Siimon, K., Reemann, P., Põder, A., Pook, M., Kangur, T., Kingo, K., Jaks, V., Mäeorg, U., and Järvekülg, M. (2014). Effect of glucose content on thermally cross-linked fibrous gelatin scaffolds for tissue engineering. Mater. Sci. Eng. C. Mater. Biol. Appl. *42*, 538–545.

III. Pook, M., Tamming, L., Padari, K., Tiido, T., Maimets, T., Patarroyo, M., Juronen, E., Jaks, V., and Ingerpuu, S. (2014). Platelets store laminins 411/421 and 511/521 in compartments distinct from α- or dense granules and secrete these proteins via microvesicles. J. Thromb. Haemost. *12*, 519–527.

IV. Pook M., Teino I., Maimets T., Ingerpuu S., and Jaks V. (2015). Changes in laminin expression pattern during early differentiation of human embryonic stem cells. Manuscript.

My contribution to these articles:

Ref. I – contributed to the conceptual and experimental design; participated in immunofluorescence analysis and in preparation of the manuscript

Ref. II – contributed to the conceptual and experimental design; participated in immunofluorescence analysis and in preparation of the manuscript

Ref. III – contributed to the experimental design and data analysis; performed immunofluorescence analysis and electron microscopy analysis, participated in writing of the article

Ref. IV – contributed to the experimental design and data analysis; performed immunofluorescence and western blot analyses; participated in writing of the article

LIST OF ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
DMSO	dimethyl sulfoxide
ECM	extracellular matix
FACIT	fibril-associated collagens with interrupted triple-helices
FTIR	Fourier transform infrared spectroscopy
hESC	human embryonic stem cells
HSPG	heparan sulfate proteoglycan
IP	immunoprecipitation
LM	laminin
MEF	mouse embryonic fibroblasts
mESC	mouse embryonic stem cells
OCT4	octamer-binding transcription factor 4
PDGF-AA	platelet-derived growth factor with two A chains
TSP-1	thrombospondin-1
VWF	von Willebrand factor

1. INTRODUCTION

Animal cells, whether growing *in vivo* or *in vitro,* interact extensively with their surrounding environment. These interactions determine many key aspects of cell fate. Signals originating from the extracellular space can induce controlled cell death, or guide their differentiation and migration. The extracellular matrix (ECM) is usually the main component of the intimate surrounding of a cell. *In vivo*, the ECM is a very complex structure that consists of large collection of different components. By contrast, the complexity of the ECM produced by cells *in vitro* is significantly lower. Artificial ECM can be manufactured with synthetic materials or purified native ECM components. In fact, even when cells are grown on artificial scaffolding, both synthetic and naturally produced components are typically found in the matrix as many cells secrete native ECM proteins themselves. Using complex 3D environments composed of synthetic and native materials as scaffolds for cell growth *in vitro* has enabled researchers to mimic the native ECM environment more precisely and given them an opportunity to use such culturing techniques in regenerative medicine. For this practical application, studies characterizing the pathways by which different surfaces and ECM components modulate cell behavior are of utmost value.

The present thesis is focused on characterization of the interactions between the ECM and cells. This includes analyses of cell growth modulation by topographical changes in silica-based surface structure or by electrospun scaffolds containing gelatin and glucose. In the first study, it was established that senescence in primary dermal fibroblasts is induced not only by impaired attachment to a growth surface but also by growth on large round structural features. The second study demonstrated that gelatin-based scaffolds containing glucose are suitable for growth of primary dermal fibroblasts, and could be potentially used in tissue engineering. In addition, expression of ECM proteins belonging to the laminin family was characterized. Specifically, the analysis focused on laminins containing the α5 chain, as they have been reported to possess the ability to support stem cell growth. It was found that gelatin-based scaffolds can induce low-level expression of the laminin α5 chain in primary dermal fibroblasts. As blood platelets also contain laminins with the α5 chain and play important roles during wound healing, we were interested in studying storage and secretion of these laminins by platelets. Our investigation revealed that platelets do not store laminins 411/421 and 511/521 in typical alpha granules, and that these proteins are secreted via microvesicles but not via exosomes when platelets become activated. Finally, we elucidated the role of α5 chain laminins during early differentiation of human embryonic stem cells. We observed that the relative amount of laminin-511 increases while the amount of laminin-521 decreases during early differentiation induced by retinoic acid. Additionally, we found that human embryonic stem cells express a wider range of different laminin chains than previously described, and that this was independent of the differentiation status of these cells.

2. LITERATURE OVERVIEW

2.1. Extracellular matrix

The extracellular matrix (ECM) is the non-cellular component of a tissue that consists of proteins and polysaccharides. ECM is present in all tissues and contains water whose amount depends greatly on tissue type. ECM remains in close contact with all cells in solid tissues either at different phases of their lives or continuously throughout their life-cycle (Frantz et al., 2010; Hynes, 2009). ECM provides structural and mechanical support for tissues and organs by acting as a scaffold for cell layers or individual cells (Hynes, 2009). In addition to its structural importance, ECM also guides cell migration and regulates differentiation processes (Reilly and Engler, 2010; Sheetz et al., 1998). Furthermore, as some growth factors interact specifically with ECM components, it actively regulates their distribution in a tissue by acting as a growth factor reservoir or barrier (Matsuo and Kimura-Yoshida, 2014). The interaction and adhesion of cells to ECM is mediated by specific cell-surface receptors, with the members of the integrin family playing the most definitive roles. Moreover, ECM also transmits chemical and mechanical signals to the cells and induces specific responses to these signals (Geiger et al., 2001). The intense cross-talk between ECM and cells regulates cellular survival, proliferation, differentiation, attachment and migration. These critical functions of the ECM are facilitated by its continuous enzymatic or non-enzymatic remodeling, as well as a variety of post-translational modifications of its components (Frantz et al., 2010). Thus, the ECM that was formerly seen as an inert aggregate of macromolecules that merely provided a mechanical support for tissues, is now known to be a dynamic structure that actively regulates numerous cellular functions.

2.1.1. Components of the extracellular matrix

There are mainly two types of macromolecules which constitute ECM: (1) proteoglycans, which carry polysaccharide chains (glycosaminoglycans) on their protein backbones and (2) fibrous proteins such as elastin, fibronectin and members of the collagen and laminin families (Alberts et al., 2014). Glycosaminoglycans have the ability to bind large amounts of water and swell to a considerable extent, forming a hydrated gel. In doing so, proteoglycans can fill up large spaces and enable the ECM to withstand compressive forces. At the same time, fibrous collagen helps the ECM to resist stretching, while elastin is important in creating resilience (Alberts et al., 2014). In addition to their structural functions, fibronectin and laminin have essential roles in regulation of cell-matrix interactions during cell adhesion, migration, growth and differentiation (Domogatskaya et al., 2012; Pankov, 2002). The physical, topological, and biochemical composition of the ECM is heterogeneous and tissue-specific (Frantz et al., 2010). For example, different laminin variants are

expressed in a tissue-specific manner, and their expression pattern changes during development (Domogatskaya et al., 2012).

The extracellular matrix composed of certain types of components and forming a distinctive layer is termed basement membrane (Figure 1). It consists of two main structural layers – basal lamina and lamina reticularis. In turn, basal lamina is also composed of two layers – clear lamina lucida and opaque lamina densa (Menter and Dubois, 2012). Basal lamina can act as the basis for the layered epithelial tissue, or surround muscle, fat and Schwann cells. Functionally, basal lamina forms a barrier between these cells and the underlying or surrounding connective tissue (Alberts et al., 2014). Its typical components are laminins, type IV collagen, nidogen and heparan sulfate proteoglycans (e.g. perlecan and agrin) (Hohenester and Yurchenco, 2013). Recent findings suggest that within basal lamina, these proteins and their domains are organized asymmetrically, which gives rise to asymmetric properties of its surfaces facing either stromal or epithelial cells (Halfter et al., 2013).

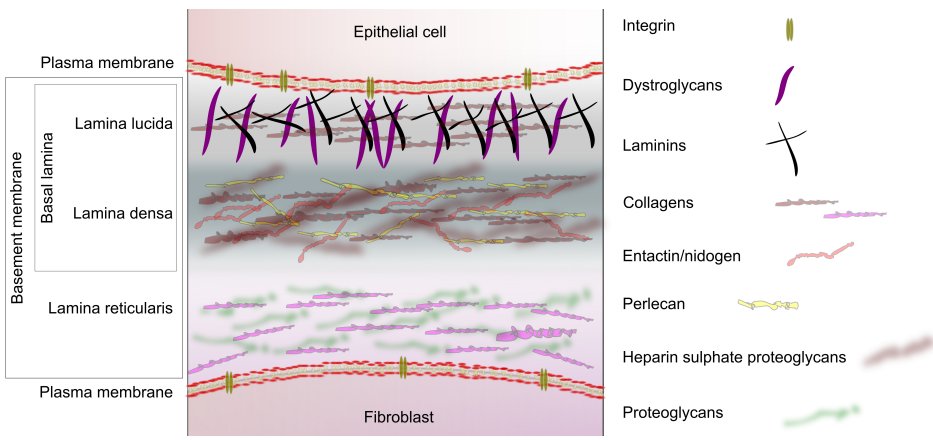

Figure 1. Schematic overview of the components of a typical basement membrane found between epithelial cells and fibroblasts.

In addition to the basement membrane, another type of extracellular matrix – interstitial connective tissue matrix – can be distinguished by its location and composition (Laurila and Leivo, 1993). The interstitial matrix, such as that found in embryonic mesenchyme and in the adult dermis, usually harbors proteoglycans, which prevalently contain chondroitin sulfates and heparan sulfates. The interstitial connective matrix also contains fibronectin, collagens I, III, V, VI, VII, XII and other proteins (Laurila and Leivo, 1993). It is important to note that the basal lamina and interstitial matrix proteins (eg laminins and fibronectin) are usually not found simultaneously in the same structures with the exception of embryonic basement membranes (Laurila and Leivo, 1993).

2.1.1.1. Collagens

Collagens are the most abundant proteins in mammals. This superfamily contains 28 members, referred to by roman numerals (I – XXVIII). However, the criteria for a protein to be classified as a collagen are not well defined. In addition to known members of the collagen family, there are many proteins that contain collagen-like domains in their primary structure but which are not classified as collagens. Collagens consist of three α chains that form a helical structure where three left-handed polyproline II helices are twisted in a right-handed triple helix. Collagens can be homotrimeric with identical α chains, or heterotrimeric, which consist of different α chains. The triple-helical sequences contain tripeptide Gly-X-Y repeats, where X and Y frequently represent proline and 4-hydroxy-proline, respectively. Depending on the collagen type, this triple-helix can expand from 10 to 96% of the protein's structure. The diversity of the collagen family is further expanded by the existence of several α chains, molecular isoforms and supra-molecular structures for any collagen type. (Ricard-Blum, 2011)

Different collagens can be divided into subgroups based on their supra-molecular structure: fibrils, beaded filaments, anchoring fibrils, and networks (Figure 2). On a larger scale, collagens can be classified as fibrillar and non-fibrillar (Chu, 2001). Collagen fibrils can be made of several different collagen types, which vary between tissues (Ricard-Blum, 2011). The most abundant collagens in organisms are fibrillar collagens type I and II (Kadler et al., 2008). Type I collagen is the main structural protein in the interstitial ECM and can be found in bone, teeth and other tissues (Boot-Handford and Tuckwell, 2003; Egeblad et al., 2010). Type II collagen is the major structural constituent of the cartilage (Boot-Handford and Tuckwell, 2003). In humans, collagens III, V, XI, XXIV and XXVII also belong to the fibrillar subclass (Exposito et al., 2010). Type III collagen can be found in a variety of internal organs and in skin. Interestingly, it has been reported that this collagen is essential for proper formation of type I collagen fibrils (Liu et al., 1997). Type V collagen forms heterotypic fibrils with collagen I and is also known to be important in the formation of fibrils (fibrillogenesis) (Sun et al., 2011). Type XI collagen normally interacts with type II and type IX collagens, and is responsible for the unique tensile strength of cartilage. Mutations in the gene encoding type XI collagen can lead to increased degradation of type II collagen in articular cartilage (Rodriguez et al., 2004). Collagen XXIV is produced during osteoblast differentiation and mature bone formation (Matsuo et al., 2008), while type XXVII collagen appears to be important during calcification of cartilage and transition of cartilage into bone (Hjorten et al., 2007). Fibrillar collagens can be found in almost all animals, where they form the well-studied striated fibrils. As an exception, type XXVII collagen can form thin nonstriated fibrils that are structurally different from the classical collagen fibrils (Exposito et al., 2010; Plumb et al., 2007).

