MOLECULAR MECHANISMS OF LTBP4-RELATED CUTIS LAXA

by

Chi-Ting Su

M.D., School of Medicine, Kaohsiung Medical University, Taiwan, 2003

MPH, University of Pittsburgh, 2012

Submitted to the Graduate Faculty of

the Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2014

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

This dissertation was presented

by

Chi-Ting Su

It was defended on

April 9, 2014

and approved by

Dissertation Advisor: Zsolt Urban, PhD, Associate Professor, Departm1ent of Human Genetics, Graduate School of Public Health, University of Pittsburgh

Daniel Weeks, PhD, Professor, Department of Human Genetics and Biostatistics, Graduate School of Public Health, University of Pittsburgh

Yadong Wang, PhD, Professor, Department of Bioengineering, University of Pittsburgh

Quasar Padiath, PhD, Assistant Professor, Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh

Zsolt Urban, PhD

MOLECULAR MECHANISMS OF LTBP4-RELATED CUTIS LUXA

Chi-Ting Su, PhD

University of Pittsburgh 2014

ABSTRACT

Structural proteins of the extracellular matrix (ECM) and associated proteins build up a complex network that is abundant in the human body. In addition to serving key biomechanical roles, ECM proteins play an important role in storage, presentation and contextualization of growth factors, including transforming growth factor-β (TGFβ). This work focused on investigating the biomechanical consequences of and molecular disease mechanisms leading to cutis laxa (CL), a rare inherited disorder characterized by loose skin and frequently associated with systemic involvement, including aortic aneurysms, pulmonary artery disease, and emphysema.

A DermaLab suction cup device was used to evaluate the mechanical properties of the skin in CL patients. The results showed significant reduction of elastic and viscoelastic moduli (VE). VE appeared to be a reliable measurement of biomechanical aging of the skin and also offered a predictive value in distinguishing cases from controls.

To study molecular disease mechanisms in CL, control and *LTBP4*-mutant human dermal fibroblasts were used to investigate TGFβ activity and signaling. In *LTBP4*-mutant cells, despite

elevated extracellular TGFß activity, downstream signaling molecules of the TGFß pathway were markedly suppressed. TGFß receptors (TGFBR1 and TGFBR2) were reduced at the protein, but not at the RNA level. Treatment with exogenous TGFβ1 led to a further decrease in downstream signaling and receptor abundance. Upon treatment with TGFBR1 kinase inhibitor, endocytosis inhibitors or a lysosomal inhibitor, the levels of TGFBR1 and TGFBR2 were normalized. Antisense mophorlino oligonucleotide-mediated knockdown of LTBP4 reduced TGFβ receptor abundance and signaling in normal cells and supplementation of recombinant LTBP4 enhanced these measures in mutant cells. I conclude that, in the absence of LTBP4, TGFBR1 and TGFBR2 are internalized and degraded by lysosomes in a ligand-dependent and receptor kinase activity-dependent manner. Thus, LTBP4 is a key molecule required for the stabilization of the TGFβ receptor complex.

Increased TGFβ levels have been found in patients with cardiomyopathy, diabetic nephropathy, cancer, and lung fibrosis, in all cases correlating with disease severity. The discovery of a new mechanism for TGFβ receptor regulation will be helpful in developing novel therapeutic reagents for systemic diseases of major public health impact.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Zsolt Urban for invaluable advice and support throughout my degree. I appreciate the valuable comments and suggestions by my committee members, Dr. Daniel Weeks, Dr. Yadong Wang and Dr. Quasar Padiath. I thank Dr. Beth Kozel, Joshua Danback, Juliann McConnell, and Robert Wilson for Dermalab skin elasticity data, Dr. Suneeta Madan-Khetarpal, Meghan Mac Neal and Kara Levine for clinical data, Elizabeth Lawrence, Dr. Bert Callewaert and Dr. Anne De Paepe for mutational data and fibroblasts, and Christine Weckenmann and Amelie Bauer, for generating HEK293 cell lines with LTBP4 overexpression.

Thanks to my family and especially my parents and husband for support and encouragement.

ABBREVIATIONS

ADCL	autosomal dominant cutis laxa
ALK	activin receptor-like kinase
ANOVA	analysis of variance
ARCL	autosomal recessive cutis laxa
ATCC	American Type Culture Collection
ATS	arterial tortuosity syndrome
AUC	area under curve
BMP	bone morphogenetic protein
cDNA	complementary DNA
CHX	cycloheximide
CL	cutis laxa
CO MO	control morpholino oligo
Co-SMAD	common mediator SMAD
DBS	De Barsy syndrome
DEPC	diethyl-pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	elastic modulus ; Young's modulus
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBN	fibrillin
FBS	fetal bovine serum
FN	fibronectin
GDF	growth differentiation factor
GO	geroderma osteodysplasticum
HEK 293	human embryonic kidney 293 cells
HS	heparan sulfate

HSPG	heparan sulfate proteoglycan
I-SMAD	inhibitory-SMAD
LDS	Loeys–Dietz syndrome
LLC	large latent complex
LOX	lysyl oxidase
LTBP	latent TGFβ binding protein
MACS	macrocephaly alopecia cutis laxa scoliosis syndrome
MAGP	microfibril-associated glycoprotein
MAPK	mitogen-activated protein kinase
MDC	monodansylcadaverine
MFS	Marfan Syndrome
MIF	Müllerian inhibitory factor
MLEC	mink lung epithelial cell
MMPs	metalloproteinases
MO	morpholino oligonucleotide
NMD	nonsense-mediated decay
NO	nitric oxide
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pERK	phosphorylated ERK
PG	proteoglycan
PI3K	phosphoinositide-3-kinase
pSMAD2	phosphorylated SMAD2
PTC	premature termination codon
pTGFBR1	phosphorylated transforming growth factor β receptor 1
PVDF	polyvinylidene difluoride
Q-PCR	quantitative PCR
RER	rough endoplasmic reticulum
RFP	red fluorescent protein
RGD	arg-gly-asp
RNA	ribonucleic acid
ROC	receiver operating characteristic
R-SMAD	receptor-specific SMADs
RT	retraction time

SARA	SMAD anchor for receptor activation
SDS	Sequence Detection System
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLC	small latent complex
TAK1	TGFβ-activated kinase 1
TDGF	teratocarcinoma-derived growth factor
TGFBR1	transforming growth factor β receptor 1
TGFBR2	transforming growth factor β receptor 2
TGFBR3	transforming growth factor β receptor 3
TGFβ	transforming growth factor β
TRAF	Tumor necrosis factor receptor-associated factor
Transferrin R	transferrin receptor
URDS	Urban-Rifkin-Davis syndrome
VE	viscoelastic modulus
XLCL	X-linked cutis laxa

1. AIMS

Cutis laxa (CL) is a group of connective tissue disorders characterized by loose, inelastic and redundant skin with manifestations involving multiple additional organs, including nervous, musculoskeletal, cardiovascular, pulmonary, gastrointestinal, and urinary systems. Despite extensive locus heterogeneity, altered structure and function of elastic fibers is a major shared mechanistic connection between different types of CL. The diagnosis for cutis laxa is made based on physical examination of the skin, aided by consideration of other systemic involvement, and pathological and molecular genetic data, if available. However, objective methods to measure skin laxity have not been applied to the diagnosis of CL yet. **I hypothesized that a simple suction cup device for the measurement of skin elasticity can provide and objective, quantitative approach to improve the diagnosis of CL.**

The second part of my dissertation work focused on a specific type of CL, autosomal recessive CL type 1C, caused by mutations in the gene for the latent transforming growth factor-beta binding protein 4 (LTBP4). Based on published data on knockout mouse models[1],[2] and previous studies in our laboratory[3], **I hypothesized that LTBP4 is a multifunctional protein, involved in the assembly of elastic fibers and in the regulation of transforming growth factor-beta (TGFβ) signaling.**

1.1. AIM 1: EVALUATE THE UTILITY OF BIOMECHANICAL MEASUREMENTS OF THE SKIN FOR THE DIAGNOSIS OF CL

A DermaLab suction cup device was used to measure mechanical properties of the skin in CL patients and control individuals. The elastic modulus and viscoelastic modulus parameters and control variables were used to identify measures that could distinguish between patients and controls with the highest specificity and sensitivity.

1.2. AIM 2: THE IMPACT OF LTBP4 MUTATIONS ON ECM ASSEMBLY, TGFB SIGNALING AND TGFB RECEPTORS

Human dermal fibroblasts from *LTBP4*-mutant patients and control individuals were cultured to study elastic fiber deposition into ECM, extracellular TGFβ activity and intracellular TGFβ signaling. In addition, TGFβ receptor complexes on the cell surface were studied in order to understand, if LTBP4 deficiency affected the turnover of TGFβ receptors. To confirm our findings in mutant cells, *LTBP4* was depleted in human dermal fibroblasts by using antisense mophorlino oligonucleotide (MO) treatment. To rescue the consequences of LTBP4 deficiency, *LTBP4*-mutant dermal fibroblasts were treated with recombinant LTBP4 protein.

2. INTRODUCTION

2.1. PUBLIC HEALTH SIGNIFICANCE

Patients with CL present multiple manifestations clinically involving different body systems. Abnormal extra cellular matrix assembly contributes to abnormal skin laxity with altered dermal mechanical properties. In *LTPB4*-related to cutis laxa, cardiovascular, pulmonary and gastrointestinal abnormalities can be involved. Additionally, LTBP4 acts as a chaperone for secreted TGFβ, composed of a latent form and extracellular matrix serves as a reservoir of TGFβ and other grow factors. Marfan syndrome is a good example, a genetic disease caused by mutations in *FBN1*, a gene encoding fibrillin-1, a major structural protein in ECM. The implication of extracellular matrix-related regulation of TGFβ signaling has been demonstrated in Marfan syndrome[4]. An accumulating body of evidence points to the central contribution of abnormal TGFβ signaling to a broad range of cardiovascular and pulmonary diseases, fibrosis, diabetic nephropathy and rheumatoid arthritis[5-7]. In fibrotic disorders overproliferation of fibroblasts was identified as a key factor[8]. Skin fibroblasts from *LTBP4*-mutant cutis laxa patients provide a useful cellular model system to investigate the molecular regulatory mechanisms of TGFβ signaling. Discovery of novel molecular mechanisms TGFβ regulation will be instrumental for the development of future therapeutic reagents broadly applicable to diseases of major public health concern.

2.2. DERMAL STRUCTURE AND MECHANICAL PROPERTIES

Epidermis, dermis and subcutaneous tissue are three major components in human skin (Figure 2.1). This complex organ provides protection against external stimulation and mechanical forces and enables reversible deformation[9,10]. Epidermal layers are covered partially by keratinized cells and dermis layers are composed of abundant collagens with elastic fibers as well as lymphatic elements. The mechanical properties of the skin include both elastic and viscous elements which are related to its complex structure[11]. Abundant collagen and elastic fibers in the dermis layers form a supportive structure and provides elasticity. Proteoglycans provide a ground substance that resists deformation via sliding of the fibrous protein network and lead to the viscous properties of skin. Elasticity and viscosity of skin can vary from different part of the human body, external environment, age and diseases. Thus, biomechanical tests of human skin can detect skin diseases and help quantify the effectiveness of dermatological interventions or treatments[10].

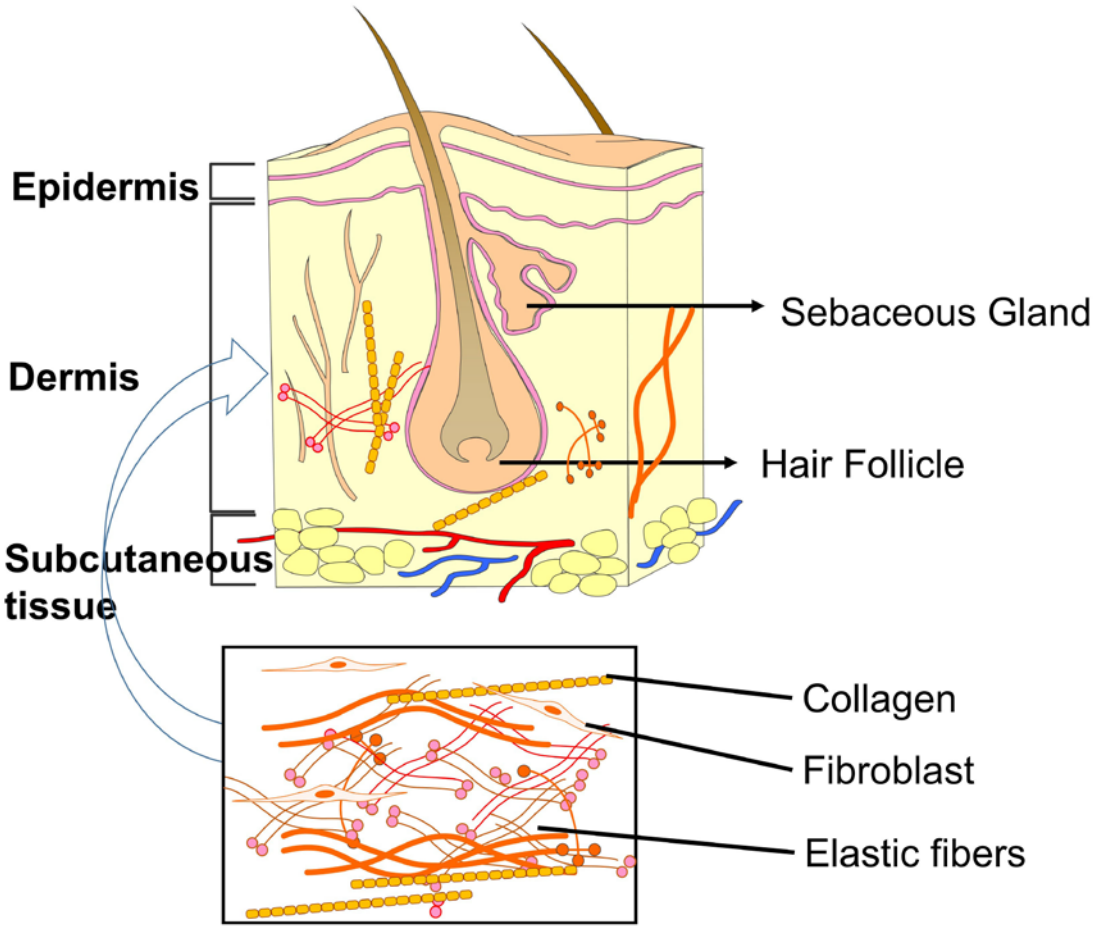


Figure 2.1 The structure of the human skin

Three layers of skin, including epidermis, dermis and the subcutaneous. Collegen fibers and elastic fibers are abudnant in the dermis, and provide elasticity to the skin.

Human skin is a highly specialized mechanical structure, which enables reversible deformities after being extremity stretched and still maintains its original phenotypic properties[10,12]. The nature of skin to be stretched extremely are attributed to a molecular network, including chemicals related to extracellular, cytoplasmic and nuclear membranes[13]. Expansion of skin can initiate a series of reactions including molecular signaling to increase cellular mitosis and synthesis of collagen fibers. Those signaling events can be mediated by ion channels, intergrins, growth factor receptors mitogen-activated protein kinase (MAPK), nitric oxide (NO) and phosphoinositide-3-kinase (PI3K)[14-16]. However, extreme stretching over human skin may result in its mechanical property loss, leading to damage of the skin and consequently irreversible deformation[17].

The elasticity of skin results from interaction among several dermal elements. The elastic properties can be applied to the mechanical laws by using the modulus of longitudinal elasticity, the Young's modulus (abbreviated to E). Based on the Hooke's Law, strain is defined as Young's modulus/stress[17], so the Young's modulus is the relation between stress and strain over the skin. There is linear relation between stress and strain applied to the skin in the elastic range. The description of the Young's modulus has been applied extensively in literature to evaluate the mechanical properties of skin.

The most acceptable methods to investigate the mechanical properties of skin are mainly based on suction[18], torsion[19] and traction[20]. The Young's modulus (E) has been used commonly in studies to describe the longitudinal elasticity, which can characterize the resistance of skin to

elastic elongation upon to stretching[10]. However, in the literature, there have been large discrepancies in the estimates of the Young's modulus (E) resulting from different tests by various methodologies. Despite the variation, studies show that skin tension and the elastic modulus are correlated with age[10,21].

2.3. EXTRACELLULAR MATRIX

The extracellular matrix (ECM) provides a supportive scaffold between cells and makes an available biological environment for cell proliferation, migration and differentiation. Additionally, ECM plays an essential role in reservoirs or activation of cytokines and interacts with proper adhesion molecules dynamically between cell surface and extracellular spaces. The major components in extracellular space are fibronectin, microfibrils, elastic fibers and collagens, which provides the resilience and elasticity in the tissues. Besides those major structural proteins, the ECM is a highly specialized area for the reservoir of soluble factors involved in inter-cellular communication[22]. Additionally, the significance of extracellular space in cell adhesion and conducting downstream intracellular signaling through adhesion receptors such as integrins has been extensively studied[23-25] and mechanical properties of the matrix, such as stiffness, deformability, have also been received much attention related to cell behavior[26,27].

Collagenous protein and non-collagenous proteins, such as elastin and fibronectin are composed of ECM. There are 5-10% sugar contents in most of them with glycosylated[28].

Moreover, a remarkable content of proteoglycans, such as perlecan has been found as an important component in the ECM[29,30]. The impacts of growth factors, catecholamine and numerous cytokines on the synthesis of those proteins are predominant. On the other hand, matrix metalloproteinases (MMPs) degrade the ECM by cleaving proteins into a range of fragments, some of which also have signaling activities[28].

Some of ECM proteins interact with growth factors and with sequential signaling. Fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs) are the most representative examples. A component of several proteoglycans (PGs), heparin sulfate, can bind to FGFs and VEFGs. Moreover, proteoglycans have been thought as a reservoir of many growth factors and also key determinants of developmentally important growth factor gradients[31] and prevent their premature degradation[32] as well as may be helpful to establish a stable ECM network[33]. There is a ligand-specific receptor manner for some growth factors to conduct downstream signaling and bind to heparin sulfate in the receptor complex. FGF binding to its receptor (FGFR) relies on the simultaneous binding of a heparan sulfate chain[34]. Additionally, transforming growth factor β (TGFβ) ligands initiate the interaction with membrane PGs that help concentrate the ligands at the proximity of their receptors on the cell membrane[35].

There are several mechanisms by which ECM molecules signal to cells and conduct downstream signaling. First, in ECM, transmembranous proteins, such as integrins and discoidin domain tyrosine kinase receptors interact with specific motifs within matrix molecules in a ligand-dependent manner[23,33]. Second, accumulating evidence suggests cross-talk and synergistic

influences between signaling by intergrins and by growth factors receptors[36]. However, it is not easy to clarify the involvements membrane-proximal interactions or connections between in the downstream signaling molecules in those proteins cross-talk. Laminins, tenascins and fibrillins and many other ECM proteins contain multiple epidermal growth factor (EGF)-like domains, which may interact with EGFR as solid-phase ligands to modulate downstream signals[37-39]. Similarly, other intrinsic domains in ECM proteins may serve as active ligands for canonical growth factor receptors and trigger the signals[33]. Thus, the ECM has been recognized as a storage of bound or intrinsic growth factors and important to the intercellular interaction via those soluble molecules in a ligand-specific manner.

The ECM is also important for the storage and regulated release of the TGFβ family of growth factors. It is one of best-studied examples of the multi-faceted interactions between the ECM and growth factors. There are three isoforms of TGFβ and a furin protease cleaves pro-TGFβ to form the mature TGFβ and its propeptide. The structure of latency-associated peptide (LAP) include the mature TGFβ and its associated propeptide. LAP and TGFβ are non-covalently bound to form a complex, known as the small latent complex (SLC). In this form, TGFβs are inactive[40,41]. Disulphide bonds connect TGFβ and LAP to form a dimeric SLC, which is bound by further disulfide bonds to the latent TGFβ binding proteins (LTBPs), called a large latent complexes (LLCs) (Figure 2.2). LTBPs can act as a chaperone, ensuring the proper folding of LAP and TGFβ. LTBPs can bind to ECM proteins, such as fibronectin and fibrillins to incorporate TGFβ into extracellular matrix in the latent form. LTBP-related incorporation in to

the ECM is required for TGFβ activation and ECM degradation release TGFβs. Mutations of ECM proteins have been reported to be related to markedly increased TGFβ activity. Marfan syndrome (MFS), caused by mutant fibrillin-1 is a well-studied example. In mouse models of MFS, aortic aneurysms could be ameliorated by treatment with TGFβ antagonists[42,43]. Many other inherited diseases have been suggested to be the consequence of abnormal TGFβ signaling secondary to the ECM defects (Table 2.1).

Table 2.1 Human disorders and animal models associated with TGFβ signaling

Gene	Human Syndrome	Animal Models	TGFβ signaling
Extracellular regulation of TGFβ signaling			
FBN1 (fibirillin-1)	MFS	Ruptured aortic aneurysm, altered pulmonary development, lethal at brith [44,45]	↑
FBN2 (fibrillin-2)	Contractural arachnodactyly	Bilateral syndactyly, involving both and hard tissues [46]	↑
FN (fibronectin)	Ehlers–Danlos syndrome, type X	Defective vascular system development, defective neural tubes [47]	↑
LTBP1L	No available publish	Impairments of cardiac valves and vessels formation, persistent trucus arteriosus, interrupted aortic arch and outflow [48]	±
LTBP4	ARCL1C/URDS	Emphysemia, colon prolapse [1,3]	±
TGFB1	Camurati–Engelmann disease	KO: Embryonic lethal with vascular defects, infiltration of immune system cells [49,50] Gain function: NA	±
TGFβ receptors			
TGFBR1 (ALK5)	LDS	Lethal at birth, impaired vascular formation [50]	↑
TGFBR2	LDS	Angiogenesis defects, lethal at birth [49,51]	↑
TGFBR3 (betaglycan)	Unknown	Abnormal cardiac septal formation, defect ventricular walls [52,53]	±

LDS, Loeys–Dietz syndrome; MFS, Marfan syndrome; TGFβ, transforming growth factor-β; TGFBR, TGFβ receptor.
NA: not available

The structural and functional complexity of the ECM is crucial to maintain proper functions in the microenvironment of cells. Accumulating evidence supports the role of ECM proteins and cytokines in tumor metastasis[54,55], stem cell niches[56], fibrosis, and immunity. Thus, investigations of molecules in the ECM related to regulate cell signaling are warranted and relevant to a range of disease states.

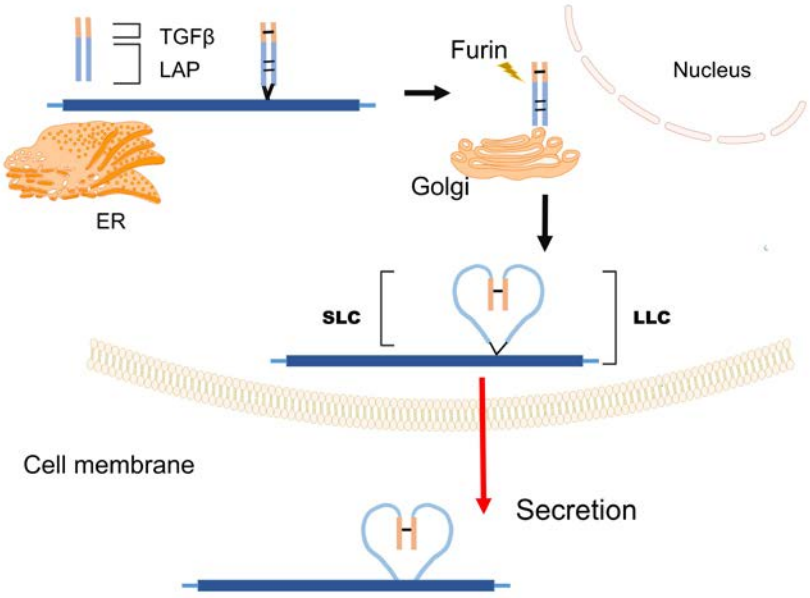


Figure 2.2 Formation of TGFβ, SLC and LLC.

In the rough endoplasmic reticulum (ER), two TGFβ pro-peptide monomers form the dimeric pro-TGFβ, which is correctly folded in the presence of LTBPs, with disufide bonds formed between the SLC and the second 8-cysteine domain in LTBP to form LLC. In the golgi, a furin cleaves proTGFβ into dimeric TGFβ and LAP. SLC: small latent complex; LLC: large latent complex.

2.4. ELASTIC FIBERS

In the ECM of the skin, lungs, large arteries, ligaments and auricular cartilage, elastic fibers are insoluble components. Elastic fibers provide the mechanical properties required to enable skin expansion repeatedly through life. In different tissues, there are different arrangement of an integrated network of mature elastic fibers to tolerate various intensity of tissue stretching. This tissue-specific structural arrangement reflects different requirements of elasticity[57]. In the skin, the elasticity is conferred by an integrated network of elastic fibers, which is anchored to the dermal-epidermal junction by fine branches running perpendicular to the surface of the skin in the papillary dermis and robust, thick fibers running parallel to the surface in the deep dermis. In pulmonary tissue, elastic fibers build up fine networks necessary to respiratory expansion and contraction[58].

2.4.1. Elastin

Elastin is the major component protein of elastic fibers. Elastogenic cells, including fibroblasts, smooth muscle cells and auricular chondrocytes, produce a secreted and soluble precursor, tropoelastin, encoded by a gene on chromosome 7q11.2. Tropoelastin is an asymmetric molecule, which contains a multi-domain structure, including lysine-alanine and lysine-proline cross-linking domains. In N-terminal parts of tropoelastin, it has spring-link properties. On the other

hand, C-terminal regions have been suggested being crucial to cell adhesion via binding αvβ3 integrin[59] and cell surface proteoglycans[60].

2.4.2. Fibrillin

Fibrillin-rich microfibrils are abundant in connective tissues and enable the interaction with cross-linked elastin in an elastic fibril network or elastin-free macroaggreates[58,61,62]. In most mammals, three fibrillin genes have been found, each encoding a 350kDa multi-domain glycoprotein[63]. Fibrillin is composed of multiple domains, including 43 calcium-biding epidermal growth factor-like (EGF) domains, 5 EFG-like domain, seven 8-cysteine-containing (TB) motifs and 2 hybrid regions in similarities to TB and EGF-like domains. Fibrillin-1, encoded by a gene on chromosome 15q21.1 is the most distributed fibrillin and it is required for microfibril homoeostatsis.

2.4.3. Auxiliary molecules associated with microfibrils and elastic fibers

Besides elastin and fibrillins, there are some auxiliary molecules interacting with microfibrils and elastic fibers related to heritable diseases with abnormal elastic fiber assembly and molecular function. Those molecules associated with microfibirls include latent TGFβ binding proteins (LTBPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family members and microfibril-associated glycoprotein (MAGPs). Moreover, fibulin3, fibulin4, fibulin5 and lysyl oxidase (LOX) family members are also elastic fiber-associated molecules [57].

2.4.4. Assembly of elastic fibers

2.4.4.1. Microfibril formation, fibronectin and heparan sulphate

In the processing in the formation of ECM, fibronectin serves as a platform for the deposition of microfibrils[64,65]. However, in some circumstances, it may not be required in many lower organisms and in fibronectin-null culture[66,67]. Those evidences indicate that fibronectin is an enhancer rather than obligatory requirement of microfibril assembly.

Fibrillin monomers interact with each other in a head-to-tail alignment, which forms a "beads-on-a-string" appearance of microfibrils[68-70]. The self-association results in longitudinal polymerization but also through lateral biding, which leads to micrifibril formation. Assembled microfibrils consist of fibrillin monomers and the untensioned periodicity of individual microfibrils in tissue electron microscopic preparations is 50-60nm[65,71]. The precise arrangement and interaction of fibrillin molecules within microfibrils remains unclear and requires further study.

Nonetheless, expect a self-assembly process of microfibril formation, it has been suggested cellular involvements are required. Kinsey et al. reported that fibronectin arg-gly-asp (RGD)-dependent α5β1 integrins were necessary for microfibril assembly in fibroblasts[64]. In addition, Fibrillin-1 can interact with cells via integrins α5β1, αvβ3 and αvβ6 in epithelial cells[72,73]. It remains unclear the precise role in the direct interaction between fibrillin-1 and cells for microfibril assembly and the plausibility of certain tissue-specific functions of microfibrils.

In addition, heparan sulfate (HS) glycosaminoglycans are important in microfibril assembly. HS is an element of syndecan and glypican receptors as well as of proteoglycan, perlecan, in the basement membrane[57]. Heparin ablates microfibril assembly in cell cultures[74,75]. At least six high-affinity binding regions of Fibrillin-1 can interact with heparin[74-77]. Although it remains unknown how HS precisely facilitates microfibril assembly, results to date suggest that it is essential. Moreover, MAGP-1 and tropoelastin can bind fibrillin-1, similar to HS and the competition among those molecules has been found[76]. Thus, HS may regulate elastic fibers deposition onto microfibrils..

2.4.4.2. Microfibril-associated molecules

Many microfibril-associated molecules have been identified, including LTBPs, ADAMTS family members and MAGPs. LTBPs are important to proper localization and deposition in the ECM via interactions with fibronectin and fibrillins as well as several extracellular proteins. LTBPs and fibrillins are highly homologous proteins but fibrillins, in comparison with LTBPs, are in the larger size, closed to 350kDa, with double the number of EFG-like and 8-cys domains (Figure 2.3). LTBP1 deposition in the ECM depends on fibronectin rather than on fibrillin-1 or fibrillin-2. Nevertheless, LTBP1 does colocalize with microfibrils both in culture and in tissue samples[78]. Fibrillin-1 is required for the incorporation of LTBP3 and LTBP4 into the ECM properly. In addition, fibronectin is also necessary for proper LTBP4 deposition in the ECM and

LTBP4 may serve as an adhesion molecule[79]. Taken together, fibronectin and microfibrils necessary for the extracellular deposition of LTBPs in a family member specific manner.

The ADAMTS family are related to microfibril biology in genetic and structural functions[80]. ADAMTS-10 mutations cause autosomal recessive Weill-Marchesani syndrome (WMS)[81], a disease that can also be caused by mutant fibrillin-1[82]. ADAMTSL-5 interacts with fibrillin-1, facilitating co-localization with microfibrils[83]. In addition, ADMATSL-4 and ADMATSL-6 promote fibrillin-1 assembly in the ECM[84,85]. MAGP-1 binds to the N-terminal rewgion of fibrillin-1, inhibiting the interaction between fibrillin-1 monomers and interfering with the fibrilin-1 self-associated assembly. Additionally, MAGP-1 can interact with fibronectin[86] and elastin and potentially be related to elastin deposition on microfibrils[87,88] It has been suggested that MAGP-2 has covalent, periodic interaction with microfibrils in certain tissues[89-91]. Moreover, MAGP-1 is able to bind to TGFβ and BMP-7 [92]and activate TGFβ signaling in culture[93]. However, observations of the molecular and phenotypic characteristics of Magp1-/- mice do not support a role for Magp1 in microfibril or elastic fiber assembly[92].

