Role of fibronectin in cyclic strain-induced extracellular matrix remodeling processes

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Role of fibronectin in cyclic strain-induced extracellular matrix remodeling processes

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**Abstract**

Mechanical forces are essential in tissue development and maturation, which requires orchestrated extracellular matrix remodeling. Fibroblasts are the most abundant cells in connective tissue and lay down a provisional matrix rich in fibronectin before tissue maturation. This thesis, consisting of two main projects, focuses on studying the role of fibronectin in matrix remodeling by using fibroblasts exposed to uniaxial cyclic strain. The first sections focus on how human foreskin fibroblasts change their matrix remodeling from a fibronectin-rich matrix (early matrix) to a collagen type I-decorated matrix (more matured matrix) under uniaxial cyclic strain. The second part investigates the importance of the presence of pericellular fibronectin by rescuing the matrix remodeling behavior of fibronectin knockout with exogenous fibronectin.

First, how fibroblasts alter their matrix remodeling capacity during tissue maturation under dynamic mechanical loading was studied, i.e. in the transition from a fibronectin-rich extracellular matrix to the type I collagen-decorated matrix. Matrix remodeling was traced, using fluorescently labeled fibronectin, soluble matrix metalloproteinase activities (including gelatinases) were measured, gene expressions were monitored by quantitative Reverse Transcription-Polymerase Chain Reaction and the conformational changes of fibronectin in extracellular matrix were studied by Förster Resonance Energy Transfer. It was found that human foreskin fibroblasts deposit fibronectin matrices along the minimum strain direction and the directionality of the fibers remains highly correlated with the orientation of actin stress fiber under uniaxial cyclic strain. When continuously supplemented with plasma fibronectin, human foreskin fibroblasts do not significantly remodel matrices with or without cyclic stretching, but the accumulation of a fibronectin-rich matrix from little matrix rapidly increase the matrix degradation capacity of the cells. As the extracellular matrix is matured from a fibronectin-rich matrix to collagen-decorated matrix, the presence of monomeric type I collagen peptides in the matrix reduces the soluble matrix metalloproteinase secretion and transmembrane type II matrix metalloproteinase (matrix metalloproteinase-15) gene expression. Fibronectin conformation studies for cells under static culturing condition showed that a fibronectin matrix is less unfolded once type I collagen is present in the matrix,
indicating a partial loss of tension in the matrix. Hereby, this study suggests that the presence of fibronectin and type I collagen, not cyclic strain, determines the remodeling capacity of fibroblasts by regulating MMP activity, possibly related to the fibronectin conformational states in the extracellular matrix.

In the first part of the study, it has been shown that fibronectin is essential in determining the matrix remodeling capacity of human foreskin fibroblasts. Therefore, in the second part, how the presence and remodeling of pericellular fibronectin matrix contributes to the cyclic strain-induced mechanosensing was studied. In addition to using the established tools from the previous project, fibronectin knockout and floxed mouse embryonic fibroblasts were employed, together with fluorescently labeled fibronectin and MMP assays, the formation of actin stress fibers and focal adhesion was observed by immunofluorescence staining and measured cell traction forces on nanopillar substrates. It has been shown that under uniaxial cyclic strain, exogenous Fn in the medium is essential for the formation of actin stress fibers and the maturation of focal adhesions, restoring Fn unfolding in the ECM and elevating MMP activities. Moreover, it has been demonstrated that full length fibronectin in the medium is essential in restoring cell traction force, as , unlike full-length fibers, the N-terminal 40k, 70k and C-terminal 120k fibronectin fragments did not increase the cell-generated force to the normal level. However fibronectin knockout fibroblasts lack strain-avoidance responses including strain-induced re-orientation, polarization and leave the pre-existing matrix behind even with the addition of exogenous pFn. Disrupting the cell-matrix dynamics by inhibiting matrix degradation did not abolish the cyclic strain-induced cell re-orientation. Taken together, it is apparent that the presence of exogenous fibronectin up-regulates (but does not rescue completely) the mechanosensitive response of fibroblasts, exposed to cyclic mechanical strain.


Im ersten Teil der Studie wurde gezeigt, dass Fibronectin essentiell für die Fähigkeit des Matrix-„Remodeling“ in humanen Vorhautfibroblasten ist. Daher untersuchten wir im zweiten Teil der vorliegenden Arbeit, auf welche Weise die Anwesenheit der perizellulären Fibronectinmatrix sowie ihr „Remodeling“ sich auf das durch zyklische Belastung induzierte Mechanosensing der Fibroblasten auswirkt.


Es konnte aufgezeigt werden, dass unter uniaxialen, zyklischem Stress exogenes Fibronectin im Medium essentiell für die Entwicklung von Aktinstressfasern, für die Maturation fokaler Adhäsionen, für die Wiederherstellung der FN-Entfaltung in der ECM und für den Anstieg der MMP Aktivität ist. Weiterhin wurde demonstriert, dass Fibronectin in voller Länge eine essentielle Funktion zukommt, die zelluläre Zugkraft wieder herzustellen. Nur das Anbinden der Zellen an eine ungekürzte Fibronectinversion resultierte in einer Wiederherstellung der zellulären Zugkräfte auf ein wildtypähnliches Niveau, im Gegensatz zu den N-terminalen 40k-, 70k-Fragmenten sowie dem C-terminalen 120k-Fragment. Fibronectin-Knockout Fibroblasten jedoch scheint die Fähigkeit auf belastungsvermeidende Maßnahmen zurückzugreifen zu fehlen. Dies schließt die
durch mechanischen Stress induzierte Ausrichtung der Zellen in eine belastungsminimale Orientierung genauso mit ein, wie die Polarisationsfähigkeit der Zellen und Interaktionen zwischen der Zelle und der Matrix, auch wenn exogenes Plasmafibronectin zugeführt wird. Wurde die Zell-Matrix-Dynamik per se unterbrochen, indem die Fähigkeit zur Matrixdegradation inhibiert wurde, so hatte dies keine Auswirkung auf die Fähigkeit der Zellen sich durch Umorientierung an zyklische Belastung anzupassen.
Zusammengenommen konnte aufgezeigt werden, dass die Präsenz exogenem Fibronectins die mechanosensitive Antwort von zyklischer, mechanischer Belastung ausgesetzten Fibroblasten hochreguliert, wenn auch nicht komplett rettet.
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Chapter 1: Motivation and specific aims

Mechanical forces acting on cells and tissues affect many physiological processes, such as tissue maturation and remodeling. Fibroblasts are the most abundant cell type in connective tissues and are the primary cells that lay down the first provisional ECM that is rich in fibronectin (Fn). During tissue maturation, the fibronectin matrix is being degraded and replaced by a collagen matrix. However, the exact role of fibronectin in cyclic strain-induced matrix remodeling processes is not known.

In this thesis, we first investigated how human foreskin fibroblasts remodel extracellular matrix under dynamic loading during the transition from a fibronectin-rich matrix to a collagen-decorated matrix. Then we focused on how pericellular fibronectin helps Fn knockout fibroblasts to restore their abilities to sense external mechanical loading and remodel the extracellular matrix (ECM).

1.1. Aim 1 (Chapter 5): Studying the influence of matrix composition in ECM remodeling under uniaxial cyclic strain

With excessive tissue tension, neo-tissue formation and remodeling can be associated with fibrosis and excessive wound contracture. We speculate that the different tissue maturation stages, characterized by the composition of the extracellular matrix, relate to fibroblast behavior and specifically their remodeling capacity. This chapter addresses the question of how Fn and/or type I collagen (Col1) regulate the early onset of matrix remodeling by human foreskin fibroblasts under dynamic mechanical loading, specifically during the transition of fibronectin-rich to collagen-decorated matrix.

To study the influence of matrix composition (Fn and Col1) in extracellular matrix remodeling under uniaxial cyclic strain, we addressed the following three aspects:
1. How do human foreskin fibroblasts assemble and remodel the fibronectin matrix under cyclic strain?

Fibroblasts are shown to re-orient along the direction of minimum strain under uniaxial cyclic strain. We speculated that human foreskin fibroblasts assemble and remodel the Fn matrix under cyclic strain along the minimum strain axis, due to a re-organized actin network. We used fluorescently labeled Fn to monitor the assembly of the Fn matrix, and immunofluorescent staining to monitor the alignment of strain-induced actin stress fibers.

2. How does the presence of Fn, or both Fn and Coll, differentially affect the matrix degradation?

We speculated that different matrix compositions determine the cellular remodeling potential of the ECM by de novo synthesis. We used a quantitative real time polymerase chain reaction (qRT-PCR) to study the gene expression of type I collagen, type III collagen and fibronectin, matrix metalloproteinase -14, -15, and tissue inhibitors of matrix metalloproteinase-1, -2, -3.

3. How does the presence of collagen alter the conformational state of the Fn matrix and potentially affect the matrix remodeling mechanism?

During tissue maturation, a provisional Fn matrix is interlaced and later replaced by a collagen matrix associated with tissue stiffening. It was hypothesized that collagen deposition depends on the existing Fn matrix and increases the matrix tension. The proposed questions were answered by utilizing fluorescently labeled Fn and collagen, confocal microscopy and Forster Energy Transfer, a mechanosensitive Fn-construct that is sensitive to the change of mechanical strain of the Fn within the ECM. Furthermore, the matrix metalloproteinase activity by MMP assays and gelatin zymography were monitored.
1.2. Aim 2 (Chapter 6): Identify the role of fibronectin in uniaxial cyclic strain-induced ECM remodeling

Fn is required in RhoA acitivation in fibroblasts exposed to equiaxial cyclic strain. However, how Fn contributes to the cellular capacity and the physical remodeling of a matrix by fibroblasts subjected to uniaxial cyclic strain has not been studied.

To answer this question, Fn knockout (Fn−/−) and Fn floxed mouse embryonic fibroblasts (Fn^{floxed} MEFs) were obtained to address the following three aspects:

1. How does exogenous Fn determine the cyclic strain-induced mechanosensing?

It was speculated that Fn is necessary for mechanosensing in fibroblasts subjected to uniaxial cyclic strain. It was hypothesized that the lack of Fn results in low cell traction forces, cell contractility, and weaker cell-ECM adhesion. To answer this question, traction force measurements utilizing nanopillar substrates, and immunofluorescent staining were employed, in combination with confocal microscopy to monitor the cellular changes.

2. How do the presence of the Fn matrix and the application of uniaxial cyclic strain affect the matrix degradation processes?

Cyclic strain is known to increase the cellular capacity of matrix degradation by matrix metalloproteinases (MMPs) in collagen lattices. It was hypothesized that the combination of fibronectin and cyclic strain can alter the MMP activity in fibroblasts. An MMP assay was employed to monitor the soluble MMPs in supernatant and gelatin zymography was utilized to study the contribution of gelatinases.
3. How do the pericellular Fn matrix and the application of uniaxial cyclic strain affect the matrix remodeling processes?

To differentiate whether MEFs re-orient along the direction of minimum strain under uniaxial cyclic strain by sensing external mechanical stimuli via cell-substrate or cell-ECM interactions, the mechanical strain in the matrix was analyzed by probing the conformational state of Fn in ECM assembled by Fn-/- MEFs using Förster Energy Transfer Microscopy. The interaction between Fn-/- MEFs and the assembled matrix was explored using fluorescently labeled Fn. And finally, how the degradation of pericellular Fn determined uniaxial cyclic strain-induced cell re-orientation by Fn rescue and MMP inhibition studies was observed.
Chapter 2: Background

In this chapter, the general background of mechanobiology is introduced, with a special emphasis on the effect of cyclic forces on cells and the remodeling of their surrounding extracellular matrix (ECM). Here the focus is on the role of fibronectin (Fn), an important ECM protein in the development of provisional matrices, and on fibroblasts, the cells that are responsible for assembling the Fn matrices in the connective tissue and their response to uniaxial cyclic strain.

2.1. Mechanical forces and uniaxial cyclic mechanical strain

Unlike cells cultured in static petri dishes, cells in vivo are exposed to various external mechanical forces. On the macro-scale, different functionalities of the organ dictate their responses to mechanical stimuli. For instance, skin, the largest organ in human body, resists external stretching and compressing forces during daily activities. During physical exercise, the human body encounters periodical forces. For example, skeletal muscle stretches upon repetitive resistance training, while kneecap cartilage and the leg bones withstand periodic compression during running (Wang and Thampatty, 2006).

Advances in microscopy techniques and computer simulations reveal that even on the nano-scale, forces applied directly to single proteins can convert mechanical signals into biochemical cues, a process termed mechanotransduction. It has been shown that external tension transmitted from the ECM into the cell activates the binding of cytoplasmic vinculin to talin-integrin complex and thus elicits intracellular signaling cascades (del Rio et al., 2009). Further study suggests that cyclical stretching of talin by acto-myosin contraction is the driving force for the binding of vinculin (Hytonen and Vogel, 2008, Margadant et al. 2011). Recent study showed that the application of mechanical forces to Fn-integrin α5β1 increases the bond life 100 fold, thereby directly affecting the bond strength (Kong et al., 2013). Sections 2.2-2.4 looks in further detail at mechanosensing involving ECM protein, integrin and adaptor proteins.
In addition to passively responding to external forces, cells also actively generate forces to maintain tissue homeostasis (Tomasek et al., 2002). Studies reveal that upon loss of tension in a wound site, fibroblasts transform to a more contractile proto-myofibroblast phenotype enriched with cytoplasmic F-actin and enlarged focal adhesions (FAs) when compared to the quiescent fibroblasts (Tomasek et al. 2002). Further exposure to an elevated level of cytokines such as tumor necrosis factor-α (TNF-α), tumor growth factor-β (TGF-β) (Goldberg et al, 2007) and cellular Fn (cFn) containing Extra Domain A (EDA) (Kohan et al., 2010) irreversibly differentiates proto-fibroblast to myofibroblasts, assisting the closure of the wound (Desmouliere et al., 2005). Therefore, aside from soluble cues, cell-generated force is important in regulating ECM remodeling (Stavrou, 2009).

Uniaxial cyclic strain-induced cell re-orientation

The remodeling of many connective tissues is recognized by the rearrangement of matrix fibers, which are critical to the biomechanical functions of the tissues. Tissue remodeling assumes a cumulative process with periodically reaching and balancing the force equilibrium within the ECM (Brodland et al., 2007, Ranft et al., 2010, Hannezo et al., 2012). Apart from sensing the static tension, cyclic force application requires the multicellular structure to balance the cortical tension and adhesion to the ECM (Käfer et al., 2007) therefore maintaining the state of minimum energy (Steinberg, 1963, Foty et al., 1996, Krieg et al., 2008).

One of the most interesting behaviors of cells is the uniaxial cyclic strain-induced re-orientation. Conversely to the alignment along the stretching direction under static and quasi-static stresses (Eastwood et al., 1998, Collinsworth et al., 2000), multiple cell types show the ability to re-orient away from the stretching direction under dynamically varying stresses, such as arterial smooth muscle cells (Dartsch, Hammerle, Betz, 1986.), endothelial cells and fibroblasts (Kanda, Matsuda, 1993, Kemkemer et al., 1999, Wang et al., 2001, Neidlinger-Wilke et al., 2002, Moretti et al., 2004, Jungbauer et al., 2008, Goldyn et al., 2009, 2010, Deibler, Spatz, Kemkemer, 2011), and human melanocytes(Wang, Boissy, Grood. 1995).
Mathematical models predict that cells with polarized shapes re-orient away from the strain axis as a result of finding the energy minimum between cell-generated force dipole and external mechanical force via cell/matrix adhesion (Fig. 2.1) (De et al. 2007, De et al. 2008). Depending on the types of fibroblasts, however, the characteristic re-orientation time may vary (Jungbauer et al. 2008), showing the complexity in predicting cellular behaviors in vitro. Furthermore, although the cyclic-strain-induced cell re-orientation response is not the same as a spontaneous motile response, it is in large part determined by the same factors, such as cell-substratum adhesiveness and actin-myosin contractility (Ngu, et al., 2008; Mol Cell Biomech 2008, 5(1), 69). Recently, it was reported that the changes in the gene and protein expressions of mesenchymal stem cells differentiated to tenocytes were strongly correlated to cell orientation angle (Morita, Watanabe, Ju, Xu, 2013; Acta Bioeng Biomech, 2013, 15, 71)

Figure 2.1: Simplified model to predict the orientation of isolated cells subjected to uniaxial cyclic strain. The reaction stress, $R$, in the matrix arises from the contractile activity of the cell, characterized by the force dipole, $P$, determines the direction of minimum energy. Figure adapted from De, Zemel, Safran, 2007.

In this thesis, the focus is on studying the contribution of the exogenous Fn to ECM remodeling by fibroblasts exposed to uniaxial cyclic strain. It is, therefore, important to eliminate cell-cell interactions. Hence, the uniaxial cyclic strain-induced cell orientation depends on the external mechanical stress, the contractility of cells and the interaction between cell and ECM, as suggested in the model shown in Fig. 2.1.
2.2. Extracellular matrix

The extracellular matrix (ECM) not only provides the structural support for the tissue integrity, but also processes the ligand binding sites specific to cell-surface receptors (Cox, Erler, 2011, Frantz, Stewart, Weaver, 2010). Furthermore, the remodeling of the ECM allows cells to release growth factors and cytokines anchored in the fibrillar network in a spatially and temporally controlled manner to guide cell behavior (Cox, Erler, 2011, Frantz, Stewart, Weaver, 2010). In this thesis, two important ECM proteins namely were studied, namely fibronectin (Fn) and type I collagen (ColI).

Fibronectin

Fibronectin (Fn) is an important ECM molecule for early steps in wound healing. It consists of two almost identical Fn monomers, each approximately 220-250kDa in molecular weight (Pankov and Yamada, 2002). The C-termini of the two Fn monomers are covalently linked via disulfide bonds, while the N-terminal RGD sequence is one of the main regions to bind Fn specific integrin receptors, namely α5β1 and αvβ3 (Antia et al., 2008) (Fig. 2.2).

![Figure 2.2: Possible binding sites on Fibronectin. Fn can bind to several surface integrins, and the two most prominent ones are α5β1 and αvβ3 , which are located within the type III modules and near the N-terminus respectively. Figure adapted from Vogel, 2006.](image)

Structurally, fibronectin is composed of three distinct modules: 12 type 1
modules (FNI), 2 type 2 modules (FNII) and 15-17 type 3 modules (FNIII). These modules consist of two sheets of anti-parallel beta strands. In contrast to FNI and FNII modules, FNIII modules do not contain stabilizing intra-module disulfide bonds, which allow FNIII to unfold more easily under mechanical tension (Gee, Ingber, Stultz, 2008, Chabria et al., 2010).

Although all Fn is the product of a single gene, Fn has more than 20 variants due to alternative splicing (Hynes, 1985). Secreted by hepatocytes, plasma fibronectin (pFN) lacks the two extra domain FNIII modules Extra Domain A and B (EDA and EDB). Fibroblasts secrete cellular fibronectin (cFn) that contains EDA domain. It has been reported that in order to close a wound, dermal fibroblasts transiently differentiate to more contractile myofibroblasts upon binding to cFn (Tomasek et al., 2002, To and Midwood, 2011). It is not surprising, therefore, that mature tissue, unless diseased, lacks of EDA (Pankov and Yamada, 2002, Muro et al., 2003). The EDA region in cellular Fn provides binding sites for integrins α4β7 (Kohan et al, 2010) and α9 (Sun et al, 2013), as opposed to α5β1 and αvβ3, the classic receptors for Fn may associate with distinct intracellular signaling from binding plasma Fn. The frequent appearance of cFn in embryonic development confirms its important role in mechanosensing during embryogenesis, vascular remodeling, tissue development and cell differentiation (Hynes, 2007, Chauhan et al., 2004, Peters and Hynes, 1996).

Various binding sites have been reported along the Fn, including ECM proteins, growth factors, and cell surface receptors (Vogel, 2006) (Fig. 2.2). Among these, the integrin binding mediates the connection between cells and fibronectin (Johansson et al., 1997). The FNIII modules 9 and 10 in addition to the area stretching from module 14 into the V region have been singled out as the main binding sites for integrins (Pankov, Yamada, 2002). Most of the reported integrins binding to FN utilize the RGD sequence on FNIII10 together with the synergy site on FNIII9. Among the integrins recognizing the RGD sites are the integrins α3β1, α5β1, αVβ1, and αVβ3 (Johansson et al., 1997). It has been suggested that the presence of EDA induces a conformational change within the fibronectin module, increasing integrin binding to an exposed RGD loop (Manabe et al., 1997).

Fn contains cryptic binding sites that can be exposed by cell-generated tension and consequently alter Fn functionality (Zhong et al, 1998, Lemmon et al., 2011,
How the conformation of Fn unfolds in ECM is, however, still under debate (Johnson et al., 1999, Erickson, 1994, Baneyx, Baugh, Vogel, 2001, Baneyx, Baugh, Vogel, 2002, Ohashi, Kiehart, Erickson, 1999, Abu-Lail et al., 2006, Smith et al., 2007, Chabria et al., 2010, Lemmon et al., 2011, Ohashi, Erickson, 2011). One model proposes a quaternary structure unfolding, suggesting that a cell-generated force can separate the two monomers in Fn and therefore leave the tertiary and secondary structures intact (Ohashi, Kiehart, Erickson, 1999, Abu-Lail et al., 2006, Lemmon et al., 2011). Another model proposes secondary structure unfolding, showing that cell-generated force further extends the secondary structures in Fn by possibly unfolding the FNIII modules (Baneyx, Baugh, Vogel, 2001, Baneyx, Baugh, Vogel, 2002, Smith et al., 2007, Klotzsch et al., 2009). Despite different theories regarding how Fn unfolds, it is accepted that Fn matrices in the ECMs undergo conformational changes due to cell traction forces.

Figure 2.3: Mechanism of Fn Fibrillogenesis. FN ligation and interaction with an intact actin cytoskeleton are required for α5β1 integrin translocation. Translocating α5β1 integrins induce initial Fibronectin fibrillogenesis by transmitting cytoskeleton-generated tension to extracellular Fibronectin molecules. Figure adapted from Pankov et al, 2000.

The assembly of Fn dimers into a fibrillar matrix, termed Fn fibrillogenesis, is
a force-dependent process. The force responsible for Fn fibrillogenesis in static cell culture is generated by actomyosin contraction, and the direction of Fn fibrillogenesis is determined by the translocation of integrin α5β1 from FA (Fig. 2.3) (Pankov et al., 2000). Previous studies showed that the mere binding of Fn to cell surface integrins is not sufficient to restore cellular mechanosensing, indicating that Fn fibrillogenesis is crucial in mechanotransduction (Sottile, Hocking, Langenbach, 2000, Hocking, Sottile, Langenbach, 2000). One hypothesis is that the Fn in the ECM not only provides integrin binding sites, but more importantly the fibrillar matrix controls binding with spatial precision. It has been shown that fibroblasts on a physisorbed N-terminal 70kDa- Fn substrate do not form α5 integrin clustering (Tomasini-Johansson, Annis and Mosher, 2006). Furthermore, the formation of vinculin and integrin-β3 rich periphery FAs on RGD-coated gold nano-patterns depends on pattern density (Cavalcanti-Adam, et al. 2007). Another study showed that the correct RGD to RGD spacing for FAs determines the signaling efficiency and migratory ability (Guillaume Le Saux et al. 2011).