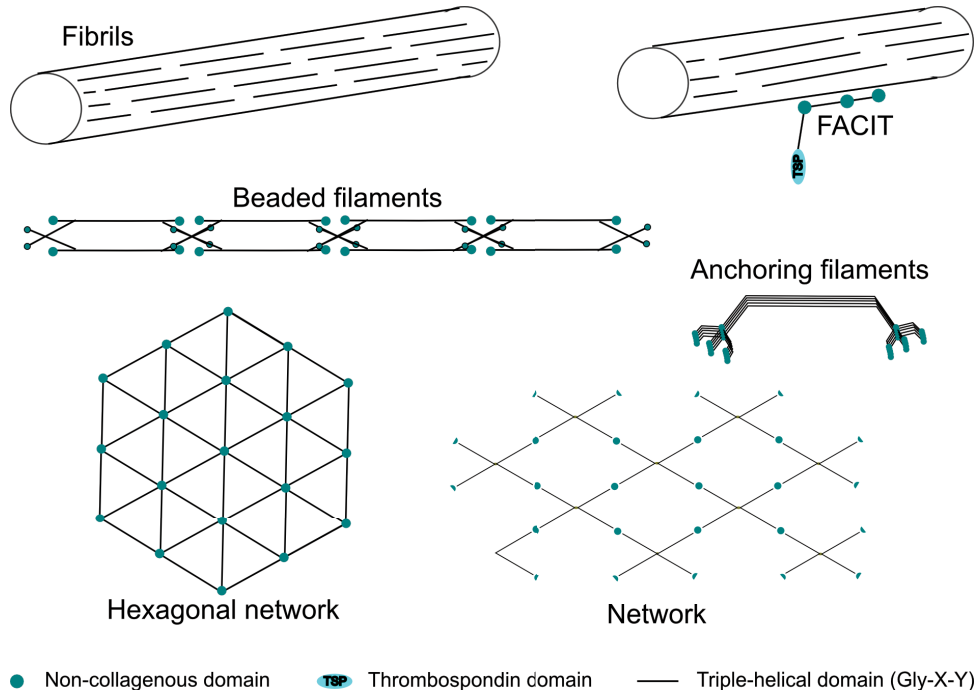

Figure 2. Common supramolecular structures of collagens.

Fibril-associated collagens with interrupted triple-helices (FACITs) form a subclass of nonfibrillar collagens. This group is comprised of collagen types IX, XII, XIV, XVI, XIX, XX, XXI, XXII (Ivanova and Krivchenko, 2014). While they do not form fibrils themselves, these collagens associate with the surfaces of preexisting collagen fibrils (Ricard-Blum, 2011). Unlike fibrillar collagens, the collagens of this subclass contain several short triple-helical collagen-like domains instead of one major domain, which results in an increased flexibility of collagen α-chain molecules. FACITs are minor components of connective tissue in multicellular animals and are important in the regulation of banded collagen fibril size. Some of the FACIT collagens have been shown to interact with various components of ECM. Moreover, some can bind to cell adhesion recepors (Ivanova and Krivchenko, 2014).

A few types of collagens tend to form networks instead of fibrils. One of these is type IV collagen, which has six different α chains and is expressed exclusively in basement membranes. Interestingly, type IV collagen can form only three distinct heterotrimers. The genes of its α chains are differentially expressed during embryonic development, which leads to formation of specific collagen IV networks in different tissues (Khoshnoodi et al., 2008). Type IV collagen is essential for stability of basement membranes, but has been found to be dispensable for membrane assembly during early development (Pöschl et al., 2004). Type VIII and X collagens can also form networks, but unlike collagen

IV these networks are hexagonal and can be found in Descemet's membrane and in hypertrophic cartilage, respectively (Ricard-Blum, 2011). Another type of network is formed by collagen VI and appears as beaded filaments in many tissues (Keene et al., 1988). Finally, type VII collagen anchoring fibrils can be found as extended networks adjacent to the lamina densa of many epithelia (Keene et al., 1987).

2.1.1.2. Laminins

Laminins are one of the major constituents of basement membranes. These multidomain heterotrimeric glycoproteins are composed of three different chains (α,β,γ) that are joined in an α-helical coiled-coil manner into a cross-like structure (Beck et al., 1990; Colognato and Yurchenco, 2000). In vertebrates, at least 16 different laminin isoforms have been identified and named according to their chain composition (e.g. laminin-111 contains one α1, one β1 and one γ1 chain) (Aumailley et al., 2005; Domogatskaya et al., 2012). Among the different laminin chains described to date, there are 5 α-, 4 β- and 3 γ-chains, although the β4-chain is yet to be found in trimeric laminin complex (Domogatskaya et al., 2012). The variation in laminin isoforms is greatly expanded by the fact that different alternative splice variants exist for some chains (Airenne et al., 1996; Ferrigno et al., 1997; Hamill et al., 2009; Hayashi et al., 2002). A good example of this is the α3 chain where the synthesis of different splice variants is relatively well-studied(Aumailley et al., 2005; Domogatskaya et al., 2012).

Laminin-111, found in Engelbreth-Holm-Swarm sarcoma, was the first to be identified (Timpl et al., 1979). Laminin trimers generally form a cross-like structure and thus, in addition to chain composition, laminins can be grouped according to the differences in short arm structures (Figure 3) (Beck et al., 1990; Colognato and Yurchenco, 2000). Laminin-111, -121, -211, -221, -213, -3B11, -511, -521 and -523 exhibit the full cross-like structure, while laminin-3A11, -3A21, -411, -421 and -423, which have a shorter α-chain, form a "topless" shape. Laminin-3B32 contains truncated β- and γ-chains, while all the chains are truncated in laminin-3A32.

The results of studies that examine the expression patters of different laminin isoforms in various tissues have been difficult to interpret. Due to the trimeric nature of laminins, using laminin chain-specific antibodies or knockout mice lacking just one laminin chain may affect multiple laminin isoforms at the same time (Domogatskaya et al., 2012). The importance of laminins in early developmental stages when the first basement membranes appear (embryonic basement membrane and Reichert's membrane) has been studied using mice harboring laminin chain gene knockouts. The results of such investigations suggest that laminin-111 is indispensable for the formation of Reichert's membrane. In the absence of laminin-111, formation of the embryonic basement membrane can be partly rescued by an α5-containing laminin, such as laminin-511 (Miner et al., 2004). In the case of most laminin chains, loss of functional protein results in early lethality or severe pathology, which can be seen in a

variety of animal species (Domogatskaya et al., 2012). The importance of different laminin variants in distinctive developmental stages was suggested already when only a few of different laminin variants were known (Engvall, 1993). Laminin-111 and -511 appear to be the most important laminins during early developmental stages, while the rest of these proteins have prevalently tissue- and cell-type specific roles in assembly of properly functioning tissues (Miner and Yurchenco, 2004).

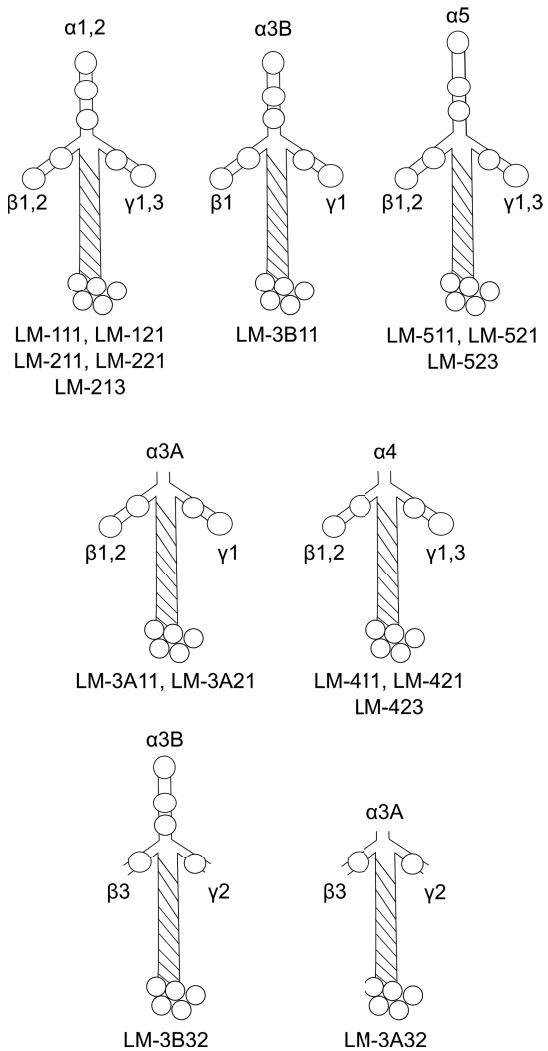

Figure 3. The family of heterotrimeric laminin isoforms.

Specific laminin isoforms co-orchestrate tissue development. For example, in mammalian neuromuscular system, many different laminin isoforms, which contain all known α-chains, are expressed. However, the pattern of their expression, as well as their assembly and tissue distribution are regulated differentially during development (Patton, 2000). An example of such tissue specificity is provided by the laminin β1- and β2-chains: While the two are mutually exclusive in respect of their presence in different types of neuromuscular basal laminae, the embryonic muscle tissue is an exception to this pattern (Patton, 2000). In addition, the distinctive distribution of different laminin variants in the same tissue compartment may reflect the need for a specific molecular function of one or another laminin at that particular location. A good example of this are blood vessels, where laminins with α4 and α5 chains are the predominant isoforms in the basal lamina of vascular endothelial cells. While the α4 chain is expressed ubiquitously throughout different developmental stages, the prominent expression of α5 chain appears postnatally and its distribution varies with vessel type (Yousif et al., 2013). The laminin α5 chain has an inhibitory effect on leukocyte transmigration through vascular basal lamina (Wu et al., 2009), while the absence of the α4 chain reduces the ability of immune cells to penetrate the vessel wall (Kenne et al., 2010). Together, these observations demonstrate how spatial and temporal distribution of laminins enables blood vessels to perform their diverse functions.

The basement membranes of epithelial cells are highly enriched in laminin isoforms that contain the α3-chain. For example, laminin-332 is important in linking epithelial cells to the stroma (Rousselle et al., 1991). In addition, laminin α1 and α5 chains can be found in some ephitelia (Ekblom et al., 1998). Expression of laminin α1 and α3 chains is largely limited to the epithelia, while the α5 chain has a broader expression pattern (Ekblom et al., 1998). Expression of the laminin α2 chain is crucial for muscle formation. Laminin-211 is expressed in normal healthy muscle, while loss of its α2 chain leads to congenital muscular dystrophy (Patton et al., 1999).

As the main binding sites for various cell receptors localize to laminin α-chains (Domogatskaya et al., 2012) their presence or absence in various tissues represents well the overall complexity of laminin expression patterns. A comprehensive analysis of adult mice and embryos has shown that several basal laminae contain multiple α-chains simultaneously, although some of these demonstrate developmentally regulated changes in their expression patterns. The adult laminin expression patterns are established by E15.5 with a few exceptions. The laminin α5 chain has been found to have the broadest expression pattern in adult mice, while expression of the α1 chain is narrowly restricted. The α4 chain is also broadly expressed, whereas the α2 chain is abundant in adult mesenchymal tissues and the α3 chain – in basal laminae of epithelia. (Miner et al., 1997)

2.1.2. Physical properties of the extracellular matrix

The physical response of tissues to mechanical forces is largely dependent on the composition of the ECM. The collagen and elastic fibers are thought to play the main role in this process (Muiznieks and Keeley, 2013). Collagen provides tensile strength to tissues, limits tissue deformation, and prevents rupture of the softer and weaker elastin fibres. The latter function in association with collagens to provide soft, reversible elasticity (e.g. in skin and elastic cartilage) (Gosline et al., 2002). In addition, proteoglycans that maintain hydration of the matrix act to resist compressive forces (Culav et al., 1999).

It has been difficult to study the biophysical properties of native basement membranes due to technical problems in obtaining suitable preparations that are free of adjacent interstitial connective tissue (Candiello et al., 2007). Research conducted on retinal basement membranes has shown that these are surprisingly thick, and their thickness and stiffness increase during early embryonic development (Candiello et al., 2007). It was demonstrated that the thickness of basement membranes (40 – 120 nm for various types of basement membranes) was underestimated since the dehydration, which is caused by the sample preparation process can shrink these structures up to 87% (Candiello et al., 2007).

The inherent complexity of the ECM makes it complicated to study the influence on the cell exerted by a single physical property of the ECM. To circumvent these difficulties, researchers have utilized artificial matrices that allow selective varying of just a few physical parameters of the ECM at a time. In parallel, 3-dimensional (3D) culture conditions can mimic cells' native environment more accurately, but it is difficult to examine single parameters in these systems. Furthermore, it has been demonstrated that some properties, e.g. elasticity of the matrice influence cell behavior differently depending on whether they are studied in a 2-dimensional (2D) or 3D setting (Janson and Putnam, 2014).

An important property of the extracellular matrix is its stiffness, which can be defined as measure of the ability of a material to resist deformation (Mason et al., 2012). This characteristic of the ECM can have profound effects on the surrounding cells. For instance, it has been found that ECM stiffness can affect stem cell differentiation program. Experiments with naive mesenchymal stem cells cultured on matrices with different stiffness values (polyacrylamide gels coated with type I collagen) demonstrated that softer matrices are neurogenic, stiffer ones are myogenic, and comparatively rigid matrices are osteogenic (Engler et al., 2006). Conversely and unexpectedly, substrate porosity and matrix protein tethering (coupling of fibrous protein to the surface of the underlying substrate) have no major impact on the differentiation programme (Wen et al., 2014).

