# Novel Insights into Integrin α11 Expression and Function

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Thesis for the degree of Philosophiae Doctor (PhD)
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## Scientific environment

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Animal experiments were performed at the Laboratory Animal Facility, Department of Clinical Medicine, Faculty of Medicine, University of Bergen.

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October 2019, Bergen

# Abbreviations

| | |
|---|---|
| ADAM | A disintegrin and metalloprotease |
| ADMIDAS | Adjacent to metal ion-dependent adhesion site |
| CAF | Cancer-associated fibroblast |
| CXCL12 | C-X-C motif chemokine 12 |
| CXCR4 | C-X-C chemokine receptor type 4 |
| D | Dimension |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's modified Eagle's medium |
| Dok1 | Docking protein 1 |
| EBS | ETS-binding site |
| ECM | Extracellular matrix |
| EGF | Epidermal growth factor |
| EGFP | Enhanced green fluorescent protein |
| EMT | Epithelial-mesenchymal transition |
| EndMT | Endothelial-mesenchymal transition |
| ERK | Extracellular signal-regulated kinase |
| FACS | Fluorescence-activated cell sorting |
| FAK | Focal adhesion kinase |
| FAP | Fibroblast activation protein |
| FSP1 | Fibroblasts-specific protein 1 |
| FITC | Fluorescein isothiocyanate |
| hGF | Human gingival fibroblast |
| HNSCC | Head and neck squamous cell carcinoma |
| IAC | Integrin adhesion complex |

| | |
|---|---|
| iCAF | Inflammatory cancer-associated fibroblast |
| ICAP-1 | Integrin cytoplasmic domain-associated protein 1 |
| IL | Interleukin |
| JNK | C-Jun N-terminal kinase |
| LOX | Lysyl oxidase |
| mAb | Monoclonal antibody |
| MAP | Mitogen-activated protein kinase |
| MDG1 | Mammary-derived growth inhibitor |
| MEF | Mouse embryonic fibroblast |
| MIDAS | Metal ion-dependent adhesion site |
| MMP | Matrix-metalloproteinase |
| MRC5 | Medical Research Council cell strain 5 |
| MSC | Mesenchymal stem cell |
| myCAF | Myofibroblastic cancer-associated fibroblast |
| NG2 | Neuron-glial antigen 2 |
| NOF | Normal fibroblast |
| NSCLC | Non-small cell lung carcinoma |
| pCAF | Pancreatic cancer-associated fibroblast |
| PDAC | Pancreatic ductal adenocarcinoma |
| PDGF | Platelet-derived growth factor |
| PDL | Periodontal ligament |
| PSC | Pancreatic stellate cell |
| PSI | Plexin-semaphorin-integrin |
| PTEN | Phosphatase and tensin homolog |
| PyMT | Polyoma middle T antigen |

| | |
|---|---|
| RD | Rhabdomyosarcoma |
| SBS | Sp1-binding site |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SFK | Src family kinase |
| SHARPIN | Shank-associated RH domain-interacting protein |
| siRNA | Silencing RNA |
| SyMBS | Synergistic metal ion-binding site |
| TCGA | The Cancer Genome Atlas |
| TGF-β | Transforming growth factor β |
| TIMP | Tissue inhibitor of MMP |
| TM | Transmembrane |
| TME | Tumor microenvironment |
| TSS | Transcriptional start site |
| WT | Wild type |
| αSMA | α-Smooth muscle actin |

# Abstract

Integrins are a major group of cell surface receptors, which link the extracellular matrix with the cell cytoskeleton. They are heterodimeric proteins consisting of non-covalently bound α- and β-subunits. By regulating mechanotransduction at cell-ECM communication sites, integrins can also activate many intracellular signaling events, which are essential for cell proliferation, cell migration and gene regulation. Integrin α11β1 is a collagen-binding integrin, which expressed on subsets of fibroblasts. Recent data demonstrated that integrin α11β1 is involved in myofibroblast differentiation and in wound healing, but also is pro-tumorigenic in the tumor stroma and pro-fibrotic in fibrosis. However, detailed molecular insights underlying integrin α11β1 expression, distribution and function in the context of tissue reorganization remains to be determined.

In this thesis, we used three different approaches to further characterize integrin α11 expression and function at both cellular and molecular level. Firstly, we investigated the functional role of integrin α11 cytoplasmic tail by deleting last 17 carboxyterminal amino acids in the α11 protein. We found that the cytoplasmic tail of α11 is important for collagen-dependent focal contact formation, collagen remodeling, cell proliferation and cell migration **(Paper 1).** Later, we generated and characterized a novel transgenic (ITGA11-Cre) mouse strain. Our results demonstrate that the activity of the 3 kb *ITGA11* promoter driven Cre-recombinase in the ITGA11-Cre mouse is sufficient to replicate the endogenous expression of integrin α11 both during embryonic development and in fibrotic conditions including cardiac fibrosis and wound healing, respectively **(Paper 2)**. Finally, we used newly developed monoclonal antibody (mAb) to human integrin α11 chain to demonstrate that α11 expression is present on different subset(s) of CAFs in the tumor microenvironment and α11β1 is involved in collagen remodeling and CAF migration in vitro **(Paper 3).** In summary, this thesis provides new understanding of integrin α11β1 functions in different subsets of fibroblasts in the context of tissue reorganization events including fibrosis and tumor-stroma interactions.

# List of Publications

**Paper 1:** Pugazendhi Erusappan, Jahedul Alam, Ning Lu, Cédric Zeltz and Donald Gullberg. **Integrin α11 cytoplasmic tail is required for FAK activation to initiate 3D cell invasion and ERK-mediated cell proliferation.** *Scientific Reports, In Press, September 2019*

**Paper 2:** Jahedul Alam, Musiime Moses, Andreas Romaine, Mugdha Sawant, Arne Olav Melleby, Ning Lu, Beate Eckes, Geir Christensen and Donald Gullberg. **Characterization of an integrin ITGA11-Cre mouse strain with Cre recombinase expression restricted to fibroblasts.** *Manuscript.*

**Paper 3:** Cédric Zeltz, Jahedul Alam, Hengshuo Liu, Pugazendhi M. Erusappan, Heinz Hoschuetzky, Anders Molven, Himalaya Parajuli, Edna Cukierman, Daniela-Elena Costea, Ning Lu and Donald Gullberg. **α11β1 Integrin is Induced in a Subset of Cancer-Associated Fibroblasts in Desmoplastic Tumor Stroma and Mediates In Vitro Cell Migration.** *Cancers* **2019**, *11*, 765

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# 1. Introduction

## 1. The Extracellular matrix

The extracellular matrix (ECM) is a non-cellular three-dimensional structure, which is composed of a mesh of macromolecules with different biochemical and physical properties. The ECM maintains tissue structures. It is not only necessary for the tissue integrity but also required for cellular process including cell adhesion, cell migration, cell differentiation, apoptosis, and tissue morphogenesis and homeostasis [1].

Based on composition and structure, ECM can the divided into two major groups: basement membranes and interstitial matrices. Basement membrane is a thin sheet-like structure underneath epithelial cells and endothelial cells; and surrounding adipocytes, muscle cells and Schwann cells, where it separates cells from the stroma and regulates certain signaling events [2]. The basement membrane is composed of a collagen type IV network and a laminin network linked together by nidogen. It also contain proteoglycans (PGs) and in some tissues collagen types XV and XVIII [3]. The major constituents of interstitial matrices are different fibrillar collagens, PGs and fibronectin, which surround cells and build a form of 3D lattice within the interstitial space [4].

### 1.1 ECM remodeling and disorders

ECM is subjected to remodeling through degradation and modifications of its components. Lysyl oxidases (LOX) catalyzes the cross-linking of collagen and elastin, which leads to increased matrix stiffness and tissue tensile strength [5]. In addition, different proteases such as matrix-metalloproteinases (MMPs), members of a protein family with a disintegrin and metalloprotease domain (ADAMs), cathepsin K and elastases are involved in degradation of ECM proteins. Tissue inhibitors of MMPs (TIMPs) balance the MMP activities, and an inequality between MMPs and TIMPs may lead to tissue fibrosis [6]. Exogenous stimuli including cytokines, oxidative stress and mechanical stress are also engaged in the ECM turnover process.

Transforming growth factor β (TGF-β), a well-known cytokine, has been widely reported to be involved in stimulating ECM synthesis [7].

## 2. Fibroblasts

Fibroblasts were originally defined as cells localized in connective tissue which synthesize ECM proteins. Fibroblasts are non-immune and non-epithelial spindle-shaped cells of mesenchymal origin, which are embedded within the interstitial ECM lattices. Usually, they are considered to be in a quiescent state with limited transcriptional and metabolic activity in normal tissues. They are among the most robust cells and have been said to be the cockroaches of the body [8]. Fibroblasts not only produce ECM proteins but also play vital roles in the maintenance and reabsorption of ECM, and are important in wound healing, inflammation, tissue fibrosis, angiogenesis and cancer progression. Fibroblasts become activated by a variety of growth factors that promote cellular differentiation and proliferation [9]. Many different biomarkers have been used to identify fibroblasts in specific tissues, including α-smooth muscle actin (α-SMA), vimentin, fibroblast activation protein (FAP), fibroblasts specific protein 1 (FSP1), discoidin domain receptor 2, PDGF receptors, integrins and pro-collagens, however, none of these markers detect only fibroblasts. Fibroblasts are heterogenous in nature and this heterogeneity in part depends on the ancestry of precursor fibroblasts [8, 10]. The heterogeneity of fibroblast also persists in the same organ, but the balance between subtypes might change in pathological situations. Distinct origins of fibroblast subgroups have recently been determined in the mouse skin and heart, respectively [11, 12].

## 3. Myofibroblasts

As mentioned above, quiescent fibroblasts become activated by a variety of factors and differentiate into so-called myofibroblasts. Besides fibroblasts, other cell types such as vascular pericytes, smooth muscle cells, bone marrow-derived cells, mesenchymal stem cells and endothelial or epithelial cells are also able to

differentiate into myofibroblasts [13]. Common factors that initiate myofibroblast differentiation include cytokines such as TGF-β and mechanical stress of the ECM [14, 15]. The characteristic phenotype of myofibroblasts includes the increased expression of αSMA which is incorporated into stress fibers and to enhance their contractility [16]. Myofibroblasts play fundamental roles in both normal physiological and pathological conditions. Under normal physiological conditions, they persist transiently and are then lost via apoptosis, such as during wound healing. However, under pathological conditions, myofibroblasts continuously persist with chronic contractile activity [17]. In addition, myofibroblasts also play a crucial role in tumor-stroma- interactions in the tumor microenvironment (TME) [8].

## 4. Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) or carcinoma-associated fibroblasts (when referring to fibroblasts associated with epithelial tumors) are the fibroblast-like cells of various lineages, which are located in the TME. CAFs are heterogenous and different subtypes have been identified within tumor stroma [18]. Two main subtypes of CAFs have been named myofibroblastic CAFs (myCAFs) and inflammatory CAFs (iCAFs) in pancreatic cancers [19]. Four main subtypes of CAFs (CAF-S1-S4) have been identified in breast cancer, which are marked by different αSMA and FAP expression patterns [20, 21]. The CAF subclasses do not usually represent fixed cell phenotypes, but have been suggested to more often represent fibroblast states [22]. Epigenetic changes have however been shown to direct the phenotypes of CAFs [23]. Some subtypes of CAFs act tumor-suppressive, on the other hand others act as tumor-supportive [24, 25]. CAFs can thus regulate different aspects of tumorigenesis via paracrine signaling [26], but also act together with growth factor receptors on CAFs to affect CAF function, and via integrin-mediated ECM remodeling change the stiffness of the TME [27].

### 4.1 Biomarkers for CAFs

CAFs biomarkers can be categorized into different subtypes including membrane proteins, intracellular proteins, cytoskeletal proteins and nuclear proteins [23]. Integrins, FAP, cadherin-11 and PDGFRβ are examples of CAF membrane proteins [23, 28-32]. Cytoskeletal proteins include αSMA, vimentin and FSP1, and secreted proteins include tenascins, osteopontin, periostin and clusterin, all have been reported to serve as biomarkers for different subtypes of CAFs [33-36]. All the above-mentioned biomarkers are also present on other cell types in the TME [23]. Using a combination of markers and analyzing tissue morphology can overcome some of these problems. Although there might not be a unique CAF biomarker given the high degree of heterogeneity more biomarkers for CAF subclasses are clearly needed.

### 4.2 Origin and heterogeneity of CAFs

The origin of CAFs varies just as like the origin of myofibroblasts. Different type of cells (**Figure 1**) such as resident fibroblasts, ADAM12$^{+}$ perivascular cells and pericytes can differentiate into CAFs [37, 38]. Tumor cells can regulate CAF differentiation through secreting cytokines and growth factors including PDGFs, prostaglandins, insulin like growth factors, TGF-β and interleukins (IL-4 and IL-6) [39, 40]. Another source of CAFs are epithelial cells, since they can convert into fibroblasts-like cells through epithelial-mesenchymal transition (EMT) in cancers [41]. It is increasingly being recognized that EMT can occur to varying degrees, and the term "partial EMT" has been introduced [42]. Mesenchymal stromal cells derived from bone marrow, have also been reported as precursors of CAFs subpopulations [30]. Similarly, cell lineage tracing in mouse models of pancreatic and gastric cancers have demonstrated that bone marrow derived mesenchymal stem cells can be enrolled into the tumor niche and differentiated into CAFs in response to cytokine signaling [43]. Endothelial cells can be converted into CAFs through endothelial-mesenchymal transition (EndMT) in response to autocrine and paracrine TGF-β signaling [44].

Other sources of CAFs include adipocytes, stellate cells (pericytes of pancreas and liver) and fibrocytes (circulating macrophage-like cells) (**Figure 1**) [39, 45].

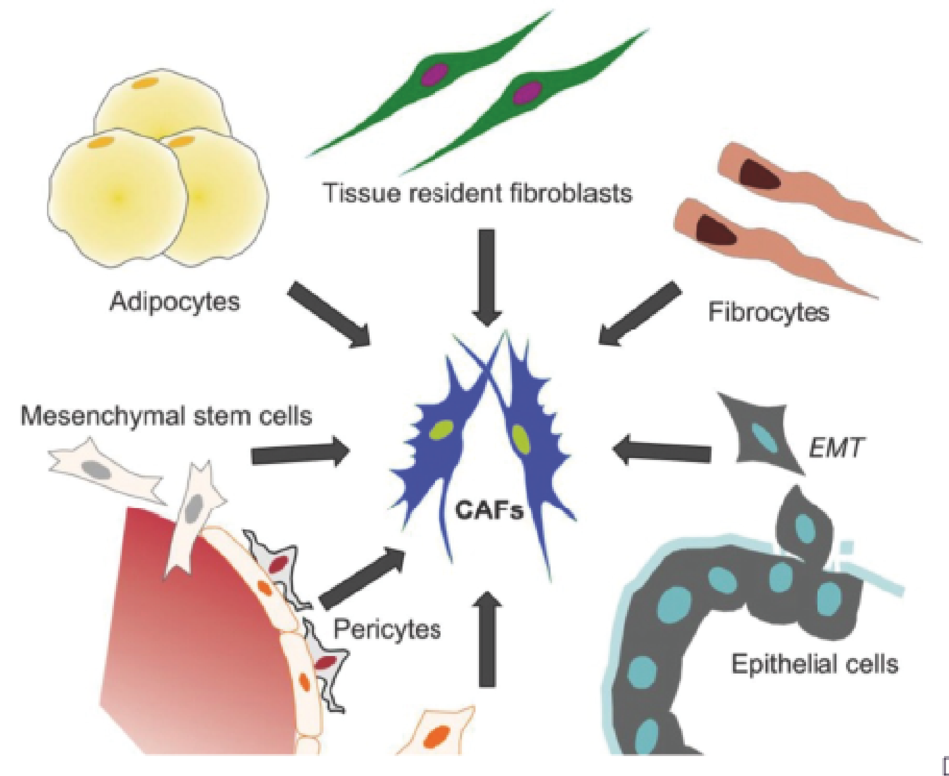


**Figure 1**: Origins of CAFs. Adapted from Alkasalias et al with permission [45].

### 4.3 Role of CAFs in tumorigenesis

Several studies have demonstrated the fundamental roles of CAFs in initiating tumorigenesis, tumor growth, tumor invasion and metastasis in the tumor stroma through secretion of growth factors, cytokines and modulation of the ECM [46-48]. The contribution of CAFs to tumor initiation, have been highlighted in multiple studies comparing the effects of CAFs isolated from human tumors and fibroblasts isolated from normal organs. It was for example demonstrated that when immortalized prostate epithelial cells were inoculated into mice with a mixture of either CAFs or normal fibroblasts that only the CAFs, and not the normal fibroblasts,

led to the emergence of the tumorigenic features [49]. In non-small cell lung carcinoma (NSCLC), CAFs are more effective in initiating tumorigenesis than normal fibroblasts and this effect is partly regulated by CAF secreted cytokines and growth factors [50]. Additionally, different experimental designs, such as gene modification, have been used to illustrate the contribution of CAFs in tumor initiation. For instance, loss of phosphatase and tensin homolog (PTEN) in stromal fibroblasts can induce tumor transformation, initiation and growth in a mouse model of mammary adenocarcinoma, coinciding with increased remodeling of ECM and immune cell infiltration [51]. Liver kinase B1 inactivation in fibroblasts has also been reported to enhance gastrointestinal cancers through induction of IL-11 production and activation of JAK/STAT3 pathway [52]. The above-mentioned studies demonstrated that the transition of natural stroma into CAF-containing tumor stroma is one of the crucial steps in tumor development.

CAFs can not only induce tumor initiation but also promote tumor progression. To promote tumor growth, CAFs can induce both autocrine and paracrine activities. For example, CAFs are involved in secreting C-X-C motif chemokine 12 (CXCL12), which collaborates with C-X-C chemokine receptor 4 (CXCR4) to activate downstream signaling pathways that induces tumor cell proliferation and motility [53-55]. In addition, CXCL14 autocrine signaling promoted tumor growth by interacting with the activation of nitric oxide signaling in CAFs in a prostate cancer study [56]. Moreover, inflammatory cytokines including IL-4 and IL-6 secreted by CAFs have been found to induce tumor growth in models of endometrial cancer and melanoma, respectively [57, 58]. In addition, activated fibroblasts secrete ECM-degrading proteases including the MMPs [59, 60]. MMPs can facilitate the motility and invasion of tumor cells by promoting EMT [61].

CAFs are essential mediators at the metastatic site as a result of secondary tumor growth. At the primary tumor site, CAFs can induce metastasis by secreting cytokines and growth factors into the circulation, which directly or indirectly promote the common features of tumor cells at distant site [62]. CAFs can alter ECM stiffness, which can lead to the generation of ECM tracks to promote cancer cells invasion [63,

64]. Tenascin-C and VEGFA-expressing CAFs have been shown to play a fundamental role in the spreading of breast cancer cells to the lung [65]. Another example includes two separate colorectal cancer studies where TGF-β1 and PDGF both stimulated CAFs to enhance the formation of distant metastasis [66, 67].

# 5. Integrins

Integrins are a major group of cell surface receptors, which are type 1 transmembrane proteins. Integrins link the ECM with cell cytoskeleton but also mediate cell-cell interactions [68]. They are heterodimeric proteins consisting of a α-subunit and a β-subunit, which dimerize non-covalently. In vertebrates, the integrin family is composed of 18 α- and 8 β-subunits that form 24 distinct integrins, which can be classified according to their ligand specificities and subunit composition (**Figure 2**) [69, 70].