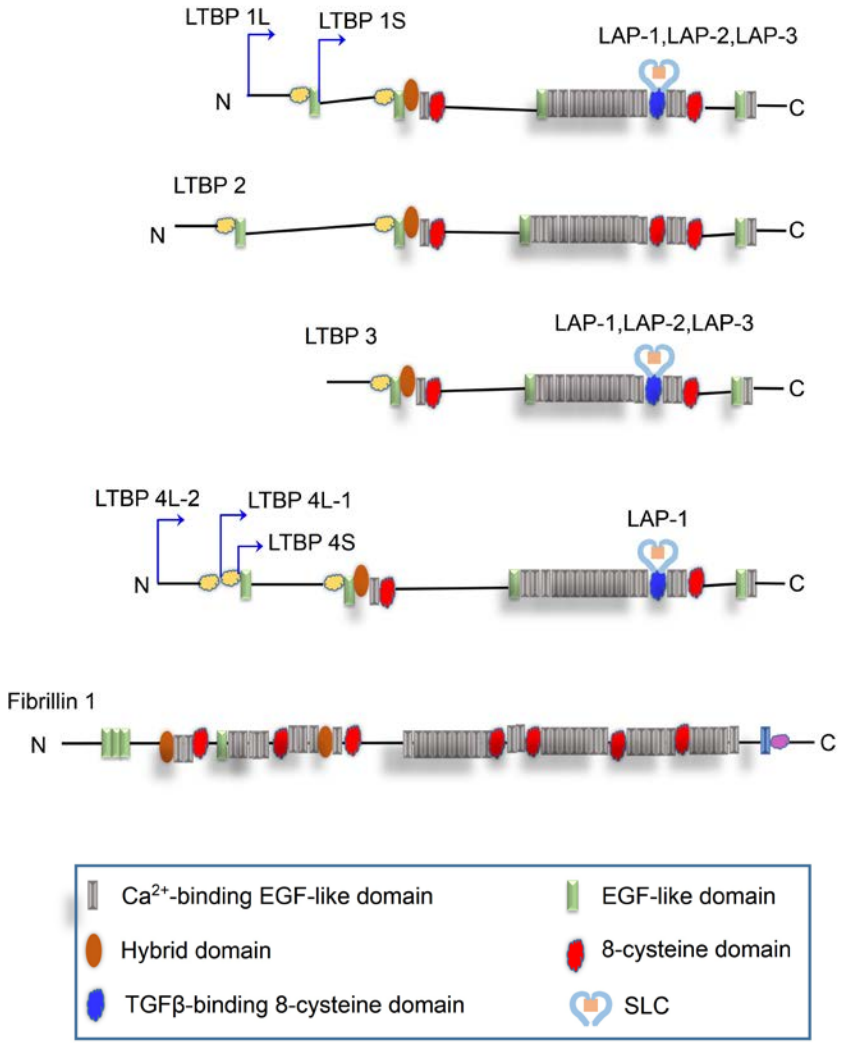


Figure 2.3 The structures of LTBP/Fibrillin-1 family members

LAP: latency-associated protein ; SLC: small latent complex; LLC: large latent complex.

2.4.5. Elastic fiber formation

Coacervation, the self-aggregation of tropoelastin monomers is thought to be the first primary stage of elastic fiber assembly[94]. The soluble (~15nm) monomers self-aggregate to form progressively larger spherical globules[95,96], steps which may be assisted by glycosaminoglycans, such as HS, on the cell surface[97]. The tropoelastin coacervates attach to integrins and glycosaminoglycans[60] until deposition onto microfibrils[95,98]. Microfibrils act as a scaffold that leads properly incorporation in the extracellular matrix and elastic fibers formation[95,99,100]. Once deposited onto the microfibril platform, the globules are stabilized by forming lysine-derived interlinks catalyzed by interaction with the lysyl oxidase enzymes (LOX) or LOX-like protein 1 (LOXL1). The localization of lysyl oxidases to microfibrils is facilitated by fibulin-4 or fibulin-5. The so-called “macroassembly” form the insoluble elastin core[94,101,102] (Figure 2.4).

Fibulin-3, -4 , and -5, are significant regulators of elastic fiber assembly[103,104]. Fibulin-4 can bind to fibrillin-1 in vitro[105] and other evidence shows colocolization between fibulin-4 and microfibrils[106]. Fibulin-4 depletion in mice abolishes elastogenesis but not microfibril formation[106], which supports a crucial role of fibulin-4 in elastic fiber assembly, in part by recruiting LOX to elastic fibers[107]. Moreover, fibulin-5 can recruit LOXL1[98,108]. Both LOX and LOXL1 oxidatively deaminate specific tropoelastin lysine residues to allysines[109]. The intra and intermolecular cross-links allow elastic fibers to distribute into three-dimensional networks. The cross-linked fibers are protease-resistant[110] and stable under mechanical stretching[111].

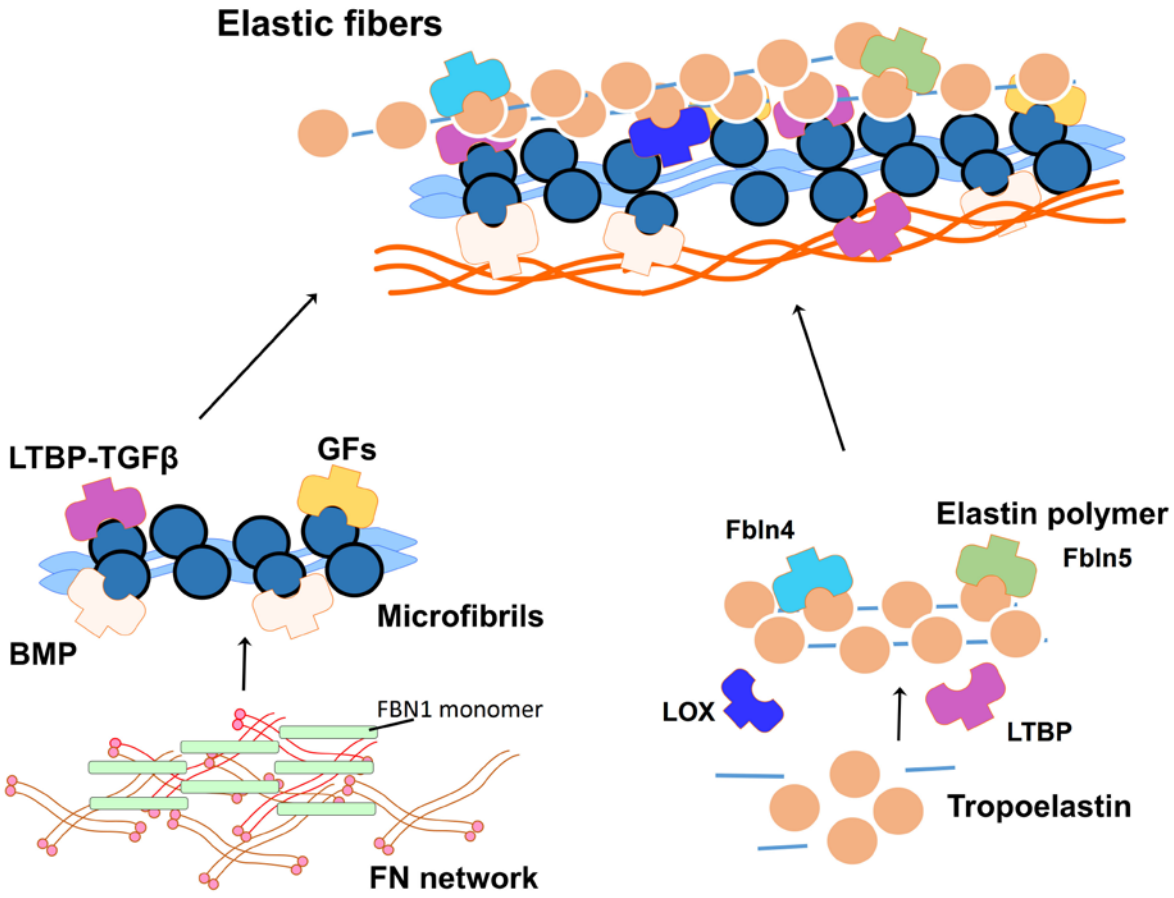


Figure 2.4 A working model of microfibrils and elastic fiber assembly.

FN: fibronectin; FBN1: fibrillin-1; BMP: bone morphogenetic protein ;GF: growth factor; Fbln4: fubulin-4; Fbln5: fubulin-5; LOX: lysyl oxidase

2.5. THE LTBP FAMILY AND TGFβ SIGNALING

The transforming growth factor β (TGFβ) pathway is essential for controlling cellular growth, differentiation and proliferation[112]. TGFβ is a ubiquitously expressed cytokine playing an important role in the synthesis of extracellular matrix (ECM) molecules, contributing to fibrotic disorders and regulating the immune system[113]. TGFβ has various and profound effects on multiple developmental phases as well as retaining biological functions required in an adult life. Latent transforming growth factor beta binding proteins (LTBPs) represent a group of matrix proteins that direct important and complicated functions in the formation of the ECM. LTBPs are recognized to regulate and facilitate TGFβ singling through several mechanisms.

2.5.1. Latent transforming growth factor beta binding proteins (LTBPs)

The LTBPs are secreted glycoproteins and homologous to fibrillins with several conserved domains. Calcium-binding epidermal growth factor-like domains with 6-cysteines, and TB domains containing 8-cysteines are primary repeats structurally among LTBP proteins[114]. LTBP1, LTBP3 and LTBP4 bind latent TGFβ, whereas LTBP2 does not[115,116] (Table 2.2). Triggering with different promoters, alternative splicing various, and proteolytic processing generate multiple LTBP isoforms from each gene. Differential promoter and exon use contribute to long and short form of LTBP1 and LTBP4. Compared to other LTBPs, LTBP4 displays a weaker binding capacity and only binds TGFβ1 LAP[116,117], which indicating that LTBP4 might

have distinct regulation from other LTBPs. The major structural domains of LTBPs are shown in Figure 2.3 .

Table 2.2 Interactions between LTBPs and TGFβ-LAP isoforms

	TGFβ1-LAP	TGFβ2-LAP	TGFβ3-LAP
LTBP1	+	+	+
LTBP2	−	−	−
LTBP3	+	+	+
LTBP4	+	−	−
Fibrillin1	−	−	−

LAP: latency-associated protein

In the absence of LTBPs, misfolded SLCs secreted abnormally slow because the reactive cysteine (Cys33) within LAP forms inappropriate disulphide bonds with free cysteines in other proteins[40]. LTBPs act as chaperones for SLC and modulate extracellular TGFβ levels[118]. Secreted LLCs, composed of SLCs and LTBPs, incorporate into the ECM with interacting via LTBPs and multiple extracellular molecules, primarily fibronectin and fibrillins. Latent TGFβ is sequestered within the extracellular space until required and it is released from its pro-peptide in the route of latent TGFβ activation[22]. Several mechanisms of TGFβ activation have been demonstrated, including cleavage of LTBPs and LAP by proteases, physical force applied by integrins, and liberation of active TGFβ from latent complexes by thrombospondin-1, F-spondin, neuropilin-1,

reactive oxygen species (ROS) and alternative pH in microenvironment. Integration of LTBPs into the ECM is a critical step to regulate the reservoir and activation of latent TGFβ. Moreover, LTBPs interact with many ECM proteins, including fibronectin and fibrillins, essential for proper microfibrillar localization and function[119]. N-terminal domains of LTBPs bind heparan sulfate proteoglycans and fibronectin to facilitate incorporation into the ECM[79]. The TB domains (8-Cys) binding sites near the C-terminus of LTBP-1 and LTBP-4 assist in ECM binding[118,119].

Both knockout mouse models and inherited human disorders caused by mutations in LTBP genes have been useful to uncover the functions of LTBPs *in vivo*. The mouse and human phenotypes caused by LTBP gene mutations is summarized in Table 2.3. Several of these phenotypes have been associated with dysregulation of TGFβ signaling except in *Ltbp2* knockout mice[120]. However, in TGFβ1 null mice[121-123], premature death, gastrointestinal and colorectal abnormalities, multi-organ inflammation have been reported. In TGFβ2 null mice, cardiovascular, pulmonary and spinal cord defects have been found[124], and cleft plate was noted in TGFβ3 null mice[125]. The observation that each LTBP null phenotype displays only a subset of abnormal phenotypes found in TGFβ (TGFβ1, TGFβ2 or, TGFβ3) null mice supports the notion that LTBPs are responsible for tissue-specific localization and activation of TGFβ. In addition, as some phenotypes observed upon the loss of individual LTBPs are not observed in TGFβ (TGFβ1, TGFβ2 or, TGFβ3) knockout mice, provides evidence for some LTBPs having additional, TGFβ-independent functions

The complex scaffold of the ECM is assembled with collagens, fibronectin, elastin and other many other associated molecules and proteoglycans. Elastic fibers are primarily composed of elastin in approximate 90% and fibrillin-rich microfibrils contribute the rest of parts, providing tissues, such as blood vessels, with elasticity. In addition to influencing the mechanical properties of tissues, many elastic fiber proteins serve as ligands for specific cell membrane receptors, mainly integrins. LTBPs are crucial to maintain the ECM structures in proper functions. The levels of latent complexes of TGFβ1 increase in parallel with the enhanced production of soluble LTBPs integrated into the ECM. In addition, cultured human lung fibroblasts deposit LTBP4 starting at day 7 of culture, accompanied by initial co-localization with fibronectin[118]. After a prolonged period of culture, the localization of LTBP4 initiates to become distinct from that of fibronectin and sequentially, LTBP4 is incorporated into microfibrillar networks on top of fibronectin templates. In *Ltbp4S*$^{-/-}$ mice, defective elastogenesis likely exists because elastin cannot interact properly with microfibril bundles to form elastic fibers[2]. LTBP4 has been suggested to have a dual role in lung development by regulating TGFβ activity and elastic fiber formation[2]. However, the activities of LTBP4 are as poorly understood in other tissues as the relative contribution of these functions to the manifestations of disease in patients with *LTBP4* mutations.

Table 2.3 Human and mouse phenotypes related to LTBP genes mutations

Mutant Gene Mouse/Human	Knockout mouse phenotype	Human phenotype
Ltbp1L*/*LTBP1	Lethal at birth, defective cardiovascular developments, malformation of valves[48]	No related phenotype[126]
Ltbp2*/*LTBP2	Embryonic lethal[120]	Congenital glaucoma, impaired lens development[127]
Ltbp3*/*LTBP3	Skeletal abnormality, osteoarthrisis, decreased body size, pulmonary alveolar septal defects[128,129]	Oligodontia, short stature, scoliosis[130]
Ltbp4s*/*LTBP4	Rectal prolapse, pulmonary emphysema, impaired elastic fibers[1,2]	Pulmonary emphysema, gastrointestinal and urinary tract abnormalities, cutis laxa[3,126]

2.5.2. TGFβ signaling and the TGFβ receptor system

TGFβ family members bind type 1 and 2 cell-surface receptors (TGFBR1 and TGFBR2, respectively). TGFBR1 and TGFBR2 receptors carry intracellular serine-threonine kinase domains and form heteromeric complexes upon the binding of dimerized ligands to their extracellular domains. Soluble ligands first bind to the constitutively active TGFBR2, followed by the interaction and phosphorylation of the GS (glycine/serine)-rich domain within TGFBR1 to produce an activated ligand-receptor complex. The cell-surface TGFBR2 receptors are constitutively active dimers, capable of autophosphorylation[131].

TGFβ binding stabilizes and upholds the interaction of the TGFBR2/TGFBR1 receptor heterotetramer and TGFBR1 phosphorylates the downstream effector SMADs. Both TGFBR1 and TGFBR2 are phosphorylated at tyrosine and serine/threonine residues, enabling complex regulatory interactions with a variety of signal transduction pathways[132]. The molecules primarily involved in the canonical TGFβ pathway are listed in Table 2.4.

Table 2.4 The canonical TGFβ signal transduction pathway

Molecule category	Molecules
Ligands	TGFβ1, TGFβ2, TGFβ3
Type I receptors (TGFBR1)	TGFBR1 (ALK5), ALK1(ACVRL1 or SKR3)
Type II receptors (TGFBR2)	TGFBR2
Type III receptors (TGFBR3)	TGFBR3 (betaglycan), endoglin
R-SMADs	SMAD2, SMAD3
Co-SMAD	SMAD4
I-SMADs	SMAD7

Abbreviation: ALK: activin receptor-like kinase; R-SMAD: receptor-specific SMADs; Co-SMAD: common mediator SMAD; I-SMAD: inhibitory-SMAD

2.5.2.1. Canonical, SMAD-dependent, pathway

The SMAD family of proteins is essential for intracellular TGFβ signaling. SMADs are subdivided into three groups based on their function: R-SMADs (receptor-associated SMADs), Co-SMADs (co-operating SMADs) and I-SMADs (inhibitory SMADs)[133,134]. After phosphorylation of TGFBR1, the activated receptor complex transduces intracellular signaling by phosphorylating R-SMADs. In humans, five different R-SMADs have been reported and are substrates for activated TGFβ receptors. SMAD2 and SMAD3 are substrates for receptors activated by TGFβs, activins and nodal, whereas SMAD1, SMAD5 and SMAD8 mediate pathways activated by bone morphogenetic proteins (BMPs), growth differentiation factors

(GDFs) and Müllerian inhibitory factors (MIFs).[132] Upon ligand activation of the receptor complex, the phosphorylated R-SMADs and Co-SMAD (SMAD4) form a complex, which shuttles into nucleus, where it interacts with other transcription factors to activate or suppress target genes[35]. Activated genes include I-SMADs, SMAD6 and SMAD7 and the expression of these inhibitory SMADs provide a negative-feedback regulation of TGFβ signaling[135,7] (Figure 2.5).

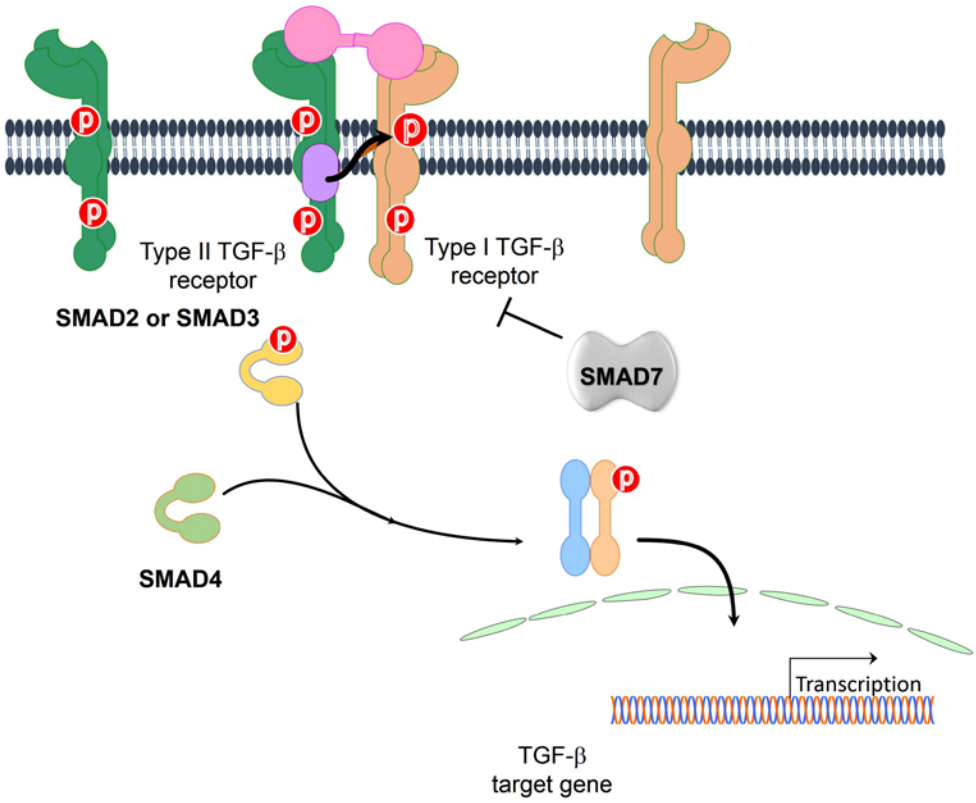


Figure 2.5 A schematic representation of the SMAD-dependent TGFβ signaling pathway.

2.5.2.2. Non-canonical, SMAD-independent, pathway

TGFβ ligands enable mitogen-activated protein (MAP) kinase activation, including extracellular signal-regulated kinase (ERK), p38 and JNK kinases[136,137]. Activation of the ERK kinase has been suggested to be important in facilitating SMADs signaling cascade and diverse TGFβ-mediated responses[131]. TGFβ triggers the MAP kinase activation, including phosphorylation of p38 MAP kinase and other kinases, such as MKK3, MKK6 and TAK1[138]. The activation of TAK1 upon TGFβ stimulation requires the interaction of ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) with the TGFβ receptor complex[139]. Activated TGFβ-activated kinase1 (TAK1) in turn leads to activation of p38 MAP kinase and JNK kinase, indicating that TRAF6 is a crucial factor in the TGFβ induced activation of the MAP kinase pathways. In addition, those non-canonical signals can crosstalk with the canonical, SMAD-dependent, signaling and mutually restrain each other. Certainly, both pathways can interact with or be affected by other pathways, including WNT, RAS, Notch and interferon pathways[140] (Figure 2.6).

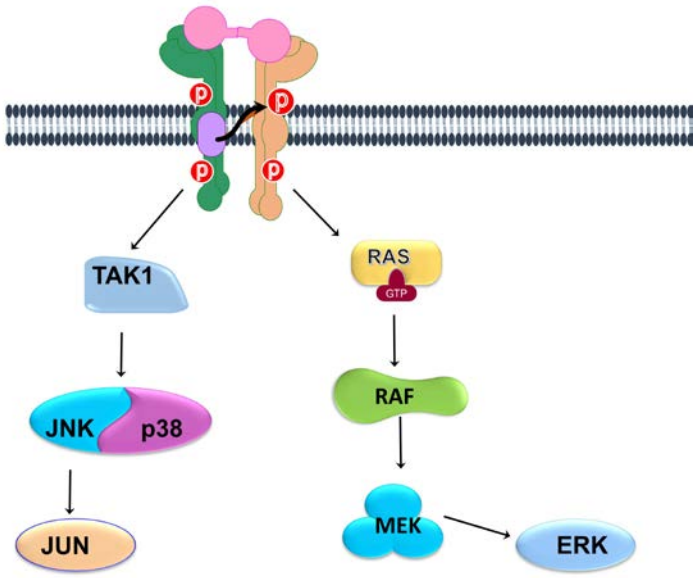


Figure 2.6 Schematic overview of non-canonical pathways and crosstalk with other pathways.

2.5.2.3. Endocytosis of the TGFβ receptors

The endocytosis of TGFβ receptors is dependent on receptor internalization and recycling routs. Clathrin-mediated endocytosis and caveolin-mediated endocytosis contribute to internalization of TGFβ receptor complexes[141,142]. Different endocytotic routes may affect the post-translational modification of the receptor complex and regulate downstream signaling.

Clathrin-mediated endocytosis of TGFβ receptors

In clathrin-mediated endocytic routs, TGFβ receptor complexes internalize through clathrin-coasted pits, subjected to TGFB2 cytoplasmic motifs[143]. Mitchel et al. reported, after internalization, Rab11-dependent recycling routes through which TGFβ receptor complexes enter early endosomes, with EEA-1 (+) and Rab5 (+), and then recycle back to the cell surface for sequential use[141,142]. This pathway is thought to facilitate TGFβ receptor signaling, specifically TGFβ-induced SMAD activation[143]. However, some results show that clathrin-mediated endocytosis may prevent TGFβ-induced SMAD activation[141,144]. The clathrin-associated AP2 adaptor complex at the plasma membrane can interact with TGFβ receptors and facilitate clathrin-mediated endocytosis[145]. In addition, SMAD anchor for receptor activation (SARA) is another adaptor protein and enable to enhance the stabilization between the interaction with TGFBR1 and SMAD2 as well as is distributed in EEA-1 (+) early endosomes[146].

Some studies demonstrated proteins-involved in the activation of SMADs colocalized with clarthrin-coated vesicles[141,144,146].

Caveolar endocytosis of TGFβ receptors

On the cell surface, TGFβ receptors can be found in lipid rafts, dynamic micro-domains of protein and lipids[108] and in non-lipid raft regions of the plasma membrane. Caveolae are enriched in lipid-raft domains on the plasma membrane and play an essential role in endocytosis[147]. Caveolin-1 and lipid-raft-medicated endocytosis leads TGFβ receptors to sequential degradation intracellularly[141]. Specifically, caveolin-1 can interact with TGFBR1, which leads the inhibition of TGFβ-induced-SMAD signaling[147,148]. Moreover, inhibition of caveolin-1 dependent endocytosis can increase the half-life of the TGFβ receptors[141]. Smad7 was found to be colocalized with caveolin-1 further contributing to the abrogation of TGFβ signaling[141]. Taken together, enhancement of caveolar endocytosis can facilitate the degradation of TGFβ receptors. In contrast, inhibition of this pathway can stabilize the receptors and allows for increased SMADs activation.

However, there are conflicting data showing both clathin-mediated and caveolin-mediated endocytosis can be related to TGFβ receptor degradation[142]. In addition, reduced internalization of TGFBR2 can be blocked by clathrin expression inhibition rather than caveolin-1-mediated inhibition[142]. The half-life of receptor was enhanced by the blocking of clathrin-dependent

internalization. Rajagopal *et al*. demonstrated SARA and SMAD7 in early endosomes with EEA-1 (+) and Rab5 (+), indicating that SMADs activation and TGFβ receptors inhibition can both be related to clathrin-mediated endocytosis[149]. The clathrin-mediated and caveolin-1-mediated endocytic pathways are summarized in Figure 2.7.

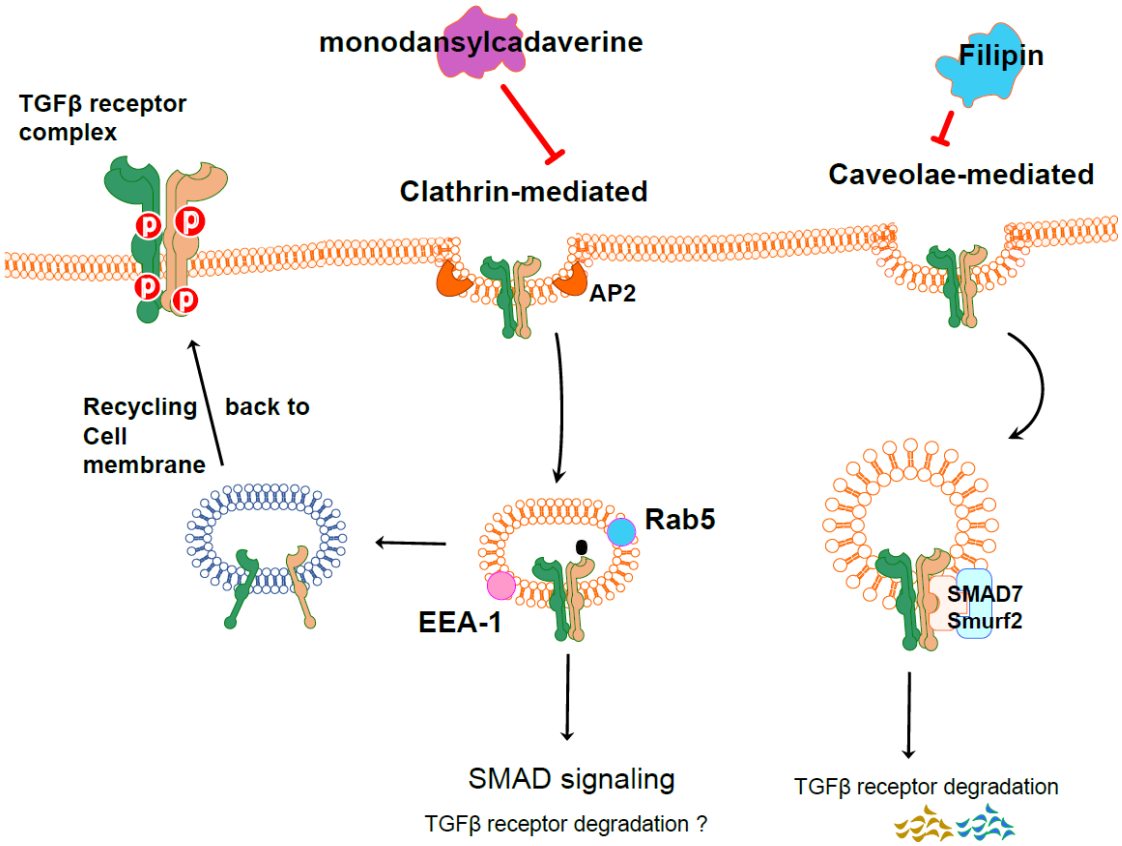


Figure 2.7 Schematic representation of the endocytic routing of TGFβ receptors

2.6. CUTIS LAXA

Cutis laxa (CL) is a rare disorder with inherited and acquired/late-onset forms. It is characterized by loose skin and may be associated with systemic involvement, including aortic aneurysms, pulmonary artery disease, and emphysema. Inherited forms include autosomal dominant, autosomal recessive and X-linked recessive inheritance patterns[150]. A shared pathological feature of cutis laxa syndromes is abnormal elastic fiber deposition in the affected tissues. Human genetic studies to date have identified 9 genes in various forms of cutis laxa, highlighting the diversity of molecular pathways required for elastic fiber biogenesis. These genes encode structural and accessory proteins of elastic fibers, including elastin (ELN) fibulin-4 (FBLN4/EFEMP2), fibulin-5 (FBLN5) and LTBP4, proteins of the secretory pathway involved in sorting of cargo molecules, and mitochondrial enzymes involved in proline metabolism. The summary of types of CL and clinical manifestation is in Table 2.5.

.