Changes in the extracellular matrix topography can provoke cell shape alterations, indicating that this parameter of the ECM is a potent regulator of cell growth and differentiation (Guilak et al., 2009). Experimental evidence suggests that depending on cell type, nanotopographical variability of the ECM

can influence cell adhesion and proliferation, but mainly affects cell shape and morphology, migration and differentiation (Janson and Putnam, 2014). Many different types of topographical features, including wells, pits, pillars and grooves, have been used to study the influence of surface topography on cell behavior. Interestingly, almost all cell types align along grooves, while all other responses to variations in the ECM topography seem to be cell type specific. Nevertheless, experimental evidence makes it clear that cells do sense changes in the topography of their physical environment (Martínez et al., 2009).

2.1.3. Formation of the extracellular matrix

The various components of the ECM are produced and organized into a proper structure mainly by the cells that are in close contact with the matrix (Alberts et al., 2014). Various cell types are able to synthesize ECM proteins. However, it is important to keep in mind that the composition and structure of the ECM produced depends on the surrounding environment and can therefore vary largely between *in vivo* and *in vitro* conditions. Furthermore, expression and deposition of ECM constituents produced by the same cells under different conditions *in vitro* can differ to a large extent (Streuli and Bissell, 1990).

In the following sections, the roles of selected cell types in ECM biogenesis are discussed. It is important to keep in mind that this complex process has been studied mostly in cell culture, and thus little is known about the mechanisms underlying ECM formation in the context of intact tissues.

Fibroblasts are known to produce and arrange ECM proteins into a properly organized matrix (Beacham et al., 2007). Fibronectin – an ECM protein – is deposited into the matrix in a pattern that mirrors the fibrillar structure of intracellular actin filaments (Hynes and Destree, 1978), indicating a transmembrane relationship between these entities. Additionally, collagen is needed for proper elongation of fibronectin fibrils (Dzamba et al., 1993). Furthermore, evidence indicates that fibronectin, fibronectin-binding and collagen-binding integrins and collagen V are needed for the formation of collagen I-containing fibrils (Kadler et al., 2008). These findings show that the deposition of different ECM constituents is interconnected.

In the skin, fibroblasts and keratinocytes are mainly responsible for secreting components of the basement membrane (Marinkovich et al., 1993). Interestingly, different subpopulations of fibroblasts in the skin have different expression profiles of ECM components (Sorrell and Caplan, 2004). Studies in cell culture have shown that only a subset of the basement membrane components are expressed by both cell types. Such findings, however, greatly depend on the culture conditions used. For instance, nidogen was initially found to be secreted only by fibroblasts (Fleischmajer et al., 1995), whereas laminin-332 was mainly thought to be expressed by keratinocytes (Marinkovich et al., 1993), and both cell types were found to express type IV and VII collagens (Marinkovich et al., 1993). However, a more recent study observed that keratinocytes express

different ECM components including laminin-332, 511/521, nidogen, uncein, and type IV and VII collagens only in the presence of fibroblasts or specific growth factors (El Ghalbzouri et al., 2005). These results indicate that fibroblasts and keratinocytes co-cultured *in vitro* mutually regulate the expression of ECM proteins, which supports the prevailing idea that the interaction of these two cell types is needed for the correct formation of the basement membrane. The interplay between fibroblasts and keratinocytes in skin is a good example of the cooperative interaction of mesencymal and epithelial cells in secreting and organizing the ECM.

Some blood cell types (megakaryocytes, platelets, monocytes, neutrophils, lymphoid cells) express ECM proteins such as laminin, suggesting a possible contribution of these cells to the formation or remodeling of the ECM (Geberhiwot et al., 1999, 2000, 2001; Nigatu et al., 2006; Pedraza et al., 2000; Wondimu et al., 2004). Surprisingly, even the smallest blood cells, platelets, contain ECM proteins: mass spectrometry of the platelet proteome indicates that platelets contain basic ECM proteins such as laminin, fibronectin, vitronectin, fibulin, tenascin, biglycan and a subset of collagen chains (Burkhart et al., 2012). At present, however, the functions of these proteins in platelets are poorly described. With few exceptions, it is also not clear whether the ECM components found in these cells are primarily expressed in megakaryocytes or whether they are endocytosed by mature platelets from plasma. This is more clear in case of platelet laminins whose origins have been traced to megakaryocytic cell lines (Geberhiwot et al., 2000; Nigatu et al., 2006). This suggests that these laminins are synthesized in megakaryocytes and later transferred into forming platelets. As an example of another mechanism, a specific receptor-mediated endocytosis uptake by platelets has been shown for fibirinogen (Handagama et al., 1993) – a glycoprotein that plays an important role in the formation of hemostatic plugs and acts as an adhesion molecule for non-activated platelets (Zaidi et al., 1996). During wound closure, fibrinogen is converted to fibrin, which then binds to another ECM protein – fibronectin. This interaction is essential for the formation of a provisional matrix, which promotes cell adhesion and migration during wound healing (Makogonenko et al., 2002). Fibronectin has been described as a “master organizer” of the matrix as it can interact with many different ECM proteins and form a link between cell surface receptors and the ECM (Halper and Kjaer, 2014). Fibronectin is expressed by megakaryocytes and is deposited in platelet α-granules (Schick et al., 1996). Interestingly, megakaryocytes and platelets are among the few adult cell types that express a fibronectin splice variant named EIIIB (Schick et al., 1996) or EDB (Pankov, 2002) in addition to its main isoform. The latter variant is associated with embryogenesis, neoangiogenesis and cancers, and is structurally exceptional in that it allows simultaneous binding of at least two integrins (Schiefner et al., 2012). Platelets can attach to fibronectin, laminin, vitronectin and collagen via integrins (Bennett et al., 2009)

The known laminin isoforms secreted by activated platelets are laminin-411, -511 and -521 (Geberhiwot et al., 1999; Nigatu et al., 2006). There is some

evidence for the presence of laminin-421 in platelets, however it was only detected at a very low level in a single study (Nigatu et al., 2006). The same study also showed that the common endothelial laminin-411 and -511 are not activating the platelets but function only as adhesive proteins (Nigatu et al., 2006). Vitronectin is another adhesive ECM protein that does not bind specifically to non-activated platelets. Instead, it is released from thrombin-activated platelets and binds to their surface, facilitating their aggregation and clot formation (Asch and Podack, 1990). Among ECM proteins, collagen appears to be the most prominent activator of platelets (Baumgartner and Haudenschild, 1972). However, the role of the collagen storage present inside platelets (Burkhart et al., 2012) and its potential in regulating platelet functions have not been elucidated yet.

While a properly formed ECM facilitates normal growth of the surrounding cells, a deregulated and disorganized ECM promotes cell transformation and metastasis and thus actively advances cancer progression. Additionally, abnormal ECM regulates the behavior of stromal cells and promotes tumor-associated angiogenesis and inflammation, thereby creating a pro-tumorigenic microenvironment (Lu et al., 2012). Interestingly, evidence exists that certain ECM compositions can decrease the tumorigenic potential of cancer cells. For instance, co-culturing breast cancer cells with embryonic mesenchyme from early stage (E12.5–13.5 mice) mammary glands results in a decrease in cancer cell proliferation and an increase in differentiation. This effect is dependent on the composition of the ECM produced by the mesenchyme cells. At least in part, expression of biglycan is essential in this antitumorigenic activity (Bischof et al., 2013).

Many cell lines isolated from human cancers display specific extracellular matrix production patterns. For instance, tumor cell lines of mesenchymal origin secrete interstitial types (I and III) of collagen and fibronectin, while carcinoma cell lines express prevalently basement membrane proteins such as type IV collagen, laminins and fibronectin (Alitalo et al., 1981). Conversely, some cell lines (e.g. IMR-32 from neuroblastoma) possess a heterogeneous pattern of ECM protein expression (Alitalo et al., 1981). Thus, accumulation of specific ECM proteins may indicate presence of cancer cells in normal tissue. For example, accumulation of versican but not decorin in breast cancers is a marker for the presence of cells that have undergone malignant transformation (Skandalis et al., 2011). Metastatic cancer cells also possess distinct characteristic ECM protein expression patterns (Naba et al., 2014). In turn, malignant cells influence the spectrum of ECM proteins expressed by the surrounding stroma (Naba et al., 2014). It has been proposed that the accumulation of ECM proteins this micro-environment is an important factor in the appearance of drug-resistant cancer cells. For instance, overexpression of type VI collagen in ovarian cancer can contribute to the tumour's cisplatin resistance (Sherman-Baust et al., 2003).

Human embryonic stem cells (hESC) share some similarities with cancer stem cells (Unai Silván, Alejandro Díez-Torre, Lucía Jiménez-Rojo, 2011). The

ECM protein-rich niche combined with the presence of specific integrins and growth factors is important in determining stem cell identity (Brizzi et al., 2012). While a number of studies have reported conflicting observations, there is a general consensus about some features of the ECM produced by embryonic stem cells. Laminin-511 is agreed to be the dominant laminin expressed by hESC (Evseenko et al., 2009; Miyazaki et al., 2008; Rodin et al., 2010; Vuoristo and Virtanen, 2009). Additionally, expression of laminin-521 has been reported in some studies (Miyazaki et al., 2008; Rodin et al., 2010), although others have failed to detect the β2 chain, which is a necessary component of this isoform (Evseenko et al., 2009; Vuoristo and Virtanen, 2009). Multiple studies also support existence of laminin-111 in the hESC ECM (Miyazaki et al., 2008; Rodin et al., 2010; Vuoristo and Virtanen, 2009). Laminin-511 and -521 have been demonstrated to support growth of pluripotent hESC in long-term culture (Rodin et al., 2010, 2014), while the combination of laminin-521 and E-cadherin is more potent in supporting clonal survival of hESC (Rodin et al., 2014). On the other hand, laminin-111 does not appear ideally suited to supporting pluripotent embryonic stem cells in culture. While it is widely used for that purpose, a study using mouse embryonic stem cells (mESC) showed that laminin-111 alone, as well as the commonly used ECM protein mixture Matrigel that contains laminin-111, are considerably less efficient in supporting growth of pluripotnent embryonic stem cells compared to laminin-511 (Domogatskaya et al., 2008).

Other ECM components secreted by hESC have not been studied as extensively. hESC colonies grown on mouse embryonic fibroblast feeder (MEF) layers have been shown to contain collagen type IV, nidogen-1 and heparan sulphate proteoglycans (Evseenko et al., 2009). Others have reported that collagen type IV is exclusively produced by MEF, while laminin and type I collagen can be found predominantly nearby MEF but also between hESC (Braam et al., 2008). Additionally, vitronectin was found to be expressed in the centers and at the edges of hESC colonies – locations where differentiation of hESC largely takes place (Braam et al., 2008).

2.1.4. Biological functions of the extracellular matrix in development and during cell differentiation

ECM is a dynamic structure that guides cell differentiation and hence development of an organism. ECM is involved in regulating many aspects of developmental processes: presenting and storing growth factors, providing adhesive substrates, contributing structural elements and environment for sensing and transducing mechanical signals (Rozario and DeSimone, 2010). Extracellular matrix-associated heparan sulfate proteoglycans (HSPGs) play important roles in controlling dispersal of growth factors (Matsuo and Kimura-Yoshida, 2014). For example, HSPGs can reshape fibronectin, after which it assumes an extended conformation and exposes binding sites for growth factors such as

PDGF-AA (platelet-derived growth factor with two A chains) (Symes et al., 2010). It has been proposed that this mechanism guides the migration of mesendodermal cells towards ectoderm during Xenopus gastrulation (Symes et al., 2010). A basic function of many ECM proteins is to provide an adhesive surface for cells, with cell receptors – mainly integrins – acting as mediators of this interaction (Danen and Sonnenberg, 2003). Presence or absence of such interactions can determine whether the cell survives or undergoes apoptosis, and these decisions depend on the exact makeup of the ECM as well as the integrin expression profile of the cell (Vachon, 2011). Specific composition of the ECM can also direct the cell to differentiate towards certain tissue types (Flaim et al., 2005; Goetz et al., 2006). For instance, type I collagen can induce osteoblastic differentiation of bone marrow cells (Mizuno et al., 2000), while type IV collagen has been found to direct mouse embryonic stem cells towards mesodermal lineages and to promote differentiation of mESC into trophectoderm (Schenke-Layland et al., 2007). Additionally, differentiation can be regulated by the cell's sensing of mechanical signals from the ECM. Tensile forces spreading from the ECM to the cellular cytoskeleton are important mediators of such signals. This mechanism has been shown to regulate epithelial branching and angiogenesis during lung development (Moore et al., 2005).

Much valuable information about the importance of different ECM components has been obtained by analyzing ECM loss-of-function phenotypes. Gene knockouts of most ECM components are lethal, highlighting the importance of ECM proteins in regulating proper cellular differentiation (Table 1). Lethality occurs at various timepoints during development of the embryo or after birth, demonstrating that different ECM components are important during different developmental stages. Gene knockouts of a smaller subset of ECM components lead to birth of viable animals with abnormalities, and only a few ECM genes can be lost without any apparent consequences (Table 1).