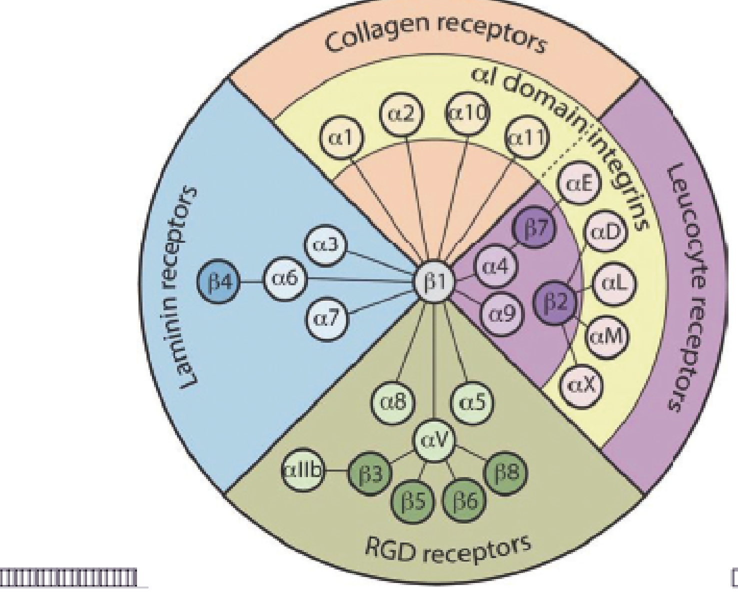


**Figure 2**: **Schematic illustration of the integrin family**. α and β subunit associations of the 24 distinct integrin heterodimers, belonging to the vertebrate integrin family. Figure used with permission [70].

Integrins play a vital role in cell adhesion to the ECM, as they make a physical connection between the cytoskeleton and the ECM. By regulating mechanotransduction at cell-ECM communication sites, integrins can also activate many intracellular signaling events, which are necessary for cell proliferation, cell migration and gene regulation [31, 71].

### 5.1 Integrin structure

Integrin heterodimers are composed of several distinct domains with flexible linkers between them. Extracellular domains of α and β-subunits contain around 1000 and 750 amino acids respectively, whereas the cytoplasmic tail is shorter; approximately 15-65 amino acids following a transmembrane domain of around 20 amino acids [72]. The α-subunit determines integrin ligand specificity and its extracellular domain consist of a seven-bladed β-propeller linked to a thigh, a calf-1, and a calf-2 domain. In addition, a domain of around 190 amino acids can be inserted between blades 2 and 3 of β-propeller, which is known as the α-I domain (**Figure 3**). Nine out of the eighteen-integrin α-chains contain this domain. The α-I domain containing integrin binds to the ligand by an even site, the metal ion-dependent adhesion site (MIDAS) [70]. The β-subunit contains 7 domains: a hybrid domain, a plexin-semaphorin-integrin (PSI) domain, a β-I domain, and four cysteine-rich epidermal growth factor (EGF) repeats. The β-I domain, homologous to the αI domain, is inserted into the hybrid domain and contains an $Mg^{2+}$-coordinating MIDAS and two extra adjacent metal sites; adjacent to metal ion-dependent adhesion site (ADMIDAS) and synergistic metal ion-binding site (SyMBS) [69, 72]. The ADMIDAS site binds an inhibitory $Ca^{2+}$, whereas the SyMBS site binds a synergistic ion which may promote integrin conformational changes resulting in a stabilized active form of integrin [73, 74]. In the integrins lacking the α-I domain, the β-propeller of the α chain and the β-I domain of the β chain are mainly involved in ligand binding. There is no striking homology among the α-subunit cytoplasmic tails except for a conserved GFFKXR motif, while the tail of the β-subunit is relatively homologous between the different β-chains except β8 [69].

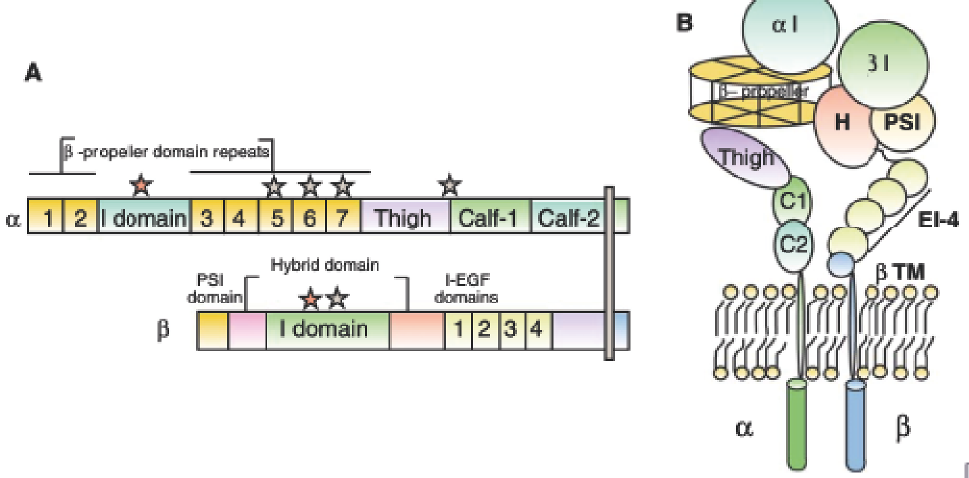


**Figure 3**: **Integrin structure**. **A)** Different domains composing on α-I domain containing integrin. **B)** Representation of domain arrangement on α-I domain containing integrin [70].

### 5.2 Integrin activation

Integrin activation is a process which involves alteration of the integrin conformation from bent (inactive) to fully extended integrin (active) [75]. Integrins can be activated in two different ways: inside-out or outside-in signaling. In inside-out signaling, cytoskeletal proteins called talins and kindlins bind to the β-cytoplasmic tail via the proximal and distal NPXY motif and break the β and α cytoplasmic tails salt bridges. This interaction initiates conformational change in the transmembrane domain (TMD) of the β-unit resulting in activation of the integrin [76, 77]. In the outside-in signaling, various ligands interact with integrin leading to a conformational change and an activation of the integrin (**Figure. 4**) [74, 78].

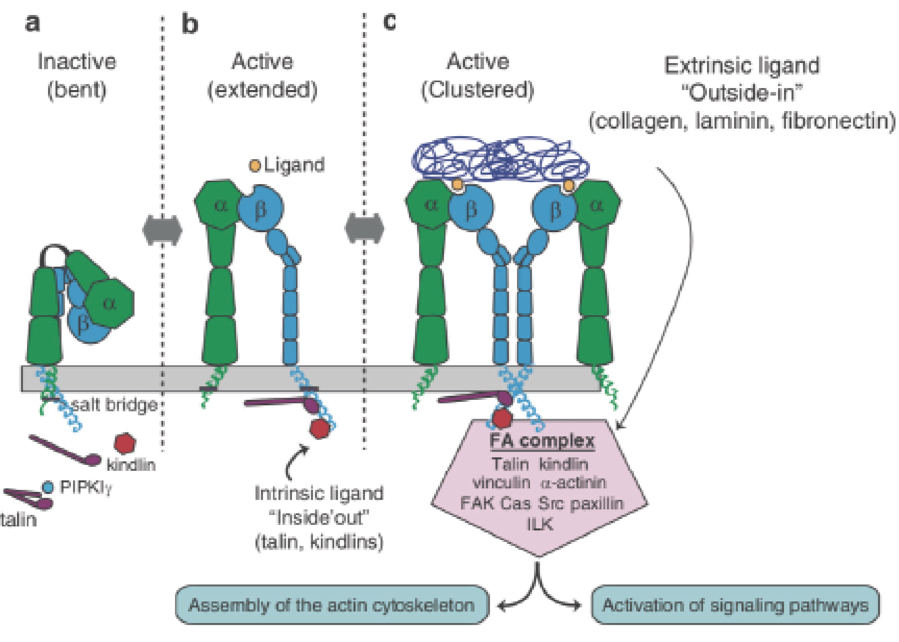


**Figure 4: Schematic illustration of integrin activation and signaling mechanisms.** The bent form corresponds to an inactive form. After integrins are activated from the ECM or the cytosol, they assume a fully extended and open form. Original figure used with permission [78].

### 5.3 Integrin signaling

Integrins can engage multiple proteins and initiate intracellular signaling pathways, although by themselves have no enzymatic activities [79]. Integrins transmit chemical signals into the cell cytoskeleton after the recruitment of approximately one hundred proteins and the formation of the integrin adhesion complex (IAC) [80]. When cells interact with different ligands, adaptor proteins (talin and vinculin) mediate connections between the cytoskeleton and integrins. Thess connections will lead to a reinforcement of the link between ECM and the cytoskeleton and thus promote the recruitment of other cytoskeletal and signaling proteins [79, 80]. Focal adhesion kinase (FAK) is one of the first signaling proteins recruited by the integrins clustering in the IAC. IAC works as a phosphorylation-regulated signaling scaffold and initiates several intracellular signaling mechanisms [81]. Autophosphorylation of FAK at Y397 generates a docking site for SH2-domain- containing proteins like Src-family

protein tyrosine kinases (SFKs) [82, 83]. Src is activated and then phosphorylates additional tyrosine residues in FAK, resulting in the recruitment of other scaffolding proteins such as paxillin and p130Cas, thereby leading to activation of signaling pathways such as Rho GTPase signaling [79, 84, 85]. Rho GTPase downstream signaling is essential in regulating cytoskeletal remodeling during cell adhesion, cell migration and cell spreading [85, 86]. In addition, mitogen-activated protein (MAP) kinase signaling is another common integrin dependent FAK-Src signaling pathway. In MAP kinase family of kinases, MAP kinase (MAPK), MAP kinase kinase (MAPKK) and MAP kinase kinase kinase (MAPKKK) are central in this pathway. Extracellular signal-related kinase (ERK), p38 mitogen-activated protein kinase (p38) and c-Jun N-terminal kinase (JNK) are the three primary MAPK family members, which play a vital role in integrin-dependent cellular functions such as cell proliferation, cell differentiation and apoptosis [87].

### 5.4 Integrin cytoplasmic tails in signaling

Integrin cytoplasmic tails are essential for integrin activation and signaling as well as being involved in structural cytoskeletal consolidations [82, 88]. The integrin α-subunit cytoplasmic tails have no striking homology except for a conserved GFFXR motif [89, 90]. Cytosolic proteins like SHARPIN (Shank-associated RH domain-interacting protein), Nischarin and MDG1 (Mammary-derived growth inhibitor) have been reported to interact with the conserved GFFXR motif of integrin α-subunits to affect integrin activation [82, 91]. Mutation of the Arginine (R) in this conserved motif renders the integrin an inactive conformation, which occurs due to a breakage of the salt bridge between the α and β subunits [92, 93]. In the β-subunit, the cytoplasmic domains are relatively similar in comparison with the highly heterogenous α-subunit cytoplasmic tails [82, 88]. As mentioned above, the conserved NPXY sequence found in the β-subunit tails is a crucial interacting site for cytoskeletal proteins such as talin and kindlins which are involved in integrin activation and signaling [77, 94]. In addition, other cytoskeletal proteins like Dok1, integrin cytoplasmic domain-associated protein 1 (ICAP-1) and Filamin-A interrupt

integrin activation by masking both talin and kindlin binding to the corresponding NPXY motifs [82]. Further, the proximal NPXY motif is suggested to play a vital role in the localization of integrins into fibrillar adhesions by interacting with the focal adhesion protein, tensin [95]. Recently, it has been demonstrated that α-subunit cytoplasmic tails contribute to several integrin-cytoskeletal interactions, previously thought to be mediated solely by binding to the integrin β-subunit tails [96-98].

**Figure 5: Sequence homologies of integrin cytoplasmic tails**. Conserved amino acids in the α and β cytoplasmic tails are coloured. Additionally, the tyrosine and threonine phosphorylation regions are highlighted in red and magenta respectively. Original figure [89] used with permission.

## 5.5 Collagen binding integrins

Collagens constitute 30% of the total protein in humans. There is a total of 28 collagens in the collagen family which can be further subdivided into subfamilies, including fibrillar collagens. Fibrillar collagens are mainly secreted and synthesized by fibroblasts [2]. Cells interact with collagens through collagen-binding integrins, α1β1, α2β1, α10β1 and α11β1 of which all contain an α-I domain in their respective α subunits [70]. These integrins have the ability to recognize a GFOGER-like sequence in native collagens [99, 100].

### 5.5.1 Integrin α1β1

Integrin α1β1 was first identified in a subset of T-cells [101]. It is expressed on mesenchymal cells, including vascular smooth muscle cells [102-104]. Integrin α1β1 expression has also been reported in neuronal cells, endothelial cells, white blood cells, bone marrow mesenchymal cells, mesangial cells, chondrocytes, pericytes and fibroblasts [101, 102]. Ligands for α1β1 include collagens I, III, IV and IX. However, α1β1 has higher affinity for collagen IV than fibrillar collagens [105] [102]. Other α1β1 ligands include various laminin isoforms, matrilin-1, semaphorin 7A, and galectins 1, 3 and 8 [70, 102].

The cytoplasmic tail of the α1 subunit contributes in α1β1 mediated MAPK signaling, which is involved in cell migration and proliferation [106]. Integrin α1-null mice show increased retinal degeneration, osteoarthritis and some other mild phenotypes at a later age [102, 103]. Moreover, in mouse tumor models, α1 deficient mice display reduced tumor angiogenesis which leads to smaller tumors in comparison with wildtype mice [102].

### 5.5.2 Integrin α2β1

The discovery of integrin α2β1 took place around the same period as the discovery of integrin α1β1 and it was originally identified as an ECM receptor for collagens

and/or laminins [101]. It is primarily expressed on fibroblasts, endothelial cells, epithelial cells, T-cells, platelets and myeloid cells [102, 107]. Fibrillar collagens are the main ligands for integrin α2β1, more specifically collagens I, II, III, V, XI and XVI, however, other collagens such as collagen IV and VI also act as ligands for integrin α2β1 [107-110]. Integrin α2β1 has also affinity for other ligands like laminins, and small PGs such as lumican and decorin [107].

The cytoplasmic tail of the integrin α2 chain has been reported to activate p38α MAPK inside a collagen gel in α2 overexpressing human osteosarcoma cells [111, 112]. Moreover, the α2 cytoplasmic tail was found to participate in protein phosphatase 2A activation to control cell proliferation via Akt dephosphorylation [113].

Integrin α2-null mice display a subtle phenotype with abnormalities in mammary gland branching, a platelet aggregation defect and induced neoangiogenesis in wound healing[114-117]. Interestingly, α2 knock out mice exhibit increased metastasis in one breast cancer model [118]. In addition, integrin α2-deficient mice have been reported to have other phenotypes such as age-related osteoporosis [119].

### 5.5.3 Integrin α10β1

Integrin α10β1 was first characterized in cartilage tissues [120]. It is generally expressed in chondrocytes or chondrogenic mesenchymal stem cells, some junctional fibroblasts in the bones and ligaments, ribs and vertebrae [121, 122]. The common ligands that bind to integrin α10β1 include collagen II and XI [100]. Further, integrin α10β1 also binds to collagen IX in vitro [123]. The α10 knock-out mice exhibit a mild phenotype with growth plate chondrocytes defect [124]. Interestingly, a chondrodysplasia-like phenotype was observed in two dog strains with truncated ITGA10 mRNA [125]. Fibroblasts growth factor 2 (FGF2) has been reported to upregulate ITGA10 mRNA levels in mesenchymal stem cells [100, 121]. In addition, integrin α10β1 also influences melanoma cell migration [126]. Recently, it has been

demonstrated that integrin α10β1 can regulate cell proliferation, migration and survival in a glioblastoma context [127].

### 5.5.4 Integrin α11β1

Integrin α11β1 is a collagen-binding integrin and the most recent addition to the integrin family [128]. Structurally, it is similar to integrin α10, but has high affinity for collagen type I [129].

#### 5.5.4.1 Structure of ITGA11

The gene *ITGA11* has been mapped to human chromosome 15, whereas the mouse integrin α11 gene (*Itga11*) is located on chromosome 9 [130, 131]. *ITGA11* contains 30 exons and 29 introns and covers about 122 kb of genomic sequences (**Figure 6**) [132]. The transcript of integrin α11 gene covers a 30-nucleotide (nt) 5' untranslated region, a 3564-nt open reading frame, a 329-nt 3' non-coding sequence with a PolyA tail and a 3' untranslated region.

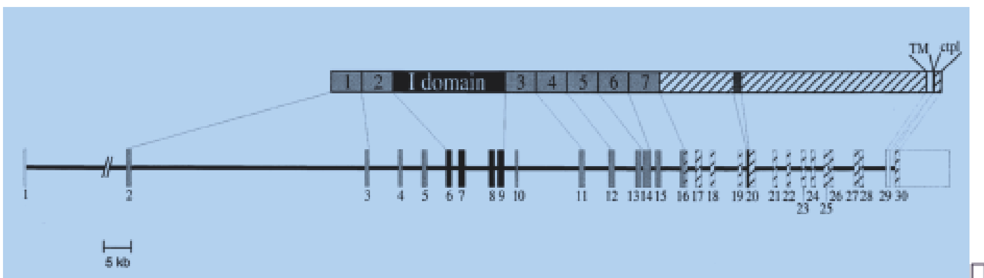


**Figure 6: Schematic overview of human *ITGA11* and integrin α11 protein**. Upper part shows marked region of the I-domain within seven FG-GAP repeats (1-7), transmembrane region (TM) and cytoplasmic tail (ctpl) in integrin α11 protein. Lower part shows the organization of *ITGA11* overview with its 30 exons. Original figure used with permission [132].

#### 5.5.4.2 ITGA11 promotor regulation

The promotors of integrin α chains frequently have TATA- and CCAAG- boxes in the proximal promoter region but the *ITGA11* promotor lacks it. Instead, *ITGA11* has two Sp1 binding sites (SBSs) and an ETS-binding site (EBS) found close to the transcriptional start site (TSS); which has been suggested to regulate integrin α11 transcription in mesenchymal cells [133]. SBSs and EBS both appears to have crucial functions in activating the proximal *ITGA11* promoter [132, 133].

TGF-β is involved in the regulation of genes by activating transcription factors binding to specific regions in their promoters [134]. TGF-β1 has been shown to induce integrin α11 expression in human skin fibroblasts and MRC-5 lung fibroblasts; as well as in the HT1080 fibrosarcoma cell line. It has been demonstrated that TGF-β1 responsive element depends on both a Smad binding element, SBE2, and a Sp1-binding site, SBS1, which are located in the proximal promoter at nt -182/-176 and -140/-134, respectively. Additionally, other elements needed for TGF-β1 responsiveness are mapped upstream in the promoter region at nt -2962/-330 [134].

Integrin α11 expression has been shown to be up-regulated in mouse embryonic fibroblasts grown on attached collagen lattices. Molecular studies revealed that TGF-β family member Activin A was involved in the regulation of integrin α11 within mechanosensitive manner under the control of Smad3 transcription factor [135].

