Advancing regenerative medicine by generating knowledge about the nature of cadherins in human mesenchymal stem cells

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CONTENTS

1

Introduction

Regenerative medicine is an interdisciplinary field that aims to regrow, repair, or replace damaged or diseased cells, organs, or tissues. This field has the potential to cure chronic diseases and repair tissue damage resulting from injury or disease. Moreover, the field has promised that engineered tissues and organs could potentially be available in large supply. This is especially relevant as the world's population grows older and the clinical demand for tissue replacement rises. To this end, the main goal of regenerative medicine is to direct cell behavior to build a tissue for therapeutic impact. Tissue engineers need tools to direct cell behavior. Typically, this entails designing an engineered microenvironment comprising a biomaterial incorporating biological cues such as growth factors. To date, material scientists and tissue engineers have designed many sophisticated materials. These materials sometimes behave as they were intended, but they sometimes fail to positively influence cell behavior.

The mixed success of tissue engineering teaches us that a more sophisticated biological toolbox is needed because our understanding of the basic biology underlying cell behavior is still far from exhaustive. This thesis is aimed at improving our understanding of how the molecular machinery of cells drives regeneration so that scientists could be more successful by taking lessons from cell–cell adhesion proteins, more specifically cadherin biology, as they are a major driving force in tissue formation and because they can influence important cell behavior. This is complementary to most efforts to date that have been inspired by cell–extracellular matrix interactions.

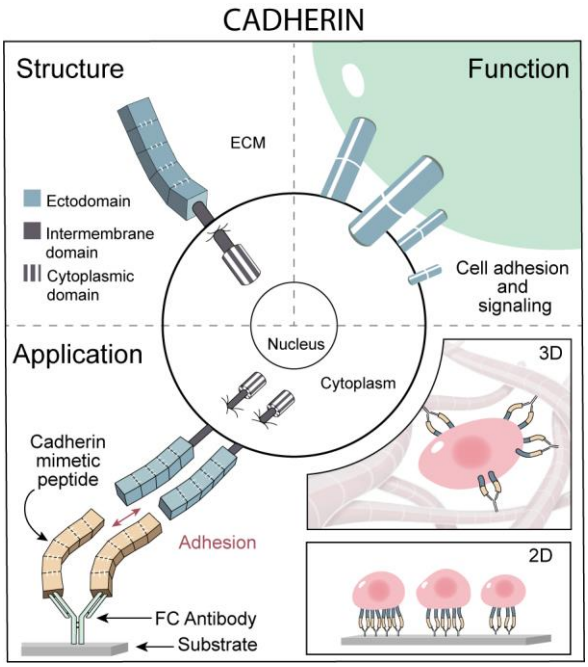

Figure 1. Overview of chapter 2

This chapter is a perspective in which we give an overview of the diversity of the cadherin family, discuss the key characteristics that make cadherins ideal for tissue engineering approaches and also elaborate on the functional significance of cadherins in the context of tissue engineering.

In **Chapter 2**, we introduce the family of proteins called the cadherins, which are the focus of this thesis. The chapter is a perspective geared towards coaxing the regenerative medicine and tissue engineering community to divert their attention towards cadherins and the role they play in cell signaling to make more successful biomaterials (Figure 1). We give an overview of the diversity of the cadherin family and then dive into the physical aspects of cadherins and discuss the key characteristics that make them ideal for tissue engineering approaches. We then discuss the functional significance of cadherins in the context of tissue engineering and describe their role in cell–cell adhesion, where we compare and contrast two major classes of cadherins: one that has been widely studied and one that has been largely ignored. We also describe the role of cadherins in cell proliferation and

differentiation, both of which are of great interest to the regenerative medicine and tissue engineering community, and are also relevant to the research described in Chapters 3 and 4. In terms of functional significance, we then discuss how modulating cadherin activity can also influence important signaling pathways. The bulk of this chapter focuses on providing a contemporary overview of exemplary studies that have used cadherins to influence cell behavior and we show tissue engineers that they already have the necessary tools to incorporate this knowledge into their designs.

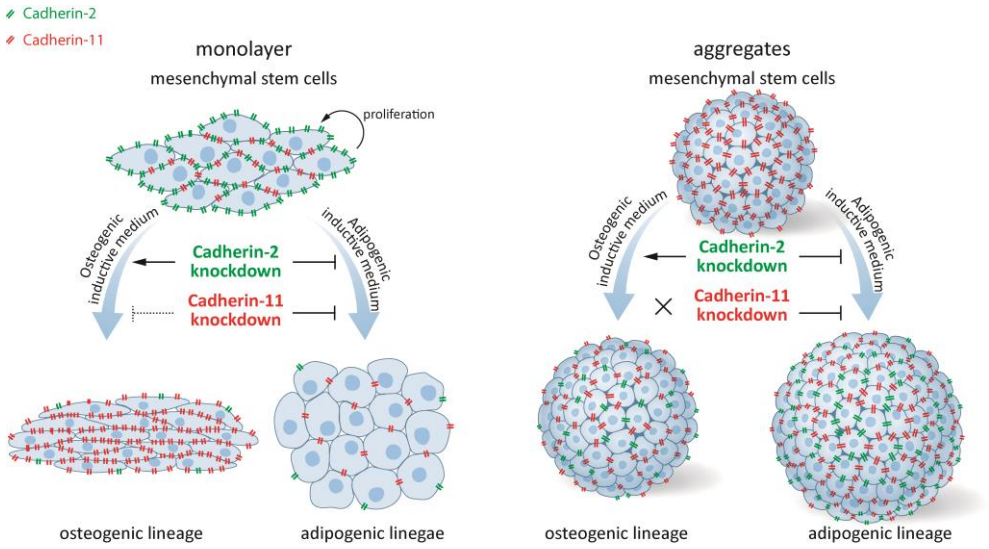

Figure 2. Overview of chapter 3

Cadherin-2 and cadherin-11 expression in an aggregate culture are different from monolayer culture. Cadherin-2 knockdown enhances mineralized matrix deposition whereas cadherin-11 knockdown diminishes it. Both cadherin-11 and cadherin-2 knockdowns disrupt adipogenic differentiation potential

It has been a long-standing goal of regenerative medicine to move towards the third dimension to develop more robust organotypic models *in vitro* and to improve outcomes *in vivo*. Great interest exists in identifying what it is about aggregate cultures that often lead to superior results over monolayer cultures. Based on the suggestions we make in Chapter 2, we formulated our own hypothesis about how

cadherins could help in achieving this goal. In **Chapter 3**, we dissect the role of two important cadherins (cadherin-2 and cadherin-11) in the differentiation of human mesenchymal stem cells (hMSCs) into two lineages relevant for regenerative medicine: the adipogenic (fat) and osteogenic (bone) (Figure 2). One of our main goals was to investigate how these cadherins differ in their contributions to hMSC differentiation depending on their culture as monolayers or as aggregates. Our research corroborates that cell culture dimensionality influences cell fate, but we also go on to show the important role of cadherin-mediated signaling. Using knockdowns, we showed for the first time that both cadherin-2 and cadherin-11 are indispensable for adipogenic differentiation in monolayer and aggregate culture, while cadherin-11 is dispensable for osteogenic differentiation in aggregates. We also showed that osteogenic differentiation is favorably affected by low levels of cadherin-2. Overall, this work showed us that cell culture dimensionality plays a critical role in hMSC fate through cadherin signaling.

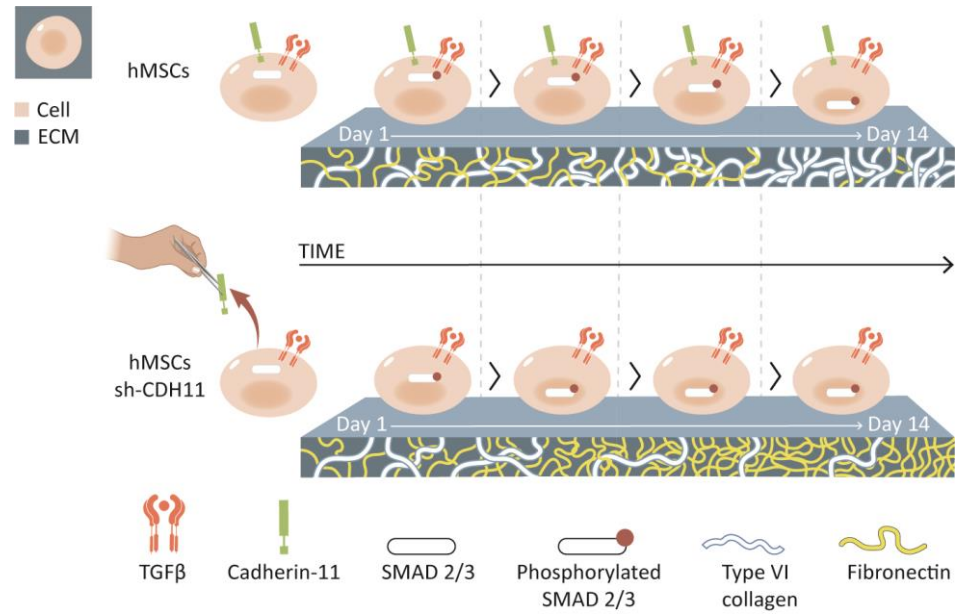

Figure 3. Overview of chapter 4

This study demonstrates that cadherin-11 regulates the ECM by temporally controlling the TGFB pathway.

The work of **Chapter 4** was inspired by that of Chapter 3, as we wanted to understand the mechanisms that regulate hMSC fate commitment with evidence to explain how knocking down cadherin-11 leads to changes in their differentiation potential (Figure 3). For most clinical applications, hMSC differentiation towards the adipogenic lineage is an undesired outcome. Therefore, understanding the mechanisms that regulate their commitment towards the adipogenic lineage might open up new avenues for fine-tuning hMSCs for regenerative medicine applications. The idea for the experiments came from the field's long obsession with targeting the receptors of the extracellular matrix (ECM) to activate specific signaling pathways and direct cell behavior. The importance of the ECM in activating specific signaling pathways to direct cell behavior is undisputed and the field of regenerative medicine often targets or mimics cell–ECM interactions. We set out to first explore whether knocking down cadherin-11 had an influence on the ECM composition, thereby affecting adipogenic differentiation. Western blotting and immunofluorescence was used to study the ECM composition of cells lacking cadherin-11. Their expression was observed at an early and a later time-point. In this study, we not only provide evidence that cadherin-11 regulates collagen, but also show for the first time that it regulates fibronectin. Cadherin-11 has no known intrinsic signaling activity, therefore we also identified a possible crosstalk with the TGFβ1 pathway through which cadherin-11 modulates the ECM. The role of TGFβ1 in hMSC differentiation and ECM synthesis was previously known, but we uncovered a new crosstalk with cadherin-11. We show evidence that changes in the TGFβ1 pathway via SMAD2/3 explain the changes in ECM composition. By implicating cadherin-11 in the regulation of the ECM, we add to the evidence of the cadherin–integrin crosstalk mechanism. Overall, this work showed that cadherin-11 plays a role in cell fate determination by modulating the ECM.

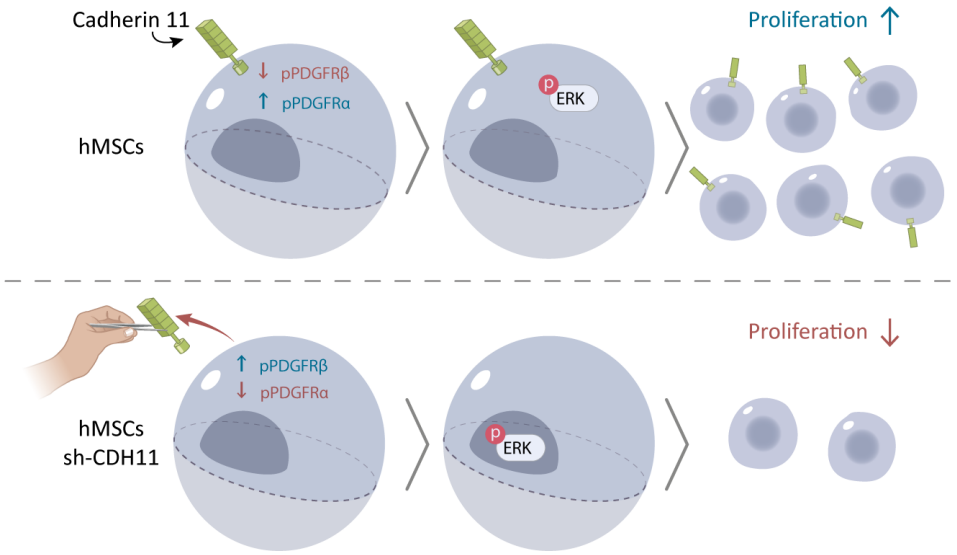

Figure 4. Overview of chapter 5

This study demonstrates the complex nature of cadherin-11-RTK crosstalk in controlling cell behavior.

Chapter 5 followed from Chapter 4, as we were curious to find out how cadherin-11 was bringing about changes in other signaling pathways despite it having no intrinsic signaling activity (Figure 4). Receptor tyrosine kinases (RTKs) are transmembrane protein that affect a wide array of cellular functions; therefore, we wanted to know if cadherin-11 has crosstalk with RTKs. We used array membranes to study which RTKs are present in hMSCs and how they change in cells that lack cadherin-11. We hypothesized that cadherin-11 physically binds to PDGFRβ, which was the RTK most highly expressed by the hMSCs, and thereby brings about changes in cellular function. By knocking down cadherin-11, we discovered it regulates not just PDGFRβ, but also other RTKs. We show evidence that changes in the RTK profile leads to downstream changes in the MAPK pathway which in turn influences cellular functions.

The work described in **Chapter 6** started out as a collaborative education project with students from the PRO3011 course (Science Research Project) at the University Collage Maastricht and was our foray into the world of biomaterials. Building on our work in Chapter 3, we wanted to know how three-dimensional aggregate cultures affect cellular behavior in the context of hydrogel encapsulation. We knew of many reports of improved outcomes from aggregating cells, but it was often unclear whether that was a specific effect of the aggregation itself or whether it had to do with different cell numbers or densities. We looked at scaffold-free self-assembled aggregates of varying cell number and compared the results to cells embedded in alginate hydrogel with and without arginine-glycine-aspartic acid (RGD) peptides. We explored how these different culture systems performed over a prolonged period in terms of cell viability and proliferation using various qualitative and quantitative assays.

In **Chapter 7**, the major findings of the thesis are highlighted and discussed in the form of four questions that further explore the insight that cadherin biology could provide for regenerative medicine. The chapter concludes with recommendations for future work. Finally, we close off in the impact chapter, **Chapter 8**, where we provide a wider context for the research, establishing its contribution to regenerative medicine and tissue engineering.

2

Sticking together: harnessing cadherin biology for tissue engineering

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ABSTRACT

Directing cell behavior and building a tissue for therapeutic impact is the main goal of regenerative medicine, for which scientists need to modulate the interaction of cells with biomaterials. The focus of the field thus far has been on the incorporation of cues from the extracellular matrix but we propose that scientists take lessons from cell–cell adhesion proteins, more specifically cadherin biology, as these proteins make multicellularity possible. In this perspective, we re-examine cadherins through the lens of a tissue engineer for the purpose of advancing regenerative medicine. Furthermore, we summarize exciting developments in biomaterials inspired by cadherins and discuss some challenges and opportunities for the future.

AN INCENTIVE TO INCORPORATE CADHERINS IN REGENERATIVE MEDICINE APPLICATIONS

Building a tissue for therapeutic impact is the goal of tissue engineering and regenerative medicine. Most efforts are either centered on the use of small molecules and soluble growth factors based on our understanding of signaling pathways, or aim to influence cell–biomaterial interactions based on our understanding of cell–extracellular matrix (ECM) interactions.[1] We propose that cell–cell interactions need to gain more attention from tissue engineers as they are a major driving force in tissue formation, because cellular adhesions are a fundamental structural feature of a multicellular organism, and because they can influence important cell behavior. Therefore, cadherins, the major protein family in cell–cell interactions should be considered by tissue engineers trying to build hierarchical structures that resemble native tissues.

Cadherins are a diverse family of cell–cell adhesion molecules that comprise over 100 members. They can be identified in almost all vertebrate cells because their evolution contributed to the generation of diversity among animal species. In the early 20th century a fascinating discovery was made by zoologists where they dissociated cells from a frog embryo which then self-assembled into an aggregate with architecture resembling the original embryo.[2] The authors of the paper described a non-static bond between cells in the aggregate that was less rigid and more complex than antigen-antibody interaction. In their paper, they also mentioned that the bond between cells is static if the aggregate consists of one kind of cell. This led scientists to the conclusion that cells have an innate ability to recognize one another and self-organize into tissues. Modern observations have summed up that the bonds that help cells selectively adhere to one another is due to cadherins.[3] Since then, we have learned that cadherins are calcium-dependent

transmembrane proteins that dynamically interact with their partners in a homophilic or heterophilic manner.

The cadherin family not only maintains the structural integrity of cells and tissues, but also controls a wide array of cellular behaviors. A wealth of evidence suggests that modifying materials with biological elements can influence the properties of cells such as adhesion, survival, and signaling. For decades, tissue engineers have incorporated the knowledge of integrins into their design principles. Integrins, which influence cell behavior through the formation of transient focal complexes that initiate a signaling cascade, can be activated by incorporating integrin-binding peptides into materials. The use of integrins in tissue engineering has been extensively reviewed so we need not review it here.[4] Since adhesion does not end with integrins, tissue engineers need to evolve their designs beyond the integrin family to fully realize the potential of biomimetic approaches. In all, the literature suggests that investigating cadherins in the context of tissue engineering and regenerative medicine could prove to be a potent tool while designing materials to achieve complex tissue architecture.

First, we dive into the physical aspects of cadherins and discuss the key characteristics that make them ideal for tissue engineering. Second, we discuss the functional significance of cadherins in the context of tissue engineering and regenerative medicine. Finally, we provide a state-of-the-art overview of exemplary studies that have used cadherins to influence cell behavior. Combined, this review will be useful for any scientist who wishes to explore new biomimetic approaches to influence cell fate by engineering the microenvironment.

A PHYSICAL MODEL OF CADHERINS

One way of generating advanced biomaterials that can influence cell behavior is to incorporate a biologically active component into the material of interest. If appropriately designed and tethered, these components retain their bioactivity and drive specific cellular behaviors. To effectively use cadherins in materials of interest to influence cell behavior, the understanding of the basic structure, as well as the diversity of the cadherin family, is essential.

The cadherins are classified into four subfamilies based on their structure (Figure 1). First, there are the classical cadherins (type I and type II) that are involved in intracellular signaling pathways, including Wnt, Ras, and RhoGTPases signaling.[5] Second, are the desmosomal cadherins that comprise desmocollin and desmoglein, which help form the desmosomal junctions.[6] Third are the protocadherins, the largest group in the cadherin family, that are predominantly expressed in the nervous system.[7] And finally, the atypical cadherin-like domain–containing proteins that play a role in intercellular adhesion.

Structurally, the classical and desmosomal cadherins consist of an ectodomain with five tandem repeats (EC1–EC5) rigidified by the binding of calcium, a single-pass transmembrane domain that acts as the anchorage point, and a cytoplasmic domain with conserved catenin binding sites.[8] While the cytoplasmic domain of classical cadherins enables their interaction with the actin filaments, the cytoplasmic domain on desmosomal cadherins interacts with the intermediary filaments. In contrast, protocadherins have more than five EC repeats in the ectodomain and their cytoplasmic domain is structurally diverse but relatively less is known about their cytoplasmic binding partners.

The cytoplasmic domain of classical cadherins is important for strong cell adhesions because of their interacting partners, the catenins, namely α, β, and p120. Beta and p120 catenin bind directly to the cytoplasmic domain, while α-catenin binds to β-catenin and also to the actin filaments.[9] The binding of β-catenin to α-catenin decreases α-catenin's affinity for actin filaments.[10,11] This is mitigated by the fact that mechanical tension acting on α-catenin while it is still bound to β-catenin causes α-catenin to unfold, exposing its actin filament binding site.[12] The unfolding of α-catenin recruits vinculin to the cadherin–catenin binding site, which further prevents the refolding of α-catenin.[13] The force-based coupling of actin filaments to α-catenin is reversible, meaning that if the tension across the cadherin junction were to decrease, the α-catenin will refold.[14] While signaling is localized to the cytoplasmic domain, selectivity and adhesion are features of the ectodomain that makes it an attractive candidate for tissue engineering applications by way of a recombinant protein or synthetic peptide of the cadherin ectodomain.

The cadherin superfamily is defined by similarities in the ectodomain and is also divided into subfamilies based on the differences in the EC1 repeat.[15] The ectodomain is rigidified and stabilized by calcium ions, which give it a curved structure.[16–19] Type-I classical cadherins have the highly conserved HAV (histidine alanine valine) tripeptide sequence in the EC1 repeat (Figure 1) while the type-II classical cadherins lack the HAV sequence. The HAV sequence is an example of a cadherin mimetic peptide implemented to engage cadherins just as RGD is used to engage integrins.

The established mechanism for cadherins on opposing cells to bind involves the formation of a strand-swapped dimer.[20] The strand-swapped dimer is formed when the tryptophan at position 2 (W2) on the EC1 repeat from one cadherin intercalates into the hydrophobic pocket of the EC1 repeat of the opposing cadherin.[21,22] The

HAV sequence forms part of the hydrophobic pocket containing the W2 amino acid.[23] The W2 amino acid is conserved across all classical cadherins within the same repeat. In addition to lacking the HAV sequence, the type-II classical cadherins need an additional tryptophan at position 4 (W4), inserted into the hydrophobic pocket to form the strand-swapped dimer.[24] While the differences between type-I and type-II classical cadherins make them incompatible for binding with each other, the similarities between members of the same subfamily increase the likelihood of cross-adhesion.

That being said, the EC1 repeat in the ectodomain of classical cadherins is necessary for binding specificity. For example, swapping the EC1 repeats of two distinct cadherins is sufficient to switch their binding selectivity. However important the EC1 repeat might be, studies have shown that cells with just the EC1 and EC2 repeats have weak cell–cell adhesion, and the additional repeats are important for full adhesive activity, especially to elicit changes in cell behavior.[19,25,26] Furthermore, recent studies have revealed that cadherins form uniform lateral clusters in the plasma membrane, about 50–60 nm in size with varying numbers of cadherins per cluster.[27,28] This lateral clustering increases the adhesive strength of cadherins but is not a prerequisite for adhesion.[20]

It is crucial for tissue engineers to understand that a single site is not responsible for cadherin adhesion and selectivity because multiple EC repeats contribute to cadherin adhesion. The EC1 repeat cannot be treated as physically independent from the other EC repeats. During cell–cell adhesion, there is an initial rapid stage for selectivity through the outermost ectodomain repeats (EC1 and EC2) and then a slower second stage that requires EC3 to form strong adhesive bonds through the activation of the full ectodomain.[29,30] Therefore, while trying to mimic cadherin

binding, a mimetic peptide might not be sufficient to achieve the desired effect like in the case of mimicking integrin binding.

Another interesting fact for tissue engineers to know is that classical and desmosomal cadherins are synthesized with a prodomain. Prodomains are cleaved off before the cadherin matures in the Golgi. The prodomains prevent the W2 residue on one EC repeat from forming and bond with that on the adjacent repeat.[31] These prodomains could be used by tissue engineers if they wish to control the activation of engineered cadherins.

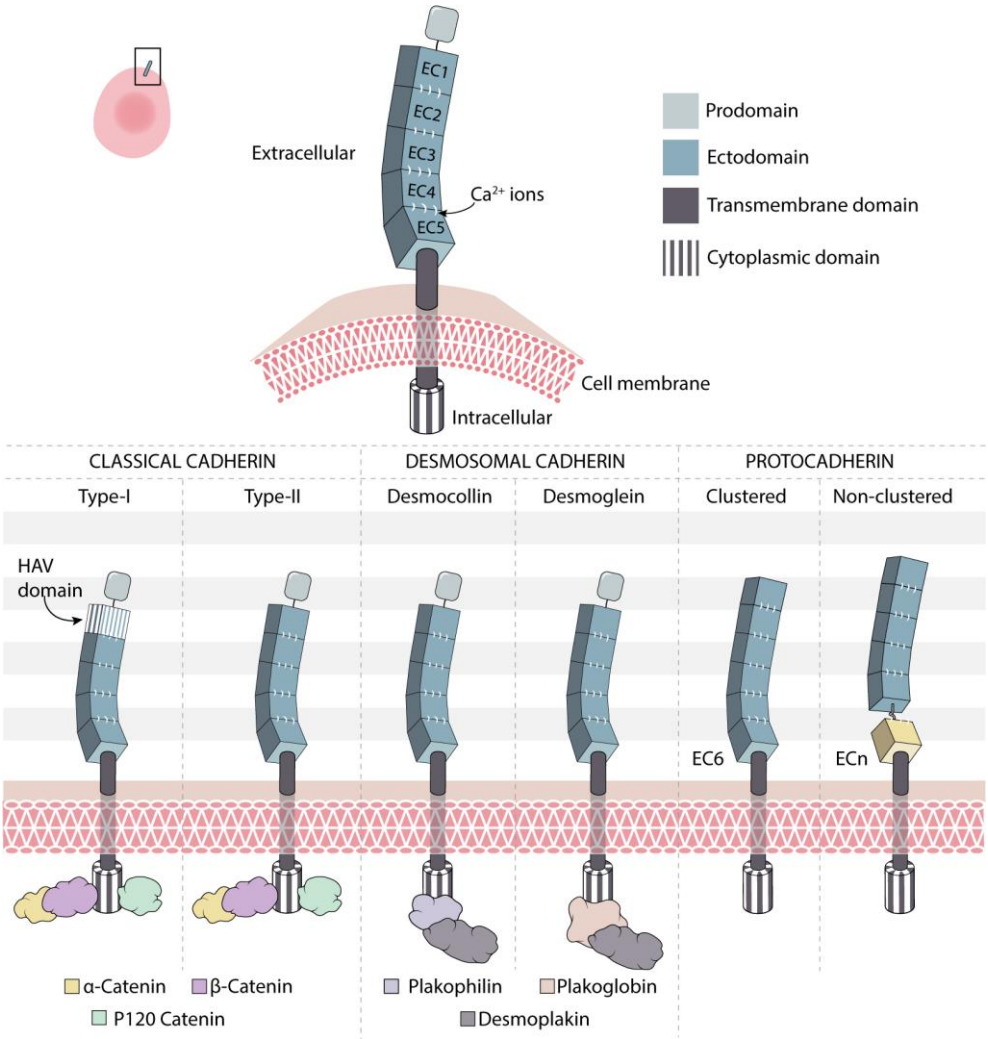

Figure 1. Structure and diversity of cadherin superfamily

Schematic overview of the cadherin family of cell adhesion molecules.[15] All cadherins possess the ability to bind calcium ions. Most cadherins are transmembrane proteins with a cytoplasmic domain capable of binding to different proteins. Classical and desmosomal cadherins feature a cleavable prodomain, an ectodomain with five EC repeats, and a cytoplasmic domain that binds to various catenins. Clustered protocadherins have six EC repeats and variable cytoplasmic domains. Nonclustered protocadherins have variable EC repeats as well as variable cytoplasmic domains.

FUNCTIONAL SIGNIFICANCE FOR TISSUE ENGINEERING

It is tempting to view the cadherins in simple, structural terms as a glue that holds cells together, but as is often the case with biology, cadherins are far more complex. They are involved in processes such as tissue organization, cell proliferation, and differentiation. Since cadherins act as the link between the neighboring cell and the cytoskeleton, it seems natural that these complexes act as a nexus to a variety of signaling pathways that drive a wide variety of cellular functions.[32] The diverse palette of cadherin proteins gives rise to different functions upon cell–cell contact, from adhesion to repulsion and everything in between. Recent advances have given us a more comprehensive description of how cadherins function and so we will explore the functions important from a tissue engineering perspective.

The most prominent function of cadherins is that they are responsible for cell–cell adhesion, cell–cell recognition, and cell segregation, which are all important functions from a tissue engineering perspective. The ability of type-I classical cadherins to mediate the segregation of different cell populations as well as mediate interactions between different cell populations is well documented.[33,34] Any given cell generally expresses multiple cadherin subtypes, and different cell types are in contact with each other in tissues. In culture, while mixing two cell types, we should be aware that cells with different cadherin subtypes do not segregate unless their expression levels are quantitatively different (Figure 2A).[35,36] If two different cell types express the same levels of cadherin subtypes, then they might undergo homophilic or heterophilic adhesion depending on the respective cadherins (Figure 2A). This can have important functional implications. For example, the homophilic interaction of cadherin-2 (also known as N-cadherin) a type-I classical cadherin between mesenchymal stem cells and human islets in mice resulted in enhanced insulin secretion by the islets.[37] It was widely assumed that cadherins only form

homophilic bonds, but researchers studying classical cadherins have shown that cadherins form heterophilic bonds.[38,39] For type-II classical cadherins, heterophilic interactions were preferred over homophilic interactions (Figure 2B).[40] These heterophilic interactions were found to be selective and unique to members of specific subsets within type-II classical cadherins.[40]

While type-I classical cadherins and desmosomal cadherins are primarily found in tissues where a high degree of cell cohesion and adhesivity is necessary, type-II classical cadherins and protocadherins are expressed where cells are more motile and cell–cell interactions are transitionary. Furthermore, it is the ectodomain, not the cytoplasmic domain, that is responsible for the differences in the degree of adhesiveness.[41] Cell–cell adhesion mediated by type-II classical cadherins has a higher rate of turnover compared to that mediated by type-I classical cadherins.[42] Type-II classical cadherins are expressed in complex patterns in the nervous system which implies that they also have an important role in organization and connectivity. A recent study unveiled that type-II classical cadherins are responsible for the organization of motor neurons into pools and this was only evident in the absence of cadherin-2.[43] From cancer research, we have learned that loss of cadherin-1 (also known as E-cadherin), a type-I classical cadherin, followed by an increase in cadherin-11 (also known as OB-cadherin), a type-II classical cadherin, results in an invasive phenotype.[44] This suggests that type-I classical cadherins may maintain a basal adhesive level and are mostly involved in the formation and maintenance of cohesive tissue, while type-II classical cadherins play a more intricate role in tissue orchestration, and are more involved in functions such as migration and differentiation. The knowledge of cadherin expression levels, adhesive specificity, and crosstalk between type-I and type-II classical cadherins are crucial for tissue engineers to consider in their substrate design.

Cadherins are also known to regulate cell proliferation. Cadherin-1, regulates cell proliferation by recruiting signaling proteins and actin regulators such as Rho family GTPases.[45] Cadherin-2 overexpression inhibits proliferation *in vivo* and *in vitro* by reducing the expression of Wnt-responsive genes such as c-myc and cyclin D1.[46] Using the right cadherins could help in the expansion of cells to produce the large quantity needed to regenerate tissue defects.

Another cell behavior the cadherins influence is differentiation.[47] We know that under the physiological state, cells tend to express specific cadherin proteins to maintain tissue integrity, while under pathological conditions, cells express inappropriate cadherins, which has a significant effect on cell behavior. For example, a requisite for apical abscission, a critical step during neurogenesis, is the loss of cadherin-2 and when cadherin-2 is persistently expressed it inhibits the apical cell–process withdrawal.[48] Cadherin-11, is implicated in adipogenic differentiation because the loss of cadherin-11 inhibits the mesenchymal stem cells from differentiating towards the adipogenic lineage.[47] The involvement of cadherins in crucial differentiation steps makes them vital for timely use to exploit the differentiation potential of stem cells for regenerative medicine.

Furthermore, modulating cadherin activity can also influence important signaling pathways. For example, when β-catenin is bound to the cadherin, it regulates cell adhesion and also acts as a transcriptional regulator downstream of the canonical Wnt pathway.[49] p120-catenin affects Rho family GTPase activity and stabilizes classical cadherins at the plasma membrane.[50] In the absence of p120-catenin, cadherins are internalized through endocytosis. Integrins connect the cytoskeleton of the cells to the ECM in protein complexes called focal adhesions. Vinculin binds to talin and actin in focal adhesions and binds to α-catenin and actin at the adherens junction based on site-specific tyrosine phosphorylation.[51] The adherens junctions

and focal adhesions are both linked to the actin cytoskeleton, and they recruit many more common signaling proteins like focal adhesion kinase (FAK) and Rho family GTPases, creating permanent crosstalk between the two systems.[52,53]

Many growth and proliferation promoting signals are initiated at the cell surface by receptor tyrosine kinases (RTK), which are activated by soluble growth factors such as VEGF, EGF, and FGF. Cadherin–catenin complexes can physically interact with RTKs and impact their signaling capabilities.[54] For example, cadherin-1 is known to localize and activate EGFR to maintain barrier function.[55] Similarly, cadherin-2 activates FGFR, stabilizes it, and inhibits its degradation.[56]

In endothelial cells, in the presence of VEGF, FAK binds to cadherin-5 (also known as VE-cadherin) and phosphorylates β-catenin, which leads to a disrupted barrier function.[57] This particular example of the barrier function demonstrates opposing functions of adherens junctions and focal adhesions that allow cells to actively adapt to changes in their environment.[58] Other signaling pathways that crosstalk with cadherins include the transforming growth factor beta (TGFβ) pathway that includes bone morphogenetic proteins (BMPs). For example, BMP preserves a population of undifferentiated mouse embryonic stem cells by maintaining high levels of cadherin-1.[59] BMP6 promotes the internalization of cadherin-5 causing endothelial cell permeability via Src phosphorylation.[60] A combination of soluble growth factors, integrin activation, and targeting the appropriate cadherins could give tissue engineers more powerful tools to control cell fate.