Table 2.5 Summary of types of cutis laxa and clinical manifestation

Subtype	Mutant genes	Clinical manifestation					
		Skin laxity	***Growth and developmental delay***	***Emphysema***	***CV***	***GI***	***Bone***
ADCL[151,152]	*ELN*	+	-	+	+	-	-
	FBLN5	+	+	+	+	-	-
ARCL1A[153]							
	FBLN4	+	+	+	+	-	-
ARCL1B[154-156]	*EFEMP2*						
ARCL1C/ URDS[3]	*LTBP4*	+	+	+	+	+	-
ARCL2A[157]	*ATP6V0A2*	+	+	-	+	-	-
ARCL2B[158,159]	*PYCR1*	+	+	-	+	-	+
XLCL[160-162]	*ATP7A*	+	-	-	-	-	+
DBS/ ARCL3[163,164]	*ALDH18A1*	+	+	-	+	-	-
GO[165]	*GORAB*	+	-	-	-	-	+
MACS[166]	*RIN2*	+	-	-	-	-	+
ATS[167]	*SLC2A10*	+	-	-	+	-	-

CV: cardiovascular, GI: gastrointestinal; ADCL, autosomal dominant cutis laxa; ARCL, autosomal recessive cutis laxa; URDS, Urban–Rifkin–Davis syndrome; XLCL, X-linked cutis laxa; DBS, De Barsy syndrome; GO, geroderma osteodysplasticum; MACS, macrocephaly alopecia cutis laxa scoliosis syndrome; ATS, arterial tortuosity syndrome.

Similar to CL, cardiovascular abnormalities are major manifestations in several inherited autosomal dominant connective tissue disorders, such as Marfan syndrome, Loeys-Dietz syndrome, arterial tortuosity syndrome and aneurysms-osteoarthritis syndrome. A shared clinical abnormality in these disorders is aortic root dilatation, a loss of elastic fibers in

abnormal smooth muscle cells in tunica media and anomalous structures and functions of the ECM. Several of these disorders are caused by heterozygous loss-of-function mutations in regulators and molecular elements related to TGFβ signaling. Intriguingly, however, there is evidence of elevated TGFβ signaling in tissues and cells from these patients [168,169] The precise mechanisms of this paradoxical activation of TGFβ signaling pathway are largely unknown.[170]

2.7. TGFβ SIGNALING AND LTBP4 MUTATIONS IN CUTIS LAXA

2.7.1. TGFβ and cutis laxa

TGFβ up-regulates many genes for components of the elastic fibers, including fibronectin[171], LTBPs[172,173], ELN[174,175], LOXs[176,177], and FBLN5[178] by transcriptional or porstranscriptional mechanisms. In cardiac fibrosis, TGFβ up-regulates ECM genes by reducing the expression of the miR-29 family of micro-RNAs, which otherwise inhibit ECM genes[179].

Loss of function mutations in genes related to TGFβ sequestration, including *FBN1* and *LTBP4*, contributed to abnormally elevated TGFβ signaling[2,3,180]. However, elevated TGFβ activity was also noted in several types of CL caused by genes, necessary for elastogensis but not directly involved in TGFβ sequestration, including ELN[151,152], fibulin-4[156,181] and ATP6V0A2[182]. Therefore, impaired elastic fiber function may lead to up-regulation of TGFβ activity indirectly, possibly through other molecules associated with ECM, such as integrins[183]. These findings also highlight the complex and reciprocal connections between the ECM and TGFβ signaling.

2.7.2. LTBP4-related cutis laxa

Autosomal recessive CL type 1 C (ARCL1C) / Urban-Rifkin-Davis syndrome (URDS; MIIM613177) is a disease with severe gastrointestinal, pulmonary and urinary involvements, resulting from mutation in LTBP4 gene[3]. Respiratory complications, including emphysema, atelectasis and tracheomalacia are usually lethal during infancy. Gastrointestinal distension, stenosis and tortuosity as well as hydronephrosis and bladder diverticulosis are found in patients[3]. *LTBP4* deficiency leads to altered production of active TGFβ and an abnormal formation of elastic fibers in the ECM. However, the mechanisms and downstream consequences of TGFβ dysregualtion in this disease remain incompletely understood.

2.8. SUMMARY

ECM and associated proteins form a complex network involving numerous macromolecules, which performs abundant mechanical, chemical and biological functions. It has been suggested that collagenous and elastic fibers provide tensile strength and elasticity in tissues. On the other hand, in extracellular space, structural glycoproteins maintain cellular cohesiveness[119]. ECM molecules can interact with cells and with each other to regulate of several processes, including TGFβ signaling. Members of the TGFβ family are multifunctional and pleiotropic growth factors, associated with cell proliferation, migration, and differentiation as well as extracellular matrix formation. Secreted latent TGFβ complexes are consistent of mature dimeric growth

factor, dimeric LAP and a LTBP molecule. The latent complex subsequently is required to be activated and this complex is secreted in a specific and targeted manner. Liberation from the latent complex enables the mature TGFβ to trigger the downstream signaling by interacting with its signaling receptors. Thus, ECM networks serve as major reservoirs for the storage of LTBPs and latent TGFβ. The complex regulation of TGFβ signaling by ECM molecules, specifically by LTBP4, is poorly understood.

For proper TGFβ function, intergradation TGFβ complexes into the extracellular matrix is obligatory. The ECM contains several molecules, including LTBPs, which interact with latent TGFβ complexes and have potentially modulated TGFβ activation and signaling. Since TGFβ regulates the expression of multiple ECM components, TGFβ directs the generation of associated molecules that adjust its accessibility and signaling. Thus, the ECM does not act as a reservoir for TGFβ-mediated elements but a platform at which the availability of cytokines or associated is modulated to ensure proper interaction between TGFβ -responsive cells and the generation of TGFβ-responsive genes. However, there is a “TGFβ paradox” that loss-of-function mutants in genes, encoded certain TGFβ-mediated matrix proteins that lead to increased, rather than declined, TGFβ levels. These observations highlight the crucial and complicated roles of TGFβ. Further investigation of the interaction between matrix and cells in LTBPs mutants may shed light on the intriguing connections between the ECM and growth factor signaling.

3. MATERIALS AND METHODS

3.1. BIOMECHANICAL PROPERTIES OF THE SKIN IN CUTIS LAXA

3.1.1. Enrolment and disease diagnosis

This study was conducted in accordance with the IRB protocols at Washington University School of Medicine and the University of Pittsburgh. One hundred and thirty six control individuals were enrolled from Washington University clinical and research populations. Cutis laxa patients (n=22) were enrolled during Cutis Laxa Research Clinics at the University of Pittsburgh. At the time of evaluation, participants, or their parents if the enrolled individual was a minor, completed a questionnaire providing demographic information and skin elasticity was measured using the DermaLab suction cup device (Cortex Technology, Denmark). The diagnosis of cutis laxa was based on physical examination, medical history, and whenever possible, based on positive mutational testing results.

3.1.2. Skin Elasticity Measurements

The suction cup was placed on the volar surface of each forearm, midway between the wrist and the elbow using a 2-sided adhesive sticker. Measurements were taken on both arms and the mean of the two measurements were used for analysis. The instrument applies vacuum in increasing increments to a small patch of skin (10 mm in diameter) under the suction cup, causing the skin

to be lifted up into the suction cup (Figure 3.1). There are two light beams in the suction cup and there is 1.5 mm between them. While the skin was lifted up at the level of the first beam and second beam by vacuum, the level of pressures are recorded as P_1 and P_2 respectively. After that, the skin is released. The device measures the amount of time required for the skin to cross the distance between two beams.

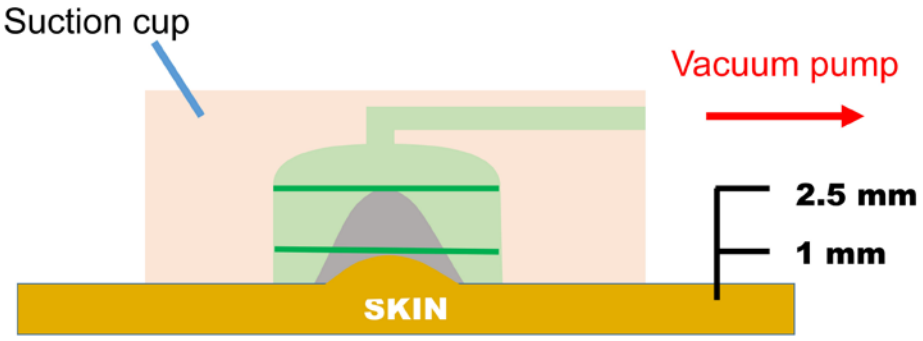


Figure 3.1 A schematic demonstration of the DermaLab suction cup device

Two light beams are in the suction cup and there is 1.5 mm between them. While the skin was lifted up at the level of the first beam and second beam by vacuum, the information including pressures and retraction times are recorded.

The output from the device is ΔP (P_2-P_1, measured in mBar) and RT (retraction time, measured in ms). This cycle is repeated two subsequent times. Data from cycles 2 and 3 showed similar characteristics to cycle 1, but lower effect sizes with respect to cutis laxa. There was a slight group and age effect for differences in RT by cycle, but this did not reach statistical significance (data not shown). Therefore, report cycle 1 data (dP_1 and RT_1) was used in analysis.

The elastic modulus (E) is calculated by the DermaLab software in MPa units by solving the following equation:

$$\Delta x = \psi \times \Delta P_1 \times r^4 / (E \times s^3)$$

Δx: skin displacement (0.0015 m for this probe)

Ψ: an instrument constant

ΔP_1: pressure difference as described above, converted to MPa units

r: the radius of the skin patch displaced (0.005 m)

s: the thickness of the skin, estimated to be 0.001 m.

Thus, E, as measured by the DermaLab instrument, is an approximate value assuming uniform skin thickness across all participants. Using 1 mm skin thickness and other probe constants, the formula used by the instrument is as follows:

$$E = 0.3125 * \Delta P_1/1.5$$

An approximate value for skin viscoelasticity (VE) is calculated by the instrument using the following formula:

$$VE = E / RTn$$

RTn is a normalized recoil time obtained by dividing RT_1 with 260 ms, the average control underarm recoil time.

3.1.3. Quality control and statistical analysis of Dermalab data

To ensure the reliability of the data, the following quality control measures were taken. If the measurements were only available on one arm or the difference of measurements over two arms were over two standard deviations of the age adjusted population mean, the participants were excluded. If the measurements of RT exceeded 10,000ms, the data were excluded from the study.

Chi-square tests were used to evaluate categorical factors and independent t-tests for continuous data for initial analysis. Pearson's correlations were calculated among age, E, VE and RT. Body mass index did not correlate significantly with any of the skin data and therefore was not included in any regression models. Logistic regression was used to determine the strength of each individual biomechanical variable in predicting affected status. Step-wise logistic regression was used to obtain a multivariate model for affected status, with age, sex, E, VE and RT in the initial model. Receiver operating characteristic (ROC) curve analysis was used to evaluate the power of the regression models to determine affected status, as quantified by the area under the ROC curve (AUC). Differences between logistic regression models were tested using ANOVA. For the potential overfitting in models, 4/5 samples, the training sub-dataset, was used to generate models while 1/5 samples, the validation or testing sub-dataset, was used to test those

models. This approach was repeated 20 times and the average AUC of ROC analysis in model 1 and model 2 were calculated. Descriptive and multivariate statistics were carried out with SPSS software (IBM, version 21.0) and R software (version 2.14). P values < 0.05 were considered statistically significant.

3.2. LTBP4 REGULATES EXTRACELLULAR MATRIX ASSEMBLY, TGFβ SIGNALING AND ITS IMPACT TO TGFβ RECEPTOR COMPLEX

3.2.1. Cell culture conditions

Human fibroblasts were harvested from patient skin tissues by biopsy. HEK 293 cells were purchased from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin, 10% fetal bovine serum (FBS), 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 45 mM sodium bicarbonate **(**$NaHCO_3$). All cells were maintained at 37°C in a humidified environment containing 5% CO_2. The media was exchanged at least every 48 hours. To subculture cells, 0.05% ~ 0.25% trypsin /ethylenediaminetetraacetic acid (EDTA) solution was used. Skin biopsies were collected via standardized protocols approved by the University of Pittsburgh Institutional Review board. Informed consent was obtained from all participants.

3.2.2. TGFβ activity assay

Cultured reporter mink lung epithelial cells (MLECs)[184] were used to detect the activity of TGFβ in media or supernatant of cultured human fibroblasts. This method we used has been published in our paper, Human Mutation 2013 Jan; 34(1): 111-21[185].MLECs were stably transfected with a plasmid construct with a TGFβ-responsive plasminogen activator inhibitor-1 promoter connected to a luciferase cDNA. Human dermal fibroblasts cells were co-cultured with the same number of MLECs (2 x 10^5/ml) in Dulbecco's modified Eagle's medium (DMEM) containing 2.0% fetal bovine serum (FBS) and were plated at 100μl per well in 96-well plates for overnight. The media samples was collected for measurement. Additionally, to check TGFβ activity in the supernatant of fibroblasts, cultured MLECs were suspended at 2 x 10^5 per ml in DMEM containing 2.0% FBS and seeded 100μl per well in 96-well plates. Six hours later, the cells were treated with conditioned media from human dermal fibroblast cultures incubated with the media overnight.

A standard curve was constructed with a decreasing TGFβ1 (PeproTech; Recombinant Human TGFβ1) concentration series from 1000 pg/ml in DMEM with 2.0% FBS and performed 2:1 serial dilution in DMEM containing 2.0% FBS. To activate latent TGFβ, samples were heated at 100°C for 10 minutes. The TGFβ1 concentration series, serum or plasma samples and heat-activated samples of conditioned media were added to triplicate wells in triplicates. Cells were incubated for 1 hour. To control for viable reporter cell numbers, In Vitro Toxicology Assay

Kit (Sigma) was used. The XTT reagent, a (2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt)-based solution, was prepared according to the instructions and added to the culture medium. Measurement of the absorbance at 450nm was performed after 2 hours-XTT incubation. The supernatant, culture medium with XTT solution, was discarded and cells were washed with PBS (phosphate-buffered saline). Cells were lysed in Reporter Lysis Buffer (Promega) and assayed for luciferase activity by Luciferase Assay System (Promega). Luminescence units, normalized to viable cell numbers as measured by the XTT assay were converted to TGFβ concentration with the use of a standard curve.

3.2.3. RNA isolation

For large-scale isolation, total RNA was extracted using TRIzol reagent (Invitrogen) from dermal fibroblasts. TRIzol separates cell lysates, after adding in bromochloroporpane (BCP) and centrifugation, into three compartments: a lower red organic phase, an interphase containing DNA, and a colorless, upper aqueous phase with RNA. The procedures were conducted under the chemical hood and all surfaces, pipettes and gloves were sprayed with RNase Zap, a spray of RNase-inactivating solution, in order to prevent RNA degradation.

Ten mL TRIzol reagent was add onto the surface of cells in a 15 cm culture dish after cells were washed by sterile PBS. Thorough washing of the cell layer and mixing with repeated pipetting was required to homogenize the lysate. BCP (2mL) was added, and the cell lysate was shaken vigorously. After 10 min storage at room temperature, the suspension was centrifuged at

10,000g for 15 min at 4 °C. The upper phase was transferred in to a new tube and mixed with 5mL RNase-free 100% isopropanol and incubated for 30 min. An RNA pellet was generated after centrifugation at 10,000g for 10 min at 4 °C. The pellet was washed with 10mL 75% ethanol and 25 % diethyl-pyrocarbonate (DEPC)-treated water. The ethanol was removed by centrifuging the samples at 7500g for 5 min and air-drying the pellet. The RNA pellet was dissolved in 100µl DEPC-treated water and the concentration of the RNA was measured by UV spectrophotometry using a Nanodrop instrument.

RNeasy Mini kit (Qiagen) was used to isolate RNA from a small amount of cells. Buffer RLT (350 µL) was added to cells growing in each well of 6-well plates. Centrifugation and buffer washes were performed according to the manufacturer's instructions. The RNA was eluted in 50µL and the concentration of RNA was measured by UV spectrophotometry using a Nanodrop instrument.

3.2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Complementary DNA (cDNA) was prepared using a SuperScript III Reverse Transcriptase kit (Invitrogen), following the manufacturer's instructions. To each RNA sample (1µL), random hexamers (1µL, 50ng/µL), dNTPs (1µL, 10 mM), and DEPC-treated water (up to 10µL) were added before a 5 min incubation at 65 °C. Before PCR, 10X RT Buffer (2µL), $MgCl_2$ (4µL, 25mM), dithiothreitol (DTT, 2µL, 0.1M), RNase OUT (1µL, 40U/µl), SuperScript III Reverse

transcriptase (200U/µl; 1µl) were added to the mix. After incubations for 10 min at 25°C, 50 min at 50°C and 5 min 85°C, the reactions were chilled on the ice, briefly centrifuged, 1µl RNase H was added to each sample and the reactions were incubated at 37°C for 20 min. The cDNA reactions were stored in -20°C and 0.5 µl of this reaction was used in quantitative polymerase chain reaction (Q-PCR).

3.2.5. Quantitative PCR

To determine the expression of *LTBP4*, *TGBR1*, *TGFBR2* and *TGFB1*, Q-PCR was used in control human dermal fibroblasts and *LTBP4*-mutant human dermal fibroblasts. I performed qRT-PCR using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). A customized quantitative real-time-PCR assay (TaqMan® Gene Expression Assay) was used to assess gene expression. The reaction dilution was prepared accorded to manufacturer's instructions, including 0.5µl cDNA, 10µl TaqMan Universal Master Mix II 2X, RNase-free water (up to 20ul). The reaction in 20µl, cDNA and master mix solution, was transferred to appropriate wells of 96-well TaqMan® Array plate. The plate was briefly centrifuged to bring the solution to the bottom of the wells (1000rmp for 1min) before Q-PCR reactions (Table 3.1).

Table 3.1 Q-PCR conditions

Cycles		Temperature (°C)	Time
1		50	2 minutes
1		95	10 minutes
40	Melt	95	15 seconds
	Anneal/Extend	60	1 minutes

Sequence Detection System (SDS) Version 2.4 included with the ABI Prism 7900HT SDS and Microsoft Excel were used to perform the analysis of Q-PCR data. Experimental samples were run in triplicates where Ct measurements per samples were normalized by an endogenous housekeeping control gene, GAPDH. The relative expression of control and patient fibroblasts was determined using the comparative Ct method ($2^{-\Delta\Delta Ct}$) [186]. *LTBP4*, *TGFBR1*, *TGFBR2*, *TGFB1*, and *FBN1* expression was compared between *LTBP4*-mutant and control human dermal fibroblasts.

3.2.6. Protein extraction

Conditioned media samples were prepared by incubating cultured dermal fibroblasts overnight with serum-free media. Protease inhibitor cocktail (1:200; Sigma) was added. Amico Ultra-15 centrifugal filter units (Millipore) were used to concentrate media samples by centrifugation for 40 min, at 6000g, 4°C. To study intracellular pathways, fibroblasts were lysed in CelLytic™M

(Sigma) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Glass homogenizers were used facilitate complete cell lysis, and the lysates were incubated on ice for 30 min before centrifugation for 10 min, at 16000g, 4°C.

To study membrane proteins, the samples were prepared by using the Mem-Per Plus Membrane Protein Extraction Kit (Thermo Scientific) with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Cultured, adherent cells were washed using the Cell Wash Solution twice in the plates and detached using cold scrapers in 3mL Cell Wash Solution. Cell pellets were collected by centrifugation (300g, 5 min at room temperature) and resuspended in 500 µL Permeabilization Buffer by vigorous vortexing. Incubation followed at 4°C for 10 min with intermittent mixing. After incubation, the suspension was centrifuged at 16000g, 4°C for 15 min. This step left cytosolic proteins in the supernatant. The pellets, containing membrane proteins were resuspended in Solubilization Buffer (150µL-250µL) and then incubated for 30 min at 4°C with intermittent mixing. The mixture was centrifuged at 16000g and 4°C for 15 min. Supernatant contained the membrane proteins. The protein concentration of the extract was quantified using the Bradford assay[187]. 10µg of cytosolic protein and 8µg of membrane protein per sample were used for immunoblotting.

3.2.7. Immunoblotting

Protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Millipore). Equal

amounts of cell lysate or media samples were diluted with LDS Sample Loading Buffer (4X) (a nonreducing lithium dodecyl sulfate sample loading buffer, Thermo Scientific Pierce) or Laemmli Sample Buffer (2x, Bio-Rad) containing 1% β-mercaptoethanol was used. To prepare membrane proteins, samples were mixed with proper volume of loading buffer with β-mercaptoethanol and incubated at room temperature for 30 min. For cytosolic proteins (cell lysates) or secreted proteins (conditioned media), the mixture was heated at 98°C for 5 min or 70° C for 15 min. The protein samples were chilled before loading onto 6% - 10% SDS-PAGE gels. The gel was subjected to electrophoresis at 80V for 90 min to 120 min. The gel was positioned between packing pads, filter papers and polyvinylidene difluoride (PVDF) membrane and then placed into a Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell for 90 min at 80V or 16 hours at 30V, 4°C. The PVDF membranes were removed and washed in PBST (1x PBS, 0.1% Tween-20). Nonspecific binding sites were blocked by incubation in PBS-0.2% Tween 20 containing 8% dried skim milk at 4°C overnight or 2 hours at room temperature. Following blocking, the membranes were incubated in PBS-0.1% Tween 20 containing the appropriate primary antibody (Table 3.2) overnight at 4°C or 1.5 hours at room temperature. After incubation, membranes were washed by PBS-0.2% Tween 20. The incubation with the secondary antibodies (Table 3.2) was for 1 hour at room temperature. Immunoreactive signals were detected by using SuperSignalWest Pico Chemiluminescent Substrate (Thermo Scientific) or Luminata Forte Western HRP substrate (Millipore).

Table 3.2 Antibodies for Western blots

Antibody		Species	Dilution	Manufacturer/Cat. No.
Primary	LTBP4	Rabbit polyclonal	1:500	Thermo;PA5-21378
	Fibrillin-1	Rabbit polyclonal	1:500	Dr. Robert Mecham
	Fibronectin	Mouse monoclonal	1:1000	Takara; M002
	pSMAD2	Rabbit polyclonal	1:1000	CellSignaling; 4695
	pERK	Rabbit polyclonal	1:1000	CellSignaling; 4377
	SMAD6	Mouse monoclonal	1:500	Sigma; SAB1406098
	SMAD7	Mouse monoclonal	1:500	Millipore; ST1625
	Tubulin	Mouse monoclonal	1:1000	Sigma; T6793
	TGFBR1	Rabbit polyclonal	1:1000	CellSignaling; 3712
	pTGFBR1	Rabbit polyclonal	1:500	Abcam; ab112095
	TGFBR2	Rabbit polyclonal	1:500	Sigma; SAB4502958 or Abcam; ab61213
	Transferrin receptor	Mouse monoclonal	1:1000	Introgen; 136890
Secondary	Anti-rabbit	Goat polyclonal	1:7500	Thermo; PAI-20391
	Anti-mouse	Goat polyclonal	1:10,000	Jackson & Immunology

3.2.8. Enzyme-linked immunosorbent assay (ELISA)

TGFBR1 and TGFBR2 levels in the cell lysates were measured using commercial ELISA kits (DY241 R&D Systems and SEA397 Hu Cloud-Clone Corp., respectively). The procedures were performed according to the manufacturer's instructions. All of the measurements were performed in triplicate for each sample. The optical density at 450 nm (OD450) was measured using an ELISA reader.

3.2.9. Immunofluorescent staining

The following quoted material has been published in our paper, Human Mutation 2013 Jan; 34(1): 111-21[185]. *"Cultured human skin fibroblasts were seeded onto 22 mm × 22 mm square glass coverslips in 6-well tissue culture dishes at a density of 3,000 cells/cm^2 and cultured in 2 mL DMEM with 10% FBS,"* or in 4-well chamber slides in 0.5mL growth media. Cells were stained 4 weeks after confluence for LTBP4, LTBP1, FBN1 and FN. *"Briefly, cells were rinsed with PBS, fixed with PBS containing 4% paraformaldehyde (ElectronMicroscopy Sciences, Hartfield, PA) at room temperature for 15 min and rinsed three times with PBS for 5 min. The cells were incubated overnight in 3% BSA with 20% donkey serum and 0.3 M glycine to prevent background and nonspecific staining. The coverslips were incubated with anti-LTBP4, anti-fibrillin-1, mouse anti-LTBP1, anti-fibulin-5, and anti-fibronectin primary antibodies at room*

temperature for 1 hr and were then washed three times with PBS at 5 min intervals. Incubation time for secondary antibodies was 1 hr at room temperature. After counterstaining with Hoechst 33258 (Sigma, St Louis, MO), slides were rinsed again three times with PBS and mounted with Cytoseal-60 mounting media (Thermo Scientific) after being completely dried. Specimens were examined and photographed using a fluorescence photomicroscope (Leica DM5000B, Leica Microsystems, Richmond, IL).″

Table 3.3 Antibodies used for Immunoflurescent staining

Antibody		Species	Dilution	Manufacturer/Cat. No.
Primary	LTBP4	Rabbit polyclonal	1:200	Thermo; PA5-21378
	LTBP4	Goat polyclonal	1:200	R&D; AD2885
	Fibrillin-1	Rabbit polyclonal	1:400	Dr. Robert Mecham
	Fibronectin	Mouse monoclonal	1:400	Takara; M002
	TGFBR1	Rabbit polyclonal	1:100	Sigma; SAB4502958
	TGFBR2	Rabbit polyclonal	1:50	Abcam; ab61213
Secondary	Mouse Alexa Fluor 594	donkey IgG	1:500	Life Technologies; A21203
	Rabbit Alexa Fluor 488	donkey IgG	1:500	Life Technologies; A21206
	Goat Alexa Fluor 594	donkey IgG	1:500	Life Technologies; A11058
	Goat Alexa Fluor 488	donkey IgG	1:500	Life Technologies; A11055

3.2.10. Treatments with drugs and chemicals

TGFβ1 (PeproTech, Rocky Hill, NJ) and TGFBR1 inhibitor (LY364947; Calbiochem) were prepared in tissue culture medium at concentrations of 5 ng/mL and 25 μM, respectively, and cells were treated for 90 minutes. Endocytosis inhibitor treatments lasted 30 minutes. Filipin (caveolin-mediated endocytosis inhibitor; Sigma) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 mg/mL and prepared fresh at a concentration of 1 μg/mL in media. Monodansylcadaverine (MDC; clathrin-mediated endocytosis inhibitor; Sigma) was dissolved in DMSO at a concentration of 50 mM as a stock solution to make a final concentration of 100 μM in media. To block protein synthesis, cycloheximide (CHX; Sigma) was added to the culture medium at a final concentration of 20 μg/mL for the indicated times. Proteasome inhibitor (lactacystin) and lysosome inhibitor (ammonium chloride) were purchased from Sigma and added to media at final concentrations of 10 μM and 10 mM, respectively, for 8 hours. Cells were treated with the various reagents for the indicated times and were processed for immunoblotting or ELISA. Cells were cultured in 10-cm culture dishes or in 6-well platea for two weeks after confluence.

3.2.11. *LTBP4* knockdown

To silence *LTBP4* expression in human dermal fibroblasts, an antisense mophorlino oligonucleotide (MO), was designed to target the donor splice site of exon 5 (based on transcript

NM003573), an exon present in all common isoforms of *LTBP4*. The sequence complementary to the intron and exon shown in normal type and italic, respectively: 5'-GGCCACCCTTTCCTCAC*CGTGCTCG*-3'. Treatment with this mophorlino was expected to result in the skipping of exon 6, leading the elimination of the first epidermal growth factor-like domain of LTBP4, thought to be essential for the correct folding of the protein. A control MO was designed by mutating key residues in the LTBP4 MO, as shown by lower case letters: 5'-GGCgAgCCTTTCgTCAg*CcTGCTCG*-3'. MOs were delivered into human dermal fibroblasts from a healthy individual using the Endo-Porter peptide delivery system (Gene Tools, Philomath, OR). For single delivery, cells were treated with 4 μM Endo-Porter and 4.5 μM MO for 2 days, given fresh media for 2 days prior to collecting the samples. For double delivery, cells were treated with the first dose for 2 days, given fresh media for 2 days, treated with a second dose for 2 days and given fresh media for 1 day prior to collecting samples (Figure 3.2). Media, membrane protein lysate and cytosol protein lysates were used for immunoblotting or ELISA.

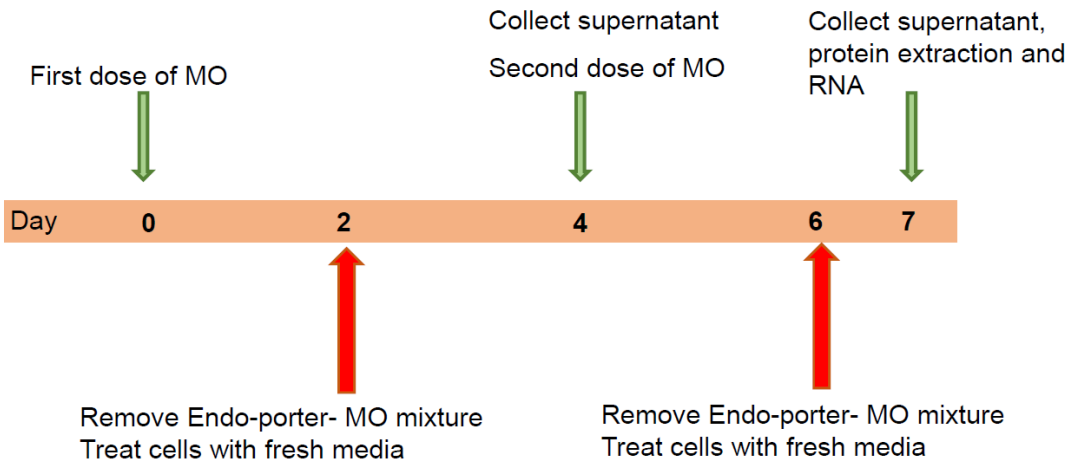


Figure 3.2 The timeline for knocking downed *LTBP4* in human dermal fibroblasts using antisense mophorlino oligos (MO)

Endo-porter-MO: the mixture of Endo-Porter peptide delivery system and MO

3.2.12. Rescue of *LTBP4*-mutant human dermal fibroblasts using recombinant LTBP4

Control lentiviral construct pLOC-TurboRFP and pLOC-LTBP4 containing the cDNA corresponding to reference sequence NM_003573 were purchased from Thermo Scientific (Pittsburgh, PA). A Myc-$(His)_6$ tag was engineered to the 3'-end of the pLOC-LTBP4 by PCR (Appendix, page 134). The resulting pLOC-LTBP4-MycHis construct was transfected in to HEK293 cells and subjected to selection with 8 μg/mL blasticidin for 3 weeks. Using limiting dilution, 32 clones were isolated from the stably transfected cells and conditioned media and cell lysates from the clones were tested by ELISA for the expression of LTBP4 and the Myc tag. Eight clones showing the highest expression by ELISA were further tested by immunoblotting for LTBP4 and the Myc tag, and clone C13 was selected for subsequent experiments. HEK293 cells stably transfected with pLOC-TurboRFP served as negative controls. The confirmation of the LTBP4 expression in clone C13 compared to HEK293 cells stably transfected with pLOC-TurboRFP was performed by immunoblotting. Conditioned media of clone C13 was used to treat LTBP4-mutant dermal fibroblasts to exam the further changes of protein expression of TGFβ receptor and TGFβ.