Table 1. ECM loss-of-function viability phenotypes. The table is assembled from data published by T. Rozario and D. W. DeSimone (Rozario and DeSimone, 2010)

Embryonic lethal	Perinatal lethal	Death at birth	Death just after birth	Death after birth	Post-natal death	Viable	Viable and normal
Fibronectin Laminin α1 Laminin α5 Laminin β1 Laminin γ1 Collagen ColI Collagen ColIV Collagen ColV Perlecan (40%) Versican	Fibulin-1 Fibulin-4	Collagen ColII Collagen ColXI	Laminin β3	Perlecan (60%)	Laminin α2 Laminin α3 Laminin β2 Laminin γ2 Collagen ColIII Collagen ColVII Collagen ColXVII Collagen ColXIX Elastin Fibrillin Fbn1	Laminin α4 Collagen ColVI Collagen ColVIII Collagen ColIX Collagen ColXII Collagen ColXIV Collagen ColXV Collagen ColXVIII Fibrillin Fbn2 Fibulin-3 Fibulin-5 Tenacin Tn-C Tenacin Tn-R Aggrecan Neurocan Brevican	Vitronectin Tenacin Tn-X

2.1.4.1. Laminin-511 and -521 in development and cell differentiation

Due to the heterotrimeric composition of laminins, it has been difficult to address the specific functions of laminin-511 and -521 isoforms in development. A number of studies address this problem by studying expression of specific laminin chains. It is known that the laminin α5 chain, a common component in isoforms 511 and 521, is required during ebryogenesis, and laminin α5 chain knockout mice are embryonic lethal before E17 (Miner et al., 1998). The α5 chain deficiency causes a number of developmental abnormalities such as failure of anterior neural tube closure (exencephaly), failure of digit septation (syndactyly), and dysmorphogenesis of the placental labyrinth (Miner et al., 1998). During early development, the α5 chain is mainly incorporated into the embryonic basement membrane. As the β2 chain is not found there, one can conclude that laminin-511 but not -521 is present in the embryonic basement membrane (Miner et al., 2004). The laminin α5 chain is present in most of the basal laminae of early somite stage embryos. Its expression becomes more restricted as development proceeds and it can be found in specific basal laminae, such as those of the surface ectoderm and placental vasculature (Miner et al., 1998). In basal laminae of an adult organism, the α5 chain is the most widely expressed α chain (Miner et al., 1997). Several studies focused on laminin-511 and -521 have highlighted some intriguing features of the expression patterns of these laminins. For example, a transition in relative abundance from laminin-511 to -521 is a characteristic event in development of the kidney glomerular basement membrane (Miner, 2011; Miner et al., 1997). A somewhat similar change has been observed in synaptic basal laminae during development of the neuromuscular system (Patton et al., 1997). Laminin-521 has also been reported to act as a stop signal for growing axons (Patton et al., 1997). Differential expression patterns of these laminins have been also identified in vascular basal laminae in muscle tissue, where laminin-511 is specifically expressed in venous and laminin-521 in arteriolar basal laminae (Patton et al., 1997). Taken together, it is conceivable that laminin-511 and -521 have distinct roles in development and cell differentiation. Unfortunately, less is known about the distinct functions of these laminins during stem cell differentiation.

2.2. Artificial surfaces for cell growth

Broadly, artificial surfaces for cell growth can be divided into three categories based on the material to which cells adhere: i). materials that contain proteins or other substances obtained from living organisms; ii). manufactured materials that do not contain any substances isolated from a living organism; and iii). materials that contain a mixture of both.

The first category includes simple 2-dimensional (2D) systems, in which growth substrate layers are coated with biological material such as ECM proteins, as well as complex systems where fibrous scaffolds are coated with

natural materials producing 3D environments (Gluck et al., 2013). In order to mimic native 3D structure of the ECM, electrospun scaffolds can be made from biological or non-biologiacal materials by applying a high electrical field to a droplet of fluid, which acts as one of the electrodes. This leads to droplet deformation and formation of continuous fibers towards the other electrode (Kanani and Bahrami, 2010). In a recent study, common ECM proteins such as collagen IV, laminin, fibronectin and vitronectin were used either to coat cell culture dishes or to create electrospun scaffolds to generate 2D and 3D growth environments, respectively. Surprisingly, the 2D and 3D growth matrices had distinct effects on growth of mouse embryonic stem cells: during the culturing mESC had higher proliferation rates in 2D cultures at early time points and in 3D cultures at later time points (Gluck et al., 2013). Common natural polymers used for electrospining are collagen and its denatured form gelatin, chitin and its N-acetyled derivative chitosan, and fibronectin (Kanani and Bahrami, 2010). In addition, mixtures combining several purified ECM proteins (Flaim et al., 2005), or protein extracts rich in basement membrane components have been used as coatings in 2D cell culture (Kleinman and Martin, 2005). Matrigel is known to contain the common basement membrane proteins and can also be used in electrospinning in order to enhance matrix structure and create a 3D environment for cell culture (de Guzman et al., 2010). In addition to materials isolated from living cell cultures, recombinant proteins such as laminin-511 and -521 have been purified and effectively used to support growth of pluripotent hESC (Rodin et al., 2010, 2014). Additionally, peptides derived from full-length laminins have been successfully used for culturing neural stem cells (Li et al., 2014). Since laminins are large proteins, this approach simplifies the purification procedure and thus facilitates potential use of these reagents in the future medical applications.

The second category includes growth surfaces that are often used in studies addressing topographical aspects that affect cell growth characteristics (Martínez et al., 2009). Substances like poly(methylmethacrylate), poly-dimethyl siloxane, polycarbonate, polystyrene, poly(L-lactic acid), poly(ethylene terephthalate), poly(4-bromostyrene), polycaprolactone, poly(D,L-lactic acid), polylactic-co-glycolic-acid, poly-ether-urethane, silicon, silicon dioxide, quartz and polyimide have been used to investigate the effects of micro- and nano-structured surfaces on cell proliferation and/or differentiation (Martínez et al., 2009). Compounds that belong to the family of poly(α-hydroxy esters), including poly(glycolic acid), poly(lactic acid), and their copolymer poly(lactic-*co*-glycolic acid), can be efficiently prepared as electrospun fibrous scaffolds and are commonly used in tissue engineering due to their biodegradability (Li et al., 2006b). Different synthetic materials and processing methods can be combined in order to modulate the properties of scaffolds. For instance, electrospun scaffolds with large pores can be obtained by combining poly(epsilon-caprolactone) electrospinning with electrospraying of poly(ethylene oxide), leading to increased cell infiltration of the matrix (Wang et al., 2014) Finally, while carbon-based coatings are also considered to be biocompatible and usable in

medicine (Correa-Duarte et al., 2004; Grill, 2003), toxicity concerns have been raised for some structural forms in combination with particular cell lines (Magrez et al., 2006).

The third category of artificial cell growth surfaces includes systems in which synthetic materials are combined with biological substances. The key difference between these surfaces and those in the first category is that in the latter, biological coating usually limits the exposure of the synthetic material to cells. Synthetic materials tend to have better physical properties and can be processed (e.g. electrospun) more effectively. However, they typically lack crucial features related to bioactivity and biocompatibility (Stankus et al., 2008). Thus, to combine the beneficial properties of both classes, biological and synthetic materials can been combined. For example, combination of porcine urinary bladder ECM and poly(ester-urethane)urea in electrospinning results in scaffolds with good mechanical properties and excellent biocompatibility (Stankus et al., 2008). Similarly, a mixture of collagen and poly(ethylene oxide) has been used in electrospinning in order to create a material with good biocompatibility and outstanding mechanical properties (Huang et al., 2001). Such materials have considerable potential for soft tissue engineering applications.

Artificial surfaces of any kind that can mimic the functionality of native ECM will be the ultimate tools in future medicine. In order to create artificial ECM, intensive research is needed to understand the biochemical and structural complexity of the ECM and its functions *in vivo*.

3. AIMS OF STUDY

The investigation described in this thesis was aimed at delineating the means by which interactions between cells and the extracellular matrix can regulate cell behavior and modulate the surrounding micro-environment.

1. The first objective of the study was to analyze how variation in the size of round structural elements on cell growth surface modulates cell behavior.

2. The second objective was to characterize the properties of artificial surfaces produced from gelatin by electrospinning in order to determine whether these are applicable for tissue engineering.

3. The third objective was to map the localization of the extracellular matrix proteins laminin-511 and -521 in human platelets and to study the secretion of these proteins upon platelet activation.

4. The fourth objective of this study was to characterize the expression of laminin-511 and -521 during early differentiation of human embryonic stem cells.

4. RESULTS

4.1. Micro- and nanopatterned surfaces affect growth of primary human dermal fibroblasts

When analyzing cell-substrate interactions, many different substrate features, including its topographical structure (Martínez et al., 2009), may affect cell behavior. In the first study completed as part of this thesis (**Ref. I**), we used novel sol–gel phase separation-based method to prepare silica surfaces with round structural elements that vary in size. Particularly, we were interested to see how these different surfaces would affect growth of human dermal fibroblasts. According to the results obtained by scanning electron microscopy and atomic force microscopy, the mean diameters of round structures on different surfaces were 200 nm, 500 nm, 1 μm and 10 μm **(Figure 1 in Ref. I),** while their mean heights were 90, 210, 200 and 920 nm **(Figure 2 in Ref. I)**, respectively. Primary dermal fibroblasts adhered to and proliferated on all four surfaces but showed different morphology depending on the dome size and distance between the structural elements **(Figure 3 in Ref. I)**. Some cells showed atypical morphology with either enlarged cytoplasmic compartment or a narrow shape. The number of cells growing on different surfaces was counted and compared. While the first two surfaces (domes with 200 nm and 500 nm diameters) had similar amounts of atypically narrow cells as did flat surfaces, the surfaces with larger domes (1 μm and 10 μm diameters) showed increased numbers of enlarged fibroblasts and decreased numbers of narrow cells (**Figure 4a in Ref. I**). In order to determine whether this was a sign of senescence, we stained cells for senescence-associated beta-galactosidase. We found that induction of senescence correlated with the increase in size of surface domes (**Figure 4b in Ref. I**). In concordance with this, we noted slight decreases in the numbers of cells positive for the proliferation marker Ki-67 when the fibroblasts were cultured on surfaces with large domes (1 μm and 10 μm diameter) (**Figure 4c in Ref. I**).

It was clear that the different surfaces had an effect on the ability of cells to attach to the substrate. SEM analysis of surface cross sections, obtained by focused ion beam, showed that fibroblasts were tightly attached to the surface with the smallest domes (**Figure 5a in Ref. I**). Attachment to a surface with 500nm diameter domes was incomplete (**Figure 5b in Ref. I**). This was even more evident on the surface with 1 μm diameter domes, where fibroblasts adhered mainly to the domes and not to the space between them (**Figure 5c in Ref. I**). On surfaces with the largest structural elements (10 μm diameter), the cells attached both to the domes as well as to the surface between the domes (**Figure 5d in Ref. I**). These results demonstrate that growth of fibroblasts can be affected by their differential attachment to the structural elements on their growth surface.

4.2. Production of fibrous scaffolds by electrospinning of gelatin and glucose

In the field of tissue engineering, gelatin has been efficiently used to obtain electrospun fibrous scaffolds resembling the natural ECM (Sajkiewicz and Kołbuk, 2014). In the second study included in this thesis (**Ref. II**), we were interested in optimizing the biocompatibility of this simple and cost-effective method by introducing a glucose-mediated crosslinking step into the production process. We used glucose blended with gelatin type A or type B in aqueous acetic acid solution. While both types of gelatins with increasing concentrations of glucose (up to 50%) could be efficiently used for electrospinning, the optimal scaffolds for tissue culture were obtained with up to 15% glucose content. Glucose content higher than 15% resulted in formation of stiff and fragile scaffolds after thermal crosslinking. Scanning electron microscopy showed that the diameter of fibers in scaffolds depended on glucose concentration. Starting at 5% of glucose content, the fibers became gradually larger (**Figure 1 in Ref. II**). Any other significant changes in the structure of fibers that could be attributed to the varying glucose content were not noted.

4.2.1. Glucose increases the degree of cross-linking in the gelatin-based scaffolds

The degree of cross-linking is an important parameter for the electrospun gelatin scaffolds because it determines the stability of the ECM meshwork, which would otherwise dissolve in the aqueous environment present *in vivo* (Sajkiewicz and Kołbuk, 2014). Since many chemical cross-linkers could be potentially cytotoxic, thermal cross-linking was used in our study. It was important to find out how the inclusion of glucose affected the properties of cross-linked scaffolds. We determined that scaffolds became insoluble in boiling water, glacial acetic acid and cell culture media after cross-linking at 170–175 ^{O}C. The optimal time for this process was found to be 3 h. Monitoring of the cross-linking was done by Fourier transform infrared spectroscopy (FTIR). The results suggested that cross-linking was mediated primarily by glucose molecules, as relevant spectral changes were detected for samples with high glucose content, while in low-glucose samples these changes were modest (**Figure 3b in Ref. II**). FTIR analysis also showed that the maximum extent of cross-linking was obtained when 20% glucose was used.