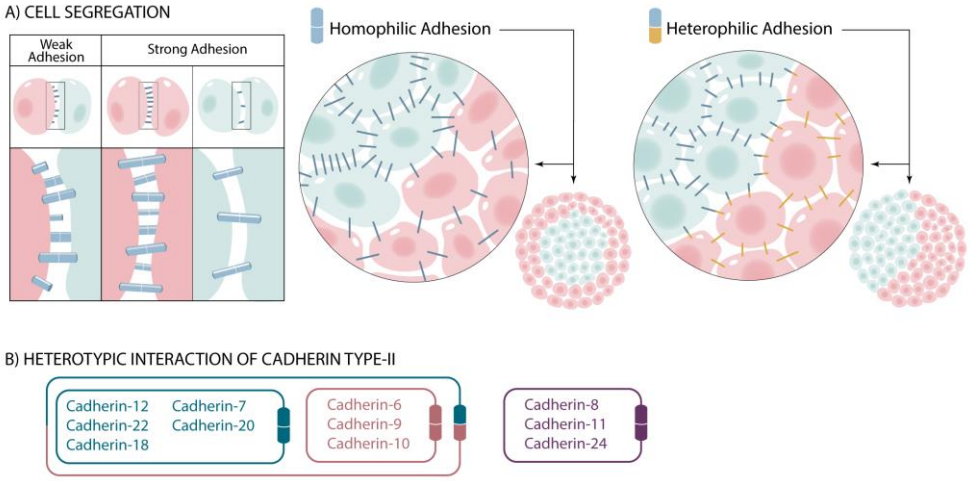

Figure 2. Functions of cadherins

(A) Strong cadherin adhesion is dependent on the cadherin expression being quantitatively similar. Purely quantitative differences in expression levels of a single cadherin suffice to produce rearrangements in cells in the absence of any difference in cadherin subtypes. On a tissue scale, high- and low-cadherin (red) populations lead to sorting into concentric shells, as low cadherin cells still prefer to bind high cadherin cells. In contrast, two cell populations that express either different cadherins (red and green) will sort into distinct compartments if these cadherins prefer homophilic adhesion to heterophilic adhesion. (B) Shapiro and colleagues showed three distinct specificity groups within type-II classical cadherins that share highly similar heterophilic binding patterns and prefer binding to one another.[40]

OPPORTUNITIES FOR ENGINEERED CADHERINS IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Knowing that cadherins are an important driving force in a variety of cellular behavior, tissue engineers can use that knowledge to engineer therapeutically relevant tissues. In recent years cadherin mimicking peptides have been used to mediate cell–biomaterial interactions for stem cell–based regeneration. For example, materials coated with cadherin-1 mimetic peptide, HAV enhanced cell adhesion of epithelial cells as well as the downstream signaling of β-catenin.[61] Hydrogels functionalized with the cadherin-2 mimetic peptide, HAV enhanced osteogenic markers in human mesenchymal stem cells (hMSCs) in both *in vitro* and *in vivo* conditions.[62] The hypothesis is that the cadherin-2 mimetic peptide emulates cadherin-2 mediated cell–cell adhesion that occurs during mesenchymal condensation, and therefore promotes the osteogenic differentiation of the hMSCs. The cadherin-2 mimetic peptide, HAVDI (histidine-alanine-valine-aspartic acid-isoleucine), which has a greater affinity for cadherin-2 than HAV alone, enhances chondrogenesis and modulates matrix mechanosensing and fate commitment of MSCs.[63,64] Likewise, chondrogenesis can be enhanced with the peptide by reducing the nuclear translocation of β-catenin, thereby inhibiting Wnt/β-catenin signaling.[65]

In some cases, synthetic peptides are capable of mimicking the function of their full-length endogenous counterparts, but when it comes to cadherins, the mimetic peptides are usually not equal to the cadherin protein. Fortunately for tissue engineers, fragments of the full-length protein can be effective if chosen wisely. For example, the full-length ectodomain, cadherin-2-EC1-5, has the greatest impact on nuclear localization of YAP/TAZ proteins and myogenic differentiation of MSCs compared to cadherin-2-EC1-2 and the HAVDI sequence.[26] The higher efficiency of the protein-based adhesion may result in higher intracellular force, which better

activates YAP/TAZ. Similarly, a full-length ectodomain, cadherin-1-EC1-5, promotes efficient cell spreading when compared to cadherin-1-EC1-2.[66]

To improve the quality of engineered tissue, using protein-tagged cadherins eg, IgG fragment crystallizable region (Fc)-tagged or SNAP-tagged cadherin is worth considering over direct chemical conjugation of cadherin proteins (Figure 3A).[66,67] Tissue culture substrates can be functionalized with Fc-tagged cadherin fusion proteins to influence cell behavior and theoretically so can a 3D hydrogel matrix (Figure 3B). Similar to culture dishes coated with Fc-cadherin-2 fusion protein showed an increased expression of cadherin-2 during neural differentiation, whereas the expression of cadherin-1 decreased.[68] The presence of Fc-cadherin-2 fusion protein on surfaces increased the yield for mouse pluripotent stem cell–derived neural progenitor cell differentiation by the suppression of Rho/ROCK signaling.[69] Similarly, Fc-cadherin-1 fusion protein incorporated on poly(lactic-co-glycolic acid)-microparticles enhanced cell proliferation and cytokine secretion in 3D MSC aggregates.[70] Mouse embryonic stem cells cultured on surfaces coated with Fc-cadherin-1 fusion protein, maintained their morphological features and, when compared to cells grown under standard conditions, had a higher proliferative ability.[71] Mouse embryonic stem cells grown on Fc-cadherin-5 fusion protein, coated surfaces maintained their pluripotency by activating the Stat3 signaling pathway that promotes self-renewal and prevents differentiation.[72] Materials designed for human pluripotent stem cell propagation should consider using cadherin-mediated adhesions rather than only integrin-mediated adhesions to preserve pluripotency.

Fc-tagged cadherin fusion proteins can also be used to prevent undesired cellular behavior. Vertical surfaces coated with the Fc-cadherin-1 fusion protein that was used in a wound-healing assay behaved as three dimensional tissue that the healing tissue could self-heal against and later terminate the healing.[73] This stop signal

achieved by using cadherin-1 coating was later used as a technique to functionalize titanium surfaces with Fc-cadherin-1 fusion protein to prevent epidermal ingrowth around percutaneous implants.[74] Surfaces modified with Fc-cadherin-1 fusion protein could also inhibit cell apoptosis under serum-deprived conditions.[75]

Furthermore, cadherin mimetic peptides coupled to porous biomaterials can be used to mediate paracrine signaling. For example, porous materials promoting cadherin-2 mediated cell–cell adhesion, enhanced paracrine signaling of rat MSCs.[76] MSCs encapsulated in nanoporous hydrogels with the HAVDI peptide showed heightened paracrine activity in response to IGF-1 (insulin-like growth factor 1).[77] This is important for tissue engineering because paracrine signaling helps MSCs orchestrate desirable functions.

Modulating cadherins can also be combined with the modulation of integrin-based signaling (Figure 3c). Studies conducted with cadherin-2 coated surfaces revealed that fibronectin matrix polymerization disrupts cadherin-2 and affects its ability to mediate adhesion.[78] Functionalized surfaces with a protein chimera integrating the functional unit of fibronectin and cadherin-11-EC1-2, was beneficial for hMSC adhesion, proliferation, and differentiation compared to the fibronectin or cadherin-11 alone.[79,80] Functionalized micropatterned surfaces comprising alternating stripes of type-VI collagen and Fc-cadherin-1 fusion protein showed that type-VI collagen was required for cell migration, while Fc-cadherin-1 fusion protein dampened lamellipodia activity.[81] Another patterned surface study of RGD and histidine-cadherin-1 fusion protein provided the optimal conditions to promote hMSC differentiation.[82]

However, our knowledge of signaling interdependencies between cell–cell and cell–ECM interaction is limited, and we require more studies to have a deeper

understating of its mechanics. Surface protein density can modulate different cellular responses (Figure 3c). For example, varying combinations of Fc-cadherin-2 fusion protein promote neuronal differentiation and maturation of neuronal stem cells. Low concentrations of Fc-cadherin-2 fusion protein enhanced neuronal differentiation and survival of neuronal stem cells compared to higher concentrations of Fc-cadherin-2 fusion protein.[83] Finally, optimizing the timing of the presentation of proteins can regulate cell behavior. HAV peptide with a cleavable-ADAM10 domain regulated chondrogenic differentiation within a hydrogel indicating that the dose and timing of the peptide are crucial.[84]

Antibodies, antibody fragments, and aptamers have also been used to modulate cadherins to achieve a therapeutic solution or serve as an imaging modality. An antibody specifically targeting the EC1 ectodomain in cadherin-3 (also known as P-cadherin) a type-I classical cadherin, disrupts cell adhesion.[85] Similar antibodies are available to target other cadherins. The epitopes that antibodies can target to block cadherin adhesion have already been mapped.[86] Recently, a DNA aptamer targeting cadherin-2 has shown efficiency in detecting circulating tumor cells, thereby providing a cost-effective method to improve cancer diagnostics.[87] A study showed that using hydrogels functionalized with both immobilized RGD peptide and antivascular endothelial growth factor receptor-2 DNA aptamer enhanced the angiogenic potential of endothelial cells.[88] There is no reason why similar solutions, namely combining cadherin mimetic peptides and DNA aptamers cannot be used in tissue engineering approaches.

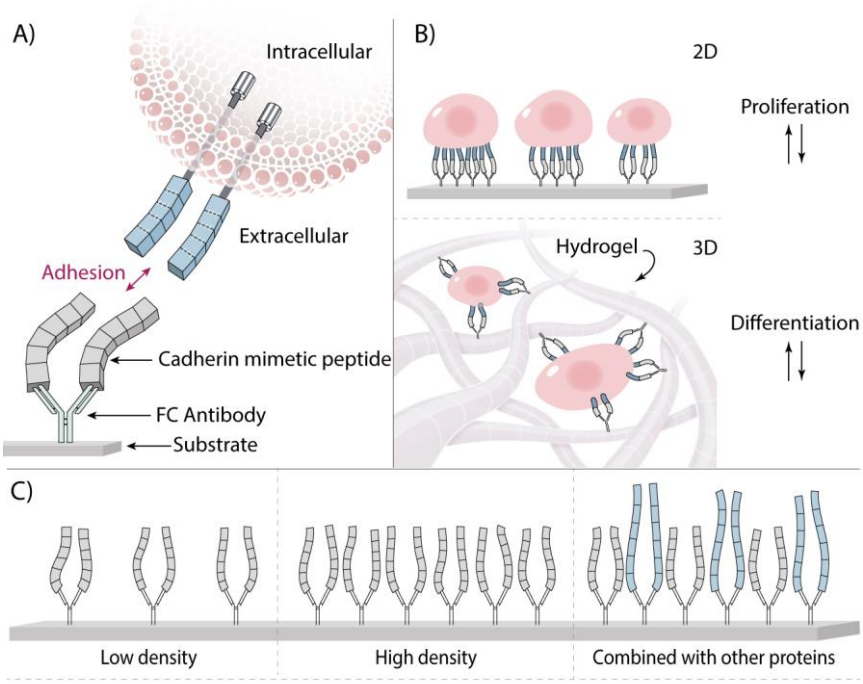

Figure 3. Opportunities for engineered cadherins in tissue engineering and regenerative medicine

(A) Synthetic peptides are capable of mimicking the function of their full-length endogenous counterparts. (B) Tissue culture substrates can be functionalized with cadherins to influence cellular behavior. (C) Surface protein density can modulate different cellular responses. Modulating cadherins can also be combined with modulation of integrin-based signaling.

BIOLOGICAL TOOLS TO MODULATE CADHERIN EXPRESSION

Recent advances in stem cell engineering have enabled scientists to create complex multicellular structures *in vitro* that resemble their physiological counterparts. The development of organoids derived from human pluripotent stem cells holds great promise to restore the functionality of a compromised organ or tissue during disease progression. Recreating a human tissue containing a high degree of structural and functional resemblance requires a precise structural organization within the multicellular heterogeneity. Therefore, the creation of organoids heavily relies on intrinsic self-organization capabilities. Interestingly, recent bioengineering approaches have been developed to direct the self-organization properties of cells by modulating cell–cell interactions.[89] These interactions are primarily mediated by the cadherins, which have been reported to be essential for the spatial organization of cells.[89] They promote selective recognition of cells by expressing certain subtypes that are specific for a cell population within a multicellular system, eventually resulting in an organized cell assembly.[90] Moreover, the sorting of cell populations can also be directed by the differential expression levels of cadherins in specific cell types, resulting in a reduced cell–cell contact favoring cell migration and self-patterning.[91] The rise of precise genome-engineering approaches, such as CRISPR/Cas9, has paved the path to manipulate cell–cell contacts to steer tissue morphogenesis.[92] The importance of the cadherins in this approach has been highlighted by recent studies where different cell populations were modified to express different cadherin subtypes.[93] An elegant study created a mosaic knockdown population for cadherin-1 using CRISPR interference, resulting in an alteration in self-organization capabilities and therefore changes in cellular patterning.[93] A similar observation was made by studying the patterning activity in 2D and 3D populations by modifying the expression level of specific cadherins using

an overexpression system.[94] It is clear that modulating the expression levels of cadherins result in a system containing self-assembling properties being able to better control for symmetry breaking and self-organization.[95] These advances highlight the importance of cell adhesion molecules, such as cadherins, in bioengineering strategies to more efficiently steer self-organization and to direct human organoid morphogenesis.

CONCLUDING REMARKS

The prize for overcoming challenges in regenerative medicine and tissue engineering goes to biomimicry. This trend was spearheaded by integrins and the discovery of ECM mimetic peptides such as RGD. However, in recent years, modulation of cadherins has also garnered a great deal of interest. For cadherins, bio-functionalized synthetics can help improve reproducibility between batches that can be produced at an industrial scale. From the reviewed studies, we have seen that biomaterial surfaces are generally randomly decorated with the cadherin peptides. However, adherens junctions usually comprise mosaics of cadherin clusters. Scientists need to make use of the existing technology to develop a controlled presentation of cadherins peptides on biomaterial substrates, which could very well be used for multicellular organization and improved cellular function. As researchers expand the toolbox of peptides and proteins, they should also deepen their knowledge of specific molecular regulators. To build hierarchical structures for a proper multicellular organization using cadherins, we need to investigate the temporal dynamics of classical cadherin expression, the quantitative level of cadherin expression, and the cross-talk between type-I classical cadherins and type-II classical cadherins.

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3

Cell culture dimensionality influences mesenchymal stem cell fate through cadherin-2 and cadherin-11

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ABSTRACT

The acquisition of a specific cell fate is one of the core aims of tissue engineering and regenerative medicine. Significant evidence shows that aggregate cultures have a positive influence on fate decisions, presumably through cell-cell interactions, but little is known about the specific mechanisms. To investigate the difference between cells cultured as a monolayer and as aggregates, we started by looking at cadherin expression, an important protein involved in cell adhesion, during the differentiation of bone marrow-derived human mesenchymal stem cells (hMSCs) in aggregate and monolayer cultures. We observed that proliferating hMSCs in monolayer culture expressed lower levels of cadherin-2 and increased cadherin-11 expression at cell-cell contact sites over time, which was not evident in the aggregate cultures. By knocking down cadherin-2 and cadherin-11, we found that both cadherins were required for adipogenic differentiation in a monolayer as well as aggregate culture. However, during osteogenic differentiation, low levels of cadherin-2 were found to be favorable for cells cultured as a monolayer and as aggregates, whereas cadherin- 11 was dispensable for cells cultured as aggregates. Together, these results provide compelling evidence for the important role that cadherins play in regulating the differentiation of hMSCs and how this is affected by the dimensionality of cell culture.

INTRODUCTION

For regenerative medicine, cells are typically studied as a monolayer on a flat surface, which does not reflect the conditions most cells experience *in vivo*. Studying cells on flat surfaces strips them of many cell-cell interactions and introduces them to a foreign adherent environment. For example, monolayer culture systems, while perfectly suitable for studying some processes in the dermal epithelium, can fall short for studying developmental processes that do not involve cells in a monolayer.[1] As regenerative medicine relies on accurately recapitulating these developmental processes for organ regeneration, culturing or even transplanting aggregates of cells is considered a promising strategy due to enhanced cell-cell interaction and because aggregates more closely mimic the natural environment of a tissue.[2,3] Indeed, cell behavior in aggregate culture systems is different from that in monolayer systems[4], and studies have revealed molecular differences between monolayer and aggregate culture systems.[5–7] For example, it has been shown that AKT and mTOR signaling is drastically reduced in aggregate culture systems.[5]

Differences in the dimensionality and geometry of cells between monolayer and aggregate culture systems can lead to different cellular responses.[8] Aggregate culture systems not only influence the spatial organization of the cell surface receptors engaged in interactions with surrounding cells, but they also induce physical constraints to cells.[9] These systems can affect signal transduction and ultimately influence gene expression and cellular behavior. For example, it has been shown that cadherin-based cell-cell interaction increases compared to integrin-based cell-matrix adhesion in aggregates.[10,11] Adhesion formation on flat surfaces with a focus on integrins is well documented[12,13] and also studied in aggregate systems, where it was suggested that the type of integrin employed by the cell is

differentially specified by the dimensionality of the microenvironment.[14] Like integrins, biomaterials can also be modified to target cadherins as a way of creating a more physiologically relevant setting and influencing cell fate.[15,16] Compared to integrin-based adhesion, less is known about cadherin-based adhesion in aggregate cultures and how this can be used in the design of biomaterials.

To this end, we investigated the influence of cadherin-mediated, cell-cell interaction on the differentiation of bone marrow-derived human mesenchymal stem cells (hMSCs) in monolayers and aggregates. hMSCs are nonhematopoietic multipotent cells that have the potential to differentiate into a variety of cell types, including, but not limited to, osteoblasts and adipocytes.[17] The human cadherin superfamily comprises of over 100 different proteins.[18] In an adult, cadherins bind cells with each other in the presence of calcium ions that give form to different tissues. Cadherins not only maintain tissue integrity but also have a role in diverse biological processes such as differentiation, proliferation, polarity, and stem cell maintenance.[19–21] The diversity of the cadherin family makes them capable of taking on such varied functions.[22,23] Different types of cadherins are expressed in different types of cells; cadherin-2 and cadherin-11 are expressed in mesenchymal-type cells.[24–26] Cadherin-2 has been implicated in neurogenesis[27], synaptogenesis[28], lens cell differentiation[29], and the development of an osteoblastic phenotype in rats [30,31] to name a few. Cadherin-11, on the other hand, has been implicated in osteogenic, chondrogenic, and myogenic differentiation.[32,33] Alterations of cadherin-2 and cadherin-11 expression are associated with malignant mesenchymal tumors.[34] Increased cadherin expression is observed in aggregated cardiac cells compared to cells cultured as a monolayer.[35] Because cadherin-2 and cadherin-11 are implicated in cell differentiation, and cadherins are implicated in the differential adhesion hypothesis[36], we hypothesized that cadherin-2 and cadherin-11 could influence

osteogenic and adipogenic differentiation differently in monolayer and aggregate cultures.

We show that cadherin-2 and cadherin-11 maintain their expression over time in aggregate cultures, whereas their expression switches from cadherin-2 to cadherin-11 in monolayer culture. Knockdown of cadherin-11 increases cadherin-2 but not vice versa suggesting a one-directional relationship between the cadherins. Functionally, knockdown of cadherin-2 and cadherin-11 led to alterations in the potential of hMSCs to differentiate towards the osteogenic and adipogenic lineages, underscoring the critical role of cell-cell interaction in directing cell fate. While cadherin-2 enhanced mineralization in both culture formats, cadherin-11 interfered with mineralization only in monolayer culture, which corroborates that monolayer and aggregate cultures yield different cell behavior.

RESULTS

1. Phenotype identification and trilineage differentiation potential of hMSCs.

We first determined the phenotype of hMSCs by assessing them at passage 5 for representative markers by flow cytometry, and by determining their trilineage differentiation potential by using established protocols, all according to the International Society for Cellular Therapy standard.[37] The cultured hMSCs were positive for mesenchymal markers CD73 (97.9%), CD90 (99.6%), and CD105 (95.2%) (Figure S1 A-C) and negative (≤0.014% positive) for hematopoietic markers CD45, CD34, CD11b, CD19, and HLA-DR (Figure S1D). The cells successfully differentiated into osteogenic, adipogenic, and chondrogenic lineages, which were determined using histological staining with Alizarin Red S (Figure S2A), Oil Red O (Figure S2B) and Safranin O (Figure S2C), respectively.

2. Cadherin-2 expression decreased over time in monolayer cultures.

To investigate whether cadherin expression changes over time when hMSCs are cultured as a monolayer, we measured cadherin-2 and cadherin-11 levels at days 1, 5, 7, and 10 (Figure 1A). In monolayer cultures, hMSCs expressed significantly decreased cadherin-2 levels over time, whereas cadherin-11 levels remained similar across the four time points (Figure 1 B and C). Similarly, to investigate whether cadherin expression changes over time when hMSCs were cultured as aggregates, we measured cadherin-2 and cadherin-11 levels at days 1, 5, and 10. (Figure 1D). In aggregate cultures, cadherin-2 expression remained low at all time points, and cadherin-2 expression was lower than cadherin-11 expression (Figure 1E). The levels of both cadherin-2 and cadherin-11 remained similar across the three time points (Figure 1E). These findings show that cadherin-2 expression remains low over time in culture in aggregates, in contrast to a monolayer where the cadherin-2 expression decreases over time.

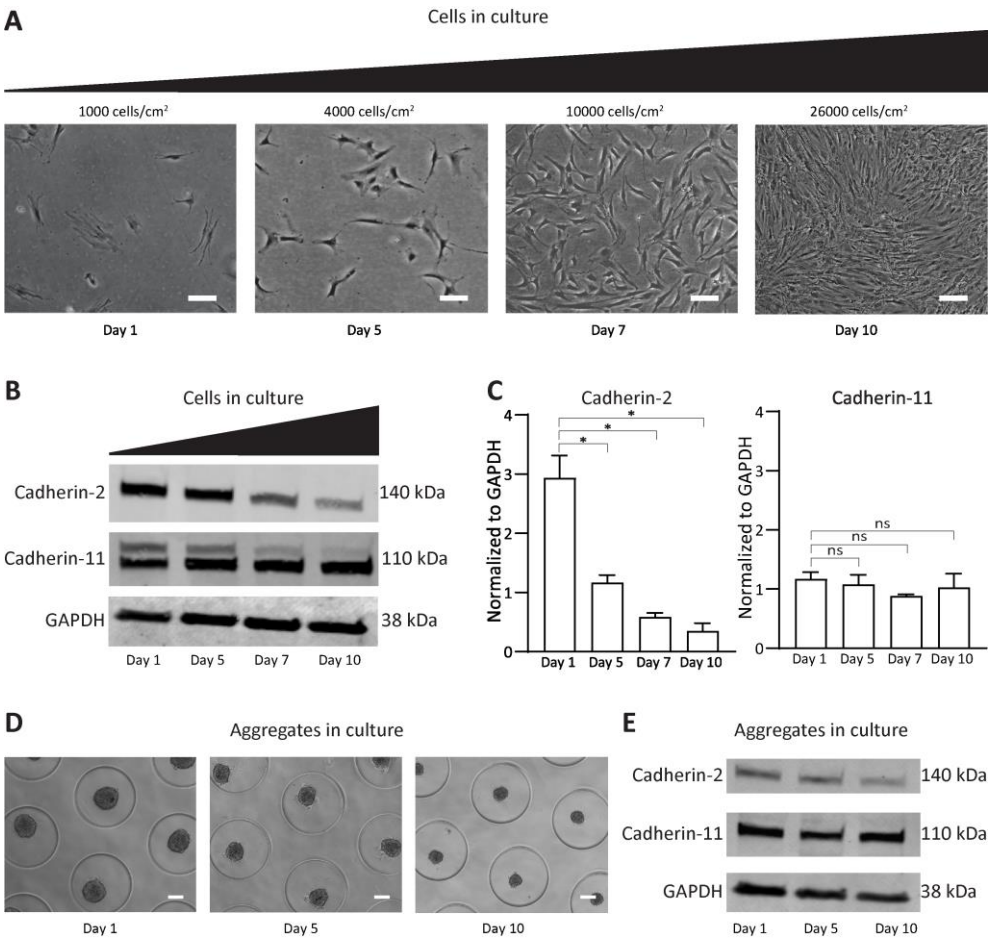

Figure 1. Cadherin-2 levels are inversely related to hMSC cell density in monolayer cultures

HMSCs were seeded at 1 × 10^4 cells/cm² and evaluated after days 1, 5, 7, and 10. (A) Phase contrast micrographs showed increasing cell density from day 1 to day 10. Scale bars represent 100 μm. (B) Western blots showed decreasing cadherin-2 and stable cadherin-11 expression over time. GADPH is shown as a loading control. (C) Quantification of Western blots normalized to GAPDH showed that cadherin-2 significantly decreased over time while levels of cadherin-11 remained the same. (D) Phase contrast micrographs showed aggregates cultured in microwells from day 1 to day 10. Scale bars represent 100 μm. (E) Western blots showed cadherin-2 and cadherin-11 expression at days 1, 5, and 10. GAPDH is shown as a loading control. Error bars show ± SD. Data are representative of at least three independent experiments with similar results. Statistics were determined using one-way ANOVA with Holm-Sidak's test for multiple comparisons: $^*p < 0.01$; ns, not significant.

3. Cadherin-2 differentially expressed in hMSCs in cultures of different dimensionality.

hMSCs were seeded as a monolayer and as aggregates, and cadherin levels were measured after hMSCs attained confluency in monolayer (approximately 5 d) or formed aggregates (24 h) in aggregate cultures. This timepoint, denoted day 0, indicated the start of hMSC differentiation (Figure 2 A and B). We found that hMSCs in aggregate cultures expressed significantly lower levels of cadherin-2 compared to those cultured in monolayers (Figure 2 C and D). In comparison, cadherin-11 levels were similar for both monolayer and aggregate cultures at day 0 (Figure 2 C and D). In monolayer culture on day 1, cadherin-2 and cadherin-11 were localized at cell-cell contact sites (Figure 2E). Over time, on day 21, cadherin-2 expression substantially decreased in culture, while the expression of cadherin-11 increased at the cell-cell contact sites (Figure 2E). The varied distribution of cadherin-11 did not affect the total levels of cadherins in monolayer culture in growth medium (Figure S3A). In aggregate culture, hMSCs expressed cadherin-2 as well as cadherin-11, which was consistent over time (Figure 2F).

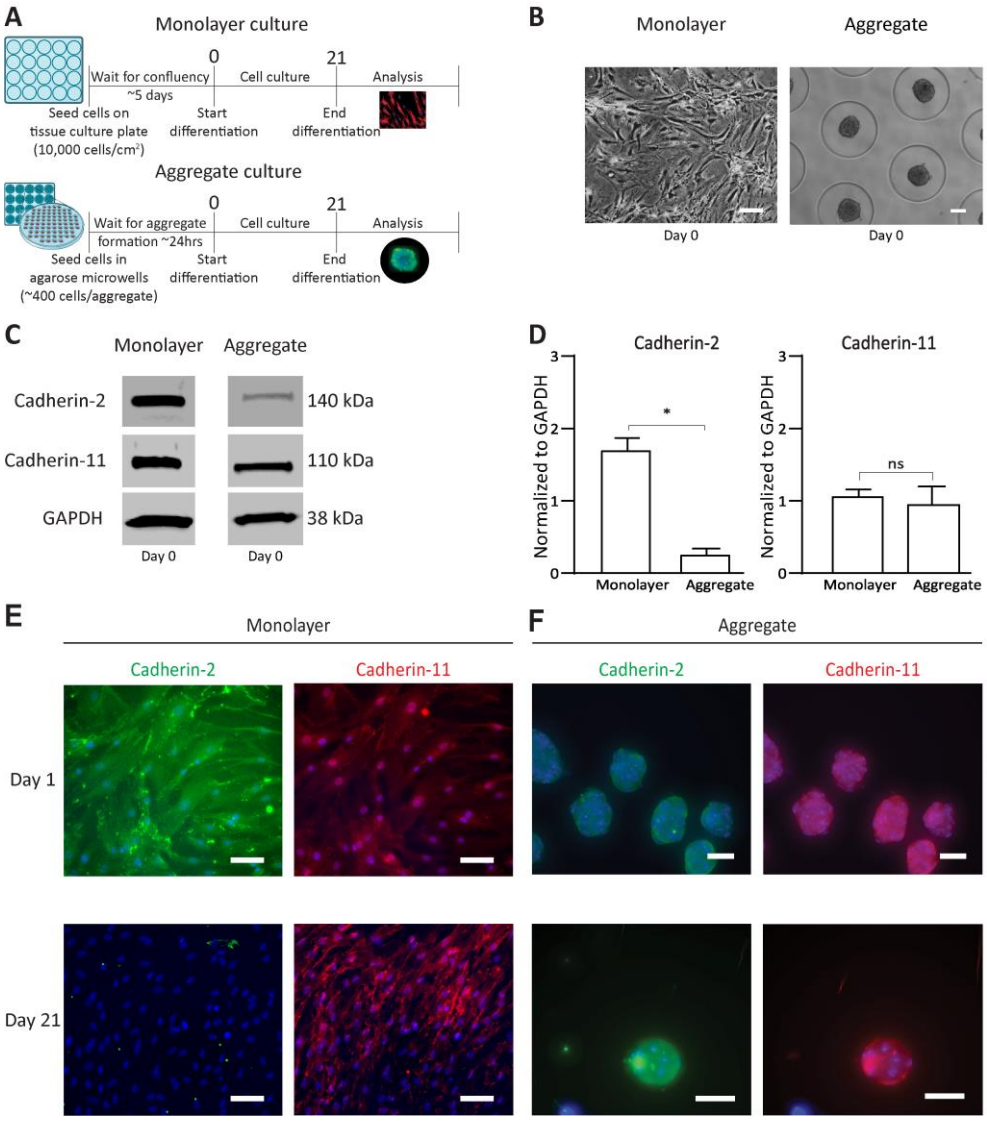

Figure 2. hMSC cultures and expression of cadherin-2 and cadherin-11

(A) Schematic timeline of the cells cultured as a monolayer and as aggregates. (B) Phase contrast micrograph of hMSCs plated as a monolayer and seeded as aggregates in microwells at day 0. Scale bars represent 100 μm. (C) Western blot indicates lower cadherin-2 expression in aggregate culture compared to monolayer culture at day 0, while cadherin-11 expression was similar. GAPDH is shown as a loading control. (D) Quantification of Western blots normalized to GAPDH expression showed a significant decrease in cadherin-2 in aggregate culture compared to monolayer culture, while levels of cadherin-11 showed no significant difference. Error bars show ± SD. Data are representative of at least three

independent experiments with similar results. Statistics were determined using one-way ANOVA with Holm-Sidak's test for multiple comparisons: $*p < 0.01$; ns, not significant. (E) Fluorescence micrographs of immunostained hMSCs, cultured as a monolayer with cadherin-2 (green, left column) and cadherin-11 (red, right column), and counterstained with DAPI (blue) showed that cadherin-2 expression decreased between day 1 (top row) and day 21 (bottom row). (F) Fluorescence micrographs of immunostained hMSCs, cultured as aggregates with cadherin-2 (green, left column) and cadherin-11 (red, right column), and counterstained with DAPI (blue) showed that cadherin-2 and cadherin-11 expression were expressed similarly across both timepoints, different from their expression in the monolayer culture.

4. Differentiation pathways influenced cadherin levels in monolayer culture but not in aggregate cultures.

To investigate whether cadherin expression changed as hMSCs were cultured in differentiation medium, the cells were cultured in osteogenic inductive medium and adipogenic inductive medium and immunostained for cadherin-2 and cadherin-11 at days 1 and 21 for cells cultured as a monolayer, and days 1 and 10 for cells cultured as aggregates. Cadherin expression in cells cultured as a monolayer was influenced by inducing differentiation. On day 1, cadherin-2 was expressed at the cell-cell contact sites (Figure 3 A and E), which significantly decreased by day 21 in both inductive medium conditions (Figure 3 B and F). In agreement, we found decreased levels of cadherin-2 at day 21 (Figure S3A). Cadherin-11 at day 1 was expressed around the nucleus (Figure 3 C and G). On day 21, we observed different levels of cadherin-11 expression over time, depending on the medium in which the hMSCs were cultured in monolayer (Figure 3 D and H). Namely, osteogenic induction medium resulted in substantially higher levels of cadherin-11 at the cell-cell contact sites (Figure 3D) compared to cells in adipogenic inductive medium (Figure 3H). Western blots showed decreased cadherin-11 expression on day 21 compared to day 1 in cells cultured in adipogenic medium (Figure S3A). These data show that for the monolayer culture, cadherin-2 levels decreased over time, while cadherin-11 levels changed depending on the medium in which they were cultured.

Unlike the situation in monolayer culture, it appeared that cadherin expression in aggregates was uninfluenced by differentiation. In aggregate cultures, cadherin-2 showed similar immunostaining at both time points and with both differentiation media (Figure 3 I, J, M, N), which was also observed with cadherin-11 (Figure 3 K, L, O, P); these observations indicate differentiation media did not affect cadherin expression in aggregate cultures. Western blots confirmed that the cadherin expression remained consistent over time in aggregate culture, unlike in monolayer culture (Figure S3B).

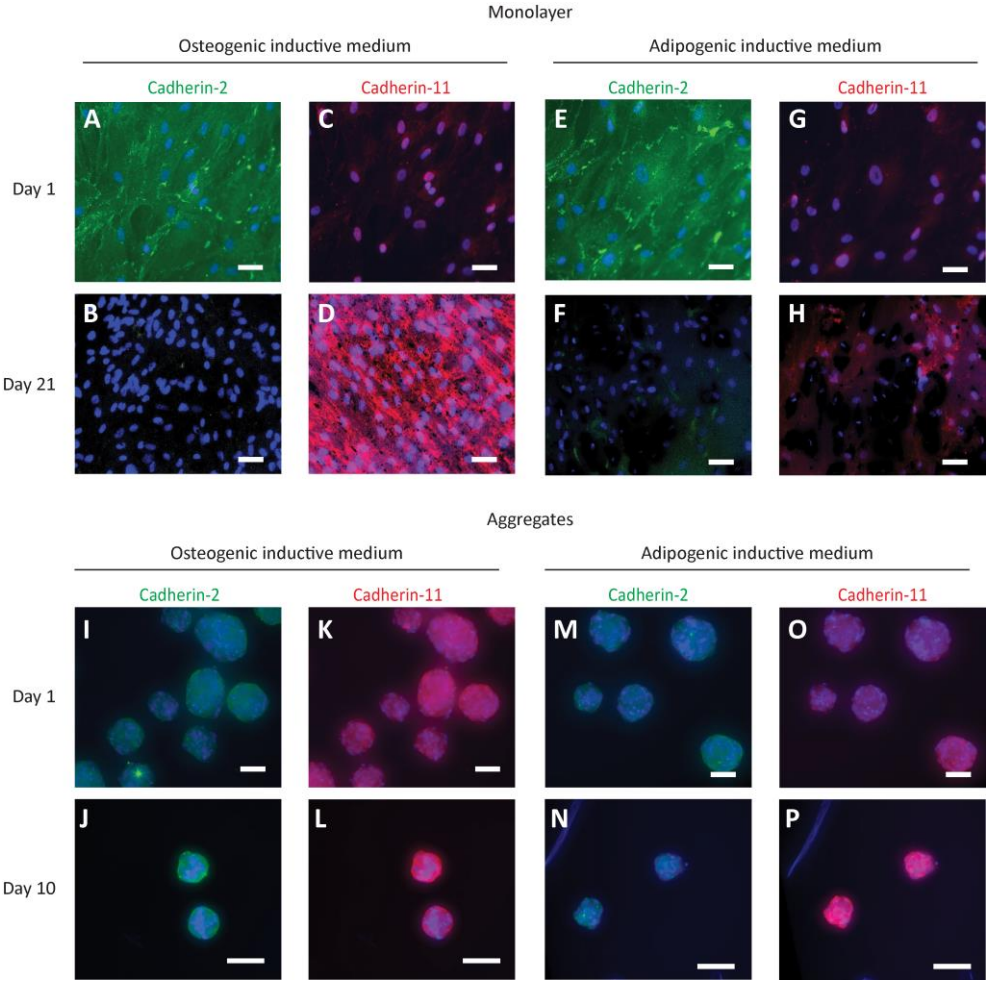

Figure 3. Differentiation medium conditions induced variation in cadherin-2 and cadherin-11 levels in hMSCs

Fluorescence micrographs of hMSCs immunostained with cadherin-2 (green, A, B, E, F, I, J, M, N) and cadherin-11 (red, C, D, G, H, K, L, O, P) and counterstained with DAPI (blue). Cells were grown in a monolayer (A–H) and as aggregates (I–P) in osteogenic (two left columns) or adipogenic (two right columns) induction medium. In monolayers, cadherin-2 expression decreased over 21 days in both differentiation media, whereas cadherin-11 expression increased over 21 days in osteogenic induction medium only. In hMSC aggregates, the cadherin expression on day 10 did not differ from the expression on day 1 in either differentiation media. Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

5. Cadherin-11 knockdown increased cadherin-2 expression

To explore whether the changes in cadherin-2 and cadherin-11 expression over 21 days in monolayer culture were interrelated, we performed lentiviral transduction with cadherin-11 shRNA, cadherin-2 shRNA or scrambled shRNA on hMSCs cultured in growth medium as a monolayer. Western blot analysis and qPCR confirmed a knockdown efficiency of 80% for cadherin-11 (Figure 4 A and B) and 84% for cadherin-2 (Figure 4 C and D) 7 days after selecting for positively tranduced hMSCs. The knockdown efficiency was maintained after 21 days in culture (Figure 4F).