Conditioned media was collected from transfected HEK293 cells 5 days after confluence, and centrifuged to remove the cell debris. Before collecting the conditioned media, cells were washed with sterile PBS twice to remove blasticidin and treated with DMEM with 10% FBS overnight to obtain contidioned media, which was then mixed with fresh growth media 1:1

and used to feed *LTBP4*-mutant dermal fibroblasts for seven days (Figure 3.3). The mixture of media was changed every day. By 2, 4, and 7 days of treatment, membrane protein and cytosol protein extracts were collected as described above.

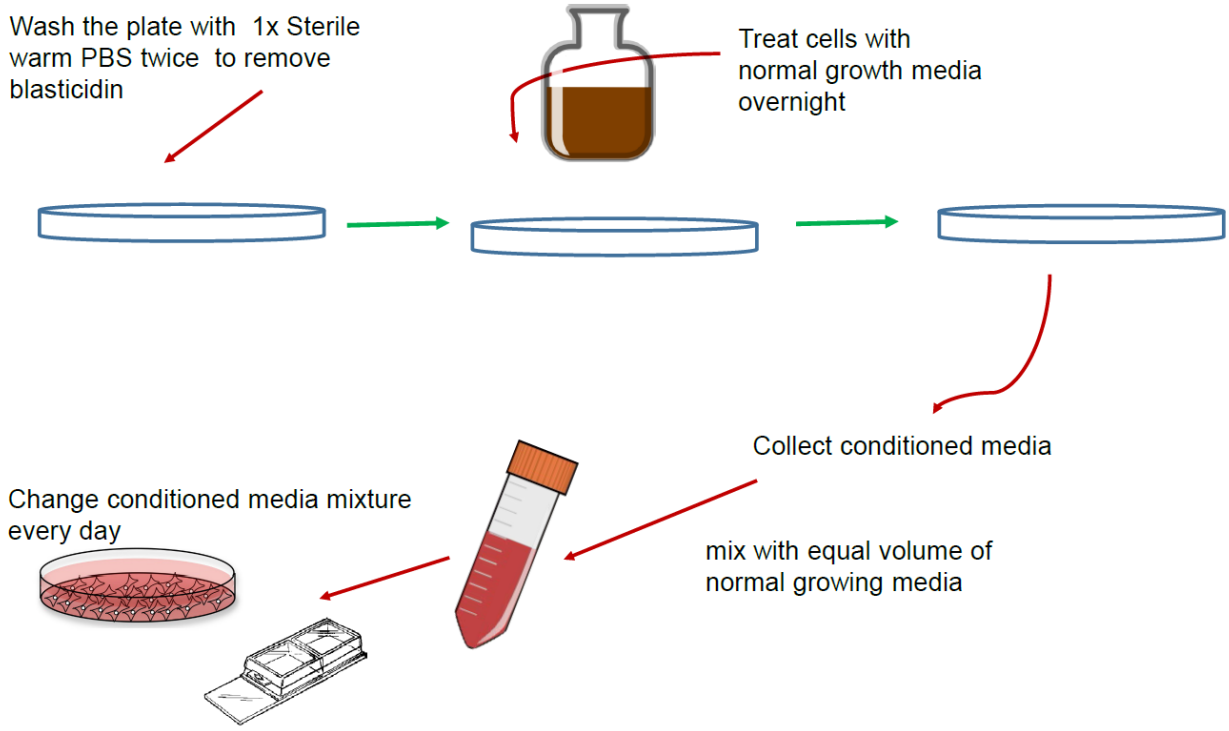


Figure 3.3 Collecting conditioned media for rescue

3.2.13. Statistical analysis for study of molecular mechanisms (chapters 4.2 and 4.3)

P values were calculated by analysis of variance (ANOVA) with a post-hoc test or independent *t*-tests, as indicated in the figure legends. Analysis was performed with SPSS (Statistical Package for the Social Sciences) software (IBM, v.21) or Microsoft Excel. Values are expressed as the mean ± standard error mean (SEM). Results with p values < 0.05 were considered to be statistically significant.

4. RESULTS

4.1. BIOMECHANICAL PROPERTIES OF THE SKIN IN CUTIS LAXA PATIENTS

An essential function of human skin is to protect against external mechanical insults, which enables a reversible deformation of its structure. In cutis laxa (CL) patients, multiple manifestations can be present according to different causative genes, however, loose skin is a share phenotype. The details about mechanical properties have been discussed in section 2.1 (page 3). This association and observational study was conducted to evaluate the predictive value of skin mechanical properties, such as elasticity, generated from a DermaLab suction device in control individuals and CL patients. The information in section 4.1 has been submitted to Journal of Investigative Dermatology and is under revision.

4.1.1. Demography and mutation status of participants

From the 136 control individuals who participated in this study, one was excluded because of no measurements of the left arm, and 17 were excluded because of significant differences between measurements of the right and left arms, yielding 118 individuals for final analysis. From 22 CL patients who had undergone Dermalab testing, three were excluded because the difference between measurements of the two arms were large and two were excluded because of RT

measurements greater than 10,000 ms. Thus, 118 control individuals and 17 cutis laxa patients were subjected to further analysis. There was no significant difference in age or gender distribution between cases and controls (Table 4.1). Eight CL patients were positive for mutations in known CL genes with *LTBP4* mutations in 2, *ELN* mutations in 3 and *ATP6V0A2* mutations in 3 patients (Table 4.2). Moreover, nine patients with unknown mutational status were enrolled (Table 4.3), including 3 individuals with congenital and 6 subjects with late-onset CL. Because skin elasticity measurements did not show significant differences among patients, the data of all the CL patients were pooled into one case group.

Table 4.1 Age and gender in the participants

	Controls (n=118)	**Patients (n=17)**	***p*-value**
Age (years)#	33.22 ± 1.58	29.21 ± 5.47	0.490*
Gender (male; %)	37.3%	35.3%	0.874**

Continues variables: mean ± standard error mean; *: independent t-test; **: Chi-square test; #: year of age when dermal mechanical parameters were taken.

Table 4.2 Enrolled cutis laxa patients with known mutations

Study ID	Age (years)[#]	Gender	Disease Onset	Mutant Gene	Type of CL	OMIM
Patient 1	14	F	Congenital	*LTBP4*	ARCL1C	613177
Patient 2	23	F	Congenital	*LTBP4*	ARCL1C	613177
Patient 3	27	M	Congenital	*ELN*	ADCL1	123700
Patient 4	16	M	Congenital	*ELN*	ADCL1	123700
Patient 5	66	F	Late-onset	*ELN*	ADCL1	123700
Patient 6	23	M	Congenital	*ATP6V0A2*	ARCL2A	219200
Patient 7	3	F	Congenital	*ATP6V0A2*	ARCL2A	219200
Patient 8	5	M	Congenital	*ATP6V0A2*	ARCL2A	219200

ARCL1C: autosomal recessive cutis laxa type 1C, ADCL1: autosomal dominant cutis laxa type 1, ARCL2A: autosomal recessive cutis laxa type 2A, OMIM: Online Mendelian Inheritance in Man phenotype ID number. #: year of age when dermal mechanical parameters were taken.

Table 4.3 Enrolled cutis laxa patients with unknown mutations

Study ID	Age (years)#	Sex	Disease Onset
Patient 9	30	M	Late-onset
Patient 10	4	F	Congenital
Patient 11	42	F	Late-onset
Patient 12	68	F	Late-onset
Patient 13	1.6	F	Congenital
Patient 14	41	M	Late-onset
Patient 15	17	F	Congenital
Patient 16	58	F	Late-onset
Patient 17	58	F	Late-onset

#: year of age when dermal mechanical parameters were taken.

4.1.2. Elasticity parameters generated by the DermaLab device

All participants underwent testing by using a DermaLab skin elasticity module, a suction cup device. During testing, the device applies vacuum to patch of skin and measures the pressure difference (△P) required to lift up the skin to a height of 1.5 mm and the time required for the skin to return to the original position, the retraction time (RT). The elastic modulus (E) can be calculated from this pressure difference, assuming uniform skin thickness (1mm). The machine calculates the viscoelastic modulus (VE) from E and RT as variables. Elasticity parameters generated from tests by using DermaLab device are summarized in Table 4.4. CL patients had significantly lower E, higher RT and lower VE ($p < 0.05$) than controls. Furthermore, there was no statistically significant difference between congenital and acquired patients in the VE (p=0.083), E (p=0.537) or RT (p=0.144).

Table 4.4 Elasticity parameters in participants

	Controls (n=118)	Patients (n=17)	*p*-value
E (MPa)	11.61 ± 0.15	7.85 ± 0.60	< 0.0001*
Retraction time (ms)	622.82 ± 21.20	1152.82 ± 211.21	0.024*
VE (MPa)	5.35 ± 0.14	2.51 ± 0.32	< 0.0001*

E, elastic modulus; VE, viscoelastic modulus.

Theoretically, mechanical properties of the skin includes elastic and viscous properties. Viscous properties of skin are related to delayed recovery from deformation and Young's modulus (E) is the ratio of stress over strain, indicating the elasticity of the skin. The skin is a complex tissue and these dermal mechanical parameters can be related to each other physiologically. It has been suggested that these parameters can change with age. Thus, I checked the correlation between elasticity parameters and the age that the measurements were taken at (Table 4.5, Figure 4.1).

Table 4.5 Correlation matrix (Pearson *r*) among age and dermal elasticity parameters

		Controls			Cases		
		E	RT	VE	E	RT	VE
Age	*r*	-0.052	0.770	-0.801	-0.105	0.541	-0.461
	p-value	0.574	<0.001	<0.001	0.689	0.025	0.063
E	*r*		0.090	0.246		0.160	0.068
	p-value		0.333	0.007		0.541	0.795
RT	*r*			-0.898			-0.806
	p-value			< 0.001			< 0.001

RT and VE were significantly correlated with age but E was not. In CL patients, VE was marginally correlated with age. Scatter plots between age and elasticity parameters are shown in Figure 4.1. Moreover, the slope estimations in linear regression lines (VE vs age & RT vs age) were statistically significant between control and patient groups ($p < 0.05$). VE decreased in controls more rapidly than in patients, but the increase in RT was slower in controls than in patients.

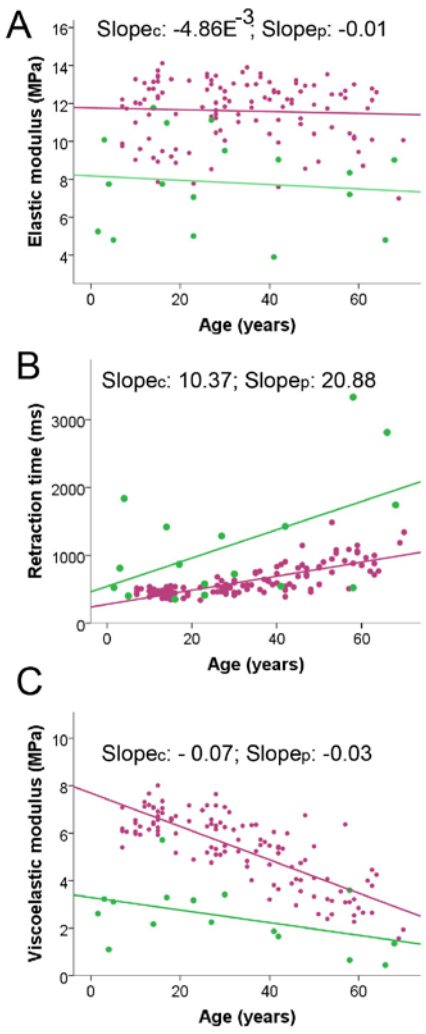


Figure 4.1 Mechanical properties of the skin in control and CL patients.

Elastic modulus (A), retraction time (B) and viscoelastic modulus (C) are plotted as a function of age for controls (magenta dots) and CL patients (green dots). Linear regression lines are shown for each variable in each group (CL: green, controls: magenta); $Slope_C$: slope of the linear regression line in controls; $Slope_P$: slope of the linear regression line in patients. In panel (B) and (C), there were significant difference of slopes between slopes ($p < 0.05$) and in panel (A), there was no difference.

4.1.3. Multivariate logistic regression analysis of skin elasticty in CL

Logistic regression analysis was used to determine the strength of each individual biomechanical variable to predict affected status. Step-wise logistic regression was used to obtain a multivariate model for predicting affection status, with age, sex, E, VE and RT in the initial model. Age (p=0.005, odds ratio (OR): 1.21, 95% confidence interval (CI): 1.06-1.37), VE (p=0.002, OR: 16.39, 95% CI: 2.85- 95.18) and E (p=0.043, OR: 3.55, 95% CI: 1.04-12.15) were significant predictors of disease status. VE was the strongest predictor and one unit reduction in VE increased the odds of CL 16.39-fold. Moreover, the receiver operation characteristic (ROC) was calculated for each variable, including E, VE and RT (Figure 4.2A). VE performed best, with ROC area under curve (AUC) reaching 0.908 among all participants in the study.

Pearson correlation r was limited between VE and E: 0.246 and 0.068 in controls and patients, respectively. In addition, RT strongly correlated with VE because of RT is a denominator in the equation to generate VE. There was no difference of age between controls and patients. Thus, E was added in to the logistic regression model to compare the reliability and stability between models, adjusted with age. Model 1 (AUC=0.992), incorporating age, VE and E, performed significantly (ANOVA, p=0.0026) better than model 2 (AUC=0.958), including age and VE in distinguishing cases from controls (Figure 4.2B). Moreover, bootstrap analysis has been performed to check the conclusion from the small sample size of patients would met in the large sample size. Based on 1000 bootstrap samples, model 1 performed better than model 2

(p=0.005, 95% CI: 0.45-130.810) and based on 5000 bootstrap samples, model 1 has higher predictive value than model 2 (p=0.009; 95% CI: 0.41-97.220).

The performance of a model on the same data that was used to fit the model would give an overly optimistic measure of performance. To test if such overfitting or over-estimating could have occurred, cross-validation tests were performed. In 20 tests, the averages of AUC in ROC analysis of were 0.951 and 0.901 in model 1 and model 2, respectively (Table 4.6). As cross-validation, on average resulted only modest decrease in AUC values, overfitting was not a major issue in this data, and similar sensitivity and specificity values can be expected in future replication studies to our present results.

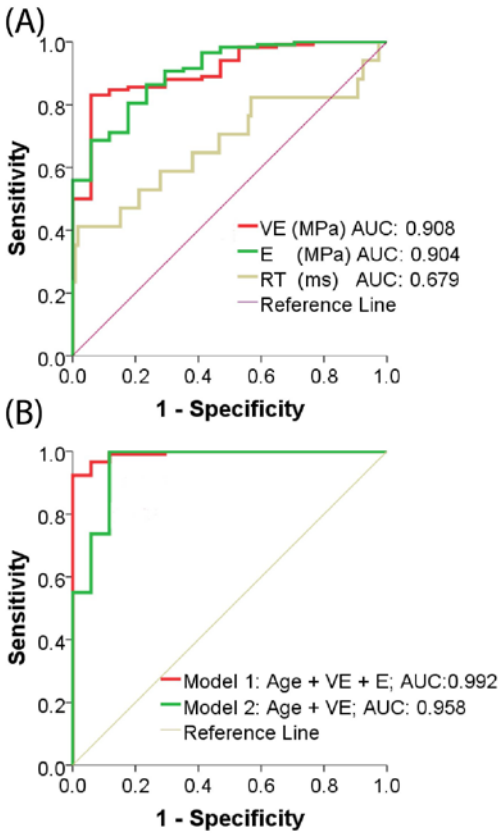


Figure 4.2 ROC analysis of biomechanical and composite variables as diagnostic measures

Viscoelastic modulus is more effective than elastic modulus or retraction time in differentiating cases from controls as indicated by receiver operating characteristic (ROC) curves. (A) ROC curve with AUC among all participants. (B) Composite variables under Model 1 (Age + VE + E) perform better than Model 2 (Age + VE).

Table 4.6 Results of cross-validation tests

	AUC of Model 1	AUC of Model 2
Testing 1	0.992	1
Testing 2	0.96	0.98
Testing 3	0.993	0.972
Testing 4	1	1
Testing 5	0.92	0.8
Testing 6	0.556	0.542
Testing 7	0.969	0.985
Testing 8	1	1
Testing 9	0.92	0.8
Testing 10	0.917	0.75
Testing 11	1	1
Testing 12	0.85	0.75
Testing 13	1	1
Testing 14	1	1
Testing 15	0.97	0.985
Testing 16	0.983	1
Testing 17	0.992	0.992
Testing 18	1	1
Testing 19	0.993	0.972
Testing 20	1	0.5
Average AUC	**0.95075**	**0.9014**

In each test, traninig dataset was included in 4/5 participants and Model (Age+VE+E) and Model (Age+VE) were generated. Models then fitted in testing dataset with 1/5 participants and the computed AUC values are shown in the table. Model 1: logistic regression model incorporating Age, VE and E. Model 2: logistic regression model incorporating Age and VE. AUC: area under ROC (receiver operating characteristic) curve.

A predictive variable (**D**) can be calculated from logistic regression analyses using the following device-specific formula:

$$\mathbf{D} \cong -27.570 + 0.187 \times \text{Age} + 2.795 \times \text{VE} + 1.267 \times \text{E}$$

where “Age” is in years, VE is the viscoelastic modulus and E is the elastic modulus, both in MPa units. If an individual’s D is less than 2.538, the probability of the individual having CL is 99.2%. D distinguished cases from controls with 100% specificity and 91.5% sensitivity.

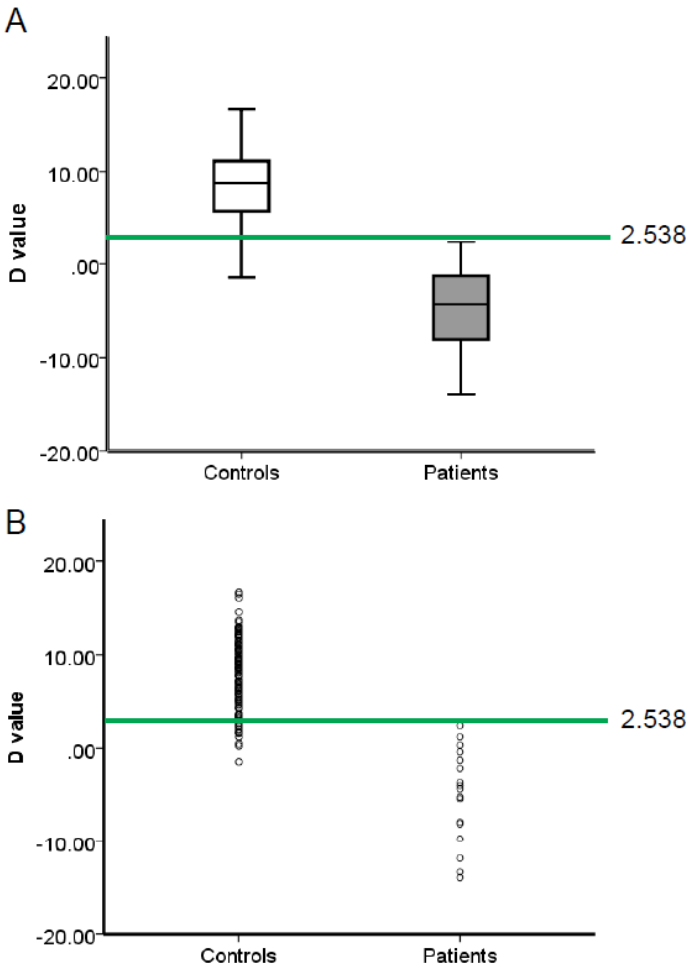


Figure 4.3 Box plots and scatter plots of D values in controls and patients.

(A): box plots; (B): scatter plots. Green lines indicate the reference “D” value of 2.538.

The skin was visually loose in cutis laxa patients with decreased VE values (Figure 4.4A). There were an insufficient number of individuals within each type of inherited cutis laxa to allow for subgroup analysis. However, individuals with ADCL caused by *ELN* mutations showed the greatest degree of variation in VE (Figure 4.4B) compared to those with other mutations, consistent with previous reports of variable expression of the skin phenotype in ADCL patients[188].

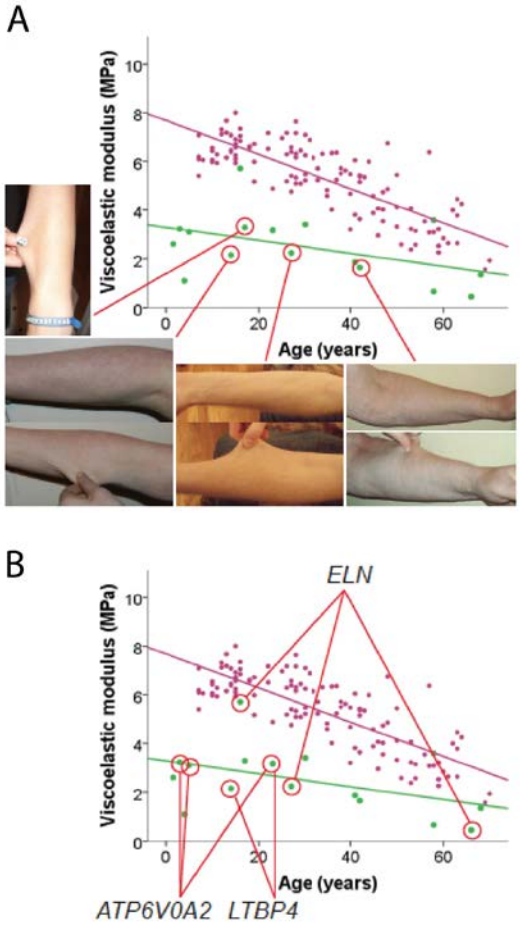


Figure 4.4 Viscoelastic modulus in relation to visual skin laxity and the causative gene mutation

(**A**) Images showing loose and wrinkled skin at the inner lower arm of several representative cases. Red tie-lines identify the data points on the viscoelastic modulus (VE)/age plot corresponding to each set of images. (B) VE values of individuals with known gene mutations. Note that one individual with *ATP6V0A2*-related CL had similar age and VE data to another participant with *LTBP4*-related CL, resulting in overlapping data points.

4.1.4. Discussion

The results in this study show significant reduction of the elastic (E) and viscoelastic (VE) moduli and significant increase of the retraction time (RT) of the skin of CL patients irrespective of the etiology of the disease. Individuals with acquired or late onset CL had similar reductions in VE compared to controls as individuals with *ELN*, *LTBP4* or *ATP6V0A2* mutations, suggesting that the disruption of elastic fibers leads to similar biomechanical alterations independent of the precise molecular disease mechanisms. VE showed significant inverse correlation with age in both CL and control individuals, but in controls the decline started from a higher level and was thus steeper. Therefore, VE appears to be a good measure of biomechanical aging of the skin, and our observations suggest that CL results in similar changes in skin mechanics to aging. VE also offers the best specificity and sensitivity in distinguishing cases from controls among the individual variables measured.

The elastic modulus (E) was significantly lower in CL patients compared to controls. We saw no correlation of E with age. This is consistent with previous reports that showed steady E values with age with an increase past 70 years[189,190], an age range not covered by either enrolled cases or controls. The absolute value of E obtained by the DermaLab instrument (E = 5-14 MPa) is not directly comparable to E values obtained by other earlier designs of a suction cup device with E of 0.05MPa to 0.15MPa[18,191]. It is plausible that the DermaLab instrument interrogates a different part of the stress-strain curve. However, based on the previous studies, E obtained with

torsion tests ranged between 0.42 MPa and 0.85MPa[192], and between 0.6Mpa and 20MPa for tensile tests[193]. Indeed, mechanical testing of human skin is challenging because of the complexity of skin layers, which may be differently engaged by various devices.

RT showed age dependence and significant difference between cases and controls. In the calculation of VE, E is divided by a normalized RT value, as RT is thought to be proportional to the viscosity of the skin. However, RT is not only proportional to the viscosity of the skin, but also is inversely proportional to the recoil property of the skin and we propose that RT is increased in CL patients because of decreased recoil in the absence of functional elastic fibers, rather than because of increased viscosity. As a result of such complex biophysical contributions to VE, measurements obtained with the DermaLab suction cup device are not directly comparable to measurements of skin biomechanics with other devices.

This study assumes uniform skin thickness (1 mm) in calculating the E and VE moduli. This is consistent with relatively constant skin thickness in the age range of 15-70 years by a variety of methods, including caliper (0.8-1.4 mm)[190] or by ultrasound measurements (0.6-0.9 mm)[189]. Furthermore, previous studies found no difference in the thickness of skin between individuals with CL and controls[194]. Although the results presented here demonstrate that a composite variable incorporating Age, E and VE can distinguish between CL patients and controls with high specificity and sensitivity, additional studies will be needed to determine how to best distinguish CL cases from other connective tissue syndromes.

4.2. LTBP4 REGULATES EXTRACELLULAR MATRIX ASSEMBLY AND TRANSFORMING GROWTH FACTOR-β SIGNALING

In CL patients, loose skin is a common phenotype in each subtype, caused by different mutant genes related to elastic fibers, provide elasticity and enable reversible deformation of the skin in response to external forces. In addition to determining the structure and mechanical properties of tissues, elastic fibers are also essential for the regulation of the activity of growth factors and hence are required for the proper development of multiple organs. In the second part of my work I focused on the function of one of the key proteins of the elastic fibers, LTBP4. In patients with *LTBP4* mutation, skin laxity, emphysema, hypotonia, and gastrointestinal abnormality are major manifestations. LTBP4-mutant skin fibroblasts from patients were used to understand the role of LTBP4 in the formation of the elastic fibers and in regulating the activity of TGFβ and the contribution of the loss of these functions to the pathogenesis of CL.

4.2.1. Effects of mutations on LTBP4 synthesis, and deposition in to the ECM

In this subsection 4.2.1, the results have been published in our paper, Human Mutation 2013 Jan; 34(1): 111-21[185].

Dermal fibroblasts of CL patients with different *LTBP4* mutations were used to detect the abnormal fibril networks and truncated protein expression. The mutations in these *LTBP4*-

defiecent fibroblasts are illustrated in Figure 4.5. All, except one (p.C1286S), of these mutations were predicted to result in premature termination of the open reading frame (Table 4.7).

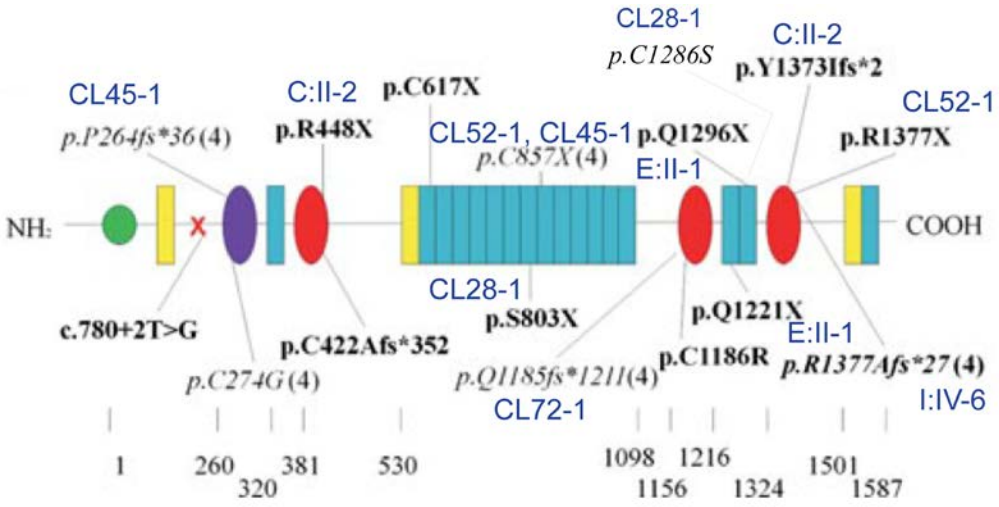


Figure 4.5 Schematic representation of LTBP4, with mutation of dermal fibroblasts used in the study

Table 4.7 *LTBP4* mutations in cutis laxa patients

Patient ID	cDNA	Protein	Type	Domain
C:II-2	c.1342C>T	p.Arg448X	Nonsense	First 8-Cys domain
	c.4115dupC	p.Tyr1373IlefsX2	Frameshift—PTC	Third 8-Cys domain
E:II-1	c.3661C>T	p.Gln1221X	Nonsense	Second 8-Cys domain
	c.3886C>T	p.Gln1296X	Nonsense	Fourteenth EGF-like domain
I:IV-6	c.4127dupC	p.Arg1377AlafsX27	Frameshift—PTC	Third 8-Cys domain
	c.4127dupC	p.Arg1377AlafsX27	Frameshift—PTC	Third 8-Cys domain

PTC, premature truncation; EGF, epidermal growth factor; LTBP4: latent transforming growth factorβ protein 4.

Consistent with expectations, the lack of secreted LTBP4 in the media was observed by immunoblotting (Figure 4.6) in two mutant fibroblasts, presumably as a result of nonsense-mediated decay of the mutant mRNA. However, LTBP4 was detected in the media from cultured mutant fibroblasts in patient I:IV-6 (homozygous for mutation p.Arg1377AlafsX27) using an antibody directed against the N-terminal half of LTBP4. In addition, LTBP4 antibodies against the C-terminus failed detect LTBP4 in the conditioned media from the same fibroblasts, indicating that escaping nonsense-mediated decay, a truncated, mutant protein was produced. The results in Figure 4.6 (p.88), Figure 4.7 (p.89) and Figure 4.8 (p.90) have been included in our published paper, Human Mutation 2013 Jan; 34(1): 111-21.