#### 5.5.4.3 Cre mediated recombination

The Cre-recombinase system is an intrinsic experimental tool for developing animal models with cell-type-specific gene activation. Cre-recombinase is a 38 kDa recombinase protein from bacteriophage P1, which mediates site specific excision or recombination between loxP sites. LoxP is a 34-bp recombination site consists of two 13-bp inverted repeats divided by an 8-bp asymmetric spacer region. Hence, Cre-recombinase is a simple form of the Xer system [136, 137]. When the Cre-

recombinase protein is expressed with a tissue-specific promoter of a transgene it will remove or invert loxP-flanked genomic segments in this tissue only. Using this Cre-recombinase, it is possible to produce desired alterations of the mouse genome, which can be used as a tool in conditional transgenesis or knock-outs and genetic activation or inactivation switch systems [137]. Currently there is no good Cre driver mouse strain for deleting genes in a completely fibroblast-specific manner. With the increasing realization about fibroblast heterogeneity, it will be important to generate new fibroblast transgenic mouse strains with specificity for distinct fibroblast subtypes.

In a previous study, a 3 kb human integrin α11 promoter reporter mouse with a mesenchymal signature has been characterized [132]. The 3kb *ITGA11* promoter was shown to drive fibroblast-restricted expression in the ITGA11-LacZ transgenic mouse embryos [134]. Based on these data, we chose to use the 3 kb promoter region and to conjugate it to Cre-recombinase in **paper 2**. We predict that ITGA11- Cre transgenic mice will be a highly useful tool for the ablation of genes in a pattern restricted to α11-expressing fibroblasts in the developing mouse as well as in models of fibrotic disease such as dermal fibrosis, heart fibrosis and tumor fibrosis.

#### 5.5.4.4 Expression of integrin α11

Integrin α11 was first identified as a major integrin in cultured human fetal muscle cells *in vitro* [138]. mRNA analysis of human adult tissue demonstrated the expression of integrin α11 was highest in the uterus, followed by heart and skeletal muscle, but later confirmed that the expression of α11 in skeletal muscle cells was an *in vitro* cell culture artefact and it's *in vivo* expression was limited to mesenchymal non-muscle cells [129, 130]. In human embryos, the expression of α11 is noted in fibroblasts around developing cartilage in ribs, vertebrae, in intervertebral discs and in keratocytes of the cornea [129]. In the mouse embryo, α11 expression appears restricted to mesenchymal non-muscle cells (**Figure 7**) including craniofacial neural crest cells, periodontal ligament cells (PDL), tendon fibroblasts, intestinal villi

fibroblasts, and mesenchymal cells in the periosteum, perichondrium around developing cartilage in ribs, sternum, vertebrae, intervertebral discs and limbs [139]. Low expression of α11 integrin protein has been shown in adult mouse tissues including intestine, ear, eye, tongue, skin, lung and spleen. High α11 integrin expression was observed in the heart, skeletal muscle, smooth muscle and skin in a transgenic mice which overexpressed α11 integrin using the β-actin promoter [140]. Additionally, α11 integrin is identified in human molar PDL fibroblasts *in vitro* and in mouse incisor PDL fibroblasts *in vivo* [139, 141]. Integrin α11 was also detected in CAFs isolated from lung carcinoma and pancreatic cancer [142, 143].

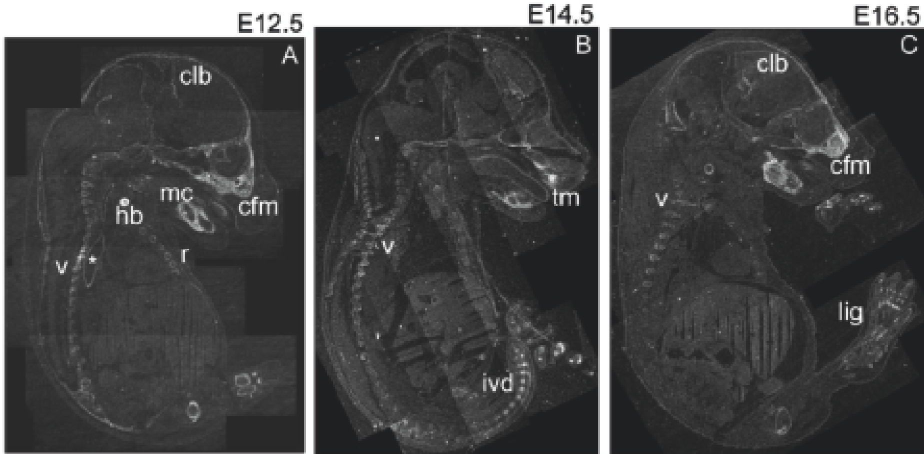


**Figure 7: α11 mRNA localization in mouse embryos.** Sagittal sections of mouse embryos at different ages after in-situ hybridization using an antisence RNA probe against mouse α11. In E12.5-E16.5, α11 mRNA expression is observed in the fibroblasts of skeletal muscle including around calvarian bone (clb), hyoid bone (hb), vertebrae (v), Meckel cartilage (mc) and ribs (r), and in craniofacial mesenchyme (cfm), intervertebral discs (ivd), tongue mesenchyme (tm) and ligament (lig). Figure used with permission [139].

#### 5.5.4.5 Function of integrin α11 *in vitro*

*In vitro* studies demonstrated that the integrin α11β1 is a functional collagen receptor that promotes cell attachment and migration. α11β1 mediates the contraction of a collagen gel and this feature resembles the collagen reorganization mediated by activated fibroblasts in tissue remodeling events, fibrosis and wound repair [129].

α11β1 displays a preference for collagen type I compared to collagen type IV. Integrin α11β1 has in one study been proposed to act as a receptor for tenascin-X and in a recent study also for osteolectin [144, 145].

#### 5.5.4.6 Function of integrin α11 *in vivo*

*In vivo* analyses using α11 integrin knockout mice have partially elucidated the function of integrin α11 (**Figure 8**). A relatively mild phenotype is observed in the α11-null mouse but the mutant mouse is dwarfed compared to their wild type littermates. Delayed incisor eruption and altered tooth shape are thought to be the reason for the reduction in size, also thought to contribute to malnutrition [141, 146].

In wounded skin, the α11 integrin KO mice showed a defect in myofibroblast differentiation and impaired JNK activation by TGF-β signaling, which supports the the *in vitro* findings that the integrin α11 chain contributes to myofibroblast differentiation [135, 147]. Integrin α11 has been reported also to be induced in pathological conditions involving fibroblast functions such as fibrosis and tumor progression [142]. In a model of diabetes-induced heart fibrosis, integrin α11 has been implicated in myofibroblast differentiation and ECM formation [148, 149]. In further support of a pro-fibrotic role of α11, a recent study has demonstrated that cardiac overexpression of integrin α11 in transgenic mice exhibit an induction of cardiac fibrosis and left ventricular hypertrophy [140]. Finally, integrin α11 has recently been reported to be required for osteogenesis in response to osteolectin in a subset of bone marrow derived mesenchymal stem cells [145].

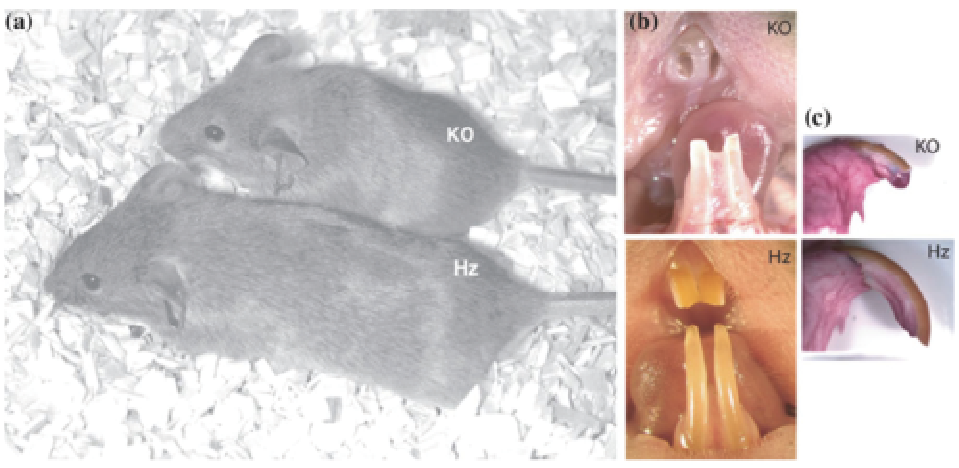


**Figure 8: Phenotype of integrin α11-deficient mice.** a Homozygous KO ($\alpha11^{-/-}$) mouse was smaller than heterozygous Hz ($\alpha11^{+/-}$) littermate. b Defect in upper incision teeth eruption was seen in KO mouse. c Altered incisors tooth shape in KO compared to Hz mouse. Original figure taken [146] with permission.

#### 5.5.4.7 Function of integrin α11 in TME

Relatively few studies have been performed concerning the role of the integrin α11 subunit in the context of tumors. In 2002, *ITGA11* was found to be part of a five novel candidate gene signature for lung adenocarcinoma [150]. It has later been demonstrated that integrin α11 is up-regulated in the CAFs in non-small cell lung cancer [142, 151]. Co-implantation of α11 knock-out MEFs and NSCLC tumor cells in a xenograft model significantly reduced NSCLC tumor growth, these data resulted in a model where stromal α11β1 regulates secretion of IGF-2 [152]. Recently, α11β1 has been reported to mediate NSCLC tumor growth and invasion by regulating a matrix cross-linking enzyme, LOXL1 (**Figure 9**) [23, 151]. In breast cancer, α11 works by a different mechanism, in the PyMT model α11β1 has recently been shown to modulate PDGFRβ/JNK signaling in CAFs and to promote cancer progression [27]. In addition, a pro-tumorigenic effect of stromal integrin α11 was suggested in a study of triple negative breast cancer [153]. In an *in vitro* based breast tumor spheroid model, integrin *ITGA11* was considered as one of seven genes that regulated cell

migration of tumor cells that had assumed a mesenchymal phenotype without undergoing EMT [154]. In pancreatic cancer, integrin α11β1 has been suggested to be a major stromal integrin, which can regulate differentiation of pancreatic stellate cells (PSCs) into CAFs and induce tumor cell invasion and metastasis [143]. To expand our knowledge integrin α11 in tumorigenesis, we examined the expression of α11 in desmoplastic tumor stroma in different types of cancers in **Paper 3.**

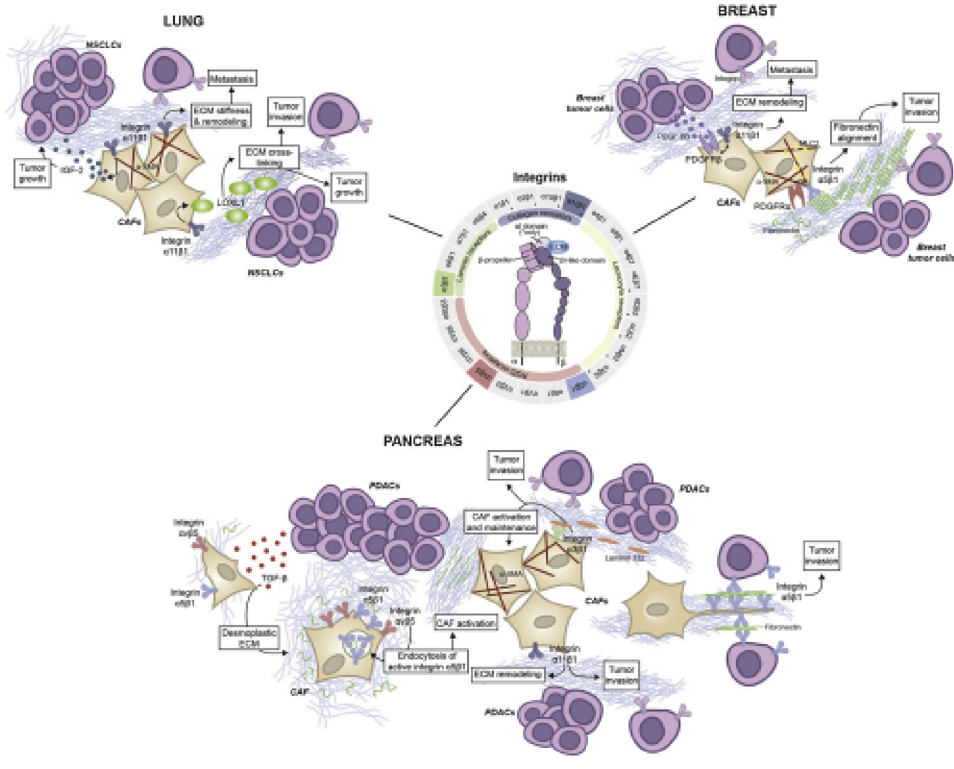


**Figure 9:** Schematic representation integrin interactions of cancer-associated fibroblasts in the TME of lung, breast and pancreatic cancers. Original figure used with permission [23].

# 2. Aims of the present study

Integrin α11β1 is a collagen-binding integrin and the last addition to the integrin family [128]. Recent data suggests that α11, in addition to playing a fundamental role in granulation tissue formation and myofibroblast differentiation in dermal fibroblasts, also is pro-tumorigenic in the non-small cell lung cancer stroma and pro-fibrotic in diabetic hearts, suggesting that α11 is a biomarker for a subset of pro-fibrotic fibroblasts in some tissues and tumors [142, 147]. However, the detailed molecular mechanisms underlying integrin α11β1 expression and function in the context of tissue and tumor fibrosis remains to be determined.

The overall aim of present study was to further characterize integrin α11 expression and function at both cellular and molecular level.

## 2.1 Specific objectives

A. To investigate the functional role of integrin α11 cytoplasmic tail.
B. To characterize a novel transgenic (ITGA11-Cre) mouse strain.
C. To analyze α11 integrin expression in the TME of different tumor types.

# 3. Summary of results

**Paper 1**

**Integrin α11 cytoplasmic tail is required for FAK activation to initiate 3D cell invasion and ERK-mediated cell proliferation**

In this paper, we generated a mutant variant of human integrin α11 ($_{Hu}\alpha$11-1171) by deleting the 17 carboxyterminal amino acids residues in the intracellular part of integrin α11 protein to investigate the role of the integrin α11 cytoplasmic tail. Constructs containing the coding sequence of either wild-type α11 ($_{Hu}\alpha$11-WT) or tail-less α11 ($_{Hu}\alpha$11-1171) were expressed in mouse satellite cells C2C12 lacking endogenous collagen-binding integrins. Both $_{Hu}\alpha$11-WT and $_{Hu}\alpha$11-1171 expressing cells attached similarly to collagen I- and fibronectin-coated surface in culture, suggesting that cytoplasmic tail has no effect in integrin α11 activation. Curiously, $_{Hu}\alpha$11-1171-expressing cells showed fewer focal adhesions, and reduction of cell proliferation and cell migration on collagen I, in comparison with $_{Hu}\alpha$11-WT expressing cells. Additionally, $_{Hu}\alpha$11-1171-expressing cells also displayed a significant reduction of collagen gel contraction than $_{Hu}\alpha$11-WT-expressing cells.

We next investigated the role of α11 cytoplasmic tail in FAK and MAPK signaling. $_{Hu}\alpha$11-1171-expressing cells demonstrated less $FAK^{Y397}$ and ERK activation compared to $_{Hu}\alpha$11-WT-expressing cells following cell attachment to collagen I. In addition, knockdown of integrin α11 in primary human gingival fibroblasts (hGFs) showed reduction of FAK and ERK activation.

We then examined the role of FAK and ERK activation in the α11 cytoplasmic tail-mediated cell functions on collagen I. The results revealed that inhibition of $FAK^{Y397}$ activation by PF57228 and small molecule inhibition of ERK activation (U0126), impaired cell proliferation in both $_{Hu}\alpha$11-WT and $_{Hu}\alpha$11-1171-expressing cells, suggesting that both FAK and ERK activation are needed for α11-mediated cell proliferation. In addition, we investigated the overlapping function of FAK and ERK.

Inhibition of $FAK^{Y397}$ impaired ERK activation, whereas inhibition of ERK had no effect on $FAK^{Y397}$ activation, indicating that ERK activation is FAK dependent. Surprisingly, inhibition of ERK had no effect in spheroid cell migration for either $_{Hu}\alpha$11-WT or $_{Hu}\alpha$11-1171-expressing cells, however $FAK^{Y397}$ inhibition completely impaired migration for both $_{Hu}\alpha$11-WT- and $_{Hu}\alpha$11-1171-expressing cells. Finally, we investigated the effect of FAK activation in focal adhesion formation, since FAK is localized in focal adhesion. $_{Hu}\alpha$11-1171 cells displayed reduced amounts of phosphorylated $FAK^{Y397}$ positive adhesions than $_{Hu}\alpha$11-WT cells in agreement with our previous results. In brief, our results suggest that the cytoplasmic tail of α11 integrin is crucial for stabilization of focal adhesions, which contributes to $FAK^{Y397}$-dependent cell proliferation and cell migration.

**Paper 2**

**Characterization of an ITGA11-Cre mouse strain with Cre recombinase expression restricted to fibroblasts.**

Previously, a 3kb *ITGA11* promoter was shown to drive a fibroblast-specific reporter gene expression (*LacZ*) in ITGA11-lacZ transgenic mouse embryos. In paper 2, using the same 3kb *ITGA11* promoter, we have generated an ITGA11-Cre mouse stain, and characterized Cre expression driven by the *ITGA11* promoter. To construct the ITGA11-Cre mouse stain, we used the site-specific integrase-mediated transgenesis method. The successfully generated ITGA11-Cre mouse strain was then crossed with the lacZ-reporter mouse strain Rosa26R (R26R) to get the *Cre+/-;lacZ+/-* mice where the Cre, once expressed, can remove the loxP-flanked DNA STOP sequence that prevents the expression of the lacZ gene, resulting the expression of lacZ. Thus, the tissue/cellular expression pattern of Cre can be monitored by lacZ expression using X-gal staining. Whole-mount X-gal staining was performed on embryos from ITGA11-Cre and R26R breeding at E13.5. Positive X-gal staining was only observed in Cre-postive embryos identified by genotyping.

Further characterization of Cre-recombinase expression was performed by X-gal staining on embryo sections, due to a technical problem of poor reagent penetration with X-gal whole-mount staining. In the embryo sections, Cre-recombinase activity, indicated by positive X-gal staining, was mainly noted in fibroblasts of musculoskeletal system including the periosteum and cartilage primordium of vertebrae, ribs, intervertebral disc, hyoid bone and scapula, and in mesenchyme in forming digits, Meckel cartilage and dental follicle. This staining pattern collectively recapitulated the endogenous α11 expression at these sites. In addition to the observed staining in mesenchymal cells in the forming muscoskeletal system, X-gal staining was also noted in meningeal fibroblasts covering the brain and in the cardiac epicardium, again reflecting previously reported endogenous integrin α11 expression. The staining seen in mature chondrocytes and inside the brain did not agree with endogenous α11 expression but was regarded as an X-gal staining artifact rather than mis-expression of the transgene. Endogenous β-galactosidase activity was also noted in intestine and skin epidermis.

Further, we also examined Cre-recombinase activity by X-gal staining in mouse embryonic fibroblasts (MEFs) isolated from α11-Cre+; R26R embryos. Interestingly, around 60% of MEFs showed positive X-gal staining while the rest were shown to be negative.

We subsequently investigated Cre-recombinase expression in adult mouse tissues. Western-blotting demonstrated low expression of β-galactosidase in the different mouse tissues in agreement with endogenous integrin α11 expression.