Morphological changes were observed in cadherin-11 knockdown cells compared to the wild type, and the scrambled (Figure 4E). Under cadherin-11 knockdown conditions, cells proliferated slower and hence were less confluent and more spread-out compared to cadherin-2 knockdown, wild type, and the scrambled control (Figure 4E). Surprisingly, we observed that knocking down cadherin-11 caused an upregulation of cadherin-2 expression, which was 4 times higher compared to wild-type (Figure 4 F and G). In comparison, cadherin-11 levels did not change in cadherin-2 knockdown cells (Figure 4 F and H). These observations indicate that cadherin-2 and cadherin-11 expression in monolayer culture is interrelated and that their expression change over time (Figure 1–3) seems to be influenced by cadherin-11 but not by cadherin-2.

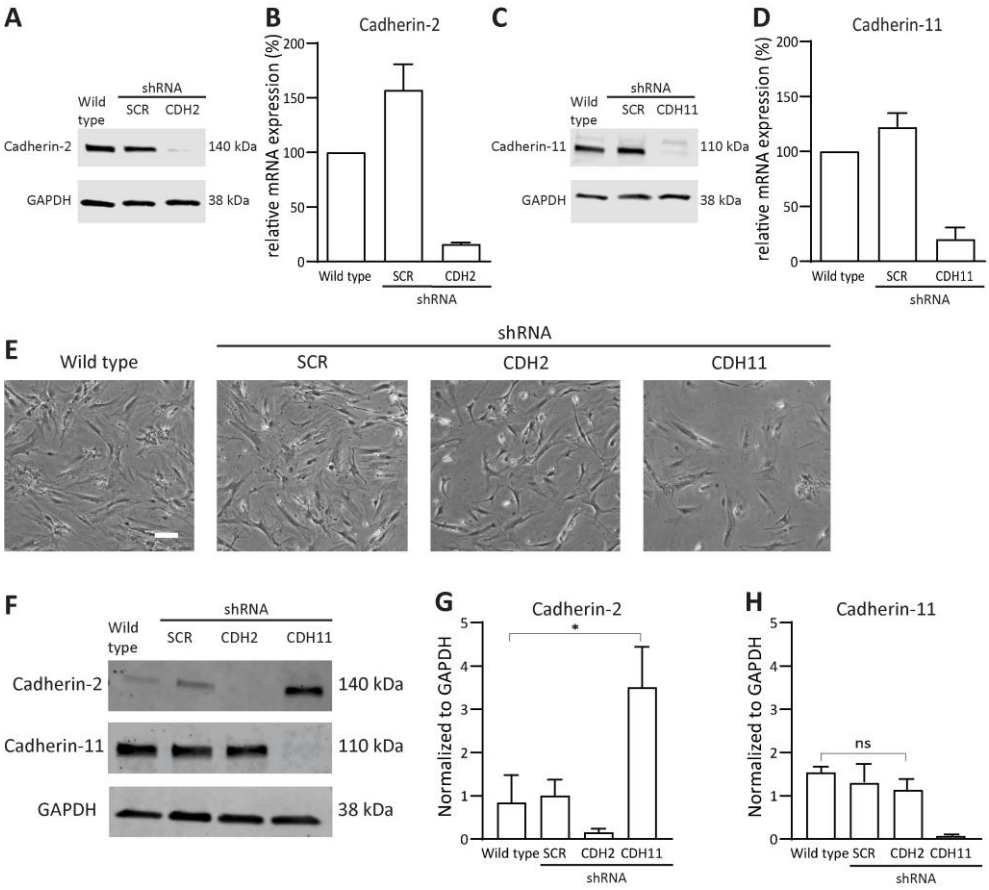

Figure 4. Cadherin-11 knockdown resulted in increased cadherin-2 expression

(A, C) Western blot analysis of cadherin-2 (A) and cadherin-11 (C) showed decreased protein expression by the respective shRNA knockdowns. GAPDH is shown as a loading control. (B, D) Quantification of relative mRNA expression demonstrated 84% knockdown efficiency for cadherin-2 (B), and 80% knockdown efficiency for cadherin-11 (D) compared to controls. (E) Phase contrast micrographs of hMSCs taken 7 days after cadherin-2 and cadherin-11 knockdown revealed morphological changes in cadherin-11–knockdown cells compared to all other conditions. Scale bars represent 100 μm. (F) Western blots showed an upregulation of cadherin-2 in cadherin-11–knockdown cells. In comparison, cadherin-11 expression in cadherin-2 knockdown cells was not affected. GAPDH is shown as a loading control. (G) Quantification of Western blots normalized to GAPDH showed that the increase in cadherin-2 expression in cadherin-11–knockdown cells was significant, while cadherin-11 expression in cadherin-2–knockdown cells was similar to wild-type controls. Error bars show ± SD. Data are representative of at least three independent experiments with similar results. Statistics were determined using one-way ANOVA with

Holm-Sidak's test for multiple comparisons: $*p < 0.01$; ns, not significant. For all panels, SCR indicates hMSCs transduced with a scrambled shRNA knockdown as a negative control.

6. Increased mineralized matrix formation in cadherin-2–knockdown hMSCs

Given our observations on differential cadherin expression levels between monolayer and aggregate cultures, we sought to determine whether cadherin-2 and cadherin-11 levels affected osteogenic differentiation. The cadherin-11– and cadherin-2–knockdown cells along with scrambled and wild-type cells were subjected to osteogenic inductive medium for 21 days in both aggregate and monolayer culture. After 21 days in culture, the cells were stained with Alizarin Red S to visualize the mineralized matrix. In both monolayer and aggregate cultures, cadherin-2–knockdown cells showed enhanced mineralized matrix formation compared to the controls (Figure 5 A and B). Cadherin-11–knockdown cells showed decreased mineralized matrix in monolayer cultures (Figure 5A), but similar mineralized matrix in aggregate cultures (Figure 5B) compared to the controls. These observations indicate that low cadherin-2 level enhances the deposition of the mineralized matrix during osteogenic differentiation in both culture formats, whereas cadherin-11 expression is important for osteogenic differentiation in monolayers only.

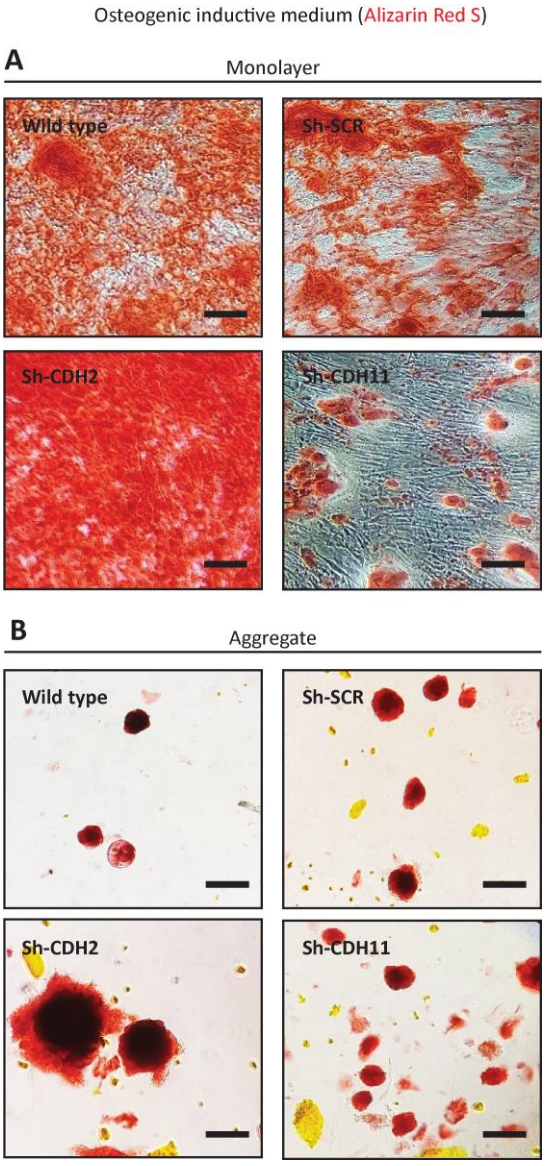

Figure 5. Increased mineralized matrix formation with cadherin-2 knockdown

Brightfield micrographs of hMSCs after 21 days in osteogenic inductive medium as monolayer (A) and aggregate (B) culture. Cadherin-2 and cadherin-11 knockdowns were induced into the osteogenic lineage for 21 days and stained with Alizarin Red S to visualize mineralized matrix. In both monolayer and aggregate culture, cadherin-2 knockdown (Sh-CDH2) resulted in increased mineralized matrix formation compared to wild-type controls. In comparison, cadherin-11 knockdown (Sh-CDH11) reduced mineralized matrix in monolayer but had no effect on matrix mineralization in aggregate culture

compared to wild-type controls. Mineralized matrix following knockdown with a scrambled shRNA (Sh-SCR) is shown as a negative control. Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

7. Disrupted adipogenic differentiation in cadherin-2– and cadherin-11–knockdown hMSCs

Next, we investigated how cadherin-2 and cadherin-11 expression influenced adipogenic differentiation. The cadherin-11– and cadherin-2–knockdown cells along with scrambled and wild-type cells were subjected to adipogenic inductive medium for 21 days in both aggregate and monolayer culture. After 21 days in culture, the cells were stained with Oil Red O to visualize the degree of lipid accumulation. Lipid accumulation was substantially reduced in cadherin-2- and cadherin-11-knockdown hMSCs in both monolayer and aggregate culture compared to scrambled and wild-type hMSCs (Figure 6 A and B). These observations indicate that both cadherin-2 and cadherin-11 are critical for adipogenic differentiation regardless of the dimensionality of the cell culture.

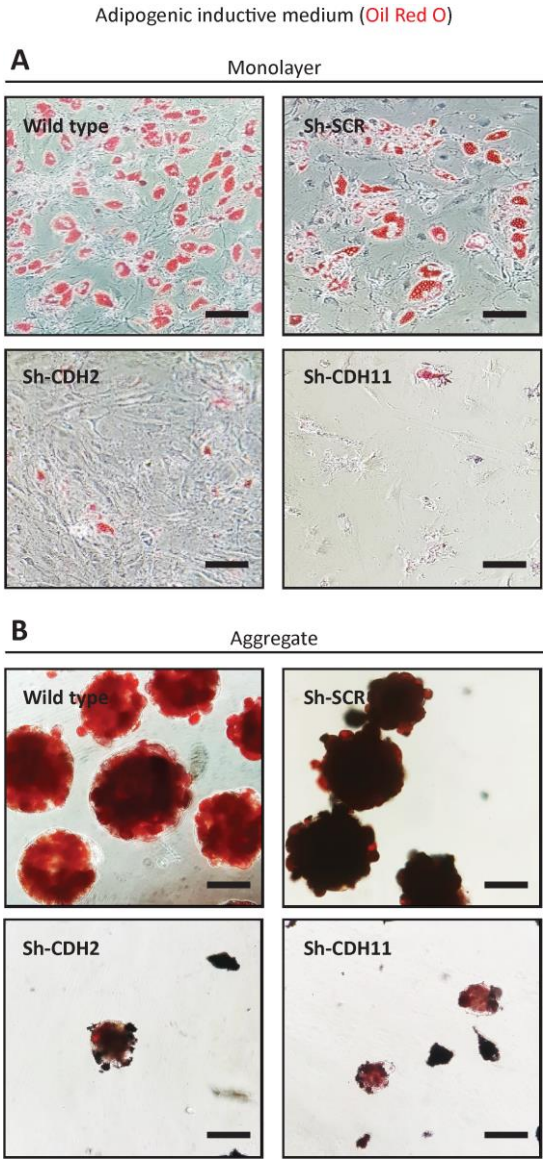

Figure 6. Disrupted adipogenic differentiation potential in hMSCs with cadherin-2 and cadherin-11 knockdowns

Brightfield micrographs of hMSCs after 21 days in adipogenic inductive medium as monolayer (A) and aggregate (B) culture. Cadherin-2 (Sh-CDH2) and cadherin-11 (Sh-CDH11) knockdowns were induced into the adipogenic lineage for 21 days and stained with Oil Red O to visualize lipid accumulation. In both monolayer and aggregate culture, cadherin-2 and cadherin-11 knockdown resulted in a reduced lipid accumulation compared to wild-type controls, indicating diminished differentiation potential. Lipid

accumulation following knockdown with a scrambled shRNA (Sh-SCR) is shown as a negative control. Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

DISCUSSION

The influence of the cell–material interface on cell fate has been an area of significant research in regenerative medicine, but comparatively little is currently known about cell-cell interactions. Furthermore, there is significant evidence that for regenerative medicine, three-dimensional aggregate cultures of hMSCs positively influence fate decisions[38,39], pointing to a role for cell-cell contact. Our aim was to look at cadherin expression and develop a better understanding of their role in cell fate decisions in monolayer and aggregate cultures.

We began by examining the levels of cadherin-2 and cadherin-11 expressed by hMSCs. During early embryogenesis, mesenchymal tissues have higher expression of cadherin-11 and a comparatively lower cadherin-2 expression[40], which correlates with what we observed during early aggregate formation (Figure 2). However, in monolayer culture, we found that cadherin-2 expression was greater than cadherin-11 and that these levels decreased with higher cell density over time, while cadherin-11 did not change (Figure 2). Mesenchymal condensation or mesenchymal cellular aggregation is a critical step for organogenesis. High levels of cadherin-2 have been reported prior to mesenchymal cellular aggregation.[41,42] The ease of condensation in aggregate cultures compared to monolayer culture could explain the differential cadherin expression. However, previous studies on mesenchymal stem cells have shown an increase in both cadherins with increasing cell density in culture.[33] Here we have shown that high protein levels of cadherin-2 correspond with a low density of hMSCs (Figure 1) and do not necessarily indicate engagement of cadherin-2 molecules with their counterparts on the surface of neighboring cells. Our findings suggest that hMSCs have a mechanism that helps regulate cadherin-2 expression based on the proximity of one cell to another. Specifically, as cell-cell interaction increases, cadherin-2 expression decreases. Even in aggregates, we have shown a

decrease in cadherin-2 expression 24 hrs into aggregate formation (Figure 2C). The striking observation was that even with ~85% knockdown of cadherin-2 and cadherin-11, hMSCs were able to aggregate, which suggests other, additional mechanisms of cell-cell adhesion. This observation contrasts with a previous study where cadherin-2-depleted cells did not form aggregates[43], which could indicate that even a small amount of cadherin-2 is sufficient for initiation of cell aggregation.

Our observations and other studies have indicated that osteogenesis is favorably affected by low levels of cadherin-2. Knockdown of 84% mRNA enhanced mineralized matrix formation (Figure 5). In rat MSCs, overexpression of cadherin-2 has been reported to inhibit osteogenesis.[44] However, the conditional deletion of cadherin-2 in mice has a negative effect on bone growth because it reduces β-catenin abundance at cell-cell contacts.[45] Germline cadherin-2 null mutation is lethal and hence does not allow for the precise understanding of the biological role of cadherin-2 at different stages of osteogenesis *in vivo*.[46]

Similar to the mRNA knockdown of cadherin-2, an 80% mRNA knockdown of cadherin-11 interfered with the differentiation ability of hMSCs. It reduced mineralized matrix deposition in monolayer culture, in agreement with studies in which cadherin-11–null mutant mice have reduced bone density.[47] The effect of the knockdowns on mineralized matrix deposition was more apparent in monolayer culture compared to aggregate cultures, again revealing the important differences cells experience in these two environments. For adipogenesis, both cadherin-2 and cadherin-11 expression were critical (Figure 6). Changes in cadherin expression during differentiation have been demonstrated in previous studies.[20,48] Interestingly, we observed a change from cadherin-2 to cadherin-11 expression in monolayer but not in aggregate cultures (Figure 1). The upregulation of cadherin-11 was more pronounced when hMSCs were in osteogenic inductive media. This agrees with the

finding that cadherin-11 is highly expressed in cells of the osteogenic lineage.[49] However, in aggregate culture, we found no evidence of changes in cadherin expression during differentiation, as a co-expression of the cadherin-2 and cadherin-11 was observed. These results indicate that cells express different molecules in interactions with their neighbors in monolayer and aggregate cultures.

While cadherin-2 and cadherin-11 are independent members of the cadherin superfamily, our studies and others indicate a relationship between them. For example, the function of cadherin-11 may be in part compensated by cadherin-2 and *vice versa* in mouse models.[45,50] The knockdown of cadherin-11 significantly increased cadherin-2 levels (Figure 4 F and G). However, this relationship seems to be one-directional, as cadherin-2 knockdown did not affect cadherin-11 levels (Figure 4 F and H). The upregulation of cadherin-2 as a result of cadherin-11 depletion could be the reason for reduced mineralized matrix deposition in monolayer cultures (Figure 5A), as overexpression of cadherin-2 inhibits osteogenesis.[44] Whether cadherin-11 depletion also upregulates cadherin-2 expression in aggregate cultures remains to be determined. We hypothesize that the upregulation of cadherin-2 in aggregate cultures may not be to the same deleterious levels, because the cadherin-2 expression was much lower in aggregates compared to monolayers (Figure 1) and mineralized matrix deposition was not affected (Figure 5B). Here we cannot dismiss the possibility of compensation by other cadherins as well. It has previously been shown that cells compensated for the loss of cadherin-2 by upregulation of other cadherins but were unable to compensate for cadherin-2 functionally.[51]

Our studies focused on two cadherins, whereas the entire superfamily of 100 cadherins play important roles in tissue integrity and may functionally compensate for one another. Indeed, disrupting cadherins might have a subtle effect on tissue

integrity. Conditional knockdown of cadherin-1 or cadherin-3 did not disrupt epidermal integrity in the mouse skin, while germline depletion of both cadherin-1 and cadherin-3 caused defects.[52] Furthermore, the upregulation of cadherin-2 can functionally compensate for the lack of cadherin-1 in embryonic stem cells.[53] These data suggest that individual cadherin species may not be solely responsible for tissue integrity because of compensation by other cadherins. Together, our findings indicate that the nature of cadherin-mediated adhesion is crucial for cell fate determination. Just by looking at two cadherins, we see that cadherins, although very influential in monolayer culture, might not be as critical for aggregate culture. Hence, for regenerative medicine, incorporating cadherins in biomaterial design could be beneficial.[15,16]

CONCLUSION

Achieving a specific cell fate is one of the core aims of tissue engineering and regenerative medicine. Our studies provide insight into the critical role that culture dimensionality plays on cell fate through cadherin signaling. We demonstrate that during MSC differentiation, there was a switch from cadherin-2 expression to cadherin-11 in cells cultured as a monolayer, but the expression of both cadherins remained consistent in aggregates. We also show that the loss of certain cadherins influences cell fate, which was also affected by culture dimensionality. Knowing the differences between cells cultured as a monolayer or as aggregates is crucial for biomaterial design, as it could be helpful for scientists aiming to design materials to influence stem cell fate.

MATERIALS AND METHODS

Monolayer cell culture

Bone marrow-derived hMSCs (PromoCell) were obtained at passage 1. Mycoplasma testing was performed using the mycoplasma detection kit from BD Biosciences. The cells were maintained in growth medium composed of minimal essential medium (MEM α, Gibco) supplemented with 10% fetal bovine serum (FBS). The medium was changed every 2 days, and the cells were maintained at 37°C in 5% CO_2 in a humidified incubator. Upon reaching 80% confluence, cells were detached by incubating with 0.05% trypsin-EDTA and replated for continuous passage. The cells were used at passage 5 for all experiments.

Microwell formation

Agarose microwell arrays were prepared as previously described[54,55] and inserted into 12-well plates. Each microwell array contained 450 microwells with a diameter of 400 μm.

Aggregate formation

To form hMSC aggregates, 180,000 cells in a 400 μL suspension in growth medium were seeded into one microwell array. The plate was subsequently centrifuged at 300 × *g* for 5 min to allow the cells to settle into the microwells, after which an additional 2 mL of growth medium was added to each well. The cells clustered spontaneously within 24 h to form aggregates of approximately 400 cells in each microwell. The medium was changed every 2 days.

Induction and evaluation of adipogenic and osteogenic differentiation

hMSCs in monolayer culture were seeded at 10,000 cells/cm² and expanded to confluency prior to differentiation, while cells in aggregate culture were seeded at

approximately 400 cells per microwell and cultured in growth medium for 24 h. All supplements are from Sigma-Aldrich unless mentioned otherwise. To induce osteogenic differentiation, the medium was changed every second day with osteogenic inductive medium composed of growth medium supplemented with 0.01 M β-glycerophosphate, 0.2 mM ascorbic acid, and 0.1 μM dexamethasone. To induce adipogenic differentiation, the medium was changed every second day with adipogenic inductive medium composed of Dulbecco's modified Eagle medium (high glucose, no sodium pyruvate; Gibco) supplemented with 10% FBS, 40 mM indomethacin, 83 mM 3-isobutyl-1-methylxanthine, 10 mg/mL insulin and 0.1 mM dexamethasone. The cultures were maintained for 21 days, after which they were evaluated by Alizarin Red S or Oil Red O staining. Cells were washed twice with phosphate-buffered saline (PBS), fixed in 4% (wt/vol) paraformaldehyde for 15 min at ambient temperature, and washed three times with distilled water. For hMSCs cultured in osteogenic inductive medium, the mineralized extracellular matrix was stained with 2% (wt/vol) Alizarin Red S (VWR) solution in distilled water (pH 4.2) for 15 min. For hMSCs cultured in adipogenic inductive medium, the intracellular lipid accumulation was stained with 0.2% (wt/vol) Oil Red O solution in 60% isopropanol for 15 min.

shRNA lentiviral transduction

pLKO.1 plasmids containing short hairpin RNA (shRNA) sequences targeting cadherin-11 or cadherin-2 were obtained from Sigma-Aldrich together with a scrambled negative control. These plasmids were co-transfected with third-generation lentiviral packaging and envelope vectors; pMD2.G, pRSV-Rev, and pMDLg/pRRE (Addgene plasmid #12259, #12253 and #12251, respectively, into HEK-293T cells using PEIpro (Polyplus) transfection reagent. The viral supernatant used to transduce hMSCs was collected 24 h after transfection. Forty-eight hours after transduction, positive cells were selected with 2 μg/ml puromycin

dihydrochloride (Sigma-Aldrich) in growth media for 5 days. The knockdown efficiency was assessed by qPCR and Western blot after 7 days.

Immunofluorescence

hMSCs were washed twice with PBS and fixed in 4% paraformaldehyde for 15 min at ambient temperature. For monolayer cultures, fixed cells were washed three times with PBS for 10 min, permeabilized with 0.2% Triton X-100 for 20 min, washed three more times, blocked in 1% goat serum in PBS for 1 h, and incubated with primary antibodies in 0.1% goat serum at 4°C overnight. The cells were washed three times, incubated with secondary antibodies in 0.1% goat serum for 2 h at ambient temperature. After 3 washes, samples were quenched with 0.5% (wt/vol) Sudan Black B (Sigma-Aldrich) for 10 min, and then the nuclei were counterstained with DAPI (0.1 μg/mL) for 10 min. For aggregate cultures, fixed cells were washed three times with PBS by centrifugation at 300 × *g* for 5 min, permeabilized with 0.5% Triton X-100 for 1 h, washed three more times, blocked in 1% goat serum in PBS for 1 h, and incubated with primary antibodies in 0.1% goat serum at 4°C overnight with gentle shaking. The cells were washed three times, incubated with secondary antibodies and DAPI in 0.1% goat serum at 4°C overnight with gentle shaking. All samples were mounted in ProLong Gold (Thermo Fisher Scientific), and fluorescence images were acquired on a Nikon E600 inverted microscope. Primary antibodies used were rabbit polyclonal anti-cadherin-11 and mouse monoclonal anti-cadherin-2 (3B9) (both 1:100; Thermo Fisher Scientific). Secondary antibodies used were goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 647 (both 1:500; Thermo Fisher Scientific).

Western blotting

hMSCs were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Thermo Fisher

Scientific). Total protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific). 10 μg of total protein lysate was supplemented with Laemmli buffer, reduced with 5% 2-mercaptoethanol (Sigma-Aldrich), and separated on 4–15% TGX gel (Bio-Rad) followed by transferring to a PVDF membrane (Bio-Rad) using the wet transfer method. Membranes were blocked in 5% milk in Tris-buffered saline (TBS) with 0.1% Tween-20 (Sigma-Aldrich) for 60 min before overnight incubation at 4°C with primary antibodies: rabbit polyclonal anti-cadherin-11, mouse monoclonal anti-cadherin-2 (3B9), (both 1:1000, Thermo Fisher Scientific) and mouse monoclonal anti-GAPDH (D4C6R, 1:1000, Cell Signaling Technology). Secondary antibodies used were IRDye 680RD goat anti-mouse IgG and IRDye 800CW donkey anti-rabbit IgG (both 1:15,000; LI-COR Biotechnology). Membranes were imaged on an Odyssey infrared imaging system (LI-COR Biotechnology). Band intensities were determined by quantifying the mean pixel gray values using the ImageJ 1.52b software. Mean pixel gray values were measured in a rectangular region of interest and normalized to GAPDH.

qPCR

hMSCs were lysed with Trizol (Thermo Fisher Scientific) followed by chloroform phase separation. The aqueous phase was diluted with 70% ethanol in a 1:1 ratio, loaded on an RNA microcolumn (RNeasy mini kit, Qiagen), and RNA extraction was performed according to the manufacturer's protocol. Subsequently, 600 ng total RNA was converted to cDNA with iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed in 20 μL reactions using the iQ SYBR Green Supermix (Bio-Rad) and a Real-Time PCR Detection System (Bio-Rad). The cycling conditions were as follows: enzyme activation at 95 °C for 3 min followed by 38 cycles at 95 °C for 12 s and at 58 °C for 30 s. Specific transcripts were detected with the primers listed in Supplementary Table S1 following evaluation for their amplification efficiency.

Statistical analysis

Statistics were determined using one-way ANOVA with Holm-Sidak's test for multiple comparisons. p values < 0.01 were considered significant. Statistical tests were performed with GraphPad Prism 8.

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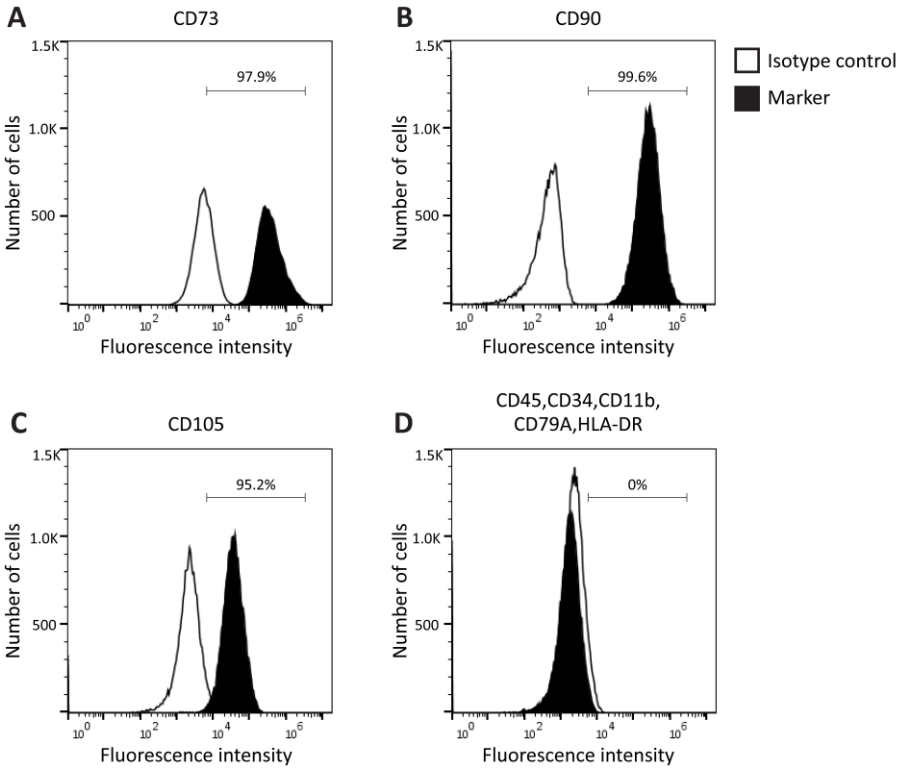
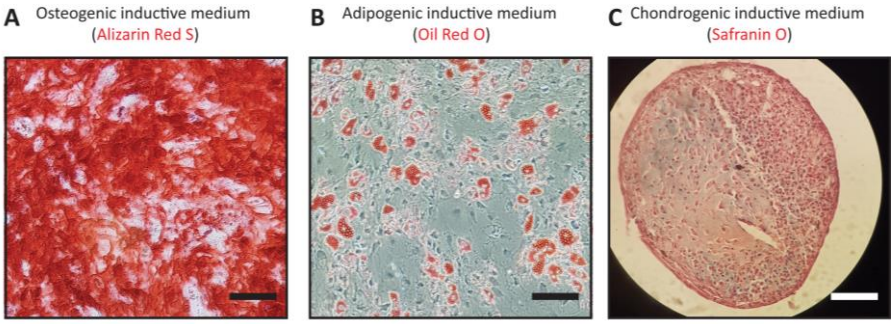
SUPPLEMENTARY INFORMATION



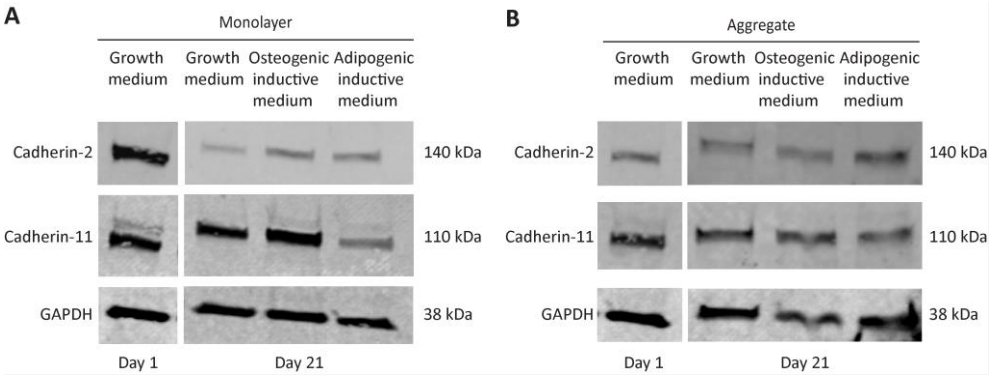
S1. Characterization of hMSC markers

Flow cytometry analysis indicated that the cultured hMSCs expressed surface markers, including CD73 (A), CD90 (B), and CD105 (C). Hematopoietic markers CD45, CD34, CD11b, CD19, and HLA-DR (D) considered to be negative mesenchymal markers, were not expressed in hMSCs. The white area indicates the isotype control, and the black area shows specific signal from the antibody.



S2. hMSC multipotency

hMSC multipotency was confirmed by histochemical staining of osteogenic differentiation by Alizarin Red S (A), adipogenic differentiation by Oil Red O (B), and chondrogenic differentiation by Safranin O (C) staining.



S3. Cadherin-2 and cadherin-11 expression in monolayer culture is different from aggregate culture

Western blots show the expression of cadherin-2 and cadherin-11 in monolayer (A) and aggregate (B) culture at day 0 in growth medium and day 21 in growth medium, osteogenic inductive medium, and adipogenic inductive medium. GAPDH is shown as a loading control.

Table S1

Primer sequences for RT-PCR

Gene	Forward primer sequence (5′-3′)	Reverse primer sequence (5′-3′)
Cadherin-11 (CDH11)	AGAGGTCCAATGTGGGAACG	GGTTGTCCTTCGAGGATACTGT
Cadherin-2 (CDH2)	AGCCAACCTTAACTGAGGAGT	GGCAAGTTGATTGGAGGGATG
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

4

Cadherin-11 influences differentiation in human mesenchymal stem cells by regulating the extracellular matrix via the TGFβ1 pathway

This chapter has been submitted for publication and is under review:

Passanha FR, Geuens T, LaPointe VL. Cadherin-11 influences differentiation in human mesenchymal stem cells by regulating the extracellular matrix via the TGFβ1 pathway. (2021)

ABSTRACT

For regenerative medicine, directing stem cell fate is one of the key aims. Human mesenchymal stem cells (hMSCs) are versatile adult stem cells that have been proposed for several clinical applications, making directing their fate of utmost importance. For most clinical applications, their differentiation towards the adipogenic lineage is an undesired outcome. Understanding the mechanisms that regulate hMSC commitment towards the adipogenic lineage might help open up new avenues for fine-tuning implanted hMSCs for regenerative medicine applications. We know that cadherin-11 is required for hMSC commitment to the adipogenic lineage; therefore, we sought to investigate the mechanisms through which cadherin-11 regulates adipogenic differentiation. We observed that hMSCs lacking cadherin-11 had decreased expression of type VI collagen and fibronectin. We provide evidence of increased transforming growth factor beta 1 and the subsequent translocation of phosphorylated SMAD2/3 into the nucleus by cells that lack cadherin-11, which could be attributed to the changes in extracellular matrix composition. Taken together, our study implicates cadherin-11 in regulating extracellular matrix production and thereby helping improve cell- and material-based regenerative medicine approaches.