**FnDA**

![Figure 2.4: Fn conformation is unfolded in denaturant due to ionic interactions. FnDA is double labeled with Alexa Fluoro 488 and Alexa Fluoro 546 (Invitrogen, Switzerland) as donor and acceptors. Donor fluorophores are specifically labeled to type III cysteins (indicated by orange module surrounded by yellow sphere), while acceptors are randomly labeled throughout the entire Fn on amines. Figure adapted from Smith et al., 2007.](image)

FnDA is a mechanosensitive Förster Energy Transfer (FRET) Fn construct that enables the monitoring of the Fn conformational changes within the ECM (Baneyx, Baugh, Vogel, 2001, Baneyx, Baugh, Vogel, 2002, Smith et al., 2007, Little
et al., 2008, Kubow et al., 2008). Fn has a compact conformation in physiological buffer (Johnson, Sage, Briscoe, Erickson, 1999), but can be unfolded in a denaturant due to ionic interactions (Baneyx, Baugh, Vogel, 2001, Smith et al., 2007). The unfolded conformation of FnDA has also been observed in the ECM as a result of cell-generated tension (Baneyx, Baugh, Vogel, 2002), and increasing mechanical strain progressively unfolds FnDA (Smith et al., 2007, Little et al., 2008), showing that FnDA is a sensitive construct to study Fn conformation in matrix and a good indicator to study ECM tension.

The unique feature of FRET identifies whether the donors are close to the acceptors and whether they are moving towards or away from each other (Clegg, 1995, Periasamy, 2001, Sekar, Periasamy, 2003, Gadella (Ed), 2009). Energy transfer between a single donor-acceptor pair depends on the dipole-dipole interaction between the two fluorophores (or phosphors), which is inversely proportional to the 6th power of the distance between donor and acceptor (Foerster, 1951) (Equation 2.1). Therefore, FRET is sensitive to the change of distance between donor and acceptor.

\[ k_t = \frac{1}{\tau_D} \left( \frac{R_0}{r_{DA}} \right)^6 \]  

where \( k_t \) = rate constant for transferring energy from donor to acceptor,
\( \tau_D \) = fluorescence lifetime of donor without acceptor,
\( r_{DA} \) = distance between donor and acceptor,
\( R_0 \) = distance at which the energy transfer efficiency is 50%

**Type I Collagen**

Collagen is the major ECM protein in adult connective tissue, and can be found in skin, tendon, ligaments and bones (Gelse, Poeschl, Aigner, 2003, Alberts, Bruce, 2002). During tissue maturation, the Fn-rich matrix is interlaced and replaced by type I collagen (Col1), which contributes to high mechanical strength in the ECM (Roeder et al., 2002, Muiznieks, Keeley, 2012). Each Col1 fiber is roughly 300nm in length and 1.5nm in diameter and consists of triple right-handed helical strands (Alberts, Bruce, 2002). Single collagen fibers can self-assemble and form fibrillar collagen that is 50-200nm in diameter and several micrometers in length with high resistance towards tensile strength (Alberts, Bruce, 2002). Atomic force microscopy
experiments showed that *in vitro* electrospun Col1 nanofibers can be stretched 33% before breakage and demonstrate significant softening upon an increase in strain (Carlisle, Coulais, Guthold, 2010). Unlike the single collagen fiber, the macroscopic fibrillar collagen network stiffens under strain (Storm et al., 2005), showing that matrix anisotropy is essential to determining the macroscopic mechanical properties in tissue.

Collagen is synthesized as procollagens and transported into a rough endoplasmic reticulum, where procollagen chains undergo glycosylation and form triple-helical structures via intrachain disulfide bonds (Alberts, Bruce, 2002). Col1 is secreted to the ECM by exocytosis, during which the N-terminal and C-terminal propeptides are removed by procollagen peptidases to form triple-helical collagen fibers (Alberts, Bruce, 2002). In fibroblast cultures, ascorbate is required in the synthesis of hydroxyproline and hydroxylysine in collagen, which is necessary for the stabilization of the collagen triple helix and crosslinking between collagen fibrils (Murad et al., 1981). However, ascorbate can also stimulate the mRNA level of procollagen and other ECM gene expressions (Belin et al., 2010, Duarte, Cooke, Jones, 2009). Therefore, to mimic a collagen-rich environment similar to the connective tissue, monomeric Col1 is more often used in cell culture than the simulation of procollagen synthesis by ascorbate.

### 2.3. Focal adhesion and integrins

External mechanical forces are transmitted from the ECM to the intracellular space mediated by focal adhesions/integrins, acting as a mechanotransducer. The following sections focus on the force-dependent growth of focal adhesions (FA), the force activation of vinculin and the force-induced activation and clustering of integrins.

**Focal complexes, focal adhesions and fibrillar adhesions**

In the case of adherent cells, the bi-directional force transmission across the
cell membrane through FA requires temporal and spatial regulation of the binding and dissociation of cells from the substrate (Kanchanawong et al. 2010). Depending on the force levels, adhesion sites can be categorized into three stages: The smallest by size, focal complexes, are difficult to visualize under light microscope. They are dot-like adhesion sites usually smaller than 1µm, exclusively found at the apical front of lamellipodia (Nobes, Hall 1995). Because of the ease of formation and dissipation due to its small size, focal complexes are dynamic structures with a high turnover rate (Alexandrova et al., 2008). Focal adhesion kinase (FAK) and paxillin are found in focal complexes by Rac and cdc42 activation, but not by Rho (Ren et al., 2000). FAs (3-10µm in length) larger than focal complexes are associated with the appearance of actin stress fibers (Zamir et al., 2000), showing the importance of Rho-dependent actomyosin tension (Ren et al., 2000). The largest cell-ECM adhesion structures are the fibrillar adhesions, which are elongated steak-like structures rich in tensin and α5β1 and exist only in highly contractile cells (Katz et al., 2000).

By using 3D super-resolution fluorescence microscopy, FA are shown to be highly structured complexes (Kanchanawong et al., 2010). Between cytoskeletal actin and cytoplasmic integrin tails, there is 40nm of vertical separation: FAK and paxillin, the closest to integrin cytoplasmic tails, are suggested to contribute to the activation of integrin. Talin and vinculin, located further away are important in mediating force-transduction. Zyxin, vasodilator-stimulated phosphoprotein (VASP) and α-actinin, being in proximity to actin, are shown to regulate actin polymerization (Kanchanawong et al., 2010).

**Vinculin – tension regulated adaptor protein**

Vinculin is an important mechano-coupling protein in focal adhesion. Single vinculin is made of 1066 amino acids with a molecular weight of 117kDa, and it can be divided into three regions; the head domain (D1-D4), the flexible hinge domain and the tail domain (D5) (Bakolitsa et al., 2004). Because the ability to link integrin to cytoskeletal actin filaments at sites of cell-matrix adhesion, vinculin is a considered as a mechano-coupling protein. Vinculin interacts with several other focal adhesion proteins such as talin, paxillin, tensin, Vasodilator-stimulated phosphoprotein
Vinculin recruitment to focal adhesions and the transmission of mechanical stress across focal adhesions are regulated separately (Mierke et al., 2008, Grashoff et al., 2010). To be more specific, the activation state of vinculin is regulated by cellular tension, which depends on actomyosin contraction (Carisey et al., 2013). When inactivated, the head domain interacts with the tail domains to adapt an autoinhibited conformation (Johnson, Craig, 1995). Vinculin binding to either talin or F-actin cannot activate vinculin, but the combinatorial binding of the head domain to talin and the tail domain to F-actin can activate vinculin (Chen, Choudhury, Craig, 2006). It was shown that mechanically stretching the talin rod exposes cryptic binding sites for vinculin binding and recruitment (Del Rio et al., 2009). Vinculin expressing cells generate forces seven times higher than Vinculin knockout cells (Mierke et al., 2008). And it is suggested that vinculin slows down the F-actin flow in maturing focal adhesion, which promotes the force generation locally (Thievessen et al., 2013).

Tension periodically rises and falls across vinculin, due to cyclic contraction generated by actomyosin machinery. It is known that the recruitment of vinculin depends on the tension applied at focal adhesions (Galbraith, Yamada, Sheetz, 2002) (Fig. 2.5). The active form of vinculin is crucial in maintaining other focal adhesion proteins such as paxillin, talin and vinexins in the focal adhesion site even when actomyosin contraction is disrupted, while inactive vinculin releases those proteins and leads to tension-dependent disassembly of focal adhesion (Carisey et al., 2013). A steered molecular dynamics study suggests a biphasic model for the activation of talin’s vinculin binding site under cellular tension: Force leads to a partial unfolding of talin, therefore presenting binding sites for vinculin. Then, further unfolding of talin results into a straightened polypeptide chain, inactivating the binding site and ultimately preventing vinculin recruitment (Hytönen and Vogel, 2008).
Despite the evidence, whether mechanical force is necessary in vinculin activation is still under debate. It has been suggested that phosphatidylinositol-4,5-bisphosphate (PIP2) binds to the tail of vinculin and activates the vinculin by disrupting the intramolecular interaction of the autoinhibited conformation without mechanical force (Huttelmaier et al., 1998). Others have shown that the binding of talin, α-actinin and invasin IpaA to inactive vinculin can disrupt the conformation of the vinculin N-terminal four helical bundle which triggers the opening of the vinculin tail domain from the autoinhibited conformation (Nhieu and Izard, 2007).

**Integrins – the transmembrane linkage between ECM and cytoskeleton**

Integrins mediate the physical connection between the extracellular matrix and the cytoskeleton of a cell. They are not only involved in the activation of various signaling pathways, but also transfer forces generated within the cell to its surroundings or take up a mechanical stimulus and forward it into the cell.

Integrins in general are heterodimers, consisting of an alpha and a beta unit. So far 8 beta units and 18 to 19 alpha units have been discovered, with 24 to 25 subunit combinations described (Hynes, 2002, Wolfenson et al., 2013). It has been
shown that an integrin dimer can exist in an open or a closed conformation, with the conformational state having a direct impact on the ligand binding affinity (Fig. 2.6).

Integrins are characterized by their ligand specificity. One distinguishes between ECM protein and IG receptors (for example the α4β1 integrin), the Laminin receptors (for example α6β4 integrin), the leukocyte specific receptors (for example αLβ2 integrin), the Collagen receptors (α1β1, α2β1 integrin among others) and the RGD receptors (αVβ1 integrin and others). The RGD receptors are named after the amino acid sequence that is the binding target for these integrins. The RGD sequence can be found on the 10th FNIII module of fibronectin.

Within a cell, integrins are connected to cytoskeletal components such as actin fibers via adaptor proteins (Schwarz and Gardel, 2012). They form large complexes termed focal adhesions, which have been reported to be mechanosensitive (Katoh et al., 2008).

Figure 2.6: Integrins exist in a closed (A) and an open (B) conformation. The alpha subunit is shown in blue, the beta subunit in red. Picture derived from the review by Hynes, 2002: “Integrins: Bidirectional, allosteric signaling machines”

Force transmitted from the ECM to cells can activate integrins, regulate the adhesion strength and determine the lifetime of the bond between the cell and the
ECM. For example, during fibroblast migration on a Fibronectin-coated substrate, cell-generated force is able to activate integrin α5β1 by stretching it to an open conformation (Askari et al. 2010). Further studies reveal that, under low tension integrin α5β1 binds primarily to the RGD site on Fibronectin but under high tension the binding of α5β1 to Fibronectin requires the association of both the RGD and synergy site to stretch-activate α5β1 (Friedland, Lee, Boettiger 2009). The high tension applied at a focal adhesion also prolongs the lifetime of the bond between Fibronectin and α5β1, showing the catch-bond behavior of this linkage (Kong et al., 2013).

Once integrin is activated, adaptor proteins in the molecular clutch can amplify force signals. Many mechanosensitive cellular components are now identified at the site of focal adhesion, including focal adhesion kinase (FAK), mitogen-activated proteins kinases (MAPKs), paxillin, talin, vinculin, tensin and others (Kreis, Vale (Ed), 1999).

2.4. Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases

ECM remodeling is regulated by degradation enzymes, including heparanase, cathepsins, hyaluronidases, matriptases, various serine and threonine proteases (Roycik et al., 2009), and metzincins, including ADAMs (a disintegrin and metalloproteinases), ADAMTSs (ADAMs with thrombospondin motifs), and matrix metalloproteases (MMPs) and their inhibitors - tissue inhibitor of MMPs (TIMPs) (Birkedal-Hansen, 1993, Mott and Werb, 2004, Nagase, 2006, Page-McCaw, Ewald, Werb, 2007, Gialleli et al., 2011). Since MMPs regulate the temporal and spatial degradation of ECMs, they have long been considered pharmacological targets for better wound healing (Lee and Murphy, 2004, Gill and Parks, 2008). Out of all twenty-three currently known human MMPs, thirteen interact with Fn, namely MMP-2, -3, -7, -8, -9, -11, -12, -13, -14, -15, -16, -25 and -26 (Klein and Bischoff, 2011). While MMP-1 to -13 are secreted as solublezymogens, MMP-14 to -28 are activated membrane-anchored endopeptidases due to intracellular cleavage by furin-like protein convertases (Sohail et al., 2008, Itoh, Seiki, 2006, Murphy, Nagase, 2011), so
membrane-type matrix metalloproteinases (MT-MMPs) can rapidly digest localized ECM molecules (Lee, Murphy, 2004). Hence MT-MMPs play important roles in regulating cell migration and tumor invasion (Chen, Parks, 2009).

**Gelatinases**

Gelatinase-A&B (MMP-2&9) cleave various substrates including ECM proteins, such as collagen, gelatin and fibronectin (Roy, Yang, Moses, 2009, Kessenbrock, Plaks, Werb, 2010). It has been shown that human fibronectin type II modules compete with MMP-2 for Coll binding sites to control the activation of MMP-2 (Stanton et al., 1998, Steffensen et al., 2002). In a diseased state such as cancer, fibronectin is shown to up-regulate MMP-2 expression (Moroz et al., 2012), MMP-9 expression and coordinated expression of MMP-2 in human T lymphocyte cell lines (Esparza et al., 1999). In addition to ECM degradation, MMP-9 is known to release embedded growth factors such as VEGF, TGF-β and FGF-2 to promote tumor invasion (Bergers et al., 2000, Yu, Stamenkovic, 2000, Ardi et al., 2009).

The secretion of MMP-9 by a human keratinocyte monolayer seeded onto a Coll-coated surface can be induced by cyclic mechanical strain (Reno, Traina, Cannas, 2009). Cyclic strain-induced MMP-2 expression in bovine aortic endothelial cells is shown to depend on p38 and extracellular signal-regulated kinase (ERK) signaling (von Offenberg Sweeney et al., 2004). Others have shown that cyclic mechanical stress up-regulated MMP-2 expression in human umbilical vein endothelial cells is likely by TNF-a via JNK pathway (Wang et al., 2003). Using mouse models, it has been found that the activation of latent MMP-2&9 is regulated differently in cardiac vascular remodeling: Whereas pro-MMP-9 increases in a mouse with exercise after 15 days, pro-MMP-2 levels decrease below the control group after 30 days (Bellafiore et al., 2013). A simultaneous increase of MMP-2&9 expression has been observed previously, however, in vascular pathologies and is likely due to high oxidative stress, which can be inhibited by antioxidant and catalase treatment (Rajagopalan et al., 1996, Uemura et al., 2001).
**Tissue inhibitors of matrix metalloproteinases**

MMP activities can be inhibited by four tissue inhibitors of metalloproteinases (TIMPs), which are highly conserved in structure (Visse, Nagase, 2003). TIMP-2 and TIMP-3 but not TIMP-1, are effective inhibitors of the MT-MMPs (Brew, Dinakarpandian, Nagase, 2000). TIMPs form a 1:1 complex with MMPs when inhibiting MMPs by blocking water molecules from entering the active Zn pocket (Brew, Dinakarpandian, Nagase, 2000). Due to their highly flexible C-Terminus their inhibition of MMPs is often unspecific (Bode, 1999, Lee and Murphy, 2004, Gill and Parks, 2008). Out of four TIMPs found in vertebrates, TIMP-3 is ECM-bound whereas TIMP-1, 2, and 4 are freely soluble (Visse, Nagase, 2003). The difference in location is believed to be advantageous for TIMP-3 to inhibit a broader profile of substrates other than MMPs such that it can influence the release of ECM-bound growth factors and cytokines (Mohammed, Smookler, Khokha, 2003). By using TIMP-3 knockout fibroblasts, it has been shown that the loss of TIMP-3 can accelerate the activation of proMMP2 (English et al., 2000). Unlike the other TIMPs, TIMP-3 is a good inhibitor of tumor necrosis factor-α converting enzyme (TACE) (Amour, 1998). Regulation of TIMP-3 in particular is associated with TNF-α levels and apoptotic events in cardiac remodeling (Lovelock et al, 2005).

There are several TIMP-MMP interactions that are proved to exist. All four TIMPs are structurally conserved, and they bind MMPs via their three C-terminal disulphide-bonded loops (Visse, Nagase, 2003). MMP-3 and TIMP-1 act as a complex, as well as MMP-14 and TIMP-2. The C-terminal domain of TIMP-2 is responsible for the formation of the complex with MMP-14. Therefore the N-Terminus of TIMP-2, which is the inhibitory domain, can bind, to MMP-14 on the cell surface. This process, which is independent from TIMP-2, activates pro MMP-2. Furthermore, TIMP-1 interacts with proMMP-9, TIMP-2 does interact with MMP-2 and TIMP3 can regulate either proMMP-2 or MMP-9. MMP-9 and TIMP-1 interact together too, whereby the interaction is modulated by thrombospondin (Lee and Murphy, 2004)
2.5. Fibroblasts

**Human Foreskin Fibroblasts**

As fibroblasts synthesize the extracellular matrix, exert forces on their environment and are critical for tissue remodeling (Lee et al., 2013, Shaw, Martin, 2008, Gabbiani, 2003), they are prime targets to study the influence of mechanical force on ECM remodeling.

In this thesis, first matrix composition (Fn and/or collagen) and how it regulates the early onset of ECM remodeling under dynamic mechanical loading is studied. To mimic tissue maturation in human tissue, human dermal fibroblasts isolated from juvenile foreskin (HFFs) are used (NHDF, Promocell, Germany). These cells have been used in studying wound healing, tissue engineering scaffolds, and tissue regeneration researches (Zhou et al, 2004, Carlson et al., 2009).

**Fibronectin knockout and floxed mouse embryonic fibroblasts**

As fibroblasts possess the ability to produce fibronectin, they can mask the effect of exogenous fibronectin on cell behavior. In order to elucidate the role of Fn on fibroblast under mechanical stress, the presence of Fn has to be eliminated.

Mouse embryonic fibroblasts with a knocked out fibronectin gene (Fn<sup>−/−</sup> MEFs) from Rainhard Fässler and his group, who established a conditional fibronectin gene knockout were therefore used (Sakai et al., 2001). The conditional knockout technique allows for the fibronectin gene to be knocked out at nearly any time point and any tissue desired, avoiding the embryonic lethality.
Figure 2.7: Overview of the Cre-lox recombination. A mouse containing a floxed target gene is crossbred with a mouse with a Cre recombinase DNA under the control of a tissue specific promoter. Although both mice are healthy, their offspring lacks the target gene in the tissue type, in which the Cre recombinase is active, as it excises the DNA sequence between the LoxP sites. By combining the Cre recombinase with an inductor (a chemical which activates the recombinase) one can control the timepoint of the knockout. Figure adapted from “The mouse ascending: perspectives for human disease models” by Rosenthal and Brown, 2007. (internet source of the picture as of February 2014: http://www.nature.com/ncb/journal/v9/n9/fig_tab/ncb437_F2.html)

For that, the target sequence is modified by inserting two flox (=flanked by LoxP) sites at both ends of the sequence. Mice containing such a modification in their fibronectin sequence display no detectable abnormalities. By crossing these mice with a mouse strain containing an inducible Cre recombinase, offspring with both the floxed target gene and the inducible Cre recombinase gene can be obtained. If the Cre recombinase is preceded by a cell type specific promoter, the recombinase will become active only in the desired cell type upon induction.

After induction the Cre recombinase recognizes the two LoxP sites and excises the sequence in between, thereby physically removing the target DNA from the genome.

For Fn knockout (Fn<sup>-/-</sup>) and floxed (Fn<sup>f/f</sup>) MEFs from Professor Haessler’s Lab, embryonic fibroblasts from the kidney were isolated and immortalized at stage E13.5. Then those MEFs were immortalized with SV40 large T antigen (simian virus)
to keep cells with continuous proliferation. Finally, immortalized MEFs were introduced to adeno-virus containing cre-recombinase DNA, which is a more efficient way of introducing cre-recombinase than the traditional chemical-induction, such as using tamoxifen, or tissue-specific promoter (Sakai et al., 2001).

References


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Steinberg, M.S., Reconstruction of tissues by dissociated cells. Some morphogenetic tissue movements and the sorting out of embryonic cells may have a common explanation. Science, 1963. 141(3579): p. 401-8.


Chapter 3: Cell stretcher

The previous chapter introduced the importance of mechanical forces in regulating tissue homeostasis, tissue remodeling and cell behaviors. In order to apply dynamic mechanical loading to cells several automated cell stretchers were compared and STREX ST-140 (B-bridge International, U.S.A) was chosen to apply uniaxial cyclic strain. In this chapter current self-built and commercially available mechanical cell stretching devices are introduced, and their advantages and disadvantages regarding the design of this thesis are compared. In addition, two substrate-coating methods (chemical covalent attachment and physisorption) are evaluated to maximize the cell attachment for stretching experiments.

3.1. Overview

While 3-dimensional stretching systems have been among the popular choices for tissue engineers to mimic the physiological loads to scaffolds, such as applying axial compression to osteocytes and chondrocytes embedded collagen gels (Klein-Nulend, Bacabac, Mullender, 2005, Wang and Detamore, 2007, McCoy and O'Brien, 2010, Schulz and Bader, 2007, Darling and Athanasiou, 2003, Grodzinsky et al., 2000), many studies have used elastic substrate to decipher molecular mechanisms under dynamic loading (Zhong et al., 1998, Sawada and Sheetz, 2002, Smith et al., 2007, Hirata et al., 2008). Elastic substrates can be stretched in one direction (Uniaxial strain) or equally stretched in all directions (Equiaxial strain). Substrates can be deformed by mechanical forces, electrical-magnetic forces or pressures either from fluid or air (Fig. 3.1).