Therefore, we characterized ITGA11-Cre expression in two mouse models; a heart fibrosis model and a skin wound healing model. In both cases, integrin α11 have been shown to be upregulated [140, 147, 148], and it was interesting to determine whether Cre expression would be regulated the same way as for endogenous α11 in thesw models. Aortic banding was performed to induce heart fibrosis and β-galactosidase expression was monitored in the fibrotic regions of transgenic mouse heart sections. Cre-recombinase expression was observed to be induced in fibrotic

regions of aortic-banded hearts compared to sham-operated control mouse hearts. Whereas western blotting detected protein levels of β-galactosidase in both sham-operated and aortic banded hearts, integrin α11 was clearly noted to upregulated in aortic-banded hearts.

To investigate ITGA11-Cre expression during wound healing, Cre-recombinase activity during excisional wound healing in a skin model of injury was examined. X-gal staining was noted in the wound of the ITGA11-Cre+; R26R mice but not in the control mice. We further confirmed X-gal staining by Western-blotting.

**Paper 3**

**α11β1 Integrin is Induced in a Subset of Cancer-Associated Fibroblasts in Desmoplastic Tumor Stroma and Mediates In Vitro Cell Migration**

In this paper, we investigated the expression and distribution of integrin α11 in different human tumor types. For this purpose, we have developed a new anti-human α11 mouse monoclonal antibody (mAb), mAb 203E3. In parallel, we generated a function blocking anti-human α11 antibody, mAb 203E1, to test the role of α11β1 as a collagen receptor in the tumor microenvironment. Both mAbs are mono-specific and no cross reactivity with the β1 subunit or other tested integrin α-chains was noted. The specificity of mAbs was confirmed by flow cytometry, immunostaining and immunoprecipitation. The effectiveness of function blocking antibody, mAb 203E1, was examined by cell functional assays under both two- and three-dimensional culture conditions.

To examine integrin α11 expression in different tumor types, a tumor tissue array with their corresponding normal tissue, was examined using α11 mAb 203E3. We found no detectable expression of integrin α11 in any of the normal tissues, except for the kidney tissue. In tumor tissue array, the breast, lung, pancreas, liver, ovary and uterus showed upregulated integrin α11 expression. Co-staining with cytokeratin antibody revealed a specific expression of α11 in the stroma. A modest to low

expression of α11 in the stroma of small intestine, stomach adenocarcinoma, skeletal muscle rhabdomyosarcoma and skin squamous carcinoma, was also observed.

To further characterize the expression of α11 in the stroma of tumor tissue array, co-staining of α11 subunit with three stroma markers, namely FSP1, αSMA and vimentin, were performed. The results showed that co-localization of integrin α11 with FSP1, vimentin or αSMA varied within different tumors. α11 with FSP1 was poorly co-expressed in the stroma of tumors, whereas α11 co-stained with αSMA to a variable degrees in areas with activated stoma, which suggested the expression pattern of integrin α11 in the stroma cells corresponding to myCAFs [19]. Finally, integrin α11 staining overlapped with vimentin staining in the stroma of most tumors. Interestingly, α11 expression was also noted in stoma cells with nen-detectable vimentin expression.

Real-time quantitative polymerase chain reaction (RT-qPCR) data with RNA isolated from normal tissues and carcinomas in breast, pancreas and lung confirmed upregulation of integrin α11 in tumors as indicated by our immunostaining results.

After screening the tissue array, we characterized integrin α11 expressing cells in tumor sections of pancreatic ductal adenocarcinoma (PDAC) and head and neck squamous cell carcinoma (HNSCC). For this purpose, α11 mAb 203E3, was used to co-immunostain with six different stroma markers. The results showed that fibroblast-activation protein (FAP) co-localized with α11, which was concentrated to the peritumoral region, whereas, FSP1 and NG2 (pericyte marker) appeared not to be co-immunostained with α11. In addition, the other two CAF markers, αSMA and PDGFRβ, were co-localized with integrin α11 to a larger extent in stroma regions. Interestingly, vimentin expression displayed different expression pattern compared to α11 expression.

To test the role of α11β1 as a collagen receptor in the TME, we examined a number of CAFs and normal fibroblasts for α11 expression and its function with collagen I using western-blotting and functional assays, respectively. Two additional collagen-binding integrin subunits α1 and α2, were also analyzed. The results revealed that the

function blocking α11 mAb, 203E1, inhibited cell-collagen interactions in the functional assays. Especially, mAb 203 was more effective in blocking 3D spheroid migration in PDAC CAFs, in which integrin α5 had been knocked down (P CAF KD α5). The degree of inhibition was in a pattern that appeared to depend on both the level of α11 expression and the presence of other collagen-receptors.

# 4. Discussion

## 4.1 Methodological considerations

### 4.1.1 Generation of the ITGA11-Cre transgenic mouse strain

The ITGA11-Cre transgenic mouse strain was generated at Transgenic Facility of Stanford University by the integrase-mediated site-specific transgenesis method as they described previously [155]. This method offers higher integration efficiency for transgene integration, and most importantly, the integration is happened in a site-specific manne at a pre-decided locus (in our case at H11 locus). Compared with conventional transgenesis methods, the site-specific transgenesis has the advantage of minimizing some common problems caused by the random integration of transgene, such as inactiving critical genes or transgene silencing due to the flanking sequences at the integration site. To perform the transgenesis, mice were modified at *Hipp11 (H11)* or *Rosa26* loci by knocking in tandem attP sites, and the homozygous mice for the modified loci served as zygote donors. We have choosen to use mice with modified *H11* loci since they gave a higher reporter gene expression than that expressed in mice with modified *Rosa26* loci, according to the results shown in [155]. Briefly, homozygous attP-containing superovulated C57BL/6 females were bred to respective males to develop homozygous attP-containing zygotes. A mixture of fresh transgene DNA/ϕC31mRNA was injected into a single pronucleus and cytoplasm of each zygote by using flow injection mode. The surviving zygotes were then inserted into oviducts of pseudo-pregnant recipient C57BL/6 females. The offspring were genotyped to screen for the founder mice with transgene insertion.

It has also been reported that integration of plasmid backbone could have negative effect in transgene expression, and we therefore used the construct where the transgene is flanked by two attB sites. In this case, the transgene can be exised by ϕC31 recombinase and inserted into the attP site of *H11* loci by cassette exchagen reaction. However, due to some technical problems, we confirmed by genotyping and sequencing that the founder mouse has the inserted transgene followed by the plasmid backbone, and that this mouse was the only one showed positive transgene insertion.

### 4.1.2 X-gal staining

To determine wheather the generated ITGA11-Cre mice revealed an active Cre-recombinase driven by *ITGA11* promoter, and furthermore, showed tissue- and cell-specific expression pattern recapulates endogenous integrin α11 expression, we bred the ITGA11-Cre mice with the reporter mice Rosa26R (R26R). In the reporter mice, a loxP-flanked DNA STOP sequence was inserted upstream of lacZ gene, preventing its expression. Once Cre recombinase is present, the STOP sequence will be removed and lacZ can then be expressed to give the gene product β-galactosidase[156]. In this way, the activity of Cre can be reflected by the expression of β-galactosidase monitored using X-gal staining.

X-gal whole-mount embryo staining was performed as described previously by Lu *et al* [134]. Closer examination in a stereomicroscope revealed that X-gal staining was largely restricted to dermis and did not extend to internal organs. We reasoned that this most likely was due to an accessibility problem and that tissue sections would be needed in order to visualize β-galactosidase activity in internal tissues. Thus, we parformed X-gal staining on sections instead of whole-mount staining.

### 4.1.3 Spheroid preparation and migration assay in 3D collagen gel

We used spheroid assays to investigate both the role of function-blocking antibody **(Paper 3)** and integrin α11 cytoplasmic tail **(Paper 1)**, in cell migration. Single cell type spheroids (homospheroids) were prepared by the hanging drop method, as described earlier [157]. Hanging drop method is a straightforward technique, which allows better control for unform spheroid size and composition. Cell droplets are suspended in an adherent cell culture lid, where gravity derives the aggregation of cells at the bottom of the droplet and forms spheroid [158]. Embedding spheroid within 3D collagen matrix is a versatile method, which provides a means of developing tissue-like cellular aggregates for analysis biomechachemical properties in a physiological-like 3D environment.

## 4.2 General discussion

The overall aim of this thesis was to further characterize integrin α11 expression and function at both the cellular and molecular level.

Integrin cytoplasmic tails are crucial for integrin activation and signaling as well as being involved in structural cytoskeletal consolidations [82]. Independent studies on deleted and mutated cytoplasmic tails have demonstrated differing results for different integrin α-chain cytoplasmic tails [90, 159, 160].

In **Paper 1** we show that the cytoplasmic tail of α11 is essential for collagen-dependent focal adhesion formation, collagen gel contraction, cell proliferation and cell migration, but not for cell adhesion.

Deletions of integrin α subunit tails after the conserved GFFXR sequence have been shown to have different effects on individual collagen-binding integrins. For integrin α1 chain, deletion of the short cytoplasmic tail in one study did not affect cell attachment to collagen IV in fibroblastic 3T3 cells, but the deletion was shown to result in ligand-independent focal adhesion localization [161]. In a separate study deletion of α1 integrin cytoplasmic tail in endothelial cells was shown to impair cell adhesion to collagen IV [106]. Similarly, α2 cytoplasmic tail deletion in RD rhabdomyosarcoma cells was shown to impair cell adhesion to collagens I and to reduce α2 focal contact localization [162]. When considering the function of integrin domains, it is also important to recognize that cell type can influence the activity status of integrin. Expression of integrin α2 in K562 cells yields inactive integrin [163], but in C2C12 cells used in our studies the equilibrium between active and inactive integrins is shifted towards the active forms. In a recent study the effect of integrin α chains in activating integrins was tested using chimeric integrins and was found to contribute differently to integrin inside-out activation in α-chain specific manner [164].

The need for integrin α11 cytoplasmic tail for the formation of focal contacts suggest that the α11 chain, together with the β1 chain, takes part in the formation of the cytoskeletal linkages.

The involvement of integrin α11 tail in collagen remodeling is in agreement with previous studies of integrin α2. In these studies replacing the α2 cytoplasmic tail with integrin α1 or α4 tails, impaired collagen remodeling as compared to the α2 1-mediated collagen gel contraction [90, 111]. These data suggest that integrin subunits with the highest affinity for fibrillar collagens (α2 and α11) also have cytoplasmic tails, able to form stable cytoskeletal linkages designed for tolerating the forces generated during collagen remodeling.

Although integrin α11 was identified more than 20 years ago, the signaling function of α11β1 has not previously been demonstrated in a system where α11β1 is the only integrin collagen receptor expressed on the cell surface. In **Paper 1** we show for the first time the contribution of α11 tail in activating ERK signaling in a FAK-dependent manner. Knockdown of integrin α11 in fibroblasts reduced the activation of FAK and ERK, which differs from human mesenchymal stem cells where it was demonstrated that the phosphorylation of ERK and PI3K occurred an in α11-dependent manner in this cell type that also expresses other collagen-binding integrins[165].

The FAK-dependent α11-mediated activation of ERK is different from results showing that integrin α1-mediated ERK signaling occurred independent of FAK in a manner occurring via Fyn-Shc-Grb2 and Ras [166]. Although integrin α1 has been reported to regulate cell cycle progression in response to FAK-independent ERK signaling via the adapter protein Shc [167], independent studies suggest that α1 also can activate FAK-dependent ERK signaling via p130$^{(CAS)}$, Crk, and Rap1 [168]. For integrin α2, the α2 cytoplasmic tail has in turn been shown to regulate the activation of P38 MAPK through Rac activation [111]. The specific residues in the α2 tail domain needed for the activation of P38 has also been identified [169].

In **Paper 1** we also examined the potential effect of ERK in integrin α11-dependent cell migration. Surprisingly, pharmacological inhibition of ERK had no effect in a spheroid assay of cell invasion. The lack of effect of ERK inhibition could be due to the presence of indirect compensatory mechanisms in the C2C12 cells overexpressing α11. One study of α2-mediated cell migration in smooth muscle cells demonstrated involvement of a G-protein-dependent mechanism [170].

Our results obtained from **paper 1** suggest that the α11 cytoplasmic tail has multiple functions which are important for integrin α11 functions including focal contact formation, collagen remodeling, cell proliferation and cell migration, and which most likely involve novel integrin α11- cytoskeletal interactions waiting to be identified.

Fibroblasts in different tissues and in tissues undergoing reorganization during wound healing, fibrosis and tumor growth are heterogenous in nature [23, 100]. The lack of fibroblast-specific markers complicates investigatation of the role of fibroblasts under these conditions. We previously characterized 3 kb of the promoter of the human integrin α11 gene (*ITGA11*) in a reporter mouse and noted a mesenchymal signature during mouse embryonic development [132]. Based on these earlier studies, we have now used the same 3 kb of the *ITGA11* promoter region to drive Cre-recombinase expression in **paper 2**. To analyze ITGA11Cre functional activity we crossed the Cre driver mouse with the Rosa26R reporter mice.

Our analysis of ITGA11-Cre in embryo demonstrated strong expression in fibroblasts of the developing musculoskeletal system, supporting previously observed endogenous expression of integrin α11 at these sites [139, 141]. In addition, positive staining was also noted in meningeal fibroblasts and in the cardiac epicardium, which is also in agreement with previously noted expression of α11 mRNA and α11 protein observed at these sites [139]. ITGA11-Cre expression in the intestine and skin epidermis of embryos was considered to be non-specific since control (Cre negative) embryo displays a similar staining pattern. To better characterize ITGA11-Cre functional activity in embryo, it would be interesting to cross the ITGA11-Cre driver mouse strain with another reporter mice; Rosa-CAG-LSL-tdTomato.

Further, analysis of ITGA11-Cre in adult mouse tissues supports endogenous low-level expression of α11 in these organs [140]. However, the Cre expression in kidney did not agree with the observed expression of endogenous α11 in mouse kidney. Instead the observed expression of β-galactosidase in kidney reflects endogenous expression. Interestingly, in another study (**Paper 3**) we observed immunoreactivity of integrin α11 mAbs with myofibroblasts in the human kidney glomeruli array [171]. If a similar expression of α11 exists in the mouse embryo kidney, the specific ITGA11 driven Cre expression as determined by immunostaining is probably masked by the endogenous β-galactosidase activity.

In excisional mouse skin wounds, activated NG2-positive pericytes have in one study been shown to contribute to around 30% of the myofibroblasts-like cells [172]. In two separate mouse studies ADAM12$^{+}$/PDGFRα$^{+}$ perivascular cells and Gli$^{+}$ MSCs have been reported to play important roles during wound healing [38, 173]. Our results obtained from *ITGA11*-Cre analysis in excisional skin wound in paper 2 showed fairly restricted X-gal staining in deeper regions of granulation tissue. In a separate study (**Paper 3**), we have shown that no or little co-localization of α11 and NG2 expression can be observed in CAFs in the stroma of various tumor types, suggesting that pericytes are not a source of CAFs expressing α11 integrin [171]. Further analysis is required to better understand the identity of cells expressed by ITGA11-Cre in skin wound and to determine potential co-localization with markers such as NG2 and PDGFRα.

Analysis of ITGA11-Cre in healthy and aortic-banded fibrotic hearts demonstrated a weak expression of Cre-recombinase in the healthy heart, but induction in areas of cardiac fibrosis, as judged by histological staining and western blotting. This finding is in agreement with the previously observed induction of integrin α11 in the challenged heart [140]. In cardiac fibrosis, resident fibroblasts fill up the damaged tissue regions and these fibroblasts upon activation may express αSMA and become contractile during the repair phase. Interestingly, unlike in dermal fibrosis where fibroblasts then undergo apoptosis, activated cardiac fibroblasts can lose their αSMA

expression [174]. Similar to skin wound healing, $Gli^+$ MSCs have been shown to contribute to cardiac fibrosis [173]. The fairly limited expression of α11 observed here again suggest α11 expression in a subset of fibroblasts and further experiments are needed to better understand the identity of this subset of cardiac fibroblasts.

Our findings also demonstrate that about 60% of MEFs expressed ITGA11-Cre activity, supporting our previous observation that integrin α11 is present in only subsets of fibroblasts. The finding that 40% of MEFs lacked ITGA11-Cre activity is interesting, since we have observed that α11 is often induced in cultured cells. It will be interesting to further study Cre activity during cell passages to determine if Cre is induced in later passages. The heterogenous expression in freshly isolated MEFs also suggest that MEFS can be separated by flow cytometry based on their repertoire of collagen-binding integrins. This would also allow analysis to determine if these subsets display functional differences with regard to cell adhesive activities.

In summary, our data from **paper 2** demonstrate ITGA11-Cre expression replicates endogenous expression of α11 during development, as well as in cardiac fibrosis and wound healing. *ITGA11*-Cre driver mouse strain could be a useful tool to further investigate the role of fibroblasts subsets in tissue reorganization events.

CAFs are a major cell type in the stroma of solid tumors and play fundamental roles in initiating tumorigenesis, tumor growth, tumor invasion and metastasis [23]. Whereas the majority of integrins are expressed on multiple cell types [70], the expression of integrin α11β1 in contrast is restricted to subsets of CAFs in NSCLC and HNSCC, as noted by immunostaining with an α11 polyclonal antibody [152, 175, 176]. In lung cancer, a NSCLC xenograft study in mice demonstrated that α11β1 is involved in mediating ECM reorganization, paralleled by increased stiffness of the tumor stroma [142]. Later studies from the same group has demonstrated that α11β1 can upregulate the collagen cross-linking enzyme, LOXL1, which is suggested to be responsible for some of the observed stiffness increase [151]. Further studies are needed to understand α11β1 mediated molecular mechanism in regulating collagen stiffness.

In **Paper 3** we further investigated the expression and distribution of integrin α11 in different human tumor types. A major problem with using polyclonal antibodies is the non-specific background staining we observed in immunostaining of adult tissues including tumor tissue. This problem was overcome by generating, characterizing and using new anti-human α11 mouse monoclonal antibodies (mAbs); 203E1 (function-blocking) and 203E3 (immunostaining).

Our results show high expression of integrin α11 protein in CAFs in invasive ductal mammary carcinoma, pancreatic carcinoma and ovary cyst adenocarcinoma. This is in agreement with available TCGA data, demonstrating high α11 mRNA expression in these tumors (TCGA Research Network: http://cancergenome.nih.gov/). Since breast tumor stroma is often stiff and desmoplastic, a strong expression of α11 in stroma of invasive ductal mammary carcinoma supports our earlier observation of increased α11 expression in areas subjected to high mechanical stress [129]. α11β1 has recently been reported to modulate PDGFRβ/JNK signaling in CAFs and to promote cancer progression in a study performed in a PyMT breast cancer model [27].

In pancreatic adenocarcinoma high α11 mRNA expression has been noted in clinical samples (TCGA Research Network: http://cancergenome.nih.gov/) which is in agreement with our immunostaining data. Recently, a largely *in vitro* based study of PDAC stellate cells suggested integrin α11β1 to be a major stromal integrin in pancreatic cancer, able to mediate differentiation of pancreatic stellate cells into CAFs and induce tumor cell invasion and metastasis [143]. However, *in vivo* data using animal models are needed to support the *in vitro* data obtained with human stellate cells in the above-mentioned study. Although the prostate tumor in the tumor tissue array showed lack of α11 expression, α11 expression was previously noted in prostate cancer using our α11 polyclonal antibody [177], and further studies are needed to examine α11 expression in a larger set of samples.