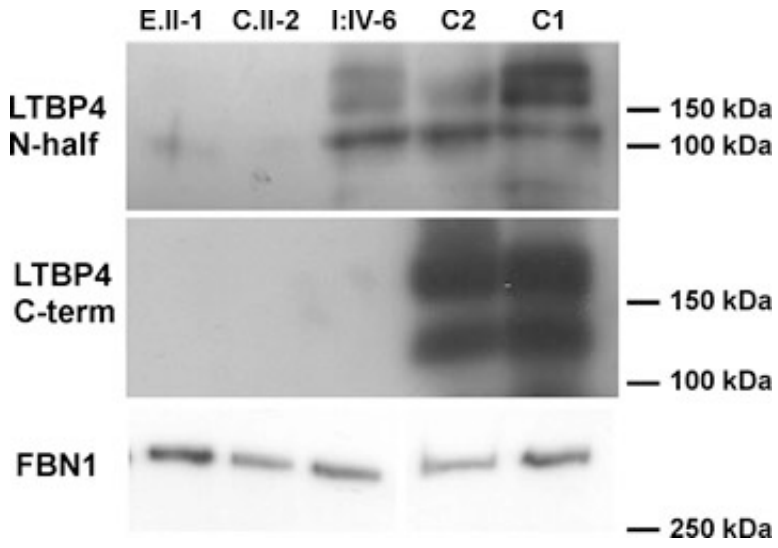


Figure 4.6 Protein expression of secreted LTBP4 and fibirllin-1 in conditioned media upon cultured control and *LTBP4*-mutant dermal fibroblasts

FBN1: fibrillin-1; C1 & C2: control 1 & control 2[185]

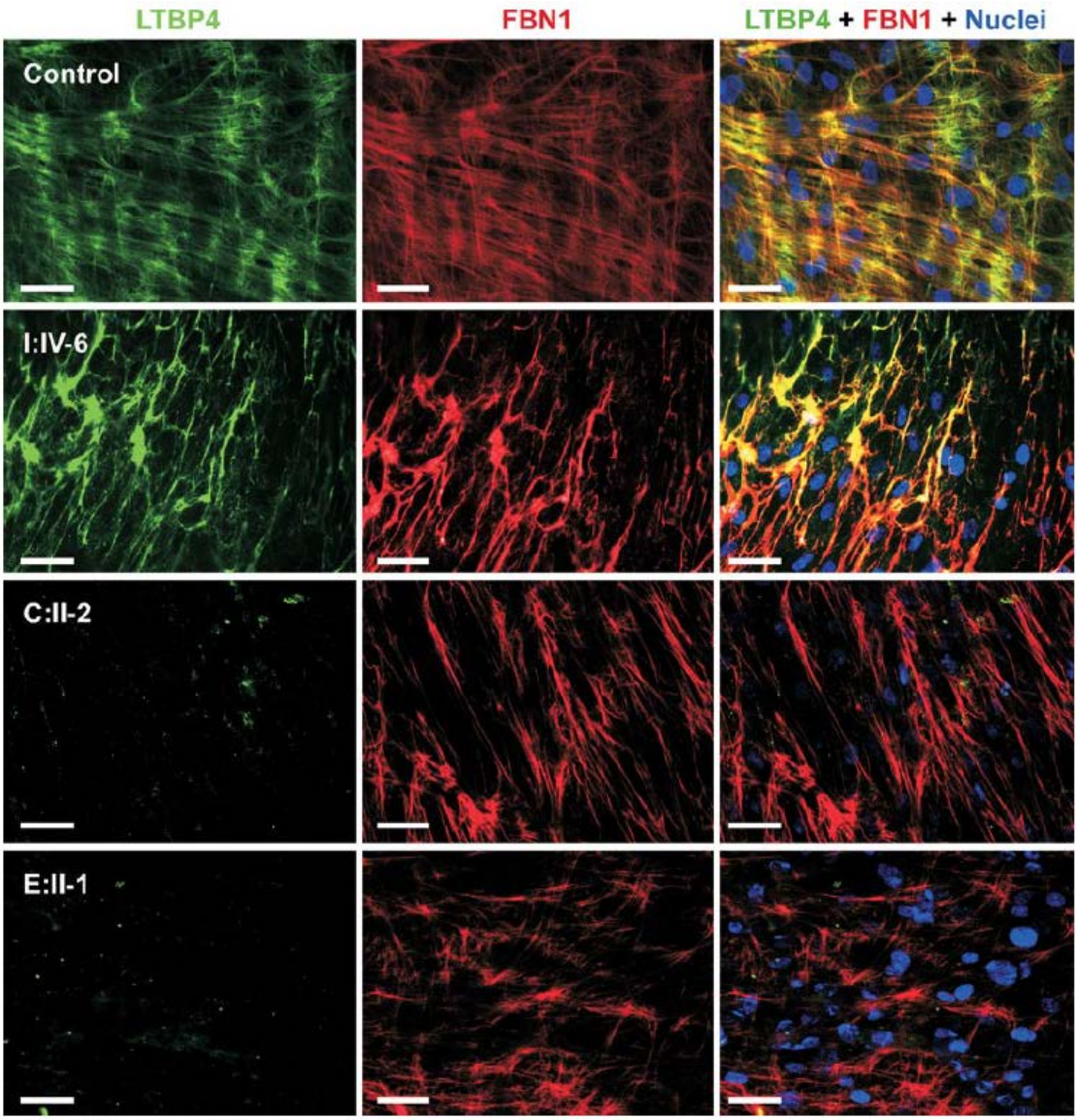


Figure 4.7 Immunofluorescence staining for LTBP4 and fibrillin-1 (FBN1).

Scale bar: 50μm. LTBP4: in green; FBN1: in red. Nuclei were counterstained in blue.[185]

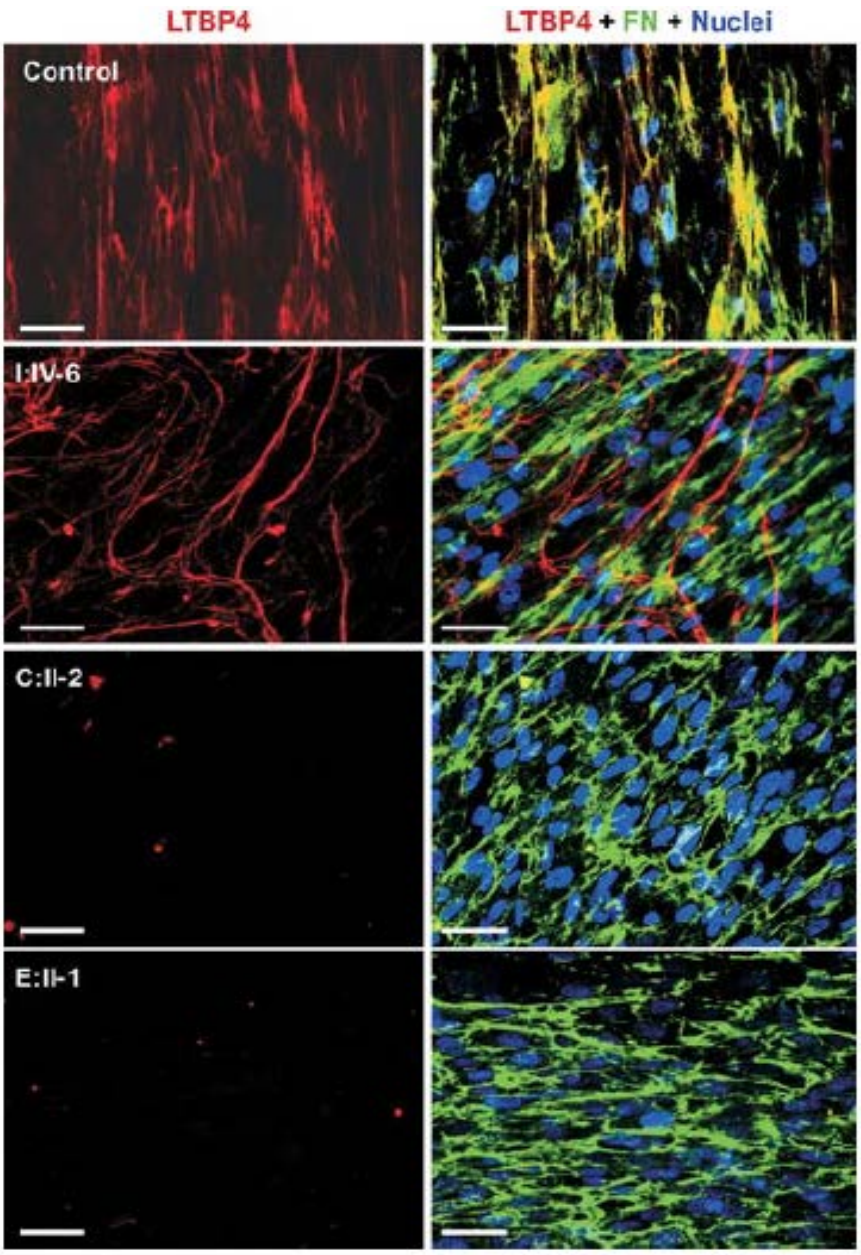


Figure 4.8 Immunofluorescence staining for fibronectin (FN) and LTBP4.

Nuclei were counterstained in blue. Scale bar: 50μm. FN: in green; LTBP4: in red[185]

Immunofluorescence staining results were consistent with the immunoblotting data. LTBP4 was undetectable in the ECM of mutant fibroblasts C:II-2 and E:II-1 (Figure 4.7 & Figure 4.8). However, using an N-terminal half-specific antibody, LTBP4 was detectable in the ECM in patient I:IV-6 with thicker and wavy fibrillin-1 and LTBP4-containing fibers compared to those in controls. In control dermal fibroblasts, patchy co-localization between fibrillin-1 and LTBP4 as well as fibronectin and LTBP4 were revealed. Interestingly, the distribution of LTBP4 and fibronectin in patient I:IV-6 was distinct, with little co-localization.

4.2.2. TGFβ activity in *LTBP4*-mutant dermal fibroblasts

This subsection 4.2.2, the results has been published in our paper, Human Mutation 2013 Jan; 34(1): 111-21[185].

Co-culture of reporter mink lung epithelial cells (MLECs)[184] and fibroblasts showed significantly increased extracellular TGFβ activity (p <0.001) in two fibroblast lines with greatly diminished LTBP4 expression, Interestingly, TGFβ activity in fibroblasts from patient I:IV-6, who expressed a C-terminally truncated LTBP4, was not statistically different from the controls (Figure 4.9; p=0.108).

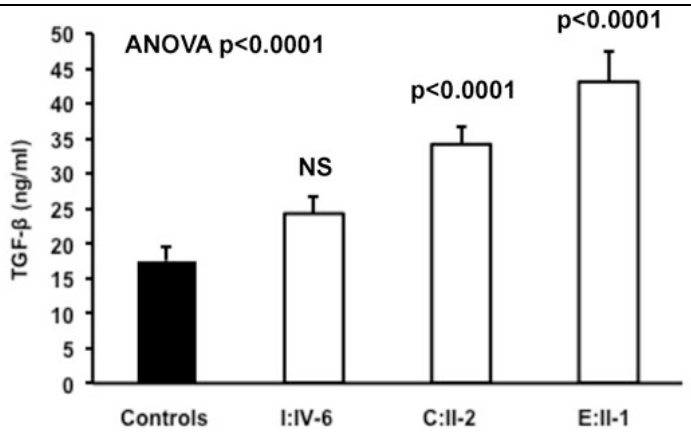


Figure 4.9 TGFβ activity in controls (n=4) and mutant fibroblasts in co-culture experiments

Measurements were doned for three times and in each experiments, tripilcation for TGFβ activity measurements in each cell line has been performed[185].

The observation of mutation-specific effects on extracellular TGFβ activity guided my subsequent experiments. To eliminate mutation-dependent variability, a set of mutant cells, (CL28-1, CL45-1, CL52-1, and CL72-1) which had homozygous or compound heterozygous loss-of-function mutations was selected (Table 4.8 and Figure 4.5).

Table 4.8 *LTBP4* mutation in cutis laxa subjects

Patient ID	cDNA	Protein	Type	Domain
CL45-1	c.791delC	p.P264fsX300 p.C857X	Nonsense	Hybrid domain
	c.2570_2571delGCinsAA		Nonsense	11th EGF-like domain
CL 52-1	c.2570_2571delGCinsAA	p.C857X p.P1376fsX1403	Nonsense	11th EGF-like domain
	c.4128insC		Frameshift-PTC	3rd 8-Cys domain
CL 72-1	c.3554delA	p.Q1185fsX1211	Frameshift-PTC	2nd 8-Cys domain
	c.3554delA	p.Q1185fsX1211	Frameshift-PTC	2nd 8-Cys domain
CL 28-1	c.3856T>A	p.C1286S p.E793fsX797	Point mutation	14th EGF-like domain
	c.2377insA		Frameshift-PTC	10th EGF-like domain

PTC, premature truncation; EGF, epidermal growth factor; LTBP4: latent transforming growth factorβ protein 4

Conditioned media samples were collected from 4 mutant and 4 control fibroblast lines to measure active and total TGFβ levels. To assay for total TGFβ, samples were heated (100°C, 10 min) to activate latent TGFβ prior to adding them to reporter cells. TGFβ activity measurements were normalized by fibroblast cell numbers. Active TGFβ results were subtracted from total TGFβ to obtain a calculated value for the latent form of TGFβ. Active, latent (not shown) and total forms of TGFβ were elevated in the conditioned media from *LTBP4*-mutant cells (Figure 4.10A,B, $p < 0.001$). Additionally, the expression of *TGFB1*, the major TGFβ isoform in skin fibroblasts, was not altered significantly at the mRNA level by qPCR (Figure 4.10C).

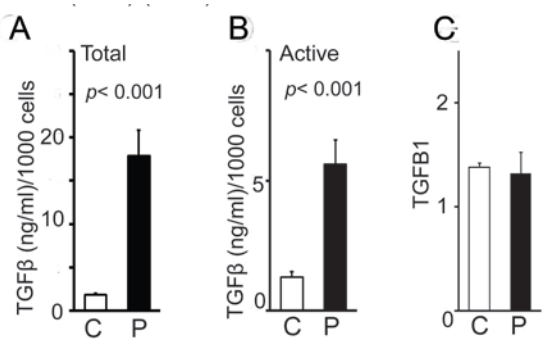


Figure 4.10 TGFβ activity in media (A, B) and mRNA level of *TGFB1* (C) of cultured dermal fibroblasts

C: controls (n=4); P: patients (n=4). *p*-value: obtained from t-test.

4.2.3. Intracellular TGFβ signaling pathway in *LTBP4*-mutant cells

To understand the downstream signaling triggered by increased extracellular TGFβ, both the canonical (SMAD-dependent) and the non-canonical (SMAD-independent) pathways were studied. Surprisingly, despite elevated extracellular TGFβ activity, mutant cells had decreased levels of SMAD2 phosphorylation compared to the controls (Figure 4.11A; p=0.014). TGFβ supplementation led to a further relative decrease in SMAD2 and ERK phosphorylation (Figure 4.11B) in patients vs. controls. (p <0.05) TGFBR1 inhibitor treatment eliminated this relative reduction (Figure 4.11C).

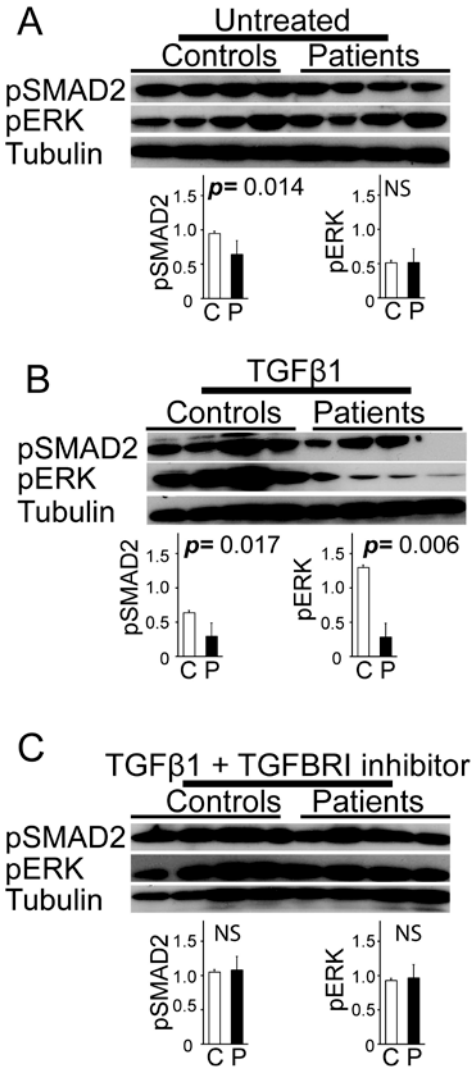


Figure 4.11 Immunoblots probing pSMAD2, pERK and tubulin as a loading control and quantitative analysis of the blots.

A: baseline; B: TGFβ1 stimulation for 90min; C: TGFβ1 + TGFβR1 inhibitor for 90 min. Immunoblots were quntified by by densitometry. pSMAD2: phosphorylated SMAD2; TGFBR1 inh.: TGFβ receptor 1 inhibitor. NS: non-significant. *p*-value: obtained from t-test. C: contols (n=4); P: patients (n=4)

To investigate the mechanisms of reduced TGFβ signaling in *LTBP4*-mutant dermal fibroblasts, I checked the expression of feedback inhibitory by inhibitory SMADs. However, reduced pSMAD2 levels were not accompanied with increased abundance of either SMAD7 or SMAD6 (Figure 4.12). Therefore, the observation of high extracellular TGFβ activity with reduced intracellular TGFβ signaling cannot be explained by increased negative feedback from inhibitory SMADs. In fact, both inhibitory SMAD levels were significantly decreased in mutant cells. As the expression of both SMAD6 and SMAD7 are under positive transcriptional control of the TGFβ signaling pathway, these results indicate that TGFβ-dependent transcriptional regulation is also depressed in mutant cells.

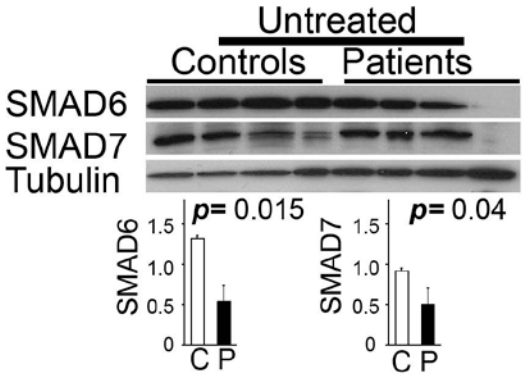


Figure 4.12 Expression of inhibitory SMADs in *LTBP4*-mutant fibroblasts
Tubulin served as a loading control. *p*-value: obtained from t-test. C: contols; P: patients

.

4.2.4. Discussion

4.2.4.1. LTBP4 mutations impair ECM assembly

> The following quoted statements have been published in our paper, Human Mutation 2013 Jan; 34(1): 111-21[185].

All, except one (p.C1286S), of these mutations were predicted to result in premature termination of the open reading frame. The results showed different microfibril assembly would happen in different mutant loci in LTBP4. *"A mutation, p.Arg1377Alafs*27 (Patient I:IV-6), partially escaped nonsense-mediated decay (NMD), producing significant amounts of truncated LTBP4. According to immunofluorescence staining, this C-terminally truncated protein altered the structure of microfibril bundles by producing thicker and wavier structures. In addition, the*

*colocalization patterns of the mutant LTBP4 with both fibrillin and fibronectin were abnormal. In normal fibroblasts, LTBP4 showed patchy colocalization with both fibronectin and with fibrillin-1. In contrast, truncated LTBP4 showed more uniform colocalization with fibrillin-1 microfibrils and reduced colocalization with fibronectin. These findings suggested that the C-terminal region of LTBP4 may be required for binding fibronectin but was not necessary for interacting with fibrillin-1 microfibrils. Loss-of-function mutations did not cause alterations in fibrillin-1 microfibril morphology. It is plausible that the C-terminal truncation mutation produced these alterations in a gain of function manner. Although the exact molecular mechanism remains unclear, uniform binding of truncated LTBP4 to fibrillin-1 microfibrils may enhance lateral association with microfibril bundles or impede their normal turnover leading to the observed thickening of the bundles. Because the C-terminus of LTBP4 is known to be required for cell attachment,[79] the interaction of these abnormal bundles with cells may also be impaired. Interestingly, in the family of patient I:IV-6, which carried the p.Arg1377Alafs*27 mutation, all patients had severe gastrointestinal involvement. Thus, fibrillin-1 bundle-size or the ability of microfibrils to support cell attachment via LTBP4 may be particularly important for gastrointestinal development."*

4.2.4.2. Elevated extracellular TGFβ activity *with paradoxically reduced intracellular TGFβ signaling*

I was surprised to find decreased intracellular TGFβ signaling in *LTBP4*-deficient human dermal fibroblasts despite abnormally elevated extracellular TGFβ activity. The binding of TGFβ to TGFβ receptors on the cell surface triggers intracellular TGFβ signaling pathways. This ligand-receptor interaction bridges extracellular and intracellular signals. I hypothesize that TGFβ receptor complexes on the cell surface may play a role to attenuate down-stream molecular signaling in micro-environments lacking LTBP4. To date, most of the therapeutic approaches to connective tissue disorders are designed to block TGFβ signaling but this approach may not be applicable to treating *LTBP4*-related CL patients, as such treatments may further reduce an already diminished TGFβ pathway. Thus, it is crucial to discover the mechanisms of TGFβ dysregulation in the absence of LTBP4 at the translational or post-translational levels.

4.3. LTBP4 REGULATES TRANSFORMING GROWTH FACTORβ RECEPTOR STABILITY

In ARCL1C/URDS patients, elevated TGFβ activities, in total, latent and active forms, have been detected in my previous experiments (Figure 4.9, above), consistent with previously published data[3]. Loss of cysteine residues were shown to impair functions in both LTBP1 and fibrillin proteins[195,196]. It may link to the dysregulation of TGFβ associated signaling to the pathogenesis

of phenotypes in patients with cutis laxa [197,198] and other inherited connective tissue disorders, such as the Marfan[180] and Loeys-Dietz syndromes[199]. Thus, it is important to understand TGFβ signaling furthermore in ARCL1C patients, especially reduced intracellular TGFβ signaling found in my results shown above (Chapter 4.2).

4.3.1. The expression of TGFβ receptors at the mRNA and protein levels

To test the hypothesis that TGFβ receptors play an essential role in altered TGFβ signaling in *LTBP4*-mutant fibroblasts, the expression of TGFBR1 and TGFBR2 at the mRNA and protein levels were investigated. There was no significant difference in the expression of TGFBR1 and TGFBR2 mRNAs (Figure 4.13, controls *vs* patients; $p > 0.05$).

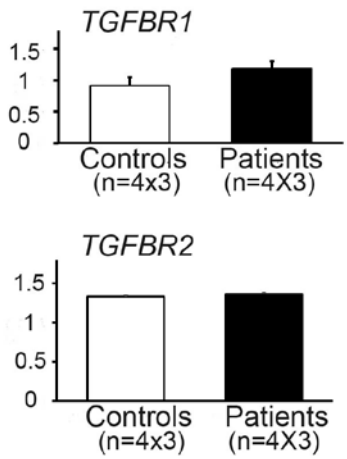


Figure 4.13 RNA levels of *TGFBR1* and *TGFBR2* among patients and controls

Quantitative RT-PCR shows similar *TGFBR1* and *TGFBR2* mRNA expression in control and patient dermal fibroblasts. Three replicates of RNA samples were used from each individual. The reference gene was *GAPDH*. Annotions of "n=4x3" indicate triplication of samples from four patients.

Immunoblotting was performed to assess the abundance and phosphorylation status of TGFβ receptors. Membrane extracts were prepared from fibroblasts and processed for immunoblotting to probe for TGFBR1, TGFBR2, TGFBR3 and pTGFBR1. TGFBR1 levels were decreased in *LTBP4*-mutant cells at baseline (Figure 4.14A) and showed a dramatic reduction (Figure 4.14B) upon TGFβ supplementation. Although the lower abundance of TGFBR2 in mutant cells at baseline was not significantly different from controls, TGFβ treatment resulted in a significant reduction of receptor levels (Figure 4.14B). TGFBR1 inhibitor treatment rescued depressed TGFBR1 and TGFBR2 levels in mutant fibroblasts, showing that the elimination of TGFBR1/TGFBR2 complexes in mutant cells was dependent on TGFBR1 activity (Figure 4.14C). There was no significant difference in TGFBR3 levels between control and patient groups under any of the treatment conditions.

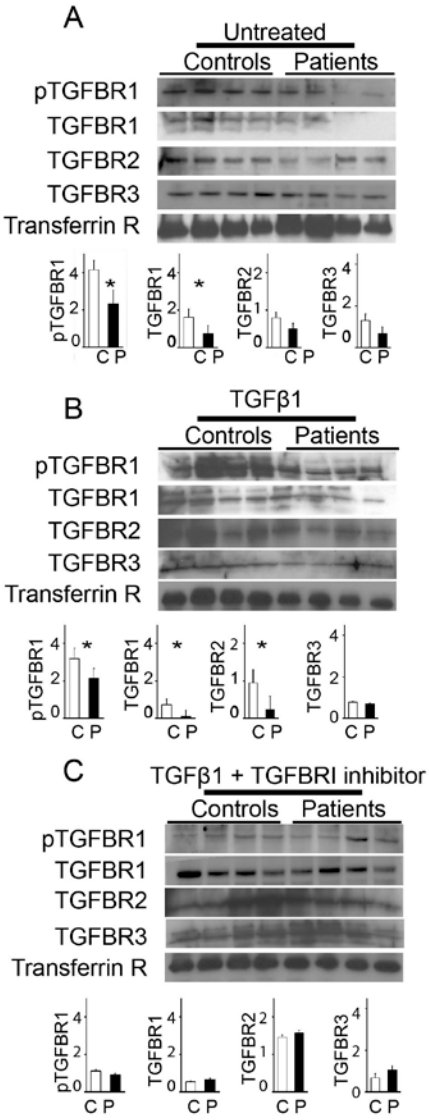


Figure 4.14 Reduced pTGFBR1, TGFBR1, TGFBR2 and TGFBR3 in protein level among *LTBP4*-mutant fibroblasts

Immunoblotting of membrane protein extracts from dermal fibroblasts left untreated (*A*), treated with TGFβ1 (*B*) or with TGFβ1 and TGFBR1 inhibitor (*C*). Immunoblots were quantified by densitometry. Transferrin receptor (Transferrin R) served as a loading control. Bar graphs show the mean ± SEM, pTGFBR1: phosphorylated TGFBR1. Quantifications are shown below the blots, which were normalized by the loading control. *: Statistically significant as demonstrated by $p < 0.05$ obtained with a *t*-test. C: controls (n=4); P: patient (n=4).

Phosphorylated TGFBR1 was less abundant in mutant cells both under baseline and TGFβ supplementation conditions, but pTGFBR1 levels normalized upon inhibitor treatment (Figure 4.14). To determine if pTGFBR1 reduction was caused by an overall reduction of the entire TGFBR1 pool or by reduced phosphorylation, I calculated the pTGFBR1/TGFBR1 ratio (Figure 4.15), which showed similar proportion of the receptor pool phosphorylated in mutant and control cells under baseline conditions as well as significantly higher proportion of the receptor pool phosphorylated upon treatment with additional TGFβ1. Thus, TGFBR1 phosphorylation occurred appropriately in *LTBP4*-mutant dermal cells, and the main reason for reduced TGFβ signaling was an overall reduction of the TGFBR1/TGFBR2 pool.

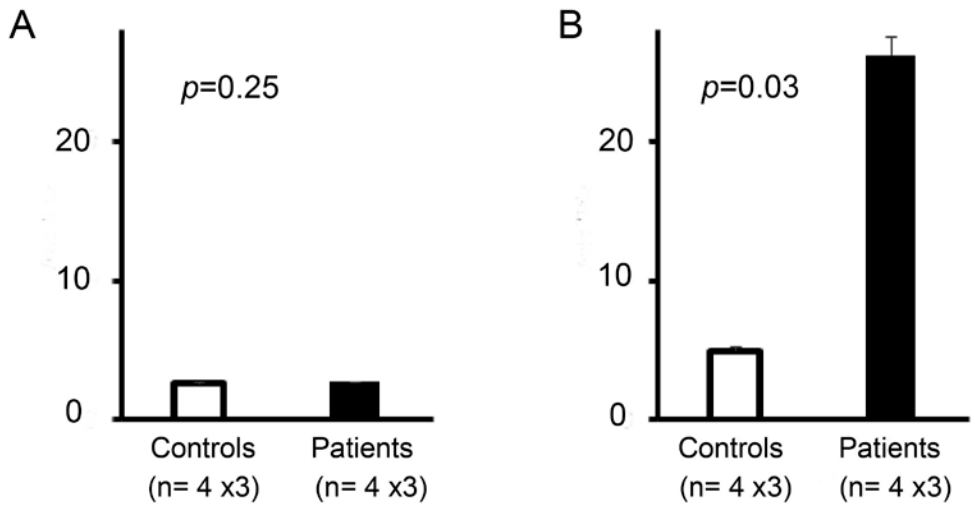


Figure 4.15 The proportion of pTGFBR1/TGFBR1 levels.

Untreated human dermal fibroblasts cells: (A); cells treated with TGFβ1: (B). Bar graphs show the mean ± SEM. *P*-value: obtained with *t*-test.

