Matrix Stiffness-dependent Regulation of Integrin α11β1 Expression

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This thesis is submitted in partial fulfilment of the requirements for the degree of Master in Biomedical Sciences

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University of Bergen

2023

Acknowledgements

I am truly thankful towards my supervisor Professor Donald Gullberg for allowing me to conduct my project in his research group. His impressive knowledge within the field has aided me with my writing and presentation skills within the lab, and improved my ability to be more efficient within my work. His guidance has also allowed me to become a more self-sufficient individual. Our meetings have always been productive, with his main goal of assuring that whatever problem I might have is delt with swiftly such that I can produce the best results possible. For this, I am greatly appreciative.

Throughout my experiments, my co-supervisors Cédric Zeltz and Moses Musiime mentored me with patience and the aim of assuring that I truly understood the fundamentals of what was being done. No matter the issue, they would allow me to try and find the answer myself, and award me for it. My ability to conduct this thesis would have not been possible without their guidance and support.

As the first person to teach me several important lab techniques, I hold Mona Grønning in high regard as always being there when I needed assistance. In addition, her ability to remember birthdays is unmatched.

I would also like to thank Professor Marion Kusche-Gullberg for her feedback on several pieces of work I have done including my 8-week project conducted in her lab, this thesis and my lab meeting presentations.

During my project, Jana arrived for a five month internship at our lab. With her here, there was always a feeling of healthy competition which motivated me further. In the lab, something definitely felt missing when she left.

Through the fun talks we had during the final stages of my project, and cheering me on as I wrote, I would also like to thank Sabrina. Even though you were in a different city, you still demanded the best from me.

Finally, I must express my profound gratitude towards my father Henrik, my mother Sudi, and my brother Armand for their unconditional love and support through these years of my higher education. This thesis is in dedication to them.

Abstract

Stiffness regulation within the tumour microenvironment plays a pivotal role in tumorigenesis. In this context, integrin α11β1 is a collagen receptor expressed on cancer-associated fibroblasts (CAFs) involved in matrix reorganization. Integrin α11 expression is often upregulated in the stroma of desmoplastic cancers, where integrin α11 can promote tumour progression. The present study aimed at understanding how matrix stiffness regulates integrin α11 expression in fibroblasts. For this purpose, we took advantage of polyacrylamide hydrogels functionalized with matrix ligands of varying stiffness. Surprisingly, BJ fibroblasts and lung CAFs seeded on soft collagen matrices displayed higher integrin α11 expression compared to cells seeded on stiff matrices. Moreover, we found that integrin α11 expression was controlled at the transcriptional level in a collagen I-dependent manner. Our data furthermore suggests that the TGF-β, FAK and Erk signalling pathways are involved in the stiffness-dependent modulation of integrin α11 expression. Altogether, these results highlight a novel regulation of integrin α11 expression, which contributes to our understanding of its role in tissue and tumour fibrosis.

Contents

Acronyms and Abbreviations

A/A	Antibiotic-Antimycotic
ANOVA	Analysis of Variance
APS	Ammonium Persulfate
ATCC	American Type Culture Collection
αSMA	α-Smooth Muscle Actin
CAF	Cancer-Associated Fibroblast
cCAF	Cycling Cancer-Associated Fibroblast
cDNA	Complementary DNA
dCAF	Developmental Cancer-Associated Fibroblast
DDR2	Discoidin Domain Receptor 2
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-to-Mesenchymal Transition
Erk	Extracellular Signal-Regulated Kinase
FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FAP	Fibroblast Activation Protein
FBS	Foetal Bovine Serum
GPa	Gigapascal
HCC	Hepatocellular Carcinoma
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase
hTERT	Human Telomerase Reverse Transcriptase
iCAF	Inflammatory Cancer-Associated Fibroblast
I-domain	Inserted Domain
IL-6	Interleukin 6
IL-10	Interleukin 10
KO	Knockout
kPa	Kilopascal
L-DOPA	L-3,4-dihydroxyphenylalanine
LOX	Lysyl Oxidase
LOXL1	Lysyl Oxidase-Like 1
mCAF	Matrix Cancer-Associated Fibroblast
MAPK	Mitogen-Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
MEK	Mitogen-Activated Protein Kinase Kinase
MIDAS	Metal Ion-Dependent Adhesion Site
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9

MMP-13	Matrix Metalloproteinase -13
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stem Cell
MT1-MMP	Membrane-Type 1 Matrix Metalloproteinase
myCAF	Myofibroblastic Cancer-Associated Fibroblast
NSCLC	Non-Small Cell Lung Cancer
O/N	Overnight
Pa	Pascal
PBS	Phosphate Buffered Saline
PDL	Periodontal Ligament
qPCR	Quantitative Real Time-Polymerase Chain Reaction
R/T	Room Temperature
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Smad	Suppressor of Mothers Against Decapentaplegic
SV40	Simian Virus 40
TBS-T	Tris Buffered Saline-Tween
TEMED	N, N, N′, N′-Tetramethylethylenediamine
TGF-β	Transforming Growth Factor-β
TGF-βR	Transforming Growth Factor-β Receptor
TAZ	Translational Coactivator with PDZ-Binding Motif
TKR	Tyrosine Kinase Receptor
TME	Tumour Microenvironment
vCAF	Vascular Cancer-Associated Fibroblast
WT	Wild Type
YAP	Yes-Associated Protein

1 Introduction

1.1 The tumour microenvironment

The tumour microenvironment (TME) plays a critical role in the progression of tumour development, providing an environment, which supports tumour growth, cancer cell invasion and metastasis[1]. In order to fully grasp how this microenvironment propels tumour growth forward, the different aspects involved must be discussed and their role understood. Several factors are involved in the formation of this environment, including different cell types (immune cells and different types of stromal cells), the extracellular matrix (ECM) as well as altered cellular signalling[2] (**Figure 1.1**).

Immune cells are critical in the TME, where they can either promote or suppress tumour growth. Among the immune cells, cytotoxic T-cells are detectors of tumour antigens expressed on cancer cells and target them for degradation. Therefore, the presence of T-cells in the TME is often correlated with a positive prognosis in cancer patients[3]. B-cells have also been found to play a role in tumorigenesis, through for example production of interleukin 10 (IL-10) and transforming growth factor-β (TGF-β)[4]. Stromal cells present in the TME include fibroblasts, vascular endothelial cells, pericytes and adipocytes. They can secrete different factors which influence angiogenesis, tumour cell proliferation and metastasis[5]. When the TME becomes hypoxic, endothelial cells increase angiogenesis, which is the formation of new blood vessels. This then allows for nutrient and water delivery to the tumour, assisting in growth[6].

1.1.1 Fibroblasts

During normal development, fibroblasts have been shown to be the main cell type responsible for the ECM production of connective tissue[7,8]. During tissue repair, fibroblasts produce and activate the growth factor TGF-β, to assume a contractile phenotype through the expression of α-smooth muscle actin (αSMA). In this activated state, they are termed myofibroblasts[9]. Myofibroblasts have an active role in ECM deposition and reorganization. Fibroblasts express cell surface receptors such as integrins, which gives cells the ability to sense the extracellular environment and to regulate specific signalling pathways through mechanotransduction[10]. When fibroblasts are present in the TME, they are named cancer-associated fibroblasts (CAFs).

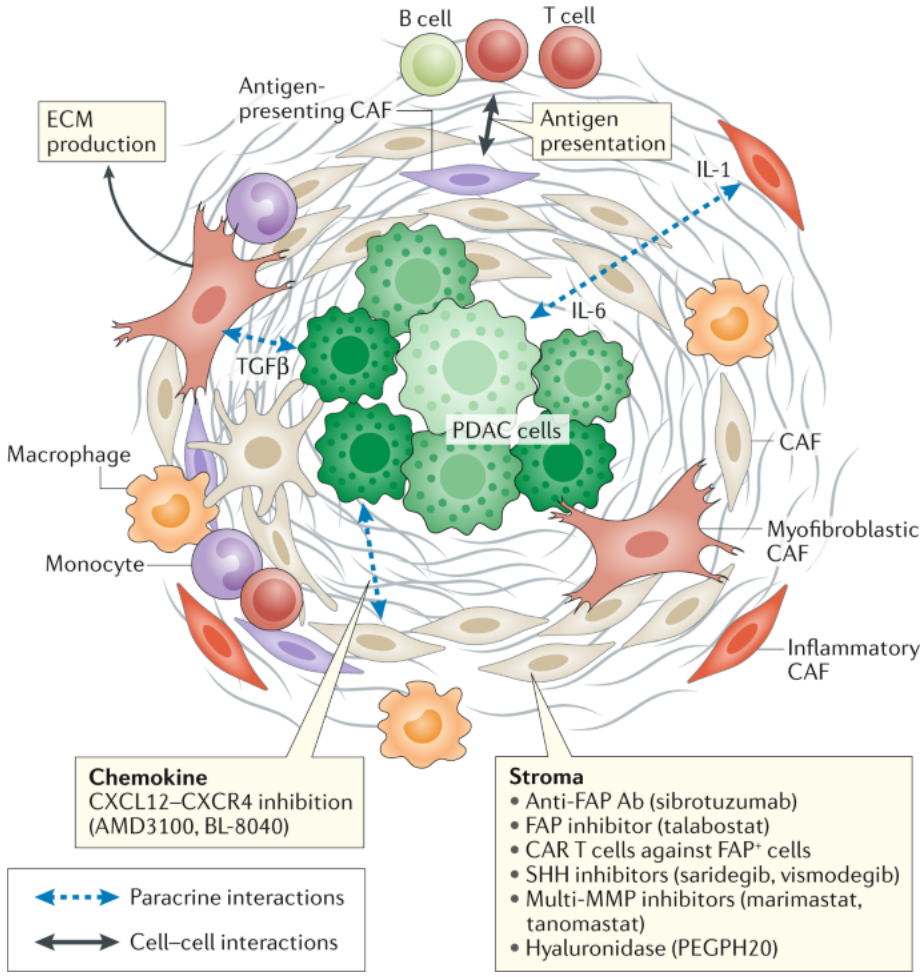

Figure 1.1: The role of the stroma in promoting or resisting tumour progression regarding surrounding signals. *Modified from W. Ho et al.* [11].