Integrin α11 expression co-localized with vimentin expression in all tested sections. Interestingly, in some part of the tumor sections α11 staining displayed different

expression pattern compared to vimentin staining. Vimentin has been widely recognized as a common marker for stromal cells, however low expression of vimentin has been observed in resident mesenchymal stem cells (MSCs) [32, 178]. The apparent different staining pattern could also be due to differential immunoreactivity of the two antibodies, and not reflect real differences in protein expression. Further studies are needed to determine the nature of the α11-expressing CAFs in these tumors.

In **Paper 3** we also show that αSMA does not systemically co-localize with α11 expression, suggesting that α11 expression occurs in different subtypes of CAFs. High αSMA expression has been observed in a subset of contractile CAFs in both mouse and human pancreatic cancer, localized peritumorally and known as myCAFs [19]. Interestingly in our immunostainings of PDAC and HNSCC tumor integrin α11 was often found to localize to peritumoral CAFs.

Our *in vitro* data with α11 function-blocking antibody, mAb 203E1, suggests that integrin α11β1 is involved in collagen remodeling and CAF migration. The degree of cell-collagen inhibition occurred in a manner that seemed to depend on both the level of α11 expression and the presence of other collagen-binding integrins, including α2β1. Interestingly, the combined effect of α11β1 and α2β1 function-blocking antibodies in blocking cell-collagen interactions was lower than the effect of anti-β1 integrin antibody, which indicates that other β1 integrins could be involved in mediating indirect interactions with collagen I [100, 128]. One potential integrin that mediates such an indirect interaction is α5β1, since fibronectin most likely is synthesized during the long-term assays used to monitor the collagen remodeling and spheroid migration.

Taken together, our data demonstrate that CAFs are heterogenous with regard to integrin α11 and αSMA expressions, which suggest that α11 expression is present on different subset(s) of CAFs in the TME. These subsets remain to be better characterized *in vitro* and *in vivo*.

# 5. Conclusion

This thesis aimed to gain further insights into integrin α11 function and distribution at both the cellular and the molecular level. We show that the α11 cytoplasmic tail is important for integrin α11 functions including focal contact formation, collagen remodeling, cell proliferation and cell migration. In addition, the generation and characterization of the ITGA11-Cre mouse strain resembled showed endogenous α11 expression during development, as well as in wound healing and cardiac fibrosis, emphasizing that this mouse strain will be a useful tool in further studies of fibroblast functions. Furthermore, our data demonstrate that CAFs are heterogenous with regard to α11 expression on different subset(s) of CAFs in the TME.

# 6. Future perspectives

Further studies are still needed to be better understand integrin α11 function. In particular, the interaction of potential unidentified partners in the formation of integrin α11-cytoskeletal interactions remain to be identified and characterized. All experiment in **paper 1** were done in *vitro*, it would be interesting to repeat some of these data *in vivo*.

To better analysis of ITGA11 Cre functional activity, it would be interesting to cross the Cre driver mouse strain with another reporter mouse strain such as Rosa-CAG-LSL-tdTomato strain. It is hoped that the ITGA11-Cre driver mouse strain will be a useful tool to further investigate the role of fibroblasts subsets in tissue reorganization events in skin wounds and cardiac fibrosis.

Since CAFs are heterogenous and α11 expression is localized on different subset(s) of CAFs, further studies are needed to better characterize the origin and functions of integrin α11-positive CAFs subsets in *vitro* and in *vivo* in different tumor types.

# 7. References

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III

*Article*

# α11β1 Integrin is Induced in a Subset of Cancer-Associated Fibroblasts in Desmoplastic Tumor Stroma and Mediates In Vitro Cell Migration

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**Abstract:** Integrin α11β1 is a collagen receptor that has been reported to be overexpressed in the stroma of non-small cell lung cancer (NSCLC) and of head and neck squamous cell carcinoma (HNSCC). In the current study, we further analyzed integrin α11 expression in 14 tumor types by screening a tumor tissue array while using mAb 203E3, a newly developed monoclonal antibody to human α11. Different degrees of expression of integrin α11 were observed in the stroma of breast, ovary, skin, lung, uterus, stomach, and pancreatic ductal adenocarcinoma (PDAC) tumors. Co-expression queries with the myofibroblastic cancer-associated fibroblast (myCAF) marker, alpha smooth muscle actin (αSMA), demonstrated a moderate level of $\alpha11^{+}$ in myCAFs associated with PDAC and HNSCC tumors, and a lack of α11 expression in additional stromal cells (i.e., cells positive for fibroblast-specific protein 1 (FSP1) and NG2). The new function-blocking α11 antibody, mAb 203E1, inhibited cell adhesion to collagen I, partially hindered fibroblast-mediated collagen remodeling and obstructed the three-dimensional (3D) migration rates of PDAC myCAFs. Our data demonstrate that integrin α11 is expressed in a subset of non-pericyte-derived CAFs in a range of cancers and suggest that α11β1 constitutes an important receptor for collagen remodeling and CAF migration in the tumor microenvironment (TME).

**Keywords:** tumor microenvironment; tumor stroma; extracellular matrix; fibrillar collagen; cancer-associated fibroblasts; integrin alpha11

## 1. Introduction

The importance of the tumor microenvironment (TME) for the growth and spread of tumors is being increasingly recognized. In addition to serving as a structural scaffold, the extracellular

matrix (ECM) serves as a reservoir of growth factors and cytokines that take part in the bidirectional communication between the stroma and the tumor cells [1,2]. The major cell types in the tumor stroma of solid tumors include cancer-associated fibroblasts (CAFs) of varying origin, endothelial cells, pericytes, mesenchymal stem cells, and immune cells [3,4]. CAFs represent a major cell type within the stroma contributing to ECM synthesis and ECM remodeling, and they also take part in the paracrine signaling, which affects the growth and invasive properties of the tumor cells, in chemoresistance and in the establishment of metastatic niches [3–5]. Importantly, a specific subset of myofibroblastic CAFs (myCAFs) has been implicated in the production of collagen [6]. CAFs produce collagen crosslinking enzymes of the lysyl oxidase (LOX) family, which increase the stiffness of the ECM and thereby affects the growth and invasion of tumor cells [7,8]. Fibroblastic cells thus constitute a group of mesenchymal cells of varying origins, some of which (i.e., myCAFs) share characteristics with the myofibroblasts that are found in granulation tissue during wound healing and tissue fibrosis [9].

In the context of pathological tissue and tumor fibrosis, the mesenchymally derived CAF population is thought to constitute a more heterogeneous cell mixture than the resident tissue fibroblasts in "resting" tissue. The balance among cells of different origins is dynamic in tissues showing tissue regeneration/fibrosis. In tissue fibrosis, genetically based cell linage tracing and a stringent use of antibodies have resulted in the characterization of activated fibroblasts that are derived either from endogenous fibroblasts [10–12], Gli+-positive mesenchymal stem cells (MSC) [13], or pericytes [14,15].

Pericytes exist as a major cell type in the pancreas and liver in the form of stellate cells [16,17], which proliferate and become activated in fibrosis models. The careful study from Öhlund et al. has defined a peritumoral alpha smooth muscle actin ($\alpha$SMA)$^{high}$ CAF population, termed myofibroblastic CAFs or myCAFs, which differ from a CAF population characterized by Il-6 production and referred to as inflammatory CAFs (iCAFs) [6].

The major sources of CAFs in tumors and tumor fibrosis are the endogenous tissue fibroblasts, pericytes, and ADAM12$^{+}$ perivascular cells [15,18,19], and recently cell lineage tracing methods applied to transgenic polyoma middle T oncogene (PyMT) mice has somewhat surprisingly demonstrated a contribution from mesenchymal, non-hematopoietic bone marrow stromal cells to a PDGFR$\alpha$-negative, clusterin-positive breast cancer CAF subpopulation [20].

Epithelial-mesenchymal transition (EMT) appears to be especially important in contributing to an invasive mesenchymal tumor cell type and creating niches for cancer stem cells [21], but these EMT processes in tumors have indirect consequences for the stroma. EMT has recently been studied in detail in *Lgr5CreER/Kras*$^{LSL-12GD}$*/p53*$^{fl/fl}$, genetic mouse model of squamous cell carcinoma (SCC) in which the tumors undergo spontaneous EMT [22]. These studies convincingly demonstrated that EMT occurs in a stepwise manner, which leads to the generation of subpopulations of tumor cells in different intermediate states between epithelial and mesenchymal. Interestingly, as the cells progressed towards EMT [22], the bona fide stroma changed in parallel, with regard to their composition, localization, and the presence of immune cells.

A detailed in vitro study using breast cancer cell spheroids identified a switch of tumor cells state into a mesenchymal invasive state without the tumor cells actually undergoing EMT [23]. The cells leading the way in this initial invasive migration, the "trailblazer cells", were characterized by a mesenchymal seven-gene signature that was composed of *DOCK1*, *ITGA11*, *DAB2*, *PDGFRA*, *VASN*, *PPAP2B*, and *LPAR1* [23].

$\alpha11\beta1$ integrin is a collagen-binding integrin that is expressed in mesenchymal cells identified as fibroblasts, myofibroblasts, and mesenchymal stem cells [24–27]. Relatively little is known about this protein in the context of tumors, but non-small cell lung cancers (NSCLC) and head and neck squamous cell carcinomas (HNSCC) express the $\alpha$11 chain in activated stroma, where it has potential for serving as a biomarker for activated CAFs [28–30]. In the current report, we investigate the expression of integrin $\alpha$11 chain in different tumor types and try to determine whether the expression of $\alpha$11 subunit within a certain tumor type is able to mark a subpopulation of CAFs. We have generated and characterized

an anti-human α11 mouse monoclonal antibody (mAb), mAb 203E3, for this purpose. In parallel, we have developed a function blocking α11 antibody, mAb 203E1, to test the functional involvement of α11β1 in collagen remodeling on CAFs.

## 2. Results

### *2.1. Generation and Characterization of Integrin α11-Specific Monoclonal Antibodies (mAbs)*

Integrin α11 mAbs were generated at nanoTools, Germany (http://www.nanotools.de/), as described in Material and Methods, by immunizing mice with soluble human α11β1. Multiple-step screenings for binders of human α11β1 not cross-reactive with human α2β1 were performed while using the Luminex Assay and flow cytometry. The latter was used to select the clones that produced mAbs specific to human α11 while not recognizing human β1 or human α2 integrin chains. In this characterization, the previously described mouse C2C12 cell lines overexpressing human integrin α11, C2C12-huα11 (in C2C12-huα11 cells, human α11 chain heterodimerizes with mouse β1 integrin chain), and C2C12-huα2, were central [25]. To exclude cross-reactivity of the antibodies with the related α2 integrin chain, the mAbs were tested for reactivity with C2C12-huα2 cells, with no reactivity observed. To exclude reactivity with β1 integrin chain or other integrin α chains, hybridoma supernatants were screened against human A431 cells, which lack the expression of α11, but express human β1 chain and α2, α3, α5, and αv integrin chains [31]. In summary, no cross-reactivity with other integrins tested was noted. Two of the hybridoma clones producing mAbs 203E1 and 203E3, were further characterized and mAbs were affinity-purified. Both mAb 203E1 and mAb 203E3 caused a clear shift in the fluorescence intensity of the C2C12-huα11 cells in flow cytometry as compared with the non-expressing C2C12 cells (negative control; Figure 1a). The immunoprecipitation of α11 using mAbs 203E1 and 203E3, followed by Western-blotting with a polyclonal α11 antibody [32] confirmed the specificities of both antibodies for the 155 kD α11 band (Figure 1b), while the immunoctytostaining of C2C12-huα11 cells that were grown on collagen I showed the expected focal adhesion staining pattern (Figure 1c). Finally, the use of mAb 203E1 in cell attachment to collagen I and in cell spheroid migration assays in collagen gels demonstrated the effectiveness of mAb 203E1 in blocking α11-mediated adhesion both under two-dimensional (2D) and three-dimensional (3D) conditions (Figure 1d,e). In summary, the hybridoma clone 203E1 was identified as producing the blocking antibody mAb 203E1, while the clone 203E3 was identified as producing mAb 203E3 suitable for immunostaining. The immunoglobulin subtype and affinity determinations established that mAb 203E1 and mAb 203E3 are both of the IgG1 subtype (Hoschuetzky, H., nanoTools, Teningen, Germany, personal communication 2019), with affinities in the pM range (Figure S1).

### *2.2. Integrin α11 Expression in a Panel of Normal and Tumor Human Tissue Sections Using a Tissue Array*

To screen for the expression of the α11 subunit in different tumor tissues, a tissue array with sections from 14 different tumor types and from corresponding normal tissues (Table 1) was screened using α11 mAb 203E3. The cytokeratin antibodies (anti-keratin 7 and 18) were used to distinguish epithelial/tumor cells from stromal cells. In agreement with previous studies in adult mouse tissues [33], the integrin α11 subunit levels in normal human tissues were low or below the detection limit in all of the normal tissues tested, except for the kidney specimen, where strong immunoreactivity was observed in the glomeruli, in a pattern that was compatible with positive mesangial cells (Figure 2, α11 expression in the normal kidney tissue section indicated by arrowheads).

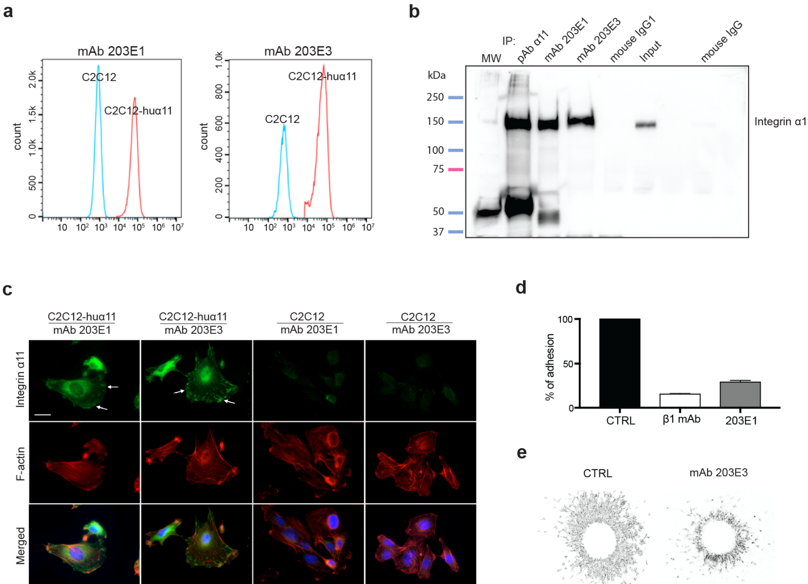


**Figure 1.** Characterization of the α11 mAb 203E1 and the mAb 203E3. (**a**) Characterization by flow cytometry. C2C12 cells and C2C12 expressing human α11 integrin (C2C12-huα11) cells were subjected to flow cytometry using the 203E1 and 203E3 mAbs. Only the C2C12-huα11 cells incubated with the 203E1 and 203E3 mAbs displayed a fluorescence shift. (**b**) Characterization by immunoprecipitation. Integrin α11β1 was immunoprecipitated with the 203E1 and 203E3 mAbs. The polyclonal α11 antibody (pAb α11) was used as a positive control, whereas mouse IgG1 was used as a negative control. Loading of the cell lysis (input) has been included to appreciate the efficiency of the immunoprecipitation. Immunoprecipitated proteins were detected with a rabbit polyclonal antibody to human α11. The full-size Western blotting is presented, MW: molecular weight marker. (**c**) Characterization by immunocytochemistry. C2C12 and C2C12-huα11 cells were plated on collagen I and immunostained using mAbs 203E1 and 203E3. Both antibodies immunostained focal adhesions (arrows). Scale bar: 20 µm. (**d**) Characterization in cell adhesion assay. C2C12-huα11 cells were incubated with either β1 mAb or 203E1 mAb and allowed to adhere to collagen I. (**e**) Characterization in invasion assay. Homospheroids composed of C2C12-huα11 cells were embedded in collagen I gel and treated with either the mouse IgG1 isotype control or 203E1 mAb at 10 µg/mL. Spheroid migration was analyzed after 24 h.

Of the tumor tissues in the array, the breast, liver, lung, pancreas, ovary, and uterus tumors stood out as having markedly upregulated integrin α11. Co-staining with cytokeratin indicated the exclusive expression of α11 in the stroma. Weaker α11 expression was noted in the stroma of the small intestine and stomach adenocarcinomas. The skin squamous carcinoma section showed notable α11 chain expression, but it was restricted to a small region, which was perhaps due to the limited area of the section and the size spotted in the tissue array. Likewise, the immunostaining of the skeletal muscle rhabdomyosarcoma tissue was diffuse and will need to be confirmed in further sections. (Figure 2, integrin α11 expressions in the different tumor sections are indicated by arrows). In summary, four of the 14 tumor tissues analyzed lacked a specific integrin α11 signal, namely the stroma of the brain oligodendroglioma, the colon adenocarcinoma, the renal cell carcinoma and the prostate adenocarcinoma, whereas the majority of the carcinomas/adenocarcinomas showed upregulated integrin α11 expression in the stroma cells as compared with the normal tissues.

**Table 1.** Donor and patient information on the tissue array sections.

| Table | Age | Sex | Pathological Diagnosis | Differentiation | TNM or Stage |
|---|---|---|---|---|---|
| Brain | 70 | F | Normal | | |
| Brain Tumor | 36 | F | Oligodendroglioma | N/A | Stage III |
| Breast | 40 | F | Normal | | |
| Breast Tumor | 47 | F | Invasive Ductal Carcinoma | N/A | $T_{unknown}N_0M_0$ |
| Colon | 87 | F | Normal | | |
| Colon Tumor | 70 | M | Adenocarcinoma, Mucuous | Moderately | $T_2N_0M_0$ |
| Skeletal Muscle | 79 | M | Normal | | |
| Skeletal Muscle Tumor | 50 | M | Rhabdomyosarcoma | Poorly | $T_3N_0M_0$ |
| Kidney | 44 | M | Normal | | |
| Kidney Tumor | 37 | M | Renal Cell Carcinoma | Moderately | $T_3N_0M_1$ |
| Liver | 64 | M | Normal | | |
| Liver Tumor | 44 | M | Hepatocellular Carcinoma | N/A | $T_3N_0M_0$ |
| Lung | 83 | F | Normal | | |
| Lung Tumor | 70 | M | Adenocarcinoma | Moderately | $T_{unknown}N_0M_0$ |
| Pancreas | 86 | F | Normal | | |
| Pancreas Tumor | 53 | M | Adenocarcinoma | Poorly | $T_{unknown}N_0M_0$ |
| Prostate | 50 | M | Normal | | |
| Prostate Tumor | 66 | M | Adenocarcinoma | N/A | Gleason 4 + 3 = 7 |
| Skin | 61 | F | Normal | | |
| Skin Tumor | 48 | M | Carcinoma, Sweat Gland | N/A | $T_1N_0M_0$ |
| Small Intestine | 70 | F | Normal | | |
| Small Intestine Tumor | 68 | M | Malignant Mesenchymoma | Well | $T_2N_0M_1$ |
| Stomach | 56 | M | Normal | | |
| Stomach Tumor | 54 | M | Adenocarcinoma, Ulcer | Moderately | $T_2N_0M_0$ |
| Ovary | 37 | F | Normal | | |
| Ovary Tumor | 54 | F | Cystadenocarcinoma, Serous | Poorly | $T_2N_0M_0$ |
| Uterus | 68 | F | Normal | | |
| Uterus Tumor | 55 | F | Adenocarcinoma | Poorly | $T_{unknown}N_0M_0$ |

N/A, not available; M, male; F, female.