Uniaxial strain (sometimes referred as simple elongation) is one of the most frequently applied strains in cell culture. An elastic substrate such as silicone sheet (Smith et al. 2007) and polydimethylsiloxane (PDMS) (Jungbauer et al., 2008, Goldyn et al., 2009, Deibler et al., 2011) can be uniaxially strained in two ways. One, tensile force is applied at one end while the opposite end is clamped (Fig. 3.1A). Two, equal tensile forces are applied at both ends but in opposite directions (Fig 3.1B). Owing to the Poisson effect, while substrate is strained in one direction it transversely
compresses in the orthogonal direction. Therefore, the combination of tension in the stretching direction and the compression perpendicular to the stretching direction affect the direction of minimum strain (De, Zemel, Safran, 2007). To avoid compression due to the Poisson effect, one can restrain the substrate perpendicular to the stretching direction by clamping (Fig. 3.1C). However, once clamping is used, there is often the “grip-end effect”, meaning that cells close to the grip end experience disturbed local strains that are different from the desired uniaxial strain and may ultimately change to other phenotypes.

**Uniaxial strain**

![Uniaxial strain diagram](image)

**Equiaxial strain**

![Equiaxial strain diagram](image)

*Figure 3.1: Simplified drawings of uniaxial, biaxial and equiaxial strains. (A-C). Uniaxial strain: stretching force is applied to one end or equally to both ends. While the elastic substrate elongates along the stretching direction, the material perpendicular to the strain direction compresses due to Poisson effect. (D-G). Equiaxial strain: deformation of substrate is equal in all directions. Equiaxial strain can be achieved by various displacement methods such as applying mechanical forces and changing fluid or air pressures. Substrate can stay in or out of plane after deformation.*

Equiaxial strain means that the substrate is deformed equally in all directions, and it can be achieved by solid platen (Fig. 3.1D), or be air or liquid-mediated (Fig. 3.1D and 3.1E) displacing the substrate membrane out of the initial plane. Flexcell systems introduced the in-plane distension by only applying a vacuum to the outer ring of the substrate (Fig. 3.1F). All such systems, however, suffer membrane/platen
friction which give rises to different behavior of a sub-population of cells located in this area compared to those that are subject homogenous equiaxial strain. The improved design, following several systemic studies of strain anisotropy and heterogeneity under radial strain (Gilbert et al., 1994, Almekinders, Banes, Ballenger, 1993), suggests a thinner substrate membrane and larger radius to minimize the sub-population (Fisher (Ed), 2013). Alternatively, one can clamp the substrate and apply mechanical equiaxial stretching using motor-generated forces (Fig 3.1G).

3.2. Automated uniaxial stretcher from b-bridge

STREX from B-Birdge International applies uniaxial strain to the Polydimethylsiloxane (PDMS) chambers where cells have been previously adhered to the coated substrate. Due to the success of the pilot experiment on a STREX from Prof. Marcy Zenobi-Wong’s Lab, the STREX ST-140 (Fig. 3.2A) was purchased. A customized list of strain profiles was acquired (Fig. 3.2B), so that the program provided sufficient variation of strain profiles for this study. However, in theory, the STREX has the potential to be re-programmed by an external controller. In addition, extra strain chamber brackets for two chamber sizes were acquired, namely the 4cm² (2cm×2cm) chambers for microscopy imaging purposes and the 10cm² (3.33cm×3.33cm) chambers which allow us harvesting enough cells for biochemical essays (Fig. 3.2C). In addition, the STREX holds multiple chambers (5× 10cm² chambers, 6× 4cm² chambers) therefore allowing parallel conditions within one set of experiments. PDMS chambers were made by mixing the elastomer and curing agent at 10:1 (w/w) ratio (SYLGARD® 184 silicone elastomer kit, Dow Corning, U.S.A.). The mixture was degassed for 1 hour, and then injected into the in-house fabricated molds (blue prints courtesy of Stephen Wheeler, Institute for Biomedical Engineering, ETH Zurich) (Fig. 3.2C). The PDMS was baked at 80°C for 4 hours to ensure complete crosslinking. After baking, the chambers were rigorously rinsed with 70% ethanol and Nanopure water (D11031, Thermo Scientific, U.S.A.) and further sterilized by autoclaving at 121°C for 20 minutes.

Three details need to be carefully checked before and during stretching experiment to ensure the reproducibility of the results. (1.) Prior to stretching the
flatness of the substrate membrane of each chamber needs to be checked, as any PDMS wrinkle will give rise to an anisotropic strain field. (2.) The stretching chambers should be attached symmetrically in the strain chamber brackets to ensure the mechanical stability of stretching plate during experiments. (3.) The stepping motor in the stretching unit that is placed inside the incubator during the stretching experiment needs to be water-cooled.

**A. STREX ST-140**

![STREX ST-140](image)

**B. Strain parameters**

<table>
<thead>
<tr>
<th>Digit</th>
<th>Program</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10,000 seconds cycle one wave</td>
<td>Hold, Slowest stretch (S%/s%) hold, Slowest contraction (S%/s%) repeat</td>
</tr>
<tr>
<td>1</td>
<td>1,000 seconds cycle one wave</td>
<td>Hold, Slowest stretch (S%/s%) hold, Slowest contraction (S%/s%) repeat</td>
</tr>
<tr>
<td>2</td>
<td>100 seconds cycle one wave</td>
<td>Slowest stretch (S%/s%) hold, Slowest contraction (S%/s%) repeat (small stretch distances may have an initial and mid cycle hold period)</td>
</tr>
<tr>
<td>3</td>
<td>10 seconds cycle one wave</td>
<td>Stretch 10 sec - contract 10 sec - repeat</td>
</tr>
<tr>
<td>4</td>
<td>10 seconds cycle one wave</td>
<td>Stretch 5 sec - contract 5 sec - repeat</td>
</tr>
<tr>
<td>5</td>
<td>5 seconds cycle one wave</td>
<td>Stretch 2 sec - contract 2 sec - repeat</td>
</tr>
<tr>
<td>6</td>
<td>5 seconds cycle one wave</td>
<td>Stretch 1 sec - contract 1 sec - repeat</td>
</tr>
<tr>
<td>7</td>
<td>1 second cycle one wave</td>
<td>Stretch 0.5 sec - contract 0.5 sec - repeat</td>
</tr>
</tbody>
</table>

**C. Chamber on brackets**

![Chamber on brackets](image)

Figure 3.2: STREX ST-140 from B-Bridge International. (A.) The STREX ST-140 consists of three main units – the stretching unit, the controller and the water-cooling tank. (B.) Customized strain parameters. Strain amplitude expands from 2-20% for 4cm² chambers and 1.3-12.7% for 10cm² chambers. Range of frequency covers 1 to 10⁴Hz. (C.) Top: 4cm² and 10cm² PDMS chambers inserted to chamber brackets. Bottom: PDMS chambers next to their molds.

### 3.3. Cell stretcher CS-10

Applying uniaxial strain, the CS-10 was first introduced to the lab by Dr. Ruth Schwartlander in search of a computer-controlled device to stretch Fibronectin fibers. The stretcher was designed and improved by Axel Gerstmair in the Dietl Lab at university of Ulm, Germany (Gerstmair et al., 2009). The chamber design (Fig. 3.3A) allows the use of commercially available silicone sheets, which have a more defined membrane thickness compared to home-made PDMS chambers. Silicone grease is applied at the silicone sheet-metal holder interface to prevent growth media from
leaking (Fig. 3.3B). To ensure cell seeding onto a flat bottom membrane, stretching chambers were pre-stretched for 5% and placed in the pre-strain holder (Fig. 3.3C) in the petri-dish.

![Figure 3.3: (A) Schematic drawing of the cell stretcher CS-10 developed in the Dietl Lab. By using the motion compensation function, the device keeps the cell of interest in the field of view during stretching. Red arrows indicate the stretching direction. (B and C) Separated and assembled membrane chamber (grey) and prestrain holder (black). Image adapted from Gerstmair et al., 2009](image)

Similar to the STREX ST-140, the CS-10 comes with stepping motors, which cannot apply a cyclic stretching frequency higher than 10 Hz. The self-written controller interface gives users the freedom to customize strain profiles. To implement the motion compensation function that keeps the cells of interest in the field of view during stretching, the stretcher is built on two independent base plates (Fig. 3.3A). The cell stretcher CS-10 came with separate software that was not yet integrated to the microscope software, manual synchronization of the start of stretcher and microscopy imaging was required before experiment.

The stretcher is compact and has the potential for live cell imaging, if the stretcher is mounted properly on an environmental-controlled inverted microscope. However, due to leakage and media evaporation from the small stretching holder, stretching cells while live imaging for more than 20 minutes proved to be extremely
challenging. As the CS-10 was still under improvement, the STREX ST-140 was chosen over the CS-10.

### 3.4. Microfluidic cell stretcher

A microfluidic (µFluidic) cell stretcher was kindly provided by Professor Beth Pruitt from Stanford Microsystems Lab. It consists of three channels: two outer deformation channels and one inner channel in which cells can be cultured (Fig. 3.4A). Deforming the outer channels by changing air or fluid pressure causes the uniaxial stretching of the inner channel substrate (Fig. 3.4B). As the name indicates, µFluidic cell stretcher operates on the micron scale. The cell-culturing channel of the µFluidic cell stretcher is 150µm in width while two deformation channels are 50µm in width. The slightest variation in the substrate due to the manufacturing process can cause a difference in the local strain field during stretching experiments. Therefore, for each µFluidic cell stretcher, strain amplitude needs to be calibrated before starting the actual experiment.

![Image of µFluidic stretcher from Stanford Microsystems Lab.](image)

*Figure 3.4: µFluidic stretcher from Stanford Microsystems Lab. (A.) µFluidic stretcher consists of three channels. Channel 1 and 2 are used to apply vacuum pressure to displace the substrate of the cell stretching channel. (B.) A schematic drawing of channel 1, 2 and the cell stretching channel.*

The µFluidic cell stretcher shares the same advantages and disadvantages as other µFluidic devices. It is a compact, re-usable device, flexible enough to culture various types of cells. On the other hand, the µFluidic cell stretcher suffers from a
high area/volume ratio, meaning without perfusion cells cultured inside the channels for long term experiments often lack nutrition. Moreover, the small culturing volume made it impossible to seed cells at sub-confluent density to avoid cell-cell contact. Although this stretcher has a lot of potential for live imaging, we did not use it for studying cell-matrix interactions and matrix remodeling in this study.

3.5. Substrate coating methods

Unless treated, fibroblasts barely adhere to the hydrophobic PDMS surface. Two ways to coat the PDMS surface were tested. One is physisorption and the other is covalent attachment (Wipff et al., 2009). Briefly, for physisorption, 25µg/mL plasma fibronectin was incubated at 37°C for 3 hours. For covalent conjugation, PDMS chambers were first plasma activated for 1 minute to expose hydroxyl groups. Then 800µl 1% v/v 3-aminopropyltriethoxysilane (APTES, Sigma, Switzerland) in EtOH was added into 4cm² chambers and left at room temperature in the hood for reaction. After that, the salinized chambers were immersed in 10% v/v glutaraldehyde (Fluka, Switzerland) in PBS for 20 min, followed by a thorough rinse for the total removal of any glutaraldehyde residue.

Physisorbing vs. covalent attaching fibronectin

![Figure 3.5: Fibroblasts grown on Physisorbed vs. covalent attached fibronectin substrates. Human foreskin fibroblasts (HFFs) were adhered overnight to the two substrates in HFF growth media and cyclic stretched at 10% 1Hz for 8 hours. After stretch, HFFs on covalently attached Fn substrate showed long protrusions (indicated by arrows) whereas HFFs on physisorbed Fn substrate rarely had long protrusions.](image)
Human foreskin fibroblasts (HFFs) were adhered to the two substrates in HFF growth media overnight and cyclically stretched at 10% 1Hz for 8 hours. After the stretch, HFFs on covalently attached Fn substrate showed multiple long protrusions (Fig 3.5, indicated by arrows) whereas HFFs on physisorbed Fn substrate rarely had long protrusions, indicating healthier cultures on physisorbed substrates.

3.6. Cell viability test

Cell stretching experiments were performed under physiological conditions at 37°C. The STREX ST-140 uses a stepping motor to regulate the stretching frequency, and the motor dissipates heat which may raise the temperature inside the stretching unit over time. For shorter stretching times of less than 4 hours, the stepping motor can be water-cooled by the external cooling tank. For longer experiments, however, the maximum stretching time before cells show signs of decreasing viability needs to first be determined. Guided by Dr. Isabel Gerber, the cell viability was determined after stretching by using MTT and neutral red assays.

MTT and Neutral red assays

Viable cells convert yellow tetrazolium dye MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan. The insoluble formazan can be dissolved in methanol, ethanol, dimethyl sulfoxide (DMSO) or acidified isopropanol (Berridge, Herst, Tan, 2005), and the amount of formazan can be used to quantify cell viability. When using an MTT assay to measure cell viability, it is assumed that all tested cells have constant mitochondrial activity. Therefore, the change in number of viable cells can be linearly related to the overall mitochondrial activity, indicated by the concentration of formazan product in supernatant. The optical density (OD) of the formazan solution can be quantified by measuring the absorbance spectrum at 550nm.

Neutral red assay in addition to MTT measurement was used to complimentarily test the cell viability after stretching. Neutral Red is a eurhodin dye used for quantitatively estimating cell viability/toxicity. Most healthy cells can
incorporate neutral red and bind it in the lysosomes (Repetto, del Peso and Zurita, 2008). However, if cells are damaged, neutral red uptake will be significantly reduced.

After 24 hours of stretching at 10% 1Hz, cells showed decreased MTT activity and reduced NR uptake, indicating lower viability, whereas 4 and 12 hours of stretching did not have such effects (Fig. 3.6). In the given period, no significant cell detachment due to stretching was observed under the stereoscope. Compared to the static samples, no significant change in cell proliferation rate was observed, indicating that the cells are metabolically similar. Based on these observations, samples were stretched no more than 12 hours/day for this project. However, the ST-140 does have the potential for several-day duration stretching experiments, once allowing sufficient time for the motor to cool properly. Based on imperial observation of the cooling tank temperature, stretching of no more than 8 hours/day is suggested if experiments require more than 5 days to complete.

**Figure 3.6: Cell viability tests-MTT and Neutral red (NR) assays.** Human foreskin fibroblasts (HFF) with plating density of 2,500cells/cm² were adhered to Fibronectin (25µg/mL in PBS) coated PDMS chambers for 16 hours and then cyclically stretched for 4, 12, 24 hours at 10% 1Hz. Samples were then incubated with MTT (0.5mg/mL, 2mL/10cm²) and NR (0.5mg/mL, 2mL/10cm²) for 3 hours. For MTT spectral readings, the absorbance of the supernatant was read at 550 nm against a DMSO blank. For NR samples, cells were first fixed by 5% v/v glutaraldehyde in PBS at room temperature for 2min and washed twice with PBS. Absorbance of the cellular extract (300µL/10cm²) from the NR samples was read at 540 nm against the extraction buffer (1% acetic acid in 50% ethanol). (A.) After 24-hour stretching, HFFs showed lower MTT activity. (p<0.05, ANOVA) (B.) After 24-hour stretching, HFFs
significantly reduced neutral red uptake compared to the unstretched samples. \( p<0.05 \), ANOVA (C.)

Compared to the unstretched samples, phase contrast images of cells containing neutral red lysosomes after 4-hour stretching showed no significant visual difference.

### 3.7. Summary

This chapter introduced three stretchers that we encountered during this project, namely the STREX ST-140 from b-bridge International (used in this thesis); the mechanical stretcher from the Dietl Lab, the CS-10; and the µFluidic stretcher from the Pruitt Lab. It compared the physisorbed and covalently attached Fn substrates for cell stretching experiments and found that fibroblasts adhered better on physisorbed PDMS substrate. Last but not least, the maximum stretching time (8 hours) was determined by testing cell viabilities for our stretching system by using MTT and neutral red assays.

### References


Chapter 4: Fibronectin purification, labeling and storage

Fibronectin (Fn) exists in human blood at concentrations as high as 300-400µg/mL (Perttilä, Salo, Peltola, 1990, Mosher, 2006), and can be purified from blood plasma by using gelatin affinity chromatography (Vuento, Vaheri, 1979, Retta, Ferraris, Tarone, 1999, Speziale et al., 2008, Akiyama, 2001). Here, the Fn purification protocol was optimized to minimize co-purified gelatinases. Furthermore, Fn storage conditions were compared for dialyzed Fn and labeled Fn to minimize fragmentation and aggregation.

4.1. Fibronectin purification

Fn was isolated by gelatin affinity chromatography based on protocols from two publications (Pal et al., 2010, Speziale et al., 2008).

![Diagram of fibronectin purification](image)

Figure 4.1: Fibronectin (Fn) purification by affinity chromatography. Fn purification consists of 5 main steps: (1.) Column packing and activation; (2.) Elimination of unspecific adsorptions. (3.) Washing unspecific binding. (4.) Gelatinase elimination. (5.) Fn elution.
Fn purification consists of five main steps (Fig. 4.1):

1. Pack and activate columns: Two columns, namely 10mL sepharose 4B and 15mL gelatin sepharose 4B column were packed into sterilized glass columns. Beads were washed with a high concentration of urea (6M) to elute any protein residues. Then columns were activated by 2mM EDTA in PBS.

2. Eliminate unspecific adsorptions: Clear plasma was warmed up to room temperature before passing through a column containing Sepharose 4B beads (4B200-500ML, Sigma-Aldrich, Switzerland) and subsequently the column containing 2mM EDTA activated gelatin Sepharose 4B beads (45000170, Fisher Scientific, Switzerland) at speed of 1 drop/second. A higher speed would shorten the purification process but generate a lower yield.

![Graphs showing protein content at different wash steps](image)

Figure 4.2: Quality control of Fn purification. (A.) Protein content was monitored by measuring absorbance of 280nm for each washing steps. The first wash with PBS+EDTA+PMSF gradually eliminated unbound proteins. Protein content was hardly detectable by absorbance measurement during the 1N NaCl wash. (C.) 3% DMSO wash significantly reduced the bound protein (gelatinases) after 150ml of washing.
3. Wash unspecific binding: After binding, the gelatin column was washed with 500ml 1N NaCl (S9888, Sigma-Aldrich, Switzerland), 500ml Phosphate buffer saline (PBS) (P4417, Sigma-Aldrich, Switzerland) pH7.4 containing 2mM PMSF and 2mM EDTA, and 500ml 1M urea.

4. Eliminate gelatinases: Gelatinases were excluded by washing the column with 150ml 3% DMSO (Pal et al., 2010) at 2 drops/second.

5. Elute Fn: Fn was eluted with 40mL 3M urea, aliquoted and stored at -80°C.

4.2. Eliminating gelatinases

There are several methods to eliminate gelatinases co-purified with Fn. Earlier methods employ separating two proteins under non-denaturing conditions by chelating sepharose charge with Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$ or passing the elute through gel filtration chromatography (Smilenov et al., 1992, Vuento and Vaheri, 1979). Combining gelatin and heparin-cellulose affinity chromatographies together was shown to increase the binding specificity of Fn, therefore effectively eliminating gelatinase contamination (Poulouin et al., 1999). Recently, 3% of DMSO was found to efficiently wash away gelatinases while keeping the Fn intact (Pal et al., 2010). Therefore, an additional 3% DMSO washing step was added before the final elution of Fn to eliminate gelatinases co-purified with Fn (Fig. 4.2A).

Gelatin zymography showed that the 3% DMSO wash significantly reduced the co-purified MMP-9 (Fig. 4.3).

![Figure 4.3: Gelatin zymography of Fn purified with and without the 3% DMSO washing step. Lane#1 is the growth media control, which shows a small amount of MMP-2. Lane#2 is Fn purified with a 3% DMSO wash.](image-url)
DMSO wash in growth media, which also shows small amount of MMP-2. Lane#3 is the positive control of MMP-2. Lane#4 is Fn purified without 3% DMSO, which shows visible amount of MMP-9, in both active and latent forms.

4.3. Storage of dialyzed Fn

Figure 4.4: -80°C is better storage temperature to keep the dialyzed Fn in PBS. When the Fn in PBS was stored at -20°C, fragmentation to a smaller molecular weight ~150kDa was observed, while Fn stored at -80°C remained intact after 6-months.

Fn purified from a gelatin binding column was stored in 3M urea. Before its usage in cell culture, the Fn was dialyzed in PBS pH7.4 by using the 10KDa molecular weight cutoff dialysis cassette (66380, Thermo Scientific, U.S.A.) to eliminate the urea and Fn fragments. Once the Fn is dialyzed and stored in PBS, depending on the storage temperature, Fn degrades at different rate. To investigate this, two storage conditions of dialyzed Fn were tested, namely -20°C and -80°C after 6-months of freezing.

Unfragmented Fn monomer has molecular weight between 220k and 250kDa. It was observed that the Fn was more fragmented in the samples stored at -20°C, while after 6-months at -80°C there was scarcely any fragmentation observed (Fig. 4.4). Therefore, the dialyzed Fn in PBS was always kept at -80°C for long-term storage (up to 6 months).
4.4. Fibronectin labeled with single fluorophore

Fn was labeled with green fluorophore Alexa fluor 488 (A-20100, Invitrogen, Switzerland) and far-red fluorophore Alexa fluor 633 (A20105, Invitrogen, Switzerland) by using n-hydroxysuccinimide ester (NHS) chemistry.

Figure 4.5: Fn labeled with fluorescent dyes Alexa Fluor. (A.) Fn was covalently modified with Alexa Fluor fluorophores, namely Alexa Fluor 488 (Fn-488) and Alexa Fluor 633 (Fn-633). (B.) The covalent modification of Fn with Alexa Fluor fluorophore did not change tryptophan absorbance compared to unlabeled Fn.

To confirm that the Fn was successfully labeled with Fluorphores, the SDS-PAGE was run after labeling and the gels were imaged with fluorescent lamps. Figure 4.5A shows that the Fn was successfully labeled with fluorophores, which were visible under fluorescent lamps.

Furthermore, it was shown that exposure of buried tryptophan by conformational changes shift the emission spectrum 7nm to blue (Zhu, Laine, Barkley, 1990). Therefore, the tryptophan emission of Fn and Fn-488 were compared using a spectrometer (Perkin Elmer, U.S.A.) between 295nm-400nm, and found that the labeling did not shift the absorbance peak (Fig. 4.5B). This result indicates that the labeling scheme did not significantly change the conformation of Fn.