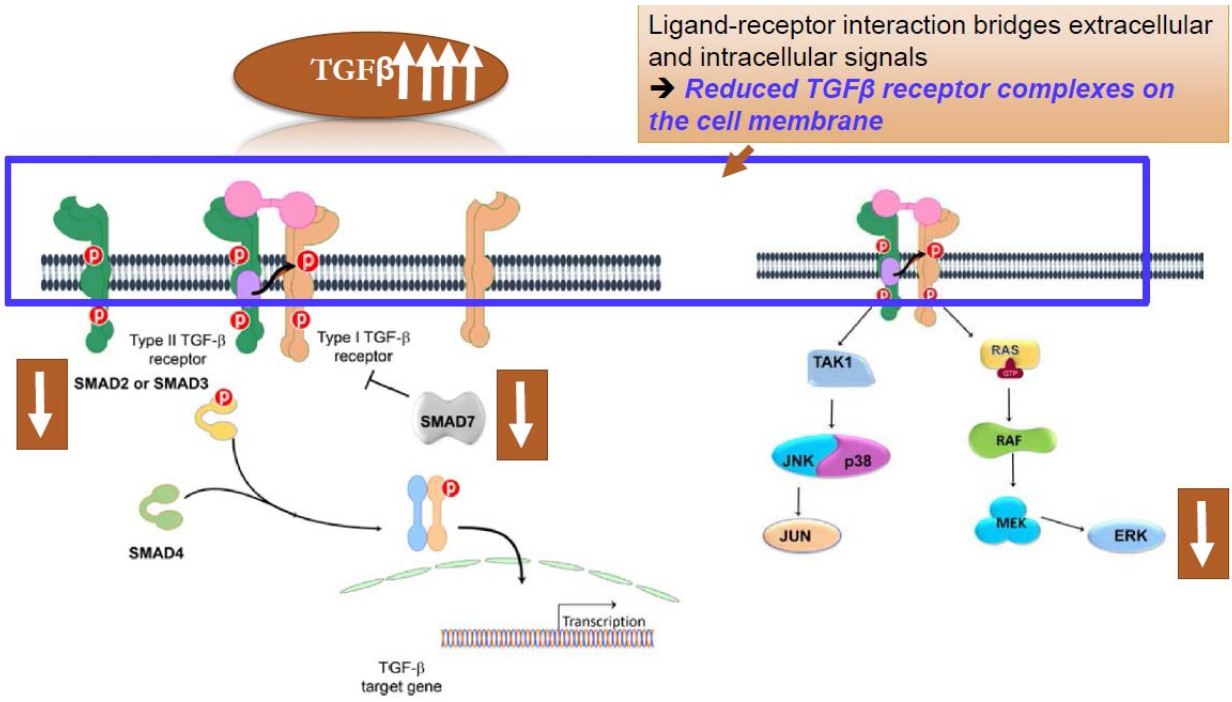


Figure 4.16 Summary of extracellular and intracellular TGFβ signaling and expression of TGFβ receptors in the *LTBP4*-mutant dermal fibroblasts.

Reduced expression of TGFβ receptors (TGFBR1 & TGFBR2) potentially leads decreased pSMAD2, pERK and SMAD7, accompanying with extracelluar TGFβ activity.

4.3.2. The molecular mechanisms of TGFBR internalization and degradation

The finding of reduced TGFBR1/TGFBR2 levels in *LTBP4*-deficient cells led me to investigate the degradation of TGFβ receptor complexes. The hypothesis was that increased endocytosis reduced the levels of TGFBR1/TGFBR2 complexes in *LTBP4*-deficient cells. For studying the half-lives of TGFBR1 and TGFBR2, cells were treated with a protein synthesis inhibitor, cycloheximide, and membrane protein extracts were collected at 2 hours, 4 hours, 8 hours and 24 hours after treatment. The responses of individual cell lines to the cycloheximide treatment varied. Therefore, immunoblots of TGFβ receptors of one control and one patient are shown as representative examples (Figure 4.17). The quantification of protein levels for four control and four *LTBP4*-mutant dermal fibroblasts based on immunoblotting experiments is shown in Figure 4.18.

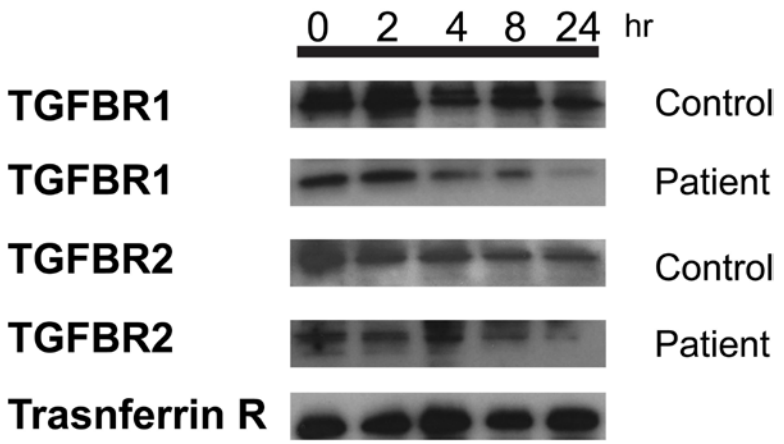


Figure 4.17 Immunoblotting for TGFβ receptors after cycloheximide treatment

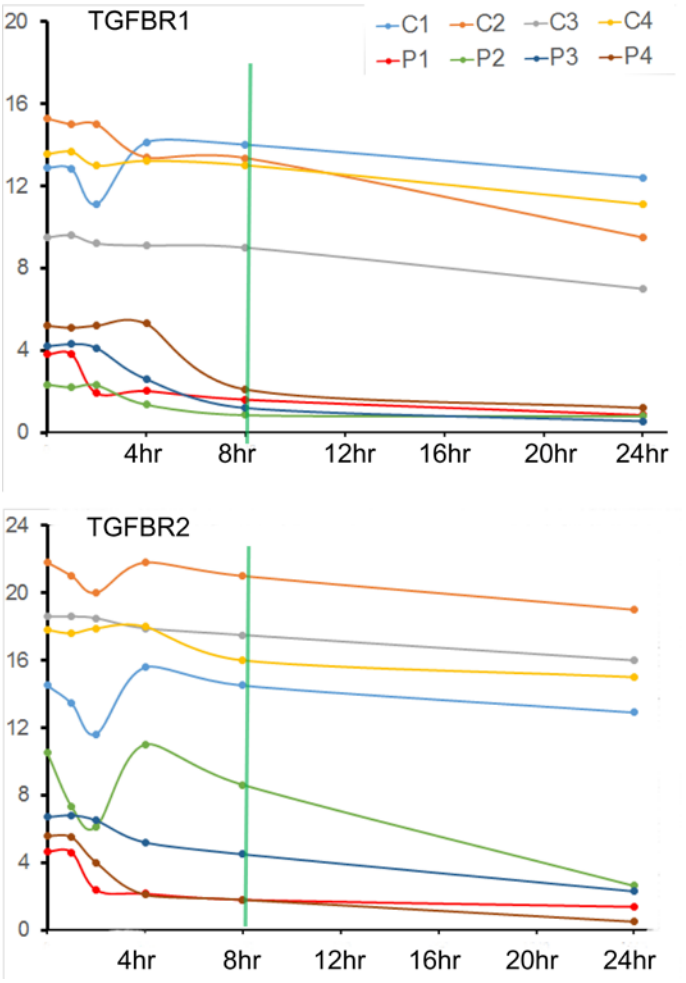


Figure 4.18 Densitometric quantification of immunoblots probing for TGFBR1 and TGFBR2 after cycloheximide treatment.

x-axis: timepoints after cycloheximide treatement. C: control; P: patient

The expression of TGFBR1 and TGFBR2 were relatively steady at 24 hours of cycloheximide treatment. At 8 hours of cycloheximide treatment, TGFβ receptor levels showed marked reduction in patients compared to controls. Thus, TGFBR1 and TGFBR2 levels were further quantified by ELISA allowing for more sensitive measurements with higher numbers of replicates. Following inhibition of protein synthesis by cycloheximide, I observed accelerated decay of both TGFBR1 and TGFBR2 in mutant cells compared to controls by ELISA (Figure 4.19).

Two primary endocytic pathways direct endocytic routs of cell surface receptors include clathrin-mediated endocytosis and lipid-raft- or caveolae-mediated endocytosis. To explore the mechanisms by of TGFBR1 and TGFBR2 degradation, cells were treated with inhibitors of clathrin-mediated (monodansylcadaverine; MDC) and caveolin-mediated endocytosis (filipin). Both treatments normalized the expression of TGFBR1 and TGFBR2 (Figure 4.20).

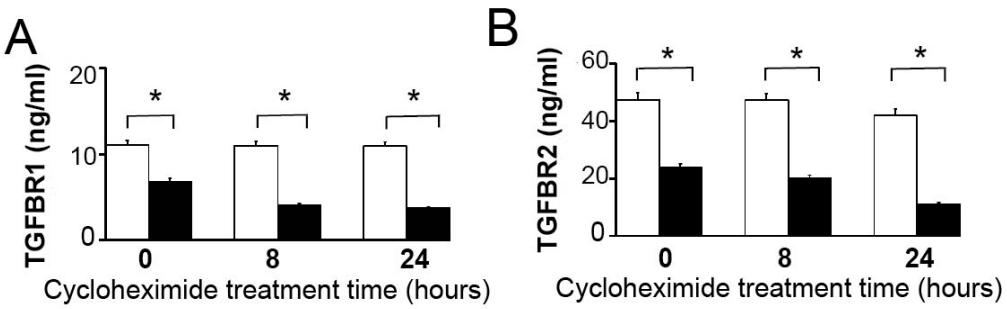


Figure 4.19 Increased turnover of TGFBR1 and TGFBR2 in *LTBP4*-mutant fibroblasts

Cells were treated with cycloheximide for 0, 8 and 24 hours. Enzyme-linked immunosorbent assays (ELISA) for TGFBR1 (*A*) and TGFBR2 (*B*) extracted from control and *LTBP4*-mutant fibroblasts, were performed. Bars indicate the mean ± SEM of three replicates samples for each of the four cases and controls. *: $p < 0.05$ (*t*-test).

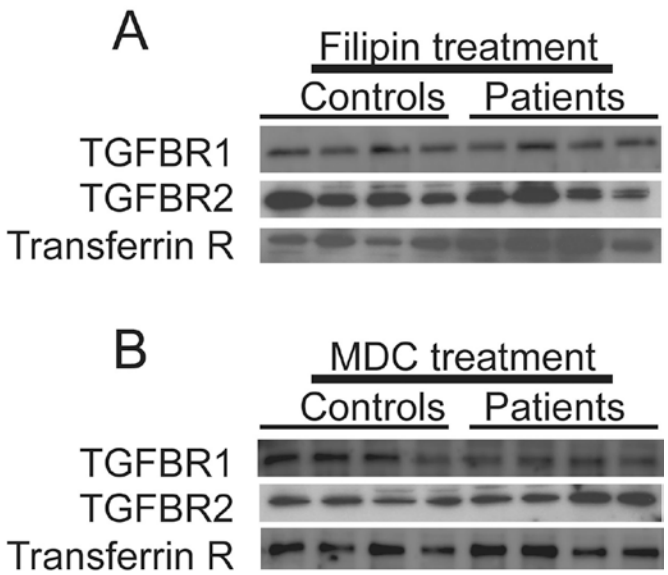


Figure 4.20 Endocytosis inhibitor treatment of control and *LTBP4*-mutant human dermal fibroblasts.

Cells were treated with filipin (A) or mododansyl cadaverine (MDC) (B) and membrane lysates were analyzed by immunoblotting. Transferrin receptor (transferrin R) served as a loading control.

TGFβ1 with or without TGFBR1 inhibition had no effect on the expression of TGFβ receptors in control and patient fibroblasts pre-treated with filipin or MDC (data not shown). As endocytosis inhibitors eliminated the differences in the expression of TGFBR1 and TGFBR2 between patients and controls, I conclude that the potential mechanism of TGFβ receptor destabilization is resulted from LTBP4-deficient microenvironments, involving internalization of TGFβ receptor complexes.

Moreover, protein degradation is primarily mediated by two routes, including the proteasome pathway and the lysosome pathway. In order to understand the sequential degradation pathways of TGFβ receptor complexes. A proteasome inhibitor, lactacystin and a lysosome inhibitor, ammonium chloride, were applied to human dermal fibroblasts. Ammonium chloride reversed the reduction of TGFBR1 and TGFBR2 proteins caused by LTBP4 deficiency (Figure 4.21 A), but lactacystin did not (Figure 4.21 B). Therefore, in LTBP4-mutant cells, TGFβ receptors are internalized and degraded by lysosomes.

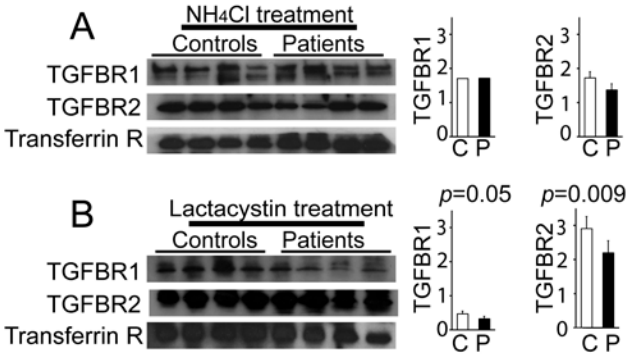


Figure 4.21 Lysosomal degradation of TGFβ receptors in mutant cells

TGFBR1 and TGFBR2 levels were normalized in mutant cells by the lysosome inhibitor ammonium chloride (NH_4Cl) (*A*) but not by the proteasome inhibitor lactacystin (*B*). Immunoblots were quantified by densitometry. The transferrin receptor (transferrin R) served as a loading control. Bar graphs show the mean ± SEM, p values represent *t*-tests. C: controls (n=4); P: patient (n=4)

4.3.3. Consequences of LTBP4 knockdown on TGFβ signaling and TGFβ receptor abundance

To generate further evidence supporting the function of LTBP4 as a regulator of TGFβ receptor stability, I experimentally depleted LTBP4 in normal cells. Antisense morpholino oligonucleotides (MO) were used to knock down LTBP4 expression in skin fibroblasts from a healthy donor. Two doses of MO created a more efficient (60%) knockdown of LTBP4 compared to a single dose of MO (8%; Figure 4.22A). In addition, immunofluorescence staining for LTBP4 showed no detectable LTBP4-positive fibers in the ECM after knockdown (Figure 4.23). Therefore, I studied the protein expression in *LTBP4* knockdown cells treated with a double dose of MO. *LTBP4* knockdown noticeably decreased the expression of TGFBR1, TGFBR2 and pSMAD2, replicating my findings in *LTBP4*-mutant cells (Figure 4.22B).

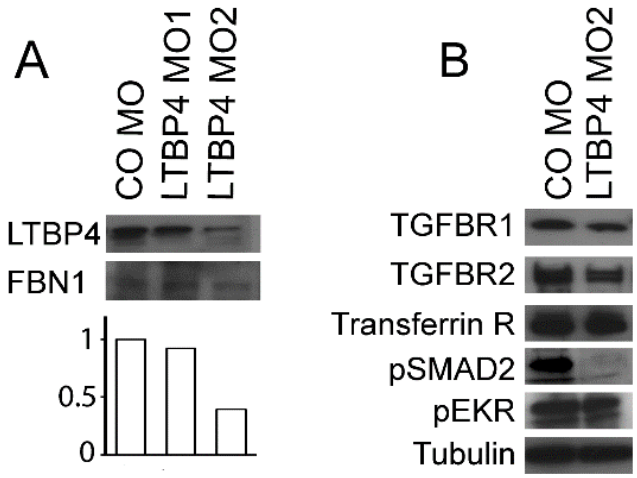


Figure 4.22 Protein expression of *LTBP4* knockdown cells.

(A) LTBP4 protein expression in media in human human fibroblasts treated with control scrambled morpholino (CO MO) or antisense LTBP4 morpholino administered once (LTBP4 MO1) or twice (LTBP4 MO2). (B) Normal human dermal fibroblasts treated by two doses of LTBP4 antisense morpholinos (LTBP4 MO2) or scrambled control morpholinos (CO MO). Immunoblots were probed by TGFBR1, TGFBR2, transferrin receptor, pSMAD2, pERK and tubulin.

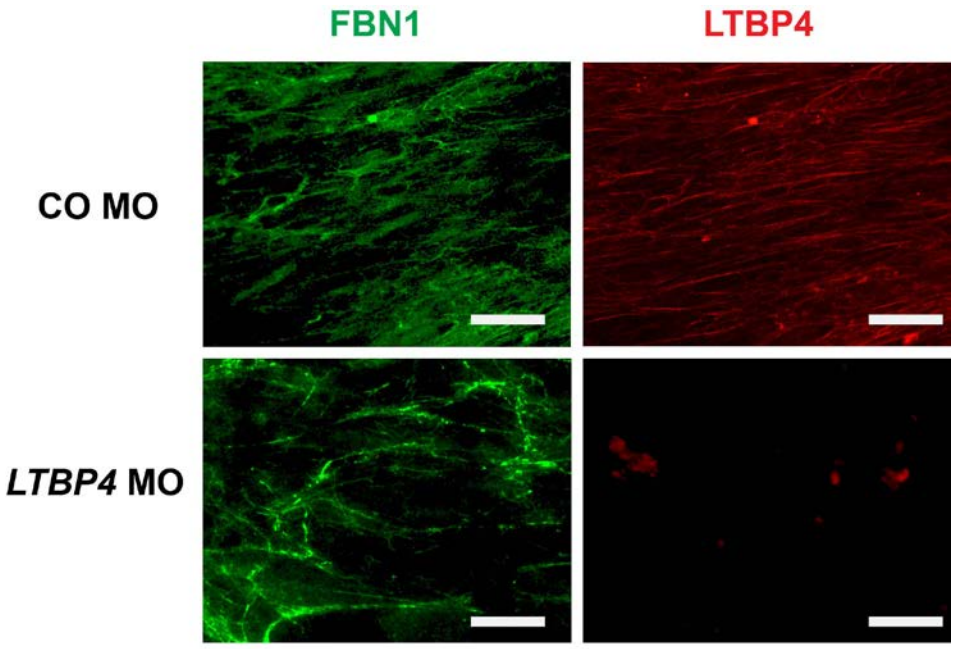


Figure 4.23 Immunofluorescence staining of the ECM in human dermal fibroblast subjected to LTBP4 MO.

CO MO: control mophorlino oligos, *LTBP4* MO: antisense *LTBP4* mophorlino administered in two doses Scale bar: 50μm

4.3.4. Rescue of mutant cells by recombinant LTBP4 treatment

I next queried if supplementation of recombinant LTBP4 would rescue the molecular phenotype of *LTBP4*-mutant cells. Thus, I overexpressed LTBP4 in HEK293 cells using a lentiviral vector, pLOC (Figure 4.24A). A vector expressing red fluorescent protein (RFP) was used to generate control HEK293 cells. Conditioned media from these two cell lines were used in subsequent experiments. To determine how long it took for *LTBP4*-mutant human dermal fibroblasts to respond to recombinant LTBP4, TGFBR2 levels were measured at different time points after treatment by using ELISA and I found that starting 2 days post-treatment, the expression of TGFBR2 increased gradually (Figure 4.24B). By 7 days, the expression peaked (Figure 4.24B). I therefore compared the expression of TGFβ receptors and signal transduction molecules by immunoblotting after 7 days of treatment (Figure 4.25). The expression of pSMAD2, pERK, TGFBR1 and TGFBR2 were elevated in LTBP4-mutant dermal fibroblasts treated with recombinant LTBP4 compared to controls (Figure 4.25). These findings provide evidence that alteration of TGFβ receptor levels in *LTBP4*-mutant cells is not an epiphenomenon, but is a consequence of LTBP4 deficiency.

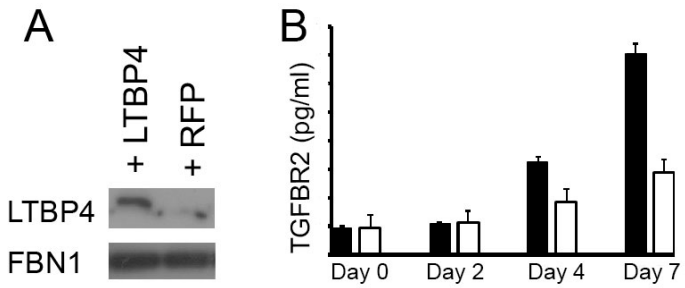


Figure 4.24 Rescue with recombinant LTBP4.

(*A*) LTBP4 expression in a HEK 293 cell clone stably transfected with a lentiviral vector expressing LTBP4 (+LTBP4) or a control vector expressing red fluorescent protein (+RFP). Blotting for fibrillin-1 (FBN1) served as a loading control (B) *LTBP4*-mutant human dermal fibroblasts were treated with conditioned media form HEK293 cells overexpressing LTBP4 (Solid bars) or transfected with a control vector expressing red fluorescent protein (empty bars) for different durations (Days 0-7).

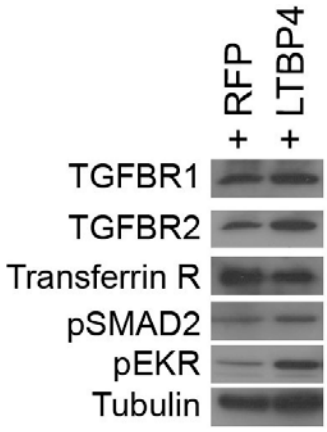


Figure 4.25 TGFβ receptors and TGFβ signaling in *LTBP4*-mutant cells after the treatment recombinant LTBP4.

LTBP4-mutant human dermal fibroblasts were treated with conditioned media form HEK293 cells overexpressing LTBP4 (+LTBP4) or red fluorescent protein (+RFP) for 7 days. Immunoblots were probed for TGFBR1, TGFBR2, transferrin receptor (transferrin R), pSMAD2, pERK and tubulin.

4.3.5. Discussion

In the present study, I investigated the role of LTBP4 in TGFβ signal transduction and in regulating the stability of TGFβ receptors. Extracellular TGFβ activity has been reported to be abnormally elevated in *LTBP4*-mutant CL patients[185], and the involvement of ECM-related regulation of TGFβ function in human genetic disease has been studied intensively, such as the *FBN1* gene mutant in Marfan syndrome. Importantly, both the canonical and non-canonical TGFβ signaling pathways were decreased in fibroblasts of *LTBP4*-mutant CL patients. Upon additional TGFβ1 stimulation, the difference in pSMAD2 expression between control and *LTBP4*-mutant cells became greater, representing a further relative decrease in SMAD2 and ERK phosphorylation. In addition, reduced amounts of TGFBR1 and TGFBR2 were detected in *LTBP4*-mutant human dermal fibroblasts compared to controls. These results suggested that the abnormally low amount of the TGFβ receptor complex attenuated the intracellular TGFβ signaling even when the cells encountered elevated extracellular TGFβ activity. These observations indicate that LTBP4 is involved in the stabilization of TGFβ receptors on the cell surface and prevents internalization. Consistent with this notion, the inhibition of endocytosis reduced the discrepancy in the TGFβ receptor expression between control and patient cells.

LTBP4 is thought to perform an essentially structural role in elastogenesis and an important functional role in regulating TGFβ signal transduction [22]. In an *Ltbp4S-/-* mouse model, lung abnormalities were partially rescued when *Tgfb2* expression was reduced, initially interpreted as

evidence for excess TGFβ signaling contributing to pathogenesis[2]. The interpretation of this finding, however, is complicated by the observation of paradoxically elevated TGFβ signaling in Tgfb2+/- mice[200]. Thus, it is possible that decreased TGFβ in Ltbp4S-/- is rescued by paradoxically elevated TGFβ signaling caused by the Tgfb2+/- genotype.

Among *LTBP4*-mutant CL patients, I found decreased intracellular TGFβ signaling despite higher extracellular TGFβ activity compared to controls. TGFβ signaling requires TGFBR1 and TGFBR2, which interact upon ligand binding, and the pathway is dynamic, with SMADs constantly shuttling between the cytoplasm and the nucleus[201]. Negative feedback mediated by Smad7 with the E3 ubiquitin ligases Smurf1 or Smurf2 has been proposed as an important mechanism to induce TGFBR1 degradation[202,203]. However, the results did not show up-regulation of either SMAD7 or SMAD6 in *LTBP4*-mutant dermal fibroblasts. These results suggested that the loss of LTBP4 may modify the receptors in a manner that would facilitate sequential degradation, contributing to the attenuation of the intracellular pathway. Thus, I reasoned that the expression of the TGFβ receptor complex may be altered in a way to explain reduced intracellular signaling instead of an upregulation of the inhibitory SMAD negative feedback pathway.

I found that reduced levels of TGFBR1 and TGFBR2 with shorter half-lives in *LTBP4*-mutant dermal fibroblasts compared to controls. Thus, LTBP4 plays a role in the stabilization and internalization of TGFβ receptors. When there is a lack of LTBP4, TGFβ receptors may become less stable and more prone to internalization, reducing the levels of TGFBR1 and TGFBR2 and,

consequently, attenuating the downstream signaling. Recently, Vizan *et al.* reported that TGFβ binding triggered the internalization of signaling-competent receptors from the cell surface and suggested that receptor dynamics regulated the long-term signaling behavior of TGFβ signaling but did not involve TGFβ-induced gene expression in a human keratinocyte cell line[204]. Unlike in Vizan's study, in our case, internalization did not occur in a context, which allowed continued signaling. Moreover, additional TGFβ1 led a further relative decrease in SMAD2 and ERK phosphorylation and enhanced the differential expression of pSMAD2 and pERK (Figure 4.11). Cell insensitivity to ligands was less likely, as exogenous TGFβ1 further decreased expression of intracellular TGFβ signal mediators. Consistent with my findings, several studies suggest that the internalization and trafficking of TGFβ receptors have important implications for disease mechanisms[205,206]. Thus, proteins that may manipulate the TGFβ receptor stability, such as LTBP4, may facilitate the development of new therapeutic approaches.

Prior evidence indicated that TGFβ receptors can be internalized via clathrin-dependent[145,207] or lipid raft/caveolae-dependent[141,208] endocytosis. Similarly, my results demonstrated that treatment with filipin and MDC eliminated the difference in the levels of TGFβ receptor expression between control and *LTBP4*-mutant cells. In addition, TGFBR1 and TGFBR2 levels were normalized in mutant cells by the lysosome inhibitor NH_4Cl but not by the proteasome inhibitor lactacystin. This finding indicates that segregation into the lysosome is the major processes involved in the degradation of TGFβ receptors in human dermal fibroblasts.

Importantly, treatment of mutant human fibroblasts recombinant LTBP4 stabilized TGFBR1 and TGFBR2. As the treatment took 7 days to reach maximal effect, I hypothesize that LTBP4 did not act in its monomeric form on a cell surface receptor. Rather, to stabilize TGFβ receptors, LTBP4 may require incorporation into the ECM, a more time-consuming process than direct receptor interaction. These results support my discovery of a new function for the extracellular matrix: regulating stability of cytokine receptors. Consistent with increased stability of TGFβ receptors, intracellular canonical and non-canonical TGFβ signaling pathways also normalized in *LTBP4*-mutant dermal fibroblasts treated with recombinant LTBP4.

In the absence of LTBP4, the dynamics of TGFBR1 and TGFBR2 are altered, and their stability is affected, potentially facilitating segregation of the receptors into the endocytic pathway. Further studies are required to investigate the interaction of LTBP4, ECM and the TGFβ receptor complex to elucidate the precise mechanism by which LTBP4 stabilizes the receptors. Many drugs that target TGFβ signaling have been developed, and some have reached phase III clinical trials for a couple of disease applications, but most of these drugs are TGFβ signaling antagonists. However, in *LTBP4*-mutant CL patients, ECM normalization may be useful as another avenue for drug development in the future.

5. CONCLUSION

The ECM regulates cellular proliferation, differentiation, homeostasis and death by interacting with dedicated membrane-bound integrin, discoidin and proteoglycan receptors and by regulating the activity of soluble cytokines. Accumulating evidence supports complex interactions between the ECM, soluble cytokines and their receptors, including ECM-dependent storage, release and diffusion of cytokines, proteolytic fragments of ECM molecules activating cytokine receptors, signaling crosstalk between ECM and cytokine receptors and multi-domain ECM molecules serving as localized, multivalent signal integrators[33]. My studies highlight a previously unrecognized activity of the ECM molecule, LTBP4, in stabilizing complexes of TGFBR1 and TGFBR2 for sustained signaling in response to TGFβ (Figure 5.1). In *LTBP4*-mutant cells, TGFβ receptors undergo ligand- and receptor kinase activity-dependent endocytosis and lysosmal degradation.

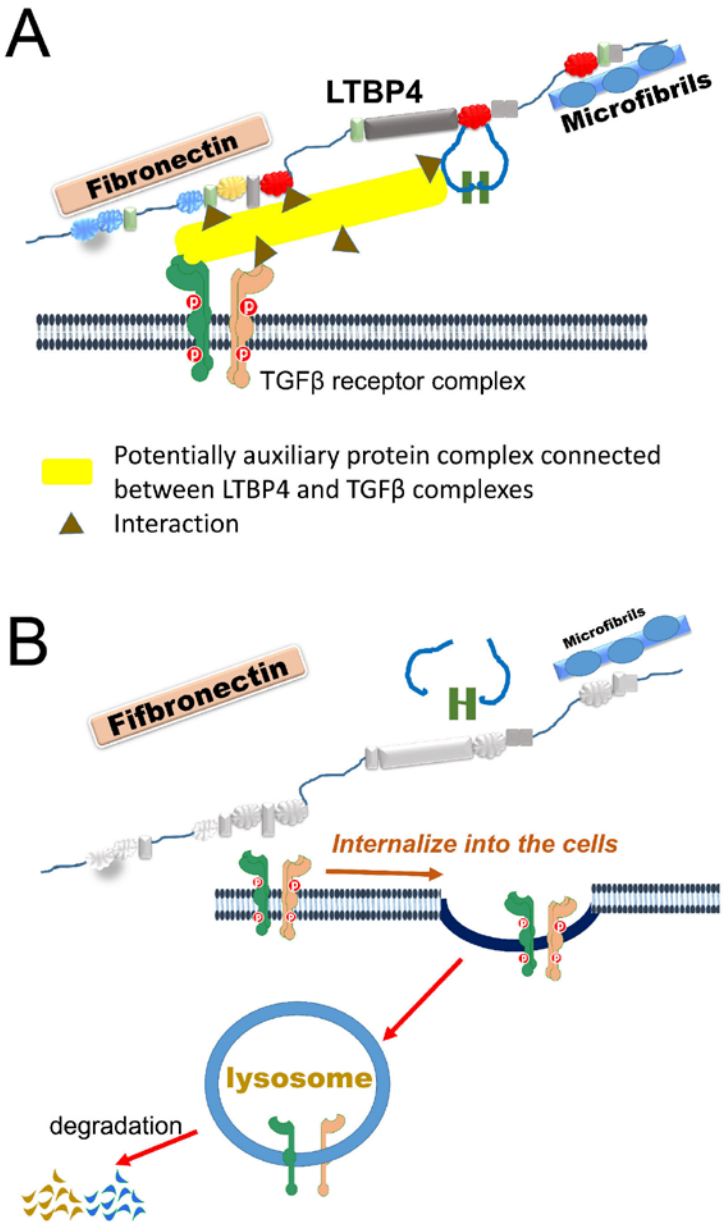


Figure 5.1 Model for potential LTBP4 stabilizing TGFβ receptors

(A) In normal cells, LTBP4 may stabilize TGFBR1 and TGFBR2 via matrix-related molecules (yellow rectangle).