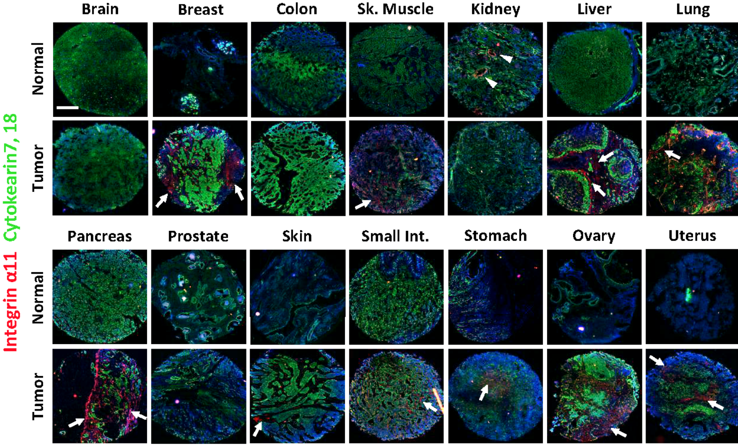


**Figure 2.** Expression of integrin α11 in array sections from normal and tumor adult human tissues. Immunofluorescence staining was performed on the sections using α11 203E3 mAb (red) and cytokeratin 7 and 18 (green). The cell nuclei were stained with DAPI (blue). In the normal tissues integrin α11 expression was only detectable in the kidney section (arrowheads), while in the tumor tissues α11 expression was detected in 10 of the 14 tumors tested (indicated by arrows in the respective tumor sections). Staining in each section was shown in a merged picture of two photos taken under a Zeiss Axioscope microscope (5×). Scale bar: 400 µm.

### 2.3. Characterization of Stromal Cells Expressing Integrin α11 Subunit in the Tissue Array Tumor Sections

Co-staining of the α11 subunit and cytokeratins were performed in combination with stromal markers to further characterize integrin α11 expression in the tissue array tumor sections. Three stromal markers were selected: fibroblast-specific protein 1 (FSP1, expressed in multiple cell types in the stroma, including immune cells [34]), αSMA (expressed in contractile activated fibroblasts, like myCAFs [6] and smooth muscle cells [35]), and vimentin (expressed in fibroblastic cells, endothelial cells, and pericytes [35]). We did not detect any α11 expression in the tumor cells (keratin-positive) of any of the sections tested, nor was the α11 chain detected in any of the larger blood vessels (αSMA-positive smooth muscle cells) using this limited set of markers. Co-localization of FSP1 with integrin α11 was only observed in breast and stomach adenocarcinoma sections, whereas αSMA and integrin α11 subunit co-localized to variable degrees in the stroma of most of the integrin α11-positive tumor tissues, which suggested that integrin α11 could be enriched in the cells corresponding to myCAFs. In the limited tissues pieces spotted on the arrays, no αSMA could be detected in either the skin sweat gland carcinoma or the stomach carcinoma (Figure 3). Finally, integrin α11 expression overlapped with vimentin expression in all sections tested, but most importantly, there were also integrin α11-positive, keratin-negative cells in the stroma with low or barely detectable vimentin expression, a phenotype that is compatible with these cells being CAFs.

To summarize this part of the investigation, the integrin α11 subunit immunostaining patterns of the tissue arrays suggest that, although α11 co-localization with FSP1, αSMA, and vimentin varies from one tumor to another, there is a trend for α11 and FSP1 to poorly co-localize in the tumor stroma, whereas α11 co-localized with αSMA to a larger extent in regions with activated stroma. Integrin α11 and vimentin also showed co-localization in the tumor stroma, but interestingly, α11 chain expression was also observed in stroma cells with no detectable expression of vimentin. A summary of the integrin α11 immunostaining and its co-localization with FSP1, αSMA, and vimentin in various tumor tissues, as seen in the screening results, is presented in Table 2.

**Table 2.** Summary of integrin α11 expression and its co-localization with other stroma markers in tumor sections of the tissue array.

| Tumor Tissue | Pathological Diagnosis | α11 Expression in Stroma | Co-Localization α11/FSP1 | Co-Localization α11/αSMA | Co-Localization α11/vimentin |
|---|---|---|---|---|---|
| Brain | Oligodendroglioma | - | | | |
| Breast | Invasive Ductal Carcinoma | +++ | + | + | ++ |
| Colon | Adenocarcinoma, Mucuous | - | | | |
| Skeletal Muscle | Rhabdomyosarcoma | ? | | | |
| Kidney | Renal Cell Carcinoma | - | | | |
| Liver | Hepatocellular Carcinoma | +++ | - | ++ | + |
| Lung | Adenocarcinoma | ++ | - | + | ++ |
| Pancreas | Adenocarcinoma | +++ | - | ++ | ++ |
| Prostate | Adenocarcinoma | - | | | |
| Skin | Carcinoma, Sweat Gland | ++ | - | - | ++ |
| Small intestine | Malignant Mesenchymoma | - | | | |
| Stomach | Adenocarcinoma, Ulcer | ++ | ++ | - | ++ |
| Ovary | Cystadenocarcinoma, Serous | +++ | - | ++ | ++ |
| Uterus | Adenocarcinoma | +++ | - | ++ | ++ |

-, no expression or co-localization; +, low expression or co-localization; ++, medium expression or co-localization; +++, high expression or co-localization; ?, uncertain expression.

### 2.4. RT-qPCR to Confirm the Immunostaining Data for the Tissue Arrays

To further verify the positive immunohistochemical data, we performed RT-qPCR to analyze levels of the integrin α11 subunit and the various markers in lung, pancreas, and skin on RNA isolated from the same tissues as used for preparing the tissue array sections. The RT-qPCR data demonstrated increased RNA levels of integrin α11 (ITGA11) in the lung, pancreas, and skin tumor tissue relative to the normal tissues, with the greatest increase in α11 RNA to be found in the pancreas tumor (Figure 4).

Interestingly, vimentin (VIM) and αSMA (ACTA2) that we showed to co-localize with integrin α11 in the pancreatic cancer tumors also displayed increased expression in this tumor tissue.

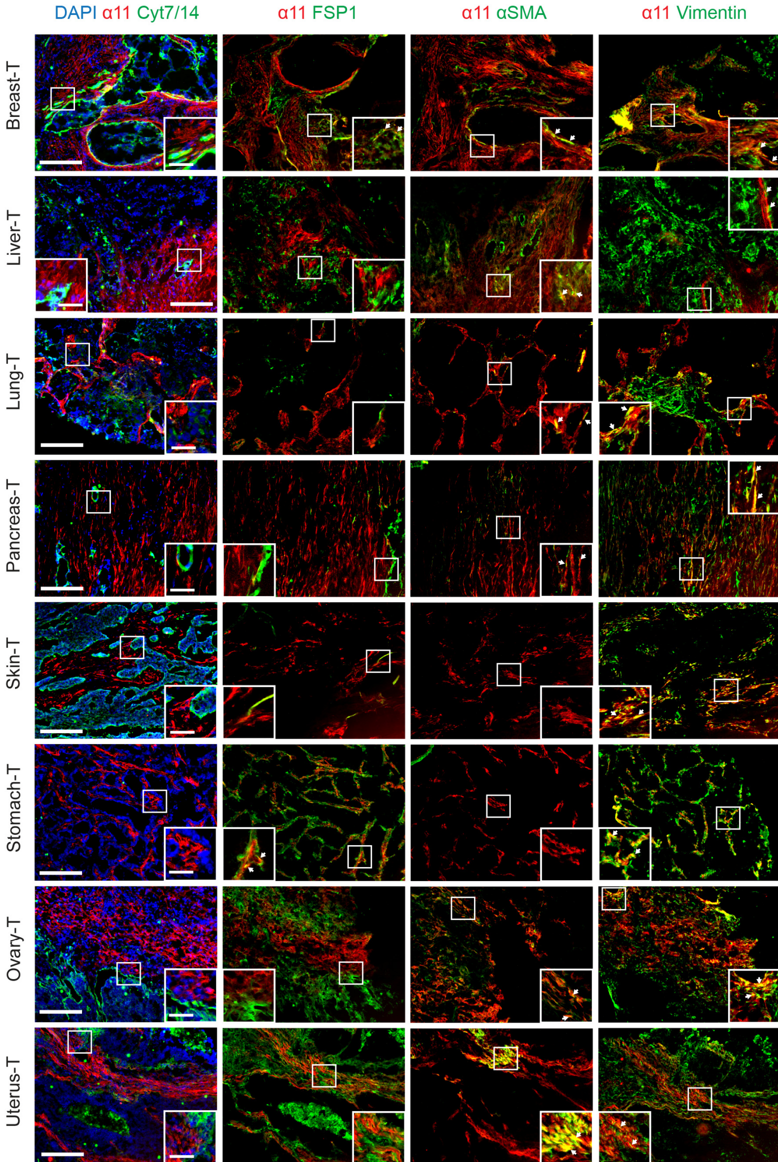


**Figure 3.** Integrin α11 co-localization with various markers in selected tumor array sections. The sections were stained with α11 203E3 mAb (red,) combined with cytokeratin 7 and 14 (green), FSP1 (green), αSMA (green), and vimentin (green), respectively, as indicated. DAPI (blue) was used for counterstaining only in the combination of integrin α11 and cytokeratin 7/14. Pictures shown were taken under Zeiss Axioscope microscope (10×). Scale bar: 200 µm.A close-up image of a region of interest is inserted in each picture (scale bar: 50 µm). Arrows denote co-localization of integrin α11 with other stroma markers.

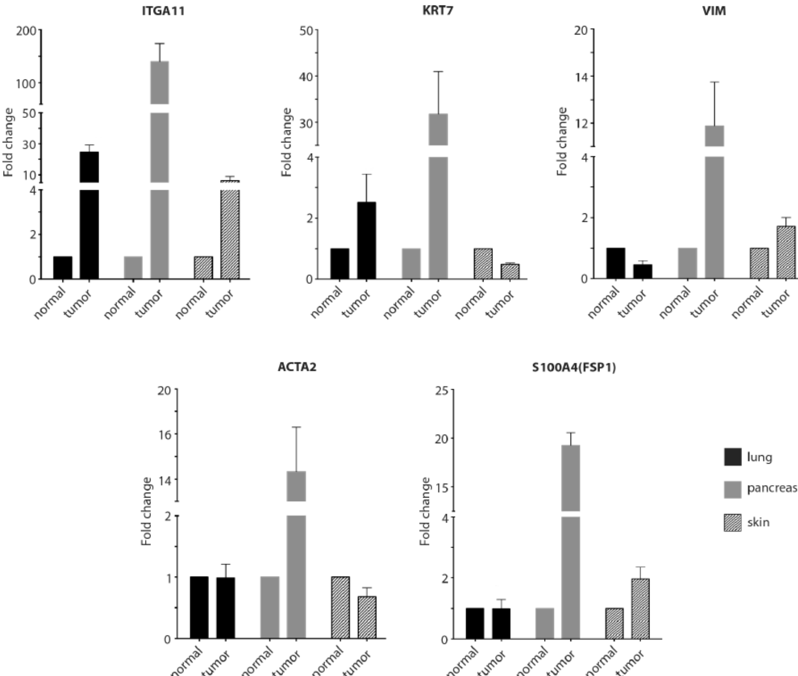


**Figure 4.** Comparison of mRNA expression of integrin α11 and various markers in selected normal and tumor tissues. Total RNA was extracted from the normal and tumorous lung, pancreas and skin tissues on which immunostaining had previously been performed. mRNA levels of integrin α11 (ITGA11), cytokeratin 7 (KRT7), vimentin (VIM), alpha smooth muscle actin (αSMA) (ACTA2), and fibroblast-specific protein 1 (FSP1) (S100A4) were analyzed by RT-qPCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a reference gene for normalization. Each gene expression level is presented as a fold change in tumor tissue relative to the normal tissue. Shown is the average fold change of the mRNA extracted from each sample, but reverse transcribed and amplified in three independent experiments. Error bar indicates the standard deviation from the average.

## *2.5. Characterization of Cells Expressing Integrin α11 Subunit in PDAC and HNSCC*

After screening the tumor tissue array, we examined the expression of α11 in the CAF subpopulations in the tumor stroma in more detail. Oncomine analyses of cancer datasets have identified ITGA11 overexpression in breast, pancreas, lung, colorectal-, and gastric cancer [36], and we have recently shown that HNSCC tumors express α11 in their stroma [30]. Based on these data, we decided to use tumor sections and isolated CAFs from PDAC and HNSCC tumors for further characterization of the expression and function of α11 using the novel α11 mAbs.

Six different stroma markers were chosen for co-staining with α11 mAb 203E3, while cytokeratin co-staining was performed to demarcate the tumor cells to better characterize the cells expressing α11. In agreement with previous data obtained with tissue sections from PDAC and HNSCC tumors, we observed α11 expression in the PDAC and HNSCC sections to be restricted to the stroma compartment and often seen peritumorally, in close in close contact with the tumor cells. The fibroblast-activating protein (FAP) staining was limited to the peritumoral region in close proximity to the tumor cells and it was extensively co-stained with α11. FSP1, on the other hand, showed little co-expression with α11, as was also the case with the pericyte marker NG2, which was expressed in distinct cell populations and appeared to not be co-expressed with α11 at all in the sections analyzed. The other two CAF markers, PDGFRβ and αSMA, were co-expressed with α11 in the majority of the stroma regions that

were observed. Curiously, vimentin expression was again widespread in the stroma of both types of tumors, but it displayed a differential expression pattern from that of α11 (Figure 5 and Figure S2).

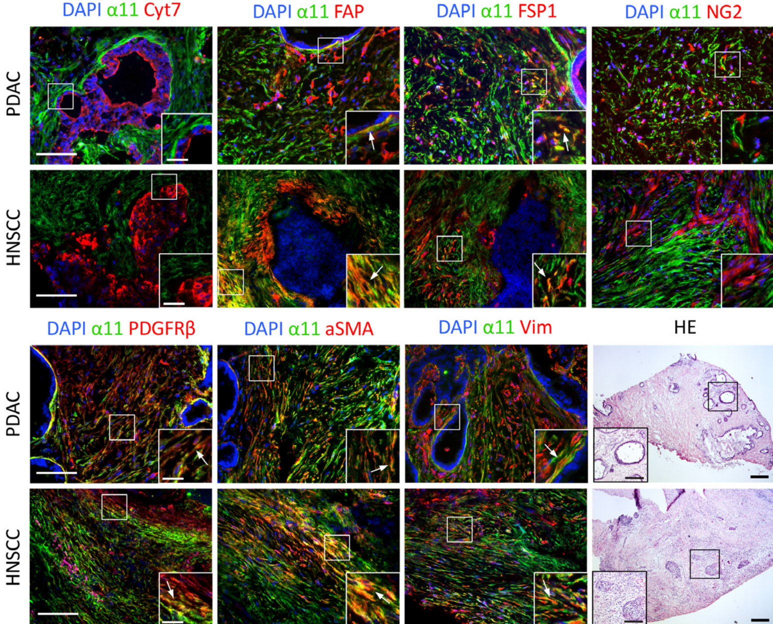


**Figure 5.** Determination of integrin α11 co-expression with various stroma markers. Fresh-frozen tumor sections from human pancreatic ductal carcinoma (PDAC) and head and neck squamous carcinoma (HNSCC) were co-stained with α11 203E3 mAb (red) and the respective tumor and stroma cell markers, as indicated (green). Cell nuclei were stained with DAPI (blue) and used as counterstaining. The photos were taken under a Zeiss Axioscope microscope (20×). Scale bar: 100 µm. A close-up image of a region of interest is inserted in each picture (scale bar: 25 µm). Arrows denote co-localization of integrin α11 with other stroma markers.HE staining of the sequential section from the PDAC or HNSCC patient was shown in parallel. The photos were taken under a Nikon Eclipse E600 microscope (5×). Scale bar: 200 µm. Inserts show higher magnification of the selected area (scale bar: 100 µm).

### 2.6. Role of Integrin α11β1 in Fibroblasts and CAFs

We screened a number of fibroblasts and CAFs for integrin α11 chain expression and its function in cell adhesive interactions with collagen I to examine the role of α11β1 as a collagen receptor in fibroblasts and CAFs (Figure 6). We also analyzed two additional collagen-binding integrin chains, integrin α1 (detected with the clone 639508 mAb) and integrin α2 (detected with P1E6 mAb and EPR 5788 mAb) chains, both dimerizing with the integrin β1 chain [37]. Human lung embryonic MRC5 fibroblasts expressed low levels of α11 in Western blotting by comparison to the α11-overexpressing C2C12 cells (C2C12-huα11, Figure 1), which served as a positive control for expression and functional analyses. The treatment with TGF-β resulted in a moderate increase in α11 levels in the MRC5 cells (Figure 6a and Figure S3). In agreement with this, the effect of 203E1 in these cells was restricted to cell adhesion, whereas collagen gel contraction and spheroid invasion were unaffected by the presence of mAb 203E1, which was presumably due to involvement of other collagen-binding integrins (Figure 6b). In agreement with the concept that the degree of inhibition is related to the degree of expression, the effect of 203 E1, and the combination of the function-blocking antibodies 203E1(α11) and P1E6 (α2) was also greater in all three functional assays (cell attachment, collagen gel contraction, and spheroid invasion) for human gingival fibroblasts (hGF) with higher α11 expression (Figure 6a,c).