4.5. Labeling FnDA for FRET studies

FnDA was double labeled with Alexa Fluoro 488 and Alexa Fluoro 546 (Invitrogen, Switzerland) as donor and acceptors. Donor fluorophores are labeled to
cysteines on type III<sub>7</sub> and III<sub>15</sub> modules, while acceptors are randomly labeled along the entire Fn on amines. Briefly, the Fn was denatured in 4M GdnHCl to allow the exposure of free cysteins to label the donor. If the concentration exceeded 2g/l, pFn the solution was diluted in the labeling buffer to 2g/l. Alexa fluoro 488 was covalently attached to the cysteines via maleimide-based conjugation (for site specific labeling, refer to the article “Efficient Site-Specific Labeling of Proteins via Cysteines” by Kim et al., 2011. Free dyes in the solution were eliminated by size exclusion columns and the Fn was renatured in 0.1M sodium bicarbonate PBS pH8.5 at room temperature for 2 hours. Acceptors (Alexa fluoro 546) were labeled via N-Hydroxysuccinimidyl ester chemistry on primary amines with average 7-8 fluorophores/Fn. Excessive dyes were excluded by size exclusion columns. Labeled FnDA was further dialyzed in PBS (pH7.4) at room temperature for 1 hour before storing at -80°C.

**4.6. Storage of labeled Fn**

![Image of a Western blot showing Fn-488 and Fn-633 aggregation](image)

*Figure 4.6: At -80°C, longer storage increases Fn-488 and Fn-633 aggregation, and Fn-633 aggregated faster than Fn-488.*

It is important to determine the storage conditions for Fn after labeling to avoid changes in the stability of the protein structure and protein aggregation. Western blotting showed that at -80°C longer storage increases Fn-488 and Fn-633 aggregation, and Fn-633 aggregated faster than Fn-488 (Fig. 4.6). Therefore, in addition to using Fn within 2 months of labeling, it is essential to centrifuge labeled Fn and only use the supernatant without aggregates for cell culture experiments.
4.7. Summary

In this chapter, an Fn purification protocol was adapted for this thesis project. It has been shown that the Fn can be purified with gelatin affinity chromatography and co-purified gelatinases eliminated by 3% DMSO wash. Regarding storage conditions, -80°C is better temperature to store dialyzed Fn. Regarding Fn single labeling, it is recommended not to exceed ~4 fluorophores/Fn. Furthermore, it has been explained how fibronectin was labeled with two fluorophores for FRET studies in this Chapter. Labeled Fn aggregates over time, therefore, it is necessary to centrifuge aliquots of labeled Fn before adding to cell cultures.

References


Chapter 5

Exposure to Type I collagen and/or Fn, not cyclic strain, affects remodeling capacity of fibroblasts by regulating MMP activity

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Author Contributions

Y.Z. and Z.L. designed and performed experiments, analyzed data. Y.Z., J.F., I.S., M.Z., and V.V. prepared the manuscript. I.S. provided tools for fiber orientation analysis. A.S. measured soluble MMP concentrations for HFFs in Fn matrix. M. W. and V.V. supervised the project.

Keywords: Fibronectin, cyclic stretching, extracellular matrix, matrix remodeling, matrix metalloproteinase
5.1. Abstract

Mechanical forces acting on cells and tissues are essential for the assembly and remodeling of connective tissue extracellular matrix during development and wound healing. However, with excessive tissue tension, neo-tissue formation and remodeling can be associated with fibrosis and excessive wound contracture. It is speculated that the different tissue maturation stages, characterized by the composition of the extracellular matrix, i.e. collagen versus fibronectin, relate to fibroblast behavior and specifically their remodeling capacity. This paper therefore addresses the question of how fibronectin and collagen regulate the early onset of matrix remodeling by human foreskin fibroblasts under dynamic mechanical loading, specifically during the transition from a fibronectin-rich to a collagen-decorated matrix.

Human foreskin fibroblasts, seeded on elastic membranes and subjected to uniaxial cyclic stain (to induce matrix remodeling), were exposed to high concentrations of fluorescently labeled collagen and/or human plasma Fn to monitor matrix assembly and remodeling. The impact of cyclic strain and addition of extracellular matrix components were evaluated against cellular capacity to assemble, degrade and remodel the matrix by utilizing confocal microscopy, matrix metalloproteinase activity assays and reverse transcription polymerase chain reaction.

Human foreskin fibroblasts were observed to respond to cyclic strain by re-aligning to the minimum strain direction, reinforcing their actin cytoskeleton and assembling new fibronectin fibrils along their long axis. Strikingly, secretion of matrix metalloproteinases by human foreskin fibroblasts was significantly increased upon the addition of exogenous Fn, but was indifferent to exposure of uniaxial cyclic strain, indicating that matrix remodeling capacity predominantly depends on the exposure to fibronectin, not cyclic strain. Cells exposed to a fibronectin matrix that was decorated with type I collagen, reduced the levels of secreted matrix metalloproteinases and matrix metalloproteinase-15 gene expression. The exposure of fibroblasts to type I collagen-decorated fibronectin matrix was also associated with a less unfolded conformation of fibronectin in the extracellular matrix, as assessed using Förster Resonance Energy Transfer, indicating partial strain release from the fibronectin matrix.
By mimicking tissue maturation during dynamic loading, this study shows how the transition from a fibronectin-rich matrix to a type I collagen matrix, not cyclic strain, determines the remodeling capacity of Human foreskin fibroblasts subjected to dynamic mechanical loading. It suggests that the remodeling capacity of dermal fibroblasts depends on the stage of tissue maturation/healing, i.e. the high remodeling capacity of cells in contact with fibronectin alone (early event) is reduced upon additional exposure to collagen (later event), associated with proceeding towards tissue homeostasis.
5.2. Introduction

Mechanical forces acting on cells and tissues affect many physiological processes from tissue development, such as the assembly and remodeling of the extracellular matrix (ECM) in connective tissue (Tomasek et al., 2002, Chiquet et al., 2009, Geiger, Spatz and Bershadsky, 2009, Cox and Erler, 2011, Kasper Dideriksen et al., 2013) to repair processes such as wound healing (Kippenberger et al., 2000, Gabbiani, 2003, Wong et al., 2011) and scar formation (Aarabi et al., 2007, Wong, Longaker and Gurtner, 2012). Fibroblasts are the most abundant cell type in connective tissues and are the primary cells that lay down the first provisional ECM that is rich in fibronectin (Fn) (Clark, 1990, Shaw and Martin, 2009). During tissue maturation, the provisional Fn-rich matrix is remodeled and later interlaced by type I collagen (Col1) fibrils (Reinke and Sorg, 2012), of which excessive bundling often leads to problematic fibrosis (Shaw and Martin, 2009, Gabbiani, 2003, Bryers, Giachelli and Ratner, 2012) and wound contracture (Montesano, 1988, da Rocha-Azevedo et al., 2013). Guiding healthy tissue maturation and remodeling in vitro, however, remains a challenge, since the specific mechanisms contributing to the different stages of the remodeling processes are difficult to disentangle, such as separating the effect of mechanical force from exposure to matrix proteins and associated matrix composition. It was speculated that the composition of the extracellular matrix, i.e. collagen vs fibronectin as a representative for different tissue maturation stages, relates to fibroblast behavior and specifically the remodeling capacity. Therefore, the effect of different ECM components (Fn and Col1), matrix conformational state and degradation capacity on the ECM remodeling by HFFs subjected to uniaxial cyclic strain was investigated.

Fibronectin is the major component of early ECM in matrix remodeling by fibroblasts (Clark, 1990, Mao and Schwarzbauer, 2005, Shaw and Martin, 2009, Singh and Schwarzbauer, 2012, Carraher and Schwarzbauer, 2013). Fn consists of two almost identical Fn monomers, each of approximately 220-250kDa in molecular weight (Pankov and Yamada, 2002). It contains cell binding sites such as the RGD sequence that is bound by surface receptor integrins (Pankov and Yamada, 2002, Mao and Schwarzbauer, 2005, Vogel, 2006, Hynes, 2009) as well as various binding sites for ECM proteins such as Fn, type I and III collagen (Pankov and Yamada, 2002, Vogel, 2006). Fn therefore affects cell signaling through binding specific integrins, namely α5β1, αvβ3, which are different from those binding to collagen associated
with α1β1, α2β1 (Humphries et al., 2006). Thereby, the attachment of cells to fibronectin vs collagen, via different integrins, provides a means to alter cellular behavior. It has therefore been hypothesized that the different tissue maturation stages, characterized by the composition of the extracellular matrix, i.e. collagen vs fibronectin, relate to fibroblast behavior and specifically their remodeling capacity.

The interpretation of the effect of the mere presence of Fn is, however, complicated by the fact that Fn interactions are co-regulated by its conformational state. In contrast to the conformation in solution, Fn becomes highly adhesive for integrins once incorporated into the ECM (Magnusson and Mosher, 1998). Mechanical forces can further trigger changes of fibrillar Fn conformation (Baneyx et al., 2002, Smith et al., 2007, Little et al., 2008, Kubow et al., 2009) and thereby either destroy or unravel existing cryptic binding sites (Zhong et al, 1998, Vogel, 2006, Lemmon et al., 2011, Little et al., 2009, Chabria et al., 2010). For example, the stretching of Fn fibrils increases the non-specific binding of albumin and casein (Little et al., 2009) and disrupts bacteria binding sites (Chabria et al., 2010). Based on these findings, it is suggested that fibrillar Fn transduces extracellular signals to cells that determine cell fate by regulating, among others, the release of growth factors, cytokines and chemokines (Vogel, 2006, Hynes, 2009). The question remaining is whether the exposure to collagen can alter the conformational state of the Fn matrix and potentially affect mechanotransduction that alters cellular behavior during stage transitions in tissue maturation.

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) play crucial roles during matrix remodeling by affecting Fn and collagen integrity and tissue tension. By regulating the degradation of the ECM, they have been considered pharmacological targets to promote healthy tissue remodeling (Lee and Murphy, 2004, Vihinen et al., 2005, Gill and Parks, 2008, Dufour and Overall, 2013). Several human MMPs interact with Fn, namely MMP-2&3, MMP-7-11, MMP-12-16 and MMP-25&26 (Klein and Bischoff, 2011) and their activities can be inhibited by four different TIMPs (Visse and Nagase, 2003). MMP-2 and -9 (gelatinase-A and −B) are secreted as inactive soluble zymogens, degrade several ECM substrates such as gelatin, collagen and Fn (Toriseva and Kahari, 2009), and have been shown to be important for connective tissue remodeling (Hayashidani et al., 2003, Romanic et al., 2002, Zamilpa et al., 2010). In addition, MMP-14 and -15 are membrane-anchored endopeptidases activated once inserted into cell membranes.
(Sohail et al., 2008, Itoh and Seiki, 2006, Murphy and Nagase, 2011) that can digest ECM molecules that are in contact with the cell membrane during cell migration (Lee and Murphy, 2004, Chen and Parks, 2009, Egeblad and Werb, 2002). But to which extent matrix composition and/or matrix conformation affects differential expression and secretion of MMPs remains elusive.

To address these questions, we explored how exposure to Col1 and/or human plasma Fn (pFn) to human foreskin fibroblasts (HFFs) affected the assembly and remodeling of the ECM. Exposing fibroblasts to different combinations of Fn and Col1 hereby mimicked the maturation of tissue from a provisional Fn-rich matrix to a Col1-rich matrix. The application of a uniaxial cyclic strain was used as means to induce ECM remodeling, since it induces fibroblast reorientation (Jungbauer et al., 2008, De et al., 2007, Faust et al., 2011), cytoskeletal reorganization (Goldyn et al., 2009, 2010, Deibler et al., 2011) and focal adhesion realignment (Carisey et al., 2013). The cellular capacity to assemble, degrade and remodel a matrix was assessed by confocal microscopy, MMP activity assays and gene expression analysis.

5.3. RESULTS
5.3.1 HFFs assemble Fn matrix fibrils parallel to their long axis when subjected to uniaxial cyclic strain.

First, Fn matrix assembly was monitored to assess the matrix remodeling behavior of the HFFs, subjected to uniaxial cyclic strain. On Fn coated substrates, unstretched cells were randomly aligned, whereas after 8 hours of uniaxial cyclic stretching at 10%, 1Hz, the orientation of the HFFs converged to 60°, i.e. the minimum strain direction (Fig. 5.3.1A), as previously observed by others (De, Zemel and Safran 2007, Jungbauer et al., 2008, Faust et al., 2011). In this case where Fn-coated PDMS substrate was uniaxially cyclically strained for 10% (Poisson’s ratio=0.5), the minimum strain direction was ~60° away from the stretching direction as a result of both stretching in the strain direction and compression perpendicular to the strain direction.

In addition to a change in cellular orientation, cyclically stretched cells show a more spindle-like morphology (Aspect Ratio_{Stretch}=1.6) compared to unstretched cells (Aspect Ratio_{Rest}=3.0) (Fig. 5.3.1B). This strain-induced change in cellular morphology confirmed previous reports (Faust et al., 2011, Deibler et al. 2011).
HFFs assemble Fn matrix fibrils parallel to their long axis when subjected to uniaxial cyclic strain. HFFs were seeded onto fibronectin (Fn) coated PDMS chambers for 16 hours to ensure firm adhesion to the substrate. The HFFs were then either exposed to cyclic stretching (stretch) or cultured at static (rest). (A.) Under resting conditions, the HFFs had random orientation. Upon uniaxial cyclic stretching of 10% 1Hz for 8 hours, the HFFs aligned to ~60°. The angle of cell orientation was obtained by fitting an ellipse to the cell. (B.) After 8 hours of uniaxial cyclic stretching at 10% 1Hz, the HFFs were more spindle-shaped (Aspect Ratio_{Stretch}=1.6) compared to unstretched cells (Aspect Ratio_{Rest}=3.0). (C.) Picture illustrates the experimental setup for monitoring Fn matrix assembly during cyclic stretching. Namely, Fn pre-labeled with fluorescent dyes (green) was added into the growth media at 50µg/mL. To observe the HFFs, the cell membrane was labeled with red membrane dye DiI (Invitrogen, Switzerland) prior to seeding. Under the resting condition, the HFFs assembled Fn matrix with random orientation. Under uniaxial cyclic stretching, majority of the HFFs aligned to 60° and assembled Fn matrix preferentially along ~60°.

To monitor the directionality of assembled Fn fibers by fibroblasts under uniaxial cyclic stretching, trace amounts of fluorescently labeled Fn (50µg/mL) were added to the cultures at the beginning of each experiment. Unstretched HFFs assembled the Fn matrix randomly, whereas cyclically stretched HFFs assembled the Fn fibrils mainly parallel to their long axis. The Fn fibril angle distribution peaked slightly below 60° away from the strain axis (Fig. 5.2.1C). These results show that HFFs react to cyclic mechanical stretching by altering not only cell alignment, but
also the orientation of the assembled Fn fibrils.

### 5.3.2 Cyclic stretching increased stress fiber formation and assembly of Fn matrix by HFFs.

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10% 1Hz

Figure 5.3.2: Cyclic stretching increased stress fiber formation and assembly of Fn matrix by HFFs. (A.) HFFs were seeded onto previously coated PDMS substrates (25µg/mL Fn at 37°C for 3 hours) for 16 hours. Then cells were stretched for 4 hours with fluorescently labeled Fn (green) at 10% 1Hz before fixation and staining for actin stress fibers. Phalloidin staining of F-actin reveals that uniaxial cyclic stretching promoted actin stress fiber formation in the HFFs. (B.) With or without stretching, the orientation of actin stress fibers in the HFFs and Fn fibrils deposited by the HFFs were closely associated with each other (R²=0.8 for rest, R²=0.8 for stretch). (C.) HFFs labeled in red were cyclically stretched for 4 and 8 hours. Compared to HFFs cultured without stretching, cyclically stretched HFFs deposited more Fn matrix within 8 hours. (p<0.05)

Since Fn fibrillogenesis is induced by translocation of α5β1 integrins along actin filaments (Pankov et al., 2000, Katz et al., 2000), the relation between Fn alignment and F-actin organization under cyclic strain was quantified next. After firm
attachment onto an Fn-coated PDMS substrate in Fn depleted growth media, HFFs were subjected to 4 hours of cyclic stretching at 10% 1Hz. Increased formation of actin stress fiber assembly was observed as visualized by antibody staining of F-actin (Fig. 5.3.2A). A strong linear correlation between the alignment of actin stress fibers and Fn fibers was detected in both unstretched (rest) samples ($R^2=0.8$) and stretched samples ($R^2=0.8$) (Fig. 5.3.2B). The strong correlation between F-actin and Fn suggests that the overall arrangement of Fn fibrils was dominated by the re-aligned actin network, not only for HFFs cultured at static conditions but also at a uniaxial cyclic strain of 10% at 1Hz.

In addition to the strain-induced assembly of aligned new matrix fibers, cyclic stretching significant increased the deposition of fibronectin by the HFFs (8 hours of uniaxial cyclic stretching, Fig. 5.3.2C). Therefore, cyclic stretching promoted both, the formation of actin stress fibers and an increased assembly of Fn matrix compared to the unstretched controls.

5.3.3 Cells predominantly assemble a new FN matrix aligned to their long axis, and minimally remodel the existing Fn matrix

Because of the strong correlation between the directionality of F-actin stress fibers and Fn fibrils, the mechanism for the HFFs change in the distribution of Fn fiber orientation was explored. To distinguish between cyclic-strain-induced remodeling of the old Fn matrix grown under static conditions and the assembly of a new Fn matrix, a pulse-chase experiment was designed in which HFFs were cultured in the presence of a green fluorescently labeled Fn under static conditions for 24 hours. Subsequently, cells were cyclically stretched for 8 hours in the presence of red fluorescently labeled Fn (Fig. 5.3.3A) by 10% at 1Hz. Images were taken before (Fig. 5.3.3B) and after stretching (Fig. 5.3.3C & D). A quantitative analysis of the angular fiber distributions was performed to detect the orientation of old and new Fn fibers for all three cases. Data revealed that new fibers (red) compared to old (green) predominantly aligned at angles around 60° (Fig. 5.3.3E-F, green versus red). As cells reorient in response to the onset of cyclic strain, they hardly remodel the existing matrix and thereby change its fiber orientation. Instead, they seem to leave the old matrix behind and assemble a new ECM with an altered Fn fiber orientation.
Cells predominantly assemble new FN matrix aligned to their long axis, and minimally remodel the existing FN matrix. (A.) HFFs were plated onto an Fn-coated PDMS substrate and then were allowed to assemble matrix with 50µg/mL fluorescently labeled Fn (green) for 24 hours. Afterwards, adherent HFFs were exposed to uniaxial cyclic strain at 10% 1Hz for 8 hours, with fresh growth media containing fluorescently labeled Fn (red). (B-F.) Fn matrix was assembled by HFFs with random alignment without stretching. After remodeling by stretched HFFs for 8 hours, the Fn fiber alignment showed slight preference towards high angles (60°-90°). The HFFs preferred assembled Fn matrix along ~60° under uniaxial cyclic strain. The merged image of an Fn matrix assembled before and during stretching showed the HFFs anchored to the old matrix while depositing a new matrix preferentially along ~60° under uniaxial cyclic stretching. (G.) Slight increase in soluble MMP secretion was observed for stretched HFFs without exogenous Fn, while this increase was not observed once 50µg/mL Fn was added to the culture media. (H.) 8 hours of uniaxial cyclic strain at 10% 1Hz did not affect the secretion of MMP-2 & -9 by HFFs. On the contrary, adding exogenous Fn significantly increased the secretion of MMP-9.
To gain insight into why cells leave the existing (old) matrix relatively untouched in response to the onset of cyclic strain, we measured the concentrations of secreted MMPs in the growth media under stretching vs resting conditions. Cyclic stretch did not result in major upregulation of soluble MMP activity as measured by a generic MMP-assay in the presence or absence of pFn (Fig. 5.3.3G, Fn+). However, in the absence of pFN a major reduction of MMP secretion was observed (Fig. 5.3.3G, Fn-).

To get more insights into the type of MMPs responsible for these differences, the activity of secreted MMP-2 and MMP-9 (gelatinases) in the growth medium was analyzed by gelatin zymography after HFFs exposed to 8 hours of uniaxial cyclic strain at 10%, 1Hz. Stretching was not observed to affect gelatinase activity (Fig. 5.3.3H), while the exposure to pFn increased MMP activity (Fig. 5.3.3G). Therefore, MMP secretion is upregulated not by cyclic strain, but depends on the exposure to pFn via the medium.

5.3.4 Exposure to Collagen (Col1) and Fn down-regulated MMP secretion by cyclically stretched HFFs.

Cyclic strain acting on fibroblasts is known to induce matrix remodeling and alter matrix composition (Foolen et al., 2012, Kanazawa et al., 2009, Gupta et al., 2006, Nguyen et al., 2009, Cha and Purslow, 2010, Shelton and Rada, 2007). However, our results so far indicate that although cyclic strain increases the assembly of Fn, it does not affect the secretion of MMPs when exposed to an Fn-rich ECM. As a further step, the way exposure to collagen, in addition to Fn, affects MMP secretion by HFFs was investigated. Cells were first allowed to assemble a Fn-matrix for 24 hours. Subsequently cells were cultured for additional 8 hours in medium that contained both pFn and soluble monomeric Col1 from a rat-tail.

Most interestingly, the MMP secretion was down-regulated when cells were exposed to Col1. HFFs secreted almost 3-fold more soluble MMPs when exposed to Fn (50µg/mL) (Fig. 5.3.4A, p<0.05), while Col1 (5µg/mL) in addition to Fn (50µg/mL) reduced MMP-levels (2-fold increase compared to control, p<0.05, Fig. 5.3.4A).

To identify the candidates of soluble MMPs that account for this observation,
the amount of MMP-2 and MMP-9 (gelatinases) on the protein level were quantified by using gelatin zymography. The secretion of both the inactive form and the active form of MMP-9 were significantly increased by introducing 50µg/mL Fn and 5µg/mL Col1 to the culturing media during 8 hours of stretching at 10% 1Hz, whilst the level of MMP-2 was unaffected (Fig. 5.3.4B). After 8 hours of cyclic stretching, MMP-9 secretion by stretched HFFs in Fn-rich matrix was superior to that resulting from a Col1-decorated matrix (Fig. 5.3.4B). Taking the soluble MMP and zymography measurements together, HFFs have a higher capacity to degrade Fn-rich matrix compared to Col1-decorated Fn-matrix.