INTRODUCTION

For regenerative medicine, directing stem cell fate is one of the key aims. Studying stem cell communication on a cellular level provides insights into how the human body forms tissues and how it functions, which in turn helps the field build highly developed tissues. Human mesenchymal stem cells (hMSCs) are versatile adult stem cells that have been proposed for several clinical applications, therefore, their preferential fate commitment to various cells of the mesodermal lineage is of utmost interest.[1–3] However, the underlying mechanisms that govern the fate commitment of hMSCs are not well understood.

Cadherin-11 is a cell adhesion molecule expressed by hMSCs, and we recently reported that it is crucial for their commitment towards the adipogenic lineage.[4] Other studies have also implicated cadherin-11 in hMSC differentiation.[5,6] HMSCs have shown their potential for the treatment of type 2 diabetes, metabolic syndrome, bone and cartilage disorders, among many others, all of which consider commitment towards the adipogenic lineage an undesired outcome.[7–9] This is because adipogenic differentiation of hMSCs occurs at the expense of osteogenic and chondrogenic lineage specificity. Adipogenic differentiation is a highly complex process and the mechanisms that govern it are ill-defined. Understanding the mechanisms that regulate hMSC fate commitment towards the adipogenic lineage might help open up new avenues for fine-tuning implanted hMSCs for regenerative medicine applications.

To this end, we set out to determine the mechanisms through which the knockdown of cadherin-11 disrupts the adipogenic differentiation potential of hMSCs. Cadherin-11 has previously been linked to tissue fibrosis, which is the excessive deposition of extracellular matrix (ECM) components.[10,11] A recent study also showed that human

fibroblasts lacking cadherin-11 had reduced collagen and elastin content.[12] Then there are numerous studies that indicate that ECM is key to fate commitment.[13,14] Linking these together, we hypothesized that the cadherin-11 knockdown alters the ECM in hMSCs, thereby disrupting their differentiation towards the adipogenic lineage.

In this study, we not only provide evidence that cadherin-11 regulates collagen, but also for the first time show that it regulates fibronectin. Cadherin-11 has no known signaling activity, therefore we also identified a possible crosstalk with the transforming growth factor beta 1 (TGFβ1) pathway through which cadherin-11 modulates the ECM. By implicating cadherin-11 in the regulation of the ECM, we add to the evidence of the cadherin–integrin crosstalk mechanism.

RESULTS

1. Cadherin-11 is necessary for adipogenic differentiation.

We performed lentiviral transduction using cadherin-11 or scrambled shRNA of hMSCs cultured in growth medium. Western blotting confirmed the knockdown of cadherin-11 on days 1 and 21 in culture (Figure 1A). Quantification of the Western blot showed that the cadherin-11–knockdown cells had an 82% lower expression of cadherin-11 at day 1 (Figure 1B) and 96% lower expression at day 21 (Figure 1C) compared to the wild type. When subjected to adipogenic inductive medium for 21 days and stained with Oil Red O, we observed that cadherin-11–knockdown cells had reduced lipid accumulation compared to the scrambled and the wild type cells (Figure 1D).

2. Cell density affects the expression of collagen.

Since cadherin-11 has no known intrinsic signaling activity, we wanted to find evidence to explain how knocking down cadherin-11 leads to changes in the adipogenic differentiation potential of hMSCs. We first hypothesized that the loss of cadherin-11 could be affecting the expression of collagen, which is known to promote adipogenic differentiation.[15–17] To test this hypothesis, we investigated various collagens that are expressed by hMSCs, namely type I collagen, type II collagen, type III collagen, and type VI collagen by immunofluorescence (Figure S2).

At the same time, having observed that knocking down cadherin-11 caused a decrease in proliferation (Figure S1A), we wanted to investigate if the changes in cell density could explain the changes in collagen expression. Among the various collagens tested, we observed that type I collagen, type II collagen, and type III collagen expression were cell density–dependent, namely they were higher in low

density cells (Figure S2A-C), meaning we could not attribute their expression directly to the cadherin-11 knockdown.

Out of curiosity, we also investigated type I collagen, type II collagen and type III collagen on day 14. Type I collagen, type II collagen and type III collagen express had no observable differences when comparing the cadherin-11–knockdown cells to the wild type at both days 1 and 14 (Figure S3A-C).

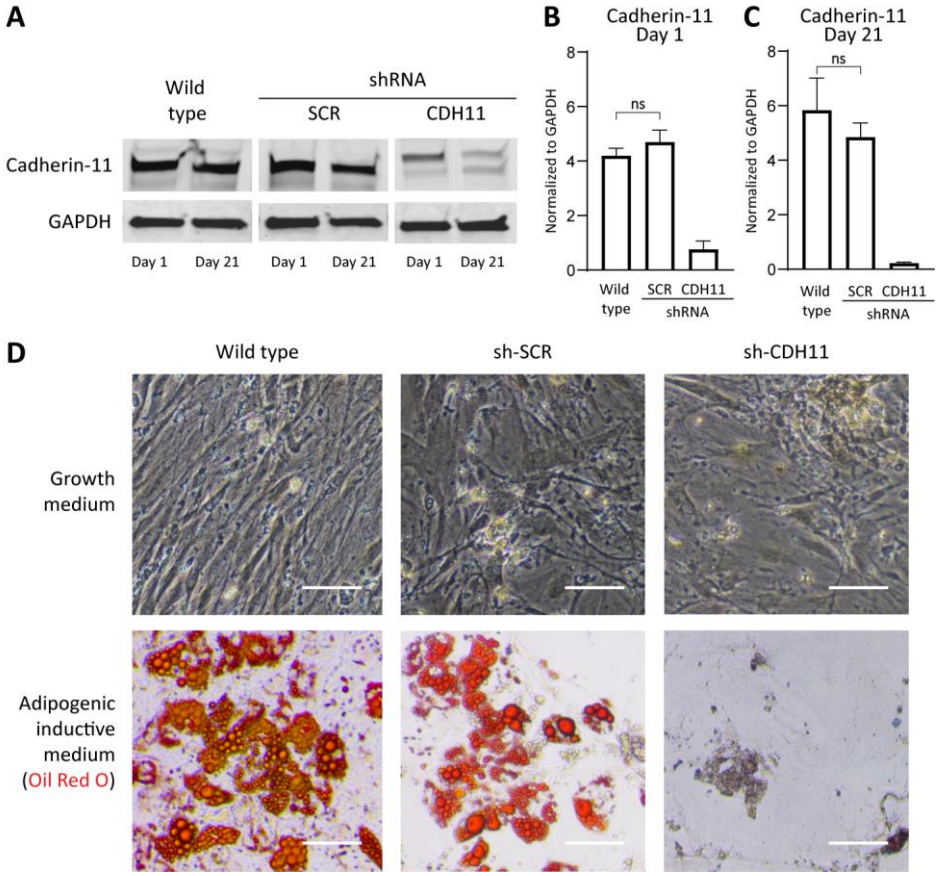


Figure 1. Loss of cadherin-11 disrupts the adipogenic potential of hMSCs

(A) Western blot analysis of cadherin-11 shows decreased expression in the cadherin-11–knockdown cells (sh-CDH11) compared to the wild type and scrambled (sh-SCR) controls at days 1 and 21. GAPDH is shown as a loading control. (B)(C) Quantification of Western blots normalized to GAPDH showed that cadherin-11 expression in sh-CDH11 cells was significantly decreased compared to the wild type and sh-

SCR controls at both days 1 and 21. Error bars show + SD. Statistical significance was determined using one-way ANOVA with Tukey's test for multiple comparisons. All comparisons are statistically significant (p<0.02) unless mentioned otherwise; (n.s, not significant.) (D) Brightfield micrographs of hMSCs stained with Oil Red O after 21 days in growth and adipogenic inductive medium show that sh-CDH11 cells had reduced lipid accumulation compared to wild type control and sh-SCR, indicating disrupted adipogenic differentiation. Scale bars represent 100 μm. All data are representative of at least three independent experiments with similar results.

3. Cadherin-11 knockdown decreases type VI collagen expression.

When we tested the various collagens, we observed that type VI collagen expression remained unchanged over the different cell densities (Figure S4A). Furthermore, type VI collagen is highly enriched in the ECM of adipocytes,[18] leading us to investigate it further. When we performed a Western, we observed that cadherin-11–knockdown cells had lower levels of type VI collagen when compared to the wild type at day 1 (Figure 2A). Immunofluorescence micrographs at day 1 confirmed the decrease in type VI collagen in cadherin-11–knockdown cells (Figure 2B). Since collagen production increases over time, we also investigated the expression of type VI collagen on day 14. Western blot analysis showed a decrease in type VI collagen at day 14 compared to the wild type (Figure 2C). This decrease in type VI collagen expression in the cadherin-11–knockdown cells was confirmed by immunofluorescence micrographs (Figure 2D).

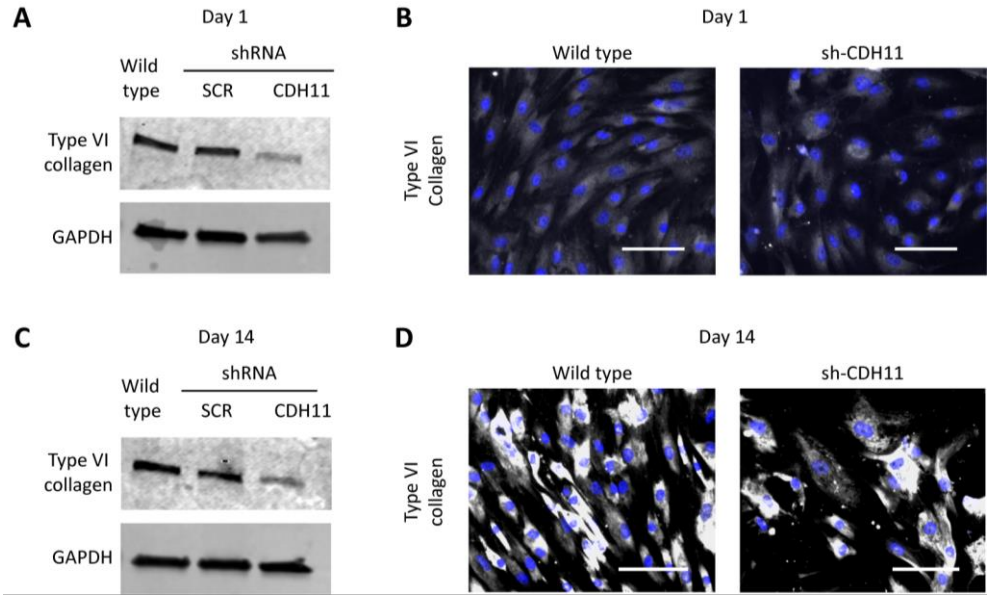


Figure 2. **Cadherin-11 knockdown reduces the expression of type VI collagen**

HMSCs were seeded at 1 × 10^4 cells/cm^2 and evaluated after 1 and 14 days. (A) Western blot analysis of type VI collagen on day 1 shows decreased expression in the cadherin-11–knockdown cells (sh-CDH11) compared to the wild type and scrambled (sh-SCR) controls. GAPDH is shown as a loading control. (B) Immunofluorescence micrographs of type VI collagen (white) on day 1 also show decreased expression in the sh-CDH11 cells compared to the wild type. (C) Western blot analysis on day 14 shows that the low type VI collagen expression persists in sh-CDH11 cells compared to the wild type and sh-SCR. (D) Immunofluorescence micrographs of type VI collagen (white) at day 14 confirm the decreased expression in sh-CHD11 cells compared to the wild type. Nuclei were counterstained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

4. Cadherin-11 knockdown increases fibronectin expression.

Given our observation of reduced type VI collagen expression, we sought to investigate if the cadherin-11 knockdown affected the expression of other ECM proteins. We first confirmed that the expression of fibronectin was not influenced by cell density (Figure S4B). Next, we investigated the expression of fibronectin at day 1 and observed an increase in cadherin-11–knockdown cells compared to the wild type (Figure 3A). Immunofluorescence micrographs confirmed the increase in fibronectin in cadherin-11–knockdown cells compared to the wild type at day 1

(Figure 3B). We also observed a change in the expression pattern of fibronectin between the wild type and cadherin-11–knockdown cells, where the cadherin-11–knockdown cells had enriched fibronectin surrounding the cell, while the wild type cells had fibronectin expression throughout their ECM (Figure 3B).

We then investigated integrin β1 to confirm the differences in the fibronectin pattern and we observed it closely followed the pattern of fibronectin (Figure S5). We also performed a Western blot after 14 days in culture and observed that cadherin-11–knockdown cells had increased fibronectin expression compared to the wild type (Figure 3C). Again, on day 14, immunofluorescence micrographs confirmed the increase in fibronectin in cadherin-11–knockdown cells (Figure 3D). The expression pattern of fibronectin in wild type cells was through the ECM with no spatial enrichment, while in the cadherin-11–knockdown cells, fibronectin was enriched surrounding the cell had a random expression pattern both within and between the cells (Figure 3D).

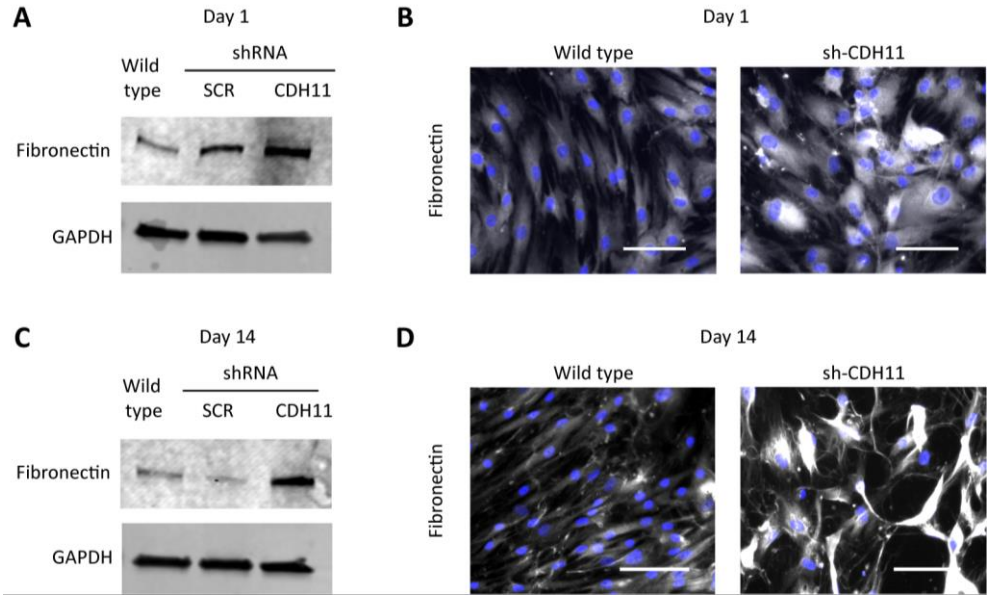


Figure 3. Cadherin-11 knockdown reduces the expression of fibronectin

HMSCs were seeded at 1×10^4 cells/cm^2 and evaluated after days 1 and 14. (A) Western blot analysis of fibronectin on day 1 shows increased expression in cadherin-11–knockdown cells (sh-CDH11) compared to the wild type and scrambled (sh-SCR) controls. GAPDH is shown as a loading control. (B) Immunofluorescence micrographs of fibronectin (white) at day 1 also show increased expression in the sh-CDH11 cells compared to the wild type. (C) Western blot analysis on day 14 shows that the fibronectin expression persists in sh-CDH11 cells compared to the wild type and sh-SCR. (D) Immunofluorescence micrographs of fibronectin (white) at day 14 confirm the increased expression compared to the wild type. Nuclei were counterstained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

5. Increased nuclear localization of phosphorylated smad2/3 in cadherin-11–knockdown cells.

To better understand how the loss of cadherin-11 changed the ECM composition of hMSCs, we looked at TGFβ1, a well-known inducer of ECM components such as collagen and fibronectin.[19] Given that the TGFβ1 pathway is implicated in ECM regulation, we hypothesised that cadherin-11–knockdown cells have enhanced TGFβ1 secretion which therefore changes the ECM.

We first wanted to investigate if cadherin-11–knockdown cells indeed secreted more TGFβ1 compared to the wild type. To this end we performed TGFβ1 ELISA on the supernatant collected from the cell culture at day 1, 2, 4, 6, 8, 12 and 14. We subtracted the baseline levels of TGFβ1 present in the medium without cells from the level of TGFβ1 measured at a particular time point. We observed an increase in TGFβ1 from a mean value of 33 pg/ml at day 1 to 595 pg/ml at day 14. There was no significant difference observed in the values between the wild type and the scrambled controls, but TGFβ1 in the cadherin-11–knockdown cells had a mean value of -118 pg/ml at day 1 and -80 pg/ml at day 14, implying there was less in the supernatant than in the medium without cells. After 6 days in culture, the amount of TGFβ1 measured in the supernatant of the cadherin-11–knockdown cells was significantly lower than both the wild type and the scrambled controls (Figure 4A; $p<0.0001$). This trend persisted at days 8, 12 and 14 (Figure 4A; $p<0.0002$). We also normalised the same data of TGFβ1 quantity to the DNA content, revealing that the average amount of TGFβ1 per cell in the cadherin-11–knockdown cells was significantly higher than in the wild type cells from day 4 onwards (Figure S6; $p<0.03$).

Since the levels of TGFβ1 themselves were insufficient to explain our observations about the ECM, we looked further at SMAD2/3, which is the downstream signalling molecule of the TGFβ1 pathway. TGFβ1 stimulation leads to phosphorylation and activation of SMAD2/3, which accumulates in the nucleus and regulates the transcription of target genes. On day 1, we observed that wild type cells had fewer nuclei positive for phosphorylated SMAD2/3 (pSMAD2/3) compared to the cadherin-11–knockdown cells, indicating that the pathway was not active in the wild type but was active in the cadherin-11–knockdown cells (Figure 4B). When this was quantified, the wild type cells had 22.8 ± 5% nuclei positive for pSMAD2/3 compared to 68.2 ±10% in cadherin-11–knockdown cells (Figure 4D; $p<0.0001$). After 14 days in

culture, all nuclei were positive for pSMAD2/3 in both the wild type and cadherin-11–knockdown cells (Figure 4C,D).

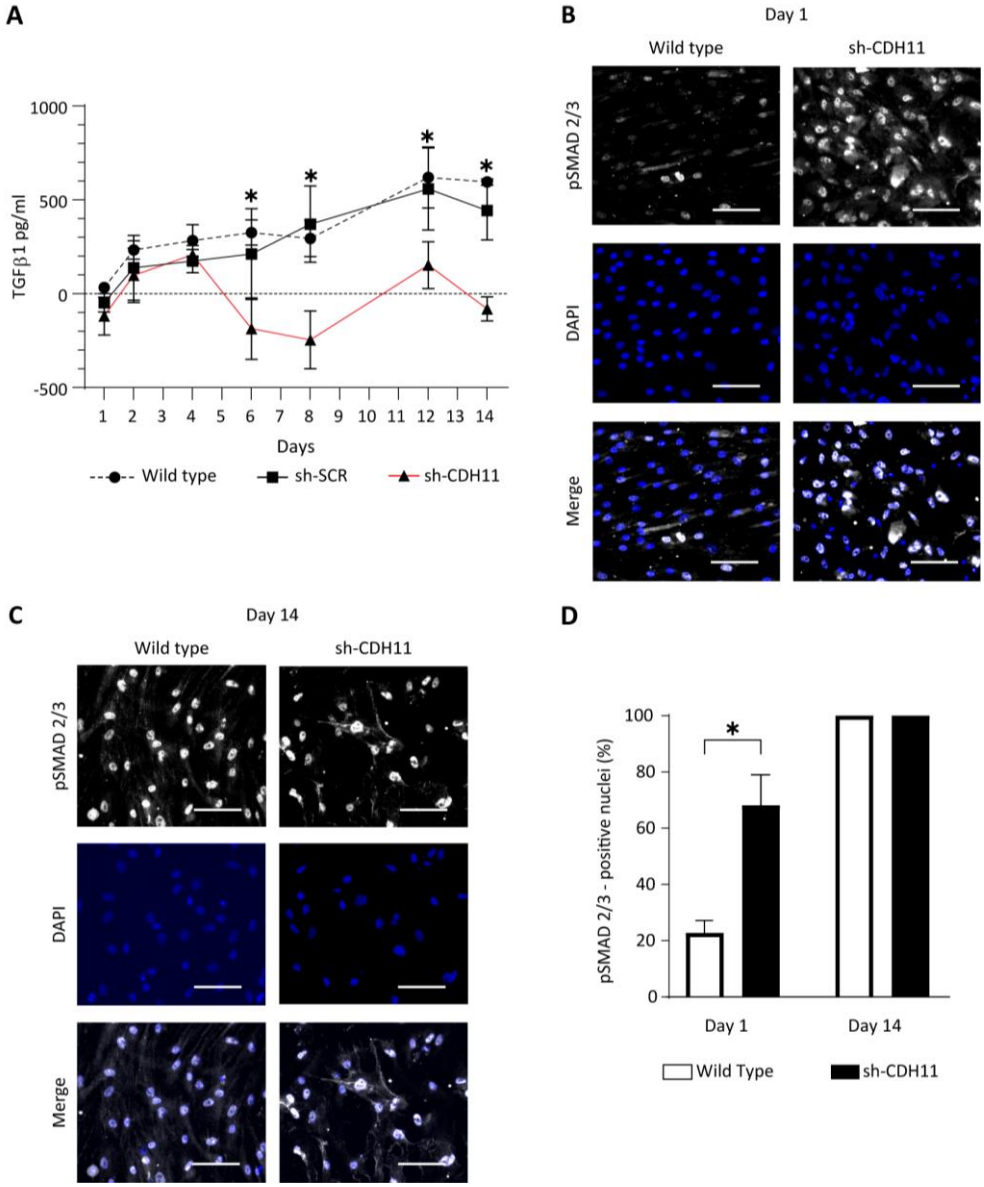


Figure 4. Cadherin-11–knockdown cells have more pSMAD2/3–positive nuclei

(A) Time course of TGFβ1 in the medium of hMSCs, where medium was collected and total TGFβ1 was measured using ELISA following acidification. The baseline level of TGFβ1 in the medium without cells is indicated with a dashed line. Compared to wild type and scrambled control (sh-SCR), less TGFβ1 was

detected in the supernatant in cadherin-11–knockdown cells (sh-CDH11). Statistics were determined using two-way ANOVA with Tukey's test for multiple comparisons. *p<0.001, sh-CDH11 compared to both wild type and sh-SCR. Error bars show ± SD. Data are representative of at least three independent experiments with similar results. Immunofluorescence micrographs at (B) day 1 and (C) day 14 of hMSCs seeded at 1×10^4 cells/cm^2 and immunostained for pSMAD2/3 (white) and counterstained with DAPI (blue). Scale bars represent 100 μm. (D) Quantification of the number of positive pSMAD 2/3 nuclei shows cadherin-11–knockdown (sh-CDH11) cells have more pSMAD2/3–positive nuclei compared to the wild type at day 1. Statistics were determined using Student's t-test: *p<0.003.

DISCUSSION

The importance of the ECM in influencing cell behaviour is unquestionable. Our study shows that cadherin-11 influences cell differentiation indirectly by regulating the ECM via the TGFβ1 pathway. Previous studies including ours have implicated cadherin-11 in osteogenic, smooth muscle cell, and adipogenic differentiation.[4,5] So far, the only evidence for signalling events involving cadherin-11 has been provided for smooth muscle cell differentiation.[5] Notably, cadherin-11 does not have any known intrinsic signalling activity, but our work reveals how knocking it down inhibits the differentiation of hMSCs towards the adipogenic lineage (Figure 1).

We were inspired by a study that showed that cadherin-11 was necessary for ECM production in fibroblasts and smooth muscle–containing tissue.[12] The authors discovered that cadherin-11$^{-/-}$ mice had significantly reduced type I collagen, type III collagen and elastin expression.[12] In order to say whether cadherin-11 influences the ECM of hMSCs, we first screened a selection of ECM components. We started by looking at collagens, as they are the most abundant ECM constituent, and compared their expression in the cadherin-11–knockdown cells to the wild type cells. Since the cadherin-11 knockdown causes the cells to proliferate more slowly (Figure S1) we chose to investigate ECM proteins with expression that was independent of cell density. We discovered that cadherin-11–knockdown cells had decreased expression of type VI collagen but an increased expression of fibronectin (Figure 2,3).

Adipogenic differentiation is usually associated with ECM remodelling, characterized by the conversion from the fibronectin and type I collagen matrix to laminin and type VI collagen.[18,20] Our current understanding of the function of type VI collagen comes mainly from the study of a mouse model with a defective type VI collagen gene, which leads to muscle myopathy that progresses with age.[21,22] A

recent study found that mature adipocyte differentiation was attenuated in type VI collagen–deficient cells.[23] Therefore the reduced expression of type VI collagen in cadherin-11–knockdown cells is related to the reduced capacity for adipogenic differentiation. Similarly, the growth of preadipocytes on a fibronectin matrix is found to inhibit adipocyte differentiation.[24] Therefore, the increased expression of fibronectin in cadherin-11–knockdown cells is likely to be related to their reduced capacity for adipogenic differentiation.

TGFβ1, a potent and pleiotropic cytokine in its biologically active form, binds to its receptor and stimulates expression of ECM components via phosphorylation of the signalling molecules SMAD2/3.[25–27] We speculated that the TGFβ1 pathway may be differentially regulated in cadherin-11–knockdown cells, resulting in changes in ECM, and we therefore investigated the expression pSMAD2/3. In our study, we provide evidence of decreased TGFβ1 in the supernatant and the subsequent translocation of pSMAD2/3 into the nucleus by cells that lack cadherin-11, which could be related to the changes in ECM composition (Figure 4). The cadherin-11 expression has been shown to be upregulated by exogenous TGFB1 supplementation in myofibroblasts.[28] In our study, showed that the TGFβ1 pathway is upregulated earlier in cadherin-11–knockdown cells compared to the wild type. TGFβ1 has also been linked to suppressed adipogenic differentiation by hMSCs when it is supplemented before commitment.[29–31] Seeing as the TGFβ1 pathway is activated prematurely in cadherin-11–knockdown cells, it is possible that a similar mechanism is observed.

Fibronectin binds a plethora of growth factors that are central in tissue repair and fibrosis, including latent TGFβ.[32,33] Seeing as pSMAD2/3 is already translocated into the nucleus at day 1, the rest of the TGFβ1 probably remains bound the ECM, which is why we saw a decrease inTGFβ1 over time. Another explanation is that hMSCs

secrete TGFβ1 in the presence of cadherin-11, as knocking down cadherin-11 reduced the levels of TGFβ1. Mouse MSCs are known to secrete TGFβ and alveolar macrophages isolated from the lungs of cadherin-11–deficient mice exhibit lower levels of TGFβ. [5,10,34] However, these studies have linked low levels of TGFβ with a decrease in pSMAD2/3, which is not in line with our study. Given the increase in fibronectin levels in cadherin-11–knockdown cells, we suggest there could be an involvement of integrin signalling. Integrins α5 and α6 have been implicated in adipogenic differentiation and fibronectin acts as a ligand for dozens of the integrin family members.[35,36]

Taken together, our study demonstrates that cadherin-11 regulates the ECM by temporally controlling the TGFB pathway. This improves the understanding of hMSC fate commitment and adds evidence to the importance of cadherin-11 in the differentiation of hMSCs.

MATERIALS AND METHODS

Cell culture

Bone marrow–derived hMSCs (PromoCell) obtained at passage 1 were maintained in growth medium composed of minimal essential medium α (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS). The medium was changed every second day, and the cells were maintained at 37°C in 5% CO_2 in a humidified incubator. Upon reaching 80% confluence, cells were trypsinized in 0.05% trypsin-EDTA and replated for continuous passage. The cells were used at passage 5 for all experiments.

ShRNA lentiviral transduction

The plasmid pLKO.1 containing short hairpin RNA (shRNA) sequences targeting cadherin-11 was obtained from Sigma-Aldrich together with a scrambled negative control. These plasmids were co-transfected with third generation lentiviral packaging and envelope vectors; pMD2.G, pRSV-Rev and pMDLg/pRRE (Addgene plasmid #12259, #12253 and #12251, respectively, which were gifts from Didier Trono[37]), into HEK-293T cells using PEIpro (VWR) transfection reagent. Lentiviral particles were harvested 48 and 72 h after transfection. Five milliliters of viral supernatant were used to transduce hMSCs seeded at 5000 cells/cm^2 in a T225 flask and incubated for 48 h. After transduction, positive cells were selected with 2 μg/ml puromycin dihydrochloride (Sigma-Aldrich) in growth media for 7 days and were then used for subsequent experiments.

Induction and evaluation of adipogenic differentiation

HMSCs were seeded at 10,000 cells/cm^2 and expanded to confluence prior to differentiation. To induce adipogenic differentiation, adipogenic inductive medium composed of Dulbecco's modified Eagle medium (high glucose, no sodium pyruvate; Gibco) supplemented with 10% FBS, 40 mM indomethacin, 83 mM 3-

isobutyl-1-methylxanthine, 10 mg/mL insulin, and 0.1 mM dexamethasone was added to the cells and refreshed every second day. The cultures were maintained for 21 days, after which they were evaluated by Oil Red O staining. Cells were washed twice with phosphate-buffered saline (PBS), fixed in 4% (w/v) paraformaldehyde for 15 min at ambient temperature, and washed three times with distilled water. The intracellular lipid accumulation was stained with 0.2% (w/v) Oil Red O solution in 60% isopropanol for 15 min and images were aquired on a Nikon eclipse TS100 inverted microscope.

Western blotting

HMSCs were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Thermo Fisher Scientific). The lysate was incubated on ice with constant mixing for 30 min, followed by sonication on ice three times for 5 sec with 10% amplitude and 30 sec between each cycle, and finally centrifuged at 16,000 × g for 20 min at 4°C. Total protein concentration was measured using the Pierce BCA protein assay kit. For separation, 20 μg of total protein lysate was supplemented with Laemmli buffer, reduced with 5% (v/v) 2-mercaptoethanol (Sigma-Aldrich) and separated on a 4–15% TGX gel (Bio-Rad) followed by transferring for 90 min to a PVDF membrane (Bio-Rad) using the wet transfer method. Membranes were blocked in 5% (w/v) milk in Tris-buffered saline (TBS) with 0.01% (v/v) Tween-20 for 60 min before overnight incubation at 4°C with primary antibodies diluted in blocking buffer. The membranes were washed three times and incubated with secondary antibodies in blocking buffer for 2 h at ambient temperature. Primary antibodies were against: type VI collagen (rabbit clone, 1:1000; Genetex, GTX109963), fibronectin (rabbit clone, 1:1000; Novus Biologicals, NBP1-91258), cadherin-11 (rabbit clone, 1:1000; Thermo Fisher Scientific, 71-7600), or GAPDH (mouse clone, 1:1000; Santa Cruz Biotechnology, SC-365062). Secondary antibodies used were: IRDye 680RD goat

anti-mouse IgG or IRDye 800CW donkey anti-rabbit IgG (both 1:15,000; LI-COR Biotechnology). The membranes were imaged on an Odyssey infrared imaging system (LI-COR Biotechnology). Band intensities were determined by quantifying the mean pixel grey values using the ImageJ 1.52b software.

Immunofluorescence

HMSCs were washed twice with PBS and fixed in 4% (v/v) formaldehyde for 15 min at ambient temperature. Fixed cells were washed three times with PBS for 10 min, permeabilized with 0.2% Triton X-100 for 20 min, washed three more times, blocked in 1% bovine serum albumin (BSA) in PBS for 1 h, and incubated with primary antibodies in 0.1% BSA at 4°C overnight. The cells were washed three times, incubated with secondary antibodies in 0.1% BSA for 2 h at ambient temperature, and the nuclei were counterstained with DAPI (0.1 μg/mL) for 10 min. Fluorescence images were acquired using a Nikon E600 inverted microscope. Primary antibodies were against: type VI collagen (rabbit clone, 1:100; Genetex, GTX109963), fibronectin (rabbit clone, 1:100; Novus Biologicals, NBP1-91258), pSMAD2/3 (rabbit clone, 1:100; R&D systems, MAB8935), type I collagen (mouse clone, 1:100; Abcam, ab6308), type II collagen (rabbit clone, 1:100; Abcam, ab34712), type III collagen (mouse clone, 1:100; Abcam, ab23445), or integrin β1 (rabbit clone, 1:100; Cell Signaling Technology, 34971S). Secondary antibodies were goat anti-mouse Alexa Fluor 647 or goat anti-rabbit Alexa Fluor 647 (both 1:500; Thermo Fisher Scientific).

TGFβ1 ELISA assay

TGFβ1 in the medium by hMSCs was quantified using a human TGFβ1 ELISA kit (antibodies online, ABIN625094). The medium was harvested on days 1, 2, 4, 6, 8, 12, and 14. The ELISA was performed according to the manufacturer's instructions. Absorbance was measured at 450 nm using a ClarioStar plate reader (BMG LabTech). Background level of TGFβ1 in the growth medium without cells was

subtracted from samples. Alternatively, TGFβ1 concentration was normalized to the total DNA content.

DNA quantification

After removing the medium, the hMSCs were washed twice with PBS and lysed with RLT lysis buffer (Qiagen). The lysate was freeze–thawed to ensure proper lysis. Samples were then diluted 50× in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and a DNA standard curve was made in the same final solution. A PicoGreen assay (Thermo Fisher Scientific) was used to quantify DNA, according to the manufacturer's protocol. The fluorescence signal (excitation: 492 nm and emission: 520 nm) was obtained on a ClarioStar plate reader.

EdU cell proliferation detection

To assess the proliferation of hMSCs, 5-ethynyl-2'-deoxyuridine (EdU) staining was conducted using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific). HMSCs were incubated with 50 μM EdU for 48 h before fixation in 4% (v/v) paraformaldehyde in PBS for 15 min at ambient temperature. Fixed samples were permeabilized with 0.2% Triton X-100 for 20 min, washed three more times, blocked in 1% BSA for 1 h, and the incorporated EdU was labeled with Alexa Fluor 647 azide for 30 min according to the manufacturer's protocol. The nuclear DNA was counterstained by DAPI (0.1 μg/ml) for 30 min. Fluorescence images were acquired on a Nikon E600 inverted microscope.