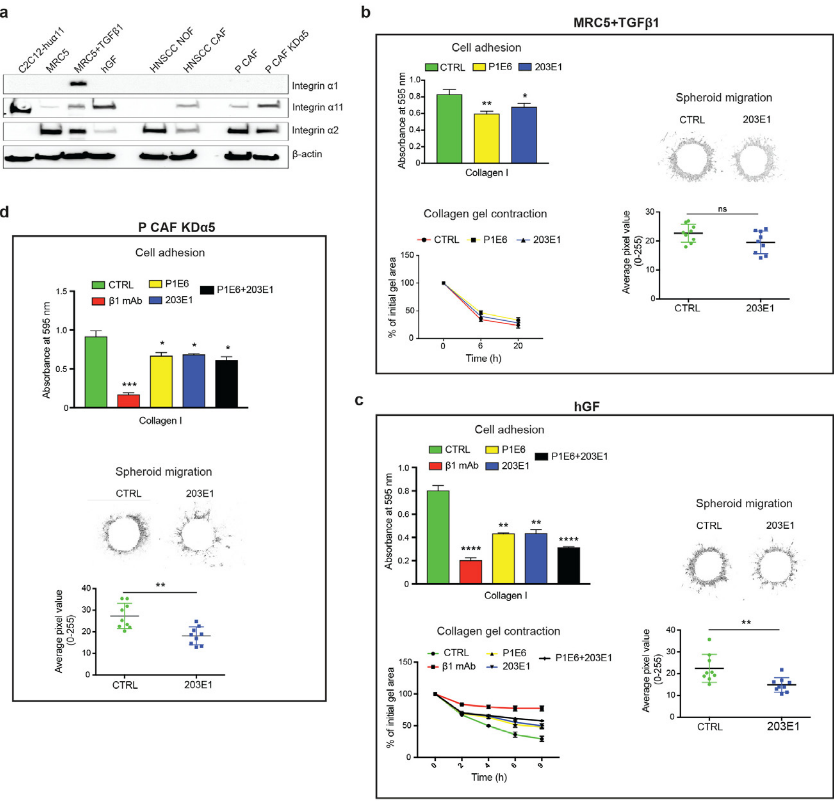


**Figure 6.** Effect of integrin α11 mAbs on cell-collagen interactions in fibroblasts and cancer-associated fibroblasts. (**a**) Western blot showing the total protein expression of integrin α1, α2, α11, and α2 in; MRC5 fibroblasts with or without TGF-β1 (the MRC5 cells were treated with 5 ng/mL TGF-β1 for 48 h to induce α11 integrin); human gingival fibroblasts (hGF); cancer-associated fibroblasts (CAFs) and normal fibroblasts (NOFs) from a head and neck squamous cell carcinoma (HNSCC) patient; CAFs from a pancreatic adenocarcinoma (pCAF) and pCAFs with integrin α5 knockdown (pCAFKDα5). C2C12-huα11 cells were used as a positive control. (**b**) Effect of integrin antibodies on α11β1-mediated cell adhesion of MRC5 cells. MRC5 cells treated with TGF-β1 were assayed in cell adhesion, collagen gel contraction and spheroid migration in the presence of control antibodies (CTRL), α2 integrin mAb (P1E6) or α11 integrin mAb (203E1). (**c**) Effect of integrin antibodies on α11β1-mediated cell adhesion of human gingival fibroblasts. hGF cells were assayed in cell adhesion, collagen gel contraction and spheroid migration in the presence of control antibodies (CTRL), α2 integrin mAb (P1E6), α11 integrin mAb (203E1), or β1 integrin mAb (mAb 13). (**d**) Effect of integrin antibodies on α11β1-mediated cell adhesion of pCAFKDα5. pCAFKDα5 cells were assayed in cell adhesion, and spheroid migration in the presence of control antibodies (CTRL), α2 integrin mAb (P1E6), α11 integrin mAb (203E1), or β1 integrin mAb (mAb 13). For cell adhesion, the cells were treated with antibodies and allowed to adhere to collagen I in serum free conditions for 50 min. For spheroid data spheroid migration was analyzed after 24 h. Results shown here are representative images of the spheroid after image processing with ImageJ. The radius of the region of interest from each individual spheroid was measured using Radial Profile plugin from ImageJ. Means ± SEM of at least three independent experiments are shown and analyzed with one tailed, unpaired $t$−test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Normal fibroblasts and CAFs from HNSCC tumors were analyzed afterwards. Here, the expression of α11 was modest in these CAFs (Figure 6a). The PDAC tumors, in turn, gave us access to two kinds

of CAFs: control PDAC CAFs (P CAFs) and CAFs, in which integrin α5 had been knocked down using CRISPER-CAS-9 (P CAF KDα5) [38]. The latter CAFs expressed α11 at a comparable level to that achieved by hGF cells (Figure 6a), but the 203 E1 antibody in these cells was mainly effective in the invasion assay, with a smaller effect of mAb being observable in the cell attachment and collagen gel contraction assays. The less efficient inhibition of 203E1 was probably partly due the to higher levels of α2 integrin in P CAF KDα5 than in the hGF cells (Figure 6c,d), but it might also reflect some involvement of other β1 integrins in the adhesion of these cells to collagen I (see Discussion).

In summary, the function-blocking assays demonstrate that α11 mAb 203E1 inhibited cell-collagen interactions in a manner that seemed to depend on the level of α11 expression, as well as on the presence of other collagen receptors. MAb 203E1 was especially efficient in blocking 3D spheroid migration in P CAF KDα5.

## 3. Discussion

Most integrins are widely expressed on multiple cell types. Among the collagen-binding integrins, the α1 and α2 integrin chains are both expressed on dermal fibroblasts [39,40], but only limited data are available regarding their expression and their function in CAFs. However, in general, the α1 and α2 integrin chains are widely expressed and they can also be detected in tumor cells as well as vascular and immune cells [41–43]. These integrins are therefore not particularly useful as biomarkers for CAFs. The collagen-binding α10 integrin is normally limited to cartilage and a very restricted subset of fibroblasts [44]. Although melanoma cells have been reported to express α10, no expression has been reported in CAFs [45]. In contrast, α11β1 integrin is expressed in a pattern that is restricted to CAFs in NSCLC and HNSCC, as observed by immunostaining while using a polyclonal antibody to α11 [28,30,46].

Data from mouse models of NSCLC show that integrin α11 expression is associated with increased stiffness of the tumors, which suggested the involvement of α11β1-mediated ECM reorganization as an underlying mechanism and resulted in stiffer and more ECM [29]. In addition to the suggested direct effect of α11β1 in mediating collagen reorganization, a correlation with lysyl oxidase-like 1 (LOXL1) expression has been noted [29,47]. This indirect mode of regulating the levels of collagen cross-linking enzymes needs further studies to directly link it to an α11β1-mediated molecular mechanism.

The current work adds to existing studies of integrin α11 expression in various human tumors. In the process of adult tissue immunostaining using the polyclonal integrin α11 antibody, we noted problems with non-specific background staining, so that it was essential to develop new and better reagents. The monoclonal antibodies mAbs 203E1 (function-blocking) and 203E3 (immunostaining) described herein are both high affinity mono-specific mouse antibodies to the human integrin α11 chain with no reactivity with either the β1 integrin chain or the human α2 integrin chain, or any other tested integrin chains.

The α11 immunoreactivity in the stroma of invasive ductal breast cancer is interesting, since breast cancer tissue is often stiff and desmoplastic [48–50], which is in agreement with our current picture of integrin α11 expression as being enriched at sites of high mechanical stress [25]. The high integrin α11 protein expression that is seen in the invasive ductal mammary carcinoma data is supported by large-scale cancer genomics data at TCGA demonstrating high α11 mRNA expression in invasive breast cancer (TCGA Research Network: http://cancergenome.nih.gov/). Similarly, the analysis of an Oncomine database (https://www.oncomine.org/) supports the expression of α11 in various forms of breast cancer. A functional role for α11 in breast cancer is likewise supported by data from a PyMT mouse model, in which the absence of α11 in the breast cancer stroma greatly attenuates breast tumorigenesis and metastasis [36]. In this context, it is also interesting to note that human breast cancer tumor cells at the invasive front in an in vitro spheroid metastasis model express integrin α11 RNA at the point in time when the cells assume a mesenchymal invasive phenotype. Integrin α11 is part of the gene signature in these "trailblazer" breast cancer cells, and it is thought to be functionally involved

in this invasion process [23]. Nevertheless, we could not observe any integrin α11 staining in the mammary cancer cells of the limited number of sections that we analyzed here.

The in vitro data that were obtained here with α11 function-blocking antibodies suggest that α11β1 has a role in CAF-mediated collagen remodeling and cell migration. Although the α11 function- blocking antibody almost completely inhibited cell-collagen adhesion interactions in α11-transfected C2C12 cells, the contribution of α11β1 to cell-collagen interactions was lower in the fibroblasts and CAFs expressing additional collagen receptors, including α2β1. Interestingly, the effect of antibodies to β1 integrin was still greater than the combined effect of the α2β1 and α11β1 blocking antibodies, which suggested the involvement of other β1 integrins in indirect cell adhesive interactions with collagen [51,52]. Integrin α5β1 is one candidate receptor for mediating these indirect interactions with the collagen matrix, since fibronectin is present during the collagen gel contraction and spheroid migration.

The strong immunoreactivity of integrin α11 protein in pancreatic carcinoma and ovarian cyst adenocarcinoma tissues is also in agreement with the TCGA expression data, where *ITGA11* expression in pancreatic cancer belongs to the top-five tumor category for all tumor types analyzed for α11 mRNA expression (TCGA Research Network: http://cancergenome.nih.gov/). A recent study of pancreatic cancer CAFs has suggested that α11 may play a role in cell migration on pancreatic CAFs [53]. However, it is important to point out that α11 has a tendency to be induced in cell culture. In our own work, we have failed to detect α11 integrin in breast cancer cells (this study and [36]) or in liver or pancreatic stellate cells [26,33], whereas work that was performed using in vitro cultured cells has suggested a role for stellate cell-derived α11-expressing CAFs in tissue and tumor fibrosis [53–55].

The lack of proper antibody controls is one weakness in some studies of integrin α11 using polyclonal antibodies. The use of polyclonal antibodies with pathological tissue sections is challenging. The monoclonal antibodies that were characterized here were generated using a soluble heterodimeric protein, which was immunized in native form, and the mAbs were screened with α11-specific reagents. No reactivity was noted with any of the other integrin α or β chains tested, and they should constitute a valuable tool for future work. Although the prostate tumor tissue that was analyzed here was negative for integrin α11 immunoreactivity, we have previously noted α11 expression in the prostate carcinoma stroma using the polyclonal integrin α11 antibody [56]. The new data agree with expression data that are available from TCGA, in which the α11 mRNA levels reported in prostate adenocarcinoma are modest. The α11 immunostaining observed in cells expressing low levels of vimentin is interesting. Vimentin has been widely regarded as a universal marker of stromal cells [4], but curiously resident mesenchymal stem cells (MSCs) have recently been reported to be characterized by a low expression of vimentin [57].

When activated, fibroblasts become contractile and they produce and remodel collagen. During the activation process, normal quiescent fibroblasts first become protomyofibroblasts and then, when fully activated, are known as myofibroblasts [58,59]. One marker myofibroblasts is αSMA. It is worth noting that data are now accumulating to suggest that αSMA is an inconsistent marker of activated collagen-producing myofibroblasts cells, at least in fibrotic conditions in the lung, kidney, and heart [60,61]. Independent in vitro data on activated fibroblasts, in tumors known as myCAFs [6], share characteristics with the CAFs that are described in this study with integrin α11 chain expression associated with αSMA expression and a myofibroblast phenotype [27,29,62–65]. Interestingly, we found here that CAFs expressing integrin α11 do not systematically co-express αSMA, since we noted a strong co-expression of integrin α11 and αSMA in CAFS around the tumor cells of the PDAC and HNSCC sections, which suggested that these α11$^{+}$-CAFs could have a role in collagen remodeling at the border of the tumor in order to facilitate tumor cell invasion. In colon cancer, the role of tumor cell αvβ3 at the tumor cell-stromal cells interfaces has been shown to be intimately connected with CAF osteopontin expression and the formation or generation of a cancer stem cell niche [66]. Recent studies using six antibody markers actually classified four different subtypes of CAFs in breast cancer TME, where the peritumoral CAFs expressed αSMA and FAP and they were found to be immunosuppressive [67]. As already mentioned, similar studies of PDAC tumors have identified a

myofibroblastic CAF subtype, myCAF, at the tumor stroma interfaces, and an inflammatory subtype iCAF at a greater distance away from the tumor cells [6].

Thus, the data that are presented here raise a number of interesting questions. One central issue concerns the origin of integrin α11-expressing CAFs in the tumor stroma and whether these have a common developmental origin. During development, integrin α11 is highly expressed in the neural crest-derived head mesenchyme, in addition to the mesenchyme contributing fibroblasts to tendons, periosteoum, and perichondrium, but also in αSMA-positive myofibroblasts in the intestinal villus cluster [25]. Villus cluster myofibroblasts are thus naturally occurring myofibroblasts. Here, we also identified certain α11-expressing cells in the kidney mesangium that are α11 positive. We suggest that these cells represent the mesangial myofibroblasts, but this will require further characterization work. In the PyMT mouse model of breast cancer, some CAFs have been shown to originate from the bone marrow (BM) compartment [20]. Once these cells from the BM have arrived in the breast cancer TME, they expand and differentiate into CAFs. Interestingly, this subset of CAFs lack PDGFαR. It will be interesting to determine the origin of the α11-expressing CAFs in breast and pancreatic cancer, especially in the light of data demonstrating the expression of α11 in a subset of mesenchymal stem cells [68].

Secondly, it will be interesting to determine which factors drive integrin α11 expression. Based on our current knowledge, it is tempting to speculate that the stiffness of the tumor tissue will be one factor, which raises the α11 expression levels via unknown mechanisms. Furthermore, the finding that integrin α11 expression is high in desmoplastic tumors raises the question of how α11β1 on CAFs contribute to collagen synthesis.

Finally, our immunohistostaining data with a limited set of markers clearly demonstrate that CAFs in the stroma are heterogeneous with regard to α11 and αSMA expression, which suggests that α11 is expressed on distinct subset(s) of CAFs. In the light of xenograft models, existing data suggest that some CAFs expressing α11β1 are tumor supportive [28,29], and future studies should be aimed at better defining the α11-expressing CAF subsets in various tumor types, including the $\alpha11^{+}/\mathrm{vim}^{high}$ and $\alpha11^{+}/\mathrm{vim}^{low}$ subsets.

## 4. Materials and Methods

### 4.1. Tissue Array Sections

Frozen Tumor and Normal Tissue Array sections from BioChain Institute Inc. (Newark, CA, USA, Cat# T6235700-5, Lot#B712100, five sections per array) were used to examine α11 expression in human normal and tumor tissues. Immunostaining was also performed on fresh-frozen tumor tissue sections from patients that were diagnosed with pancreatic ductal adenocarcinoma (PDAC) or head and neck squamous carcinoma (HNSCC), which were both obtained from Haukeland University Hospital and subject to ethical approval from the Committee for Ethics in Health Research of West Norway (permit numbers REK Vest 2013/1772 and 2010/481, respectively).

### 4.2. Cells and Reagents

The C2C12 cells stably expressing human α11 integrin or human α2 integrin subunits (C2C12-huα11 and C2C12-huα2, respectively) have been described previously [25]. MRC5 human lung fibroblasts (American Type Culture Collection) were obtained from Robert Lafyatis laboratory (University of Pittsburgh Medical Center, Pittsburgh, PA, USA), the primary hGFs were isolated from healthy gingival tissue, as described earlier [69], and the primary oral cancer-associated fibroblasts (CAFs) and the primary normal oral fibroblasts (NOFs) were isolated from the same patient that was diagnosed with HNSCC at Haukeland University Hospital. The pancreatic cancer CAFs and integrin α5 knockdown CAFs isolated from PDAC, as described in [38], were obtained from Edna Cukierman's laboratory (Fox Chase Cancer Center, Philadelphia, PA, USA). All of the cells were attested as mycoplasma-free using the Lonza Mycoalert mycoplasma detection kit (Fisher scientific,

Gothenburg, Sweden, Cat# 11630271) and they were cultured in DMEM with GlutaMAX (Gibco, Life technology limited, Paisley, PA49RF, UK, Cat# 31966-021) supplemented with 10% fetal bovine serum (Gibco, Life technology limited, Cat# 10270-106) and 1% Penicillin-Streptomycin (Sigma, St Louis, MO, USA, Cat# P4333). TGF-β1 was from PeproTech (Hamburg, Germany, Cat# 100-21C).

### *4.3. Generation of Mouse Monoclonal Antibodies Specific to the Human Integrin α11 Chain*

The integrin α11 mAbs were custom-made at nanoTools (http://www.nanotools.de/) while using established procedures. Briefly, NT-HRM mice (nanoTools Antikoerpertechnik, Germany) were immunized with soluble recombinant human α11β1 integrin protein produced in CHO cells (R&D Systems, Minneapolis, MN, USA, Cat# 6357-AB), boosted twice, and cell fusion performed on day 68. Fusion was performed from 12 mice and hybridomas were screened for α11-producing clones in several steps. Luminex beads that were coated with α11β1 integrin were used to screen the α11 binders. Supernatants from positive clones were tested in flow cytometry for a positive signal with C2C12-huα11 cells [25], but a lack of reactivity with cells not expressing human α11 (parental mouse C2C12 cells and A431 cells, which express human β1 integrin, together with a number of other human integrin α chains, including αv, α2, α3, and α5 [31]). The positive supernatants were tested for their ability to immunostain focal contacts α11- containing in C2C12-huα11 cells that were plated on collagen I and to inhibit cell attachment of C2C12-huα11 cells to collagen I, but not to fibronectin. Limited dilution further characterized and finally subcloned positive clones.

### *4.4. Flow Cytometry*

The C2C12-huα11 cells were detached and neutralized with DMEM with FBS. After being washed three times with PBS (without $Ca^{2+}$ and $Mg^{2+}$), they were blocked with 5% BSA for 30 min at room temperature (RT). They were then mixed with mAb 203E1 or mAb 203E3 (3 μg/mL each) and then incubated for 1 h at 37 °C, followed by washing three times with PBS and incubation for 1 h in RT with Alexa fluor® 647-conjugated goat anti-mouse IgG (1:400, Jackson ImmunoResearch, Cambridgeshire, UK). Finally, the cells were washed and analyzed by FACS Accuri at the Molecular Imaging Center (MIC, University of Bergen, Bergen, Norway). FLOWJO computer software was used for data analysis (FLOWJO, LLC, Franklin Lakes, NJ, USA).

### *4.5. Immunoprecipitation*

Subconfluent C2C12-huα11 cells were cultured in 10 cm Petri dishes and lysed in 1 mL lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP40, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and complete Mini, EDTA-free cocktail (Roche Diagnostics GmbH, Manheim, Germany, Cat# 11836170001) for 20 min at 4 °C on a rocker. Protein lysates were centrifuged at 13,000× *g* for 20 min at 4 °C. The supernatants were incubated with 50 μL of protein G Sepharose beads (GE Healthcare, Uppsala, Sweden, Cat# 17-0618-01) with control non-immune mouse IgG for 2 h in a rotator at 4 °C, and spun down at 5000 rpm for 2 min at 4 °C. The resulting supernatants were then collected and incubated with 5 μg/mL of primary antibody (rabbit polyclonal anti-human α11 antibody or mAbs 203E1 or 203E3) overnight at 4 °C. The samples were incubated with 50 μL of protein G Sepharose beads for 2 h at 4 °C and spun down at 5000 rpm for 2 min at 4 °C. The beads were washed twice in PBS and 50 μL of 2× sample buffer with reducing agent was added before the boiling samples for 5 min. Finally, the samples were centrifuged for 2 min at 5000 rpm and loaded onto 6% SDS-PAGE gels for the separation of proteins, which were transferred to PVDF membranes while using the iBlot® system (Invitrogen, Kyrat Shmona, Israel, Cat# IB301002). The immunoprecipitated proteins were detected by incubating the membranes with polyclonal α11 rabbit antibody [32] followed by goat anti-rabbit HRP (see Western blotting for details).

### *4.6. Western Blotting*

The cells cultured in monolayers were washed with phosphate-buffered solution (PBS, Sigma-Aldrich, St Louis, MO, USA) lysed in SDS-sample buffer (Bio-Rad, Oslo, Norway, Cat# 1610791)

with 3% of 2-β-mercaptoethanol (Sigma-Aldrich, Cat# M7154) and sonicated using a Vibra-Cell™ ultrasonic processor (Sonics and Materials, Newtown, CT, USA). The cell lysates were subjected to (6% acrylamide) SDS-PAGE electrophoresis after boiling for 5 min., and the proteins were transferred to PVDF membranes using the iBlot® system. The membranes were blocked with 5% non-fat dry milk (Marvel, UK) in Tris-buffered saline containing 0.1% Tween20 (TBS-T), incubated with primary mouse anti-human α11 antibody Mab 210F4 [70] or rabbit monoclonal anti-human α2 (EPR 5788, Abcam, Cambridge, MA, USA, Cat# ab133557) or mouse monoclonal anti-human α1 antibody (R&D Systems, Minneapolis, MN, USA, Cat# MAB 5676) and anti-β-actin (AC-74, Sigma-Aldrich, Cat# A5441) overnight at 4 °C. Following the incubations, the membranes were washed in TBS-T three times for 10 min and incubated with goat anti-mouse- or goat anti-rabbit-HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were developed while using the ECL™ western blotting systems kit (GE Healthcare) and photographed using the ChemiDoc XRS device and the Quantity One 1-D Analysis Software (Bio-Rad).