Figure 5.3.4: Exposure to Collagen (Col1) and Fn down-regulated MMP secretion by cyclically stretched HFFs. (A.) After 8 hours of uniaxial cyclic stretching at 10% 1Hz, HFFs secreted almost 3-fold more soluble MMPs when HFFs were given 50µg/mL Fn to assemble matrix (p<0.05). Decorating the Fn matrix with Col1 made HFFs secreted 2-fold more soluble MMPs when the HFFs were given 50µg/mL Fn and 5µg/mL Col1 to assemble matrix. (p<0.05). (B.) The increase in soluble MMP secretion due to Fn and Col1 addition was mainly contributed by MMP-9 not MMP-2. Again, MMP-9 level secreted by the HFFs as measured by gelatin zymography was lower for the Col1 decorated matrix than the Fn matrix (p<0.05). (C.) Under cyclic stretching, gene expression of type III collagen was decrease by ~20% upon Fn matrix assembly, while Fn expression was increased by ~30%. The expression of MMP-15 was up-regulated by ~40% (p<0.05), whereas gene expression of HFFs in the
Col1 decorated matrix did not show any significant change compared to cells growing in normal growth media.

Generic MMP assay and gelatin zymography only measure the MMPs in supernatant, hence cannot reveal the activity of membrane-anchored MT-MMPs. Therefore, gene expression levels of MT-MMPs, as well as TIMPs, Fn, collagens, were assessed using Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR). In general, changes in expression levels upon uniaxial cyclic stretching of 10% 1Hz for 8 hours were very small. Nevertheless, addition of Fn appeared to upregulate Fn and MMP-15 (transmembrane type I MMP) expression and downregulate collagen expression (Fig. 5.3.4C) by the cells. Exposure of collagen in addition to Fn appeared to abolish these effects (Fig. 5.3.4C).

5.3.5 Deposition of type I collagen co-localizes with Fn fibrils

Figure 5.3.5: Deposition of Type I collagen co-localizes with Fn fibrils. HFFs were given 50µg/mL pre-labeled Fn (green) for 30 minutes before adding 1, 5, 10µg/mL pre-labeled Type I collagen (Col1) (red). Cells were allowed to assemble matrices for 8 hours before fixation. (A-C.) The HFFs
incorporated exogenous Fn (50µg/mL) and Col1 (1µg/mL) to assemble the ECM. Cells assembled soluble Fn into a fibrillar Fn matrix. Col1 assembly at 1µg/mL was visible but the amount was low. The Col1 fibrils co-localized with the Fn fibrils. (D-F.) The HFFs assembled significantly more Col1 fibrils when the Col1 concentration in media was increased to 5µg/mL. Deposition of Col1 fibrils was co-localized to Fn fibrils. (G-I.) Increasing exogenous Col1 concentration to 10µg/mL promoted rapid deposition of Col1 matrix. Again, the Col1 fibrils co-localized to the Fn fibrils.

The transition from Fn-rich matrix to Col1-decorated Fn matrix significantly decreased the matrix remodeling capacity of the cells (Fig. 5.4), while the mechanical environment hardly induced changes. Hence, a protective effect of collagen on ECM remodeling may exist. Therefore, a colocalization study of Fn and collagen was performed to observe whether Col1 could indeed protect Fn remodeling. Pre-labeled green Fn and red Col1 were added to static HFF cultures for 24 hours, after which their localizations were visualized by confocal microscopy. Increasing Col1 concentration (1, 5 and 10µg/mL) increased the amount of Col1 incorporated into the ECM (Fig. 5.5B, E, H). In all three cases, collagen fibrils co-localized with Fn fibrils (Fig. 5.5), which indeed suggests a protective mechanism of collagen to Fn.

5.3.6 Addition of Col1 decreases HFF-induced Fn fiber unfolding.

Fn matrix conformation indicates the functional state of the ECM (Bradshaw and Smith, 2013) due to, among others, the degree of Fn stretch determines its adhesiveness to other proteins (Baneyx et al., 2002). Therefore, it was posited that the decrease in HFF capacity to remodel the ECM exposed to Col1 in combination with the possible protective effect of collagen on Fn could be explained by the conformational state of Fn in the matrix. Fluorescence resonance energy transfer (FRET)-based mechanical strain sensors were utilized (Baneyx et al., 2001, Smith et al., 2007, Little et al., 2008, Kubow et al., 2009) to probe the change in matrix conformation upon the incorporation of Col1. FRET-labeled Fn was added to static HFF cultures for 24 hours in the absence or presence of Col1 (1, 5 or 10µg/mL). Increasing concentrations of collagen increased the intensity ratio of acceptor and donor, indicating that Fn-stretch decreased with increasing Col1 decoration of Fn fibers (Fig. 5.3.6A). Overlaid histograms and box plots showed that Col1-added matrices had a higher acceptor/donor intensity ratio with values above 0.70 compared to the control sample of 0.66 (Fig. 5.3.6B). Therefore, our results indicate that the decrease in Fn unfolding in response to decorating the Fn fibers correlate with Col1 the matrix remodeling capacity of HFFs to decrease their MMP secretion activity.
Addition of COL1 decreases HFF-induced Fn fiber unfolding. HFFs were seeded in DMEM supplemented with Fn depleted 1% FBS for 30min before adding FRET-labeled Fn (Fn-DA). To prevent intermolecular FRET, 5µg/mL Fn-DA was mixed with 45µg/mL unlabeled Fn. For control samples, HFFs were supplemented with 5µg/mL Fn-DA and 45µg/mL unlabeled Fn. For COL1 samples, an additional 1, 5 and 10µg/mL COL1 were added to DMEM Fn depleted 1% FBS with 5µg/mL Fn-DA and 45µg/mL unlabeled Fn. Live images of matrix conformation were acquired after 24hours of matrix assembly (A.) Representative image of the HFF incorporating Fn-DA into Fn-rich and COL1-decorated matrix. Heat map images of the intensity ratio of acceptor and donor indicate the degree of unfolding, the more unfolded the redder the image. It showed that matrix was less unfolded once COL1 was added to the growth media compared to the control sample. (B.) Overlaid histograms and box plots show that COL1 added matrices had higher acceptor/donor intensity ratio with values higher than 0.7 compared to the control sample of 0.66. The grey box indicates the 95% confidence interval while the mean is shown by the black bar. p < 0.05
5.4. DISCUSSION

This paper addressed the question of how matrix composition affects matrix remodeling by HFFs subjected to uniaxial cyclic strain. HFFs responded to cyclic strain by re-aligning to the minimum strain direction, reinforcing their actin cytoskeleton, and assembling new Fn fibrils along their long axis (Fig. 5.3.1&2). Strikingly, MMP levels were highly dependent on cellular exposure to Fn but not on applied cyclic strain (Fig. 5.3.3). Exogenously added Col1 decorated the existing Fn matrix (Fig. 5.3.5), reduced the levels of secreted MMPs compared to the Fn matrix only (Fig. 5.3.4) and resulted in a more unfolded conformation of Fn in the ECM (Fig. 5.3.6).

Whilst the formation and orientation of new Fn fibrils along the long axis of fibroblasts is in agreement with previous findings on Fn (Antia et al., 2008) or collagen assembly (Trelstad and Birk, 1985, Birk and Trelstad, 1984, Birk and Trelstad, 1986), our pulse-chase experiments showed that cells preferentially aligned new matrix and left the existing matrix unaltered. Corresponding to the need for new matrix material, cells up-regulated Fn assembly and gene expression, which is in agreement with earlier studies in which an elevated Fn expression was found in various types of fibroblasts (Fluck et al., 2003, Garvin et al., 2003, Howard et al., 1998, He et al., 2004). During this early stage, Col1 and Col3 expression was decreased when cells were exposed to exogenous Fn. This appears to be in contrast to the general behavior that collagen levels are increased when cells are subjected to cyclic strain (Kim et al., 2002; Parsons et al., 1999; Yang et al., 2004; Wang et al., 2003; Curwin et al., 1988), however, no Fn was added in these studies. Moreover, it was observed that Col1 expression was reduced to control levels when exogenous Col1 was added. These findings indicate that cells prioritize their gene expression for the build-up of a provisional Fn matrix during the early stage of tissue formation, but only transiently until the formation of a collagen matrix starts, representative of further matured tissue.

Compared to the observed tendency of upregulated MMP levels in response to cyclic strain, which is in agreement with previous findings (Prajapati 2000), exposure of cells to Fn had a far greater effect, i.e. on MMP-9 activity and MMP-15 expression levels. Col1 in addition to Fn subsequently decreased these MMP levels. This suggests that early Fn matrix can be rapidly remodeled, but once tissue matures to include Col1, matrix remodeling capacity decreases. This hypothesis is supported by
the specificity of MMP-15 for Fn, laminin and tenasin-C but none of the collagens (O’Ortho, 1997). However, unlike cells in 3D collagen lattices (Haas, 1998, Ruangpanit et al., 2001, Lee et al., 2006, Wilkinson et al., 2012), significant up-regulation of MMP-14 expression with the increase of Col1 content was not observed.

The incorporation of Col1 into an Fn matrix also led to a more unfolded conformation of Fn in the ECM, indicating a reduction of strain in the matrix as probed by FRET. It is speculated that Col1 reduces the matrix remodeling capacity of HFFs by changing the conformational state of Fn. Fn conformation can affect cell signaling through altered binding of integrins. For example, it has been shown that the binding affinities for different integrins depend on substrate-induced conformational changes of adsorbed Fn and changes of α5β1 binding affected cell behavior (Garcia, Vega and Boettiger, 1999). Additional support for Fn to be involved in mechanotransduction comes from the recent finding that FAK activation on Fn-coated hydrogels was tension-dependent whereas activation on Col1 was tension-independent (Seong et al., 2013). However, the question of whether direct Col1 binding by α1β1 and α2β1 integrins and related signaling cascades or a shifted affinity for Fn-binding integrins underlay the changes in MMP activity remains unclear.

In summary, our study shows that exposure of cells to different matrix components (fibronectin vs collagen), rather than the mechanical loading regime, triggers matrix remodeling of human foreskin fibroblasts by regulating MMP activity. This implies that during tissue maturation, not only the capacity to remodel the ECM is altered by accumulating ECM, but also the exposure to newly synthesized ECM components such as type I collagen directly impacts the cellular capacity for matrix remodeling. When viewed in the context of wound healing and tissue maturation, the early stage of healing where dermal fibroblasts are exposed to fibronectin associates with high remodeling capacity, which at later stages where cells are additionally exposed to collagen associates reduces and proceeds towards tissue homeostasis.

5.5. MATERIALS AND METHODS

5.5.1 Cell culture

Before stretching experiments, primary normal human dermal fibroblast (NHDF) more commonly known as human foreskin fibroblasts (HFF) (C-12300, Promocell, Germany) were maintained at 37°C with 5% CO₂ in the fibroblast growth
medium (C-23010, Promocell, Germany). This fibroblast growth medium contained no serum but recombinant human fibroblast growth factor at 1ng/ml and insulin at 5µg/ml for a more consistent cell proliferation rate and better morphological maintenance. When HFFs reached 70-90% confluency, they were trypsinized by 0.05% trypsin-EDTA (25300-054, Invitrogen, Switzerland) and sub-cultured at 17,500 – 35,000 cells/ml. The recovery of cryo-preserved HFFs was carefully monitored, and they were passaged at least once before any experiments. All experiments with HFF were carried out with cells below passage 10 to eliminate the appearance of myofibroblasts at higher passage numbers.

5.5.2 Isolation of plasma fibronectin

Human plasma fibronectin (pFn) was isolated by gelatin affinity chromatography. Briefly, frozen human plasma (UniversitaetsSpital Zurich, Switzerland) was thawed in the water bath at 37°C and protease inhibitor 2mM PMSF (78830, Sigma-Aldrich, Switzerland) and 2mM EDTA (ED-100G, Sigma-Aldrich, Switzerland) were added to prevent protein degradation. Plasma was spun at 10,000rpm for 20min at 4°C to eliminate any debris and precipitation. Then, the plasma was passed through a column containing Sepharose 4B beads (4B200-500ML, Sigma-Aldrich, Switzerland) and subsequently the column containing 2mM EDTA activated gelatin Sepharose 4B beads (45000170, Fisher Scientific, Switzerland) at a rate of 1 drop/second. After binding, the gelatin column was washed with 500ml 1N NaCl (S9888, Sigma-Aldrich, Switzerland), 500ml Phosphate buffer saline (PBS) (P4417, Sigma-Aldrich, Switzerland) pH7.4 containing 2mM PMSF and 2mM EDTA, and 500ml 1M urea. Gelatinases were eluted by washing the column with 500ml 3% DMSO (Pal et al., 2010), then pFn was eluted with 3M urea and stored at -80°C. Before adding pFn to cell culture, pFn was dialyzed in PBS pH7.4 by using the 10KDa molecular weight cutoff dialysis cassette (66380, Thermo Scientific, U.S.A.) to eliminate the urea and small Fn fragments.

5.5.3 labeling of plasma Fibronectin and collagen

pFn was labeled with green fluorescent dye Alexa fluor 488 (A-20100, Invitrogen, Switzerland) and far-red fluorescent dye Alexa fluor 633 (A20105, Invitrogen, Switzerland) by using NHS chemistry. Briefly, stock pFn was first
dialyzed in 2L labeling buffer consisting 0.1M sodium bicarbonate (S5761, Sigma-Aldrich, Switzerland) PBS pH8.5 at room temperature for 1.5 hours and then further dialyzed in 1L fresh labeling buffer for 1.5 hours at room temperature. After dialysis, Fn concentration was determined by measuring the absorbance at 280nm (NanoDrop 2000, Thermo Scientific, U.S.A.). If concentration exceeded 2g/l, the pFn solution was diluted in the labeling buffer to 2g/l. Then a 20 fold molar excess of Alexa fluor 488 or 633 carboxylic acid, succinimidylic ester was added. Because pFn is sensitive to mechanical stress, mixing was done by gently inverting the tubes. The mixture was then incubated in the dark for 1 hour at room temperature. Finally, the labeled pFn was separated from free dyes by passing through the PD-10 desalting column (17-0851-01, GE Healthcare, U.K.). Absorbance at 280nm, 495nm and 632nm was measured and the labeling ratio was calculated by the following equations. A typical labeling ratio of single color labeled pFn was 4:1 (Alexa fluor dye : pFn). Labeled pFn was aliquoted and stored at -80°C till usage.

\[
\text{Molarity}_{\text{Alexa}} = \frac{A_{\text{495 or 632}}}{\varepsilon_{\text{Alexa}} \times l} \\
\text{Molarity}_{\text{Fn}} = \frac{A_{280} - A_{\text{495 or 632}} \times CF}{\varepsilon_{\text{Fn}} \times l}
\]

CF = correction factor, 0.11 for Alexa Fluor 488 and 0.55 for Alexa Fluor 633.
\( \varepsilon = \) extinction coefficient at \( \lambda_{\text{max}} \) (495nm for Alexa Fluor 488 and 632nm for Alexa Fluor 633), 71,000cm\(^{-1}\)M\(^{-1}\)for Alexa Fluor 488 and 100,000cm\(^{-1}\)M\(^{-1}\)for Alexa Fluor 633.

Plasma Fn was dual labeled with two fluorophores (FnDA) for FRET experiments. Alexa Fluor 488 fluorophores (FRET donors) were randomly labeled on amines via succinimidylic ester chemistry and Alexa Fluor 546 fluorophores (FRET acceptors) were specifically targeted to cysteins on type III Fn via maleimide chemistry (Molecular Probes), as previously described (Baneyx et al., 2002, Smith et al., 2007, Kubow et al., 2008, Little et al., 2008, Legant et al., 2012). The labeling ratio of FnDA was determined by measuring the absorbance of at 280, 498 and 556 nm and using extinction coefficients of \( \varepsilon_{280} = 8\,789 \text{M}^{-1}\text{cm}^{-1} \), \( \varepsilon_{498} = 78\,000 \text{M}^{-1}\text{cm}^{-1} \) and \( \varepsilon_{556} = 0 \text{M}^{-1}\text{cm}^{-1} \) for Alexa Fluor 488, \( \varepsilon_{280} = 12\,500 \text{M}^{-1}\text{cm}^{-1} \), \( \varepsilon_{498} = 13\,000 \text{M}^{-1}\text{cm}^{-1} \)
and \( C_{\text{FRET}} = 105'000 \text{ M}^{-1}\text{cm}^{-1} \) for Alexa Fluor 546 (Invitrogen, Switzerland) and \( C_{\text{FRET}} = 563'200 \text{ M}^{-1}\text{cm}^{-1} \) for Fn. On average, 7 donors and 3.8 acceptors were labeled on each Fn dimer. Fn-FRET was stored as 10 µl aliquots in PBS at −80°C and used upon thawing on ice. For consistent FRET results, FnDA from the same batch was used in this paper.

Collagen I (Col1) monomer from rat tails (BD Biosciences, Switzerland) was kept in 0.5 M acetic acid at 4°C till usage. The monomer solution was dialyzed in borate buffer saline (pH8.2) overnight for pH adjustment. The concentration of dialyzed Col1 was determined by using BCA protein assay (Thermo Scientific, Switzerland). Then, a 20-fold molar excess Alexa Fluoro 633 (Invitrogen, Switzerland) was added into the dialyzed Col1, completely mixed with the solution and was incubated at room temperature in the dark for 1 hour. To separate labeled Col1 from free dyes in solution as well as gradually re-nature the collagen monomers, mixture was dialyzed in 0.1N acerbic acid for 4 days at 4°C in the dark. Only freshly labeled Col1 was used for experiments.

5.5.4 Preparing chambers

Polydimethylsiloxane (PDMS) chambers were made by mixing the elastomer and curing agent at 10:1 (w/w) ratio (SYLGARD® 184 silicone elastomer kit, Dow Corning, U.S.A.). The mixture was degassed for 1 hour, and then was injected into the in-house made molds. The PDMS was baked at 80°C for 4 hours to ensure complete crosslinking. After baking, the chambers were rigorously rinsed with 70% ethanol and Nanopure water (D11031, Thermo Scientific, U.S.A.) and sterilized by autoclaving at 121°C for 20 minutes.

5.5.5 Stretching cells

Because PDMS surface is hydrophobic, 25µg/ml pFn was used to coat the chambers to enable cell adhesion. The chambers were coated at 37°C for 3 hours followed by careful PBS pH7.4 wash to eliminate Fn residues. HFFs were seeded at 5,000cells/cm² for immunofluorescence experiments and 10,000cells/cm² for MMP experiments in DMEM supplemented with 1% (v/v) FBS depleted of Fn by two passages over the gelatin Sepharose column and 1% (v/v) penicillin streptomycin (P/S) (15140-122, Invitrogen, Switzerland). HFFs were allowed to adhere and spread
overnight. To trace the deposition of the Fn matrix before and during mechanical stretching, 50µg/ml labeled pFn was added to the stretching medium accordingly. HFFs were cyclically stretched at 10% 1Hz for 8 hours at 37°C with 5% CO₂.

5.5.6 Microscopy and quantitative image analysis

Fluorescent samples were imaged by Leica SP5 microscopy system with 20X/0.7 air objective or 63X/1.4 NA oil objective. Argon/Helium and 633nm laser power was opened at 20%, and the photon multiplier tube (PMT) was adjusted to 700V. Sequential scanning was applied to avoid bleed-through between channels. Signals were collected sequentially at 498-530nm, 570-630nm and 643-800nm. For Z-stack, samples were imaged at every 300nm from top to bottom. Maximal project was employed to allow the visualization of overall staining.

Quantitative image analysis was performed using ImageJ (National Institute of Health) and custom-written software in MATLAB (MathWorks) or Mathematica (Wolfram Research). Cell orientation and elongation was analyzed by thresholding DiI fluorescent images, converting to a binary mask, and approximating the cell outline by a fitting ellipse. Aspect ratio was defined as the ratio between major and minor axis and taken as a measure of cell elongation. Cell orientation was defined as the orientation of the major axis relative to the external direction of uniaxial strain. For analyzing the orientation of actin or Fn fibers, fluorescent phalloidin/Fn grayscale images were pre-processed by normalizing their values between 0 and 1 and padding them in all directions by 2 rows/columns. Filter kernels were constructed based on normalized 5x5 Sobel kernels in x direction (Sx) and y direction (Sy). Three filtered images were created by convolution of the rescaled image with the 2nd derivative kernels Sx*Sx, Sy*Sy, and Sx*Sy. Local orientation was computed for each pixel in the image by taking the arcus tangens of the following combination

\[ \alpha = \frac{1}{2} \cdot \arctan \left( -2I[Sx*Sy]/(I[Sx*Sx]-I[Sy*Sy]) \right), \]

resulting in angles between 0° and 180°. For restricting the orientation analysis to fibers, masks were generated by applying a 3x3 Laplace filter to the grayscale fiber images, automatically thresholding the filtered images by the method of Otsu, and binarizing the result. For non-zero pixels in the masks, the angles in the corresponding orientation image were pooled. For Figs. 1B and 3F, pixel angles larger than 90° were folded back onto the range from 0° to 90° by setting the angle to 180° minus α, pixel values from several
cells were pooled and depicted as histograms. For Fig. 5.3.2B, all angles from pixels in fibers in/around a single cell were pooled into a histogram that then was fitted by a normal distribution to determine the principal orientation; orientations were projected in the range of 0° to 90°, and pairs of average actin/Fn angles for the individual cells were depicted as points in the scatter plots.

All Fluorescent Resonance Energy Transfer (FRET) images were acquired from living samples. Images were acquired with 16-bit depth on the Olympus FV1000 confocal microscope with a 40X water immersion objective NA1.25. Acceptor and donor intensities were detected using 12-nm bandwidths across the donor (514–526 nm) and acceptor (566–578 nm) emission peaks, as previously described (Smith et al., 2007). Multiple regions of interest (ROIs) were acquired from each sample to include statistical variation. In each region, FRET images were acquired throughout the entire z-stack with slice-slice distance of 1µm. The position with minimum autofocus blur was extracted for FRET analysis. Dark currents (background) for both donor and acceptor channels were acquired every 30 min. Images were processed with self-written Matlab codes, as previously described (Smith et al., 2007).

5.5.7 Generic MMP activity and zymography

Soluble MMP activity was measured by the generic MMP assay (SensoLyte, ANASPEC, U.S.A.). Gelatin and casein zymographies were performed according to the manual suggested by Invitrogen and the protocol published by Hu and Beeton (Hu and Beeton, 2010). Briefly, culturing medium of each sample was harvested and spun at 2000rpm at 4°C for 10 minutes to eliminate dead cells and debris. Supernatant was then collected for gelatin or casein zymography. Without boiling or reduction by reducing agent, samples were mixed with Tris-Glycine SDS sample buffer (LC2676, Invitrogen, Switzerland) on ice and loaded to 10% Tris-Glycine gel with 0.1% gelatin (EC6175BOX, Invitrogen, Switzerland). Gels were run in the Tris-Glycine SDS running buffer (LC2675, Invitrogen, Switzerland) using XCell Surelock Mini-Cell system (EI0001, Invitrogen, Switzerland) at constant voltage of 125V for 90 minutes. After gel electrophoresis, gels were renatured in 100ml renaturing buffer (LC2670, Invitrogen, Switzerland) with gentle agitation at room temperature for 30 minutes, followed by overnight incubation at 37°C in the development buffer. The next day, gels were carefully rinsed with deionized water three times and scanned to record the
positions of molecular standard bands before SimplyBlue staining (LC6060, Invitrogen, Switzerland). If needed, stained gels were incubated in deionized water at room temperature for 30-60 minutes. Gels were scanned and saved as tiff files, then the intensity of the bands was analyzed by ImageJ.