1.1.1.1 Cancer-associated fibroblasts

The origins of CAFs are not fully understood, and their definition is not simple. They are of a mesenchymal origin, and are non-vascular, non-epithelial and non-inflammatory[12]. There are multiple variables, which can induce this phenotype, such as inflammatory signals, physiological stress, TGF-β expression and altered ECM composition and stiffness[7,13]. An important distinction is that CAFs are not tumour cells themselves, but they play an important role in the formation of the TME to promote tumour progression. Like with differentiated fibroblasts, CAFs can also express αSMA and fibroblast activation protein (FAP)[12]. A key differentiation between fibroblasts and CAFs is the increased amount of extracellular proteins, which CAFs deposit into the ECM, leading to fibrosis[14]. It has been demonstrated that CAFs are heterogenous, mainly attributed to the many possible cellular sources[13,15]. From recent research, the majority of CAFs are derived from local fibroblasts[16]. Furthermore, mature

adipocytes have been shown to be activated by the Wnt/β-catenin pathway, causing dedifferentiation into a fibroblast-like phenotype[17].

1.1.1.2 CAF heterogeneity in the TME

As previously mentioned, CAFs are heterogeneous and it is thus important to consider that different CAF subpopulations have different roles in the TME. Nurmik and colleagues defined the different CAF subtypes as 'states' instead of a fixed cell type[18]. The cellular origin of CAFs has been suggested to define their phenotype. They can emerge from cell types including resident-tissue fibroblasts, pericytes, adipocytes, hematopoietic stem cells, mesenchymal stem cells (MSC), epithelial cells and endothelial cells[19,20].

Tissue specific CAF subpopulations have been shown to be involved in breast, colorectal and pancreatic cancer development[21–24]. In pancreatic cancer, Öhlund *et al.* proposed the existence of two mutually exclusive subtypes of CAFs, named myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs)[25]. myCAFs were defined by their high FAP and αSMA expression and are located near tumour cell nests. iCAFs, however, were defined as having low αSMA expression and high interleukin 6 (IL-6) expression, and are located in the desmoplastic area[25]. In breast cancer, Kanzaki and colleagues identified four subpopulations of CAFs; matrix CAFs (mCAFs), vascular CAFs (vCAFs), cycling CAFs (cCAFs) and developmental CAFs (dCAFs). The gene expression profile of vCAFs showed a vast number of genes functionally linked to vascular development and angiogenesis. The mCAF was enriched with transcripts in relation to the ECM and EMT. dCAFs were distinguished by the expression of stem cell related genes[26]. Li *et al.* further characterized 2 subtypes from a specimen gathered from colorectal cancer patients, naming the subtypes CAF-A and CAF-B. CAF-A expressed ECM remodelling genes including the TGF-β activator matrix metalloproteinase-2 (MMP-2). CAF-B cells expressed myofibroblastic markers including transgelin (*TAGLN)* and αSMA (*ACTA2*)[27,28].

1.1.2 The extracellular matrix

The ECM is part of connective tissues and is formed by a complex network of macromolecules such as collagens, glycoproteins, elastin and proteoglycans. It provides a physical scaffolding for the cells, but also activates important biomechanical cues for tissue differentiation and homeostasis[29,30]. The biophysical interactions between ECM proteins and cells involve specific receptors that allow cells to sense their extracellular environment and adapt accordingly

through mechanosensing via the regulation of signalling pathways[31]. Through these mechanisms, the ECM can maintain its organization in order to ensure correct organ function[30].

The ECM is extremely important in relation to oncogenesis as overexpression of ECM proteins have shown to be correlated with cancer[30]. ECM organization within the TME has also been shown to be associated with cell behaviour through its mechanical properties. The higher production of ECM proteins such as collagens and fibronectin has been linked to increased stiffness of the surrounding environment, hence altering cell signalling[32]. This increase in ECM protein deposition brings disorder to the structural organization of the ECM, increasing the possibility of intravasation and metastasis. Collagen overexpression, reorganization and cross-linking within the TME has been associated with a higher risk of cancer progression[33]. However, deletion of collagen type I in cells expressing αSMA in a pancreatic cancer mouse model increased tumour growth, supporting that the tumour stroma can also operate as a restraining barrier[34].

1.1.3 Tissue stiffness

Increased deposition and reorganization of ECM proteins can result in an increase in tissue stiffness. Tissue stiffness is seen as the measure of a tissue's resistance to deformation under stress, where these changes in the environment can have a significant impact on the development and further progression of the TME[35]. High collagen density enhances tumour incidences in mouse models, further implying that a stiff matrix as a result of increased collagen deposition is an important factor in tumour formation[36]. The literature suggests that on stiff matrices, the cells undergo a morphological change[37]. Paszek and colleagues observed that cultured epithelial cells on a matrix that mimics stiffness of tumour tissues lead to an increase in cytoskeletal tension, thus altering tissue polarity, disrupting proper lumen formation and increasing tumour growth[38]. Ondeck and colleagues further showed that mammary cell spheroids on a matrix of varying stiffness displayed morphological changes; the cells lose epithelial characteristics to partially gain a mesenchymal phenotype, similar to some morphologies observed in tumours[39]. A stiff environment is prone to promote tumour cell proliferation. High matrix stiffness has been shown to increase the proliferative ability of hepatocellular carcinoma (HCC) cells, lung, pancreatic and colorectal cancer cells[35,40–44]. Furthermore, a stiff matrix can further affect stromal cell function, like CAF differentiation, to ultimately stimulate tumour growth[45].

Cell migration and invasion are also important steps for cancer progression that can lead to metastasis, where tissue stiffness plays a role. Several studies have shown that a stiff matrix results in a migrating phenotype in osteosarcoma, HCC, lung, colorectal, breast and ovarian cancer[41,46–51]. In one study, Dai and colleagues reported that a stiff matrix triggers epithelial-to-mesenchymal transition (EMT) and subsequently facilitates the invasion of cancer cells[51]. *In vitro* migration assays further showed that cells on a stiff 2D matrix migrate more actively compared to those on a soft 2D matrix[50–53].

In HCC, lung and mammary cancer, matrix stiffening enhances the metastatic potential of tumor cells[35,47,54]. Interestingly, a stiff matrix can alter the cell surface protein expression on endothelial cells, further increasing intravasation of cancer cells and promoting metastasis[55]. It has also been demonstrated that the metabolic rewiring between CAFs and cancer cells is regulated through matrix stiffening, further increasing the possibility of metastasis[56].

TGF-β signalling is one of the main pathways that contribute to tissue stiffness. Stimulation of this pathway leads to the activation of Smad (Suppressors of mothers against decapentaplegic) proteins, which translocate to the nucleus and regulate the transcription of target genes involved in collagen synthesis and deposition[57,58].

1.2 Integrins

Integrins are transmembrane heterodimeric cell receptors involved in cell-, ECM- and pathogen-cell contacts. Integrins act as links between the ECM and the cytoskeleton of the cell. Through signalling, integrins can regulate the adhesive strength of the cell, thereby playing important functions in cell adhesion, cell migration, cell proliferation, cell differentiation and gene expression[59].

1.2.1 Structure

The integrin family of heterodimers is composed of 18 α and 8 β subunits which can dimerize to form 24 defined integrins with varying ligand specificity based upon the subunit combination[12]. Both subunits include an extracellular 'head', a rod-like 'leg', a transmembrane helix and a cytoplasmic tail[60]. The literature describes three main states of integrin conformations (**figure 1.2**); bent-closed, extended-closed and extended-open. In its bent-closed conformation, ligand binding is greatly inhibited with the head group being closed. Upon

activation, integrins undergo conformational changes. In the extended-open conformation, the receptor has a much greater affinity for binding to ligands such as collagen in the ECM[60].

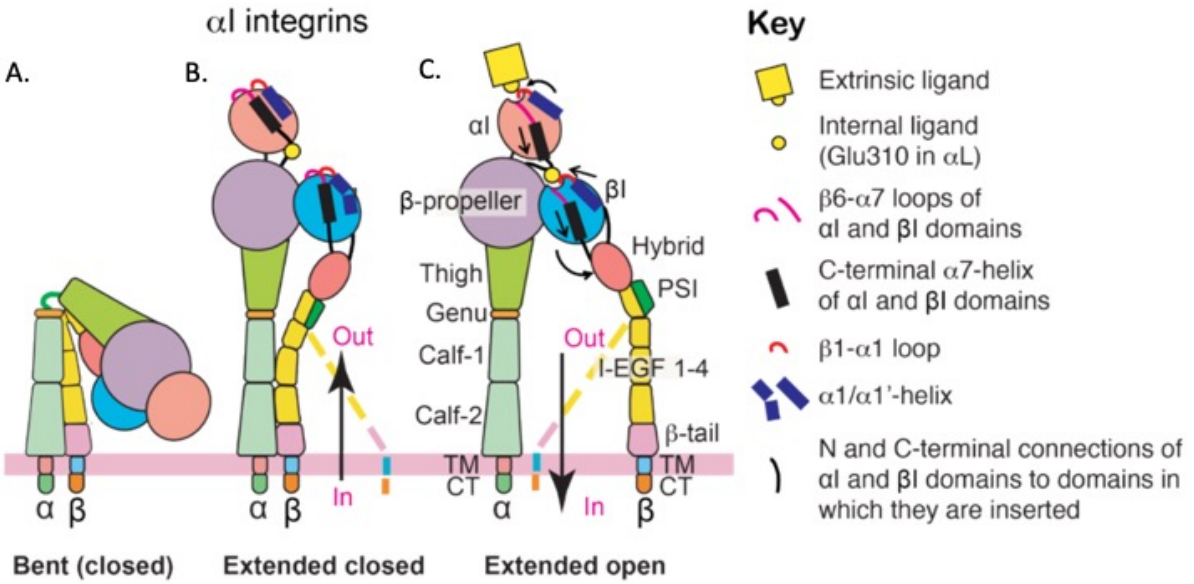

Figure 1.2: Schematic of an αI-domain integrin in different conformations*. (a): Low affinity conformation (b): Intermediate affinity conformation and (c): High affinity conformation. Modified from H. Zhang et al.* [61].

1.2.2 Signalling

Integrin signalling is bi-directional, where both outside-in and inside-out signalling is involved. In inside-out signalling, intracellular adapter proteins such as talins and kindlins are recruited to the integrin β-subunit cytoplasmic tail leading to an extended-open conformational change for higher extracellular ligand affinity[62]. In outside-in signalling, ligand binding to integrins trigger numerous intracellular signalling cascades, which are cell and context specific[63]. The focal adhesion kinase (FAK) is one of the main signalling molecules recruited following ligand binding.