4.2.2. Fibrous gelatin-based scaffolds with different glucose content affect growth of fibroblasts

Cell growth can be affected by the properties of the surface on which the cells reside (Janson and Putnam, 2014). In order to test how the properties of electrospun scaffolds impact cell growth, we evaluated the viability of fibroblasts cultured on scaffolds with different glucose content and compared it

with cell growth on glass. The number of viable cells was measured indirectly by using a luminometer-based assay that detects the amount of ATP in cell preparations. Interestingly, cell growth was most efficiently promoted by the glass surface. The amount of viable cells on the scaffolds prepared from either type A or B gelatins varied, but both showed a decrease in cell growth on scaffolds with high glucose content (25 and 30%) (**Figure 5 in Ref. II**). In the case of type A gelatin, the number of viable cells started to decrease at 25% glucose, while in the case of type B gelatin, the decrease in viable cell number was detectable at only 10% glucose (**Figure 5 in Ref. II**). Cell morphology was also affected when fibroblasts were cultured on different scaffolds. SEM analysis showed that cells formed more dendrites on scaffolds with larger pore sizes than on meshes with smaller pore sizes or on a flat surface (**Figure 6 in Ref. II**). Additionally, certain directional alignment of fibroblasts was detected on areas of scaffolds where parallel orientation of loosely-packed fibers was present (**Figure 6b in Ref. II**).

4.2.3. Laminin α4 and α5 chain are differently expressed by fibroblasts cultured on fibrous scaffolds from gelatin and glucose

As fibroblasts are primarily responsible for secreting ECM proteins in tissues, the artificial scaffolds characterized in the second publication included in this thesis (**Ref. II**) were evaluated for their ability to support this role. This property of the scaffolds is important for co-culturing other cell types with fibroblasts. Previously, laminin-511 was found to be produced by "feeder layer" fibroblasts that support growth of pluripotent embryonic stem cells (Hongisto et al., 2012). Thus, we were particularly interested in studying expression of laminins containing α4 or α5 chain, which differ structurally from each other. Laminin α4 chain production is common to skin fibroblasts, but the α5 chain is expressed mainly by keratinocyes (Fleischmajer et al., 2000; Matsuura et al., 2004). Although the results presented in this section are unpublished, they further extend the characterization of the fibrous scaffolds from gelatin and glucose as possible surfaces for tissue engineering.

First, expression of laminin was detected with antibodies specific to the β1 chain as it is present in a variety of different laminin isoforms. Fibroblasts were found to express detectable levels of laminin next day after seeding and also after 7 days of culturing. Expression of the β1 chain could be detected regardless of the growth surface type (**Figure 4**). Detection of laminin α4 and α5 chains was technically complicated, as these were present at much lower levels than the β1 chain and the scaffolds had high autofluorescence. Nevertheless, expression of laminin α4 chain could be detected after 7 days in cells grown on all surfaces (**Figure 5**). Interestingly, laminin α5 chain was detected at very low levels on fibrous scaffolds, but was almost undetectable when fibroblasts were cultured on glass surfaces (**Figure 6**). Taken together, these data demonstrate that primary skin fibroblasts can produce ECM proteins when cultured on the fibrous scaffolds described above.

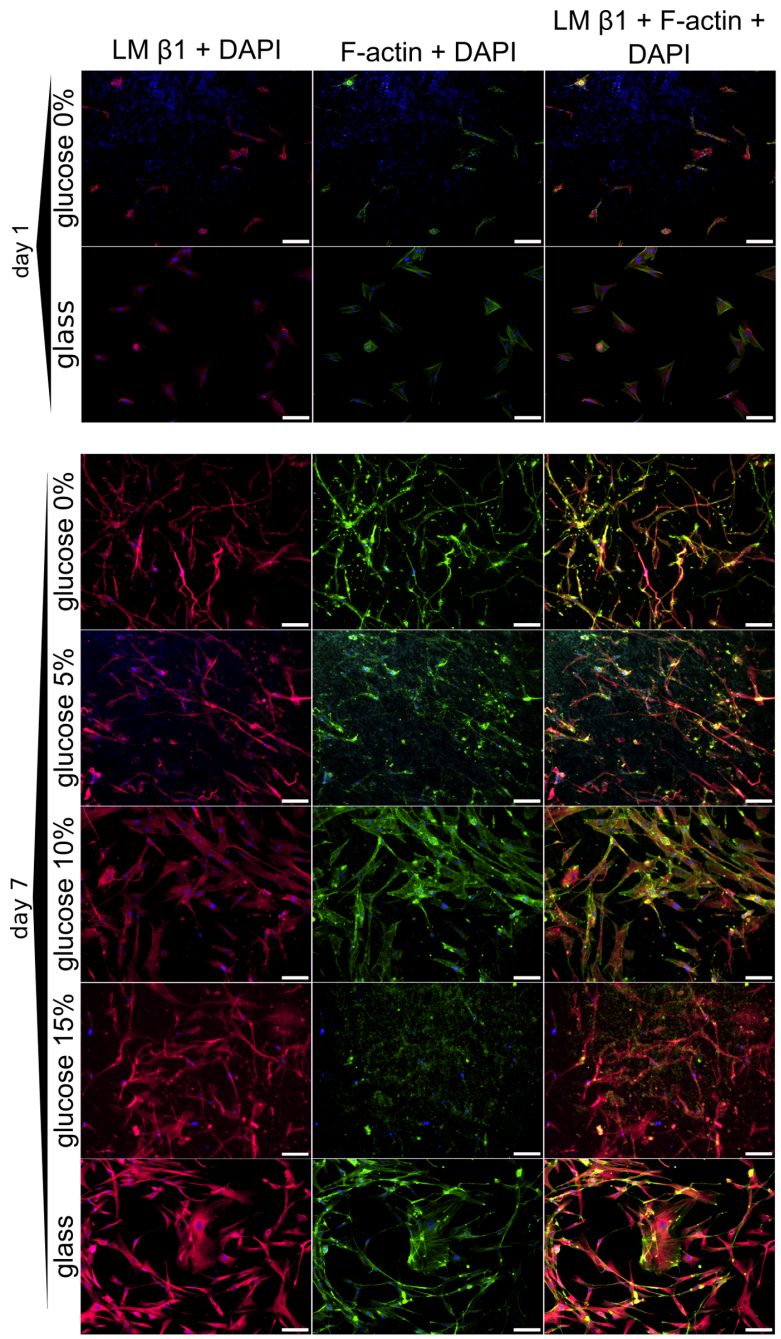

Figure 4. Laminin β1 chain expression in primary dermal fibroblasts cultured on electrospun scaffolds. Laminin (LM) β1 chain (red) was detected with chain-specific primary and Alexa-647-conjugated secondary antibody. F-actin (green) was stained with Alexa-488-phalloidin conjugate. DAPI (blue) was used to visualize cell nuclei. Scale bar: 100 µm.

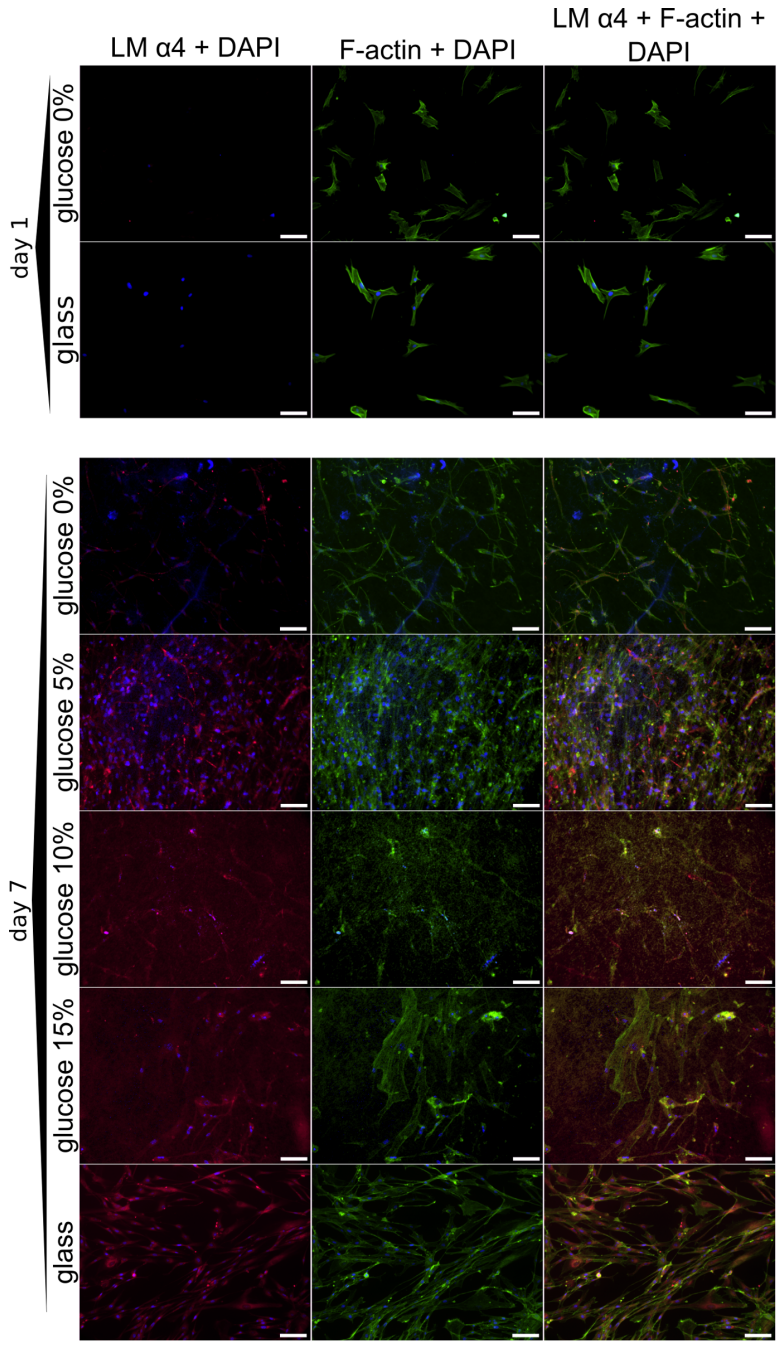

Figure 5. Laminin α4 chain expression in primary dermal fibroblasts cultured on electrospun scaffolds. Laminin (LM) α4 chain (red) was detected with chain-specific primary and Alexa-647-conjugated secondary antibody. F-actin (green) was stained with Alexa-488-phalloidin conjugate. DAPI (blue) was used to visualize cell nuclei. Scale bar: 100 μm.

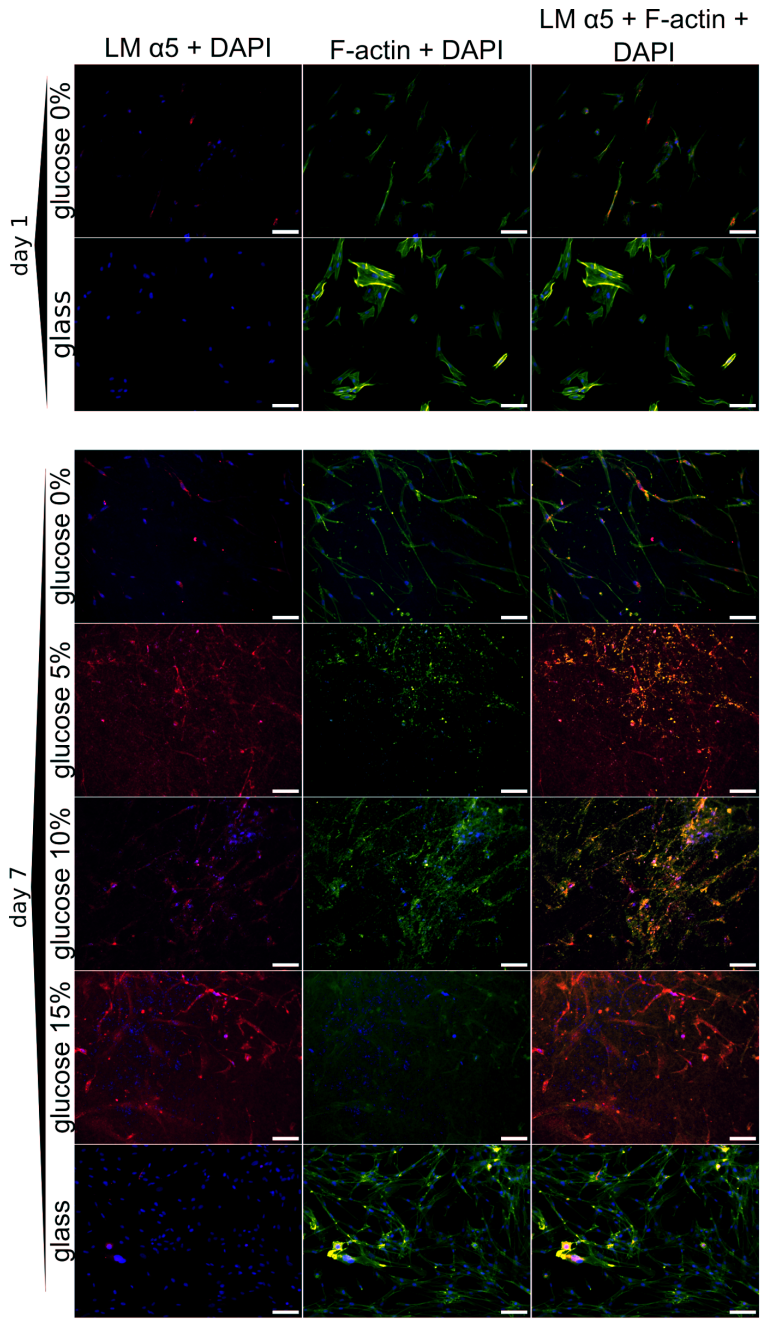

Figure 6. Laminin α5 chain expression in primary dermal fibroblasts cultured on electrospun scaffolds. Laminin (LM) α5 chain (red) was detected with chain-specific primary and Alexa-647-conjugated secondary antibody. F-actin (green) was stained with Alexa-488-phalloidin conjugate. DAPI (blue) was used to visualize cell nuclei. Scale bar: 100 μm.