(B) In the absence of LTBP4, the TGFBR1 and TGFBR2 complex become less stable and degraded by endocytosis. (▲): tan triangles represent possible binding interaction.

Experimental knockdown of LTBP4 destabilized the receptors leading to diminished TGFβ signaling, whereas supplementation with LTBP4 led to stabilization of the receptors, providing three independent lines of evidence for LTBP4 as a regulator of TGFβ receptor stability and activity. This new function complements known activities of LTBP4 in elastic fiber assembly and TGFβ sequestration[22,209].

The activity of TGFβ receptors is regulated, in part, by segregating the receptor complexes into different endocytic compartments. Endocytosis via clathrin coated pits into early endosome antigen-1 (EEA1) positive endosomes permits ongoing receptor signaling, whereas segregation into lipid rafts followed by caveolin-mediated endocytosis leads to the degradation of the receptor complex[141]. TGFBR3 enhances TGFβ signaling, by serving as a co-receptor of TGFβ and by facilitating the clathrin-mediated endocytosis of TGFBR1 and TGFBR2[210]. TGFβ receptor degradation in the absence of LTBP4 did not involve shifting the balance of the receptors from clathrin to caveolin-mediated endocytic pathways, as inhibition of either of the pathways stabilized the TGFβ receptor complex. Nor did the endocytic elimination of the receptor complex involve TGFBR3, as TGFBR3 levels remained unchanged in LTBP4 deficient cells.

The known degradation pathway of TGFβ receptors involves recruitment of ubiquitin ligases Smurf1[202] or Smurf2[203] to TGFBR1 by SMAD7, causing proteasomal and lysosomal degradation of the receptor complex. As SMAD7 is a transcriptional target of TGFβ, it serves as a negative feedback to limit the duration and intensity of signaling. As SMAD7 levels were not affected, it is plausible that TGFβ receptor degradation occurred by a different mechanism in *LTBP4* deficient

cells. Consistent with this notion, a proteasome inhibitor did not rescue TGFβ receptor loss in mutant cells, but a lysosome inhibitor did. Whereas the exact mechanism, which targets TGFβ receptors to degradation in the absence of LTBP4 remains unclear, recruitment of sorting nexins, known to traffic TGFβ receptors to lysosomes[211], is a likely candidate.

The receptor through which the cells sense the presence of LTBP4 and the precise domain of LTBP4 serving as a ligand for this putative receptor remain to be identified. Interaction of monomeric LTBP4 directly with TGFBR1 or TGFBR2 is unlikely, as the response of cells to LTBP4 supplementation takes at least 2 days, consistent with a requirement of LTBP4 to be incorporated into a complex, multimeric ECM structure. As LTBP4 is known to support cell adhesion and bind heparin [79], it may stabilize TGFβ receptors indirectly, by binding cell surface heparan sulfate proteoglycans, such as TGFBR3[210] or syndecan-2[212]. An alternative indirect mechanism may involve alterations of integrin binding to fibrillin-1 microfibrils[213] containing LTBP4, with subsequent integrin-TGFβ receptor crosstalk[214].

Those findings are relevant to understanding of the molecular mechanisms and to developing treatments for a range of inherited and complex diseases. ARCL1C is a rare recessive disorder associated with prematurely redundant and inelastic skin and disorganized ECM in the dermis. In addition to impaired assembly of the elastic fibers, the pathological alterations in these patients may be exacerbated by inadequate signaling by TGFβ, a key diver of ECM gene expression. With respect to treatment, LTBP4-like ligands may be effective in stabilizing the TGFβ receptor complex and allowing for adequate signal intensity and improved ECM production.

Several inherited aortic aneurysm syndromes, such as Marfan syndrome and Loeys-Dietz syndrome are associated with disorganized vascular ECM and excessive TGFβ signaling [215]. In Loeys-Dietz syndrome, excess TGFβ activity is considered paradoxical, as it is caused by heterozygous loss-of-function mutations in positive regulators of the pathway, such as TGFBR1, TGFBR2, TGFB2 or SMAD3. The current hypothesis to explain these observations is that reduced canonical TGFβ signaling results in the elimination of negative feedback mechanisms leading to excessive non-canonical TGFβ activity[215]. Molecular disease models and treatment approaches for these conditions may be refined by considering the consequences of these mutations and the resulting changes in the ECM for the stability of TGFβ receptors.

Tissue fibrosis is a common consequence of injury and is considered a barrier to tissue repair and functional recovery. It is characterized by overproliferation of fibroblasts, excessive TGFβ signaling and ECM production[8]. Increased tissue stiffness is known to serve as a positive feedback mechanism by enhancing integrin-mediated TGFβ activation [216]. These data, taken together with my results, suggest that increased LTBP4-mediated TGFβ receptor stabilization may be an additional positive feedback mechanism in this condition. In muscular dystrophy, myofibrillar damage results in extensive fibrosis and functional decline. Interestingly, LTBP4 has been identified as a modifying gene of muscular dystrophy in both mice[217] and humans[218], highlighting its function in modulating fibrosis.

5.1. FUTURE WORK

All results in my dissertation indicate that altered extracellular matrix lead to abnormal skin elasticity and potentially impact on abnormal TGFβ signaling I am planning to conduct the following experiments in order to extend the results we found.

5.1.1. Future work on skin elasticity

The predictive value (D) was generated from a small data set so it will be interesting to study the application and predictive value of D in other inherited connective diseases and in a larger sample of CL patients.

1. The implication of the predictive value (D) in other inherited diseases with hypoelasitc skin.
2. Apply the equation generating the predictive value (D) to other dataset including control individuals, CL patients, and other inherited diseases with altered ECM assembly.

5.1.2. Future work on the impact of LTBP4 on TGFβ signaling

It is important reproduce my findings *in vivo*, even though LTBP4 knock-down cell lines successfully reproduce my finding in *LTBP4*-mutant skin fibroblasts, including reduction of intracellular TGFβ singling and TGFβ receptor complexes. *In vivo* may be carried out using skin tissue samples from knock-out mice.

1. Interrogate the expression of pSMAD2, pERK, TGFBR1 and TGFBR2 in skin fibroblasts from *Ltbp4S*-/- mice to establish that our observations are generalizable to other mammals.
2. Obtain skin tissue of *Ltbp4S*-/- knockout mice and interrogate the expression of same proteins by immunoblotting to obtain *in vivo* evidence.

Additionally, it is highly plausible that LTBP4 in extracellular space stabilizes the TGFβ receptor complexes on the cell membrane. Therefore, it is relevant to prove direct or indirect interactions between LTBP4 and TGFβ receptors.

1. Direct interaction: Solid-phase binding essay can be used to demonstrate the potential direct interaction between TGFβ receptors and LTBP4.
2. Indirect interaction: Co-immunoprecipitation can be used. If the result of direct interaction is negative, it is plausible that an auxiliary protein may help to form a large complex, composed of LTBP4 and TGFβ receptors, to stabilize receptors on the cell membrane.

APPENDIX: PCRs for the generation of the pLOC-LTBP4-MycHis construct

Vectors designed for the generation of pLOC-LTBP4-MycHis and the schematic representation of the constructs are shown in Figure 6.1. The primers used, their sequence, melting temperature (Tm), the number of cycles for the reactions, annealing temperatures (AT) and extension durations (ED) used in the PCRs, are summarized in Table 6.1.

PCR 1: the amplification of approximately 1000 bp, a part of LTBP4 with NotI restriction site, ending at exon 35 and excluding the stop codon.

PCR 2: the amplification of a 100 bp product, including the last base pairs of exon 35, a Myc site, His tag, a stop codon and an AscI restriction site.

PCR 3: the complementary parts (exon 35) of products in PCR 1 and PCR 2, annealed and hybridized; The whole construct was amplified using short variants of the outside primers.

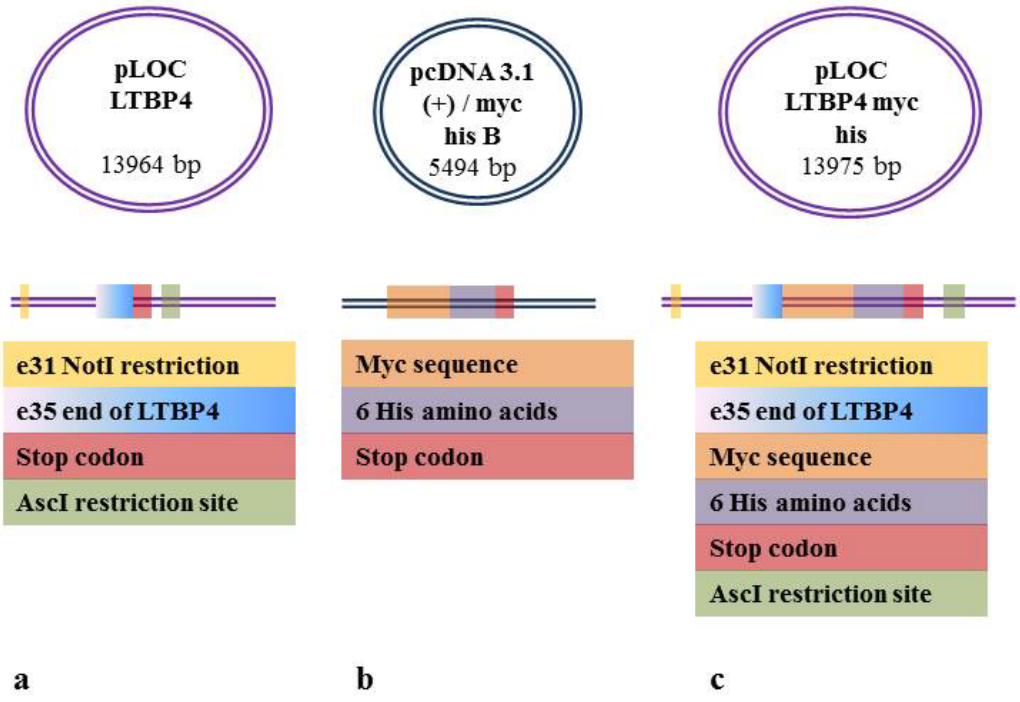
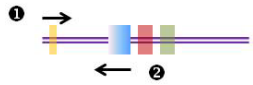
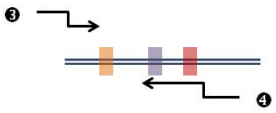
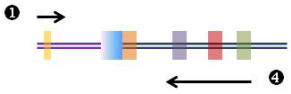


Figure 6.1 Vectors used for the creation of the pLOC-LTBP4-MycHis plasmid.

The pLOC LTBP4 plasmid with its NotI restriction site (yellow) in the LTBP4 protein (pale blue). The protein is terminated by the stop codon (red) and an AscI restriction site (lime green). (b) The Myc (orange) and His sequence (purple) of the pcDNA 3.1(+) / MycHis vector were used as template for the addition of the tag to the pLOC LTBP4 plasmid via PCR. (c) The desired plasmid, after the insertion of the tag via PCR, with all relevant elements combined. Modified from Christine Weckenmann's Master's thesis, 2012.

Table 6.1 Primers and PCR conditions for the generation of the pLOC-LTBP4-MycHis vector

Product size	Name of primers	Tm [°C]	Primers sequence (5' → 3')
 1000 bp	❶hLTBP4_e31_3s	68.6	GAGGGCGGCCGCTGTGTCAACACTGT
	❷hLTBP4_e35_3a	78.5	GGCCCGGGGCCGTGCGGGCGCACAGT
 100 bp	❸hLTBP4_e35_3s_Myc	74.7	ACTGTGCGCCCGCACGGCCCCGGGCCGAACAAAAACTCATCTCAGAAGAG
	❹pcDNA_His_pLOC_ASCI_a	69.1	GGGCGCGCCTCAATGGTGATGGTGATGATGA
 1100 bp	①hLTBP4_e31_4s	63.5	GCGGCCGCTGTGTCAACACT
	④pcDNA_His_pLOC_ASCI_2a	61.3	GGCGCGCCTCAATGGTGAT

Modified from Christine Weckenmann's Master's thesis, 2012

BIBLIOGRAPHY

1 Sterner-Kock, A. *et al.* Disruption of the gene encoding the latent transforming growth factor-beta binding protein 4 (LTBP-4) causes abnormal lung development, cardiomyopathy, and colorectal cancer. *Genes & development* **16**, 2264-2273, doi:10.1101/gad.229102 (2002).

2 Dabovic, B. *et al.* Dual functions for LTBP in lung development: LTBP-4 independently modulates elastogenesis and TGF-beta activity. *Journal of cellular physiology* **219**, 14-22, doi:10.1002/jcp.21643 (2009).

3 Urban, Z. *et al.* Mutations in LTBP4 cause a syndrome of impaired pulmonary, gastrointestinal, genitourinary, musculoskeletal, and dermal development. *American journal of human genetics* **85**, 593-605, doi:10.1016/j.ajhg.2009.09.013 (2009).

4 Carta, L. *et al.* p38 MAPK is an early determinant of promiscuous Smad2/3 signaling in the aortas of fibrillin-1 (Fbn1)-null mice. *The Journal of biological chemistry* **284**, 5630-5636, doi:10.1074/jbc.M806962200 (2009).

5 Flavell, R. A., Sanjabi, S., Wrzesinski, S. H. & Licona-Limon, P. The polarization of immune cells in the tumour environment by TGFbeta. *Nature reviews. Immunology* **10**, 554-567, doi:10.1038/nri2808 (2010).

6 Derynck, R. & Akhurst, R. J. Differentiation plasticity regulated by TGF-beta family proteins in development and disease. *Nature cell biology* **9**, 1000-1004, doi:10.1038/ncb434 (2007).

7 Akhurst, R. J. & Hata, A. Targeting the TGFbeta signalling pathway in disease. *Nature reviews. Drug discovery* **11**, 790-811, doi:10.1038/nrd3810 (2012).

8 Margadant, C. & Sonnenberg, A. Integrin-TGF-beta crosstalk in fibrosis, cancer and wound healing. *EMBO reports* **11**, 97-105, doi:10.1038/embor.2009.276 (2010).

9 Esser, P., Weller, M., Heimann, K. & Wiedemann, P. [Thrombospondin and its importance in proliferative retinal diseases]. *Fortschritte der Ophthalmologie : Zeitschrift der Deutschen Ophthalmologischen Gesellschaft* **88**, 337-340 (1991).

10 Pawlaczyk, M., Lelonkiewicz, M. & Wieczorowski, M. Age-dependent biomechanical properties of the skin. *Postepy dermatologii i alergologii* **30**, 302-306, doi:10.5114/pdia.2013.38359 (2013).

11 Payne, P. A. Measurement of properties and function of skin. *Clinical physics and physiological measurement : an official journal of the Hospital Physicists' Association, Deutsche Gesellschaft fur Medizinische Physik and the European Federation of Organisations for Medical Physics* **12**, 105-129 (1991).

12 De Filippo, R. E. & Atala, A. Stretch and growth: the molecular and physiologic influences of tissue expansion. *Plastic and reconstructive surgery* **109**, 2450-2462 (2002).

13 Silver, F. H., Siperko, L. M. & Seehra, G. P. Mechanobiology of force transduction in dermal tissue. *Skin research and technology : official journal of International Society for Bioengineering and the Skin* **9**, 3-23 (2003).

14 Wong, V. W., Akaishi, S., Longaker, M. T. & Gurtner, G. C. Pushing back: wound mechanotransduction in repair and regeneration. *The Journal of investigative dermatology* **131**, 2186-2196, doi:10.1038/jid.2011.212 (2011).

15 Simpson, C. L., Patel, D. M. & Green, K. J. Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. *Nature reviews. Molecular cell biology* **12**, 565-580, doi:10.1038/nrm3175 (2011).

16 Takei, T., Mills, I., Arai, K. & Sumpio, B. E. Molecular basis for tissue expansion: clinical implications for the surgeon. *Plastic and reconstructive surgery* **102**, 247-258 (1998).

17 Tepole, A. B., Gosain, A. K. & Kuhl, E. Stretching skin: The physiological limit and beyond. *International journal of non-linear mechanics* **47**, 938-949, doi:10.1016/j.ijnonlinmec.2011.07.006 (2012).

18 Hendriks, F. M. *et al.* A numerical-experimental method to characterize the non-linear mechanical behaviour of human skin. *Skin research and technology : official journal of International Society for Bioengineering and the Skin* **9**, 274-283 (2003).

19 Berardesca, E., de Rigal, J., Leveque, J. L. & Maibach, H. I. In vivo biophysical characterization of skin physiological differences in races. *Dermatologica* **182**, 89-93 (1991).

20 Sugihara, T., Ohura, T., Homma, K. & Igawa, H. H. The extensibility in human skin: variation according to age and site. *British journal of plastic surgery* **44**, 418-422 (1991).

21 Vogel, H. G. [Biomechanical studies of human and animal skin in vivo and in vitro]. *Zeitschrift fur Hautkrankheiten* **59**, 1098-1100 (1984).

22 Todorovic, V. & Rifkin, D. B. LTBPs, more than just an escort service. *Journal of cellular biochemistry* **113**, 410-418, doi:10.1002/jcb.23385 (2012).

23 Hynes, R. O. Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687 (2002).

24 Berrier, A. L. & Yamada, K. M. Cell-matrix adhesion. *Journal of cellular physiology* **213**, 565-573, doi:10.1002/jcp.21237 (2007).

25 Legate, K. R., Wickstrom, S. A. & Fassler, R. Genetic and cell biological analysis of integrin outside-in signaling. *Genes & development* **23**, 397-418, doi:10.1101/gad.1758709 (2009).

26 Discher, D. E., Mooney, D. J. & Zandstra, P. W. Growth factors, matrices, and forces combine and control stem cells. *Science* **324**, 1673-1677, doi:10.1126/science.1171643 (2009).

27 Geiger, B., Spatz, J. P. & Bershadsky, A. D. Environmental sensing through focal adhesions. *Nature reviews. Molecular cell biology* **10**, 21-33, doi:10.1038/nrm2593 (2009).

28 Kolacna, L. *et al.* Biochemical and biophysical aspects of collagen nanostructure in the extracellular matrix. *Physiological research / Academia Scientiarum Bohemoslovaca* **56 Suppl 1**, S51-60 (2007).

29 Pelouch, V., Dixon, I. M., Golfman, L., Beamish, R. E. & Dhalla, N. S. Role of extracellular matrix proteins in heart function. *Molecular and cellular biochemistry* **129**, 101-120 (1993).

30 Pelouch, V., Milerova, M., Ostadal, B., Hucin, B. & Samanek, M. Differences between atrial and ventricular protein profiling in children with congenital heart disease. *Molecular and cellular biochemistry* **147**, 43-49 (1995).

31 Grunert, M., Nurcombe, V. & Cool, S. M. Stem cell fate decisions: the role of heparan sulfate in the control of autocrine and paracrine signals. *Current stem cell research & therapy* **3**, 1-8 (2008).

32 Saksela, O., Moscatelli, D., Sommer, A. & Rifkin, D. B. Endothelial cell-derived heparan sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. *The Journal of cell biology* **107**, 743-751 (1988).

33 Hynes, R. O. The extracellular matrix: not just pretty fibrils. *Science* **326**, 1216-1219, doi:10.1126/science.1176009 (2009).

34 Mohammadi, M., Olsen, S. K. & Goetz, R. A protein canyon in the FGF-FGF receptor dimer selects from an a la carte menu of heparan sulfate motifs. *Current opinion in structural biology* **15**, 506-516, doi:10.1016/j.sbi.2005.09.002 (2005).

35 Shi, Y. & Massague, J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700 (2003).

36 Alam, N. *et al.* The integrin-growth factor receptor duet. *Journal of cellular physiology* **213**, 649-653, doi:10.1002/jcp.21278 (2007).

37 Panayotou, G., End, P., Aumailley, M., Timpl, R. & Engel, J. Domains of laminin with growth-factor activity. *Cell* **56**, 93-101 (1989).

38 Schenk, S. *et al.* Binding to EGF receptor of a laminin-5 EGF-like fragment liberated during MMP-dependent mammary gland involution. *The Journal of cell biology* **161**, 197-209, doi:10.1083/jcb.200208145 (2003).

39 Iyer, A. K., Tran, K. T., Griffith, L. & Wells, A. Cell surface restriction of EGFR by a tenascin cytotactin-encoded EGF-like repeat is preferential for motility-related signaling. *Journal of cellular physiology* **214**, 504-512, doi:10.1002/jcp.21232 (2008).

40 Rifkin, D. B. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *The Journal of biological chemistry* **280**, 7409-7412, doi:10.1074/jbc.R400029200 (2005).

41 ten Dijke, P. & Arthur, H. M. Extracellular control of TGFbeta signalling in vascular development and disease. *Nature reviews. Molecular cell biology* **8**, 857-869, doi:10.1038/nrm2262 (2007).

42 Ramirez, F., Sakai, L. Y., Rifkin, D. B. & Dietz, H. C. Extracellular microfibrils in development and disease. *Cellular and molecular life sciences : CMLS* **64**, 2437-2446, doi:10.1007/s00018-007-7166-z (2007).

43 Ramirez, F. & Dietz, H. C. Extracellular microfibrils in vertebrate development and disease processes. *The Journal of biological chemistry* **284**, 14677-14681, doi:10.1074/jbc.R900004200 (2009).

44 Carta, L. *et al.* Fibrillins 1 and 2 perform partially overlapping functions during aortic development. *The Journal of biological chemistry* **281**, 8016-8023, doi:10.1074/jbc.M511599200 (2006).

45 Judge, D. P. *et al.* Evidence for a critical contribution of haploinsufficiency in the complex pathogenesis of Marfan syndrome. *The Journal of clinical investigation* **114**, 172-181, doi:10.1172/JCI20641 (2004).

46 Arteaga-Solis, E. *et al.* Regulation of limb patterning by extracellular microfibrils. *The Journal of cell biology* **154**, 275-281 (2001).

47 George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H. & Hynes, R. O. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. *Development* **119**, 1079-1091 (1993).

48 Todorovic, V. *et al.* Long form of latent TGF-beta binding protein 1 (Ltbp1L) is essential for cardiac outflow tract septation and remodeling. *Development* **134**, 3723-3732, doi:10.1242/dev.008599 (2007).

49 Goumans, M. J. & Mummery, C. Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *The International journal of developmental biology* **44**, 253-265 (2000).

50 Tang, Y. *et al.* Epistatic interactions between modifier genes confer strain-specific redundancy for Tgfb1 in developmental angiogenesis. *Genomics* **85**, 60-70, doi:10.1016/j.ygeno.2004.09.003 (2005).

51 Loeys, B. L. *et al.* Aneurysm syndromes caused by mutations in the TGF-beta receptor. *The New England journal of medicine* **355**, 788-798, doi:10.1056/NEJMoa055695 (2006).

52 Stenvers, K. L. *et al.* Heart and liver defects and reduced transforming growth factor beta2 sensitivity in transforming growth factor beta type III receptor-deficient embryos. *Molecular and cellular biology* **23**, 4371-4385 (2003).

53 Pece-Barbara, N. *et al.* Endoglin null endothelial cells proliferate faster and are more responsive to transforming growth factor beta1 with higher affinity receptors and an activated Alk1 pathway. *The Journal of biological chemistry* **280**, 27800-27808, doi:10.1074/jbc.M503471200 (2005).

54 Kojima, M. *et al.* Human subperitoneal fibroblast and cancer cell interaction creates microenvironment that enhances tumor progression and metastasis. *PloS one* **9**, e88018, doi:10.1371/journal.pone.0088018 (2014).

55 Moro, N., Mauch, C. & Zigrino, P. Metalloproteinases in melanoma. *European journal of cell biology*, doi:10.1016/j.ejcb.2014.01.002 (2014).

56 Chan, T. M., Lin, H. P. & Lin, S. Z. Letter to the Editor: In situ altering of the extracellular matrix to direct the programming of endogenous stem cells. *Stem cells*, doi:10.1002/stem.1693 (2014).

57 Baldwin, A. K., Simpson, A., Steer, R., Cain, S. A. & Kielty, C. M. Elastic fibres in health and disease. *Expert reviews in molecular medicine* **15**, e8, doi:10.1017/erm.2013.9 (2013).

58 Kielty, C. M. Elastic fibres in health and disease. *Expert reviews in molecular medicine* **8**, 1-23, doi:10.1017/S146239940600007X (2006).

59 Bax, D. V., Rodgers, U. R., Bilek, M. M. & Weiss, A. S. Cell adhesion to tropoelastin is mediated via the C-terminal GRKRK motif and integrin alphaVbeta3. *The Journal of biological chemistry* **284**, 28616-28623, doi:10.1074/jbc.M109.017525 (2009).

60 Broekelmann, T. J. *et al.* Tropoelastin interacts with cell-surface glycosaminoglycans via its COOH-terminal domain. *The Journal of biological chemistry* **280**, 40939-40947, doi:10.1074/jbc.M507309200 (2005).

61 Ramirez, F., Sakai, L. Y., Dietz, H. C. & Rifkin, D. B. Fibrillin microfibrils: multipurpose extracellular networks in organismal physiology. *Physiological genomics* **19**, 151-154, doi:10.1152/physiolgenomics.00092.2004 (2004).

62 Hubmacher, D., Tiedemann, K. & Reinhardt, D. P. Fibrillins: from biogenesis of microfibrils to signaling functions. *Current topics in developmental biology* **75**, 93-123, doi:10.1016/S0070-2153(06)75004-9 (2006).

63 Piha-Gossack, A., Sossin, W. & Reinhardt, D. P. The evolution of extracellular fibrillins and their functional domains. *PloS one* **7**, e33560, doi:10.1371/journal.pone.0033560 (2012).

64 Kinsey, R. *et al.* Fibrillin-1 microfibril deposition is dependent on fibronectin assembly. *Journal of cell science* **121**, 2696-2704, doi:10.1242/jcs.029819 (2008).

65 Sabatier, L. *et al.* Fibrillin assembly requires fibronectin. *Molecular biology of the cell* **20**, 846-858, doi:10.1091/mbc.E08-08-0830 (2009).

66 Reber-Muller, S., Spissinger, T., Schuchert, P., Spring, J. & Schmid, V. An extracellular matrix protein of jellyfish homologous to mammalian fibrillins forms different fibrils depending on the life stage of the animal. *Developmental biology* **169**, 662-672, doi:10.1006/dbio.1995.1177 (1995).

67 Dallas, S. L. *et al.* Fibronectin regulates latent transforming growth factor-beta (TGF beta) by controlling matrix assembly of latent TGF beta-binding protein-1. *The Journal of biological chemistry* **280**, 18871-18880, doi:10.1074/jbc.M410762200 (2005).

68 Reinhardt, D. P. *et al.* Fibrillin-1: organization in microfibrils and structural properties. *Journal of molecular biology* **258**, 104-116, doi:10.1006/jmbi.1996.0237 (1996).

69 Baldock, C. *et al.* The supramolecular organization of fibrillin-rich microfibrils. *The Journal of cell biology* **152**, 1045-1056 (2001).

70 Wang, M. C., Lu, Y. & Baldock, C. Fibrillin microfibrils: a key role for the interbead region in elasticity. *Journal of molecular biology* **388**, 168-179, doi:10.1016/j.jmb.2009.02.062 (2009).

71 Jensen, S. A., Robertson, I. B. & Handford, P. A. Dissecting the fibrillin microfibril: structural insights into organization and function. *Structure* **20**, 215-225, doi:10.1016/j.str.2011.12.008 (2012).

72 Bax, D. V. *et al.* Cell adhesion to fibrillin-1: identification of an Arg-Gly-Asp-dependent synergy region and a heparin-binding site that regulates focal adhesion formation. *Journal of cell science* **120**, 1383-1392, doi:10.1242/jcs.003954 (2007).

73 Jovanovic, J. *et al.* alphaVbeta6 is a novel receptor for human fibrillin-1. Comparative studies of molecular determinants underlying integrin-rgd affinity and specificity. *The Journal of biological chemistry* **282**, 6743-6751, doi:10.1074/jbc.M607008200 (2007).

74 Tiedemann, K., Batge, B., Muller, P. K. & Reinhardt, D. P. Interactions of fibrillin-1 with heparin/heparan sulfate, implications for microfibrillar assembly. *The Journal of biological chemistry* **276**, 36035-36042, doi:10.1074/jbc.M104985200 (2001).

75 Ritty, T. M., Broekelmann, T. J., Werneck, C. C. & Mecham, R. P. Fibrillin-1 and -2 contain heparin-binding sites important for matrix deposition and that support cell attachment. *The Biochemical journal* **375**, 425-432, doi:10.1042/BJ20030649 (2003).

76 Cain, S. A. *et al.* Heparan sulfate regulates fibrillin-1 N- and C-terminal interactions. *The Journal of biological chemistry* **283**, 27017-27027, doi:10.1074/jbc.M803373200 (2008).

77 Cain, S. A. *et al.* Fibrillin-1 interactions with heparin. Implications for microfibril and elastic fiber assembly. *The Journal of biological chemistry* **280**, 30526-30537, doi:10.1074/jbc.M501390200 (2005).

78 Zilberberg, L. *et al.* Specificity of latent TGF-beta binding protein (LTBP) incorporation into matrix: role of fibrillins and fibronectin. *Journal of cellular physiology* **227**, 3828-3836, doi:10.1002/jcp.24094 (2012).

79 Kantola, A. K., Keski-Oja, J. & Koli, K. Fibronectin and heparin binding domains of latent TGF-beta binding protein (LTBP)-4 mediate matrix targeting and cell adhesion. *Experimental cell research* **314**, 2488-2500, doi:10.1016/j.yexcr.2008.05.010 (2008).

80 Hubmacher, D. & Apte, S. S. Genetic and functional linkage between ADAMTS superfamily proteins and fibrillin-1: a novel mechanism influencing microfibril assembly and function. *Cellular and molecular life sciences : CMLS* **68**, 3137-3148, doi:10.1007/s00018-011-0780-9 (2011).

81 Dagoneau, N. *et al.* ADAMTS10 mutations in autosomal recessive Weill-Marchesani syndrome. *American journal of human genetics* **75**, 801-806, doi:10.1086/425231 (2004).

82 Cain, S. A., McGovern, A., Baldwin, A. K., Baldock, C. & Kielty, C. M. Fibrillin-1 mutations causing Weill-Marchesani syndrome and acromicric and geleophysic dysplasias disrupt heparan sulfate interactions. *PloS one* **7**, e48634, doi:10.1371/journal.pone.0048634 (2012).