5.5.8 Reverse transcription-quantitative polymerase chain reaction

After mechanical stretching, cell pellets were immediately washed, collected and stored at -80°C. Cells were lysed, shredded by QIAshredder (79656, Qiagen, Switzerland) and RNA was isolated by the RNeasy procedure (74104, Qiagen, Switzerland). RNA quality was assessed by measuring the absorbance of each sample at wavelength of 230nm, 260nm and 280nm, and only samples with 260/280 and 260/230 values between 1.9-2.1 were used for cDNA synthesis. RNA was reverse-transcribed with iScript™ Advanced cDNA Synthesis Kit for RT-qPCR (170-8842, Bio-rad, U.S.A.). cDNA, primers and SsoAdvanced™ SYBR® Green Supermix (172-5261, Bio-rad, U.S.A.) were mixed and genes were amplified on CFX Connect™ Real-Time System (185-5200, Bio-rad, U.S.A.). Primer sequences used for the experiments were listed in the table below, and synthesized by Microsynth (Microsynth, Switzerland).

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<th>Accession No.</th>
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References


Chapter 6

Presence of exogenous fibronectin upregulates the mechanosensitive response of fibroblasts exposed to cyclic mechanical strain

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Author Contributions

Y.Z. designed and performed experiments, analyzed data and prepared the manuscript. J.S. contributed to the design and execution of the nanopillar experiments and prepared the manuscript. Z.L performed the FRET study and analyzed the data. J.S. prepared the manuscript. I.S. provided tools for fiber orientation analysis and contributed to manuscript preparation. V.V. supervised the project and Y.Z., J.F. and V.V. wrote the manuscript.

Keywords: fibronectin, uniaxial cyclic strain, ECM, matrix remodeling, cell-generated force, matrix metalloproteinase
6.1. Abstract

The fibronectin matrix plays important roles in mechanotransduction, extracellular matrix remodeling, and maintaining mechanical stability of the ECM. While mechanical forces are well known to regulate these processes, little is known about how Fn contributes to cellular mechanosensing and matrix remodeling under dynamic mechanical loading. Here, we aimed at determining how pericellular Fn contributes to the cellular involvement in cyclic strain-induced ECM remodeling.

Therefore, fibronectin knockout and fibronectin floxed mouse embryonic fibroblasts were seeded on fibronectin- or vitronectin-coated elastic substrates and exposed to uniaxial cyclic mechanical strain in the presence or absence of exogenous fibronectin. Subsequently, cellular stress fiber development and focal adhesion formation, force generation and matrix remodeling were assessed by using confocal microscopy, immunofluorescence staining and matrix metalloproteinase activity assays.

Results show that the addition of exogenous fibronectin in the medium restored the total traction force generated by the Fn knockout fibroblasts and stretched fibronectin conformation in the extracellular matrix on fibronectin-coated substrates. In addition, exogenous plasma fibronectin in the medium enhanced the formation of actin stress fibers and maturation of focal adhesions in a subpopulation of fibronectin knockout fibroblasts and significantly increased matrix metalloproteinase activities in these cells, suggesting fibronectin’s dominant role in extracellular matrix remodeling. It also shows, however, that the typical strain avoidance response was not present for Fn-treated fibronectin knockout fibroblasts, which in addition showed increased migratory behavior by escaping from pre-existing matrix during cyclic stretching. It remains to be explored whether the absence of strain-avoidance for fibronectin knockout fibroblasts relates to lack of available cellular fibronectin and its associated integrins.

In summary, fibronectin was shown to be crucial in cellular force generation and extracellular matrix remodeling capacity by increasing actin stress fiber formation, focal adhesion maturation, cellular traction force, matrix metalloproteinase activities and unfolding fibronectin in the extracellular matrix to normal levels (comparable to the level of fibronectin floxed mouse embryonic fibroblasts). These results imply that fibronectin is required for cyclic strain-induced cellular mechanosensitivity and associated capacity to remodel the extracellular matrix.
6.2. Introduction

Mechanical forces acting on cells and tissues affect many physiological processes, from tissue growth and remodeling (Carter, 1987, Eyckmans et al., 2011, Heisenberg and Bellaiche, 2013, Heisenberg and Bellaiche, 2013), to wound healing (Hinz et al., 2001, Tomasek et al., 2002, Kippenberger et al., 2000, Wong, Longaker and Gurtner 2012). Fibroblasts are the most abundant cell type in connective tissues and are the primary cells that lay down the provisional extracellular matrix (ECM) that is rich in fibronectin (Fn) (Clark, 1990, Shaw and Martin, 2009, Singh, Carraher and Schwarzbauer, 2010). The increasing expression of the Fn gene is among the first ECM genes observed in fibroblasts exposed to cyclic mechanical strain (Howard et al., 1998, Wang et al., 2007), suggesting the importance of fibronectin in early ECM remodeling under dynamic mechanical loading. Yet, how exogenous Fn contributes to the capacity and the physical remodeling of ECM by fibroblasts subjected to cyclic strain still remains elusive.

ECM remodeling is controlled by biochemical and biomechanical cues (Yu et al., 2010, Cox and Erler, 2011). For example, cell traction can alter matrix anisotropy (Lakshman et al., 2007, Meshel et al., 2005, Glaser et al., 1987, Foolen et al., 2010, Stopak and Harris, 1982, Sawhney and Howard, 2002, Harris et al., 1981), very likely through alternating lamellipodia extension and retraction (Meshel et al., 2005; Glaser et al., 1987) Besides traction-induced ECM remodeling, mechanical loading induces cell alignment/re-alignment (De, Zemel and Safran 2007, Jungbauer et al., 2008) and the deposition of ECM fibers parallel to their long axis (Trelstad and Birk, 1985, Birk and Trelstad, 1984, Birk and Trelstad, 1986). Hence, cyclic strain presents a way to first manipulate cellular orientation which subsequently causes the associated ECM anisotropy (Balestrini et al., 2010, Gould et al., 2012, Foolen et al., 2012). Strain-induced alignment of cells thus plays an important role in the mechano-guidance of tissue growth and the ECM remodeling processes. Previous studies primarily focused on collagen type I in ECM, whereas the contribution of Fn in these processes has rarely been studied. The question was, therefore, how the remodeling of exogenous Fn contributes to establishing the matrix anisotropy by fibroblasts under uniaxial cyclic strain.

Fn is a crucial component in the development of early ECM and has been suggested to act as a mechanotransducer (Vogel, 2006). Consisting of two almost identical monomers, ~220-250kDa each, Fn exists as a dimeric glycoprotein in more
than 20 variants due to alternative splicing (Kosmehl et al., 1996, Pankov and Yamada, 2002, Mao and Schwarzbauer, 2005). Recently, it was shown that force applied to Fn-integrin α5β1 increase the bond lifetime 100 fold (Kong et al., 2013), providing evidence that force can directly strengthen the bond between Fn-integrin α5β1. Furthermore, mechanical forces can expose cryptic binding sites on Fn (Zhong et al, 1998, Vogel, 2006, Lemmon et al., 2011, Little et al., 2008, Kubow et al., 2009), lead to conformational unfolding (Smith et al., 2007, Little et al., 2008, Kubow et al., 2009), or alter Fn functionality by destroying binding sites (Little et al., 2009, Chabria et al., 2010). It has also been shown that co-signaling of integrins with growth factor binding sites on Fn is affected by the mechanical strain of Fn fibers and regulates human mesenchymal stem cell differentiation (Li et al., 2013). Therefore, it has been suggested that Fn can convert extracellular mechanical signals to intracellular signals (Vogel, 2006, Hynes, 2009). Based on Fn’s crucial role in mechanotransduction, it was hypothesized that a pericellular Fn matrix is essential for the cellular capacity for mechanosensitivity and ECM remodeling under dynamic loading.

Matrix metalloproteinases (MMPs) have the capacity to degrade ECM components and therefore play a crucial role in ECM remodeling. Many human MMPs interact with Fn (MMP-2&3, 7-9, 11-16 and 25&26) (Klein and Bischoff, 2011). MMP-2 and -9 (gelatinase-A and –B) specifically target substrates such as gelatin, collagen and Fn (Toriseva and Kahari, 2009) and were shown to be required for connective tissue remodeling (Hayashidani et al., 2003, Romanic et al., 2002, Zamilpa et al., 2010). Apart from affecting cellular and matrix alignment, cyclic mechanical strain was shown to upregulate MMP levels (Yoshida et al., 2002, Prajapati et al., 2000). In addition, the previous study showed that the composition of the ECM (Fn versus collagen) has a major effect on the expression and activity of MMPs by human foreskin fibroblasts (Chapter 5). These results imply that there is a potentially cumulative effect of Fn and cyclic strain-induced cell contraction on MMP activities.

In this study it was asked how pericellular Fn contributes to cyclic strain-induced ECM remodeling by fibroblasts to gain insights into the role of exogenous Fn during the early stages of tissue repair and matrix remodeling. Therefore, Fn knockout (Fn−/−) and the Fn floxed mouse embryonic fibroblasts (Fnfl/fl MEFs) were used to study the importance of pericellular Fn on strain-induced ECM remodeling by using confocal microscopy combined with traction force measurements utilizing nanopillar
substrates and finally, by conducting MMP assays on the supernatant.

6.3. Results

6.3.1. Exogenous Fn in the medium is required for the cyclic strain-induced maturation of focal adhesions and actin stress fibers

Figure 6.3.1: Exogenous Fn in the medium is required for the cyclic strain-induced maturation of focal adhesions and actin stress fibers (A.) Experimental setup: Cells were seeded onto pFn coated (25μg/mL in PBS physisorbed at 37° for 3 hours) PDMS substrates at 75 cells/mm² in Fn-depleted medium and allowed to adhere to substrate for 16 hours. For Fn⁻ samples, Fn⁻ MEFs were stretched in Fn-depleted media for 8 hours at 10% 1Hz. For Fn⁻ +pFn and Fn⁺/⁻+pFn samples, knockout and floxed MEFs were supplemented with fluorescently labeled 50μg/mL pFn in the media during
Matrix remodeling depends on cellular forces generated by an actomyosin network (Legant, Chen, Vogel, 2012, Kirmse, Otto, Ludwig, 2012) that are subsequently transmitted through focal adhesions to external anchorage sites (Balaban et al., 2001, Tan et al., 2003, Gallant, Michael, Garcia, 2005). It was asked whether the presence of exogenous Fn in the medium might affect the development of these adhesion structures on Fn-coated surfaces. MEFs (floxed and knockout) were seeded on pFn-coated PDMS in the presence or absence of pFn in the growth medium and subjected to cyclic strain for 8 hours at 10% 1Hz (Fig. 6.3.1A), after which F-actin/actin stress fibers (SFs) and focal adhesions/vinculin (FAs) were visualized by immunofluorescence staining (Fig. 6.3.1B). Fn°° MEFs formed relatively few actin SFs and short FAs in the absence of pFn. For the quantification of actin SFs and FAs, only cells with more than 15 actin SFs (Fig. 6.3.1C) and FAs longer than 3µm were counted (Fig. 6.3.1D). The addition of exogenous pFn to the growth medium significantly increased the formation of actin SFs in Fn°° MEFs from ~5% of the cell population (no exogenous pFn) to ~25% (with exogenous pFn, Fig. 6.3.1C), and the formation of FAs from ~2% to ~20% (Fig. 6.3.1D), although the levels of Fn°° MEFs in the presence of pFn were not reached. These data show that exogenous pFn in the medium enhances cyclic strain-induced actin SF development and FA formation, however only a subpopulation of the Fn°° MEFs responded.

6.3.2. Full length exogenous Fn in the medium restores total cell traction forces in Fn°° MEFs

How does the partial enhancement of actin SF and FA formation by exogenous Fn in the medium affect the total cell traction forces? To measure traction forces generated by the cells, Fn°° and Fn°° MEFs were seeded on pFn-coated nanopillar substrates, with or without exogenous pFn in the medium (45 nM) (Fig.
Figure 6.3.2: Full length exogenous Fn in the medium but not Fn fragments restores total cell traction forces in Fn⁺⁺ MEFs generated on vitronectin-coated nanopillars (A.) Experimental setup for measuring cell traction force on nanopillar substrates: Pre-labeled MEFs (red) were seeded on pFn or vitronectin-coated (Vn-coated) nanopillar substrates (pillar-pillar distance: 800nm) for 30 minutes in Fn-depleted media. Depending on each condition, seeding medium was changed to fresh media without any Fn or with 45nM full-length pFn, or Fn fragments (40k, 70k, 120kDa Fn). Cells were given 2 hours to bind exogenous Fn (or fragment Fn) in solution before force measurement on nanopillars. (B.) Three Fn fragments were used: 40kDa Chymotryptic Fn fragment binds collagen and gelatin. N-terminal 70kDa catheptic fragment additionally contains binding sites for fibrin, heparin, tenascin and other Fn. Both 40k and 70kDa Fn bind cell surface integrin α5β1. Also generated by chymotrypsin digestion, 120kDa Fn contains various protein binding sites and more importantly RGD and synergy sites to interact with α5β1 and αvβ3 integrins. Figure adapted from Antia et al., 2008. (C.) On pFn-coated substrates: Fn⁺⁺ MEFs generated force ~30% lower than Fn⁺⁺ MEFs with 45nM exogenous pFn.
Fn\textsuperscript{+/−} MEFs, and Fn\textsuperscript{+/−} MEFs with 45nM exogenous pFn (p<0.05, ANOVA). On Vn-coated substrates: Fn\textsuperscript{+/−} MEFs on Vn-coated substrates with exogenous pFn generated force comparable to Fn\textsuperscript{+/−} MEFs with exogenous pFn on pFn-coated substrates. Fn\textsuperscript{−/−} MEFs with exogenous 70k Fn fragments generated forces ~20% lower than Fn\textsuperscript{+/−} MEFs with full-length pFn, but higher than Fn\textsuperscript{−/−} MEFs in Fn depleted medium. Fn\textsuperscript{−/−} MEFs in Fn depleted medium generated forces ~40% lower than Fn\textsuperscript{−/−} MEFs supplemented with full-length pFn (p<0.05, ANOVA). 10 cells (grey dots) were analyzed for each condition. Box plot shows the median ± 1 standard deviation.

The mean displacement of all pillars was plotted to represent the total force generated by the cell. The Fn\textsuperscript{−/−} MEFs in the absence of exogenous Fn generated 30% lower traction forces compared to the Fn\textsuperscript{+/−} MEFs, even when Fn\textsuperscript{−/−} MEFs were seeded on Fn-coated substrates (Fig. 6.3.2C, left panel). Interestingly, the exogenous pFn in medium increased the ability of Fn\textsuperscript{−/−} MEFs to apply traction forces that are similar to those generated by Fn\textsuperscript{+/−} MEFs.

Why wasn’t the cell traction force completely restored when Fn\textsuperscript{−/−} MEFs were seeded on Fn-coated substrates, but the addition of exogenous Fn in medium did? To test whether surface-adsorbed Fn plays a role in cell-generated forces, the substrate coating was changed to vitronectin (Vn) and evaluated the traction force generated by Fn\textsuperscript{−/−} MEFs with or without exogenously added pFn (Fig. 6.3.2B). Once again, it was observes that adding exogenous pFn in the medium increased traction force generated by Fn\textsuperscript{−/−} MEF’s to the same level despite of the substrate coating (Fig. 6.3.2C). Without the exogenous pFn, however, Fn\textsuperscript{−/−} MEFs on Vn-coated substrates generated force ~ 15% lower than Fn\textsuperscript{+/−} MEFs on Fn-coated substrates (Fig. 6.3.2C). These results indicate that below a threshold value, traction force generation in Fn\textsuperscript{−/−} MEFs depends on exogenous Fn concentration.

Is the binding of cell-surface integrins responsible for the restoration of cell traction forces? Although Fn fragments provide intact integrin binding regions, they cannot undergo Fn fibrillogenesis (Zhou et al., 2008). Hence, Fn\textsuperscript{−/−} MEFs were seeded on Vn-coated nanopillar substrates, and supplemented with the different Fn fragments (45nM for 2 hours, Fig. 6.3.2A).

Fn\textsuperscript{−/−} MEFs with 45nM exogenous 70k Fn fragments generated force ~20% lower than Fn\textsuperscript{−/−} MEFs with full-length pFn, but higher than Fn\textsuperscript{−/−} MEFs in Fn depleted medium. Fn\textsuperscript{−/−} MEFs in Fn depleted medium generated force ~40% lower than Fn\textsuperscript{−/−} MEFs supplemented with full-length pFn (Fig. 6.3.2C, right panel). These results show that binding Fn fragments is not enough to restore cell traction force generation. Hence, full-length pFn is required for restoring cell traction force generation, suggesting that Fn fibrillogenesis is a prerequisite.
6.3.3. The Fn<sup>−/−</sup> MEFs assemble Fn into ECM and unfold the Fn fibrils

(A.) Experimental setup of the Fn conformation study: 75 cells/mm<sup>2</sup> were first seeded onto pFn-coated substrates (25µg/mL) for 30 minutes. Fn conformational sensor, FnDA was added to growth media (5% FnDA and 95% Fn w/w, total 50µg/mL) for 24 hours so that cells incorporate FnDA into ECM. (B.) False-color image shows that Fn<sup>−/−</sup> and Fn<sup>f/f</sup> MEFs assemble exogenous pFn into ECM with L<sub>acceptor</sub>/L<sub>donor</sub> ~0.57. (C.) Histogram of L<sub>acceptor</sub>/L<sub>donor</sub> shows that Fn<sup>−/−</sup> and Fn<sup>f/f</sup> MEFs unfold matrix to the same extent. Box plot shows that Fn conformation in matrix assembled by Fn<sup>−/−</sup> MEF is not statistically different from Fn<sup>f/f</sup> MEFs (p>0.05, ANOVA).

The lack of cell-generated traction forces can be restored by full-length exogenous Fn in the medium (Fig. 6.3.2), but is the restored force functional, i.e. sufficient to unfold Fn in ECM, such as those observed in physiological-relevant ECM? The established fluorescence resonance energy transfer (FRET)-based mechanical strain sensors were employed to monitor the change in the Fn matrix conformation (Baneyx et al., 2001, Smith et al., 2007, Little et al., 2008, Kubow et al., 2009). Therefore, MEFs (floxed and knockout) were exposed to exogenous pFn where 5% was the conformation-sensitive construct, and were allowed to assemble matrices on an Fn-coated substrate under static conditions for 8 hours (Fig. 6.3.3A).
Although the Fn\textsuperscript{f/f} MEFs assembled a denser Fn matrix (Fig. 6.3.3B), the magnitude of matrix unfolding was not significantly different between floxed (0.570±0.021) and knockout MEFs (0.572±0.023) (Fig. 6.3.3). Consequently, the presence of Fn in the medium restored the force generation of the Fn\textsuperscript{+/−} MEFs as shown above (Fig. 6.3.2) and the cell-generated tension unfolded Fn fibrils of early ECM (Fig. 6.3.3).

6.3.4. Exogenous Fn in the medium increases MMP activates under uniaxial cyclic strain

In the next step, how pFn contributes to the cellular capacity for ECM remodeling by enzymatic digestion was assessed. Therefore, matrix metalloproteinase (MMP) secretion and activity were measured after exposing MEFs (floxed and knockout) to cyclic strain in the presence or absence of exogenous Fn on Fn-coated surfaces. A significant increase of soluble MMP secretion was observed in response to the exposure of both cell types to 50µg/mL of exogenous pFn in the medium (Fig. 6.3.4A). Importantly though, the amount of soluble MMPs secreted by Fn\textsuperscript{−/−} MEFs remained ~50% lower compared to Fn\textsuperscript{f/f} MEFs. Furthermore, gelatin zymography conducted on the supernatant after the cells were cyclically strained for 8 hours revealed that the addition of exogenous Fn significantly increased MMP-9, but not MMP-2 activity (Fig. 6.3.4B). Therefore, exogenous pFn up-regulates MMP secretion, including MMP-9 activity.

6.3.5. Pharmacologically inhibiting MMP activities prevents the MEFs from matrix degradation, but does not abolish the cyclic strain-induced cell re-orientation of Fn\textsuperscript{f/f} MEFs

The presence of exogenous Fn was shown here to increase the matrix degradation capacity of MEFs by up-regulating MMP activities. To assess whether these MMPs in the supernatant are involved in matrix remodeling, we added a broad-spectrum MMP inhibitor GM6001 (ilomastat) over 8 hours and monitored the degradation of fluorescently labeled Fn matrices by cells (Fig. 6.3.5A). MEFs were allowed to assemble Fn matrices over 16 hours with 50µg/mL exogenous pFn, then the medium was changed to an Fn depleted medium so that the MEFs had to utilize the pre-existing Fn in ECM. MEFs (knockout and floxed) degraded the previously assembled matrices (Fig. 6.3.5B, Ctrl), and inhibition of MMP activity by GM6001 blocked Fn degradation effectively (Fig. 6.3.5B), confirming that MMP activity is
involves involved in Fn matrix degradation.

**Figure 6.3.4: Exogenous Fn in the medium increases MMP activates under uniaxial cyclic strain**

For measuring MMP secretion and activity, 150/mm² cells were adhered onto pFn-coated PDMS in Fn-depleted media overnight. (A.) Experimental setup: MEFs were seeded onto pFn-coated (25µg/mL in PBS physisorbed at 37° for 3 hours) PDMS substrates at 75cells/mm² in Fn-depleted medium and allowed to adhere to substrate for 16 hours. Then the seeding medium was changed to fresh Fn-depleted medium, and the cells were cyclically strained for 8 hours at 10% 1Hz with or without the 50 µg/mL exogenous pFn. The supernatant of stretching medium from each sample was collected for MMP analysis. (B.) The secretion of soluble MMPs: Adding 50µg/mL exogenous Fn significantly boosted the secretion of soluble MMPs by Fn⁻/⁻ and Fn⁺⁺ MEFs, subjected to 8 hours of uniaxial cyclic strain. However, Fn⁻/⁻ MEFs with the addition of exogenous Fn secreted approximately 50% soluble MMPs compared to Fn⁺⁺ MEFs. (C.) Addition of exogenous Fn up-regulated the secretion gelatinase-B
(MMP-9) (p<0.05, ANOVA), while the level of gelatinase-A (MMP-2) remained unaltered (p>0.05, ANOVA).