4.3. Redefining localization of laminin and characterizing its secretion in platelets

Since secretion and deposition of laminins into the ECM is highly important for normal tissue formation and functioning, we became interested in the mechanisms that govern these processes. Platelets secrete and deposit laminins upon activation (Geberhiwot et al., 1999; Nigatu et al., 2006). Interestingly, out of the many laminin isoforms, only laminin 411/421 and laminin 511/521 are stored in platelets (Geberhiwot et al., 1999; Nigatu et al., 2006). Notably, it has been suggested that the laminin α5 chain within platelets is contained in α-granules (Maynard et al., 2007). However, we have previously observed that the laminin β1 chain and known α-granule marker proteins are detected as separate entities (**Figure 7**). Therefore, we set out to study in greater detail the sub-cellular localization of the α5 and α4 chains in human platelets and to determine whether laminin is secreted by platelet microvesicles or exosomes (**Ref. III**).

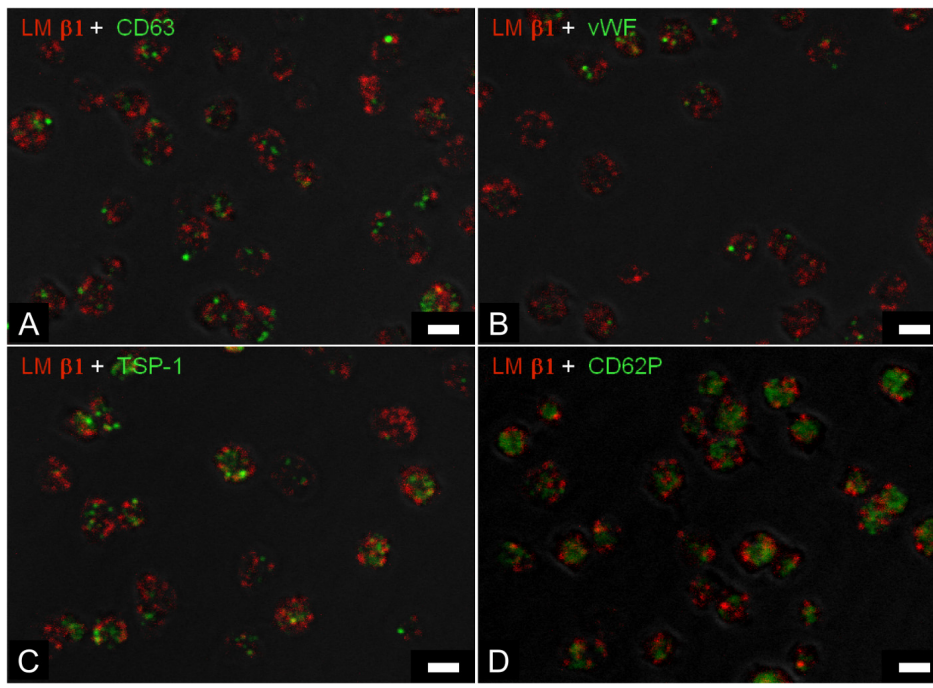

Figure 7. Distribution of the laminin β1 chain and α-granule marker proteins in human platelets as analyzed by confocal fluorescence microscopy. The laminin β1 chain (red) was detected using biotin-conjugated chain-specific antibody (DG10) followed by streptavidin-Alexa-647 conjugate. Granule marker proteins (green), CD63 (MEM-259), VWF (2F2-A9), TSP-1 (A6.1) and CD62P (1E3) were detected by indirect staining using primary and Alexa-488-conjugated secondary antibodies. Scale bar: 2 μm. TSP-1, thrombospondin-1; VWF, von Willebrand factor.

4.3.1. Laminin 411/421 and laminin 511/521 in platelets do not co-localize with common α- or dense granule marker proteins

In order to detect accurately the location of granule proteins in platelets, one has to take into account that platelets may get activated by manipulations during the separation process. Therefore, we employed formaldehyde fixing of platelets in buffy coats before separating the cells. For comparison, we used whole blood samples that were fixed with formaldehyde immediately after collection. This approach allowed us to keep platelet activation at a low level.

First, we used anti-laminin α5 and -α4 chain antibodies that detect laminin 411/421 and 511/521, respectively, in combination with antibodies to known granule marker proteins to study their possible co-localization. Proteins such as thrombospondin-1 (TSP-1) and von Willebrand faktor (VWF) are typical α-granule proteins (Italiano et al., 2008), while CD62P and CD63 also mark dense granules (McNicol and Israels, 1999). Interestingly, confocal microscopy analysis revealed that laminin α4 and α5 chains localize in distinct compartments that lack granule marker proteins CD63, vWF, TSP-1 and CD62P (**Figure 1 in Ref III**).

In order to validate these results and to further characterize the location of laminins in platelets, ultrastructural electron microscopy analysis was performed. Due to technical limitations, we were only able to stain a single antigen at a time. Nevertheless, by analyzing structural properties of different types of granules, it was possible to conclude that laminin α4 and α5 chains are not located in α- or dense granules. The structure of subcellular compartments where laminins were concentrated was clearly distinct from the compartments labeled with TSP-1 or CD63 (**Figure 2 in Ref. III**). TSP-1 clearly marked entities that morphologically resembled α-granules, while CD63 was present in compartments with a different morphology. Interestingly, laminin was mostly localized in smaller containers with barely detectable borders (**Figure 2 C´, D´ in Ref. III**) and was also present in pseudopodia of a few spontaneously activated platelets (**Figure 2 E in Ref. III**).

4.3.2. Activated platelets secrete laminin 411/421 and 511/521 via microvesicles, but not via exosomes

Upon activation with thrombin, platelets secrete mainly two types of vesicles – microvesicles, shed from cell surface, and exosomes, which are generated by exocytosis of multivesicular bodies and α-granules (Heijnen et al., 1999). Taking this into account, we activated platelets with thrombin and separated the secreted vesicles by centrifugation and filtration. These procedures enabled us to discriminate between microvesicles and exosomes. We then used CD41, CD61 and CD63 to characterize the separated vesicles (Horstman et al., 2004; Mathivanan et al., 2010) and determined that while CD41 and CD61 were present in both types of vesicles, CD63 was largely concentrated in the exosome fraction (**Figure 4 in Ref. III**). In turn, laminin α4, α5, β1 and β2 chains were

clearly concentrated in microvesicles but not in exosomes (**Figure 4 and S6 in Ref. III**). The presence of laminin in microvesicles but not in CD63-containing exosomes was further validated by immunoprecipitation (IP) of the CD63-positive exosome fraction (**Figure 4 and S6 in Ref. III**). In order to exclude the possibility that laminin adhered to vesicles' surfaces, IP of laminin β1 was performed from the microvesicle fraction and showed no detectable amount of laminin on the microvesicle membrane (**Figure S7 in Ref. III**).

The microvesicle fraction was further analyzed by detecting the laminin α5 and β1 chains as well as vesicle markers CD41, CD62P and CD63 using flow cytometry. This allowed us to characterize the microvesicle fraction with high resolution. Interestingly, we detected significant amount of heterogeneity as CD41 was present in 45% and CD62P in 40% of analyzed microvesicles, while 20% of vesicles contained CD63. Laminin was found to be present in 37-38% of microvesicles (**Figure 5 A in Ref. III**). The exosome fraction was also analyzed by flow cytometry. Due to their small size, exosomes had to be adsorbed to latex beads prior to analysis, but this made it impossible to characterize the exosome population at high resolution. Nevertheless, the flow cytometric analysis showed that only 2% of latex beads covered with exosomes contained laminin (**Figure 5 B inRef. III**). Taken together, our results conclusively show that laminin is secreted from platelets in microvesicles but not in exosomes.

4.4. Analyzing laminin expression in pluripotent and early differentiating human embryonic stem cells

The putative role of laminin in regulating growth and differentiation of embryonic stem cells has generated considerable interest. The number of studies involving laminin and hESC has grown rapidly in parallel with the need to find new defined culture conditions for hESC. While most of these studies have focused on culturing hESC as pluripotent cells (Evseenko et al., 2009; Miyazaki et al., 2008; Rodin et al., 2010, 2014; Vuoristo and Virtanen, 2009), little effort has been dedicated to characterizing different laminins as potential regulators of hESC differentiation. In the third study included in this thesis (**Ref. IV**), we examined hESC within the short time-frame when these cells downregulate their pluripotency-associated marker proteins and begin to differentiate. Based on the findings that the α5 chain-containing laminins support long-term culturing of undifferentiated hESC (Rodin et al., 2010, 2014), we were particularly interested in finding out how expression of these laminin isoforms changes during early differentiation.

4.4.1. Retinoic acid induces differentiation of hESC characterized by a decrease in OCT4 and a transient increase in β-III-tubulin expression

In order to induce hESC differentiation, we treated the cells with retinoic acid for 5 days. During this time period, we characterized the daily changes taking place in the hESC culture. We observed that immunofluorescence staining of the pluripotency marker OCT4 (octamer-binding transcription factor 4) began to decrease from day 2. This change was first detected in the centers of large colonies (**Figure 1 A in Ref. IV**), while OCT4 levels remained high in small colonies and at the edges of large colonies even on day 5 (**Figure S2 A and B in Ref. IV**). In general, expression of OCT4 decreased notably after 5 days of treatment as detected by Western blot analysis (**Figure 1 B in Ref. IV**), confirming the differentiated status of hESC at that time point. Interestingly, the ectodermal differentiation marker β-III-tubulin showed somewhat decreased expression on day 5, although it was abundantly detected on day 3 (**Figure 1 A in Ref. IV**). Moreover, expression of β-III-tubulin was weakly detectable one day after passaging, when OCT4 was still highly expressed (**Figure 1 B in Ref. IV**). Similarly, small colonies with high OCT4 levels but with low levels of β-III-tubulin were present on day 5 of treatment (**Figure S2 B in Ref. IV**). At the same time, some of the differentiated cells in large colonies showed robust expression of β-III-tubulin but weak expression of OCT4 (**Figure S2 C in Ref. IV**). These results suggest that the early differentiation of hESC is diverse depending on colony size and exposes heterogeneous expression of OCT4 and β-III-tubulin in the differentiating colonies.

4.4.2. Laminin β1 chain is a component of fiber-like structures in differentiating hESC colonies

As differentiation of hESC occurred mainly in the centers of cell colonies, we decided to characterize expression of laminin in these areas. Expression of laminin subunits that belong to the laminin 511/521 heterotrimers was revealed by immunostaining. As differentiation progressed, we observed increased levels of laminin chains coinciding with decreased OCT4 expression, as well as signs of structural alterations in the laminin-rich ECM (**Figure 2 in Ref. IV**). These changes were further studied by confocal microscopy. 3D analysis of the laminin β1 chain expression pattern revealed fiber-like structures forming a network mostly on top and around cells (**Figure 3 in Ref. IV**).

4.4.3. Laminin-511 but not -521 preferentially accumulates in hESC culture during early differentiation induced by retinoic acid

Our results obtained by immunostaining of subunits belonging to laminin 511/521 suggested that laminin increasingly accumulates in regions of differentiating hESC. However, the ratio of laminin-511 to -521 remained unclear. In order to compare the relative expressions of these two isoforms, we immunoprecipitated laminin-511 and -521 using an α5 chain-specific monoclonal antibody. We analyzed quantitatively the material obtained from hESC treated with retinoic acid for 3 and 5 days by western blotting. Laminin α5 chain expression clearly increased between the two time points (**Figure 4 and Table S5 in Ref. IV**). Moreover, the amount of the β1 and γ1 chains bound to the α5 chain increased as well, whereas the amount of the β2 chain decreased (**Table S6 in Ref. IV**). This change in the relative abundance of laminin-511 vs. laminin-521 suggests that modulation of the ratio of these two laminins may coordinate early stage of hESC differentiation.