Signalling crosstalk between integrin and tyrosine kinase receptors (TKRs) is a well-known phenomenon which plays a major role in regulating functions such as cell adhesion and migration[64]. There are direct and indirect interactions between integrins and TKRs which may occur via different mechanisms including physical association, recruitment and sharing of signalling intermediates and/or the modulation of each other's activity[65]. A good example of integrin and TKR crosstalk is through FAK signalling[12]. Integrin-mediated activation of FAK can increase the activity of the epidermal growth factor receptor (EGFR) via the phosphorylation and further recruitment of downstream signalling molecules such as the

GTPase Ras, Mitogen-activated protein kinase kinases (MEKs) and Extracellular signal-regulated kinase (Erk), where their activation can prevent apoptosis and sustains cell proliferation[66,67].

1.2.3 Mechanotransduction

Integrins act as a link between the ECM, through the plasma membrane, to the actin cytoskeleton of the cell to communicate forces[68]. Having this connection results in a force balance where equal force is exerted from the ECM and cytoskeleton on the integrin. The force transmission exerted from the ECM goes through the integrin cytoplasmic tail to actin binding adaptor proteins including vinculin and talin, which then transmit the force on the actin cytoskeleton[69]. As with forces outside the cell acting inwards, this also applies from within the cell and out, whereby the actin cytoskeleton, via polymerization or myosin contraction, can apply force to the ECM. However, if integrins are not bound by either actin or the ECM, this will cause sliding of integrins along the membrane, inhibiting force transmission[70]. Interestingly, two types of bonds are associated with the ECM-integrin interaction, "slip bonds" and "catch bonds". During applied forces, like those resulting from a higher tissue rigidity, "slip bonds" weaken, while "catch" bonds are strengthen. This leads to an interplay between integrins and the ECM as the bond between them is often a combination of both, namely a "catch-slip" bond[71,72]. Through this mechanism, it is proposed that matrix stiffness promotes integrin clustering and formation of focal adhesion (FA) complexes, further amplifying signalling pathways[73] (**Figure1.3)**.

Specific signalling pathways, such as the Hippo pathway, which encompasses the downstream transcription factors Yes-associated protein (YAP) and Transcriptional coactivator with PDZ-binding motif (TAZ), are modulated by mechanical forces[74,75]. Stiffness can contribute to the inactivation of the Hippo pathway, leading to dephosphorylation of YAP and TAZ and their translocation to the nucleus, where they drive transcriptional activity[76]. In several cancers, YAP/TAZ display an increase in nuclear activity contributing to tumour tissue growth[77].

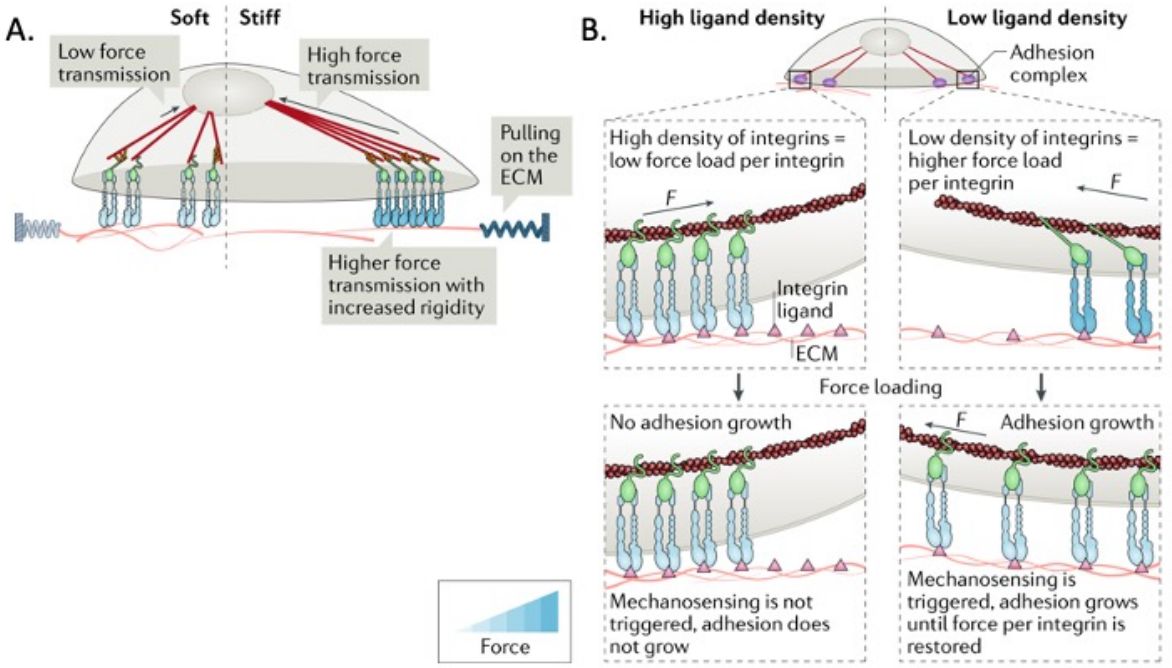

Figure 1.3: Integrins mediating responses to ECM signals such as stiffness. *A: Cells pulling on the ECM through integrin adherence and actomyosin contraction. B: A reduction in ECM ligand density decreases the amount of bound integrins hence each integrin experiencing a higher fraction of force. Modified from Kechagia et al.* [72].

1.2.4 Collagen-binding integrins

Among the 24 members of the integrin family, four integrins have a specificity for the most abundant ECM constituent, collagen, namely integrins α1β1, α2β1, α10β1 and α11β1[78]. Collagen-binding integrins interact with collagen via the inserted domain (I-domain) present in the α subunit. They recognize the specific collagenous motif, GFOGER, when collagen is in its triple-helical form[79]. Integrins α1β1 and α2β1 are expressed on a variety of cell types, whereas α10β1 expression is usually limited to chondrocytes in cartilage[80]. Integrin α11β1 is found on mesenchymal cells such as fibroblasts[81]. Although the four integrins recognize the same ligand and share the same β1 subunit, they bind with different affinity and show distinct roles[82].

1.2.5 Integrin α11β1

Integrin α11β1 is of high interest as recent research has shown that it is involved in granulation tissue formation during tissue repair, it is pro-fibrotic, and it is pro-tumorigenic in the lung and breast cancer stroma[35,83].

1.2.5.1 Structure of integrin α11β1

The *ITGA11* genes encodes an 1188 amino acid long protein. Through a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), it shows as a 150kD band, which indicates a higher level of glycosylation compared to the α2 and α10 collagen-binding integrins[84]. The extracellular domain of the α11 chain contains seven FG-GAP repeats with a 195 amino acid long I-domain inserted between repeats 2 and 3. A metal ion-dependent adhesion site (MIDAS) motif as well as three possible divalent cation binding motifs are present on the α11 I-domain. Further, the 24 amino acid long cytoplasmic tail contains the GFFRS motif rather than the more common GFFKR sequence seen in a majority of integrin α subunits[85,86].

1.2.5.2 Expression of integrin α11β1

Integrin α11β1 was first identified in cultured human foetal muscle cells[86], however, further research showed that integrin α11β1 is expressed by fibroblasts in muscle tissue. It is now clear that integrin α11β1 integrin is expressed in mesenchymal cells identified as fibroblasts, myofibroblasts and mesenchymal stem cells. In 8-week old human embryos, integrin α11β1 is present in ribs, vertebrae and intervertebral discs[87]. In mouse embryos, integrin α11β1 is localized to the ectomesenchyme in the head, tendons and intestinal villi fibroblasts[88]. In adult tissues, integrin α11β1 expression is low, but it is upregulated in some pathological conditions[89,90].

1.2.5.3 *In vitro* functions of integrin α11β1

The first study demonstrating that integrin α11β1 promotes cell attachment to collagen I was published in 2001[87]. This study also showed that integrin α11β1 displays a specificity for collagens, preferentially binding to collagen type I while it interacts with collagen type IV with a lower affinity. The I-domain of integrin α11β1 recognizes the GFOGER and the GLOGER sequences within collagen I[91,92].

Current knowledge proposes that the role of integrin α11β1 in cell migration is cell type dependent. An example of this is the disparity between C2C12 mouse myoblasts and mouse embryonic fibroblasts (MEFs). Tiger C-F *et al.* stably transfected C2C12 cells with human integrin α11 and observed more migration compared to un-transfected cells. In contrast,

Popova *et al.* studied MEFs, which were depleted in integrin α11β1, and saw an increase in migration across collagen I coatings[87,88].

Integrin α11β1 also plays a role in myofibroblast differentiation and in reorganization of collagen matrices[87,93–96]. It achieves this by interacting with other receptors such as the TGF-β receptor (TGF-βR) and to activate specific signalling pathways within the cell[8].

1.2.5.4 *In vivo* functions of integrin α11β1

To better understand the *in vivo* function of integrin α11β1, an integrin α11 knockout (KO) mouse model has been generated. When compared to wild-type (WT) mice, integrin α11β1-deficient mice are smaller[95]. Rather than structural defects in cartilage or bone formation, the dwarfism observed in the integrin α11β1-deficient mice is correlated with a delay in incisor eruption. Playing a pivotal role during incisor eruption, the incisor periodontal ligament (PDL) was observed to be thicker due to an increase in collagen levels. Messenger ribonucleic acid (mRNA) levels of matrix metalloproteinases including membrane type 1-matrix metalloproteinase (MT1-MMP) and matrix metalloproteinase-13 (MMP-13) were also shown to be down-regulated. However, integrin α11β1-deficient MEFs isolated from KO embryos demonstrated that, despite downregulation of MT1-MMP and MMP-13 mRNA, the expression of MMP-2 and matrix metalloproteinase-9 (MMP-9) remained unchanged. It was thus proposed that integrin α11β1 regulates specific matrix metalloproteinases in order to control collagen turnover within the PDL and during matrix remodelling[84].

In a study that induced excisional wounds on both WT and KO mice, it was found that integrin α11β1 expression was strongly induced in the WT mice, while the KO mice exhibited reduced formation of granulation tissue and decreased tensile strength[97]. Integrin α11β1 was hence considered to contribute to the formation and function of skin repair *in vivo*[97,98]. It is well documented that collagen reorganization is an active process during wound healing, in particular during ECM remodelling which is distinguished by scar formation[71]. Studies which focus on wound healing in integrin α11 KO mice illustrate that α11β1 is crucial in collagen reorganization *in vivo*[97]. Further *in vivo* studies have been conducted to investigate whether α11β1 has a role in tissue fibrosis. The same study referenced for wound healing also demonstrated that Itga11$^{-/-}$ mice were protected from damaging dermal fibrosis, while α1 or α2 KO mice still developed fibrosis[98].