ACKNOWLEDGMENTS

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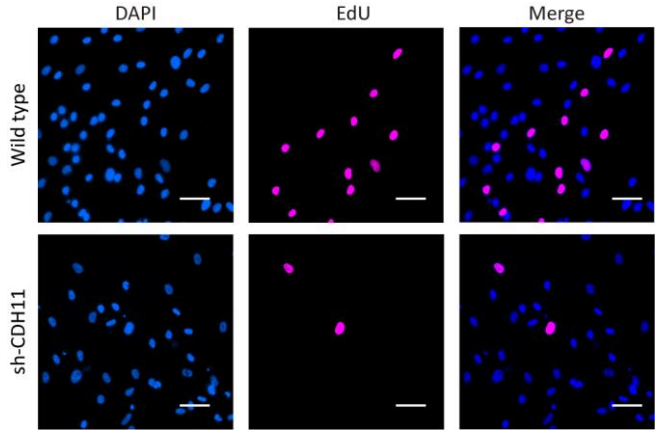
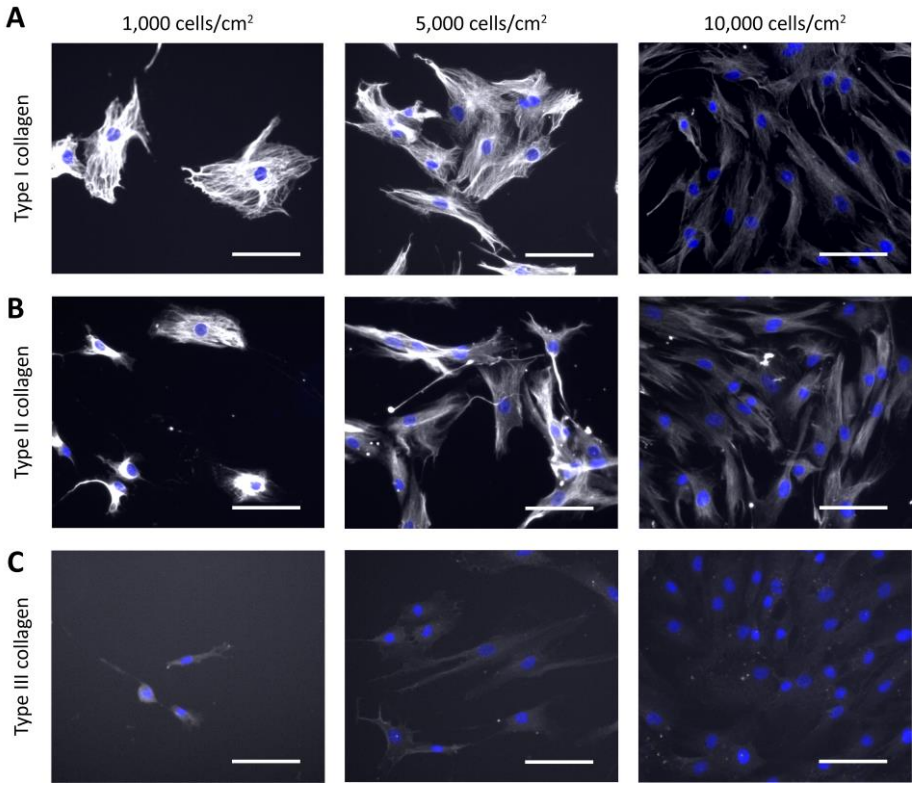
SUPPLEMENTARY INFORMATION



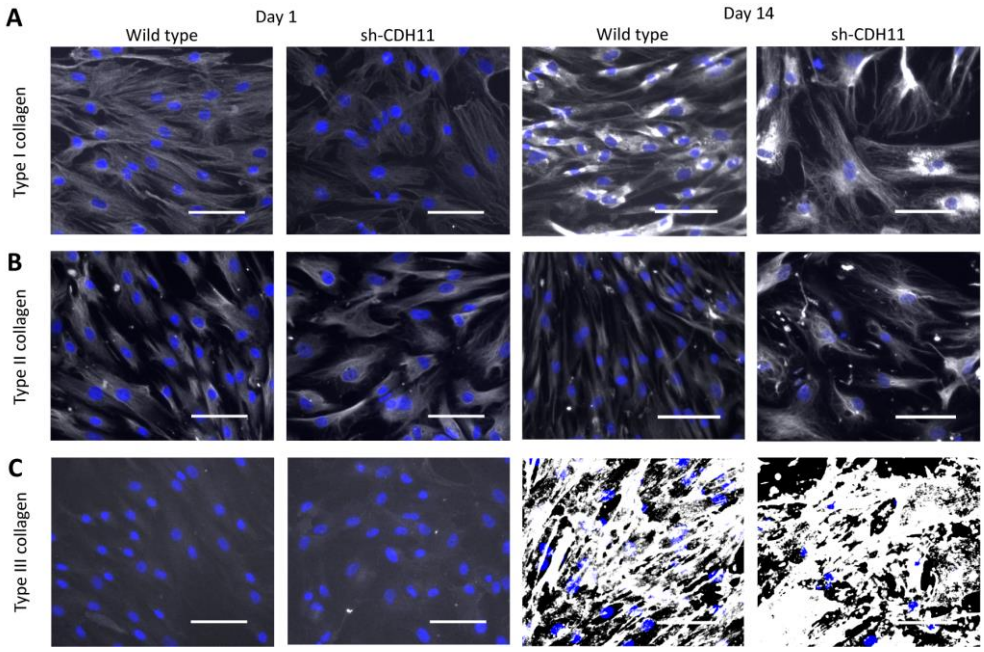
S1. Cadherin-11–knockdown cells have reduced proliferation

Immunofluorescence micrographs of hMSCs seeded at 1×10^4 cells/cm^2 show reduced EdU in cadherin-11–knockdown (sh-CDH11) cells after 2 days in culture. DAPI (blue) and EdU (magenta) staining of hMSCs incubated with EdU for 48 h in growth medium. Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.



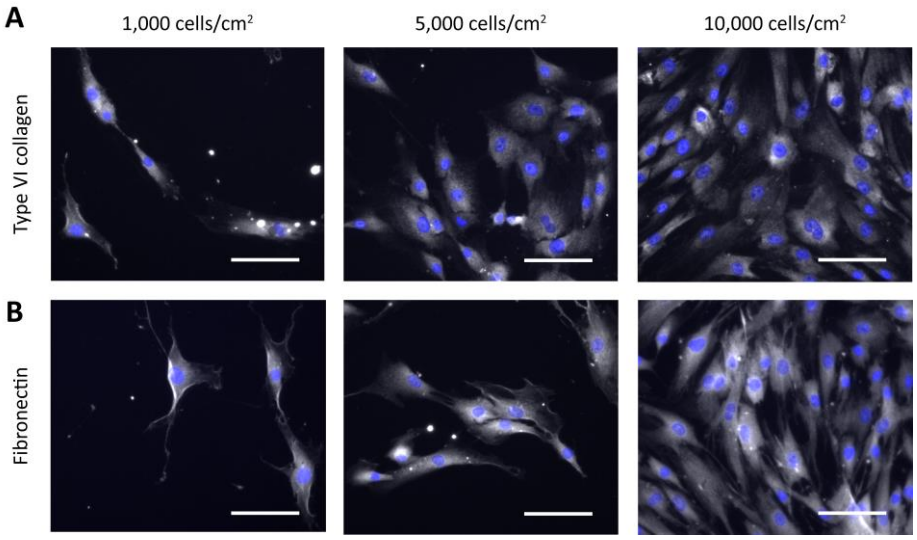
S2. Type I, II and III collagen expression decrease with increasing cell density

Immunofluorescence micrographs of hMSCs seeded at different densities: 1×10^3 cells/cm², 5×10^3 cells/cm², and 1×10^4 cells/cm² and evaluated 24 h after seeding. (A) Type I collagen (white) expression decreases with increasing cell density. (B) Type II collagen (white) expression decreases with increasing cell density. (C) HMSCs have a low expression of type III collagen (white) which decreases with increasing density. Nuclei are stained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.



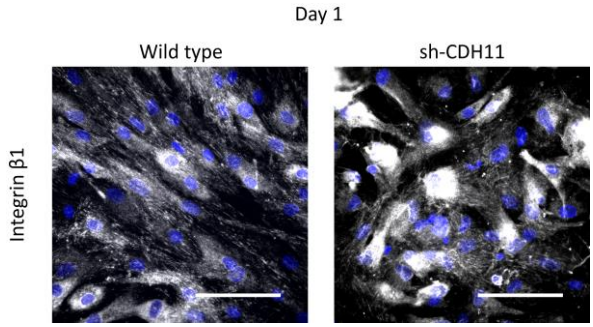
S3. Type I, II, and III collagen expression in cadherin-11–knockdown cells

Immunofluorescence micrographs of hMSCs seeded at 1×10^4 cells/cm^2 and evaluated after days 1 and 14. (A) No difference in type I collagen (white) expression was observed in wild type and sh-CDH11 cells.. (B) No difference in type II collagen (white) expression was observed in wild type and sh-CDH11 cells. (C) No difference in type III collagen (white) expression was observed in wild type and sh-CDH11 cells. Nuclei are stained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.



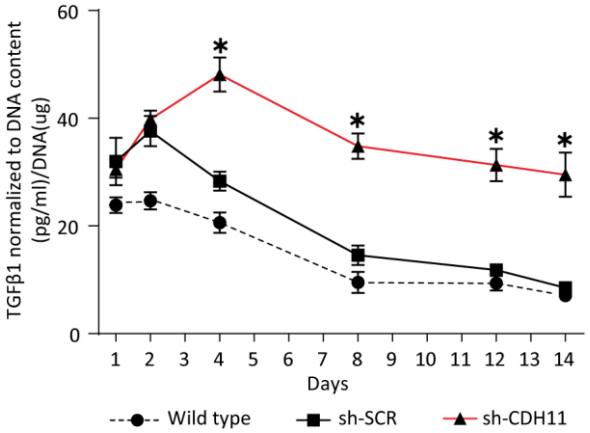
S4. Type VII collagen and fibronectin expression remain unchanged with increasing cell density

Immunofluorescence micrographs of hMSCs seeded at different densities: 1×10^3 cells/cm², 5×10^3 cells/cm², and 1×10^4 cells/cm². (A) Type VI collagen (white) expression remains unchanged with increasing density. (B) Fibronectin expression (white) remains unchanged with increasing density. Nuclei are stained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.



S5. Integrin β1 followed the pattern of fibronectin

Immunofluorescence micrographs of hMSCs seeded at 1×10^4 cells/cm^2. Integrin β1 (white) follows a pattern similar to the fibronectin. Nuclei are stained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.



S6. More TGFβ1 in the medium of cadherin-11–knockdown cells

Time course of TGFβ1 in the medium of hMSCs, where medium was collected and total TGFβ1 was measured using an ELISA following acidification. The data were normalized to the DNA content (proportional to cell number) in each sample (n=3). Compared to wild type and scrambled control (sh-SCR), less TGFβ1 was detected in the supernatant in cadherin-11–knockdown cells (sh-CDH11). Statistics were determined using two-way ANOVA with Tukey's test for multiple comparisons: *$p < 0.03$, sh-CDH11 compared to both wild type and sh-SCR. Error bars show ± SD. Data are representative of at least three independent experiments with similar results.

5

Cadherin-11 regulates cells proliferation via the RTK-ERK1/2 signaling pathway in human mesenchymal stem cells

This chapter has been submitted for publication and is under review:

Passanha FR, Divinagracia ML, LaPointe VL. Cadherin-11 regulates cells proliferation via the RTK-ERK1/2 signaling pathway in human mesenchymal stem cells. (2021)

ABSTRACT

Controlling stem cell fate is the cornerstone of regenerative medicine. Cadherins have an important role in cell fate commitment and the function of cadherin-11 in the regulation of differentiation in human mesenchymal stem cells (hMSCs) has recently come to light. To better understand how cadherin-11 regulates hMSC behavior, we explored its interaction with receptor tyrosine kinases (RTK), an important family of proteins involved in a myriad of cellular functions. In this study, we provide evidence that cadherin-11, a cell adhesion protein expressed in hMSCs, regulates the activity of several RTKs, including PDGFRβ and PDGFRα. By knocking down cadherin-11 we found that the changes in the RTK activity caused hyperactivation of the MAPK pathways, which were sustained through the phosphorylation and nuclear translocation of ERK1/2 and subsequently caused a decrease in cell proliferation. Together these results provide compelling evidence for the important role of the interaction of cadherin-11 and RTKs in the behavior of hMSCs.

INTRODUCTION

Type II classical cadherins, of which cadherin-11 is a member, are a family of cell surface glycoproteins that mediate calcium-dependent intercellular adhesion. Cancer research has found that cadherin-11 is frequently methylated and silenced in multiple carcinoma cell lines and its upregulation leads to accelerated invasion in prostate, breast, and pancreatic cancer.[1–4] Cadherin-11 has also been shown to be important for synaptic organization in the hippocampus in mice, and essential for the mechanical strength of smooth muscle tissue.[5,6] We have previously shown that loss of cadherin-11 affects cellular differentiation in human mesenchymal stem cells (hMSCs).[7] The involvement of cadherin-11 in the fate commitment of hMSCs makes it an interesting target for regenerative medicine.

HMSCs possess the ability to self-renew, differentiate into various cell types, and release soluble factors that are necessary for tissue repair and renewal.[8,9] HMSCs express different cell surface proteins including cell adhesion molecules, growth factor receptors, integrins, and chemokine reporters that make them highly responsive to diverse signals. Among the growth factor receptors, EGFR, FGFR, IGFR, PDGFR, TGFβR have been reported to be important for hMSC fate.[10–18] Apart from TGFβR, these receptors all belong to the family of receptor tyrosine kinases (RTKs).

RTK signal transduction is initiated by ligand binding to the receptor, which promotes receptor dimerization as well as phosphorylation of other substrates to ultimately convey the signal from the membrane to the nucleus.[19,20] Humans have 58 RTKs that respond to different extracellular signals and lead to an array of different outputs.[21] There are many documented cases of interactions between RTKs and other membrane proteins, such as cell adhesion molecules, GPCRs, and other

signaling receptors, all of which alter the ligand binding affinity of RTKs and further increase the diversity of downstream consequences.[22–25] We know that cadherin-11 has no known intrinsic signaling activity but recent studies have indicated that cadherin-11 interacts with various cell surface receptors including RTKs, thereby affecting cellular behavior. Specifically platelet-derived growth factor receptors (PDGFR), which are important markers for hMSCs, have recently gained traction for their interaction with cadherin-11.[26–30]

To understand the mechanisms controlling hMSC behavior, we decided to look at the interaction between cadherin-11 and RTKs. We then hypothesized that cadherin-11 binds to PDGFRβ, which leads to downstream signaling and changes in cell behavior. However, we observed that cells lacking cadherin-11 had a change in the activity of various RTKs and not just PDGFRβ. In contrast to previous studies, which have been limited to a single RTK, here we explore multiple human RTKs that are activated by hMSCs. We also show that the change in the activity of RTKs resulted in a change in the mitogen-activated protein kinase (MAPK) pathway activation. Finally, we show that a cadherin-11–mediated change in RTK suppresses proliferation in hMSCs.

RESULTS

1. Cadherin-11 forms a complex with PDGFRβ.

Recent studies have demonstrated a complex formation between PDGFRβ and cadherin-11, one similar to that between cadherin-1 and EGFR.[31,32] To explore if cadherin-11 and PDGFRβ are coexpressed in hMSCs, we performed immunostaining of the proteins and observed them using a confocal microscope (Figure 1A). The merged micrograph of cadherin-11 (magenta) and PDGFRβ (green) clearly shows regions where both proteins are expressed. A line scan of the intensity of cadherin-11 (magenta) and PDGFRβ (green) on the membrane in two locations indicated that the two proteins had a similar expression pattern (Figure 1B, C). However, we did observe a decrease in the overlap of the intensities at the cell–cell or cell–substrate interaction sites (Figure 1C).

To determine whether cadherin-11 and PDGFRβ were forming a complex and not just localized in similar areas of the plasma membrane, we performed co-immunoprecipitation experiments. Western blot analysis of the immunoprecipitation of cadherin-11 revealed that PDGFRβ co-immunoprecipitated and had formed a complex with cadherin-11 (Figure 1D). To confirm this result, we also performed immunoprecipitation of PDGFRβ, and Western blot analysis showed that cadherin-11 co-immunoprecipitated and, conversely, had formed a complex with PDGFRβ (Figure 1E).

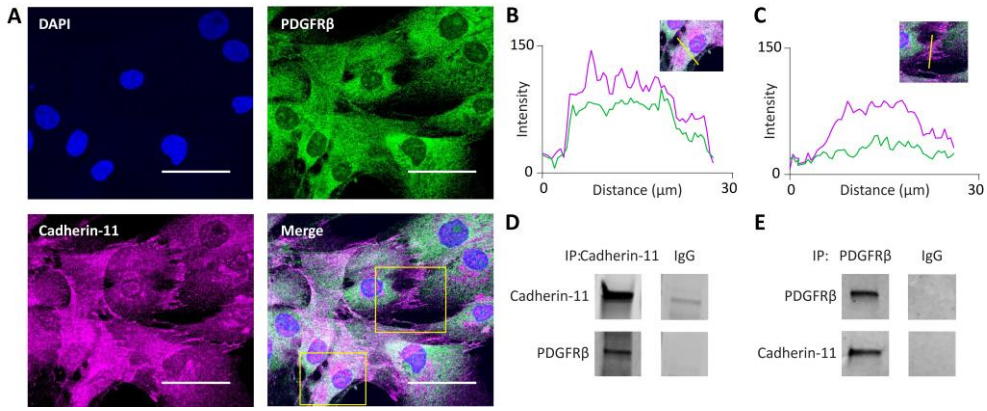


Figure 1. Cadherin-11 forms a complex with PDGFRβ

HMSCs were evaluated 48 h after seeding. (A) Confocal micrographs of DAPI (blue), PDGFRβ (green), and cadherin-11 (magenta) and their merged micrograph. Overlapping staining of PDGFRβ and cadherin-11 (white) indicated that the proteins are in close proximity. (B, C) Linescan drawn across the membrane shows the overlap of PDGFRβ (green) and cadherin-11 (magenta). (D) Cell lysates co-immunoprecipitated with antibodies against cadherin-11 on beads followed by Western blot for cadherin-11 and PDGFRβ revealed a complex of cadherin-11 with PDGFRβ. (E) Cell lysates co-immunoprecipitated with antibodies against PDGFRβ on beads followed by Western blot for PDGFRβ and cadherin-11 revealed a complex of PDGFRβ with cadherin-11.

2. Cadherin-11 knockdown changes phosphorylated RTKs.

Given that cadherin-11 and PDGFRβ form a complex, we sought to investigate other RTKs in the hMSCs. To this end, we screened for 47 RTKs to find which are active (indicated by their phosphorylation status) in hMSCs using a phosphorylated-RTK array. We observed that EGFR, HGF, RKY, PDGFRβ, PDGFRα, and AXL are phosphorylated in hMSCs (Figure 2A).

Given the evidence that cadherins can alter RTK phosphorylation, we wanted to investigate whether there were RTKs other than PDGFRβ that were also impacted by the knockdown of cadherin-11. We performed lentiviral transduction using cadherin-11 or scrambled shRNA of hMSCs. Immunofluorescent micrographs

confirmed a knockdown of cadherin-11 (Figure S1). Seven days after lentiviral transductions, the hMSCs were passaged and lysed after 48 h. We then used the phosphorylated-RTK array to identify which RTKs are differentially regulated in cadherin-11–knockdown cells. When we compared the wild type array and scrambled controls to the cadherin-11–knockdown cells, we observed a change in phosphorylated-RTKs (Figure 2B, C). The phosphorylated-RTK array heat map showed that EGFR, HGF, RKY, PDGFRα, and AXL were less phosphorylated, while PDGFRβ was more phosphorylated in the cadherin-11–knockdown cells (Figure 2D).

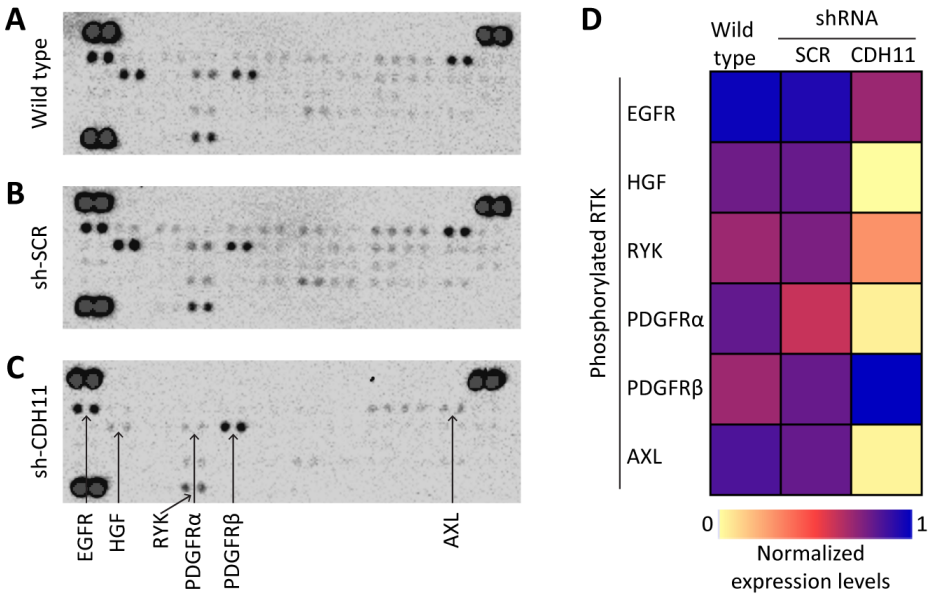


Figure 2. **Analysis of phosphorylation levels of RTKs**

HMSCs were evaluated 48 h after seeding. The samples were subjected to the Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems). Spot intensities were quantified by densitometry using ImageJ. Signals for each phosphorylated RTK are presented as a pair of duplicate spots, with three pairs of dark reference spots on the upper left, upper right, and lower left corners for alignment. (A) The phosphorylated RTK array of the wild type cells. (B) The phosphorylated RTK array of the scrambled control. (C) The phosphorylated RTK array of the cadherin-11–knockdown cells (sh-CDH11). (D) Heat maps show the average densitometric values of six phosphorylated RTKs of the wild type, scrambled

control, and cadherin-11 knockdown cells. High expression level in blue, intermediate expression level in red, and low expression level in yellow. The experiments were performed three times for the phosphorylated RTK array.

3. Cadherin-11 knockdown increases phosphorylated PDGFRβ and decreases phosphorylated PDGFRα.

Given that PDGFRs increase mesenchymal cell migration and proliferation and are critical for normal tissue development, we decided to further investigate these proteins. We first found that the immunofluorescent micrographs of PDGFRβ showed no changes in its expression when comparing the wild type to the cadherin-11–knockdown cells (Figure 3A, B). Similarly, an ICW assay showed no significant difference in the expression of PDGFRβ when comparing the wild type to the cadherin-11–knockdown cells (Figure S2A). Next, we investigated phosphorylated PDGFRβ and observed that the cadherin-11–knockdown cells had an increased expression compared to the wild type cells. When quantified using the ICW assay, we observed a significant increase in the expression of phosphorylated PDGFRβ in the cadherin-11–knockdown cells compared to the wild type ($p < 0.0001$; Figure S2B). This result was in agreement with what we observed in the phosphorylated-RTK array (Figure 3C, D).

When we investigated PDGFRα, we also observed no difference in the expression pattern of PDGFRα in the cadherin-11–knockdown compared to the wild type (Figure 4A, B). Similarly, an ICW assay showed no significant difference in the expression of PDGFRα when comparing the wild type to the cadherin-11–knockdown cells (Figure S3A). However, immunofluorescent micrographs of phosphorylated PDGFRα indicated a decrease in expression in cadherin-11–knockdown cells compared to the wild type (Figure 4C, D). The phosphorylated PDGFRα staining in the wild type cells was observed in the nuclei, indicating that

phosphorylated PDGFRα translocated to the nucleus (Figure 4C). When quantified using the ICW assay, we observed a significant decrease in the expression of phosphorylated PDGFRα in the cadherin-11–knockdown cells compared to the wild type ($p < 0.003$; Figure S3B).

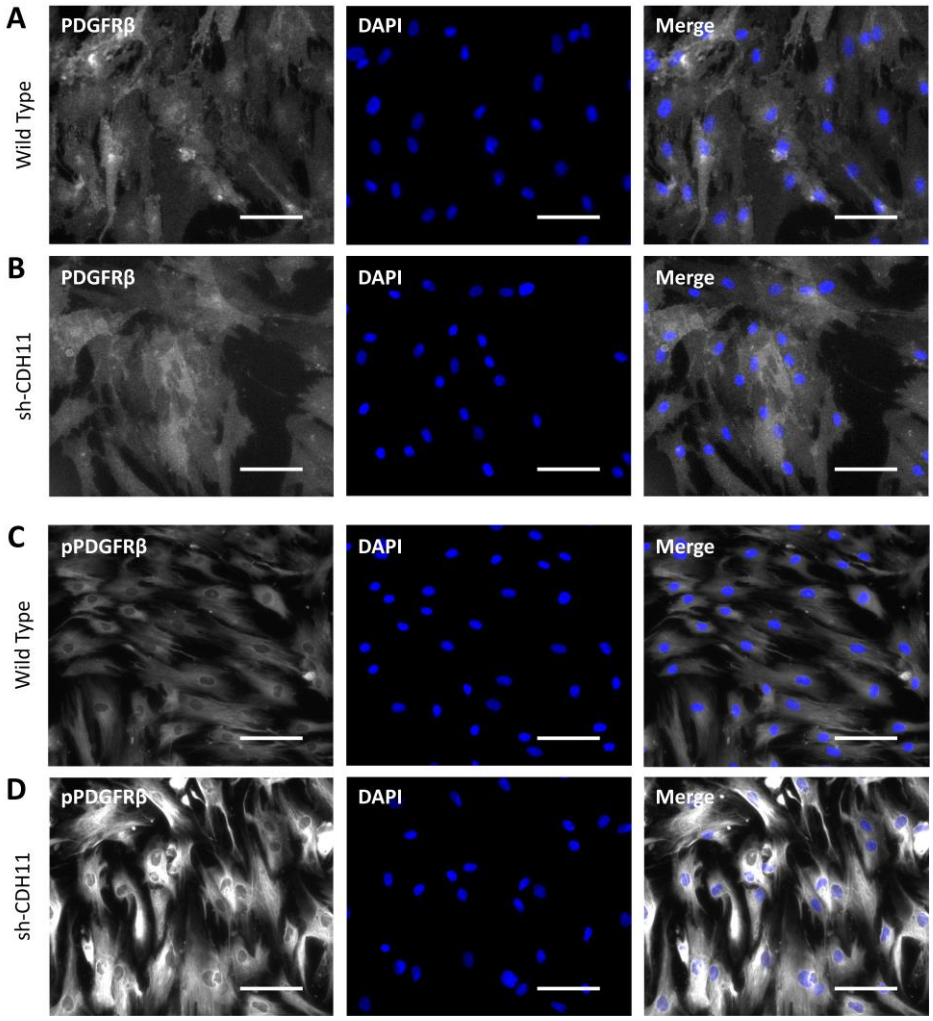


Figure 3. Cadherin-11 knockdown increased PDGFRβ phosphorylation

HMSCs were evaluated 48 h after seeding. Fluorescence micrographs of PDGFRβ (white) in (A) wild type cells and (B) cadherin-11–knockdown cells (sh-CDH11) show similar expression patterns. Fluorescence micrographs of phosphorylated PDGFRβ (white) in (C) wild type cells and (D) sh-CDH11 cells show an increased expression in sh-CDH11 cells compared to the wild type. Nuclei were counterstained with

DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

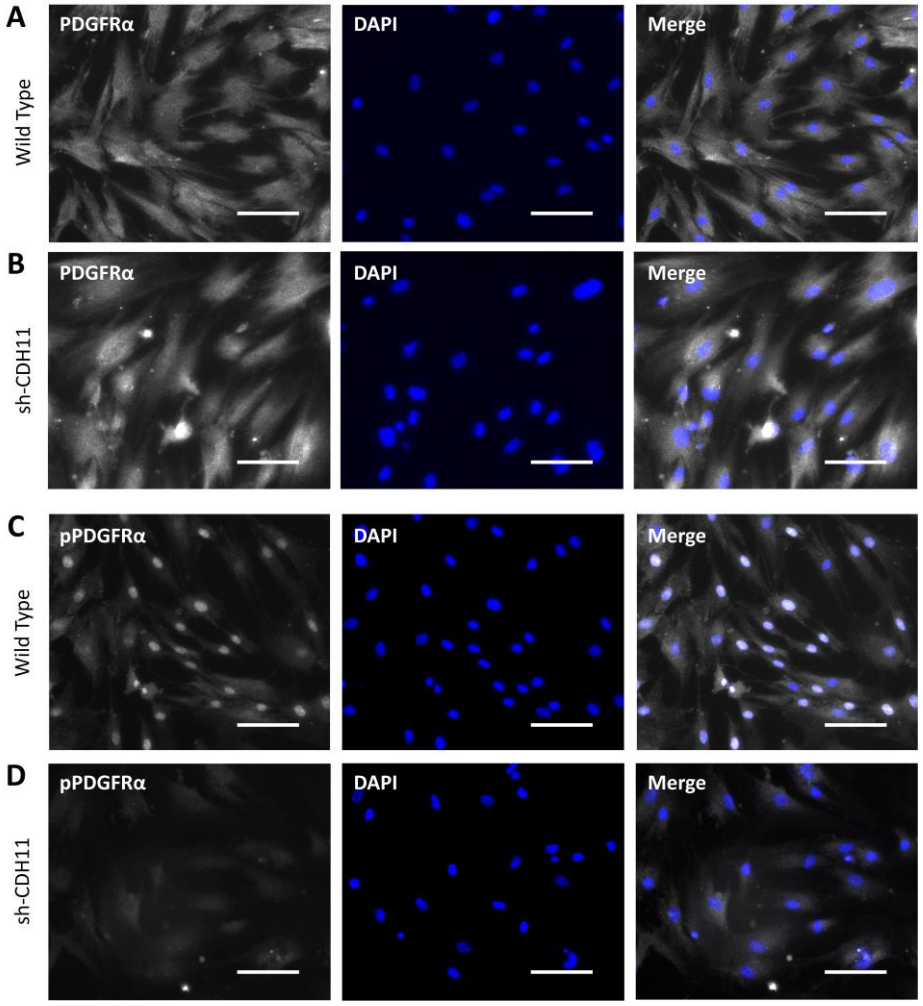


Figure 4. Cadherin-11 knockdown decreases PDGFR*α* phosphorylation

HMSCs were evaluated 48 h after seeding. (A) Fluorescence micrographs of PDGFR*α* (white) in (A) wild type cells and (B) cadherin-11–knockdown cells (sh-CDH11) show similar expression patterns. Fluorescence micrographs of phosphorylated PDGFR*α* (white) in (C) wild type cells and (D) sh-CDH11 cells show a decreased expression in sh-CDH11 cells compared to the wild type. Nuclei were counterstained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

4. Cadherin-11 knockdown causes decreased proliferation in hMSCs.

We have previously shown that cadherin-11 regulates the differentiation of hMSCs towards the osteogenic and adipogenic lineages.[7] Considering that cadherin-11 changes the RTK activation profile and RTKs are involved in differentiation as well as proliferation, we wanted to further explore the changes in cell behavior. We seeded both the wild type and the cadherin-11–knockdown hMSCs and assessed them after 48 h. When we studied the proliferation using EdU, we observed that the cadherin-11–knockdown cells proliferated more slowly than the wild type cells (Figure 5A). We quantified the proliferation by evaluating the number of EdU-positive cells and found that the wild type had 26% EdU-positive cells while the cadherin-11–knockdown cells only had 5% (p<0.0008) (Figure 5B).

Having observed a decrease in the number of proliferating cells in the cadherin-11–knockdown cells we wanted to determine how hMSCs were progressing through the cell cycle. Cell cycle analysis was done using a flow cytometer 48 h after seeding. Overall, we observed that the cadherin-11–knockdown cells had more cells (83.42 +/- 5.05%) in the G0/G1-phase compared to the wild type (69.45 +/- 0.52%) (Figure S4a and b; $p < 0.03$).

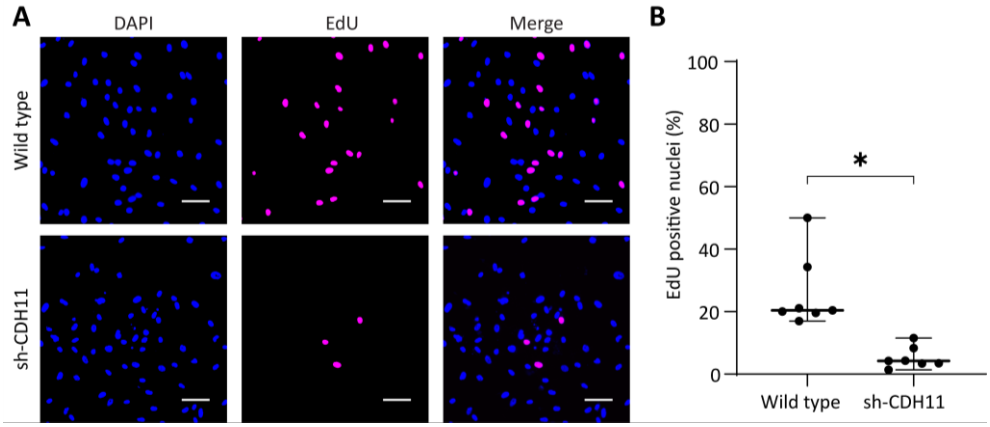


Figure 5. Loss of CDH11 reduces proliferation

(A) Fluorescence micrographs of hMSCs show reduced EdU in cadherin-11–knockdown (sh-CDH11) cells after 48 h in culture. DAPI (blue) and EdU (magenta) staining of hMSCs incubated with EdU for 48 h in growth medium. Scale bars represent 100 μm. (B) Quantification of the number of EdU-positive cells. n=7; N=3. Statistics were determined using Student's t-test: *p<0.0008.