Figure 6.3.5: Pharmacologically inhibiting MMP activities prevents MEFs from matrix degradation, but does not abolish the cyclic strain-induced cell re-orientation of Fn\textsuperscript{ff} MEFs

Experimental setup of the MMP inhibition study: Cells were seeded onto pFn-coated (25µg/mL) substrates in Fn-depleted media for 30 minutes. 50µg/mL fluorescently labeled pFn was added to media to allow matrix formation by cells for 16 hours. Then Fn-containing media was changed to Fn-depleted media with or without 20µM broad-spectrum MMP inhibitor GM6001 for 8 hours. (B.) Both
Fn<sup>−/−</sup> and Fn<sup>+/+</sup> MEFs degraded Fn matrix by utilizing MMPs. Inhibiting MMP activity by GM6001 blocked matrix degradation. (C.) With or without MMP inhibition, Fn<sup>−/−</sup> MEFs did not change the random orientation after 8 hours of uniaxial cyclic strain at 10% 1Hz. Inhibition with GM6001 did not abolish the cyclic strain-induced cell re-orientation in Fn<sup>+/+</sup> MEFs (Ctrl vs. GM6001).

But are the stabilizing interactions between cells and the ECM by inhibiting matrix degradation essential to the ability of cells to reorient in the presence of uniaxial cyclic strain on Fn-coated surfaces? Previous work showed that inhibition of MMP activity abolished cyclic strain-induced cell reorientation in 3D collagen matrices (Foolen et al., 2012). MEFs seeded on Fn-coated surfaces were supplemented with pFn (50µg/mL) overnight under static conditions, and were then subjected to uniaxial cyclic strain (10%, 1Hz) in the presence or absence of a broad-spectrum inhibitor GM6001 (Fig. 6.3.5A) for 8 hours. Inhibiting matrix degradation did not abolish the cyclic strain-induced cell re-orientation in Fn<sup>+/+</sup> MEFs (Fig. 6.3.5C, D). This result indicates that reduced matrix degradation does not interfere with strain-induced cell realignment using the current setup.

6.3.6. Cyclically-strained Fn<sup>−/−</sup> MEFs leaves pre-existing Fn matrix

Next was the exploration of whether the restoration of cellular activities (traction forces, Fn unfolding in ECM and partial rescue of actin SF and FA formation and MMP activates) by adding exogenous Fn also restored cell-matrix interactions. To distinguish between old ECM that was deposited under static conditions, from ECM assembled after the cells were exposed to cyclic strain, a pulse-chase experiment was performed by adding differently labeled Fn at different time points (Fig. 6.3.6A). Cells were first cultured in the presence of Fn labeled with green fluorescence prior to cyclic stretching (“Old matrix”) and in the presence of Fn labeled with red fluorescence during cyclic stretching (“New matrix”). Importantly, approximately 70% of Fn<sup>−/−</sup> MEFs left the old matrix behind as they moved away to the bare Fn-coated substrates when subjected to uniaxial cyclic strain (Fig. 6.3.6B, C). In contrast, more than 80% of wild type Fn<sup>+/+</sup> MEFs deposited new matrices within the boundary of the old matrix (Fig. 6.3.6B, C). The discrepancy between Fn<sup>−/−</sup> and Fn<sup>+/+</sup> MEFs indicate that Fn<sup>−/−</sup> MEFs could not maintain a strong adhesion to the pre-existing matrix under uniaxial cyclic strain.
Figure 6.3: Cyclically-strained Fn<sup>−/−</sup> MEFs leaves pre-existing Fn matrix (A.) Experimental setup to monitor cell-matrix interactions: cells were seeded onto pFn-coated (25µg/mL) substrates. 50µg/mL fluorescently labeled Fn (green, "old") was added to the growth medium so that cells assembled matrices prior to stretching. During stretching, medium containing 50µg/mL Fn with another color (red, "new") was added while cells were exposed to 8 hours of uniaxial cyclic strain. (B.) Fn<sup>−/−</sup> MEFs detached from old matrices and deposited new matrices under cyclic mechanical stretching while MEF Fn<sup>f/f</sup> remodeled the old matrices (arrow) and deposited new matrices at the same location of the old matrix. (C.) On average 67% of Fn<sup>−/−</sup> MEFs assembled matrices during stretching ("new") away from the “old” matrices assembled prior to stretching, whereas 85% of Fn<sup>f/f</sup> MEFs assembled “old” and “new” matrices at the same location.
6.3.7. *Fn* does not restore cyclic-strain-induced cell re-orientation in *Fn*<sup>−/−</sup> MEFs

The highly migratory behavior of *Fn*<sup>−/−</sup> MEFs relative to the apparent reorientation process at the original location of the *Fn*<sup>+/+</sup> MEFs, indicates that the response to uniaxial cyclic strain is distinctly different between both cell types. We therefore asked whether the inability for matrix degradation prevents MEFs from strain-avoidance, i.e. cell re-orientation and matrix remodeling.

![Experimental setup](image)

**Figure 6.3.7:** *Fn* does not restore cyclic-strain-induced cell re-orientation in *Fn*<sup>−/−</sup> MEFs (A.) Experimental setup: cells were seeded onto pFn-coated (25µg/mL) PDMS chambers in Fn-depleted medium for 30 minutes. Cells were left in the Fn-depleted medium for 16 hours before the application of 8 hours of uniaxial cyclic strain. During stretching, 50µg/mL Fn was supplemented. (B.) With or without exogenous pFn, cyclically strained *Fn*<sup>−/−</sup> MEFs showed random orientation. *Fn*<sup>+/+</sup> MEFs with exogenous pFn aligned along the direction of minimum strain (~60° away from the strain axis). (C.) Histogram shows that *Fn*<sup>−/−</sup> MEFs with exogenous Fn did not avoid uniaxial cyclic strain by re-orienting towards the minimum strain direction. Majority of *Fn*<sup>+/+</sup> MEFs after 8 hours of stretching re-oriented to ~60°. (D.) Cyclically-strained *Fn*<sup>−/−</sup> MEFs with exogenous pFn have a less polarized shape (lower aspect ratio) than *Fn*<sup>+/+</sup> MEFs (higher aspect ratio).

Since the *Fn*<sup>−/−</sup> MEFs showed a higher potential to migrate away from the old matrix assembled under static conditions, we also quantified *Fn*<sup>−/−</sup> MEF’s capacity to reorient the long axis of their nuclei in response to uniaxial cyclic strain, either in the
presence or absence of exogenous pFn (Fig. 6.3.7A). Strikingly, Fn\textsuperscript{−/−} MEFs not only did not re-orient towards the minimum strain direction (Fig. 6.3.7B, C), but also lacked strain-induced cell polarization, clearly present in Fn\textsuperscript{ff} MEFs (Fig. 6.3.7B, D).

These results show that Fn\textsuperscript{−/−} MEFs, even with presence of exogenous pFn in the medium cannot initiate uniaxial cyclic strain-induced cell polarization nor rescue the cyclic strain-induced cell re-orientation.

6.4. Discussion

This study was designed to address the question of how the presence of Fn contributes to the fibroblast’s capacity of matrix remodeling under dynamic mechanical loading. By exposing Fn\textsuperscript{−/−} MEFs to exogenous Fn in the medium, Fn was shown here to completely restore the generation of total traction force (Fig. 6.3.2) and the associated Fn conformation in the ECM (Fig. 6.3.3). The presence of exogenous Fn enhanced the development of cyclic strain-induced actin stress fibers and maturation of focal adhesion (Fig. 6.3.1), however only a subpopulation of the Fn\textsuperscript{−/−} MEFs responded to the Fn-treatment. The Fn increased the secretion and activation of MMPs, but Fn\textsuperscript{−/−} secreted 50% less soluble MMPs to the medium than Fn\textsuperscript{ff} MEFs (Fig. 6.3.4). Blocking ECM degradation by MMP inhibitors did not affect strain-avoidance by Fn\textsuperscript{ff} MEFs (Fig. 6.3.5). However, the addition of exogenous Fn in the medium did not restore the strain-induced cell re-orientation of Fn\textsuperscript{−/−} MEFs (Fig. 6.3.7), while increasing migration was observed compared to Fn\textsuperscript{ff} MEFs (Fig. 6.3.6).

This study reveals that Fn enhances the formation of thick actin SFs and the maturation of FAs (vinculin recruitment) in fibroblasts subjected to cyclic strain (Fig. 6.3.1). Recently, actin organization and vinculin recruitment for Fn-deficient fibroblasts subjected to equiaxial cyclic strain, were observed on Fn-coated substrates but not on Vn-coated substrates in Fn depleted medium (Lutz et al., 2010). Interestingly however, our results show that an abundance of Fn accessible to the cells from the medium upregulates actin SF formation and FA maturation in Fn\textsuperscript{−/−} MEFs to resemble their floxed counterparts, while in Fn depleted medium the Fn coating only induces sparse development of stress fibers and small vinculin-containing focal adhesions under uniaxial cyclic strain. Hence, conversely to equiaxial strain, uniaxial cyclically strained fibroblasts increase their cellular mechanosensitivity (SF formation and FA maturation) to the presence of soluble fibronectin accessible from the medium.
The differences in SF formation and FA length observed for Fn-coating versus Fn added to the growth medium thus agrees with the observations on nanopillars (Fig. 6.3.2). The addition of Fn to the medium fully restored total cell traction force generated by Fn−/− MEFs, while an Fn-coating alone in depleted medium resulted in significantly lower forces. This corroborates well with reduced contraction of collagen gels by embedded Fn−/− MEFs with decreasing concentrations of exogenously added Fn (Hocking, Sottile, Langenbach, 2000, Sottile et al., 2007).

Strikingly, apart from the availability of Fn, the mere presence of an Fn coating, when compared to Vn coating, resulted in the generation of higher forces (Fig. 6.3.2). This links to the fact that fibronectin is recognized by, among others, α5β1 and αvβ3 integrins, while Vn is recognized by αvβ3 integrins (Humphries et al., 2006). This specific interaction between Fn and cells via integrins correlates to the observed increase of force generation, even though focal adhesion kinase (FAK) gets activated in the absence of α5β1 integrins (Midwood et al., 2006, Friedland, Lee, Boettiger, 2009, Carraher and Schwarzbauer, 2013). Further more, the mere binding of Fn to cell surface integrins is not sufficient to restore cellular traction forces (Fig. 6.3.2). The N-terminal fragments used (40kDa and 70kDa) contain ligand-binding sites for integrin α5β1, and the C-terminal fragment (120kDa) contains both RGD-specific α5β1 and αvβ3 (Pankov and Yamada, 2002, Mao and Schwarzbauer, 2005). However, when exposing the Fn−/− MEFs to these fractions while being attached to a vitronectin coating, did not increase traction force relative to untreated controls. This agrees with the finding that increasing concentrations of added 70kDa fragment to fibroblasts embedded in collagen gels, reduces the degree of volumetric reduction of the gel induced by cell traction in time (Sottile, Hocking, Langenbach, 2000, Hocking, Sottile, Langenbach, 2000). Since the used fragments lack the ability for fibrillogenesis (Zhou et al., 2008), it is speculated that Fn fibrillogenesis is highly related to cell traction force generation in regard of Fn matrix assembly.

The hypothesis that force restoration by Fn can unfold Fn in ECM is supported by the reported FRET study, i.e. Fn−/− and Fn−/− MEFs assemble and stretch exogenously added ECM Fn fibers to equal levels (Fig. 6.3.3). Previous work has already shown that mechanical forces can change Fn conformation in Fn fibrils (Baneyx, Baugh, Vogel, 2001, Smith et al., 2007, Little et al., 2008, Kubow et al., 2009), thereby altering Fn functionalities (Little et al., 2009, Chabria et al., 2011).
Hence, as previously suggested, the Fn conformation may provide a mechanism whereby the protein can act as a mechanotransducer (Vogel, 2006, Hynes, 2009). Furthermore, the sole presence of exogenous Fn in the medium greatly enhanced secretion and activation of MMPs by the MEFs (Fig. 6.3.4). As to our knowledge, no study has shown that the presence of exogenous Fn up-regulates MMP-9 activity in fibroblasts subjected to uniaxial cyclic strain. However, Fn was observed to up-regulate MMP-9 activity in cancer cells mainly by involving integrin receptor α5β1 (Sen et al., 2010) and in T-lymphocyte cell lines likely involving α4, α5 and αv integrins (Esparza et al., 1999). Hence, the question of whether the exposure of MEFs to Fn in the medium greatly improves the potential for ECM remodeling via integrin remains unanswered. By performing pulse-chase experiments in the presence or absence of a broad-spectrum MMP-inhibitor, it was confirmed that indeed MMPs contribute to ECM degradation. This inhibitory effect was previously demonstrated to relate to the expression of MT1-MMP (Shi and Sottile, 2011). Our study further shows that MMP activity is not required for strain-induced cell re-orientation on elastic substrates.

The application of uniaxial cyclic strain is known to alter cellular mechanosensing by inducing cell reorientation and polarization along the direction of minimum strain by reorganizing the actin stress fiber network and focal adhesion sites (Goldyn et al., 2009, Chen et al., 2012, Greiner et al., 2013). Mathematical models predict that both cellular mechanosensitivity determined by cytoskeletal contractility as well as interactions between cell and its surround ECM is responsible for the strain-avoidance behavior on 2D substrates (De, Zemel, Safran, 2007, 2008, Faust et al., 2011). Therefore, it is most surprising that although these processes (F-actin development, FA formation and traction force) in Fn−/− MEFs were (partially) restored through the addition of exogenous Fn (Fig. 6.3.1, 6.3.2), the strain-avoidance feature was not (Fig. 6.3.7). Instead, increased migration was observed for Fn−/− MEFs in comparison to FnF/F MEFs (Fig. 6.3.6). These results suggest that Fn−/− in the presence of pFn is still intrinsically different from FnF/F MEFs.

The explanation for why Fn−/− MEFs do not respond to cyclic strain and display weaker association with old matrix compared to FnF/F MEFs, remains an open question. The distinct behaviors of knockout and floxed fibroblasts may be related to the differences in matrix composition. The exogenously added pFn is secreted by hepatocytes and generally lacks extra domain A and B (EDA, EDB), whereas cellular
Fn (cFn) secreted by Fn^{ef} MEFs contain the EDA (Pankov and Yamada, 2002, Schwarzbauer and DeSimone, 2011, Hynes, 2009). In addition to binding integrin α5β1 and αvβ3, studies revealed the role of EDA-Fn receptors in mediating separate signaling cascades involving force transduction through integrins α4β7 (Kohan et al, 2010) and α9 (Sun et al, 2013). Therefore, there needs to be further investigation into whether cFn in comparison to pFn results in distinct integrin activation and differential incorporation of secondary messenger proteins to restore cellular mechanosensing of uniaxial cyclic strain.

In summary, Fn was shown to be crucial in cellular force generation and ECM remodeling capacity by increasing actin stress fiber formation, focal adhesion maturation, cellular traction force, MMP activities and unfolding Fn in extracellular matrices to a normal level (Fn^{ef} MEFs). These results imply that the presence of Fn is required for cyclic strain-induced cellular mechanosensitivity and associated capacity to remodel ECM.

6.5. Materials and methods

6.5.1. Cell culture

Fibronectin-knockout (Fn^{-/}) and fibronectin floxed (Fn^{ef}) mouse embryonic fibroblasts (MEFs) were obtained from Rainhard Faessler (Max-Planck Institute for Biochemistry, Martinsried, Germany). Before stretching experiments, cells were maintained at 37°C with 5% CO₂ in the Dulbecco’s modified Eagle medium (DMEM) (31966, Invitrogen, Switzerland) supplemented with 10% fetal bovine serum (FBS) (S181H, Biowest, Switzerland). When MEFs reached 90% confluency, they were trypsinized by 0.25% trypsin-EDTA (25200-056, Invitrogen, Switzerland) and subcultured at 75 – 150 cells/mm². All MEFs used were below passage 10.

6.5.2. Isolation and labeling of plasma fibronectin

Human plasma fibronectin (pFn) was isolated by gelatin affinity chromatography. Briefly, frozen human plasma (UniversitaetsSpital Zurich, Switzerland) was thawed in the water bath at 37°C and protease inhibitor 2mM PMSF (78830, Sigma-Aldrich, Switzerland) and 2mM EDTA (ED-100G, Sigma-Aldrich, Switzerland) were added to prevent protein degradation. Plasma was spun at 10,000rpm for 20min at 4°C to eliminate any debris and precipitation. The plasma
was then passed through a column containing Sepharose 4B beads (4B200-500ML, Sigma-Aldrich, Switzerland) and subsequently the column containing 2mM EDTA activated gelatin Sepharose 4B beads (45000170, Fisher Scientific, Switzerland) at a rate of 1 drop/second. After binding, the gelatin column was washed with 500ml 1N NaCl (S9888, Sigma-Aldrich, Switzerland), 500ml Phosphate buffer saline (PBS) (P4417, Sigma-Aldrich, Switzerland) pH7.4 containing 2mM PMSF and 2mM EDTA, and 500ml 1M urea. Gelatinases were eluted by washing the column with 500ml 3% DMSO (Pal et al., 2010), then pFn was eluted with 3M urea and stored at -80°C. Before adding pFn to cell culture, pFn was dialyzed in PBS pH7.4 by using the 10kDa molecular weight cutoff dialysis cassette (66380, Thermo Scientific, U.S.A.) to eliminate the urea and small Fn fragments.

pFn was labeled with green fluorescent dye Alexa fluor 488 (A-20100, Invitrogen, Switzerland) and far-red fluorescent dye Alexa fluor 633 (A20105, Invitrogen, Switzerland) by using NHS chemistry. Briefly, stock pFn was first dialyzed in 2L labeling buffer consisting 0.1M sodium bicarbonate (S5761, Sigma-Aldrich, Switzerland) PBS pH8.5 at room temperature for 1.5 hours and then further dialyzed in 1L fresh labeling buffer for 1.5 hours at room temperature. After dialysis, Fn concentration was determined by measuring the absorbance at 280nm (NanoDrop 2000, Thermo Scientific, U.S.A.). If the concentration exceeded 2g/l, the pFn solution was diluted in the labeling buffer to 2g/l. Then a 20 fold molar excess of Alexa fluor 488 or 633 carboxylic acid, succinimidyl ester was added. Because pFn is sensitive to mechanical stress, mixing was done by gently inverting the tubes. The mixture was then incubated in the dark for 1 hour at room temperature. Finally, the labeled pFn was separated from free dyes by passing through the PD-10 desalting column (17-0851-01, GE Healthcare, U.K.). Absorbance at 280nm, 495nm and 632nm was measured and labeling ratio was calculated by the following equations. A typical labeling ratio of single color labeled pFn was 4:1 (Alexa fluor dye : pFn). Labeled pFn was aliquoted and stored at -80°C till usage.

\[
\text{Molarity}_{\text{Alexa}} = \frac{A_{495\text{or}632}}{\varepsilon_{\text{Alexa}}} \times l
\]

\[
\text{Molarity}_{\text{Fn}} = \frac{A_{280} - A_{495\text{or}632} \times CF}{\varepsilon_{\text{Fn}}} \times l
\]

CF = correction factor, 0.11 for Alexa Fluor 488 and 0.55 for Alexa Fluor 633.
\[ \varepsilon = \text{extinction coefficient at } \lambda_{\text{max}} \text{ (495nm for Alexa Fluor 488 and 632nm for Alexa Fluor 633), 71,000cm}^{-1}\text{M}^{-1}\text{for Alexa Fluor 488 and 100,000cm}^{-1}\text{M}^{-1}\text{for Alexa Fluor 633.} \]

6.5.3. Nanopillar experiments

The SU8 nanopillar arrays were fabricated by a combination of nanosphere lithography and plasma etching. First, a nanopillar template made of silicon was fabricated. Then, polydimethylsiloxane (PDMS) was filled onto the silicon template to create a nano-mold with an inversed pillar structure. The SU8 solution was filled into the PDMS mold, merged with a glass coverslip from the bottom, and degassed under vacuum. Subsequently, the SU8 was cured by UV exposure. After the PDMS nano-mold was removed the SU8 nanopillar array remained on the glass coverslip. Atomic force microscopy was used to obtain the spring constant \( k \) of nanopillars resulting in a value of 79 nN*\( \mu \)m\(^{-1}\). Nanopillar arrays allow the measurement of displacements ranging from 0.01 \( \mu \)m to 0.8 \( \mu \)m. To measure cell-induced traction forces on nanopillar arrays, each nanopillar is assumed to be an elastic spring. Cell movements induced pillar bending and therefore, traction forces within the nanopillar. To calculate the traction force \( F \), the following equations were used:

\[
F = k_{\text{bend}} \times x \hspace{1cm} \text{(1)}
\]

\[
k_{\text{bend}} = \frac{3}{64\pi ED4/L3} \hspace{1cm} \text{(2)}
\]

Thus:

\[
F = k_{\text{bend}} \times x = \left(\frac{3}{64\pi ED4/L3}\right) \times x \hspace{1cm} \text{(3)}
\]

According to equations (1) - (3), the cell-induced traction force \( F \) in the nanopillar can be calculated via the deflection of the nanopillar top \( x \) and the spring constant \( k_{\text{bend}} \).

The cell-induced deflection of the polymeric nanopillar top \( x \) was measured by confocal microscopy. In equation (1), the pillars were assumed to be fixed on the substrate. The Young’s modulus of SU8 was 600 Mpa.
According to equation (2), the bending spring constant was:

\[ k_{\text{bend}} = 101.62 \text{ nN/\text{\AA}}. \]

Importantly the deflection of nanopillars allows not only bending of the nanopillars, but also shear- and tilt movements. To account for these additional movements, the spring constant \( k_{\text{bend}} \) was adapted using a correction factor \( a \) accordingly:

\[ a = x_{\text{bend}}/(x_{\text{bend}} + x_{\text{shear}} + x_{\text{tilt}}) \]  

\[ \cdots\cdots\cdots (4) \]

Thus:

\[ k_{\text{corr}} = k_{\text{bend}} \times a \]  

\[ \cdots\cdots\cdots (5) \]

Finally:

\[ F = k_{\text{corr}} \times x \]  

\[ \cdots\cdots\cdots (6) \]

with: \( k_{\text{corr}} = 87.76 \text{ nN/\text{\AA}}. \)

According to equation (6) traction forces now take into account bend-, shear- and tilt movements of nanopillars.

The equations above are based on ideal geometric design of the nanopillar arrays. As it is not possible to fabricate nanopillars with perfect geometries, we used Atomic Force Microscopy (AFM) to get even more accurate information about the spring constant \( k_{\text{corr}} \). Using an AFM tip with a known spring constant of 62 nN/\text{\AA}, force was applied with this tip on single SU8 nanopillars. After indentation compensation, the new spring constant was calculated from the slope of the force curve: \( k_{\text{corrAFM}} = 79 \text{ nN/\text{\AA}}. \) This experimentally determined value was used to calculate the traction forces in this study.