Further, overexpression of integrin α11 induces cardiac fibrosis in mice according to Romaine *et al*[83]. This is caused by alterations in intracellular hypertrophic signalling, which increases collagen production in the heart, leading to more fibrotic tissue formation[83].

1.2.5.5 Integrin α11β1 in cancer

As a major collagen-binding integrin α chain expressed on fibroblasts, integrin α11 is overexpressed in the tumor stroma of several desmoplastic cancers[12,71].

In non-small cell lung cancer (NSCLC), integrin α11β1 regulates collagen matrix stiffness and promotes tumorigenicity. Its expression is increased in the TME of NSCLC, in correlation with the upregulation of lysyl oxidase like 1 (LOXL1)[35]. LOXL1 belongs to the family of lysyl oxidases (LOXs) that are enzymes responsible for collagen crosslinking, which stiffens tissue and influences tumour progression and metastasis[46].

Excessive fibrosis in breast tissue is correlated with an increased risk of breast cancer. Integrin α11β1 was found to be associated with platelet-derived growth factor receptor β (PDGFRβ) in histological analysis of clinical breast cancer samples and in the preclinical transgenic PymT mouse breast cancer model. This crosstalk between integrin α11 and PDGFRβ in CAFs promoted breast tumor invasion[12].

Significant overexpression of α11β1 in fibrotic environments and its role in effecting tissue stiffness and myofibroblast differentiation suggest a possible mechanism of pathogenicity and therapeutic target[35].

1.2.5.6 Integrin α11β1 and matrix stiffness

There are only few studies that relate integrin α11β1 to matrix stiffness. Carracedo and colleagues observed that integrin α11β1 expression was dynamic in simian virus 40 (SV40) immortalized MEFs under matrix reorganization. They showed that, in cells under strained conditions in a 3D environment, integrin α11β1 expression is upregulated, whereas it is decreased in cells embedded in a softer matrix[96]. During both healthy and pathological conditions, matrix stiffness influences the activation and regulation of several pathways. As mechanotransducers, integrins are vital for force transmission, however much remains to uncover regarding the effect of tissue stiffness on integrin α11β1 expression and function.

1.3 Project aims

This project aimed at understanding how matrix stiffness regulates integrin α11 expression in fibroblasts. The goals were to:

- Determine whether the regulation of integrin α11 on different stiffnesses is dependent on collagen receptors.

- Determine whether integrin α11 expression is regulated at the transcript level or at the protein level.

- Investigate the potential signalling pathways involved in the stiffness-dependent regulation of integrin α11 expression.

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

Cell line	**Origin**	**Cell type**	**Source**
BJ hTERT	Human foreskin	Fibroblast	ATCC
CAF hTERT	Human NSCLC	Fibroblast	Ming Tsao Lab, UHN, Canada[35]

2.1.2 Buffers

Solution	**Composition**
Tris Buffered Saline-Tween (TBS-T)	25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.1 % Tween-20, pH 7.4
Dulbecco's Phosphate Buffered Saline (PBS)	137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4
10 x SDS running buffer (Bio-Rad, USA)	25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3
Polyacrylamide separating gel buffer	2 M Tris-HCl pH 8.8
Polyacrylamide stacking gel buffer	0.5 M Tris-HCl pH 6.8
HEPES buffer	0.1 M HEPES/0.1 M NaCl pH 8.0
Stripping buffer	62.5 mM Tris/HCl, 2 % SDS, 100 mM β-Mercaptoethanol, pH 6.8
RIPA lysis buffer	50 mM tris pH 8.0, 50 mM NaCl, 1 % Triton-100, 0.5% sodium deoxycholate, 0.1 % SDS, 1 % complete mini protease inhibitor cocktail (Sigma-Aldrich, USA)
4 x XT Sample buffer	Bio-Rad, USA, 1610791

2.1.3 Reagents

Reagent	**Manufacturer, Lot Number**
β-Mercaptoethanol	Sigma-Aldrich, Norway, M7154

Instant non-fat dry milk	Demoulas Supermarkets Inc., USA
Acrylamide/Bisacrylamide 30 %, 37,5:1	Bio-Rad, USA, 1610158
Acrylamide 40 %	Bio-Rad, USA, 1610140
Bisacrylamide 2 %	Bio-Rad, USA, 1610142
TEMED (N,N,N',N'-Tetramethylethylendiamine)	Sigma-Aldrich, Norway, 1.10732
Ammonium Persulfate (APS)	Sigma-Aldrich, Norway, 248614
Pierce™ ECL Western Blotting Substrate	Thermo Fisher Scientific, USA, 32106
Precision Plus Protein™ WesternC™ Blotting standards	Bio-Rad, USA, 1610399
3-Aminopropyltrimethoxysilane	Acros Organics, USA, 313251000
NaOCl	Honeywell, USA, 71696
Glutaraldehyde 70 %	Sigma-Aldrich, Norway, G7776
I-3,4-Dihydroxyphenylalanine (L-DOPA)	Alfa Aesar, Germany, A11311
Trizma base	Sigma-Aldrich, Norway, T1503
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Norway, D2650
Ethanol, 99 %	VWR chemicals, Norway, 20821.330
RNeasy Mini Kit	Qiagen, Norway, 74104
MagicMark™ XP Western Protein Standard	Thermo Fisher Scientific, USA, LC5603

2.1.4 Cell culture reagents

Reagent	**Manufacturer**
Sterile 1 x PBS	Sigma-Aldrich, United Kingdom, D8537
0.05 % Trypsin-EDTA, Phenol Red	Thermo fisher scientific Gibco, USA, 25300054
DMEM	Thermo fisher scientific Gibco, USA, 31966- 021
Foetal Bovine Serum	Thermo fisher scientific Gibco, USA, A4736401
Antibiotic-Antimycotic 100 x	Thermo fisher scientific gibco, USA, 15240062

2.1.5 Primary antibodies

Target	Species	Dilution	Size (kDa)	Manufacturer	Category number
Human integrin α11	Mouse	1:100	150 kDa	Gullberg lab UIB, Norway[12]	mAb 210F4
Human β-actin	Mouse	1:5000	42 kDa	Sigma-Aldrich, Norway	A5441

2.1.6 Secondary antibodies

Antibody	Dilution	Manufacturer	Category number
m- IgGκ BP -HRP	1:5000	Santa Cruz Biotechnology, USA	sc-516102

2.1.7 Reagents for reverse transcription and PCR

Reagents	Supplier	Cat. no/Ref. no
5x iScript Reaction Mix	Bio-Rad	Cat no: 1708891
iScript Reverse Transcriptase	Bio-Rad	Cat no: 1708891
Sybr green supermix	Bio-Rad	Cat no: 1708891
Nuclease-free H_2O	Bio-Rad	Cat no: 1708891

2.1.8 qPCR primers

Primer	Sequence forward (5' – 3')	Sequence reverse primer (5' – 3')
ITGA11	CTC TCC AAA GGT GCC AGA CC	TGA ACA GGA TGA CCT TGC CC
ACTB	GGC TGT ATT CCC CTC CAT CG	CCA GTT GGT AAC AAT GCC ATG T

2.1.9 Inhibitors

Inhibitor	Target	Manufacturer
SB-505124	TGF-βR	RnD Systems

PD-098059	MEK1	Sigma-Aldrich
PF-573228	FAK	Sigma-Aldrich

2.2 Methods

2.2.1 Cell culture

BJ fibroblasts obtained from the normal foreskin of a neonatal male and CAFs obtained from non-small cell lung cancer patients were cultured with Dulbecco's modified Eagle's medium (DMEM) (Gibco®, USA) with 10 % of foetal bovine serum (FBS) (Gibco®) and 1 % Antibiotic-Antimycotic (A/A) (Gibco®) in a humidified 5 % CO_2 atmosphere at 37 °C. The cells have been tested negative for mycoplasma. The cells were cultured in either T25 or T75 cell culture flasks (Nunc, Denmark) until they reach 80-90 % confluency. Once confluent, cells were trypsinized and passaged 1/10 for future experiments. For long-term storage, cells were frozen in DMEM containing 10 % Dimethyl sulfoxide (DMSO) and 20 % FBS in DMEM and kept in cryovials (Nunc). Cryovials were then frozen down slowly to -80 °C using the CoolCell®LX (Corning life sciences, USA) cell freezing container.

2.2.2 Preparation of polyacrylamide hydrogels

2.2.2.1 Coverslip activation

Polyacrylamide hydrogels of varying stiffness were polymerized on glass coverslips. In order for hydrogels to adhere to the glass, coverslips were chemically activated. The coverslips were first washed with 20 mL of 10 % NaOCl (sodium hypochlorite; Honeywell, USA) overnight (O/N) on an orbital shaker. The solution was then replaced with 20 mL of 0.2M HCl and left for 3 hours on the orbital shaker. After the incubation, the coverslips were washed five times with de-ionized H_2O for 2 minutes and 20 mL of 0.1 M NaOH was added for 3 hours. Coverslips were washed again with de-ionized H_2O and were then incubated with 20 mL of 0.5 % aminopropyltrimethoxylilane (Acros Organics B.V.B.A., USA) in de-ionized H_2O O/N. The following morning, they were rinsed with de-ionized H_2O and incubated with 0.5 % glutaraldehyde in 1X PBS O/N. Finally, the coverslips were dried using wipes (Kimtech, USA) and ready for gel casting.

2.2.2.2 Casting of hydrogels

Polyacrylamide solutions were prepared according to **Table 1** to form hydrogels of different stiffnesses between 400 pascal (Pa) and 60 kilopascal (kPa).

Table 1: Volumes of reagents used to polyacrylamide hydrogel of different stiffness preparation

Reagents	Volume* (µL) for 400 Pa	Volume* (µL) for 2700 Pa	Volume* (µL) for 6000 Pa	Volume* (µL) for 22 kPa	Volume* (µL) for 60 kPa
40 % acrylamide	120	300	300	300	400
2 % bisacrylamide	40	28	56	200	400
10x PBS	160	160	160	160	160
MiliQ water	960	792	764	620	320
0.1 % Tetramethylethylenediamine (TEMED)	160	160	160	160	160
Total volume	1440	1440	1440	1440	1440

* Volumes are indicated to cast five coverslips before 1 % APS is added

Once the gel solutions were prepared, they were degassed for one hour. After degassing, 160 µL of 1 % APS was added into the solutions to start gel polymerisation, and immediately pipetted onto the activated coverslips. A new glass coverslip treated with a water repellent (Rain-X, ITW, UK) was put on top of each solution. After polymerisation, the top coverslips were removed. The gels were then sterilized with 70 % ethanol and kept in 2 mL of sterile PBS under a fume hood for functionalization.