5. Cadherin-11–knockdown cells have more phosphorylated ERK1/2–positive nuclei.

To confirm that changes in cell behaviour could be linked to the changes in RTK profile in the cadherin-11–knockdown cells, we explored downstream changes. We first examined the mitogen-activated protein kinase (MAPK) pathway that involves a series of protein kinase cascades that send information to the nucleus and play a critical role in regulating cell proliferation.[33–35] Platelet-derived growth factors use this pathway to relay and amplify signals in response to external stimuli. We looked at phosphorylated ERK1/2, which is the downstream signalling molecule of the MAPK pathway and accumulates in the nucleus to regulate the transcription of target genes. We observed that wild type cells had few positive nuclei positive for phosphorylated ERK1/2 compared to the cadherin-11–knockdown cells (Figure 6A, B). When this was quantified, the wild type cells had <1 % nuclei positive for

phosphorylated ERK1/2 compared to 29% in cadherin-11–knockdown cells (Figure 6C; $p<0.0001$).

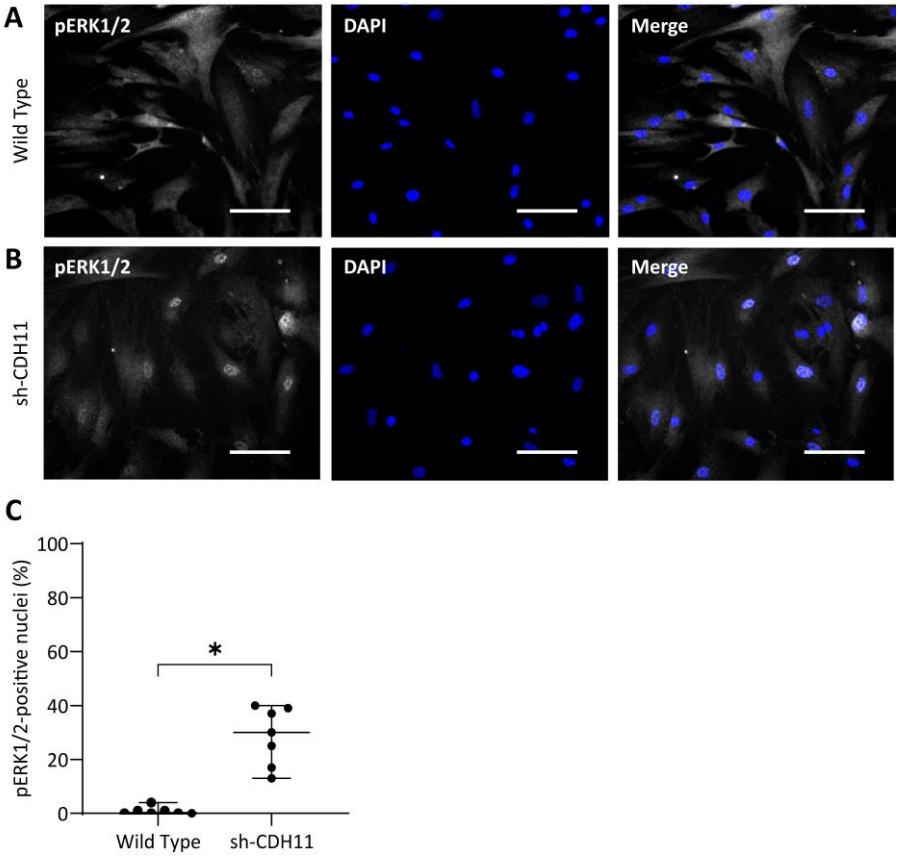


Figure 6. Cadherin-11 knockdown cells have more phosphorylated ERK1/2–positive nuclei

HMSCs were evaluated 48 h after seeding. (A) Fluorescence micrographs of phosphorylated ERK1/2 (white) in (A) wild type cells and (B) cadherin-11–knockdown cells (sh-CDH11). Nuclei were counterstained with DAPI (blue). Scale bars represent 100 μm. (C) Quantification of the number of positive phosphorylated ERK1/2 nuclei shows sh-CDH11 cells have more phosphorylated ERK1/2–positive nuclei compared to the wild type. n=7; N=3. Statistics were determined using Student's t-test: *$p<$ 0.0001.

DISCUSSION

Recent studies in fibroblast cells have shown a correlation between cadherin-11 and PDGFRs. These studies were conducted to ascertain the role of cadherin-11 cell signaling because it has no defined intrinsic signaling activity. Inspired by these studies, we aimed to study the role of cadherin-11 in hMSCs because we previously reported it influences the differentiation of hMSCs towards the adipogenic and osteogenic lineages. We based our initial hypothesis on the idea that PDGFRβ interacts with cadherin-11 thus influencing various functional changes in the hMSCs based on studies linking cadherin-1 to EGFR and cadherin-2 to FGFR.[36,37] What we observed was that cadherin-11 does not regulate just one RTK, but caused changes in multiple RTKs.

We first looked into PDGFRβ, an RTK that is considered one of the regulators of hMSC behavior.[38] We observed that not only does cadherin-11 form a complex with PDGFRβ, but in its absence, there is an increase in the phosphorylation of PDGFRβ (Figure 1, 3). A recent study also showed that cadherin-11 was complexing with PDGFRβ in fibroblasts enhances proliferation and tissue regeneration via the PDGFR-AKT signaling axis.[26] In their study the loss of cadherin-11 led to decreased PDGFRβ protein synthesis. In contrast, we did not observe a decrease in the PDGFRβ protein (Figure 3A). Our results are in line with studies that showed that cadherin-1 reduces the affinity of EGFR to its ligand thereby reducing EGFR phosphorylation.[39] Since we used co-immunoprecipitation to provide evidence for the complex formation, we cannot rule out the possibility that other proteins might also facilitate the binding of the cadherin-11 to PDGFRβ.

When we used a screening array to study the RTK profile of the hMSCs, we observed that cells lacking cadherin-11 had altered activation of multiple RTKs and not just

PDGFRβ (Figure 2). This highlights the complexity of both cadherin-11 and the RTKs. We further investigated PDGFRα and discovered that cells lacking cadherin-11 had a decreased expression of phosphorylated PDGFRα (Figure 4). A previous study showed an interaction between cadherin-11 and PDGFRα in synovial fibroblasts using co-immunoprecipitation. [27] In our fluorescent micrographs of phosphorylated PDGFRα, we observed its accumulation in the nucleus. This is in line with studies that have shown nuclear translocation of full-length receptors, including PDGFRβ, EGFR, ErbB2, ErbB3, FGFR1, FGFR2, IGF-1R, Met, VEGFR1 and VEGFR2 in response to ligand binding.[40] So far, the nuclear localization of PDGFRα has only been observed in alveolar fibroblasts in early embryonic lung development in a mouse model.[41]

The precise regulation of the ERK/MAP kinase pathway is essential for the growth and survival of eukaryotic cells. We have shown that cadherin-11–knockdown cells have more nuclei positive for phosphorylated ERK1/2 (Figure 6). One explanation could be that these cells lack feedback loops for ERK1/2 hence causing hyperactivation or prolonged activation of ERK1/2. Nuclear translocation of ERK1/2 occurs within 15 min of activation and persists during the entire G1 phase and reverses when the mitogenic stimulus is removed.[42] ERK1/2 is rapidly inactivated at the transition of the G1 to S phase.[43,44] The prolonged activation of ERK1/2 could indicate that cadherin-11–knockdown hMSCs are in the G1 phase for longer and that this transition to the S phase is delayed."

Together these findings improve our understanding of the complicated way in which hMSCs regulate their behavior. As cadherin-11 is implicated in cell fate decisions through its interaction with RTKs, a better understanding of their relationship could aid regenerative therapies using hMSCs.

MATERIALS AND METHODS

Cell culture

Bone marrow–derived hMSCs (PromoCell) obtained at passage 1 were maintained in growth medium composed of minimal essential medium α (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS)(SigmaAldrich). . The medium was changed every second day, and the cells were maintained at 37°C in 5% CO_2 in a humidified incubator. Upon reaching 80% confluence, cells were trypsinized in 0.05% trypsin-EDTA and replated for continuous passage. The cells were used at passage 5 for all experiments.

shRNA lentiviral transduction

The plasmid pLKO.1 containing short hairpin RNA (shRNA) sequences targeting cadherin-11 was obtained from Sigma-Aldrich together with a scrambled negative control. These plasmids were co-transfected with third generation lentiviral packaging and envelope vectors, pMD2.G, pRSV-Rev and pMDLg/pRRE (Addgene plasmids #12259, #12253 and #12251, respectively, which were gifts from Didier Trono[45]), into HEK-293T cells using PEIpro (VWR) transfection reagent. Lentiviral particles were harvested 48 and 72 h after transfection. Five milliliters of viral supernatant were used to transduce hMSCs seeded at 5000 cells/cm^2 in a T225 flask and incubated for 48 h. Transduced cells were selected with 2 μg/ml puromycin dihydrochloride (Sigma-Aldrich) in growth media for 7 days and were then used for subsequent experiments.

Phosphorylated-RTK array

The Proteome Profiler Human Phospho-RTK Array Kit (R&D Systems) is a membrane-based sandwich immunoassay. HMSCs were lysed in lysis buffer provided by the manufacturer supplemented with protease inhibitor (Sigma-

Aldrich). The lysate was incubated on ice with constant mixing for 30 min and then centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was transferred to a clean tube and total protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific), after which 300 μg of total protein lysate was incubated on the phospho-RTK array membrane and processed according to the manufacturer's protocol. Membranes were imaged on a ChemiDoc XRS+ System (Bio-Rad) with a 10 min exposure. Spot intensities were determined by quantifying the mean pixel grey values using the ImageJ 1.52b software.

Co-Immunoprecipitation

HMSCs were lysed on ice in 1X PBS containing 50 mM Tris-HCL, 150 mM NaCl, 1% IGEPAL CA-630 (Sigma-Aldrich), 2 mM EDTA, 1% dodecyl maltoside (Invitrogen) supplemented with protease inhibitor (Sigma-Aldrich). The lysate was incubated on ice for 30 min and centrifuged at 10,000 × g for 10 min at 4°C. Total protein concentration was measured using the Pierce BCA protein assay kit. For immunoprecipitation, 1 mg of total protein lysate in 1 ml was incubated overnight at 4°C with Dynabeads Protein G (Thermo Fisher Scientific) that were previously incubated with the 5 ug of primary antibody for 1 h at 4°C. The beads were collected by magnetic separation and washed 3 times in 1X PBS containing 50 mM Tris-HCL, 150 mM NaCl, 1% IGEPAL CA-630, 2 mM EDTA, supplemented with protease inhibitor. The beads were then boiled at 95°C in Laemmli buffer and 100 mM DTT and separated on 4–15% TGX gel (Bio-Rad) followed by transferring to a PVDF membrane using the wet transfer method. Membranes were blocked in 5% (w/v) milk in Tris-buffered saline with 0.01% (v/v) Tween-20 for 60 min before overnight incubation at 4°C with primary antibodies diluted in blocking buffer. The membranes were washed three times and were incubated with secondary antibodies in a blocking buffer for 2 h at ambient temperature. Primary antibodies were against cadherin-11 (rabbit polyclonal, 1:1000; Thermo Fisher Scientific, 71-7600) and

PDGFRβ (rabbit monoclonal, 1:1000; Thermo Fisher Scientific, MA5-15143), or cadherin-11 (mouse monoclonal, 1:1000; Thermo Fisher Scientific, 32-1700). Secondary antibodies used were: IRDye 680RD goat anti-mouse IgG or IRDye 800CW donkey anti-rabbit IgG (both 1:15,000; LI-COR Biotechnology). Membranes were imaged on an Odyssey infrared imaging system (LI-COR Biotechnology).

Immunofluorescence

HMSCs were washed twice with PBS and fixed in 4% formaldehyde for 15 min at ambient temperature. Fixed cells were washed three times with PBS for 10 min, permeabilized with 0.2% Triton X-100 for 20 min, washed three more times, blocked in 1% bovine serum albumin (BSA) in PBS for 1 h, and incubated with primary antibodies in 0.1% BSA at 4°C overnight. The cells were washed three times, incubated with secondary antibodies in 0.1% BSA for 2 h at ambient temperature, and the nuclei were counterstained with DAPI (0.1 μg/mL) for 10 min. Fluorescence images were acquired using a Nikon E600 inverted microscope or confocal microscope. Primary antibodies were against: PDGFRβ (monoclonal, 1:100; Thermo Fisher Scientific, MA5-15143), PDGFR*α* (rabbit polyclonal, 1:100; Thermo Fisher Scientific, 710169), pPDGFRβ (mouse monoclonal, 1:100; Thermo Fisher Scientific, MA5-15192), pPDGFR*α* (rabbit polyclonal, 1:100; RnD systems, AF21141), pERK1/2 (rabbit clone, 1:100; Cell Signaling Technology, 9101), pEGFR (rabbit polyclonal, 1:100; Thermo Fisher Scientific, 44-788G), cadherin-11 (rabbit polyclonal, 1:100; Thermo Fisher Scientific, 71-7600), or cadherin-11 (mouse monoclonal, 1:100; Thermo Fisher Scientific, 32-1700). Secondary antibodies were goat anti-mouse Alexa Fluor 647 or goat anti-rabbit Alexa Fluor 780 (both 1:500; Thermo Fisher Scientific).

In-Cell Western (ICW)

The ICW assay was performed using the Odyssey CLx Infrared Imaging System (LI-COR Biosciences), according to the manufacturer's instructions. Briefly, hMSCs were grown in 96-well plates, washed twice with PBS, and fixed in 4% formaldehyde for 15 min at ambient temperature. Fixed cells were washed three times with PBS for 10 min, permeabilized with 0.5% Triton X-100 for 20 min, washed three more times, blocked in 1% bovine serum albumin (BSA) in PBS for 1 h, and incubated with primary antibodies in 0.1% BSA at 4°C overnight. The cells were washed three times, incubated with secondary antibodies in 0.1% BSA as well as DRAQ5 (1:2000; Thermo Fisher Scientific) for 2 h at ambient temperature. Primary antibodies were against: PDGFRβ (rabbit monoclonal, 1:100; Thermo Fisher Scientific, MA5-15143), PDGFRα (rabbit polyclonal, 1:100; Thermo Fisher Scientific, 710169), pPDGFRβ (mouse monoclonal, 1:100; Thermo Fisher Scientific, MA5-15192), pPDGFRα (rabbit polyclonal, 1:100; R&D Systems, AF21141). Secondary antibodies were goat anti-mouse IgG IRDye 800 antibody or goat anti-rabbit IgG IRDye 680 antibody (both 1:500; LI-COR Biosciences). The 96-well plates were scanned with the Odyssey CLx Infrared Imaging System and quantified using the ImageJ 1.52b software. The relative amount of protein was obtained by normalizing to DRAQ5 in all experiments.

EdU cell proliferation detection

To assess the proliferation of hMSCs, 5-ethynyl-2'-deoxyuridine (EdU) staining was conducted using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific). HMSCs were incubated with 50 μM EdU for 48 h before fixation in 4% (v/v) paraformaldehyde in PBS for 15 min at ambient temperature. Fixed samples were permeabilized with 0.2% Triton X-100 for 20 min, washed three more times, blocked in 1% BSA for 1 h, and the incorporated EdU was labeled with Alexa Fluor 647 azide for 30 min according to the manufacturer's protocol. The nuclear DNA

was counterstained by DAPI (0.1 μg/ml) for 30 min. Fluorescence images were acquired using a Nikon E600 inverted microscope.

Cell cycle analysis

The cells were washed with PBS and trypsinized in 0.05% trypsin-EDTA 5 min at 37°C and resuspended and washed twice with ice-cold PBS. The cells were centrifuged at 300 × g and the PBS was aspirated. Ice-cold absolute ethanol was added dropwise to the cells while vortexing, in which the cells were fixed overnight at 4°C. Fixed samples were washed twice with PBS, resuspended in PBS, and treated with 10 μg/ml RNase A (Invitrogen) and 40 μg/ml propidium iodide (Sigma-Aldrich) overnight at 4°C in the dark. DNA content was determined by flow cytometry (BD Accuri C6). At least 10,000 events were acquired by pooling three samples for each experimental condition. The percentage of cells in different phases of the cell cycle was assessed using FlowJo software v10.6.0, and the detection of the G1, S, and G2 peaks was carried out using the Watson Pragmatic algorithm.

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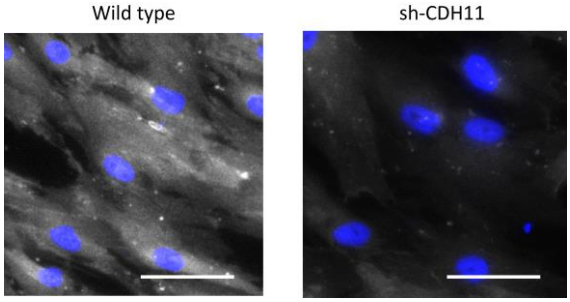
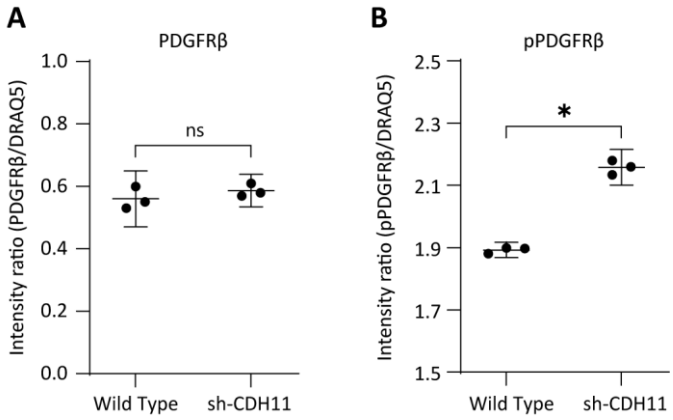
SUPPORTING INFORMATION



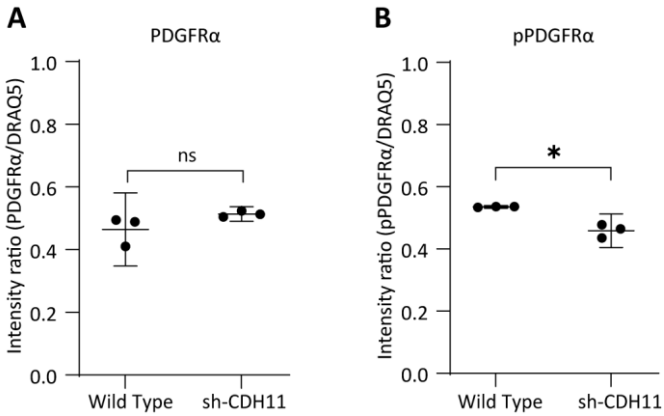
S1. Validation of knockdown

Immunofluorescent micrograph of hMSCs transduced with shRNA for CDH11 shows a decrease in cadherin-11 (white) expression compared to the wild type. Nuclei were counterstained with DAPI (blue). Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.



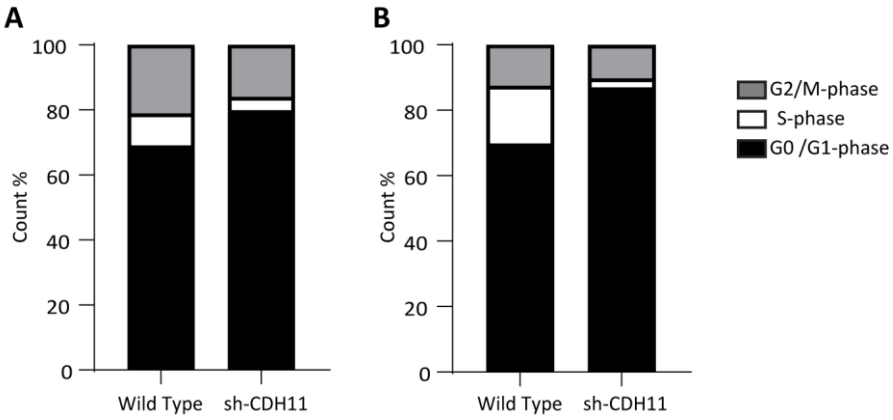
S2. Cadherin-11 knockdown increased PDGFRβ phosphorylation

Quantification of the cells stained with (A) PDGFRβ and (B) pPDGFRβ using the ICW assay normalized to the cell number (DRAQ5). Phosphorylated PDGFRβ showed a significant increase in expression in sh-CDH11 cells compared to the wild type while the levels of PDGFRβ remained unchanged. N=3. Statistics were determined using Student's t-test: *$p<0.0001$; ns: not significant.



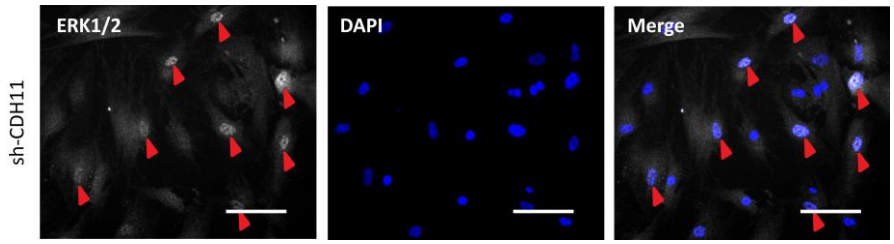
S3. Cadherin-11 knockdown increased PDGFRα phosphorylation

Quantification of the cells stained with (A) PDGFRα and (B) pPDGFRα using the ICW assay normalized to the cell number (DRAQ5). Phosphorylated PDGFRα showed a significant decrease in expression in sh-CDH11 cells compared to the wild type while the levels of PDGFRα remained unchanged. N=3. Statistics were determined using Student's t-test: *$p < 0.003$; ns: not significant.



S4: The Cadherin-11 knockdown has more cells in the G0/G1 phase compared to the wild type

HMSCs were evaluated 48 h after seeding. The samples were labelled for cellular DNA content followed by flow cytometry. Bar graph represents the quantitative measurement cell cycle phases (G0/G1, S, G2/M), where sh-CDH11 had more cells in the G0/G1 phase compared to the wild type. (A) and (B) each represent data from one independent experiment



S5. The red arrows indicate the positive nuclei counted for pERK1/2

Phosphorylated ERK1/2 is in white and nuclei were counterstained with DAPI (blue). The red arrow indicates the nuclei that were counted as positive for pERK1/2 in Figure 6C. Data are representative of at least three independent experiments with similar results. Scale bars represent 100 μm.

6

A comparative study of mesenchymal stem cells cultured as cell-only aggregates and in encapsulated hydrogels

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**These authors contributed equally.*

ABSTRACT

There is increasing evidence that cells cultured in three-dimensional (3D) settings have superior performance compared to their traditional counterparts in monolayers. This has been attributed to cell–cell and cell–ECM interactions that more closely resemble the *in vivo* tissue architecture. The rapid adoption of 3D cell culture systems as experimental tools for diverse applications has not always been matched by an improved understanding of cell behavior in different 3D environments. Here, we studied human mesenchymal stem/stromal cells (hMSCs) as scaffold-free self-assembled aggregates of low and high cell number and compared them to cell-laden alginate hydrogels with and without arginine-glycine-aspartic acid (RGD) peptides. We observed a significant decrease in the size of cell-only aggregates over 14 days in culture compared to the cells encapsulated in alginate hydrogels. Alginate hydrogels had persistently more living cells for a longer period (14 days) in culture as measured by total DNA content. Proliferation studies revealed that a weeklong culture of hMSCs in 3D culture, whether as aggregates or cell-laden alginate hydrogels, reduced their proliferation over time. Cell cycle analysis found no significant differences between days 1 and 7 for the different culture systems. The findings of this study improve our understanding of how aggregate cultures differ with or without a hydrogel carrier, and whether aggregation itself is important when it comes to the 3D culture of hMSCs.

INTRODUCTION

Therapies to repair or regenerate damaged tissue by the transplantation of stem cells are a promising approach in the field of regenerative medicine. Mesenchymal stem/stromal cells (MSCs) are one such candidate because of their ability to differentiate into various cell types, their immunomodulatory properties, their capacity to migrate to the site of injury, their low risk of teratoma formation, and that they can be derived from many (autologous) tissues[1–4]. MSC-based therapies have shown efficacy in treating patients with musculoskeletal injuries and disease, acute lung injury, traumatic brain injury, acute renal failure, cardiac injury, and other indications[5–8]. There are currently >20 ongoing Phase 3 trials using MSCs (https://www.clinicaltrials.gov/), making it reasonable to expect that more therapies will be available to patients in the near future.

To date, the MSC field continues to struggle with how to best direct the behavior of the cells, and scientists are increasingly moving towards three-dimensional (3D) culture to overcome this hurdle. In general, MSCs are reported to have improved behavior in 3D environments compared to monolayers. For example, spheroids of MSCs have higher osteogenic potential compared to cells in a monolayer both *in vitro* and *in vivo*[9]. Similarly, they also induce enhanced chondrogenic differentiation by an increased expression of TGFβ3[10]. MSC aggregates also secrete substantial quantities of potent anti-inflammatory proteins compared to monolayer cells[11] and late passage MSCs cultured as spheroids can regain their immune-modulatory factors[12]. The positive effects of 3D culture were also seen when medium conditioned by MSC spheroids effectively stimulated endothelial cell migration and proliferation compared to the medium conditioned by an adherent monolayer[13].

There are multiple ways to confer a 3D environment onto MSCs. For example, they can self-assemble into aggregates, be suspended in hydrogels, or combinations

thereof. In all cases, positive effects on cell behavior have been reported; for example, embedding cells within 3D microenvironments such as alginate hydrogels has also been shown to improve their survival and also allow the secretion of endogenous healing factors[14–16]. However, there have been few direct comparisons that would provide insight into how the behavior of MSCs is affected by the different 3D culture systems.

We sought to answer whether cells are best cultured as aggregates or encapsulated in hydrogels as a cell suspension. In the present study, we look at scaffold-free self-assembled aggregates (low and high cell number aggregates), and unaggregated cells encapsulated in alginate hydrogels with and without arginine-glycine-aspartic acid (RGD) peptides (Figure 1). We compared these systems based on cell viability, proliferation, and cell cycle analysis over a 7 day culture period in an effort to compare and contrast the different cell culture systems.

RESULTS

1. Cell-only aggregates decreased in size over time compared to cells encapsulated in alginate hydrogels.

We sought to study scaffold-free self-assembled high and low cell number aggregates, as well as cells encapsulated in alginate hydrogels with and without RGD peptides (Figure 1). The RGD peptide was selected because it is an important modifier used in polymers for tissue engineering [17]. Since it can be found in various proteins (e.g., collagens, gelatin, elastin, fibronectin, and laminins) and interacts with both α and β integrins, it can provide adhesion to non-fouling polymers such as alginate. To aggregate hMSCs, we used agarose microwells for low cell number aggregates and 15 ml polypropylene conical tubes for high cell number aggregates and allowed the cells to self-aggregate. The cell number for each of the conditions was kept constant at 100,000 cells per condition (Figure 1). Once assembled, the low and high cell number aggregates decreased in size over the first 7 days ($p < 0.0001$; Figure S1a and b). HMSCs were also encapsulated in the alginate hydrogels with and without RGD and were examined by phase contrast microscopy. There was no significant difference in the size of the alginate hydrogels over time (Figure S1c and d). They also had a similar appearance and had homogeneously distributed hMSCs after 14 days in culture (Figure S2).

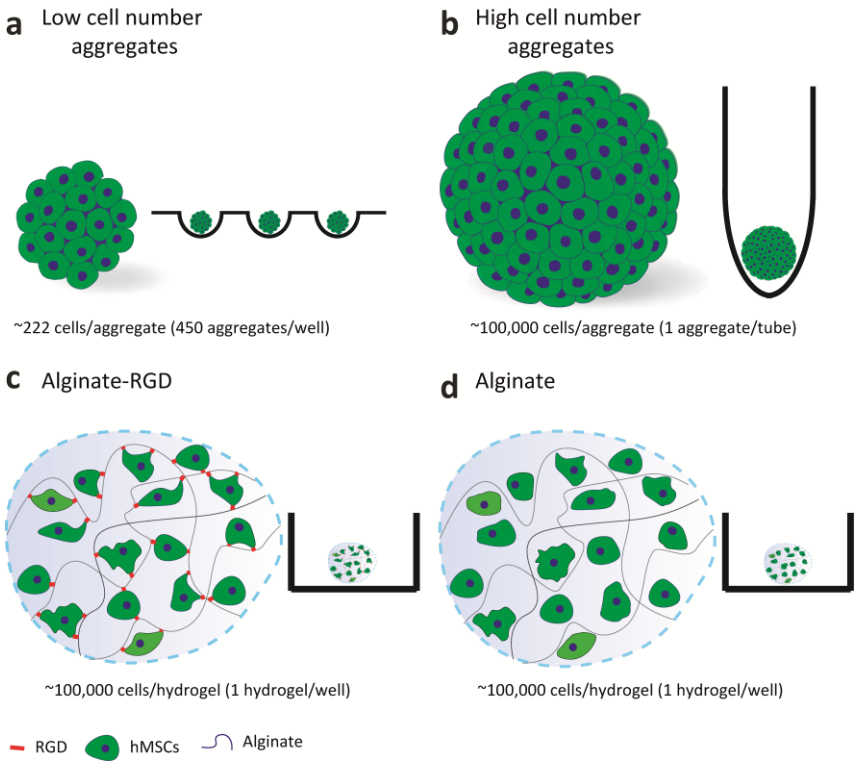

Figure 1. hMSCs in four different cell culture systems

Schematic illustration of the four 3D cell culture systems: hMSCs seeded as low cell number aggregates (a), hMSCs seeded as a high cell number aggregate (b), hMSCs encapsulated in alginate hydrogels modified with RGD (c), and hMSCs encapsulated in alginate hydrogels without modification (d).

2. DNA content decreases over time for all four culture systems.

To quantify the total amount of DNA present over time, a value directly related to the cell number, we used PicoGreen DNA quantification assay in each of the cell culture systems. To make a relative comparison, we compared the differences between the median of three independent experiments in each of the cell culture systems (each having started with 100,000 cells in total). These data were confirmed using the CellTiter-Glo 3D Cell Viability Assay (Figure S3).

On day 1, the DNA content of the different culture systems was not significantly different from each other except when comparing the low and high cell number aggregates (Figure 2a). Namely, the high cell number aggregate had significantly lower DNA content compared to the low cell number aggregates, while they both had similar number of cells while seeding ($p < 0.04$; Figure 2a). Whether cells were encapsulated in alginate or cultured as aggregates had no effect on the DNA content after 1 day. When comparing the measured DNA content to the amount that would come from the 100,000 cells that were seeded (660 ng), both the low cell number aggregates and cells in encapsulated in alginate with RGD had significantly higher DNA content ($p < 0.003$).

After 7 days of culture, a different trend was observed (Figure 2b). In all culture systems, the DNA content had decreased since day 1 ($p < 0.002$) except the cells encapsulated in alginate hydrogels without RGD (Figure 2d–g). When comparing the different culture systems, we observed that the difference in DNA content of low and high cell number aggregates was insignificant, as was the DNA content of cells encapsulated in alginate hydrogels with and without RGD (Figure 2b). The DNA content of the cells encapsulated in alginate hydrogels was significantly higher than cells as aggregates ($p < 0.002$). Compared to the DNA content of 100,000 cells, the cells encapsulated in alginate hydrogels with RGD had significantly higher DNA content, while the low cell number aggregates had significantly lower DNA content ($p < 0.01$; Figure 2b).

After 14 days of culture, there were no statistically significant differences compared to day 7 in all culture systems (Figure 2d–g). However, when comparing the different culture systems we observed that the DNA content was significantly different in all conditions except for low and high cell number aggregates (Figure 2c)., where both had significantly lower DNA content compared to cells

encapsulated with and without RGD ($p < 0.02$; Figure 2c). Compared to the DNA content of 100,000 cells, only low and high cell number aggregates had significantly lower DNA content ($p < 0.003$; Figure 2c).

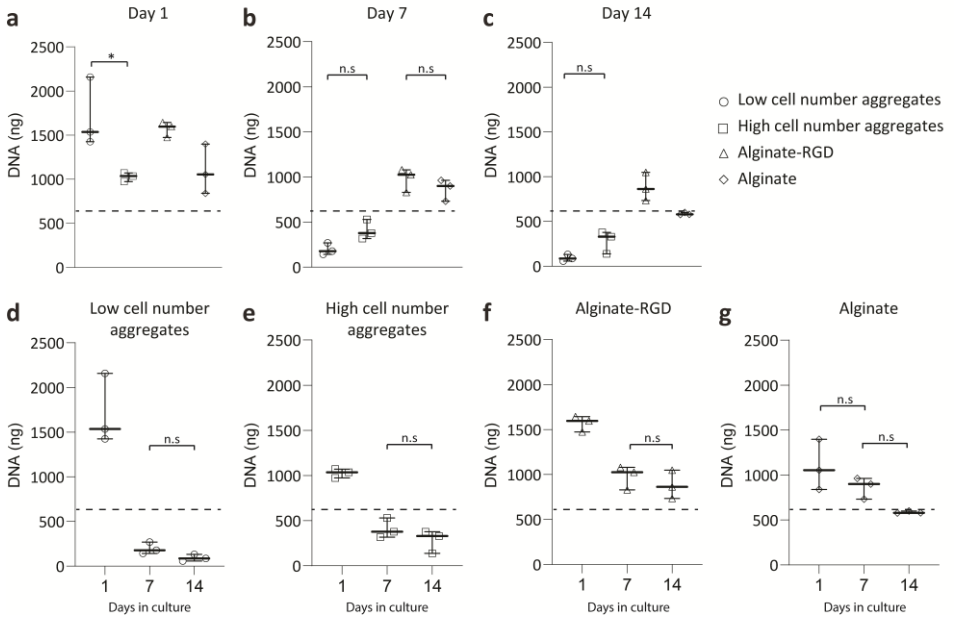

Figure 2: Alginate hydrogels have higher DNA content over time compared to cells in aggregates

HMSCs were seeded in four different cell culture systems: low cell number aggregates, high cell number aggregate, alginate hydrogels without modification and alginate hydrogels modified with RGD. The DNA content was analysed using the PicoGreen assay at day 1 (a), 7 (b), and 14 (c). The same data were also used to compare the different culture systems over time: hMSCs seeded as low cell number aggregates (d), hMSCs seeded as a high cell number aggregate (e), hMSCs encapsulated in alginate hydrogels modified with RGD (f), and hMSCs encapsulated in alginate hydrogels without modification (g). Data are from three independent experiments. The dotted line indicates the approximate DNA content of 100,000 cells. Statistical significance was determined using one-way ANOVA with Tukey's test for multiple comparisons. Except for a, all comparisons are statistically significant unless mentioned otherwise; *: $p < 0.02$; n.s: not significant. Data are represented as median with range.