MEFs are pre-labeled with membrane dye DiI (Invitrogen, Switzerland) prior to experiments. Labeled MEFs were then seeded on coated nanopillars at 7,500 cells/cm\(^2\) in DMEM (31966, Invitrogen, Switzerland) Fn depleted 1% FBS (S181H, Biowest, Switzerland) with 1% penicillin/streptomycin (P/S) (15070063, Invitrogen, Switzerland). For negative controls, Fn\(^{-/-}\) MEFs were seeded onto 10nM vitronectin (Vn) coated nanopillar substrates for 30 minutes and cultured in Fn depleted media for 2 hours before measuring the cell traction force. To differentiate the substrate Fn from the Fn in solution, Fn\(^{-/-}\) MEFs were seeded onto 10nM pFn-
coated nanopillars and adhered in Fn depleted media for 2 hours. To rescue Fn⁻/⁻ MEFs with exogenous pFn, Fn⁻/⁻ MEFs were seeded onto Fn-coated substrates in Fn depleted media for 30 minutes, then the media was changed to 45nM plasma Fn containing DMEM with 1% Fn depleted FBS and 1% P/S. For positive controls, Fn⁺⁺ MEFs were seeded onto Fn-coated substrates in Fn depleted media for 30 minutes, then the media was changed to 45nM plasma Fn containing DMEM with 1% Fn depleted FBS and 1% P/S.

To investigate the effect of fragmented Fn, 40kDa, 70kDa and 120kDa were used. Nanopillars were first coated with 10nM each of Fn fragments before seeding. Fn⁻/⁻ MEFs were then cultured in Fn depleted media for 30 minutes, afterwards the growth media DMEM with 1% Fn depleted FBS and 1% P/S was supplemented with 45nM Fn fragments and cells were allowed to bind Fn fragments for 2 hours before fixation.

6.5.4. Preparing chambers

Polydimethylsiloxane (PDMS) chambers were made by mixing the elastomer and curing agent at 10:1 (w/w) ratio (SYLGARD® 184 silicone elastomer kit, Dow Corning, U.S.A.). The mixture was degassed for 1 hour, and then was injected into the in-house made molds. The PDMS was baked at 80°C for 4 hours to ensure complete crosslinking. After baking, the chambers were rigorously rinsed with 70% ethanol and Nanopure water (D11031, Thermo Scientific, U.S.A.) and sterilized by autoclaving at 121°C for 20 minutes.

6.5.5. Stretching cells

Because the PDMS surface is hydrophobic, depending on the experiment either 10nM (for nanopillar experiments) or 25μg/ml (for other experiments) of pFn was used to coat the chambers to enable cell adhesion. The chambers were coated at 37°C for 3hours followed by a careful PBS (pH7.4) wash to eliminate Fn residues. Both Fn⁻/⁻ and Fn⁺⁺ MEFs were seeded at 15,000cells/cm² for MMP experiments and 7,500cells/cm² for other experiments in DMEM supplemented with Fn depleted 1% FBS and 1% P/S. MEFs were allowed to adhere and spread for 16 hours. MEFs were cyclically stretched at 10% 1Hz for 8 hours at 37°C with 5% CO₂.

The stretching experiments above were modified to monitor Fn assembly and
Fn matrix remodeling. Namely, the MEFs were given fluorescently labeled Fn prior to stretching to assemble the matrix for 24 hours. Then cells were given a different color labeled Fn during 8 hours of uniaxial cyclic stretching at 10% 1Hz for monitoring the matrix deposition during stretching.

6.5.6. Immunofluorescence staining

Cells in PDMS chambers were washed twice with warm PBS and fixed with 4% paraformaldehyde (P6148, Sigma-Aldrich, Switzerland) in PBS pH7.4 at room temperature for 15 minutes. The cell membrane was permeabilized with 0.1% Triton X-100 (X100, Sigma-Aldrich, Switzerland) in PBS, and then cells were incubated in PBS containing 1% BSA (85040C, Sigma-Aldrich, Switzerland) for 30 minutes to block unspecific binding. MEFs were stained with anti-vinculin 1:200 (FAK100, Merck Millipore, U.S.A.) in PBS for 1 hour at room temperature in the dark, followed by three 10 minute PBS washes. TRITC-labeled phalloidin 1:100 and Alexa Fluor 488-labeled donkey anti-mouse IgG 1:200 (A-21202, Invitrogen, Switzerland) were incubated simultaneously at room temperature for 1 hour in PBS containing 1% BSA. Cells were washed three times with PBS followed by DAPI staining 1:1000 in PBS for 5 minutes. After the final wash, samples were mounted in ProLong Gold antifade reagent (P36930, Invitrogen, Switzerland) overnight for microscopy imaging.

6.5.7 Microscopy and quantitative image analysis

Phase contrast images were acquired by a Zeiss AxioVision 1000 wide field microscope. Fluorescent samples were imaged by a Leica SP5 microscopy system with 20X/0.7 air objective or 63X/1.4 NA oil objective. Argon/Helium and 633nm laser power were opened at 20%, and the photon multiplier tube (PMT) was adjusted to 700V. Sequential scanning was applied to avoid bleed-through between channels. Signals were collected sequentially at 420-470nm, 498-548nm and 643-800nm. For Z-stack, samples were imaged at every 75nm from top to bottom. Maximal project was employed to observe overall staining.

Quantitative image analysis was performed using ImageJ (National Institute of Health) and custom-written software in MATLAB (MathWorks) or Mathematica (Wolfram Research). Cell orientation and elongation were analyzed by thresholding DiI fluorescent images, converting to a binary mask, and approximating the cell outline by a fitting ellipse. Aspect ratio was defined as the ratio between major and
minor axis and taken as a measure of cell elongation. Cell orientation was defined as
the orientation of the major axis relative to the external direction of uniaxial strain.
For analyzing the orientation of Fn fibers, fluorescent phalloidin/Fn grayscale images
were pre-processed by normalizing their values between 0 and 1 and padding them in
all directions by 2 rows/columns. Filter kernels were constructed based on normalized
5x5 Sobel kernels in x direction (Sx) and y direction (Sy). Three filtered images were
created by convolution of the rescaled image with the 2nd derivative kernels Sx*Sx,
Sy*Sy, and Sx*Sy. Local orientation was computed for each pixel in the image by
taking the arcus tangens of the following combination
\[ \alpha = 1/2 \cdot \arctan\left(-2I[Sx*Sy]/(I[Sx*Sx] - I[Sy*Sy])\right) \]
resulting in angles between 0° and 180°. For restricting the orientation analysis to fibers, masks were generated by
applying a 3x3 Laplace filter to the grayscale fiber images, automatically thresholding
the filtered images by the method of Otsu, and binarizing the result. For non-zero
pixels in the masks, the angles in the corresponding orientation image were pooled.
For Fig. 6.2C, pixel angles larger than 90° were folded back onto the range from 0° to
90°, pixel values from several cells were pooled and depicted as histograms.

Nanopillar arrays were placed on petri dishes to enable the culture of the
MEFs. The MEFs were directly seeded on SU8 nanopillar arrays coated with
Fibronectin and Fn fragments (45nM for 2 hours). The displacement x of the
nanopillar tip induced by single MEFs was measured by Diatrack 3.03 (Powerful
Particle Tracking, Semasopht) and the force calculated according to Hooke’s law:
\[ F = k \cdot x \]
For time-lapse- and force generation experiments, Vybrant/Dil (Invitrogen,
1:200)-labeled MEFs (10⁴ cells ml⁻¹) were added onto the nanopillar structures. The
imaging process was started 2h after seeding the cells on nanopillars. To calculate the
traction force exerted by the MEFs on the nanopillar surface, the deflection of the
nanopillar tip was imaged with confocal microscopy using a Leica confocal
microscope SP5 with a 63x, oil immersion, NA1.43 objective. During image
acquisition, the MEFs were kept at 37°C and 10% CO2. The following laser
wavelengths were used to acquire images of nanopillar arrays and Di-labeled cells:
488 nm (to create a DIC image of nanopillar structures) and 546 nm (DiI). Cells were
imaged for a total time of 20 min, with a scanning ratio of 30 s per frame.

To determine the location of the old and new Fn matrices in Fig. 6.2D, the
boundary of a matrix was first outlined by thresholding (ImageJ, National Institute of
And also observed was whether the center of mass of the new matrix lies inside or outside of the old matrix.

6.5.8. *Generic MMP activity and gelatin zymography*

Soluble MMP activity was measured by the EnzoLyte™520 Generic MMP assay Kit (AnaSpec, U.S.A.). Gelatin and casein zymographies were performed according to the manual suggested by Invitrogen and the protocol published by Hu and Beeton (Hu and Beeton, 2010). Briefly, a culturing medium of each sample was harvested and spun at 2000rpm at 4°C for 10 minutes to eliminate dead cells and debris. Supernatant was then collected for gelatin or casein zymography. Without boiling or reduction by reducing agent, samples were mixed with Tris-Glycine SDS sample buffer (LC2676, Invitrogen, Switzerland) on ice and loaded to 10% Tris-Glycine gel with 0.1% gelatin (EC6175BOX, Invitrogen, Switzerland). Gels were run in the Tris-Glycine SDS running buffer (LC2675, Invitrogen, Switzerland) using XCell Surelock Mini-Cell system (EI0001, Invitrogen, Switzerland) at a constant voltage of 125V for 90 minutes. After gel electrophoresis, gels were renatured in 100ml renaturing buffer (LC2670, Invitrogen, Switzerland) with gentle agitation at room temperature for 30 minutes, followed by overnight incubation at 37°C in the development buffer. The next day, gels were carefully rinsed with deionized water three times and scanned to record the positions of molecular standard bands before SimplyBlue staining (LC6060, Invitrogen, Switzerland). If needed, stained gels were incubated in deionized water at room temperature for 30-60 minutes. The gels were scanned and saved as tiff files, then the intensity of the bands was analyzed by ImageJ.

6.5.9. *MMP inhibition by GM6001*

Fn<sup>−/−</sup> and Fn<sup>+/+</sup> MEFs were plated in the Fn coated PDMS chambers at 7,500 cells/cm<sup>2</sup>. For pulse samples, cells adhered and were allow to assemble matrices in DMEM (31966-021, Invitrogen, Switzerland) with 10% FBS (S181H, S04716S181H, Biowest, Switzerland) 1% Penicillin-Streptomycin (P/S) (15070063, Invitrogen, Switzerland) media supplemented with fluorescently labeled Fn at 50µg/mL for 24 hours. Samples were fixed in 4% PFA and mounted in prolong anti-fade mounting media before image acquisition. For chase samples, cells after 24 hours of matrix
assembly were changed to Fn depleted growth media, namely DMEM with Fn depleted 1% FBS 1% Penicillin-Streptomycin so that cells degrade the matrix for 24hrs. Fn was eliminated from FBS by passing FBS through gelatin columns twice. Fn depleted FBS was sterilized by 0.22µm filtering. 20µM GM6001 (M5939, Sigma, Switzerland) was added during the phase of matrix degradation to inhibit MMP activities. For vehicle control, the same volume of DMSO was added during the phase of matrix degradation.
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References


Chapter 7: Summary

In this thesis, the role of fibronectin in a uniaxial cyclically strain-induced extracellular matrix remodeling processes was investigated. In the first project, by mimicking tissue maturation, the focus was on the question addressing how the transition of Fn-rich early extracellular matrix to a collagen type I-decorated matrix regulates the matrix remodeling by human foreskin fibroblasts under dynamic loading, i.e. uniaxial cyclic strain (Chapter 5). In the second project, the aim was deciphering the contribution of pericellular fibronectin to the cellular involvement in cyclic strain-induced mechanosensing and matrix remodeling (Chapter 6).

Unlike the conventional static cell culturing, here, automatic cell stretcher was employed to apply uniaxial cyclic strain to cells, which were seeded onto elastic substrates. The details regarding choice of the stretcher, casting stretchable chambers, and evaluating the viability of cells in the stretcher was described in Chapter 3 Cell stretcher.

Fibronectin is the main molecule of interest in this thesis. Therefore, consistent high quality fibronectin was essential in the study. Affinity chromatography was optimized for fibronectin purification and consequent gelatinase elimination, and additionally the storage and labeling conditions were further characterized to ensure Fn quality. The details of this can be found in Chapter 4 Fibronectin purification, labeling and storage.

In the first project (Chapter 5), it was shown that the matrix composition, namely Fn and collagen type I rather than cyclic strain, determines the remodeling capacity of human foreskin fibroblasts (HFFs). HFFs subjected to uniaxial cyclic strain re-align, re-organized actin stress fibers and assembled Fn matrices along the minimum strain direction. However, the remodeling capacity of HFFs is not dependent on cyclic strain, indicated by MMP activities and gene expressions. Changing the matrix composition from a Fn-rich to a Col1-decorated matrix lowers the soluble MMP secretion, MMP-9 and also MMP-15 expression, which coincides with more Fn unfolding (strain release) in Col1-decorated matrix. Therefore, it is suggested that Fn conformational change acts as a mechanotransducer affecting the matrix remodeling capacity during the different stages of tissue maturation.
The second project (Chapter 6) studied the mechanisms by which exogenous Fn restores mechanosensing activities in Fn knockout mouse embryonic fibroblasts (Fn<sup>-/-</sup> MEFs) exposed to uniaxial cyclic strain. It was shown that exogenous Fn in the medium is essential in formation of actin stress fibers, maturation of focal adhesions and MMP activity under uniaxial cyclic strain, although it only responded in a subpopulation of Fn knockout fibroblasts. Full-length Fn, not Fn fragments, rescues cell traction forces to a normal level in Fn knockout fibroblasts, indicating that Fn fibrillogenesis is essential in traction force generation. However, strain-avoidance cell re-orientation was absent even in the presence of pericellular plasma Fn for Fn knockout fibroblasts, which are more migratory than the Fn floxed MEFs (Fn<sup>f/f</sup> MEFs), suggesting an intrinsic difference between Fn knockout fibroblast with exogenous Fn and Fn floxed fibroblasts. It is, therefore, suggested that the difference may be due to the presence of cellular fibronectin.

In summary, the studies demonstrate the crucial role of Fn in cyclic strain-induced ECM remodeling, by acting as a mechanotransducer via conformational changes to determine the ECM remodeling capacity in HFFs exposed to uniaxial cyclic strain. This is important in cyclic strain-induced cell contractility, focal adhesion formation, cell traction force generation and MMP activities.
Chapter 8: Outlook

The first project (Chapter 5) showed that the presence of collagen type I lowers HFF’s matrix remodeling capacity. Fibronectin conformation was investigated and it was suggested that partial strain release from the matrix is related to the reduced matrix remodeling capacity of human foreskin fibroblasts in a collagen-decorated matrix. What remains to be elucidated is whether the increasing collagen binding to cell surface receptors (such as integrin) triggers reduced a matrix remodeling potential. Therefore it will be of interest to check if collagen type I specific integrins α1β1, α2β1 and fibronectin receptors α5β1, αvβ3 are activated differently in cyclically-strained human foreskin fibroblasts in a fibronectin-rich and collagen-decorated matrix.

The second project (Chapter 6) showed how pericellular Fn restores cyclic strain-induced actin stress fiber and focal adhesion formations, cell traction forces and MMP activities in fibronectin knockout fibroblasts, but failed to rescue the strain-avoidance cell re-orientation. It has been speculated that two reasons contribute to this observation: the deficient recruitment of integrins and/or the absence of cellular fibronectin in ECM.

To test the hypothesis of lack of integrin α5β1 recruitment, immunofluorescence staining of integrin α5β1 in cyclic-trained fibronectin knockout fibroblasts in the presence of exogenous fibronectin will provide a direct answer to the question. In addition, it should be determined whether these integrins are required in regulating strain-avoidance re-orientation in floxed mouse embryonic fibroblasts. One method would be to functionally block integrin activity. However this may not result in a complete inhibition of integrin signaling, as other integrins can compensate in the overall activity upon functional blocking of one kind. The most elegant method would, therefore be to transiently knock down integrin genes in fibronectin floxed fibroblasts using siRNA method.

Regarding cellular fibronectin, in a setup similar to the one with plasma fibronectin, cellular fibronectin could be added exogenously to check whether the strain-avoidance strain orientation is restored. However, the exact effective concentration of cellular fibronectin would first need to be determined.
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EDUCATION
Eidgenössische Technische Hochschule Zürich Zürich, Switzerland
Ph.D. candidate in Health Sciences and Technology 2009-Present

Eidgenössische Technische Hochschule Zürich Zürich, Switzerland
M.Sc. in Materials Science, GPA 5.34 / 6.0 2005-2008

Columbia University, School of Engineering and Applied Science New York, U.S.A.
B.Sc. in Biomedical Engineering, Minor in Economics 2001-2005
GPA 3.632 / 4.0 (Cum Laude)

TECHNICAL SKILLS
Applications: AutoCAD, Studio Tools, MATLAB, Mathematica, Microsoft Office, Adobe Photoshop, COMSOL Multiphysics Image J, FJI, CS ChemOffice
Operating Systems: Windows and Macintosh OS
Lab Equipment: Confocal Microscopy, Strain Gage, EKG, EMG, RT-qPCR, Spectrometer
Lab Techniques: Photolithography, Surface Silanization, basic protein conjugation, Animal Cell Culture, Immunofluorescence Staining, Cryosectioning, immunohistostaining, Liquid Chromatography, Western Blotting, SDS-PAGE, Zymography

AWARDS
• Travel Grant for Summer School Cellular Systems, University of Heidelberg (2011)
• MatLife Master Thesis Program, CCMX Switzerland (2007-2008)
• Solidarity Fund for Foreign Students, ETH Zürich (2006-2007)
• Graduation with honors, Columbia University (2005)
• Summer Undergraduate Research Fellowship, Columbia University (2004)
• Dean’s List, Columbia University (Spring 2002-Fall 2003, and Fall 2004)
• Regional Champion and National Top 16 Alliances, 2000 FIRST Robotics Competition (2000)

PUBLICATION AND PRESENTATION
• Yang Zhang, Zhe Lin, Jau-ye Shiu, Alberto Santoro, Ingmar Schoen, Viola Vogel (2014) “Presence of Fn and Type I collagen, not cyclic strain, determines the remodeling capacity of fibroblasts by regulating MMP activity” (in preparation)
• Bojun Li, Zhe Lin, Maria Mitsi, Yang Zhang, Cameron Moshfegh and Viola Vogel (2013) “Heparin induced conformational change of Fibronectin promotes hMSC osteogenic differentiation” (submitted).
• Presentation: Yang Zhang (2011) “Extracellular matrix under mechanical strain - the missing link for improving soft tissue replacements”, Cellular Systems, University of Heidelberg
• Presentation: **Yang Zhang** (2011) “Project overview: Fibronectin Matrix remodeling under cyclic mechanical strain”, D-HEST opening day, ETH Zürich
• Online publication: **Yang Zhang** and Viola Vogel (2009) “Engineering Unloading Stations for Molecular Shuttles”, CCMX Switzerland

**LABORATORY EXPERIENCE**

**Laboratory for Applied Mechanobiology**, Department of Health Sciences and Technology, Department of Materials Science, ETH Zürich, Zürich, Switzerland  
*Research and teaching assistant*, Dec. 2008-Present  
A five-year Ph.D. project to investigate how the remodeling of fibronectin and collagen, together with cyclic mechanical strain can regulate the behavior of human dermal fibroblasts to minimize scar formation.

**Laboratory for Biologically Oriented Materials**, Department of Materials Science, ETH Zürich, Zürich, Switzerland  
Combined organic and inorganic compounds to engineer microtubule-kinesin based molecular shuttle unloading stations by using photolithography and surface chemical functionalization. Further Ph.D. students attempted designing a lab-on-a-chip device based on this thesis and previous research.

Under the supervision of a postdoc in chemistry, independently carried out experiments to test and compare the differences in microtubule motility and morphology on five covalently-modified silanized glass surfaces.

Assisted a team within the Molecular Shuttles Project for Tau protein modification on microtubule behavior to enhance the understanding of Alzheimer’s disease *in vitro*.

**The Bone Bioengineering Laboratory**, Department of Biomedical Engineering, Columbia University, New York, NY, U.S.A.  
A semester of undergraduate research study, during which the team effort was focused on constructing and optimizing three-dimensional microfluidic channels using polydimethylsiloxane (PDMS) for feasible osteoblast culture environments.

**The Sheetz Lab**, Department of Biological Sciences, Columbia University, New York, NY, U.S.A.  
*Summer Research Fellow*, Jun. 2004-Aug. 2004  
Assisted postdoctoral fellow in the project “Studying Polyvalent Interactions in Cells by Using Nano-scale Bioarrays”. Independently performed photolithography fabrications and fluorescence microscopy observations to minimize non-specific binding.

**OTHER EXPERIENCE**

**ETH (Art and Science) Cortona Week**, Cortona, Italy  
Introduced the guest speaker Literature Professor Prof. Keller to an audience with mainly scientific background and led the panel discussions of her talk “Of Mice, Men and Metaphors”.

**The Department of East Asian Languages and Cultures**, Columbia University, New York, U.S.A.  
*Assistant*, Fall 2004-Spring 2005
Assisted distinguished professor De Barry and his assistants in his teaching of east Asian language and culture studies in parallel to my study of Biomedical Engineering.

**China Merchants Dichain (Asia) Limited**, Shenzhen, Guangdong, China
*Financial Intern*, Summer 2003
Assisted teams in different phases of product development in a two-month financial internship in logistics, and learned the importance of client-developer relationship in the final success of project completion.

**Columbia Video Network**, Columbia University, New York, NY, U.S.A.
*Cameraman*, Spring 2002
Attracted by the concept and the rapid development of long-distance teaching, joined the work-study program offered by Columbia Video Network.

**TEACHING EXPERIENCE**

Department of Health Sciences and Technology, ETH Zürich, Zürich, Switzerland
Designed and supervised a six-month master thesis in Biomechanics entitled “Regulation of tissue inhibitor of metalloproteinases on extracellular matrix degradation under mechanical stretching”.

**Teacher for Laboratory Course I and II**, 2010-2011
Department of Materials Science, ETH Zürich, Zürich, Switzerland
Introduced the theoretical background of soft lithography and taught undergraduate students microcontact printing and chemical etching techniques over two semesters.

**Teacher for Laboratory Course III**, 2009
Department of Materials Science, ETH Zürich, Zürich, Switzerland
Introduced undergraduate students to the concept of independent research. Practiced with students aseptic techniques, mammalian cell culture, chemical fixation and immunofluorescence staining.

**LANGUAGES**
Fluent English
Native Chinese (Mandarin)
Basic German