2.2.2.3 Hydrogel functionalization, ligand crosslinking and cell seeding

Each polyacrylamide gel was functionalized using 2 mL of 1 mg/mL of L-3,4-dihydroxyphenylalanine (L-DOPA) (Alfa Aesar, USA) in 10 mM Tris-HCl pH 8.5 for 5 minutes in the dark to minimize photosensitivity. L-DOPA was then removed, and the gels were washed three times with sterile PBS. Depending on the experiment, the gels were crosslinked either with 2 mL of 5 µg/mL fibronectin (Sigma Aldrich, Norway) O/N or with a thin layer of collagen gel consisting of 50 % DMEM, 40 % collagen I (3mg/mL) and 10 % 0.2 M HEPES pH 8. The collagen gel would coat the entire hydrogel before being immediately

taken off, forming a thin film, which was then left for 1 hour at 37 °C to polymerize. After the ECM ligand had been crosslinked, hydrogels were washed once with PBS and then incubated O/N at 37 °C with 3 mL of DMEM. The next day, cells are seeded with DMEM+10 % FBS and 1 % A/A, with a desired confluency of approximately 60-70 %. The cells are then incubated for 24 hours at 37 °C and 5 % CO_2 . A resume of the whole process is depicted in the **figure 2.1**.

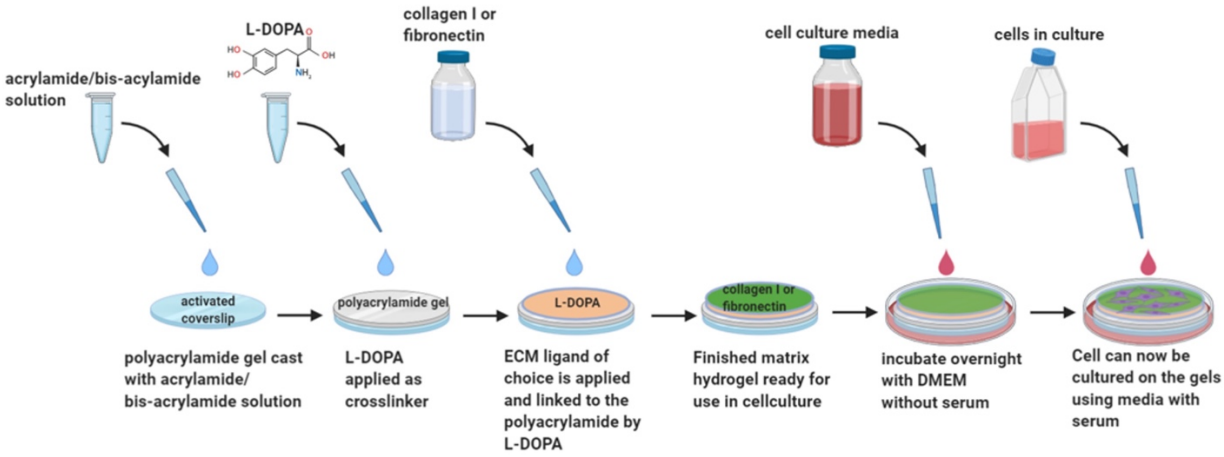

Figure 2.1: Schematic showing the different steps from the preparation of hydrogels till the culture of cells. Modified from the project report of an Erasmus student, de Ruijter [99].

2.2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting

2.2.3.1 Sample preparation

Hydrogels were washed three times with PBS before addition of 200µL/4 hydrogels of RIPA lysis buffer. Lysates were sonicated at 4 ºC (10 cycles of 30s of sonication (20-60kHz) followed by 30s of intervals) and then centrifuged for 20 minutes at 16000 g at 4 ºC. Sample buffer (4X) was then added to the supernatant with 3 % of β-Mercaptoethanol before being boiled at 95 °C for 5 minutes. The samples were then submitted to SDS-PAGE.

2.2.3.2 SDS-PAGE

Polyacrylamide gels, composed of a running gel (7.5 %) and of a stacking gel (5 %) were prepared according to **Table 2 and 3**. Briefly, the running gel was first poured into a cassette. After polymerisation, the stacking gel was then poured in the cassette on top of the running gel, and immediately a 10-well comb was put into the top of the cassette. After polymerization of the stacking gel, the SDS-polyacrylamide gels were put into a Bio-Rad tank (Bio-Rad, USA),

which was then filled with 1 x running buffer (2.1.2). Fifty microliters of the prepared sample were loaded onto the wells, and a ladder containing a mix of 2 µL MagicMark^{TM} XP (Thermo Fisher, USA) and 2 µL Precision plus protein standards (10 to 250 kDa, Bio-Rad) diluted into 46 µL of 1 x sample buffer was added into one well. The gel was run for approximately 20 minutes at 80 V to concentrate samples in the stacking gel and then 1 hour at 100 V for protein separation.

Table 2: Volumes of reagents used for the 7.5 % running gel preparation (20 mL)

Materials	Volume
Acrylamide/Bis (37:5:1, 30 %, Bio-Rad)	5 mL
Tris-HCl 2M pH 8.8	4 mL
10 % APS	100 µL
20 % SDS	100 µL
TEMED	20 µL
H_2O	11 mL

Table 3: Volumes of reagents used for the 5 % stacking gel preparation (6 mL)

Materials	Volume
Acrylamide/Bis (37:5:1, 30 %, Bio-Rad)	1 mL
Tris-HCl 0.5M pH 6.8	1 mL
10 % APS	30 µL
20 % SDS	30 µL
TEMED	20 µL
H_2O	4 mL

2.2.3.3 Western immunoblotting

After separation, the proteins were transferred from the gels onto a nitrocellulose membrane using a semi-dry transfer system (iBlot, Thermo Fisher). With this device, the transfer was set up to 20 V for 7 minutes, upon which the membrane was washed with Tris-buffered solution with 0.05 % Tween-20 (TBS-T) before blocking with a 5 % solution of non-fat dry milk in TBS-T for 1 hour at room temperature (RT). Following blocking, mouse monoclonal to human integrin α11 (mAb 210F4)[12] and mouse monoclonal β-actin (Sigma-Aldrich) were added at a 1:100 and 1:5000 dilution, respectively into 10 mL of 1 % non-fat dry milk in TBS-T. The

membranes were then left to incubate in the primary IgG solution O/N at 4 °C on a shaker. The next day, the membrane was washed three times for 10 minutes in TBS-T and then incubated with secondary antibody horseradish peroxidase(HRP)-conjugated goat anti-mouse (1:5000 in 1 % non-fat dry milk in TBS-T) for 1 hour at RT. Pierce™ ECL Western Blotting Substrate (Thermo Fisher) was used to develop the membrane.

2.2.3.4 Signalling pathway inhibition experiment

After the cells were seeded on hydrogels, 10μM of FAK or TGF-βR inhibitor or 5 μM of Mek1 inhibitor (2.1.9) was added to the culture media. The hydrogels were incubated for 24 hours at 37 °C and cell lysates were then collected using RIPA buffer before being subjected to SDS-PAGE.

2.2.4 Gene expression analysis using qPCR

2.2.4.1 RNA extraction

Cells on hydrogels were detached using 7mM EDTA in PBS and centrifuged. Cell pellets were resuspended in 300 μL of RLT lysis buffer (from RNeasy Mini Kit (Qiagen, Norway)) containing 1 % of β-Mercaptoethanol. RNA extraction was then performed following the protocol provided with the RNeasy Mini Kit. RNA concentration and purity were then measured using the NanoDrop 2000 Spectrophotometer (Thermo Fisher).

2.2.4.2 cDNA synthesis

The iScript™ cDNA synthesis Kit (Bio-Rad) was used for the reverse transcription of 1 μg RNA into cDNA as guided by the manufacturers protocol. Per reaction, 1 μL of iScript reverse transcriptase and 4 μL of 5x iScript reaction buffer were mixed with 1 μg of RNA samples. The reaction mixture was then incubated in the Thermal cycler for complementary DNA (cDNA) synthesis with the following program: primer annealing at 25 °C for 5 minutes, reverse transcription of RNA at 46 °C for 20 minutes, enzyme inactivation at 95 °C for 5 minutes. The cDNA was then diluted 1:25 using Mili-Q water for qPCR and kept on ice.

2.2.4.3 Quantitative real time-polymerase chain reaction (qPCR)

qPCR was used to monitor the amplification of the targeted cDNA. For each reaction, 12.5 μL of SYBR Green Supermix (Bio-Rad) was mixed with 1 μL of 10μM forward primer, 1 μL of

10µM reverse primer (2.1.8 for primer list), 5.5 µL H2O and 5 µL of diluted cDNA (2ng/µL). Each reaction mixture was then loaded in duplicates in a 96-well plate, before being centrifuged for 1 minute at 400 g. The qPCR was run on a LightCycler® 480 (Roche, Sweden), with the following program: DNA amplification of 45 cycles composed of denaturation for 10 seconds at 95 °C, primer annealing for 10 seconds at 60 °C and elongation for 10 seconds at 72 °C.

2.2.5 Data and statistical analysis

Data analysis has been performed for Western blotting and qPCR experiments. For analysis of the Western blotting results, band intensities were quantified using the ImageJ software. The values were normalized using β-actin band intensities. For analysis of the qPCR results, ΔCT value for each gene was log2 transformed and normalized to the *ACTB* housekeeping gene. Results of the different experiments are expressed as the mean ± standard deviation of three replicates and are representative of three independent experiments unless stated otherwise. Statistical significance was performed using unpaired Student t-test and one-way or two-way ANOVA when indicated, with $p<0.05$ being significant. Analysis was done using the GraphPad Prism software.

3 Results

3.1 Fibroblast spreading on soft and stiff collagen-coated hydrogels

To investigate the influence of ECM rigidity on integrin α11 expression in fibroblasts, we took advantage of the polyacrylamide hydrogels, which can mimic different tissue stiffnesses and can be crosslinked with an ECM ligand. We first aimed to optimize the previous hydrogel protocol adapted from the V. Weaver lab at UCSF, by replacing the monomeric collagen coating with a thin layer of fibrillar collagen. Since previous studies documented a change in cell morphology with the variation of stiffness, we examined the spreading of fibroblasts on the different hydrogels to validate our model[100]. We selected stiffnesses of 400 Pa (soft) and 60 kPa (stiff) for our experiments, which correspond to the stiffness of several healthy tissues and of severely fibrotic tissue, respectively. In addition, cells cultured on plastic (GPa) were used as a control. Using light microscopy, we observed that human lung CAFs and BJ fibroblasts on soft substrates appeared more roundish after 24 hours compared to cells seeded on stiff and plastic surfaces, where cells showed an elongated morphology (**Figure 3.1**).