4.4.4. hESC express a diverse range of laminin chains

Expression of different laminin variants in non-differentiated hESC has been characterized previously (Evseenko et al., 2009; Miyazaki et al., 2008; Rodin et al., 2010; Vuoristo and Virtanen, 2009). However, there is no universal consensus as to which laminin isoforms are expressed by these cells. Inconsistent culture conditions or different cell lines used in these studies may explain the discrepancies in the reported results. Therefore, we decided to generate qualitative estimates of expression of different laminin chains in hESC culture. We utilized RT-PCR (reverse transcriptase-coupled PCR) and western blot analysis in order to detect expression of the laminin α1-α5, β1-β3 and γ1-γ3 chains. We compared laminin expression in different culture conditions, including samples from hESC grown in differentiation media and treated with retinoic acid or with its solvent, DMSO (dimethyl sulfoxide). Cells grown under normal culture conditions on matrigel and in mTeSR1 media were used as a reference. In order to follow the gradual changes caused by differentiation, we analyzed the cells next day after passage (day 0), on day 3 and on day 5. Surprisingly, expression of multiple laminin chains was detected (**Figure 5 in Ref. IV**). Protein levels of different chains were not drastically different between various culture conditions, although some laminin chains were detected at very low levels on day 0 (**Figure 5 in Ref. IV**). Moreover, RT-PCR showed no qualitative differences in the laminin expression patterns (**Figure S3 in Ref. IV**). In addition to laminin α1, α5, β1, β2 and γ1 chains, which have been well characterized in other studies (Miyazaki et al., 2008; Rodin et al., 2010), we were able to detect the mRNA and protein of laminin α2, α3, β3, γ2, and γ3 chains in the hESC (**Figure 5 in Ref. IV**). Interestingly, we were not able to detect laminin α4 chain at the protein level.

Additionally, we identified laminin subunit variants with dissimilar electrophoretic mobility. In particular, two laminin α3 chain variants (165 kDa and 145 kDa) were present, with the smaller variant specifically enriched in hESC treated with retinoic acid (**Figure 5 in Ref. IV**). The β3 chain protein was detected in hESC samples only as a variant with a higher molecular mass, while the γ2 chain was present only as a variant with low molecular mass (105 kDa) (**Figure 5 in Ref. IV**). This suggests for the presence of specific laminin chain variants in the hESC culture.

5. DISCUSSION

The utilization of man-made matrices for tissue engineering applications to generate artificial equivalents of certain types of tissues is a promising tool in the field of regenerative medicine. It is widely accepted that studies describing cell-substrate interactions and the assembly of ECM are of utmost importance (Daley et al., 2008).

The most common approach in cell culture is to utilize flat 2D growth surfaces. In the first part of this thesis (**Ref. I**), we introduced topographical changes to the growth environment of cells by manufacturing silica-based surfaces with round nano- and microscale features. Importantly, we were able to modulate the size of these structural elements without changing the chemical properties of the growth surface. This allowed us to focus exclusively on the effects of topographical changes on cell growth. We found that both an incomplete attachment of fibroblasts and the presence of large round structural elements affect fibroblast growth and induce features of senescence. Attachment of fibroblasts to their growth surface is likely mediated by the ECM proteins that these cells produce. Fibronectin in particular has been shown to play important role in fibroblast adhesion (Grinnell and Feld, 1979). Therefore, we speculate that changes in surface topography affect the presentation of the ECM to the fibroblasts, which in turn modulates cell behavior through ECM-cell interactions. Thus, our data confirm the importance of proper growth surface topography when designing artificial matrices for cell culture.

Most cells in the human body grow in a 3D environment, interacting with the fibrous mesh-work that forms the backbone of the natural ECM (Lee et al., 2008). This contrasts strongly with growth on flat 2D surfaces employed in conventional cell culture (Lee et al., 2008). Electrospinning of gelatin is a cost-effective and simple method to produce an *in vitro* 3D environment with fibrous scaffolds that mimic the morphology of native ECM (Sajkiewicz and Kołbuk, 2014). Pure gelatin-based scaffolds are structurally unstable in aqueous environments at temperatures similar to those *in vivo*. Therefore, further stabilization of the artificial collagen mesh is needed. One way to stabilize the gelatin scaffolds is chemical cross-linking, but many chemicals used for this purpose are cytotoxic (Sisson et al., 2009). In the second part of this thesis (**Ref. II**), we described an alternative way of producing gelatin scaffolds by electrospinning. By blending gelatin with glucose and applying a thermal treatment, our method led to efficient cross-linking of gelatin-based scaffolds. Furthermore, it allowed us to control the extent of cross-linking by varying the glucose content in the fibers. With up to 15% of glucose, the scaffolds were easy to handle, supported cell growth, and could be digested enzymatically, demonstrating their supreme suitability for tissue engineering purposes.

On large scale, two types of gelatin exist: type A is isolated from pig skin by pretreatment with acid, while type B is purified from beef hides and bones via alkaline pre-treatment (Gorgieva and Kokol, 2011). Mass spectrometry analysis revealed several differences in composition of type A and B gelatins (**Table 2 in**

Ref. II). This prompted us to test the suitability of both types for constructing cell growth scaffolds. Interestingly, we found gelatins A and B to be equally suitable for glucose-mediated crosslinking and electrospinning. Nevertheless, differences were noted in their ability to support cell culture, which could be caused by their distinct contents.

During analysis of cell growth on scaffolds, we noted that the number of viable fibroblasts was smaller on artificial collagen scaffolds than on glass surfaces. While this might appear as a major drawback at first, this property of novel collagen meshes can, in fact, be useful. For instance, extensive proliferation of fibroblasts is not desirable when they are co-cultured with keratinocytes. Moreover, controlled proliferation of fibroblasts may be quite valuable in medical applications as hyperproliferation of these cells during wound healing causes the undesirable pathological condition termed hypertrophic scarring (Bellemare et al., 2005).

In addition to the basic characterization of cell growth on our collagen-based scaffolds (**Ref. II**), we also examined how this scaffold affects the cells' ECM production. Specifically, we focused on expression of the α4 and α5 chain-containing laminins. Interestingly, we found that weak expression of the laminin α5 chain could be detected in primary fibroblasts from skin only when they were grown on gelatin-based scaffolds but not on a glass surface. It is interesting to note, that in co-cultures of skin keratinocytes and fibroblasts of the dermis, the expression of the α5 chain has been reported to appear much later than the other laminin chains and was not found in monotypic fibroblast cultures (Fleischmajer et al., 2000) . Thus, our results suggest that the expression of laminin α5 chain in dermal fibroblasts may be regulated by the extracellular environment. It is known that particular collagen-derived constituents of gelatin maintain their primary structure that provides RGD (Arginine-Glycine-Aspartic) binding sites that promote integrin-mediated cell adhesion (Sajkiewicz and Kołbuk, 2014). We speculate that this feature of gelatin-based scaffolds is partly responsible for the observed production of α5 laminins. Importantly, α5 chain-containing laminins can regulate tissue-regenerative capacity of keratinocyte stem cells and early differentiating keratinocytes in organotypic cultures with dermal fibroblasts (Li et al., 2004). Thus, upregulation of laminin α5 chain expression has potential implications in co-culturing of fibroblasts and keratinocytes in order to produce skin equivalents.

In the third part of this thesis (**Ref. III**), we present evidence that laminins 411/421 and 511/521 in platelets are not stored in α-granules, in contradiction with results published earlier (Maynard et al., 2007). As we did not see co-localization of laminins with CD62P or CD63 (common platelet granule markers), we concluded that laminins are not secreted from α-granules or dense granules. Suprisingly, we found that laminins are preferentially secreted via microvesicles and are not included into exosomes of thrombin-activated platelets. Our results are in agreement with the finding that exosomes originate from α-granules and multivesicular bodies (Heijnen et al., 1999). By contrast, microvesicles bud from the plasma membrane (Heijnen et al., 1999; Raposo and

Stoorvogel, 2013), and thus we speculate that laminins are included directly into microvesicles. Interestingly, CD62P, a granule marker that did not co-localize with laminin in non-activated platelets, was present in 40% of analyzed secreted microvesicles. This suggests that there exist different types of microvesicles, including some that contain laminin but not CD62P. This remains to be clarified by upcoming studies. The mechanism of secretion from activated platelets is highly complex, and distribution of cargo between three distinct kinetical classes is thought to be heterogeneous (Jonnalagadda et al., 2012). Such heterogeneity makes it difficult to unequivocally connect a particular granule type or content with a single secretion mechanism. Nevertheless, we expect that our analysis will facilitate future elucidation of the functional role of laminins in platelets.

The exact functional role of platelet laminins is currently unknown. The α4-containing laminins may be important in angiogenesis (Abrass et al., 2010; Li et al., 2006a), while laminins with the α5 chain appear to perform a wider range of functions and have "stem cell supportive" properties (Domogatskaya et al., 2012; Paquet-Fifield et al., 2009). Activated platelets may secrete these laminins to facilitate wound healing. Given our finding that laminins are secreted via microvesicles, it is likely that platelets affect many cell types simultaneously via laminin secretion. Unfortunately, the beneficial physiological role of platelets in wound healing can be subverted by cancer cells. A number of studies demonstrate that platelets can facilitate cancer progression and promote metastasis and cancer-associated angiogenesis (Gay and Felding-Habermann, 2011). There is a good reason to believe that the α4 and α5 chain-containing laminins secreted by platelets also play a role in cancer progression and survival.

Several types of cancer cells have been described as very similar to embryonic stem cells (Unai Silván, Alejandro Díez-Torre, Lucía Jiménez-Rojo, 2011). These similarities may be imparted at least partly by the surrounding ECM. Supporting evidence for such speculation includes the observation that while α5 chain-containing laminins support growth of pluripotent hESC (Evseenko et al., 2009; Hongisto et al., 2012; Miyazaki et al., 2008; Rodin et al., 2010, 2014; Vuoristo and Virtanen, 2009), they are also associated with cancer progression (Pouliot and Kusuma, 2012). Interestingly, it has been shown that out of the two main α5 chain-containing isoforms, laminin-511, rather than laminin-521 correlates with aggressive mammary tumors (Chia et al., 2007). In the fourth study included in this thesis (**Ref. IV**), we discovered that during early differentiation of hESC, laminin-511 and -521 accumulate in the surrounding ECM at different concentrations. Our results suggest that while both of these laminins are expressed in non-differentiated hESC, the relative expression of laminin-521 decreases during differentiation. In contrast, laminin-511 is abundant during early differentiation of hESC. Thus, we speculate that early differentiation is regulated by the ratio between laminin-511 and -521 proteins. Furthermore, we found that both pluripotent and differentiating hESC express many other types of laminins. Therefore, it is possible that ratios

between many different isoforms of laminins, and not only laminin-511 and -521, are also important in defining stem cell status.

In vivo, early differentiation steps are associated with formation of laminin-containing membranes such as Reichert's and embryonic basement membranes (Miner et al., 2004). While carrying out confocal analysis of immunostained hESC, we were surprised to observe that laminin forms 3D network-like structures in the centers of differentiating hESC colonies growing on 2D substrates. This finding once again highlights the complex relationship between cellular differentiation and ECM structure. It suggests that hESC induced to differentiate in 2D cultures intrinsically construct a 3D matrix to facilitate their differentiation.

The pattern of laminin expression in non-differentiated hESC has been previously analyzed, but the results vary between studies (Evseenko et al., 2009; Miyazaki et al., 2008; Rodin et al., 2010; Vuoristo and Virtanen, 2009). These differences could be at least partly explained by the use of different culture conditions and cell lines. Our results provide an overview of the expression patterns of different laminin chains in non-differentiated H9 (WA09) hESC when grown on matrigel with mTeSR-1 medium. The H9 cell line has been intensively characterized, while matrigel and mTeSR-1 medium are commonly used culture reagents. Under these conditions, cells are routinely passaged after 3-4 days in order to maintain their pluripotent state. We followed hESC growth for 5 days and allowed spontaneous differentiation in the culture to take place. In addition to this, we used MEF-conditioned media (differentiation media) supplemented with retinoic acid to induce differentiation in hESC culture. As a control for the latter, DMSO in differentiation media was utilized. Interestingly, we did not observe gross differences in mRNA levels of different laminin chains between cells analyzed at different time points under any culture conditions. Precise measurement of such changes remains as a subject for further studies. However, the value of such exact analyses is dubious as it is very difficult to correlate the expression of single laminin chains with the abundance of different trimeric laminin isoforms.

Most of the studies performed to date have analyzed laminin expression at the mRNA level, while few data are available about relative levels of laminin proteins. To fill this gap, we examined expression of laminin-511 and -521 proteins during early differentiation, since only these isoforms are known to support pluripotent hESC growth in long term cultures (Rodin et al., 2010, 2014). Future studies that quantitatively analyze the protein levels of other laminin isoforms may provide new perspectives on our understanding of the ways in which changes in the ECM during early steps of differentiation regulate cell fate. Unfortunately, such detailed analyses are technically complicated due to limited availability of suitable antibodies.