### *4.7. Immunocytofluorescence*

C2C12 and C2C12-huα11 cells were seeded on coverslips that were pre-coated with bovine collagen I (Advanced BioMatrix, PureCol, Carlsbad, CA, USA, Cat# 5005) and cultured for 4 h. The coverslip coating was done in a 24-well plate with collagen I solution at a final concentration of 100 µg/mL, followed by incubation overnight at 4 °C. After culturing, the cells were briefly washed with PBS and fixed in 4% PFA for 10 min., washed in PBS (3 × 5 min), permeabilized, and blocked with 0.1% TritonX-100 and 1% BSA in PBS at RT for 1 h. For integrin α11 detection, the cells were incubated with affinity-purified α11 mAb, either mAb 203E1 or mAb 203E3 (0.32 mg/mL and 0.5 mg/mL, respectively, both diluted 1:200). The antibodies were diluted in 10% goat serum in PBS and supplied on coverslips in a 24-well plate, 200 µL/well. After incubation at 37 °C for 1 h, the cells were rinsed in PBS/Tween-20 (three washes 5 min each). The secondary antibody was Alexa Fluor® 488 AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, Cat# 115-545-062, 1:800) and TRITC-conjugated phalloidin (Sigma, St Louis, MO, USA, Cat# P1951, 1:100) was used to counter-stain stress fiber-associated actin. Both the secondary antibody and pahalloidin were diluted in PBS and applied to coverslips for 1 h at RT. The cells were rinsed for 3 × 5 min in PBS/Tween-20 and stained for 2 min with DAPI Nucleic Acid Stain (Molecular Probes). The staining results were recorded using a Zeiss Axioscope microscope (Zeiss, Oberkochen, Germany) that was equipped with an AxioCam camera (Zeiss) and Axiovision software (Zeiss).

### *4.8. Immunohistostaining*

The tissue array sections or fresh tumor cryosections were fixed with methanol for 8 min at −20 °C, followed by rehydration in PBS (3 × 10 min). The unspecific binding sites were blocked using 10% goat serum in PBS and the sections were incubated with primary antibody combinations, as indicated in the figures. The primary antibodies used were: mouse anti-integrin α11 mAb (mAb 203E3, 0.5 mg/mL, 1:200), rabbit anti-human cytokeratin 7 mAb (R17-S, Novusbio, Centennial, CO, USA, Cat# NBP1-30152, 1:200), rabbit anti-human cytokeratin 18 mAb (Epitomics, Burlingame, CA, USA, Cat# 1433-1, 1:400), rabbit anti-human FAP mAb (My Biosource, San Diego, CA, USA, Cat# MBS33414, 1:200), rabbit anti-FSP1 pAb (Millipore, Darmstadt, Germany, Cat# 07-2274, 1:300), rabbit anti-NG2 pAb (Millipore, Cat# AB5320), and mouse anti-αSMA FITC-conjugated mAb (1A4, Sigma, Cat# F3777, 1:400). All of the primary antibodies were diluted in 10% goat serum in PBS. After incubation at 37 °C for 1 h, the slides were rinsed in PBS/Tween-20 (three times for 5 min). The secondary antibodies Alexa Fluor® 594 AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch, Cat# 115-585-062, 1:800) and Alexa Fluor® 488 AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch, Cat# 111-545-045, 1:800) were diluted in PBS, applied to the sections, and incubated for 1 h at room temperature. The slides were then rinsed in PBS/Tween-20, the stained sections mounted in ProLong™ Gold Antifade Mountant

with DAPI (ThermoFisher, Eugene, OR, USA, Cat# P36931). The staining results were recorded using a Zeiss Axioscope microscope that was equipped with an AxioCam camera and Axiovision software.

### 4.9. Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR was performed, as previously described [27]. One microgram RNA was used along with MMLV-derived reverse transcriptase (Bio-Rad) and a blend of oligo (dT) and random hexamer primers. Next, 20 ng of reverse-transcribed cDNA was used as a template, along with 0.5 µM of each primer, in a 20 µl qPCR reaction using FastStar Universal SYBR Green Master (Roche Applied Science, Penzberg, Germany), which was in accordance with the manufacturer's protocol. RT-qPCR was performed in a Light-Cycler 480 Instrument II (Roche Applied Science). The qPCRs were performed in triplicate for each cDNA sample and negative controls where no cDNA template was included for each pair of primers. Table 3 lists the primers used for qPCR.

**Table 3.** List of Primers for the quantitative Polymerase Chain Reaction (qPCR).

| Human Gene | Forward Primer | Reverse Primer | Product Length |
|---|---|---|---|
| *ITGA11* | 5′-GTGGCAATAAGTGGCTGGTC | 5′-GACCCTTCCCAGGTTGAGTT | 122 bp |
| *KRT7* | 5′-ACTCATGAGCGTGAAGCTGG | 5′-ATCACAGAGATATTCACGGCTCC | 117 bp |
| *VIM* | 5′-TGGACCAGCTAACCAACGACAAAG | 5′-TCCTCTCTCTGAAGCATCTCCTCC | 112 bp |
| *ACTA2* | 5′-AGCCAAGCACTGTCAGGAATC | 5′-TGTCCCATTCCCACCATCAC | 192 bp |
| *S100A4* | 5′-GCAAAGAGGGTGACAAGTTCAAGC | 5′-CCTGTTGCTGTCCAAGTTGCTC | 137 bp |

### 4.10. Cell Adhesion Assay

48-well plates were coated with human plasma fibronectin (2 µg/mL: Sigma-Aldrich, Cat# F0895) or bovine collagen type I (0,5 µg/mL: Bovine PureCol®, Advanced BioMatrix, Carlsbad, CA, USA, Cat# 5005) and incubated for 2 h at 37 °C. After washing the coated plates twice with PBS, they were blocked with 2% BSA for 1 h at 37 °C, and the cells were washed twice with DMEM without FBS. $1 \times 10^5$ cells/well were incubated for 45 min at 37 °C with clone 11,711 (10 µg/mL, mouse IgG1 isotype control, R&D Systems, MN, USA, Cat# MAB002), mAb 203E1 (10 µg/mL, integrin α11 antibody), P1E6 (5 µg/mL, integrin α2 antibody, Merck Millipore, Cat# MAB1950Z), and mAb 13 (5 µg/mL, integrin β1 antibody, BD Biosciences, San Jose, CA, USA, Cat# 552828). Following incubation, the non-adherent cells were carefully removed by washing twice with PBS containing $Ca^{2+}$ (1 mM) and $Mg^{2+}$ (0.5 mM). The cells were fixed with absolute ethanol for 10 min at room temperature, washed twice with distilled water, and stained with 0.1% crystal violet for 25 min at room temperature. The plates were washed three times with distilled water and the cells were lysed with 1% Triton X-100/PBS for 15 min. The lysates were transferred to a 96-well plate and the absorbance was read at 595 nm (Spectramax® Plus 384, Molecular Devices, San Jose, CA, USA).

### 4.11. Collagen Gel Contraction

Collagen gel contraction was performed according to a previously described protocol [69]. 24-well plates were blocked with 2% BSA overnight at 37 °C and washed three times with PBS. A collagen solution was prepared by mixing 50% of DMEM 2× (SLM-202-B, Merck Millipore, Cat# SLM-202-B), 10% of 0.2M HEPES at pH 8.0, and 40% of collagen type I. The solution was then mixed with cells to obtain a final concentration of $1 \times 10^5$ cells/mL. 400 µL of cell-collagen suspension was added to each well and allowed to polymerize for 90 min at 37 °C. Antibodies were added to DMEM containing 0.5% FBS for the blocking experiments. Polymerized collagen gels were floated with 400 µL of DMEM. The gel diameters were measured using a ruler and percentage of the initial gel area was calculated at different time points.

### 4.12. Spheroid Preparation and Migration Assay in 3D Collagen Gel

Single cell type spheroids (homospheroids) were prepared by the hanging drop method, as described earlier [63]. Cells with 80% confluency were trypsinized and resuspended in a solution that

was composed of $\frac{3}{4}$ volume of DMEM with 10% FBS and $\frac{1}{4}$ volume of methylcellulose (Sigma-Aldrich) to a concentration of $1 \times 10^6$/mL. Approximately 35 drops (25 µL/drop, $2.5 \times 10^4$ cells) were placed on the lid of a Petri dish containing DMEM in the bottom. The lid was inverted over the bottom of the dish. The spheroids were cultured for one day under regular cell culture conditions (37 °C and 5% CO2). A collagen solution was prepared by mixing 50% of DMEM 2×, 10% of 0.2 M HEPES at pH 8.0 and 40% of collagen type I, and 100 µL of this solution was added onto a 96-well plate and incubated for 15 min at 37 °C. 1 spheroid was embedded per well and the collagen-spheroid solution was allowed to polymerize for 90 min at 37 °C. After polymerization, 100 µL of DMEM with antibody was added to each well to cause the collagen gel to float, before culturing for 24 h. The spheroids were examined under an inverted light microscope (Leica DMIL, Wetzlar, Germany) and photographed. The resulting images were then analyzed and processed with Fiji. The modification of the spheroids included the alteration of the type to 8-bit, adjustment of the brightness/contrast, subtraction of the background, and establishment of a threshold. The radial Profile plugin of Fiji was applied to quantify the intensity of the cells.

## 5. Conclusions

In summary, our data indicates that integrin α11 is induced in CAFs in the stroma of tumor tissues that are characterized by high tissue stiffness and desmoplasia, and the morphology of the cells in the set of tumors analyzed here suggests a reactive stromal phenotype, which is not associated with vascular structures. Functional assays with cultured fibroblasts and CAFs demonstrated a role for α11β1 in collagen reorganization and CAF invasion, lending further support to the hypothesis that α11 might be an interesting candidate for stromal-targeted therapy to increase the efficacy of immune therapy, as well as conventional therapeutic approaches.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2072-6694/11/6/765/s1, Figure S1: Determination of the integrin α11 203E1 and 203E3 mAb affinity, Figure S2: Immunostaining and H&E staining of sections from three different HNSCC patients, Figure S3: Full-size Western blots of Figure 6a and protein quantification for each blot.

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## Supplementary Materials

# $\alpha 11\beta 1$ integrin is induced in a subset of cancer-associated fibroblasts in desmoplastic tumor stroma and mediates *in vitro* cell migration

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### Supplementary Figures S1-S3

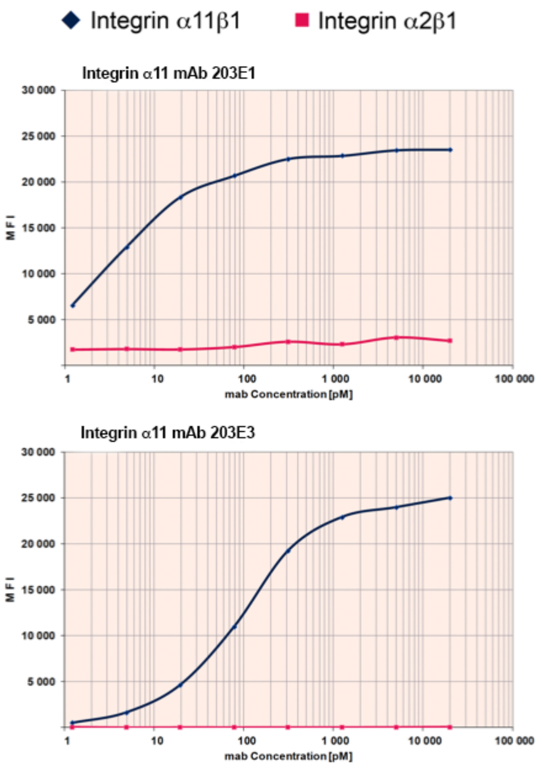


**Figure S1**. Determination of the integrin $\alpha 11$ 203E1 and 203E3 mAb affinity. The experiment was done in nanoTools using Luminex beads (Biorad) conjugated with either integrin $\alpha 11\beta 1$ or $\alpha 2\beta 1$ protein (target protein, both are from R&D Systems). Binding affinity of the mAb 203E1 and 203E3 to the target protein was indicated by the mean fluorescent intensity (MFI) at different mAb concentrations.

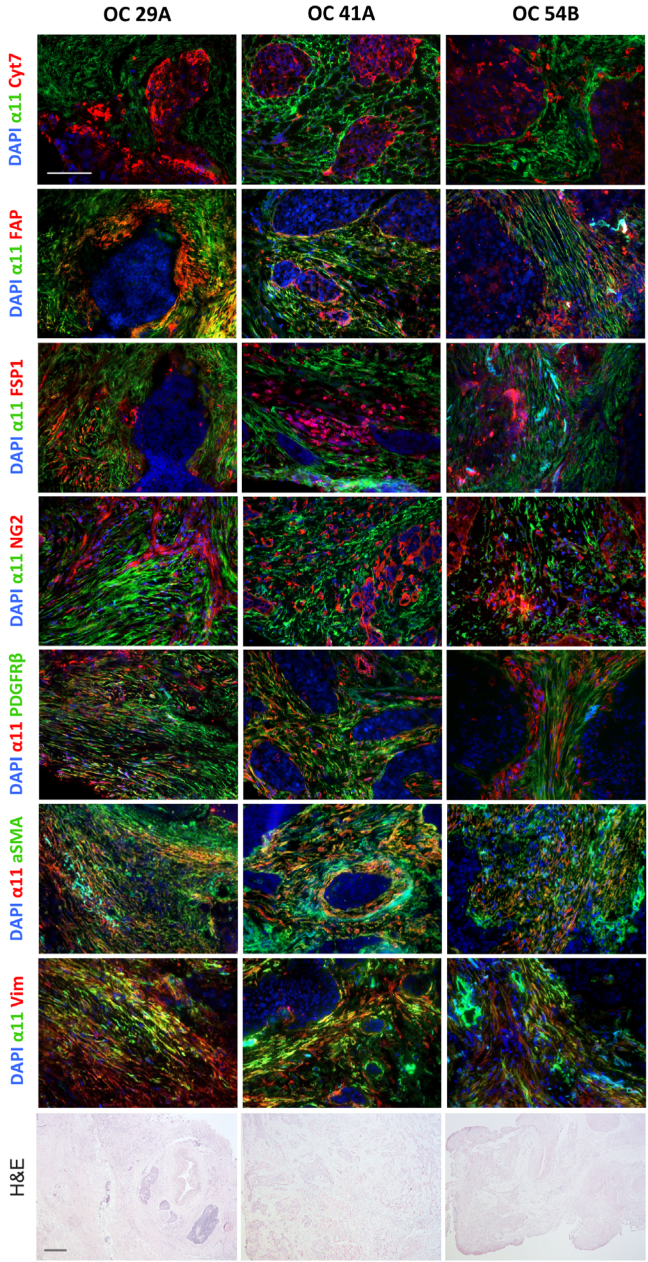


**Figure S2**. Immunostaining and H&E staining of sections from three different HNSCC patients. Immuno and H&E stainings were performed in available sections from 3 independent patients with an oral cancer (patient OC 29A, OC 41B and OC 54B). A representative staining result from OC 29A was shown in Figure 5 indicated as HNSCC, together with staining result from a PDAC patient. Scale bar: 100 µm in IF pictures and 200 µm in H&E pictures.

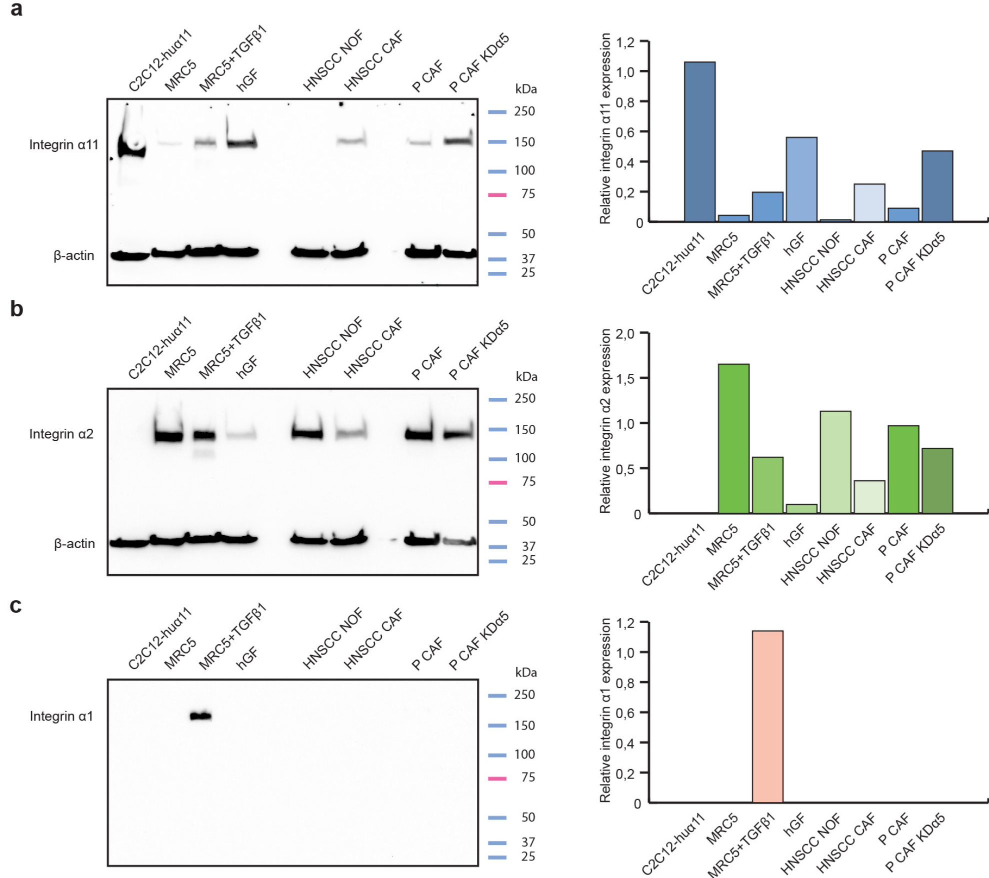


**Figure S3**. Full-size Western blots of Figure 6a and protein quantifications for each blot. Protein extracts from indicated cells were transferred to a PVDF membrane, and the membrane was blotted sequentially with antibodies to integrin $\alpha$11 **(a)**, integrin $\alpha$2 **(b)** and integrin $\alpha$1 **(c)**. The protein-antibody complexes were stripped off before each blotting. Molecular weight marker (BioRad) was used and sizes of the bands were indicated. The relative expression levels of the integrin $\alpha$ chains were normalized to β-actin on each membrane except for integrin $\alpha$1, in which the β-actin bands from integrin $\alpha$2 blot were used since the integrin $\alpha$1 blot was only incubated with $\alpha$1 but not β-actin antibody.

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