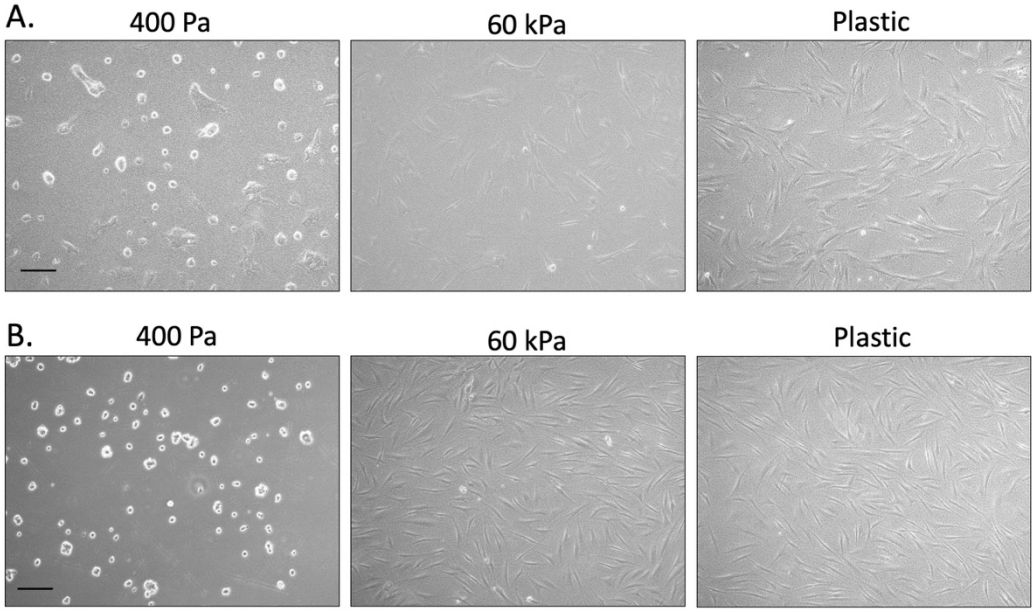

Figure 3.1: Cell morphology of BJ and CAF varies depending on the stiffness of collagen-coated hydrogels. *Morphological differences in BJ fibroblasts (A) and CAFs (B) seeded on soft (400 Pa) or stiff (60 kPa) polyacrylamide hydrogels or on plastic, coated with collagen I for 24 hours were observed under a light microscope. Scale bar: 100µm.*

3.2 Integrin α11 expression in fibroblasts cultured on collagen I-coated hydrogels

We analysed integrin α11 expression in lung CAFs and BJ fibroblasts cultured on soft (400 Pa), stiff (60 kPa), and plastic surfaces coated with fibrillar collagen I, after 24 hours using

Western immunoblotting. When cultured on plastic, where cells experience an extreme stiffness (GPa), BJ fibroblasts displayed high expression of integrin α11 (**Figure 3.2**). Surprisingly, integrin α11 levels were also significantly high in cells seeded on a soft substrate, but lower than on the stiff substrate. We observed similar results in CAFs, where integrin α11 expression also appeared upregulated on soft hydrogels (**Figure 3.3**). Further experiments were conducted with BJ cells due to their higher basal expression of integrin α11.

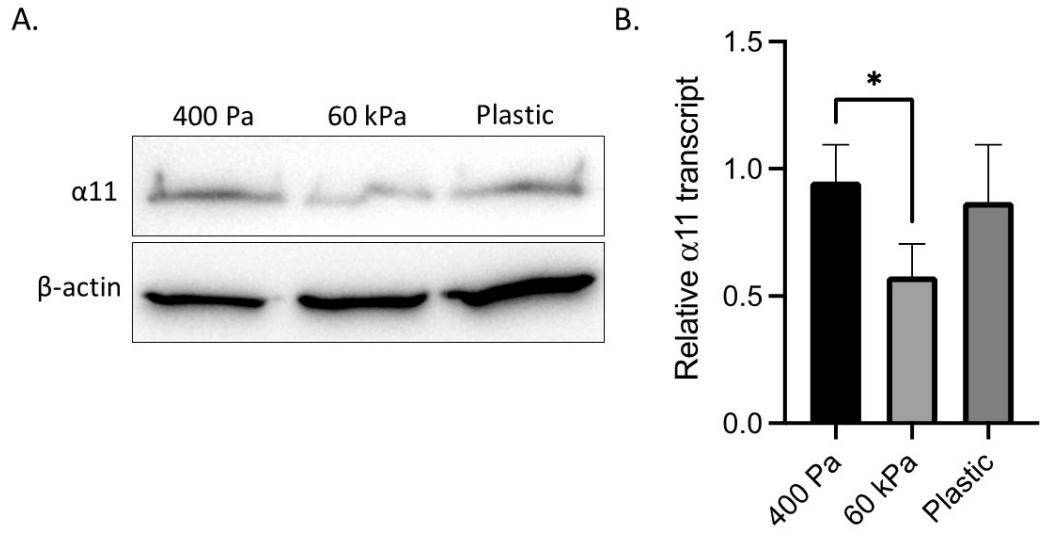

Figure 3.2: Integrin α11 expression in BJ fibroblasts on polyacrylamide gels coated with collagen I. *A: The levels of integrin α11 in BJ cells cultured on polyacrylamide gels with stiffnesses of 400 Pa and 60 kPa and plastic were analysed by Western blotting. β-actin was used as loading control. B: Quantification of integrin α11 expression by densitometry. The integrin α11 band intensity was normalized to β-actin signal. Statistical analysis was performed using an unpaired t-test from three independent experiments (*, $p<0.05$; mean±SD).*

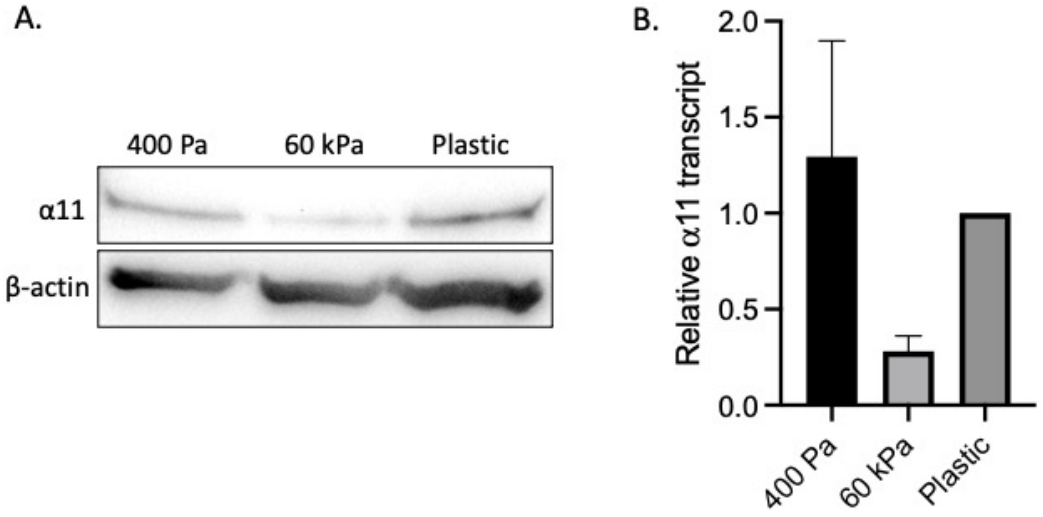

Figure 3.3: Integrin α11 expression in lung CAFs on polyacrylamide gels coated with collagen I. *A: The levels of integrin α11 in BJ cells cultured on polyacrylamide gels with stiffnesses of 400 Pa, 60 kPa and plastic were analysed by Western blotting. β-actin was used as loading control. B: Quantification of integrin α11 expression by densitometry. The integrin α11 band intensity was normalized to β-actin signal. From two experiments.*

To explore more in details the regulation of integrin α11 by stiffness, we have included three more degrees of softness, 2700 Pa, 6000 Pa and 22 kPa, in the hydrogel panel crosslinked with fibrillar collagen I. We showed that after 24 hours, expression of integrin α11 in BJ fibroblasts was negatively regulated with stiffness within the range of 400 Pa-60 kPa (**Figure 3.4**), indicating that the regulation of integrin α11 expression is dependent on the stiffness.

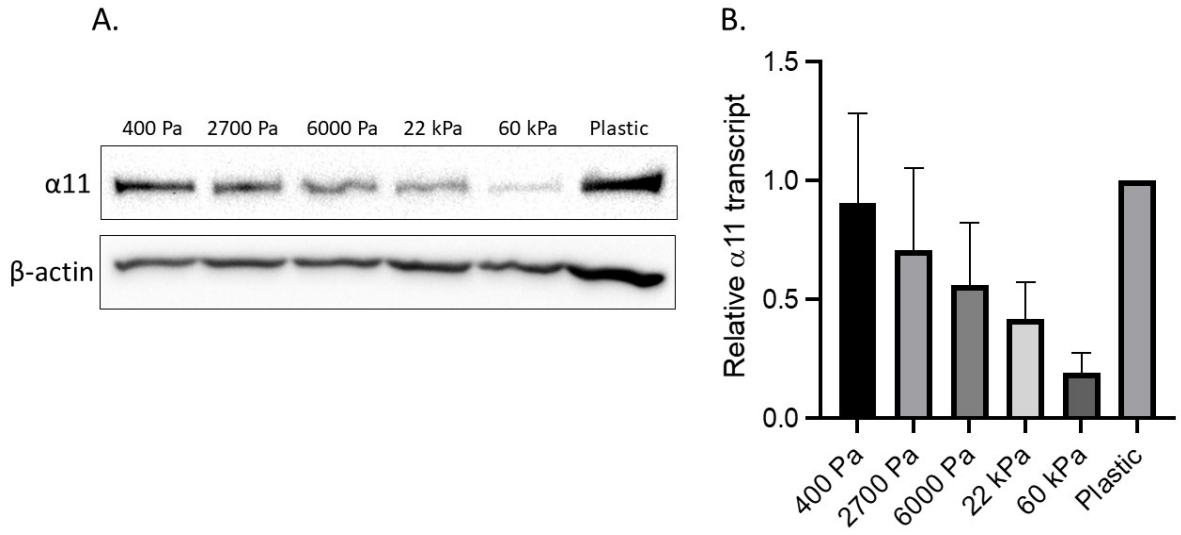

Figure 3.4: Inverse correlation between integrin α11 expression and stiffness in BJ fibroblasts seeded on collagen I for 24 hours. *A: The levels of integrin α11 in BJ cells cultured on polyacrylamide gels with stiffnesses of 400 Pa, 2700 Pa, 6000 Pa, 22 kPa, 60 kPa and plastic were analysed by Western blotting. β-actin was used as loading control. B: Quantification of integrin α11 expression by densitometry. The integrin α11 band intensity was normalized to β-actin signal. Statistical analysis was performed using one-way ANOVA, from three independent experiments (p=0.0142; mean±SD).*

The Gullberg lab has previously shown in different 3D models that mouse integrin α11 expression is upregulated by mechanical stress after one week culture[96]. Since our present results were obtained after only 24 hours incubation, we investigated whether a time extension of the culture on the hydrogels can affect the regulation of integrin α11 by stiffness. Hence, BJ fibroblasts were seeded on soft (400 Pa), stiff (60 kPa) and plastic surfaces coated with fibrillar collagen I for 7 days. After this period, we observed higher levels integrin α11 on stiff substrates compared to soft hydrogel (**Figure 3.5**). However, it is important to note that the cell culture was 100 % confluent on all substrata after 7 days, whereas it was approximately 60 % after 24 hours.