3. Cells remained viable for at least 14 days in culture.

Since the different ways of aggregating and encapsulating hMSCs could have an effect on their access to soluble gases and nutrients, we used a live/dead viability assay at days 1, 7, and 14 to determine whether spatial differences could explain the changes in cell number described in Figure 2.

In the images of low cell number aggregates, we could observe more dead cells, especially in the center of the aggregates, at day 7 (Figure 3b) compared to day 1 (Figure 3a). In the high cell number aggregates, we observed more dead cells at day 7 and 14 compared to day 1 (Figure 3d–f). Similarly, for cells encapsulated in alginate hydrogels, at the periphery, with (Figure 3g–i) or without (Figure 3j–l) RGD, we observed similar numbers and distribution of both live and dead cells at all time points. We observed more dead cells at day 7 for cells encapsulated in alginate with RGD compared to day 1, however for cells encapsulated in alginate without RGD, there was no observable difference in the number of dead cells at all three time points. This is consistent with DNA content results (Figure 2f and g).

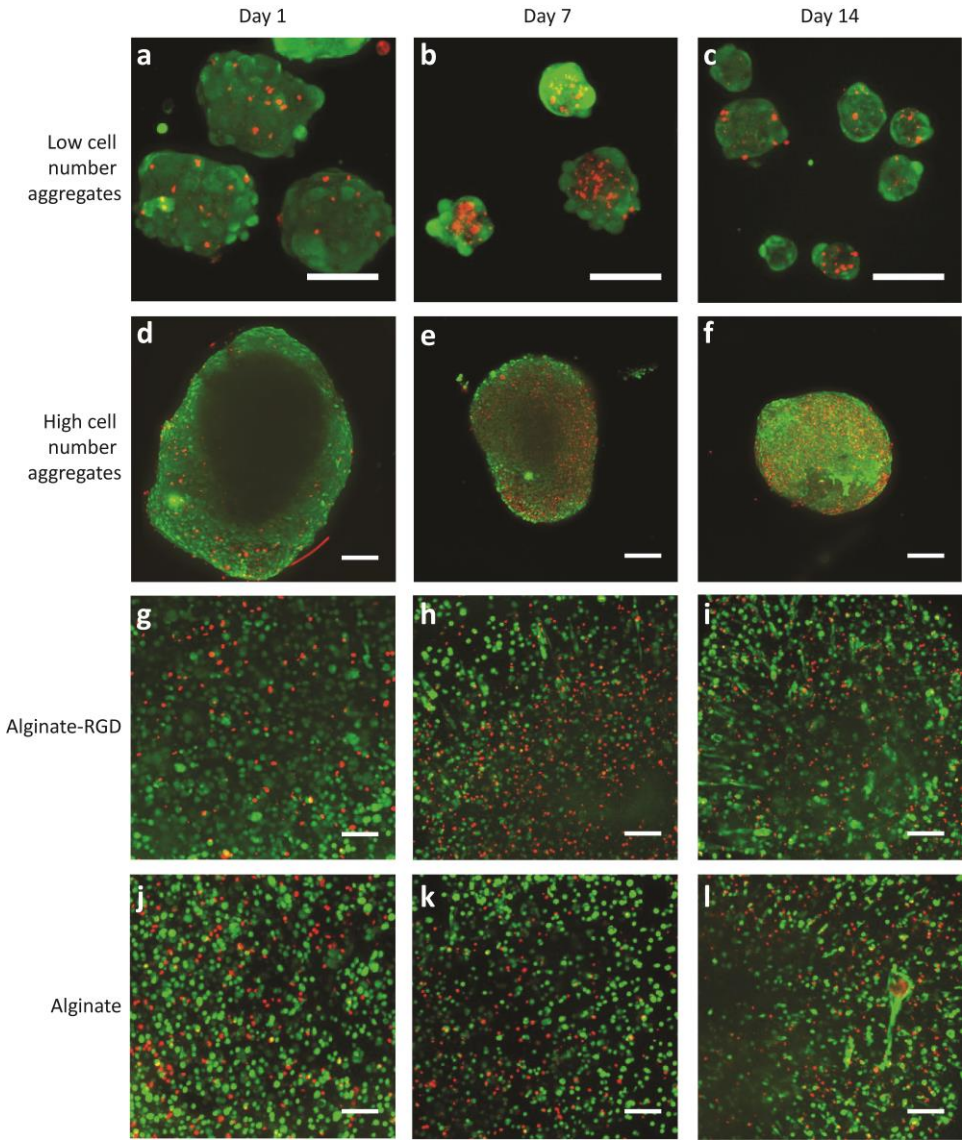

Figure 3. The cell culture systems all maintain viable cells over 14 days in culture

HMSCs were seeded in four different cell culture systems and labelled with calcein-AM (green; live) and ethidium homodimer-1 (red; dead) at days 1, 7, and 14. Fluorescence micrographs of hMSCs seeded as low cell number aggregates (a–c), a high cell number aggregate (d–f), encapsulated in alginate hydrogels modified with RGD (g–i), and encapsulated in alginate hydrogels without modification (j–l). Scale bars represent 100 μm. Data are representative of at least three independent experiments with similar results.

4. The long-term culture of hMSCs in 3D culture systems decreases proliferation.

To understand if the sharp decrease in DNA content in aggregates could be attributed to an increase in cell death or if cell proliferation played a role, we set out to investigate the differences in proliferation rates between the samples. Given that the doubling time of these hMSCs on tissue culture polystyrene was approximately 48 hours, we used a 48-hour EdU incubation to detect proliferating cells. The analysis was done at days 2, 7, and 14 on images of whole-mounted samples.

In all four cell culture systems, proliferating cells were detected at day 2 (Figure 4). The low cell number aggregates had proliferating cells in 100% the 73 aggregates analysed on day 2 (Figure 4a), but on days 7 and 14, only 7% of the aggregates analysed contained proliferating cells (Figure 4b and c). In high cell number aggregates, there were more proliferating cells visible in the periphery of the aggregates on day 2 (Figure 4d), which was notably diminished by days 7 and 14 (Figure 4e and f). Microscopy limitations prevented us from getting a clearer picture of the centre of the large aggregates.

In the alginate hydrogels either with or without RGD, there was no discernible difference between the number of proliferating cells at day 2 (Figure 4g and j). Similar to the aggregates, fewer proliferating cells were observed in the alginate hydrogels at day 7 (Figure 4h and k) and day 14 (Figure 4i and l) compared to day 1. Taken together, these results indicate that the differences noted in DNA content in Figure 2 might be due to differences in both cell death and proliferation rates.

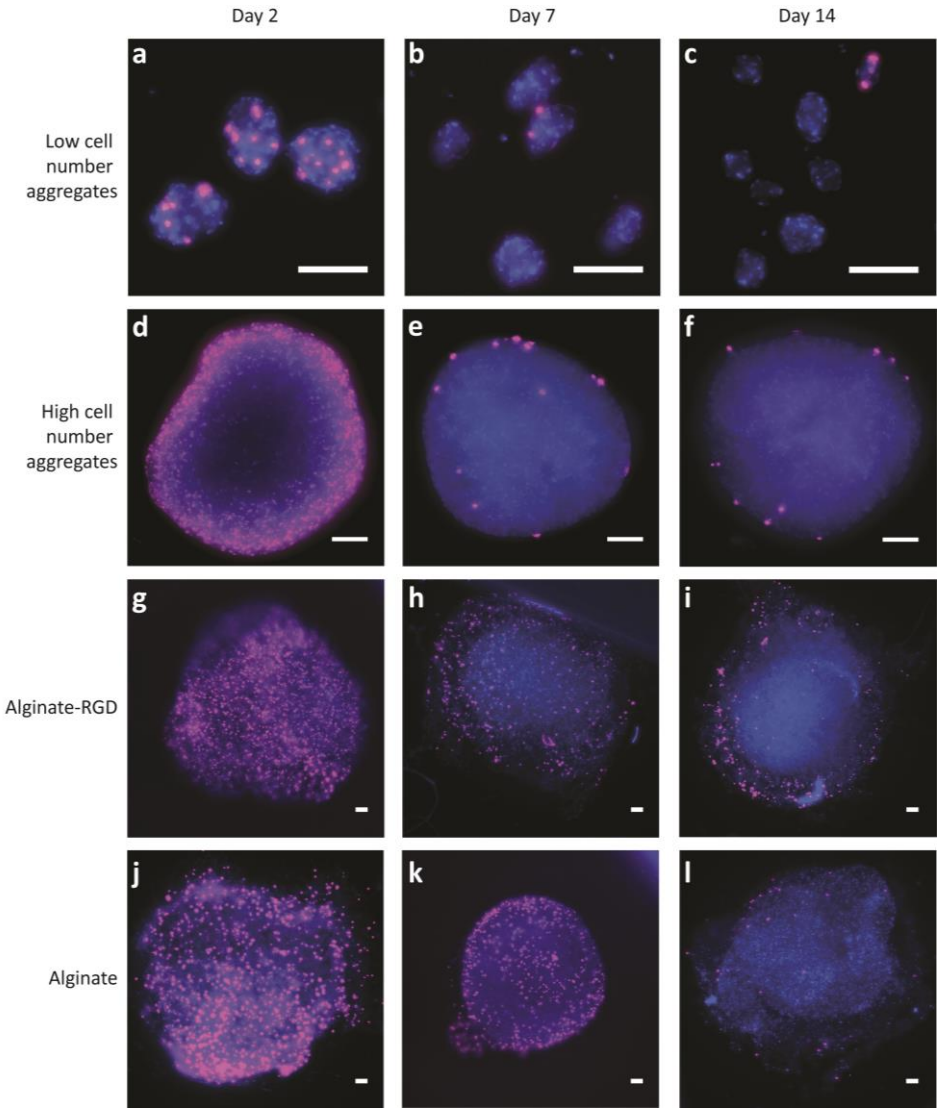

Figure 4. Long-term culture of hMSCs as aggregates and in alginate hydrogels suppresses proliferation

HMSCs were seeded in four different cell culture systems and were subjected to EdU (pink) for 48 hours prior to analysis on days 2, 7 and 14. The samples were counterstained with DAPI (blue). Fluorescence micrographs depict hMSCs seeded as low cell number aggregates (a–c), high cell number aggregate (d–f), encapsulated in alginate hydrogels modified with RGD (g–i), and encapsulated in alginate hydrogels without modification (j–l) at day 2, 7, and 14. Scale bars represent 100 μm. Data are representative of at least three independent experiments with similar results.

5. Low cell number aggregates inhibit cell cycle progression.

Having observed a decrease in the number of proliferating cells over time, we wanted to determine how hMSCs were progressing through the cell cycle in the different culture systems. Cell cycle analysis was done using flow cytometry at days 1 and 7 (Figure 5). Overall, we observed no significant differences in the number of cells in the S-phase at either day 1 and day 7 when we compared the different culture systems (Figure 5a and b), whereas differences were noted in the number of cells in the G_0/G_1-phase and the G_2/M-phase.

When observing how cells progressed through the cell cycle at days 1 and 7, there were differences that could be attributed to the culture system. On day 1, the low cell number aggregates had significantly more cells in the G_0/G_1-phase compared to cells in the alginate without RGD ($p < 0.02$; Figure 5a). Furthermore, there were significantly more cells in the G_2/M-phase in alginate with RGD compared to the low and high cell number aggregates ($p < 0.03$; Figure 5a). Later, on day 7, there were significantly more cells in the G_0/G_1-phase in low cell number aggregates compared to cells in alginate with RGD ($p < 0.03$; Figure 5b). There were more cells in alginate without RGD in the G_2/M-phase compared to in the high cell number aggregates ($p < 0.02$; Figure 5f).

In low cell number aggregates at day 1, we observed 85.5 ± 4.2% cells were in G_0/G_1-phase, 6.7 ± 5.2% in S-phase, and 7.6 ± 1.1% in G_2/M-phase (Figure 5c). On day 7, this was not significantly different, and we measured 81.3 ± 6.9% cells in G_0/G_1-phase, 9.0 ± 8.0% in S-phase, and 9.5 ± 1.1% in G_2/M-phase. This suggests that the low rate of proliferation observed was due to a large number of cells arrested in the G_0/G_1-phase after aggregate formation. In high cell number aggregates at day 1, 81.0 ± 2.0% of cells were in G_0/G_1-phase, 8.1 ± 6.3% in S-phase, and 10.1 ± 4.2% cells in G_2/M-phase (Figure 5d). On day 7, this was not significantly different, and we measured

77.1 ± 5.9% of cells in G_0 /G_1-phase, 10.5 ± 2.4% in S-phase, and 12.2 ± 6.1% in G_2/M-phase. Overall, there were no significant differences between low and high cell number aggregates in the cell cycle phases at either time point ($p > 0.05$).

Analyzing the cells encapsulated in alginate with RGD at day 1, 60.4 ± 8.9% were in G_0 /G_1-phase, 10.7 ± 12.2% in S-phase, and 28.7 ± 5.1% cells in G_2/M-phase (Figure 5e). On day 7, this was not significantly different, and we measured 48.5 ± 5.6% of cells in G0 /G1-phase, 22.2 ± 5.7% in S-phase, and 29.2 ± 5.4% in G_2/M-phase. For the cells encapsulated in alginate without RGD at day 1, 57.6 ± 9.2% were in G_0 /G_1-phase, 6.8 ± 2.5% in S-phase, and 35.5 ± 8.2% in G_2/M-phase (Figure 5f). On day 7, this was not significantly different, and we measured 46.1 ± 8.6% of cells in G_0/G_1-phase, 23.7 ± 8.5% in S-phase, and 30.1 ± 8.8% in G_2/M-phase. There were no significant differences between cells encapsulated in alginate with RGD and without RGD in the cell cycle phases at either time point ($p > 0.05$).

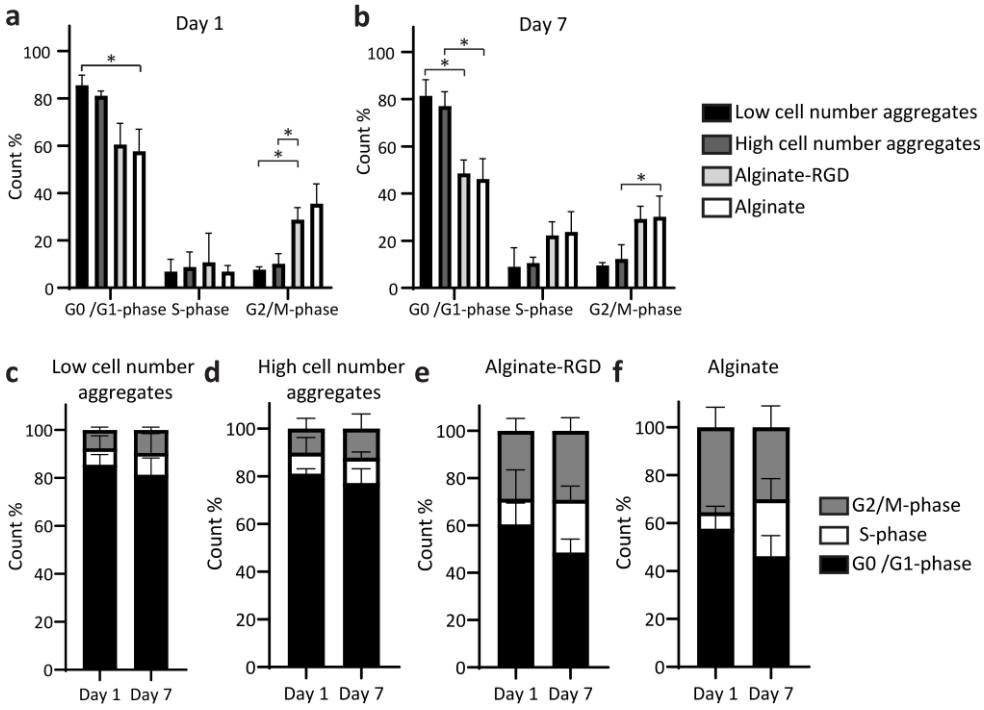

Figure 5. Low cell number aggregates have less cell cycle progression

HMSCs that were seeded in four different cell culture systems and were labelled for cellular DNA content followed by flow cytometry at days 1 and 7. Bar graph represents the quantitative measurement cell cycle phases (G0/G1, S, G2/M) at day 1 (a), 7 (b). The same data were also used to compare the different culture systems over time using stacked bars: hMSCs seeded as (c) low cell number aggregates, (d) a high cell number aggregate, (e) encapsulated in alginate hydrogels modified with RGD, and (f) encapsulated in alginate hydrogels without modification. Error bars represent mean ± SD. Data are from three independent experiments. Statistical significance was determined using two-way ANOVA with Tukey's test for multiple comparisons: *$p < 0.04$.

DISCUSSION

HMSCs are an attractive candidate for the development of regenerative therapies, and employing 3D cell culture systems is one possible way to maximize their therapeutic potential[11]. In this study, we were able to compare and contrast the cell number (DNA content), viability, proliferation and cell cycle progression of hMSCs in different 3D culture systems, namely: scaffold-free self-assembled aggregates of two sizes (termed high and low cell number aggregates) and cells encapsulated in alginate with and without RGD functionalization. Overall, we noted changes in the DNA content and proliferation, while the cell cycle progression of the hMSCs in the different culture systems remained unchanged over 7 days in culture.

A quantitative DNA assay revealed a decrease in DNA content in all culture systems over time (Figure 2). This decrease was more pronounced when cells were cultured in aggregates than when they were encapsulated in alginate. Low cell number aggregates ended with the lowest DNA content at day 14. A similar outcome was observed in a study that showed that hMSC aggregates undergo apoptosis unless they get appropriate signals for differentiation[18]. The overall decrease in the size of low cell number aggregates was also consistent with the decrease in DNA content.

Studies have shown that aggregation can keep hMSCs viable for longer periods compared to adherent cultures[11,19]. However, both studies attributed their outcomes to the use of dynamic 3D culture methods, which is in contrast to the static culture techniques used in this study. Here, we found that aggregation itself (assessed at day 1) did not reduce the cell numbers, but it nonetheless appeared that the cells lacked some survival cues (Figure 2). The decrease in DNA content we measured may be the result of poor nutrient and oxygen diffusion to all cells present in aggregates due to crowdedness. In contrast, culturing in the alginate hydrogels may

resolve this issue by providing more space between cells, allowing for more nutrients and oxygen diffusion, and thereby higher DNA content. In fact, researchers have shown that glucose, thymidine and proteins such as insulin growth factor-1 (IGF-1), growth hormone (GH) and bovine serum albumin (BSA) were able to diffuse inside alginate hydrogels[20]. For most of these molecules, 4 hours were enough to reach 80% equilibrium. Based on these studies, we conclude that our 1% alginate gels harbor similar diffusion kinetics.

To further investigate the differences in cell number and to obtain spatial information about the location of dead cells, we performed a live/dead assay (Figure 3). We observed an increase in cell death on day 7 compared to day 1 in all culture systems, which was consistent with our DNA content result as well as other studies that have shown increase in cell death due to apoptosis when MSCs are cultured as aggregates under static conditions[21]. However, it seemed that the differences in cell death could not be attributed to their spatial distribution, as a necrotic core was observed in small cell number aggregates but not in high cell number aggregates or in either alginate hydrogel systems.

To explain why aggregates had lower DNA content, we hypothesized there was an imbalance between cell death and proliferation. We demonstrated that cells proliferated in all samples until day 2, but this decreased after one week in culture and remained stable until two weeks (Figure 4). Cells at the periphery of aggregates and hydrogels were more proliferative than cells in the centers, which correlates to previous research findings stating that proliferation occurs when cells have access to appropriate nutrients, correct signaling molecules, and sufficient oxygen[22,23]. Overall, the aggregates seem to promote less proliferation compared to the alginate hydrogels. This low, but present, degree of proliferation is likely the reason why alginate hydrogels show better maintenance of DNA content over time. The overall

decrease in the size of low cell number aggregates is also consistent with the decrease in cell number, the decrease in the number of proliferating cells, and cell death. Past studies have suggested that this could be due to cell compaction[24], which was not observed in our study.

To further examine proliferation, we looked at how cells progress through the cell cycle and found no significant differences between the different time points for the different culture systems (Figure 5). This was in contrast to what we observed using the EdU proliferation assay, but was in agreement with a recent finding[21]. One explanation for this difference could be that we added EdU to the cells immediately after seeding and encapsulation but before aggregation. Since hMSCs take approximately 24 hours to form aggregates, the EdU incorporation into proliferating hMSCs occurred when they were still in a single cell suspension. This may also explain why the DNA content at day 1 increased compared to the amount we would expect from seeding 100,000 cells.

Overall, no significant differences were observed between cells encapsulated in alginate with and without RGD, which might be explained by the relatively low amount of RGD peptide incorporated into the hydrogel. A previous study has shown that increasing the density of RGD grafted onto alginate hydrogels led to more adhesion, cell spreading, and proliferation, while small amounts of RGD induced myoblasts to acquire a more rounded morphology [17]. In this study, we may have not reached a sufficiently high number of grafted peptides and, therefore, did not observe a significant difference between alginate with and without RGD. In addition, the alginate concentration may have promoted less spreading, even in the presence of the RGD peptide. Other researchers have reported that 0.5% alginate–RGD induces little to no spreading of MSCs and ADSCs, especially compared to 2% alginate–RGD [25]. Future studies with different concentrations of RGD and other

relevant adhesion motifs should be conducted to understand how these peptides influence the outcome of 3D hMSC culture systems. For instance, comparing the performances of alginate hydrogels grafted with GHK (derived from osteonectin), GFOGER (collagen type I), and IKVAV (laminin), amongst others, may allow us to better design an ideal 3D culture system [26–29].

In summary, the research performed here assessed four different 3D cell culture systems with two different variants in each to see how they influence the behavior of hMSCs: scaffold-free self-assembled aggregates of two sizes and cells encapsulated in alginate with and without RGD functionalization. From our measurements, we observed that the alginate constructs (both with and without the RGD peptide), appear to better sustain the cells over time. In conclusion, this study underlines the notion that alginate hydrogels might be able to keep hMSCs viable for a longer period compared to cell aggregates.

MATERIALS AND METHODS

Cell culture

Bone marrow–derived hMSCs (PromoCell) were obtained at passage 1 and confirmed free of mycoplasma using the mycoplasma detection kit from BD Biosciences. The cells were maintained in growth medium composed of minimal essential medium (MEM α; Gibco) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich). The cells were maintained at 37°C in 5% CO_2 in a humidified incubator and the medium was changed every two days. Upon reaching 80% confluence, cells were detached by incubating with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Thermo Fisher Scientific) and re-plated for continuous passage. The cells were used at passage five for all experiments.

Microwell formation

Agarose microwell arrays were prepared as previously described[30]. Briefly, 3% ultra pure agarose solution (Invitrogen) was cast onto a poly(dimethylsiloxane) stamp with microstructures to imprint microwells, de-molded upon solidification, cut to size, and inserted into 12-well plates. Each well of the microwell array contained 450 microwells with a diameter of 400 μm.

Low cell number and high cell number aggregate formation

HMSC aggregates were formed in two different sizes of approximately 222 cells (low cell number) or 100,000 cells (high cell number). To form low cell number hMSC aggregates, 100,000 cells in 400 μl growth medium were seeded into one microwell array. The plate was centrifuged at 300 × g for 5 min to allow the cells to settle into the microwells, after which an additional 2 ml of growth medium was added to each well. The cells clustered spontaneously within 24 h. To form a high cell number hMSC aggregate, 100,000 cells in 2 ml of growth medium were seeded into a 15 ml

polypropylene conical tube (Greiner Bio-One). The tube was centrifuged at 300 × g for 5 min to allow the cells to settle to the bottom. The cells clustered to form an aggregate within 24 h. For both aggregate cultures, medium was changed every two days.

Preparation of RGD-modified alginate

Food grade alginate (70% GG blocks; kindly provided by FMC Polymers, Norway) was purified according to a previously published protocol [31]. Briefly, the alginate was dissolved overnight in ultrapure water (18 MΩ, Milli-Q UltraPure Water System, Millipore) at a final concentration of 1% (w/v). After dissolution, 2% (w/v) activated charcoal (Sigma- Aldrich) was added under agitation for 1 h at ambient temperature. The obtained suspension was then centrifuged for 1 h at 27,000 × g. Afterwards, the supernatant was passed through a series of filters (1.2, 0.45, and 0.22 μm porous membranes; VWR) via vacuum filtration and was freeze-dried and stored at -20°C until further use. The alginate was then modified with the peptide (glycine)-4-arginine-glycine-aspartic acid-serine-proline (RGD; Genscript) to allow cell adhesion using aqueous carbodiimide (EDC) chemistry. Briefly, as described previously[32], a 1% (w/v) alginate solution was prepared in 0.1 M 2-(N-morpholino) ethane sulfonic acid (MES) buffer solution (0.1 M MES buffering salt, 0.3 M NaCl, pH adjusted to 6.5 using 1 M NaOH, Sigma-Aldrich). N-hydroxy-sulfosuccinimide (sulfo-NHS; Pierce Chemical, 27.40 mg per gram alginate) and 1-ethyl-(dimethylaminopropyl)-carbodiimide (EDC; Sigma-Aldrich, 48.42 mg per gram alginate), at a molar ratio of 1:2, were sequentially added to the solutions, followed by the addition of 16.70 mg RGD per gram alginate. The solution was stirred for 20 h at ambient temperature and quenched with 18 mg of hydroxylamine hydrochloride (Sigma-Aldrich) per gram of alginate. The final product was dialyzed (MWCO 3500, Spectra/Por, VWR) against decreasing concentrations of NaCl (7.50, 6.25, 5.00, 3.75, 2.50, 1.25 mg) in 4 l of ultrapure water for three days at 4°C, freeze-

dried, and stored at -20°C until use. The RGD concentration was 35 μM, as reported in a previous study [33].

Alginate hydrogel formation

Alginate hydrogels containing hMSCs were made by centrifuging 100,000 cells at 500 × g for 5 min and resuspending them in 10 μl of 1% (w/v) alginate (either with or without RGD) in NaCl (0.9% (w/v) in water). The alginate hydrogels were formed by dispensing the 10 μl droplet into a 100 mM $CaCl_2$ (Sigma-Aldrich) bath and allowing it to cross-link for 5 min. The crosslinking solution was then replaced by growth medium for subsequent culture in non-adherent cell culture plates (VWR). Phase contrast micrographs of hMSCs encapsulated in alginate hydrogels were taken at days 1 and 14 with a Nikon eclipse TS100 inverted microscope.

DNA quantification

A DNA quantification was conducted on days 1, 7, and 14 using the PicoGreen assay (Thermo Fisher Scientific). After measuring luminescence (for the CellTiter-Glo assay), the samples were lysed in RLT lysis buffer (Qiagen) and stored at -80°C. Samples were freeze-thawed thrice to ensure their complete lysis. The samples were diluted 1:100 in a solution of 10 mM Tris-HCl with 1 mM EDTA (pH 7.5) and were analysed using the PicoGreen assay on a ClarioStar plate reader (BMG LabTech) with the fluorescence signal (excitation: 492 nm and emission: 520 nm) used to extrapolate the DNA concentration from a standard curve.

Live/dead assay

In order to determine the location of the viable cells, a fluorescence-based live/dead viability assay was conducted on days 1, 7, and 14. Cell aggregates and the alginate hydrogels were washed with Tris-buffered saline (TBS) after which they were fully immersed in a solution of 2 μM calcein-AM ester and 5 μM ethidium homodimer-1

in α-MEM without phenol red for 30 min at 37°C before imaging directly. The fluorescence images were acquired on a Nikon E600 inverted microscope.

EdU cell proliferation detection

To assess cell proliferation, 5-ethynyl-2'-deoxyuridine (EdU) staining was conducted using the Click-iT EdU Alexa Fluor 647 Imaging Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. HMSCs were incubated with 50 μM EdU for 48 h before fixation at days 2, 7, and 14. Cell aggregates and the alginate hydrogels were washed twice in TBS with 7.5 mM $CaCl_2$, and fixed in 4% (v/v) paraformaldehyde (Sigma-Aldrich) in TBS/$CaCl_2$ for 15 min at ambient temperature. Fixed samples were permeabilized with 0.5% (v/v) Triton X-100 (VWR) in TBS for 1 h and the incorporated EdU was labeled using a click reaction with Alexa Fluor 647 azide for 30 min according to the manufacturer's protocol. The nuclear DNA was counterstained by DAPI (0.1 μg/ml) for 30 min. The fluorescence images were acquired on a Nikon E600 inverted microscope.

Cell cycle analysis

To give more information about proliferation, hMSCs were seeded as aggregates or encapsulated in alginate hydrogels in parallel, and cell cycle analysis was conducted on days 1 and 7. To give more information about proliferation, a cell cycle analysis was conducted on days 1 and 7. The cell aggregates were washed with PBS and incubated with 1 ml of Accutase (Thermo Fisher Scientific) for 30 min in a water bath at 37°C and the cells were resuspended vigorously every 10 min. The alginate hydrogels were washed with PBS and incubated with 50 mM EDTA in PBS for 10 min at 37°C. After dissociation of both the cell aggregates and alginate hydrogels, the cells were washed twice with ice-cold PBS. The cells were centrifuged at 300 × g and the PBS was aspirated. Ice-cold absolute ethanol was added dropwise to the cells while vortexing, in which the cells were fixed overnight at 4°C. Fixed samples

were washed twice with PBS, resuspended in PBS, and treated with 10 μg/ml RNase A (Invitrogen) and 40 μg/ml propidium iodide (Sigma-Aldrich) overnight at 4°C in the dark. DNA content was determined by flow cytometry (BD Accuri C6). At least 10,000 events were acquired by pooling three samples for each experimental condition. The percentage of cells in different phases of the cell cycle was assessed using FlowJo software v10.6.0, and the detection of the G1, S, and G2 peaks was carried out manually. The location of the peaks was fixed in order to have the best fit over all the samples.

CellTiter-Glo 3D Cell Viability Assay

The number of viable cells was determined using the CellTiter-Glo 3D Cell Viability Assay (Promega) based on the detection of the presence of ATP in living cells according to the manufacturer's protocol. Briefly, cell aggregates and the alginate hydrogels were transferred to a 96-well plate with 100 μl of growth medium on days 1, 7, and 14, and 100 μl of CellTiter-Glo 3D Reagent was added into each well. The plate was then placed on an orbital shaker for 5 min and incubated at ambient temperature for an additional 25 min. The luminescence was measured on a ClarioStar plate reader (BMG LabTech) with an integration time of 1 s.

Statistics

Statistics were determined using one-way ANOVA with Holm-Sidak's test for multiple comparisons for DNA content and two-way ANOVA with Tukey's test for multiple comparisons for cell cycle analysis, with p values < 0.05 considered significant. Statistical tests were performed with GraphPad Prism 8.

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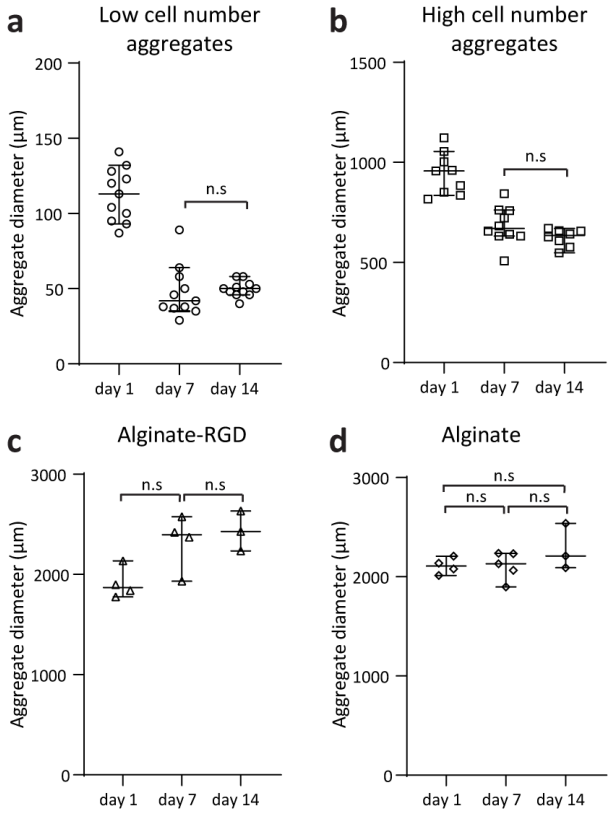
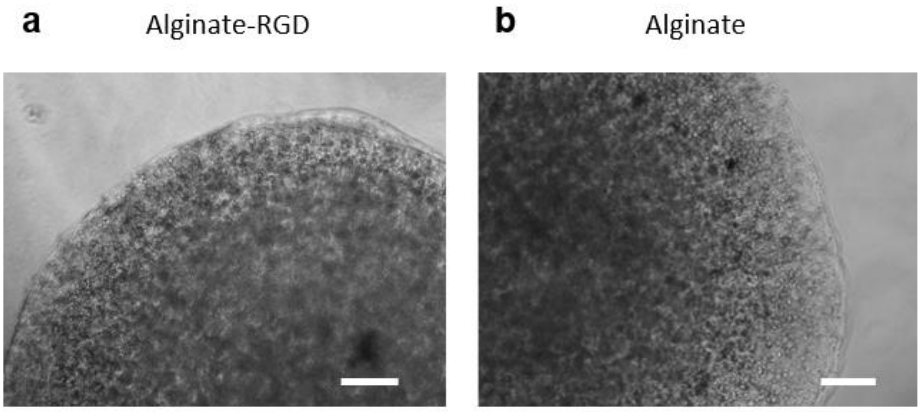
SUPPORTING INFORMATION



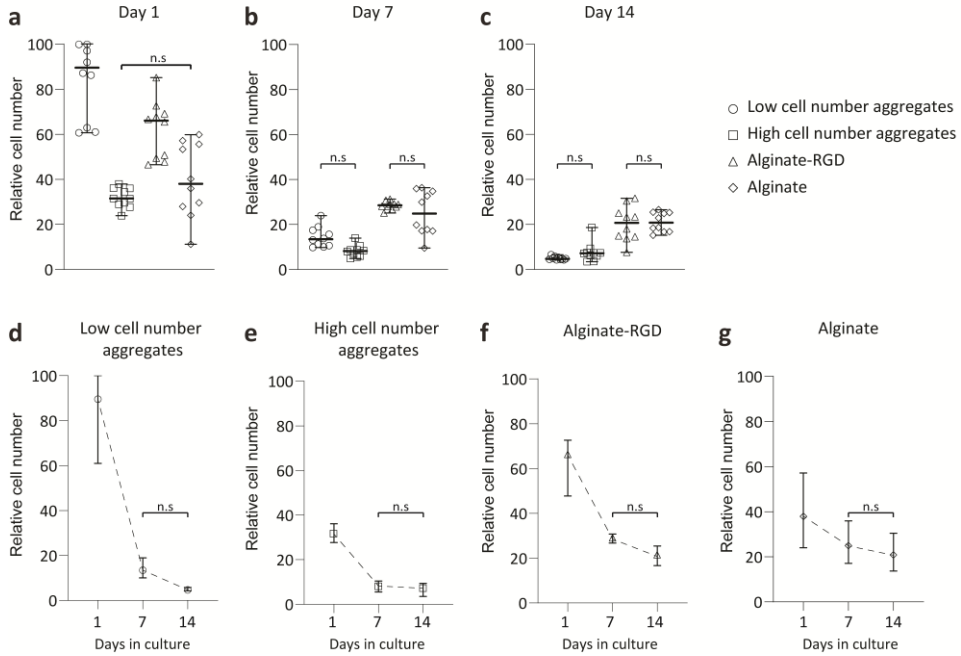
S1: Aggregates decreased in size over time for both low and high cell number aggregates

The diameter of the hMSCs seeded as low cell number aggregates (a), as high cell number aggregate (b), in alginate hydrogels modified with RGD (c), and in alginate hydrogels without modification (d) was measured from fluorescence micrographs of hMSCs stained with calcein-AM from the live/dead assay and DAPI from the EdU assay at days 1, 7 and 14. The diameters of the aggregates and alginate hydrogels were measured using ImageJ 1,52b software. Data are from three independent experiments. All comparisons are statistically significant ($p < 0.05$) unless mentioned otherwise; n.s: not significant; error bars represent median ± 95% CI.