ECM is a dynamic environment where proteolytic cleavage and conformational changes of its components add an additional dimension of functional complexity to the regulation of cell behavior (Schenk and Quaranta, 2003). Therefore, in addition to the differential expression of various laminin

isoforms in hESC culture, the presence of protein processing in the ECM is also potentially important. We found that some specific laminin variants were expressed in hESC (**Ref. IV**). Regardless of hESC differentiation status, the laminin γ2 chain was present as a 105 kDa fragment, while the larger unprocessed form was not detected. This could point to the increased migratory properties of hESC, as processing of the γ2 chain facilitates an increase in cell motility (Ogawa et al., 2004). Additionally, the laminin β3 chain was observed as a variant with low electrophoretic mobility in hESC grown under all the culture conditions. In contrast, the common protein variant with higher electrophoretic mobility was present in the control cell line A431. To our knowledge, the functional role of the shorter β3 chain variant has not been described. Additionally, we found that the 145 kDa variant of the α3 chain is specifically enriched in hESC during the differentiation induced by retinoic acid. In keratinocytes, this variant associates with assembly of hemidesmosomes but is not known to have a role in cell migration (Baudoin et al., 2005). Whether this could point to the increased role of hemidesmosomal connections of hESC remains to be solved by further studies.

In conclusion, the studies presented in this thesis have further expanded our understanding of the mechanisms by which laminin contributes to the regulation of cell function. Particularly, the importance of laminins containing the α5 chain has been elucidated in greater detail. Still, laminins constitute only a small subset of all ECM proteins that are involved in the regulation of cell fate. Therefore, studying the intricate nuances of the complex cross-talk between cells and the surrounding ECM will certainly remain one of the key topics of research in regenerative medicine.

SUMMARY

The research presented in the current thesis was designed to study the ways in which cells interact with the surrounding extracellular matrix. These bidirectional interactions both affect cell growth and allow cells to modulate their surrounding micro-environment. The studies included in this thesis investigated how these interactions depend on the surface topography or regulate the production of ECM components by the cells that adhere to these surfaces. Expression of laminins – essential ECM proteins that regulate cell behavior – was analyzed in human primary dermal fibroblasts and in human embryonic stem cells. Primary focus was on the α5 chain-containing laminins as these proteins are associated with enhanced tissue regeneration properties. Localization and secretion of laminins was also analyzed in blood platelets.

In the first set of experiments, we analyzed how the size of round structures on cell growth surface can modulate cellular behavior. Novel silica-based surfaces containing round structural elements with controlled size were manufactured for this study. Importantly, the sizes of these elements could be varied without changing the chemical properties of the growth surfaces. Surfaces with four different sizes of structural elements were produced and analyzed for their ability to support growth of human primary dermal fibroblasts. It was found that small structural elements (mean diameter 200 nm) did not alter cell growth significantly. Larger elements (mean diameter 500 nm and up) increasingly disturbed cell attachment. This was particularly pronounced when the dome diameter reached 1 μm and cells began to attach only to the domes but not to the surface between them. Interestingly, in the case of very large domes (mean diameter 10 μm), fibroblasts were able to attach to the domes as well as to the inter-dome surfaces. Overall, increasing size of these structural elements had a significant negative effect on fibroblast growth and promoted cellular senescence.

In addition to surface topography in general, cell growth can be affected by the type of material used as growth substrate and by the way in which this material is presented to the cells. The second study included in this thesis demonstrated that gelatin-based 3D growth surfaces produced by electrospinning can be effectively used for culturing of human primary dermal fibroblasts. In these experiments, novel gelatin-based scaffolds were produced by using a mixture of gelatin and glucose. Addition of glucose enabled efficient cross-linking of the scaffolds by thermal treatment, thereby stabilizing them and giving them properties suitable for use in tissue engineering. It was determined that glucose content up to 15% was optimal for producing desirable mechanical properties and tissue compatibility of the scaffolds. Additionally, expression of the laminin β1, α4 and α5 chains was evaluated in fibroblasts grown on these scaffolds. Notably, growth on the novel gelatin-glucose matrices induced low-level expression of the α5 chain in fibroblasts, whereas the same was not observed when the cells were cultured on an uncoated glass surface.

In vivo, the ECM is continuously modulated by different types of cells. Blood platelets are one cell type that can secrete some components of the ECM. In the third study included in this thesis, localization and secretion of platelet laminins was investigated. Combined results of immunofluorescence- and electron microscopy analysis revealed that laminin 511/521 and laminin 411/421 are not localized in common type of platelet alpha granules, as has been suggested. Upon activation by thrombin, platelets were found to secrete laminins via microvesicles but not via exosomes, which further confirmed our finding that platelet laminins are not stored in typical alpha-granules.

It has been established that the ECM can guide cell differentiation. The fourth study of this thesis characterized the changes in expression of α5 chain-containing laminin-511 and -521 during early steps of differentiation in human embryonic stem cells. The results suggested that the amount of laminin-511 increases while the amount of laminin-521 decreases during differentiation. The β1-containing laminins secreted by the differentiating cells was observed to be organized into fiber-like structures. Expression analysis revealed that the cells produced a wider range of different laminin chains than has been reported previously. The results of this study suggest that laminin-511 plays a role in early differentiation of human embryonic stem cells, in addition to its well-established function in supporting stem cell growth.

In conclusion, the results presented in the current thesis emphasize the role of the ECM in regulating cell behavior. These findings should prove valuable in the field of tissue engineering, where culture conditions must closely mimic the complex organization of ECM *in vivo*. These new aspects of understanding of ECM function can be used for improving the design of engineered tissues and increase hope for success of future medical attempts to regenerate functional human tissues.

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SUMMARY IN ESTONIAN

Kunstlike ja rakuvälise maatriksi valkudega kaetud pindade osa rakkude kasvu ja diferentseerumise reguleerimises

Loomade kõik rakud puutuvad oma elu jooksul kokku neid ümbritseva rakuvälise maatriksiga. See on rakkude poolt sekreteeritud vaheaine, mis peamiselt koosneb valkudest ja polüsahhariididest. Koes annab rakuväline maatriks rakkudele mehhaanilise toe ja võimaldab üles ehitada keerukaid organeid. Lisaks mõjutavad sealsed komponendid rakkude kasvu, migreerumist ja diferentseerumist. Mehhaanilise toe tagamisel on valdav roll erinevatel kollageenidel. Rakkude kasvu võivad mõjutada paljud erinevad maatriksi komponendid. Ühed enimkirjeldatud on laminiinid, mille 16 erinevat isovormi võivad kõik mõjutada rakke erineval viisil. Üha enam nähakse rakuvälises maatriksis olulist panust rakkude kasvu ja käitumise aktiivses reguleerimises, kuna on teada, et selline maatriks toimib ka mitmete kasvufaktorite reservuaarina, millest need vabanevad vastavalt vajadusele. Paljud rakud sekreteerivad ise suurema osa oma lähikeskkonna rakuvälisest maatriksist, kuid see võib moodustuda ka mitmete erinevate rakkude osalusel. Elusorganismides on rakuväline maatriks väga keerulise ehitusega ning sisaldab palju erinevaid komponente. Maatriksid võivad olla ka tehislikult saadud ning siis on tavaliselt nende keerukuse aste märksa väiksem. Tehismaatrikseid kasutatakse igapäevaselt koekultuuris erinevat tüüpi rakkude kasvatamiseks. Mida paremini suudavad tehismaatriksid jäljendada elusorganismide rakuvälise maatriksi kompleksust, seda täpsemalt on võimalik suunata rakkude kasvu.

Käesoleva doktoritöö esimeses pooles iseloomustati erinevate maatriksite mõju sellel kasvavatele rakkudele. Esmalt uuriti kuidas rakkude kasvupinna topograafilised omadused mõjutavad inimese naha fibroblastide kasvu. Kasvupinnasena kasutati ränil põhinevat materjali (n.ö tehisklaasi), mis andis võimaluse tekitada pindu, mis oli kaetud erineva suurusega ümarate struktuuridega. Selgus, et väga väikesed struktuurid (keskmine diameeter 200 nm) rakkude kasvu oluliselt ei mõjutanud, kuid kui need olid suuremad (keskmine diameeter 500 nm, 1 μm ja 10 μm), hakkas see rakkude kasvu pärssima, millega kaasnes rakkude nn „vananemine" (ingl.k. *senescence*). Avastati, et kui 500 nm ja 1 μm diameetriga struktuuride puhul oli rakkude kinnitumine raskendatud ning nad seondusid eelistatult vaid struktuuride pinnale, siis maatriksil, kus olid kõige suuremad struktuurid (keskmine diameeter 10 μm) olid fibroblastid võimelised korraga kinnituma nii ümarate struktuuride pinnale kui ka nendevahelisele alale. Kuivõrd ka viimasel juhul täheldati negatiivset mõju rakkude kasvule, viitab see asjaolule, et lisaks probleemidele rakkude kinnitumises, põhjustavad ka suured pinnastruktuurid rakkudes muutusi, mis võivad viia nende kasvu peatumiseni.

Järgnevalt analüüsiti inimese nahast eraldatud fibroblastide kasvu nn 3D „tehiskangast" kasvupindadel, mis olid saadud želatiinist ja glükoosist elektrospinnimise teel. Glükoosi lisamisega želatiinile saavutati parem struktuuride ristseondumine pärast „tehiskanga" kuumutamist. Selgus, et rakkude kasvuks

olid kõige sobivamad maatriksid need, mis elektrospinniti kuni 15% glükoosi sisaldusega lahusest. Kõrgema kontsentratsiooni puhul olid „tehiskangad" liiga jäigad ja murdusid kergesti ning lisaks oli raskendatud nende ensümaatiline lagundamine. Sobiliku glükoosi sisaldusega pinnad toetasid fibroblastide kasvu ja need võiks olla kasutatavad koetehnoloogiates. Lisaks täheldati, et eelnimetatud kasvupinnad käivitasid fibroblastides laminiini α5 ahela ekspressiooni, mida ei ilmnenud kui samu rakke kasvatati tavalistel klaaspindadel. Sellest võib järeldada, et antud kasvupindadel on bioaktiivsed omadused, mis mõjutavad rakkude kasvu spetsiifiliselt.

Lisaks ülalnimetatud katsetele kunstlike maatriksitega, uuriti ka rakuvälise maatriksi valkude, laminiinide, paiknemist ja sekreteerimisest inimese trombotsüütides. Kuna on teada, et trombotsüüdidid osalevad aktiivselt haavade parandamises, on tähelepanuväärne, et nad sisaldavad ka laminiine 511/521 ja 411/421. Aktiveerunud trombotsüüdid võivad neid laminiine sekreteerida ümbritsevasse keskkonda ja seeläbi mõjutada teiste rakkude füsioloogiat. Meie töö tulemusena selgus, et aktiveerumata trombotsüütides ladustatakse neid laminiine seniarvatust erinevalt. Kui algselt arvati, et laminiinid paiknevad trombotsüütide alfa-graanulites, siis meie katsetest selgus, et laminiinid ei paikne alfa-graanulites ega ka teistes tüüpilistes ladestusgraanulites. Lisaks saime teada, et trombotsüütide aktiveerumise korral väljutatakse laminiinid mikrovesiikulitesse pakituna, kuid mitte eksosoomide kaudu, mille kaudu toimub teiste alfa-graanulite sisaldiste väljastamine.

Doktoritöö viimases osas jätkati uuringuid nende laminiinidega, mis sisaldavad α5 ahelat. Nimelt on eelnevalt näidatud, et laminiinid 511 ja 521 toetavad pluripotentsete embrüonaalsete tüvirakude kasvu, kuid nende laminiinide ekspressioon tüvirakkude varajases diferentseerumises pole täpselt teada. Meie töö tulemusena selgus, et kui retinoolhappega mõjutatud tüvirakud on varajase diferentseerumise etapis, siis suureneb nendes laminiini 511 ja väheneb laminiini 521 hulk. Kui tüvirakke uuriti konfokaalmikroskoopia abil, siis selgus, et laminiinid paiknesid rakuvälises alas organiseeritult, mis võib mängida olulist rolli nende rakkude diferentseerumisprotsessi juhtimises. Me leidsime ka, et sõltumata diferentseerumise astmest ekspresseerub inimese embrüonaalsetes tüvirakkudes laminiinide mitmeid erinevaid ahelaid. Isegi pluripotentsusmarkereid ekspresseerivatest rakkudest oli võimalik tuvastada rohkemate laminiini ahelate ekspresssiooni kui seda oli eelnevalt näidatud. Sellest tulenevalt võib oletada, et rakkude diferentseerumise suunda võib määrata see, milline on erinevate laminiinide ekspressiooni tase ja omavaheline suhe.

Käesolevas doktoritöös käsitletud teemad on vaid osa rakuvälise maatriksi ja rakkude interaktsiooni uurimises, mida teostatakse paljude uurimisgruppide poolt üle maailma. Sellest hoolimata on iga väikegi avastus selles vallas kasulik, sest lisab väärtuslikku informatsiooni disainimaks uusi rakendusi koetehnoloogiates ja tuleviku regeneratiivses meditsiinis.

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PUBLICATIONS

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