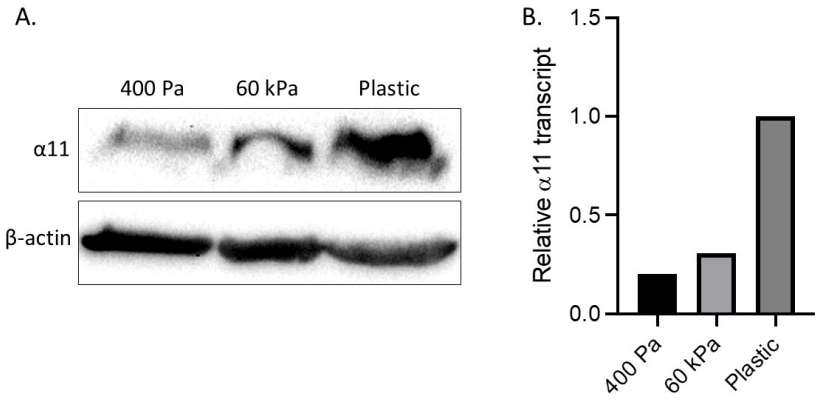

Figure 3.5: Integrin α11 expression in BJ fibroblasts on polyacrylamide gels coated with collagen I for 7 days. *A: The levels of integrin α11 in BJ cells cultured on polyacrylamide gels with stiffnesses of 400 Pa, 60 kPa and plastic were analysed by Western blotting. β-actin was used as loading control. B: Quantification of integrin α11 expression by densitometry. The integrin α11 band intensity was normalized to β-actin signal. From one experiment.*

3.3 Analysis of integrin α11 expression at the transcriptional level

To determine whether the stiffness-dependent expression of integrin α11 protein was reflective of either a regulation at the transcription level or a regulation of protein fate, we analysed the expression of *ITGA11* using qPCR. BJ fibroblasts were cultured as previously on hydrogels of 400 Pa and 60 kPa and plastic, coated with fibrillar collagen I for 24 hours. Similarly, to the protein levels, expression of *ITGA11* transcript (mRNA) was significantly higher in fibroblasts seeded on a soft substrate (**Figure 3.6**), indicating that stiffness regulates integrin α11 at the transcription level.

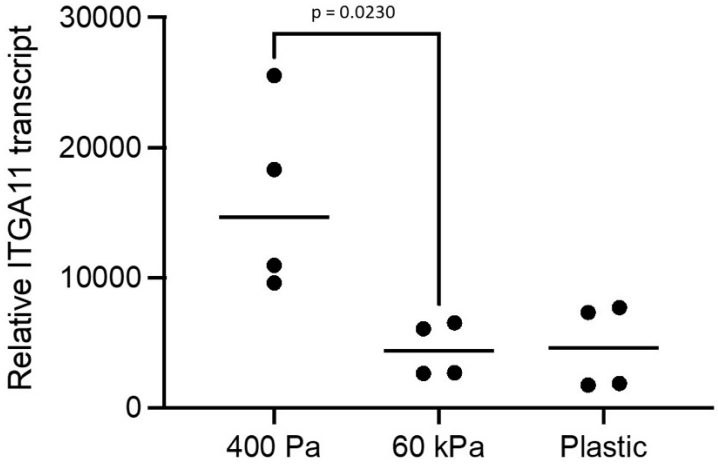

Figure 3.6: ITGA11 mRNA expression in BJ fibroblasts on polyacrylamide gels of varying stiffness coated with collagen I. *Relative ITGA11 transcript from BJ fibroblasts seeded on hydrogels of 400 Pa, 60 kPa and plastic were analysed after 24 hours by qPCR. ΔCT value for each gene was log2 transformed and normalized to the ACTB house-keeping gene. Statistical analysis was performed using an unpaired t-test from two independent experiments (p=0.023; mean is indicated for each condition).*

3.4 Stiffness-regulated integrin α11 expression and collagen I sensing

Until now, the different stiffness experiments were performed in the presence of collagen I. We thus wondered whether the regulation of integrin α11 levels was substrate specific. We

conducted the same experiment as previously, but this time with fibroblasts seeded on hydrogels coated with fibronectin instead of collagen. Analysis by Western blotting of cell lysates collected from fibronectin-coated hydrogels showed no significant differences in integrin α11 expression between the different stiffnesses (**Figure 3.7**), hence suggesting that a collagen receptor is involved in the stiffness-dependent regulation of integrin α11 expression.

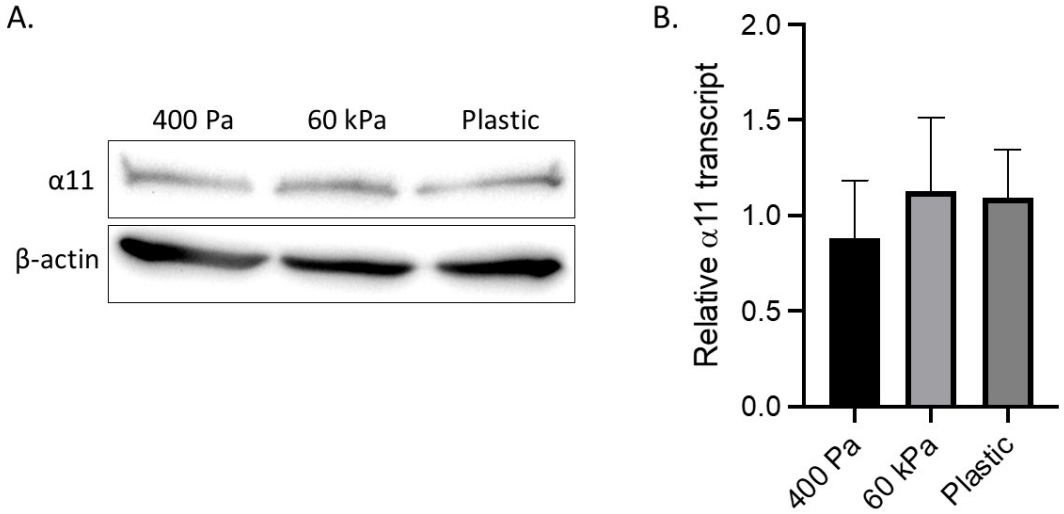

Figure 3.7: Integrin α11 expression in BJ fibroblasts on polyacrylamide gels coated with fibronectin A: *The levels of integrin α11 in BJ cells cultured on polyacrylamide gels with stiffnesses of 400 Pa, 60 kPa and plastic were analysed by Western blotting. β-actin was used as loading control. B: Quantification of integrin α11 expression by densitometry. The integrin α11 band intensity was normalized to β-actin signal. Statistical analysis was performed using an unpaired t-test from three independent experiments (mean±SD).*

3.5 Effect of inhibition of FAK, TGF-βR and Erk on integrin α11 expression

Since our previous experiments suggested that a collagen receptor, and presumably a collagen-binding integrin, was involved in the process, we used an inhibitor of FAK activation (PF-573228). In addition, we employed a TGF-βR inhibitor (SB-505124), as TGF-β is known to regulate integrin α11 expression[101]. We showed that the increase of integrin α11 expression in 400 Pa compared to 60 kPa collagen-coated hydrogels is negligible when either FAK or TGF-β signalling is inhibited (**Figure 3.8**). Furthermore, there is a significant decrease in integrin α11 expression on soft substrates after treatment with FAK inhibitor, suggesting that these pathways could play a role in the regulation of integrin α11 levels.

FAK can activate Mitogen-activated protein kinase (MAPK) signalling, including Erk signalling and the Gullberg lab has previously shown that Erk is part of integrin α11 signalling[102]. We thus inhibited MEK1 (PD-098059), an activator of Erk, using PD-098059 to determine whether Erk could also be involved in the regulation of integrin α11. We found that

the high levels of integrin α11 observed on softer surfaces was not observed when FAK or Erk was inhibited (**Figure 3.9**), suggesting that Erk might also play a role in the stiffness-dependent regulation of integrin α11.