S2. Cells encapsulated in alginate hydrogels with and without RGD modification look visually similar

Phase contrast micrographs of hMSCs encapsulated in alginate hydrogels with RGD modification (a) and without RGD modification (b) after 14 days in culture. Scale bars represent 100 μm.



S3. Alginate hydrogels have higher viability over time compared to cells as aggregates

HMSCs were seeded in four different cell culture systems: low cell number aggregates, high cell number aggregate, alginate hydrogels without modification, and alginate hydrogels modified with RGD. The number of viable cells was evaluated using the CellTiter-Glo 3D cell viability assay at days 1, 7, and 14 (a–c). The same data were also used to compare the different culture systems over time: hMSCs seeded as low cell number aggregates (d), as a high cell number aggregate (e), encapsulated in alginate hydrogels modified with RGD (f), and encapsulated in alginate hydrogels without modification (g). Data are from 10 independent experiments. Statistical significance was determined using one-way ANOVA with Tukey's test for multiple comparisons and all comparisons are statistically significant ($p < 0.03$) unless mentioned otherwise; n.s: not significant. (a–c) data are represented as median with range. (d–g) error bars represent median ± 95% CI.

7

Discussion

INTRODUCTION

The purpose of this thesis was to perform some basic research inspired by the overarching challenge in regenerative medicine: why cells respond to cues from certain materials and fail to respond to others. Although the challenge sounds simple, achieving this goal is to tissue engineers as proving string theory is to physicists. Most research in realizing this goal has been focused on cell–matrix interactions, and relatively little is known about how the cell–cell interactions may influence the response of the cell to the material. The key to driving embryonic development, collective cell migration, and also cancer metastasis, among others is the combination of cell–cell interactions with cell–matrix interactions.[1–4] This research, an attempt at leveling the playing field between cell–cell and cell–matrix interactions in understanding how cells respond to cues, not only has implications in the field of regenerative medicine for material-centric approaches but can also be useful for cell-centric approaches.

Tissue engineers must take lessons from developmental biology while trying to answer their questions. Similar to *Drosophila melanogaster*, in which biologists study how a fruit fly develops from an egg to an embryo to an adult, I decided to study how adult stem cells go from being cells with the multipotent ability to ones with specialized functions. Understanding the mechanisms of cell adhesion in adult stem cells, what triggers a particular behavior in adult stem cells, and eventually trying to harness these underlying mechanisms can help understand why and how certain materials can drive desirable cell behavior.

We know from developmental biology that cadherins, cell–cell adhesion molecules, are part of the essence of what it is to be an animal, as they maintain the structural integrity of multicellular organisms.[5] Furthermore, cadherins drive self-

organization, thereby playing a crucial role in development, and are known to influence cell phenotype.[6,7] Therefore, I decided to explore cadherin biology in the context of regenerative medicine and see if it was the answer to our larger questions about cell behavior. Although human mesenchymal stem cells (hMSC) have amassed 30 years of literature for their interesting cell biology and broad-ranging clinical potential, they are still an enigma. As we unravel the complex biology and therapeutic potential of hMSCs, there remains much to be gained in terms of scientific knowledge and clinical benefit. In my thesis, I, therefore, aimed to contribute to a deeper understanding of the nature of cadherins in hMSC behavior to generate fundamental knowledge to advance the field of regenerative medicine. To this end, I set up smaller tangible goals and discussed them in detail in Chapters 3, 4, 5, and 6. This chapter contains discussion and future research possibilities described with the help of the following four questions:

1. What does the differential expression of cadherins in 2D and 3D mean for regenerative medicine?
2. How can the role of cadherins in differentiation benefit regenerative medicine?
3. What insight does the role of cadherin-11 provide for regenerative medicine?
4. What consequences does the cadherin–RTK interaction have for regenerative medicine?

1. WHAT DOES THE DIFFERENTIAL EXPRESSION OF CADHERINS IN 2D AND 3D MEAN FOR REGENERATIVE MEDICINE?

When I began my research, there was a rise in the number of papers claiming that aggregate culture models would work better than monolayer ones. We have learned that depending on the circumstances there can be dramatic differences between monolayer and aggregate culture.[8] HMSC aggregation has been shown to improve their differentiation capacity, which is particularly evident in the case of chondrogenic differentiation because monolayer culture is non-physiological for chondrocytes as it promotes an overabundance of adhesion.[9–11] Therefore, we first wanted to understand whether the change in behavior between monolayer and aggregate cultures was driven by cadherin-mediated interactions.

In Chapter 3 we showed that when hMSCs form aggregates, they have diminished expression of cadherin-2 after 24 h in culture, while as a monolayer this change took 10 days. In contrast, the expression of cadherin-11 remained constant. Using atomic force microscopy it had been previously demonstrated that individual cadherin-11 bonds are two-fold stronger than cadherin-2 bonds.[12] Strong bonds are important to coordinate activity in aggregates, which might also be why cadherin-2 is downregulated in aggregate cultures. Changes in cadherin expression between monolayer and aggregates also lead to changes in major signaling pathways such as Wnt/β-catenin and PI3K/AKT signaling.

Over the past few decades, tremendous progress has been made in the field of tissue engineering as a result of strategies that combine cells with 3D biodegradable scaffolds to create replacement tissues. However, there are still some problems when it comes to the long-term 3D culture of hMSC which we touched upon in Chapter 6

where we compared different methods to culture hMSCs in a 3D environment. We noted that hMSCs cultured as aggregates were missing certain cues necessary for long-term survival. At the same time, multiple passages of hMSCs *in vitro* as a monolayer also result in reduced differentiation capacity, replicative senescence, and reduced paracrine capacities. It has been shown that short-term spheroid formation before monolayer culture enhances the regenerative capacity of hMSCs by increasing pluripotency markers Sox-2, Oct-4, and Nanog.[13] Furthermore, in Chapter 4 we have shown that cadherin-11 has a role in the ECM composition of the hMSCs. In aggregates, cell-secreted ECM plays a key role in cell aggregation, spherical aggregate formation, and cohesion in suspension culture systems.[14]

The classical microenvironment for hMSCs is indeed a 3D environment; therefore studying them as aggregates more accurately captures the *in vivo* scenario. If hMSCs are only studied as a monolayer then we miss crucial information that is needed to mimic these mechanisms for regenerative medicine. Even though the mechanisms seem to be fairly well understood in monolayer culture, an aggregate culture's microenvironment is different, as others have reported and we also showed in this thesis. Sophisticated material design cannot be reasonably done without a thorough understanding of cells in a 3D environment.

2. HOW CAN THE ROLE OF CADHERINS IN DIFFERENTIATION BENEFIT REGENERATIVE MEDICINE?

In Chapter 2, I focused on various ways in which tissue engineers have tried to incorporate cadherins in their design. However, most of the focus thus far has been either on cadherin-1 or cadherin-2. There is considerable diversity in the cadherin family and much to be gained from understanding the importance of this vast and diverse family. The bulk of this thesis tried to understand cadherin-11, a previously understudied cadherin. In Chapters 3, 4, and 5 I have revealed many changes caused by cadherin-11–knockdown as well as two major physiological changes: one to proliferation and the other to differentiation.

In Chapter 3 we not only showed that disrupting cadherin expression disrupts the ability of hMSCs to commit to a particular lineage, but also that this behavior is again different in 2D and 3D environments. We also studied this behavior in cells from two different donors. We know from Chapter 3 that the loss of cadherin-2 can increase osteogenic differentiation. Furthermore, we observed that the loss of cadherin-11 is compensated by an increase in cadherin-2 expression. This increase in cadherin-2 expression decreased bone matrix formation in hMSCs in 2D, while cells in 3D could differentiate normally.

We know that certain calcium phosphate materials, a class of tunable bioactive materials that have been widely used for bone tissue repair are osteoinductive, while others, often unpredictably, fail.[15] Tissue engineers working with bone have demonstrated that differences in cell–cell adhesion exist on different calcium phosphate materials.[16,17] The underlying mechanism of osteoinductivity by these calcium phosphate materials is a subject of active research.[18] Based on what we have observed in Chapter 3, a worthwhile experiment would be to explore whether

calcium phosphate materials that are known to be osteoinductive cause decreased cadherin-2 expression in hMSCs cultured on them compared to ones that fail. In this context, cadherins can hence be used as potential markers to help us select bone graft materials that induce bone formation.

A major problem in the field of regenerative medicine is the inefficient conversion of stem cells from one stage of differentiation to the next. Knowing which cadherins are expressed by cells in a 3D environment opens the possibility of engineering cells to precisely control their fate in the stem cells. Genome editing could be employed to modify the intrinsic response of cells in an organoid to external stimuli to generate cell types that would otherwise be absent. Cadherins are excellent markers for the identification of specific cell types as they are used by the cells for spatial organization in a 3D environment. For example in the human kidney where a complex patterning on cadherin expression is seen.[19–24] Therefore, the sorting of cell populations can also be directed by the differential expression levels of cadherins in specific cell types.[25] At the same time, having large numbers of undifferentiated progenitors from stem cells has a wide application in regenerative biology. It has been shown that co-culture of MSCs with β-cell progenitors promotes cell proliferation and self-renewal without differentiation.[26] The self-renewal signal provided by mesenchyme might be mediated by cadherins, which is another avenue that can be explored.

3. WHAT INSIGHT DOES THE ROLE OF CADHERIN-11 PROVIDE FOR REGENERATIVE MEDICINE?

Fetal tissue is capable of healing perfectly, while adult tissue repair has evolved to be fast rather than perfect.[27] This rapid race to close the wound has evolutionary advantages because slow healing means prolonged bleeding and chances of infection. In the fetus, this perfect repair is size- and age-dependent. In human adults, the function of injured organs is usually never completely restored. To give only a few examples, skin scars do not contain sweat glands and hair follicles, scar tissue formed on the heart after myocardial infarct does not beat, scarred lungs do not contribute to gas exchange, and sclerotic kidneys do not filter.[28–31] Notably bone and liver are some of the few tissues that can heal without forming a fibrous scar. It remains unexplained why mammals tend to have imperfect healing and scarring, rather than full regeneration in most organs. Recently it was shown in *Drosophila* that their fat body cells are motile and undertake functions to drive wound healing.[32] Research has also shown that the transformation of fibroblasts to adipocytes during wound healing can reduce scar formation.[33,34] In Chapter 3, we showed that the loss of cadherin-11 inhibited adipogenic differentiation. Seeing as cadherin-11 has an important role in adipogenic differentiation, the mechanism underlying this could potentially have implications for scar-free wound healing.

It is often challenging to study cells in a 3D environment. Therefore to evaluate what was happening in hMSCs when I knockdown cadherin-11, I decided to use monolayer culture. There was still so much to learn in a simple model before embarking on a more complex one. TGFβ1 is one example of a growth factor that has been repeatedly shown to affect stem cell differentiation. In Chapter 4, we showed that cadherin-11 modulated the downstream signaling of the TGFβ1 pathway. It does this by changing the timing of the activation of the downstream

signaling via pSMAD2/3. This temporal stochasticity changes the composition of the ECM of the hMSCs which in turn results in the changes in differentiation seen in Chapter 3.

A significant point for tissue engineering here is that the ECM is not simply a collection of proteins but that its components interact with each other in very specific ways. This is supported by the evidence that scarred tissue does not function as native tissue does. Furthermore, in Chapter 4 we have seen that that changes in the composition of ECM can lead to changes in the differentiation potential in hMSCs. Using approaches like chemical processing or other manipulations of collections of ECM components are therefore unlikely to produce the desired changes because it is difficult to recreate the complex ultrastructure and composition of the native tissues ECM. This is also because there are major gaps in our understanding of the ECM. Therefore, the more fundamental knowledge we can gather about ECM dynamics, the greater our chances of mimicking the native tissue ECM. Our knowledge when it comes to the ECM is constantly evolving and there is tremendous progress being made in decellularization techniques and optimization of recellularization strategies, thus the future of ECM biomaterials in tissue engineering and regenerative medicine applications is promising.[35]

Furthermore, there are directions that we did not explore yet. In Chapter 4, we have seen that this early translocation pSMAD2/3 leads to an increase in fibronectin in the ECM. Fibronectin alone binds a plethora of growth factors that are central in tissue repair and fibrosis, including vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and latent TGFβ1. Seeing as pSMAD2/3 is translocated into the nucleus early in cells lacking cadherin-11, the rest of the TGFβ1 probably remains bound to the ECM, which explains why we saw a

decrease in serum TGFβ1 levels over time. Given the pleiotropic nature of TGFβ1 and its central role in coordinating almost every aspect of normal tissue repair and homeostasis, special security measures may have evolved in the form of cadherin-11 to protect us against the disastrous consequences of dysregulated TGFβ1 signaling.

4. WHAT CONSEQUENCES DOES THE CADHERIN–RTK INTERACTION HAVE FOR REGENERATIVE MEDICINE?

RTKs are transmembrane protein receptors that contain intrinsic enzyme activity and help cells interact with their neighbors in a tissue.[36] When a signaling molecule binds to an RTK, the tyrosine kinase in the cytoplasmic tail is activated. This results in a series of enzymatic reactions that carry the signal to the nucleus, where it alters patterns of protein transcription. Therefore, when RTKs don't function properly, cell behavior goes rogue. For instance, many cancers appear to involve mutations in RTKs.[37] For this reason, RTKs are the targets of various drugs used in cancer chemotherapy.

It is increasingly clear that RTKs regulate communication amongst cells during the sophisticated rearrangements that drive tissue morphogenesis. We know very well that the cytoplasmic domain of the cadherin interacts with cytoplasmic proteins such as catenins, but in Chapter 5 we learn that cadherin-11 also binds to transmembrane proteins like the RTK. In Chapter 5 we provided evidence to suggest that cadherin-11 induces differentiation and proliferation through the MAPK signaling pathway which is downstream of various RTKs. Something we haven't explored in this thesis is that RTKs phosphorylate MEK which then phosphorylates ERK1/2, leading to phosphorylation and inactivation of PPARγ.[38,39] A known target of PPARγ is the adipokine adiponectin, which could explain why cadherin-11–knockdown cells could not differentiate towards the adipogenic lineage. Furthermore, we have seen in Chapter 4 that knocking down cadherin-11 increases fibronectin in the hMSC ECM. In Chapter 5 I have shown that knocking down cadherin-11 increases phosphorylated PDGFRβ. A study has shown that adhesion to fibronectin in MSCs induces PDGFRβ signaling in an $\alpha 5\beta 1$ integrin–dependent manner.[40] This adds to our understanding of the complex cadherin–RTK–ECM–integrin crosstalk.

The potential to control RTKs through cadherins rather than growth factors is enormous. Some studies show that some RTKs can dimerize in the absence of growth factor binding. In Chapter 5 we have seen that knocking down cadherin-11 does not affect just one RTK but multiple RTKs at the same time, which makes it a better target than a single growth factor. If you aim to inhibit cell proliferation via the ERK-mediated signaling pathway then it's not possible to do so via a single growth factor because the ERK signaling cascade is downstream of EGFR, PDGFR, and FGFR.[41] Also, long-term growth factor activation can decrease the cells' sensitivity to a particular growth factor. Growth factors can also degrade which can result in loss of function and therefore loss of their binding capacity.[42,43] If cadherin does change the binding affinity of RTKs to the ligands then it is possible to achieve a strictly controlled regulation of RTK signaling. Since regeneration is not the function of a single cell or single molecule, we need to focus on achieving coordinated and cooperative actions of these biological cues.

FUTURE OF REGENERATIVE MEDICINE

The tantalizing promise that the field of regenerative medicine made, to replace our damaged and diseased tissue, is far from being realized. Despite significant progress with small animal studies, its realization in the clinic has been slow. Tissue engineering, a platform of regenerative medicine, has a vast array of literature composed of a selection of sophisticated and dynamic materials that aim to promote specific cell behavior. This is not to imply that tissue engineering has to involve biomaterials, but the delivery of these signals cannot take place in a vacuum. However, seeing as there are fewer and fewer therapies making their way to the clinic it looks as though we might not achieve the goal we want with the current approaches. Something needs to change and we need new biological tools, new approaches, and a new perspective. The future of tissue engineering lies in our ability to combine lessons from developmental biology and to develop the new biological tools necessary. I argue for more fundamental research into cell behavior towards improving our understanding of underlying molecular mechanisms specifically geared towards advancing regenerative medicine.

We must try to prove the fundamental research in primary cells. We have seen that primary cells behave differently from cell lines. Primary cells are the closest representation of the human *in vivo* situation. Cell lines lack key morphological or functional features, so they might not be able to induce relevant biomarkers. This means that the results obtained with cell lines in the lab cannot be fully translated to humans. Similarly, the fundamental research conducted on cells seeded as a monolayer must also be proven in aggregate cultures as they mimic the natural environment of tissue more closely.

For these reasons, I have focused my thesis on advancing the fundamental understanding of hMSC behavior by studying cadherin-11. In conclusion, this thesis has built knowledge on how cadherin-11 influences process such as differentiation and proliferation and has unraveled part of the underlying biological mechanism.

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8

Impact

INTRODUCTION

The concept of tissue regeneration is not new; one could even say it is ancient. Skin grafts were first employed in the Indian subcontinent as early as 2500 BC to treat mutilations of the ear.[1] Since then, our fascination with the ability to regenerate tissues and organs has been unstoppable. It has led to the development of *ex vivo* products in the mid-1990s and, more recently, the *in vivo* constructs that are ushering in the new era of regenerative medicine. The field of regenerative medicine promises to improve health and quality of life by repairing or regenerating cells, tissues, and organs as a way to meet the demand for worn-out body parts as the world's population lives longer. One method of progressing in this area is to effectuate collaboration between developmental biologists and tissue engineers. In doing so, ideas regarding the specification and correct positioning of the cells of our tissues can be shared, and consequently used to repair damage caused by injury or disease. Over time, we have come to realize that the interactions that cells have with one another and with their environments are very complex, and their behavior is difficult to control.

The work in this thesis is conducted primarily on mesenchymal stem cells (MSCs), a type of adult stem cell. This thesis also focuses on cadherins, which are an adhesion receptor that can influence MSC behavior. The most commonly understood impact of cadherins lies in their contribution to the preservation of cell-to-cell cohesion in tissues. Understanding cadherins in the context of regeneration of tissue can give us the advantage we need to manipulate cell behavior. In the following sections, I explore how the knowledge generated by this thesis can be used to create an impact.

A QUESTION OF ETHICS

MSCs are adult stem cells that can be isolated from numerous sources including bone marrow, fat, and placental tissue. Their relative ease to culture *in vitro*, their ability to differentiate into several different cell types that are in short supply, and their immunomodulatory properties make them a powerful cell source for regenerative medicine.[2] According to the website *www.clinicaltrials.gov*, as of March 2021, more than 1300 trials using MSCs are underway worldwide, 390 of which are completed trials, and 20 of those completed trials were phase 3 trials. Due to the increase of degenerative diseases in the globally aging population, there is both a huge scientific and public interest to see regenerative medicine succeed. As a result, this has created a surge in the interest for MSC-based therapies.

When looking into Google Trends data of the past decade, the search count for MSC is more than double that of the other stem cells. This is in line with the increase in the number of private clinics that advertised and sold autologous-based MSC therapies to patients.[3–5] These therapies have not been published in scientific literature and hence are untested and unproven. Furthermore, there is a significant gulf between the public expectation of MSC therapies fueled by media coverage and the reality of progress that is made by early phase clinical trials.[6] Vulnerable patients and their families have bought into these therapies and have incurred the exorbitant costs as well as the uncertain risk associated with it. Although MSCs have remarkable potential, our understanding of their behavior is not necessarily ready for medical application and widespread use. Education and information generated by scientists should be targeted at the public to empower people to take responsibility for their healthcare choices.

To a large extent, education in regenerative medicine is also lagging behind the scientific advances.[7] This leaves the physicians ill-equipped to address the changing needs of patient care. Early incorporation of next-generation healthcare tools into mainstream medical education is essential in order to deliver validated and regulated regenerative medicine solutions. This could potentially prevent patients from seeking stem cell treatments outside of regulated clinics.

NEED FOR FUNDAMENTAL RESEARCH

MSC-based approaches:

Early-stage MSC trials have demonstrated safety and efficacy, but only a small number of MSC products have be commercialized, indicating that the therapeutic market for MSCs remains at an early stage. Several meta-analysis studies of these trials have revealed that MSCs therapies were effective in certain patients but not all, and the reason for this is unclear. Like with most cell-based therapies in regenerative medicine, the physical, phenotypic, and functional properties of MSCs remain ill-defined. Acknowledging the complexity of MSCs behavior and therefore a need for a better understanding of MSC biology is essential to temper the expectations placed on MSC therapies. The work discussed in Chapters 3, 4, and 5 deepens our knowledge of the inner workings of MSCs, as we have explored the effect of cadherin-11 on their differentiation and proliferation.

Differentiation and proliferation are the properties of stem cells that are of considerable interest to the field of regenerative medicine. Importantly, proliferation and differentiation, if unchecked, can be dangerous to patients, therefore the quality of their regulation is crucial. In Chapter 4, we tried to understand the mechanism that helps MSCs differentiate and commit to becoming a fat cell over a bone cell,

which could help to fine-tune MSC fate commitment for regenerative medicine applications. In Chapter 5, we showed that cadherin-11 is essential for MSC proliferation, which could help further our understanding of the mechanisms that preserve the undifferentiated stem state of the MSCs. However, additional research is required to understand if the mechanisms described in Chapters 3, 4, and 5 are robust in MSCs from various donors and if they are reproducible across different laboratories. Ultimately, the knowledge generated by these chapters indicates that cadherin-11 can be used as a tool to control MSC behavior.

Material-based approaches:

Tissue engineering literature describes a diverse selection of scaffold materials that aim to promote specific cell behavior and advance regenerative medicine. Yet, the field is puzzled over the question of why some materials succeed in directing cell behavior while others fail. For example, in the case of bone regeneration, many bone graft materials fail to replicate the fracture healing exhibited by autografts. The current toolbox used by tissue engineers has not been enough, and a better understanding of how cells respond to cues is needed for improved material design. Thanks to the research conducted in this thesis and by others, it is slowly becoming a reasonable reality that new knowledge can be used to advance the field.

We have seen in Chapters 3, 4, and 5 that MSCs lacking cadherin-11 do not proliferate and differentiate like normal MSCs, establishing the importance of cadherin-11 in MSC behavior. Proteins such as cadherin-11 can then be harnessed by tissue engineers to improve material-based therapies by incorporating this information into the design of engineered constructs to control MSC cell fate. In Chapter 2, we summed up various material-based approaches that have used cadherins to improve material design. The results in Chapter 4 suggest that the

timing of certain signaling molecules is essential for extracellular matrix deposition and the eventual MSC fate commitment. This therefore points to the importance of nuanced material design to guide cell behavior.

3D models

Many results obtained *in vitro* do not correlate to results obtained in an *in vivo* setting. We have shown in Chapter 3 that MSC behavior differs based on the cell culture dimensionality. Moreover, in Chapter 6, we explored different 3D culture methods which better mimic the *in vivo* setting and observed a dramatic decrease in cell number over long-term culture. This shows that great effort is necessary to prepare MSCs for the *in vivo* environment, which may eventually pay big dividends, as it will enhance their clinical efficacy. The findings of Chapters 4 and 5 need to be validated in a 3D model, as 3D culture has profound effects in MCSs and the results are needed for *in vivo* applications.

OUTLOOK FOR REGENERATIVE MEDICINE

Regenerative medicine solutions are meant to address the need for the replacement of damaged tissues and organs. However, while much of the current research in the field of regenerative medicine is confined to the bench rather than the bedside, clinical translation is becoming increasingly apparent. When it comes to clinical translation of fundamental research, tissue engineering should be to developmental biology what drug development is to molecular biology. Tissue engineers cannot do what they do without the knowledge of specific molecular regulators, and similarly, biologists are looking for newer tools to answer questions that cannot be done using the existing toolbox. The main focus of this thesis was to try and improve tissue engineering by taking lessons from cadherin biology. We have the technology in our arsenal, but basic biology still needs to be deciphered. Stem cell–based approaches to repair and regenerate tissue are far from being successful in the clinic because our understanding of the basic biology underlying tissue repair is still far from exhaustive. This is despite all the advancements that have been made by fundamental biologists in our understanding of biology. A strong alliance between tissue engineers and developmental biologists can catapult the field towards new discoveries. This is possible if fundamental biology questions are framed within the context of tissue engineering.

The knowledge generated by this thesis is a start to give scientists a better understanding of the microenvironment they wish to control. Furthermore, fields such as cancer biology could also use the knowledge for their questions, as there are many similarities between tissue stem cells and cancer stem cells.

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Epilogue

SUMMARY

The acquisition of a specific cell fate is one of the core aims of tissue engineering and regenerative medicine. When it comes to engineering cell environments, the focus of the field thus far has been on how cells respond to cues from the extracellular matrix, but this thesis is aimed towards generating knowledge on the importance of cell–cell adhesion proteins. More specifically, this thesis aims to better understand the role of cadherins, a family of cell–cell adhesion molecules, in the behavior of bone marrow–derived human mesenchymal stem cells (hMSCs). To this end, in Chapter 2, we examine cadherins through the lens of a tissue engineer and propose that tissue engineering could be more successful if scientists would take lessons from cadherin biology, as they are a major driving force in tissue formation and because they can influence important cell behavior. This is complementary to most efforts to date that have been inspired by cell–extracellular matrix interactions. We aim to make cadherin biology more accessible to tissue engineers by giving an overview of the diversity of the cadherin family, discussing the key characteristics that make cadherins ideal for tissue engineering approaches, and elaborating on the functional significance of cadherins in the context of tissue engineering.

There is significant evidence to suggest that aggregate cultures have a positive influence on fate decisions compared to monolayer cultures, presumably through cell–cell interactions, but little is known about the specific mechanisms. In Chapter 3, we provide insight into the critical role of the dimensionality of cell–cell interactions in determining cell fate. We demonstrate that during differentiation, there is a switch in the expression of cadherin-2 to cadherin-11 in cells cultured as a monolayer, which is not evident in the aggregate cultures. We also show that the

loss of certain cadherins influences cell fate towards the osteogenic and adipogenic lineages.

In Chapter 4, we follow up on the finding observed in Chapter 3 by providing evidence to explain how knocking down cadherin-11 in hMSCs leads to changes in the adipogenic differentiation potential. The importance of the extracellular matrix in influencing cell behavior is unquestionable, and we demonstrate that knocking down cadherin-11 changes the extracellular matrix composition via the transforming growth factor beta 1 pathway, thereby affecting cell differentiation. Chapter 5 implicates cadherin-11 in the regulation of hMSC behavior by asking how cadherin-11 brings about changes in signaling pathways despite it having no intrinsic signaling activity. We explore the cross-talk between cadherin-11 and receptor tyrosine kinases (RTKs) and we demonstrate that RTK activity changes in hMSCs lacking cadherin-11, which thereby brings about changes in cell proliferation via the mitogen-activated protein kinase pathway.

Next to studying cadherins, in Chapter 6 of this thesis, we study hMSCs as scaffold-free self-assembled aggregates of low and high cell number and compare them to cell-laden alginate hydrogels with and without arginine–glycine–aspartic acid peptides. We found that alginate hydrogels had persistently more living cells for a longer period in culture and that a weeklong culture of hMSCs in 3D culture, whether as aggregates or cell-laden alginate hydrogels, reduced their proliferation over time. The findings of this study improve our understanding of how aggregate cultures differ with or without a hydrogel carrier.

Overall, this thesis adds fundamental knowledge to our understanding of how to influence hMSC behavior and can be a stepping-stone towards improving cell- and material-based regenerative medicine approaches.

SAMENVATTING

Het verwerven van een specifiek lot van een cel is een van de kerndoelen van tissue engineering en regeneratieve geneeskunde. Bij het maken van een nagebootste biologische omgeving, lag tot nu toe de focus op hoe cellen zich gedragen onder invloed van de extracellulaire matrix in die omgeving. Het onderzoek beschreven in dit proefschrift is gericht op het genereren van kennis over het belang van cel-cel adhesie-eiwitten en hun invloed op cel gedrag. In het bijzonder beoogt dit proefschrift een beter inzicht te geven in de rol van "cadherins", een specifike familie van cel-cel adhesiemoleculen, op het gedrag van uit het beenmerg afkomstige menselijke mesenchymale stamcellen (hMSC's). In hoofdstuk 2 bekijken we "cadherins" door bril van een "tissue engineer" om met als doel de regeneratieve geneeskunde te bevorderen. Het doel is om meer inzicht te verkrijgen in de invloed van cadherins op cellen voor toepassingen in regeneratieve geneeskunde door de diversiteit van de cadherin familie te beschrijven, en daarnaast de belangrijkste kenmerken te bespreken van cadherins die een belangrijk effect kunnen hebben op cel gedrag en functie in de context van tissue engineering.

Er is significant bewijs dat suggereert dat geaggregeerde driedimensionale celkweek, cellen die in kleine bolletjes gegroeid worden, een significant positieve invloed hebben op het gedrag en differentiatie van deze cellen, in vergelijking tot dezelfde cellen die op een traditioneel twee dimensionale wijze gekweekt worden, vermoedelijk veroorzaakt doordat cel-cel interacties in de driedimensionale omgeving anders zijn dan in een twee dimensionale omgeving. Er is tot nu toe maar erg weinig bekend over de specifieke onderliggende mechanismen die hierbij een rol spelen. In Hoofdstuk 3 geven we inzicht in de cruciale rol van de dimensionaliteit van cel-cel interacties bij het dicteren van cel gedrag. We laten zien dat er tijdens

differentiatie een omslag is in de expressie van cadherine-2 naar cadherine-11 in cellen die in op een plat vlak zijn gekweekt, wat niet voor de hand liggend is in de aggregaat cel kweek. We laten ook zien dat het verlies van bepaalde cadherines het lot van de cellen beïnvloedt in de richting van de osteogene en adipogene lijn.

In Hoofdstuk 4 volgen we de bevinding uit Hoofdstuk 3 op door bewijs te leveren hoe het uitschakelen van cadherine-11 in hMSC's leidt tot veranderingen in het adipogene differentiatiepotentieel. Het belang van de extracellulaire matrix bij het beïnvloeden van celgedrag staat buiten kijf, en we tonen aan dat na het uitschakelen van cadherin-11 de samenstelling van de extracellulaire matrix verandert via de zgn TGF bèta-1 route, waardoor de differentiatie van cellen wordt beïnvloed. Hoofdstuk 5 beschrijft de rol van cadherin-11 bij de regulatie van hMSC-gedrag door onderzoeken hoe cadherin-11 veranderingen in signaaltransductie teweegbrengt, ondanks dat het zelf geen intrinsieke signaaltransductie activiteit heeft. We onderzoeken de interactie tussen cadherine-11 en receptor-tyrosinekinasen (RTK's) en we tonen aan dat RTK-activiteit verandert in hMSC's zonder cadherine-11, wat leidt tot veranderingen in celgroei via de zgn. mitogen geactiveerde eiwitkinase signaal transductie route.

Naast het bestuderen van cadherins, bestuderen we in Hoofdstuk 6 van dit proefschrift hMSC’s als zelf-geassembleerde aggregaten, zonder de ondersteuning van een op biomateriaal gebaseerde drager, met een lage en grote hoeveelheid cellen en vergelijken ze met cellen gezaaid in alginaathydrogelen met en zonder arginine-glycine-asparagine-peptiden. We ontdekten dat alginaathydrogelen meer levende cellen bevatten voor een langere periode in kweek, en dat een weeklange kweek van hMSC's in 3D, hetzij als aggregaten of in een alginaathydrogel, hun groei in de loop van de tijd verminderde. De bevindingen van deze studie geeft ons meer inzicht in

hoe het cel gedrag in aggregaat kweken verschillen met of zonder een hydrogeldrager.

Over het algemeen voegt dit proefschrift fundamentele kennis toe aan ons begrip van hoe hMSC-gedrag kan worden beïnvloed door hun directe omgeving en kan het een opstap zijn naar het verbeteren van toepassingen van cel- en materiaalgebaseerde regeneratieve geneeskunde toepassingen.

ABOUT THE AUTHOR

Fiona Rosaleen Passanha was born on 23rd August 1991 in Manipal, India. She received her Bachelor in Biomedical Engineering from Manipal University in 2013. After completing her bachelor's she moved to the Netherlands to start her Master in Biomedical Engineering at the University of Twente. For her thesis, she worked in the group of Leon Terstappen where she studied how circulating tumor cells can be isolated from blood and assessed using a self-seeding microwell chip. She obtained her master's degree in 2016. Soon after she started her doctoral work at the MERLN Institute in Maastricht University. Under the supervision of Vanessa LaPointe, she focused her research on understanding a family of cell adhesion molecules called cadherins and their role in human mesenchymal stem cells.

LIST OF PUBLICATIONS

Passanha FR, Divinagracia ML, LaPointe VL. **Cadherin-11 regulates cell proliferation via the RTK-ERK1/2 signaling pathway in human mesenchymal stem cells**. (2021) (manuscript under review)

Passanha FR, Geuens T, LaPointe VL. **Cadherin-11 influences differentiation in human mesenchymal stem cells by regulating the extracellular matrix via the TGFβ1 pathway**. (2021) (manuscript under review)

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**These authors contributed equally*

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