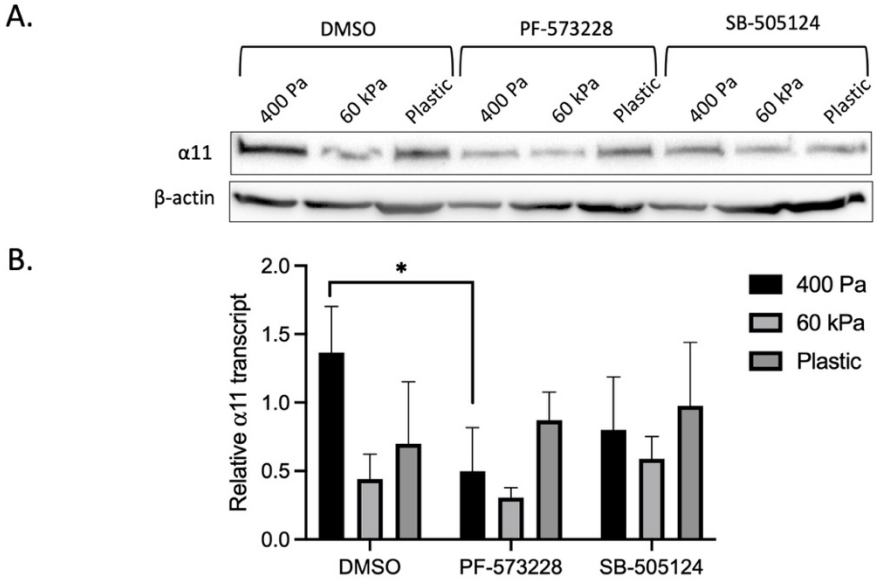

Figure 3.8: Integrin α11 expression in BJ fibroblasts on polyacrylamide gels coated with collagen I after treatment with FAK and TGF-βR inhibitors. *A: The levels of integrin α11 in BJ cells cultured on polyacrylamide gels with stiffnesses of 400 Pa, 60 kPa and plastic after addition of 10μM of FAK inhibitor (PF-573228) or TGF-βR inhibitor (SB-505124) were analysed by Western blotting. DMSO was used as control. β-actin was used as loading control. B: Quantification of integrin α11 expression by densitometry. The integrin α11 band intensity was normalized to β-actin signal. Statistical analysis was performed using an unpaired t-test from three independent experiments (*, p<0.05; mean±SD). A significance of p=0.0270 was found using a two-way ANOVA on all samples.*

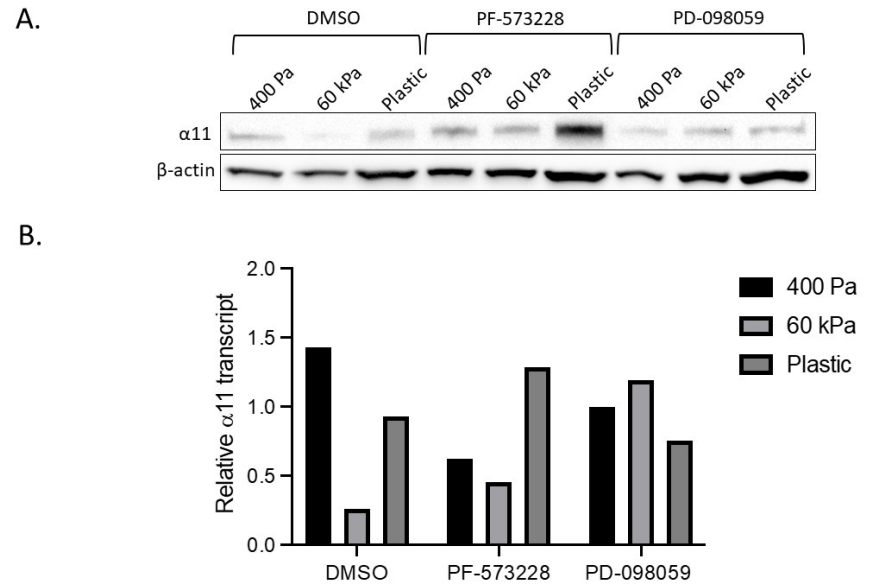

Figure 3.9: integrin α11 expression in BJ fibroblasts on polyacrylamide gels coated with collagen I after treatment with FAK and MEK1 inhibitors. *A: The levels of integrin α11 in BJ cells cultured on polyacrylamide gels with stiffnesses of 400 Pa, 60 kPa and plastic after addition of 10μM of FAK inhibitor (PF-573228) or 5μM of MEK1 inhibitor (PD-098059) were analysed by Western blotting. DMSO was used as control. β-actin was used as loading control. B: Quantification of integrin α11 expression by densitometry. The integrin α11 band intensity was normalized to β-actin signal. From one experiment.*

4 Discussion

Integrin α11 plays an important role in cell adhesion and fibroblast differentiation into myofibroblasts[96]. This study aimed to elucidate how matrix stiffness could alter integrin α11 expression for tumour fibrosis. Here, we have shown that integrin α11 expression in fibroblasts, both at the protein and mRNA level, was higher on soft collagen matrix-coated hydrogels compared to stiff hydrogels.

4.1 Integrin α11 expression in BJ fibroblasts

Integrin α11 is often found in fibrotic tissues and desmoplastic tumours, which display a stiffer environment[12,35]. Furthermore, Carracedo *et al*. observed an upregulation of integrin α11 in MEFs embedded in an attached 3D collagen matrix, which mimic a stiff environment, compared to a floated collagen lattice that represents the soft counterpart[96]. The results from our study, where α11 expression is increased in cells cultured on soft collagen-coated hydrogels seem to be in opposition to the main consensus of these previous findings. As a 3D environment better mimics physiological conditions, using a 2D environment in our experiments must be considered when discussing its potential impact on protein expression. However, a recent paper published by Fiore and colleagues reported that the fibroblastic loci, an area of active matrix deposition in fibrotic lung, was surprisingly as soft as normal lung tissue. Their model suggests that integrins drive fibroblast contraction in softer environments leading to straining of the environment, integrin upregulation and fibrotic progression[103]. This could possibly explain the morphology seen in the cells cultured on soft environments, where a rounder structure is a result of the cells ability to contract and pull on the surrounding soft environment more easily compared to a stiff environment.

In contrast to the 24 hour incubation used in this study, cells cultured on collagen-coated hydrogels for 7 days showed a higher integrin α11 expression on stiff compared to soft hydrogels. This upregulation could possibly be explained by altered integrin turnover after longer periods of time. In fibroblasts under normal culture conditions, integrins are typically quite stable with half-lives of approximately 12-24 hours[104]. However, within the BJ cell line, their recycling may be downregulated over longer periods of time, hence altering integrin α11 expression[105]. Nevertheless, after 7 days, we did not observe differences in the morphology of fibroblasts seeded on soft and stiff hydrogels, as we usually do after 24 hours. Furthermore,

cell confluency reached after 7 days enables cell-cell contacts, introducing cell-cell mechanosensing and thus "interfering" with the mechanosensing from the ECM[106].

To investigate whether integrin α11 regulation by stiffness was collagen-receptor dependent, we conducted experiments with fibronectin coated hydrogels instead of collagen I. The lack of differences in integrin α11 expression between fibronectin-coated soft and stiff hydrogels indicates that collagen receptors are indeed involved in this process. Fibroblasts express the integrins α1, α2 and α11 as collagen-binding integrins. In addition, they also express the discoidin domain receptor 2 (DDR2), another collagen receptor. DDR2 has been shown to influence the mechanotransduction of collagen-binding integrins to regulate breast tumour stiffness. DDR2 is thus a good potential candidate that may contribute to the regulation of integrin α11 expression[107].

4.2 Stiffness-dependent regulation of integrin α11 at the transcriptional level

We showed that stiffness-regulated integrin α11 expression at the mRNA level correlated with differences observed at the protein level. However, we cannot exclude a role of integrin α11 trafficking and degradation in its regulation, as shown for other integrins[108,109]. A study by Lerche and colleagues investigated the mechanism of integrin trafficking in mammary gland fibroblasts[110]. The authors found that within these cells, integrin α11 is partly regulated via lysosomal trafficking and degradation, which can also be a possible mechanism in the BJ cell line[110]. Considering that the cells cultured on 60 kPa and plastic undergoing a higher amount of mechanical stress and strain, an overall decrease in mRNA expression may occur since stiffness has been shown to alter the cell ability to maintain normal RNA levels[111].

4.3 TGF-β, Erk and FAK signalling pathways and integrin α11 expression

We investigated three signalling pathways that have previously been associated with integrin α11, TGF-β, FAK and Erk. From previous studies in the Gullberg lab, it is known that TGF-β can regulate integrin α11 expression at the protein level in a Smad-dependent manner due to increased transcriptional activity[101]. Using a TGF-βR inhibitor we observed a decrease in integrin α11 expression on soft hydrogels compared to the control, suggesting that TGF-β could be part in the regulation of integrin α11 on soft tissues. This is intriguing because activation of TGF-β is dependent on tractional forces and mechanically resistant ECM[57]. In this context, how soft substrates influence TGF-β signalling remains to be determined. Inhibition of FAK

signalling significantly reduced integrin α11 expression on soft matrices, confirming that collagen-binding integrin signalling may be involved in the regulation of integrin α11 expression. Interestingly, the cytoplasmic tail of integrin α11 has been shown to be implicated in FAK activation and FA stabilization[102]. In the same study, it was also showed that integrin α11 mediates Erk activation in a FAK-dependent process. Moreover, it is documented that the FAK-Erk axis plays an important role in the mechanotransduction of skin fibroblasts[112]. In the present study, inhibition of Erk abrogated the differences in α11 expression from stiff to soft. Further investigations are required to better understand the role of these signalling pathways in the regulation of integrin α11 expression.

4.4 Limitations of the study

There are some limitations to consider in this study. 1. We have limited several experiments to BJ fibroblasts. Fibroblast subpopulations are an important factor to consider, as they have different characteristics. Although we showed similar stiffness-dependent regulation of integrin α11 expression in BJ fibroblasts and lung CAFs, the associated mechanism could be different. Furthermore, different CAFs could display different integrin α11 regulation on stiff and soft substrata. 2. Due to time constraints, few experiments have only been performed once or twice as indicated. Thus, the conclusions we formulated from these experiments could be biased. 3. Acrylamide was a main reagent used in the making of the hydrogel cultures. Acrylamide could possibly also have altered cellular signalling compared to plastic by increasing oxidative stress[113].

5 Conclusion

We have shown that integrin α11 expression in fibroblasts is regulated by stiffness. Surprisingly, integrin α11 displayed higher expression on soft collagen matrices than on stiff. We also determined that integrin α11 expression was controlled at the transcriptional level and was dependent on a collagen receptor. Finally, we suggested that the TGF-β, FAK and Erk signalling pathways are involved in the regulation of integrin α11 expression. Additional studies must be conducted to further elucidate the stiffness-dependent regulation of α11 in different cell types.

6 Future perspectives

Several unanswered questions still remain regarding the regulation of integrin α11 by stiffness. As indicated in the limitations of the study section, it will be interesting to incorporate different CAFs in our studies and to complete some experiments. To complement our 2D hydrogel culture experiments with cells in 3D soft and stiff collagen matrices would be interesting to gain further insight into integrin α11 regulation. Studying stiffness-dependent alterations in protein degradation pathways is also of interest as it is a mechanism of integrin α11 regulation in other cell types[110]. The Hippo pathway with regard to YAP/TAZ signalling is another mechanism of interest. In this regard, it would be pertinent to investigate whether the upregulation of integrin α11 on soft substrates correlates with YAP/TAZ translocated to the nucleus.

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