83 Bader, H. L. *et al.* A disintegrin-like and metalloprotease domain containing thrombospondin type 1 motif-like 5 (ADAMTSL5) is a novel fibrillin-1-, fibrillin-2-, and heparin-binding member of the ADAMTS superfamily containing a netrin-like module. *Matrix biology : journal of the International Society for Matrix Biology* **31**, 398-411, doi:10.1016/j.matbio.2012.09.003 (2012).

84 Gabriel, L. A. *et al.* ADAMTSL4, a secreted glycoprotein widely distributed in the eye, binds fibrillin-1 microfibrils and accelerates microfibril biogenesis. *Investigative ophthalmology & visual science* **53**, 461-469, doi:10.1167/iovs.10-5955 (2012).

85 Tsutsui, K. *et al.* ADAMTSL-6 is a novel extracellular matrix protein that binds to fibrillin-1 and promotes fibrillin-1 fibril formation. *The Journal of biological chemistry* **285**, 4870-4882, doi:10.1074/jbc.M109.076919 (2010).

86 Werneck, C. C. *et al.* Mice lacking the extracellular matrix protein MAGP1 display delayed thrombotic occlusion following vessel injury. *Blood* **111**, 4137-4144, doi:10.1182/blood-2007-07-101733 (2008).

87 Jensen, S. A., Reinhardt, D. P., Gibson, M. A. & Weiss, A. S. Protein interaction studies of MAGP-1 with tropoelastin and fibrillin-1. *The Journal of biological chemistry* **276**, 39661-39666, doi:10.1074/jbc.M104533200 (2001).

88 Rock, M. J. *et al.* Molecular basis of elastic fiber formation. Critical interactions and a tropoelastin-fibrillin-1 cross-link. *The Journal of biological chemistry* **279**, 23748-23758, doi:10.1074/jbc.M400212200 (2004).

89 Lemaire, R., Bayle, J., Mecham, R. P. & Lafyatis, R. Microfibril-associated MAGP-2 stimulates elastic fiber assembly. *The Journal of biological chemistry* **282**, 800-808, doi:10.1074/jbc.M609692200 (2007).

90 Penner, A. S., Rock, M. J., Kielty, C. M. & Shipley, J. M. Microfibril-associated glycoprotein-2 interacts with fibrillin-1 and fibrillin-2 suggesting a role for MAGP-2 in elastic fiber assembly. *The Journal of biological chemistry* **277**, 35044-35049, doi:10.1074/jbc.M206363200 (2002).

91 Hanssen, E., Hew, F. H., Moore, E. & Gibson, M. A. MAGP-2 has multiple binding regions on fibrillins and has covalent periodic association with fibrillin-containing microfibrils. *The Journal of biological chemistry* **279**, 29185-29194, doi:10.1074/jbc.M313672200 (2004).

92 Weinbaum, J. S. *et al.* Deficiency in microfibril-associated glycoprotein-1 leads to complex phenotypes in multiple organ systems. *The Journal of biological chemistry* **283**, 25533-25543, doi:10.1074/jbc.M709962200 (2008).

93 Massam-Wu, T. *et al.* Assembly of fibrillin microfibrils governs extracellular deposition of latent TGF beta. *Journal of cell science* **123**, 3006-3018, doi:10.1242/jcs.073437 (2010).

94 Yeo, G. C., Keeley, F. W. & Weiss, A. S. Coacervation of tropoelastin. *Advances in colloid and interface science* **167**, 94-103, doi:10.1016/j.cis.2010.10.003 (2011).

95 Kozel, B. A. *et al.* Elastic fiber formation: a dynamic view of extracellular matrix assembly using timer reporters. *Journal of cellular physiology* **207**, 87-96, doi:10.1002/jcp.20546 (2006).

96 Clarke, A. W. *et al.* Tropoelastin massively associates during coacervation to form quantized protein spheres. *Biochemistry* **45**, 9989-9996, doi:10.1021/bi0610092 (2006).

97 Tu, Y. & Weiss, A. S. Glycosaminoglycan-mediated coacervation of tropoelastin abolishes the critical concentration, accelerates coacervate formation, and facilitates spherule fusion: implications for tropoelastin microassembly. *Biomacromolecules* **9**, 1739-1744, doi:10.1021/bm7013153 (2008).

98 Hirai, M. *et al.* Fibulin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage in vivo. *The Journal of cell biology* **176**, 1061-1071, doi:10.1083/jcb.200611026 (2007).

99 Mecham, R. P. Elastin synthesis and fiber assembly. *Annals of the New York Academy of Sciences* **624**, 137-146 (1991).

100 Pepe, A. *et al.* Dissection of human tropoelastin: supramolecular organization of polypeptide sequences coded by particular exons. *Matrix biology : journal of the International Society for Matrix Biology* **24**, 96-109, doi:10.1016/j.matbio.2005.01.004 (2005).

101 Cirulis, J. T. & Keeley, F. W. Kinetics and morphology of self-assembly of an elastin-like polypeptide based on the alternating domain arrangement of human tropoelastin. *Biochemistry* **49**, 5726-5733, doi:10.1021/bi100468v (2010).

102 Wagenseil, J. E. & Mecham, R. P. New insights into elastic fiber assembly. *Birth defects research. Part C, Embryo today : reviews* **81**, 229-240, doi:10.1002/bdrc.20111 (2007).

103 Yanagisawa, H. & Davis, E. C. Unraveling the mechanism of elastic fiber assembly: The roles of short fibulins. *The international journal of biochemistry & cell biology* **42**, 1084-1093, doi:10.1016/j.biocel.2010.03.009 (2010).

104 McLaughlin, P. J. *et al.* Lack of fibulin-3 causes early aging and herniation, but not macular degeneration in mice. *Human molecular genetics* **16**, 3059-3070, doi:10.1093/hmg/ddm264 (2007).

105 Choudhury, R. *et al.* Differential regulation of elastic fiber formation by fibulin-4 and -5. *The Journal of biological chemistry* **284**, 24553-24567, doi:10.1074/jbc.M109.019364 (2009).

106 Kobayashi, N. *et al.* A comparative analysis of the fibulin protein family. Biochemical characterization, binding interactions, and tissue localization. *The Journal of biological chemistry* **282**, 11805-11816, doi:10.1074/jbc.M611029200 (2007).

107 Horiguchi, M. *et al.* Fibulin-4 conducts proper elastogenesis via interaction with cross-linking enzyme lysyl oxidase. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 19029-19034, doi:10.1073/pnas.0908268106 (2009).

108 Liu, X. *et al.* Elastic fiber homeostasis requires lysyl oxidase-like 1 protein. *Nature genetics* **36**, 178-182, doi:10.1038/ng1297 (2004).

109 Siegel, R. C., Pinnell, S. R. & Martin, G. R. Cross-linking of collagen and elastin. Properties of lysyl oxidase. *Biochemistry* **9**, 4486-4492 (1970).

110 Romero, N., Tinker, D., Hyde, D. & Rucker, R. B. Role of plasma and serum proteases in the degradation of elastin. *Archives of biochemistry and biophysics* **244**, 161-168 (1986).

111 Bedell-Hogan, D., Trackman, P., Abrams, W., Rosenbloom, J. & Kagan, H. Oxidation, cross-linking, and insolubilization of recombinant tropoelastin by purified lysyl oxidase. *The Journal of biological chemistry* **268**, 10345-10350 (1993).

112 Pohlers, D. *et al.* TGF-beta and fibrosis in different organs - molecular pathway imprints. *Biochimica et biophysica acta* **1792**, 746-756, doi:10.1016/j.bbadis.2009.06.004 (2009).

113 Wahl, S. M. & Chen, W. Transforming growth factor-beta-induced regulatory T cells referee inflammatory and autoimmune diseases. *Arthritis research & therapy* **7**, 62-68, doi:10.1186/ar1504 (2005).

114 Todorovic, V. *et al.* Latent TGF-beta binding proteins. *The international journal of biochemistry & cell biology* **37**, 38-41, doi:10.1016/j.biocel.2004.03.011 (2005).

115 Saharinen, J. & Keski-Oja, J. Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF-beta. *Mol Biol Cell* **11**, 2691-2704 (2000).

116 Saharinen, J., Taipale, J. & Keski-Oja, J. Association of the small latent transforming growth factor-beta with an eight cysteine repeat of its binding protein LTBP-1. *EMBO J* **15**, 245-253 (1996).

117 Gleizes, P. E., Beavis, R. C., Mazzieri, R., Shen, B. & Rifkin, D. B. Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1. *J Biol Chem* **271**, 29891-29896 (1996).

118 Koli, K., Hyytiainen, M., Ryynanen, M. J. & Keski-Oja, J. Sequential deposition of latent TGF-beta binding proteins (LTBPs) during formation of the extracellular matrix in human lung fibroblasts. *Exp Cell Res* **310**, 370-382, doi:10.1016/j.yexcr.2005.08.008 (2005).

119 Unsold, C., Hyytiainen, M., Bruckner-Tuderman, L. & Keski-Oja, J. Latent TGF-beta binding protein LTBP-1 contains three potential extracellular matrix interacting domains. *J Cell Sci* **114**, 187-197 (2001).

120 Shipley, J. M. *et al.* Developmental expression of latent transforming growth factor beta binding protein 2 and its requirement early in mouse development. *Molecular and cellular biology* **20**, 4879-4887 (2000).

121 Yoshinaga, K. *et al.* Perturbation of transforming growth factor (TGF)-beta1 association with latent TGF-beta binding protein yields inflammation and tumors. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 18758-18763, doi:10.1073/pnas.0805411105 (2008).

122 Shull, M. M. *et al.* Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* **359**, 693-699, doi:10.1038/359693a0 (1992).

123 Yang, Z. *et al.* Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. *The Journal of cell biology* **176**, 787-793, doi:10.1083/jcb.200611044 (2007).

124 Sanford, L. P. *et al.* TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* **124**, 2659-2670 (1997).

125 Kaartinen, V. *et al.* Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nature genetics* **11**, 415-421, doi:10.1038/ng1295-415 (1995).

126 Robertson, I., Jensen, S. & Handford, P. TB domain proteins: evolutionary insights into the multifaceted roles of fibrillins and LTBPs. *The Biochemical journal* **433**, 263-276, doi:10.1042/BJ20101320 (2011).

127 Ali, M. *et al.* Null mutations in LTBP2 cause primary congenital glaucoma. *American journal of human genetics* **84**, 664-671, doi:10.1016/j.ajhg.2009.03.017 (2009).

128 Dabovic, B. *et al.* Bone abnormalities in latent TGF-[beta] binding protein (Ltbp)-3-null mice indicate a role for Ltbp-3 in modulating TGF-[beta] bioavailability. *The Journal of cell biology* **156**, 227-232, doi:10.1083/jcb.200111080 (2002).

129 Colarossi, C. *et al.* Lung alveolar septation defects in Ltbp-3-null mice. *The American journal of pathology* **167**, 419-428, doi:10.1016/S0002-9440(10)62986-0 (2005).

130 Noor, A. *et al.* Oligodontia is caused by mutation in LTBP3, the gene encoding latent TGF-beta binding protein 3. *American journal of human genetics* **84**, 519-523, doi:10.1016/j.ajhg.2009.03.007 (2009).

131 Kang, J. S., Liu, C. & Derynck, R. New regulatory mechanisms of TGF-beta receptor function. *Trends in cell biology* **19**, 385-394, doi:10.1016/j.tcb.2009.05.008 (2009).

132 Santibanez, J. F., Quintanilla, M. & Bernabeu, C. TGF-beta/TGF-beta receptor system and its role in physiological and pathological conditions. *Clin Sci (Lond)* **121**, 233-251, doi:10.1042/CS20110086 (2011).

133 Huminiecki, L. *et al.* Emergence, development and diversification of the TGF-beta signalling pathway within the animal kingdom. *BMC evolutionary biology* **9**, 28, doi:10.1186/1471-2148-9-28 (2009).

134 Ross, S. & Hill, C. S. How the Smads regulate transcription. *The international journal of biochemistry & cell biology* **40**, 383-408, doi:10.1016/j.biocel.2007.09.006 (2008).

135 Itoh, S. & ten Dijke, P. Negative regulation of TGF-beta receptor/Smad signal transduction. *Current opinion in cell biology* **19**, 176-184, doi:10.1016/j.ceb.2007.02.015 (2007).

136 Derynck, R. & Zhang, Y. E. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-584, doi:10.1038/nature02006 (2003).

137 Moustakas, A. & Heldin, C. H. Non-Smad TGF-beta signals. *Journal of cell science* **118**, 3573-3584, doi:10.1242/jcs.02554 (2005).

138 Yamashita, M. *et al.* TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. *Molecular cell* **31**, 918-924, doi:10.1016/j.molcel.2008.09.002 (2008).

139 Sorrentino, A. *et al.* The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. *Nature cell biology* **10**, 1199-1207, doi:10.1038/ncb1780 (2008).

140 Guo, X. & Wang, X. F. Signaling cross-talk between TGF-beta/BMP and other pathways. *Cell research* **19**, 71-88, doi:10.1038/cr.2008.302 (2009).

141 Di Guglielmo, G. M., Le Roy, C., Goodfellow, A. F. & Wrana, J. L. Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. *Nature cell biology* **5**, 410-421, doi:10.1038/ncb975 (2003).

142 Mitchell, H., Choudhury, A., Pagano, R. E. & Leof, E. B. Ligand-dependent and -independent transforming growth factor-beta receptor recycling regulated by clathrin-mediated endocytosis and Rab11. *Molecular biology of the cell* **15**, 4166-4178, doi:10.1091/mbc.E04-03-0245 (2004).

143 Ehrlich, M., Shmuely, A. & Henis, Y. I. A single internalization signal from the di-leucine family is critical for constitutive endocytosis of the type II TGF-beta receptor. *Journal of cell science* **114**, 1777-1786 (2001).

144 Penheiter, S. G. *et al.* Internalization-dependent and -independent requirements for transforming growth factor beta receptor signaling via the Smad pathway. *Molecular and cellular biology* **22**, 4750-4759 (2002).

145 Yao, D., Ehrlich, M., Henis, Y. I. & Leof, E. B. Transforming growth factor-beta receptors interact with AP2 by direct binding to beta2 subunit. *Molecular biology of the cell* **13**, 4001-4012, doi:10.1091/mbc.02-07-0104 (2002).

146 Hayes, S., Chawla, A. & Corvera, S. TGF beta receptor internalization into EEA1-enriched early endosomes: role in signaling to Smad2. *The Journal of cell biology* **158**, 1239-1249 (2002).

147 Brown, D. A. & London, E. Functions of lipid rafts in biological membranes. *Annual review of cell and developmental biology* **14**, 111-136, doi:10.1146/annurev.cellbio.14.1.111 (1998).

148 Razani, B. *et al.* Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. *The Journal of biological chemistry* **276**, 6727-6738, doi:10.1074/jbc.M008340200 (2001).

149 Rajagopal, R., Ishii, S. & Beebe, D. C. Intracellular mediators of transforming growth factor beta superfamily signaling localize to endosomes in chicken embryo and mouse lenses in vivo. *BMC cell biology* **8**, 25, doi:10.1186/1471-2121-8-25 (2007).

150 Berk, D. R., Bentley, D. D., Bayliss, S. J., Lind, A. & Urban, Z. Cutis laxa: a review. *Journal of the American Academy of Dermatology* **66**, 842 e841-817, doi:10.1016/j.jaad.2011.01.004 (2012).

151 Hu, Q. *et al.* Mechanisms of emphysema in autosomal dominant cutis laxa. *Matrix biology : journal of the International Society for Matrix Biology* **29**, 621-628, doi:10.1016/j.matbio.2010.06.005 (2010).

152 Callewaert, B. *et al.* New insights into the pathogenesis of autosomal-dominant cutis laxa with report of five ELN mutations. *Human mutation* **32**, 445-455, doi:10.1002/humu.21462 (2011).

153 Loeys, B. *et al.* Homozygosity for a missense mutation in fibulin-5 (FBLN5) results in a severe form of cutis laxa. *Human molecular genetics* **11**, 2113-2118 (2002).

154 Hucthagowder, V. *et al.* Fibulin-4: a novel gene for an autosomal recessive cutis laxa syndrome. *American journal of human genetics* **78**, 1075-1080, doi:10.1086/504304 (2006).

155 Dasouki, M. *et al.* Compound heterozygous mutations in fibulin-4 causing neonatal lethal pulmonary artery occlusion, aortic aneurysm, arachnodactyly, and mild cutis laxa. *American journal of medical genetics. Part A* **143A**, 2635-2641, doi:10.1002/ajmg.a.31980 (2007).

156 Renard, M. *et al.* Altered TGFbeta signaling and cardiovascular manifestations in patients with autosomal recessive cutis laxa type I caused by fibulin-4 deficiency. *European journal of human genetics : EJHG* **18**, 895-901, doi:10.1038/ejhg.2010.45 (2010).

157 Kornak, U. *et al.* Impaired glycosylation and cutis laxa caused by mutations in the vesicular H+-ATPase subunit ATP6V0A2. *Nature genetics* **40**, 32-34, doi:10.1038/ng.2007.45 (2008).

158 Guernsey, D. L. *et al.* Mutation in pyrroline-5-carboxylate reductase 1 gene in families with cutis laxa type 2. *American journal of human genetics* **85**, 120-129, doi:10.1016/j.ajhg.2009.06.008 (2009).

159 Reversade, B. *et al.* Mutations in PYCR1 cause cutis laxa with progeroid features. *Nature genetics* **41**, 1016-1021, doi:10.1038/ng.413 (2009).

160 Byers, P. H. *et al.* X-linked cutis laxa: defective cross-link formation in collagen due to decreased lysyl oxidase activity. *The New England journal of medicine* **303**, 61-65, doi:10.1056/NEJM198007103030201 (1980).

161 Moller, L. B. *et al.* Identification and analysis of 21 novel disease-causing amino acid substitutions in the conserved part of ATP7A. *Human mutation* **26**, 84-93, doi:10.1002/humu.20190 (2005).

162 Kennerson, M. L. *et al.* Missense mutations in the copper transporter gene ATP7A cause X-linked distal hereditary motor neuropathy. *American journal of human genetics* **86**, 343-352, doi:10.1016/j.ajhg.2010.01.027 (2010).

163 Bicknell, L. S. *et al.* A missense mutation in ALDH18A1, encoding Delta1-pyrroline-5-carboxylate synthase (P5CS), causes an autosomal recessive neurocutaneous syndrome. *European journal of human genetics : EJHG* **16**, 1176-1186, doi:10.1038/ejhg.2008.91 (2008).

164 Skidmore, D. L. *et al.* Further expansion of the phenotypic spectrum associated with mutations in ALDH18A1, encoding Delta(1)-pyrroline-5-carboxylate synthase (P5CS). *American journal of medical genetics. Part A* **155A**, 1848-1856, doi:10.1002/ajmg.a.34057 (2011).

165 Hennies, H. C. *et al.* Gerodermia osteodysplastica is caused by mutations in SCYL1BP1, a Rab-6 interacting golgin. *Nature genetics* **40**, 1410-1412, doi:10.1038/ng.252 (2008).

166 Basel-Vanagaite, L. *et al.* RIN2 deficiency results in macrocephaly, alopecia, cutis laxa, and scoliosis: MACS syndrome. *American journal of human genetics* **85**, 254-263, doi:10.1016/j.ajhg.2009.07.001 (2009).

167 Coucke, P. J. *et al.* Mutations in the facilitative glucose transporter GLUT10 alter angiogenesis and cause arterial tortuosity syndrome. *Nature genetics* **38**, 452-457, doi:10.1038/ng1764 (2006).

168 Boileau, C. *et al.* TGFB2 mutations cause familial thoracic aortic aneurysms and dissections associated with mild systemic features of Marfan syndrome. *Nat Genet* **44**, 916-921, doi:10.1038/ng.2348 (2012).

169 Goudie, D. R. *et al.* Multiple self-healing squamous epithelioma is caused by a disease-specific spectrum of mutations in TGFBR1. *Nat Genet* **43**, 365-369, doi:10.1038/ng.780 (2011).

170 Akhurst, R. J. The paradoxical TGF-beta vasculopathies. *Nat Genet* **44**, 838-839, doi:10.1038/ng.2366 (2012).

171 Ignotz, R. A., Endo, T. & Massague, J. Regulation of fibronectin and type I collagen mRNA levels by transforming growth factor-beta. *The Journal of biological chemistry* **262**, 6443-6446 (1987).

172 Ahmed, W. *et al.* Signaling pathway by which TGF-beta1 increases expression of latent TGF-beta binding protein-2 at the transcriptional level. *Connective tissue research* **37**, 263-276 (1998).

173 Weikkolainen, K., Keski-Oja, J. & Koli, K. Expression of latent TGF-beta binding protein LTBP-1 is hormonally regulated in normal and transformed human lung fibroblasts. *Growth factors* **21**, 51-60 (2003).

174 Kahari, V. M. *et al.* Transforming growth factor-beta up-regulates elastin gene expression in human skin fibroblasts. Evidence for post-transcriptional modulation. *Laboratory investigation; a journal of technical methods and pathology* **66**, 580-588 (1992).

175 Kucich, U., Rosenbloom, J. C., Abrams, W. R., Bashir, M. M. & Rosenbloom, J. Stabilization of elastin mRNA by TGF-beta: initial characterization of signaling pathway. *American journal of respiratory cell and molecular biology* **17**, 10-16, doi:10.1165/ajrcmb.17.1.2816 (1997).

176 Boak, A. M. *et al.* Regulation of lysyl oxidase expression in lung fibroblasts by transforming growth factor-beta 1 and prostaglandin E2. *American journal of respiratory cell and molecular biology* **11**, 751-755, doi:10.1165/ajrcmb.11.6.7946403 (1994).

177 Kim, D. J. *et al.* Lysyl oxidase like 4, a novel target gene of TGF-beta1 signaling, can negatively regulate TGF-beta1-induced cell motility in PLC/PRF/5 hepatoma cells. *Biochemical and biophysical research communications* **373**, 521-527, doi:10.1016/j.bbrc.2008.06.071 (2008).

178 Kuang, P. P., Joyce-Brady, M., Zhang, X. H., Jean, J. C. & Goldstein, R. H. Fibulin-5 gene expression in human lung fibroblasts is regulated by TGF-beta and

phosphatidylinositol 3-kinase activity. *American journal of physiology. Cell physiology* **291**, C1412-1421, doi:10.1152/ajpcell.00087.2006 (2006).

179 van Rooij, E. *et al.* Dysregulation of microRNAs after myocardial infarction reveals a role of miR-29 in cardiac fibrosis. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 13027-13032, doi:10.1073/pnas.0805038105 (2008).

180 Neptune, E. R. *et al.* Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. *Nature genetics* **33**, 407-411, doi:10.1038/ng1116 (2003).

181 Hanada, K. *et al.* Perturbations of vascular homeostasis and aortic valve abnormalities in fibulin-4 deficient mice. *Circulation research* **100**, 738-746, doi:10.1161/01.RES.0000260181.19449.95 (2007).

182 Fischer, B. *et al.* Further characterization of ATP6V0A2-related autosomal recessive cutis laxa. *Human genetics* **131**, 1761-1773, doi:10.1007/s00439-012-1197-8 (2012).

183 Piszczek, J., Dalton, B., Peters, T., Ruether, D. & Urbanski, S. Extensive necrotizing fasciitis associated with sunitinib therapy. *Clinical genitourinary cancer* **12**, e47-49, doi:10.1016/j.clgc.2013.11.017 (2014).

184 Abe, M. *et al.* An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Analytical biochemistry* **216**, 276-284, doi:10.1006/abio.1994.1042 (1994).

185 Callewaert, B. *et al.* Comprehensive clinical and molecular analysis of 12 families with type 1 recessive cutis laxa. *Human mutation* **34**, 111-121, doi:10.1002/humu.22165 (2013).

186 Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402-408, doi:10.1006/meth.2001.1262 (2001).

187 Zor, T. & Selinger, Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Analytical biochemistry* **236**, 302-308, doi:10.1006/abio.1996.0171 (1996).

188 Szabo, Z. *et al.* Aortic aneurysmal disease and cutis laxa caused by defects in the elastin gene. *Journal of medical genetics* **43**, 255-258, doi:10.1136/jmg.2005.034157 (2006).

189 Escoffier, C. *et al.* Age-related mechanical properties of human skin: an in vivo study. *The Journal of investigative dermatology* **93**, 353-357 (1989).

190 Grahame, R. & Holt, P. J. The influence of ageing on the in vivo elasticity of human skin. *Gerontologia* **15**, 121-139 (1969).

191 Diridollou, S. *et al.* In vivo model of the mechanical properties of the human skin under suction. *Skin research and technology : official journal of International Society for Bioengineering and the Skin* **6**, 214-221 (2000).

192 Agache, P. G., Monneur, C., Leveque, J. L. & De Rigal, J. Mechanical properties and Young's modulus of human skin in vivo. *Archives of dermatological research* **269**, 221-232 (1980).

193 Manschot, J. F. & Brakkee, A. J. The measurement and modelling of the mechanical properties of human skin in vivo--I. The measurement. *Journal of biomechanics* **19**, 511-515 (1986).

194 Grahame, R. & Beighton, P. The physical properties of skin in cutis laxa. *The British journal of dermatology* **84**, 326-329 (1971).

195 Jensen, S. A., Iqbal, S., Lowe, E. D., Redfield, C. & Handford, P. A. Structure and interdomain interactions of a hybrid domain: a disulphide-rich module of the fibrillin/LTBP superfamily of matrix proteins. *Structure* **17**, 759-768, doi:S0969-2126(09)00161-0 [pii] 10.1016/j.str.2009.03.014 (2009).

196 Lack, J. *et al.* Solution structure of the third TB domain from LTBP1 provides insight into assembly of the large latent complex that sequesters latent TGF-beta. *Journal of molecular biology* **334**, 281-291, doi:S0022283603012087 [pii] (2003).

197 Callewaert, B. *et al.* New insights into the pathogenesis of autosomal-dominant cutis laxa with report of five ELN mutations. *Human mutation* **32**, 445-455, doi:10.1002/humu.21462 (2011).

198 Renard, M., Holm, T., Callewaert, B., Adès, L., Baspinar, O., Pickart, A., Dasouki, M., Rauch, A., Coucke, P., Dietz, H., De Paepe, A., Loeys, B. *Major cardiovascular involvement in autosomal recessive cutis laxa patients confirms fibulin-4 as a key player in vascular elastic fiber formation* (Center for Medical Genetics, Ghent university Hospital, Belgium, 2010).

199 Loeys, B. L. *et al.* A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nature genetics* **37**, 275-281, doi:10.1038/ng1511 (2005).

200 Lindsay, M. E. *et al.* Loss-of-function mutations in TGFB2 cause a syndromic presentation of thoracic aortic aneurysm. *Nature genetics* **44**, 922-927, doi:10.1038/ng.2349 (2012).

201 Schmierer, B., Tournier, A. L., Bates, P. A. & Hill, C. S. Mathematical modeling identifies Smad nucleocytoplasmic shuttling as a dynamic signal-interpreting system. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 6608-6613, doi:10.1073/pnas.0710134105 (2008).

202 Ebisawa, T. *et al.* Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *The Journal of biological chemistry* **276**, 12477-12480, doi:10.1074/jbc.C100008200 (2001).

203 Kavsak, P. *et al.* Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Molecular cell* **6**, 1365-1375 (2000).

204 Vizan, P. *et al.* Controlling long-term signaling: receptor dynamics determine attenuation and refractory behavior of the TGF-beta pathway. *Science signaling* **6**, ra106, doi:10.1126/scisignal.2004416 (2013).

205 Park, I., Son, H. K., Che, Z. M. & Kim, J. A novel gain-of-function mutation of TGF-beta receptor II promotes cancer progression via delayed receptor internalization in oral squamous cell carcinoma. *Cancer letters* **315**, 161-169, doi:10.1016/j.canlet.2011.09.036 (2012).

206 Del Galdo, F. *et al.* Decreased expression of caveolin 1 in patients with systemic sclerosis: crucial role in the pathogenesis of tissue fibrosis. *Arthritis and rheumatism* **58**, 2854-2865, doi:10.1002/art.23791 (2008).

207 Anders, R. A., Arline, S. L., Dore, J. J. & Leof, E. B. Distinct endocytic responses of heteromeric and homomeric transforming growth factor beta receptors. *Molecular biology of the cell* **8**, 2133-2143 (1997).

208 Chen, C. L. *et al.* Cholesterol suppresses cellular TGF-beta responsiveness: implications in atherogenesis. *Journal of cell science* **120**, 3509-3521, doi:10.1242/jcs.006916 (2007).

209 Noda, K. *et al.* Latent TGF-beta binding protein 4 promotes elastic fiber assembly by interacting with fibulin-5. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 2852-2857, doi:10.1073/pnas.1215779110 (2013).

210 McLean, S. & Di Guglielmo, G. M. TGF beta (transforming growth factor beta) receptor type III directs clathrin-mediated endocytosis of TGF beta receptor types I and II. *The Biochemical journal* **429**, 137-145, doi:10.1042/BJ20091598 (2010).

211 Hao, X. *et al.* SNX25 regulates TGF-beta signaling by enhancing the receptor degradation. *Cellular signalling* **23**, 935-946, doi:10.1016/j.cellsig.2011.01.022 (2011).

212 Shi, Y. *et al.* Syndecan-2 exerts antifibrotic effects by promoting caveolin-1-mediated transforming growth factor-beta receptor I internalization and inhibiting transforming growth factor-beta1 signaling. *American journal of respiratory and critical care medicine* **188**, 831-841, doi:10.1164/rccm.201303-0434OC (2013).

213 Bax, D. V. *et al.* Cell adhesion to fibrillin-1 molecules and microfibrils is mediated by alpha 5 beta 1 and alpha v beta 3 integrins. *The Journal of biological chemistry* **278**, 34605-34616, doi:10.1074/jbc.M303159200 (2003).

214 Scaffidi, A. K. *et al.* alpha(v)beta(3) Integrin interacts with the transforming growth factor beta (TGFbeta) type II receptor to potentiate the proliferative effects of TGFbeta1 in living human lung fibroblasts. *The Journal of biological chemistry* **279**, 37726-37733, doi:10.1074/jbc.M403010200 (2004).

215 Lindsay, M. E. & Dietz, H. C. Lessons on the pathogenesis of aneurysm from heritable conditions. *Nature* **473**, 308-316, doi:10.1038/nature10145 (2011).

216 Wells, R. G. & Discher, D. E. Matrix elasticity, cytoskeletal tension, and TGF-beta: the insoluble and soluble meet. *Science signaling* **1**, pe13, doi:10.1126/stke.110pe13 (2008).

217 Heydemann, A. *et al.* Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. *The Journal of clinical investigation* **119**, 3703-3712, doi:10.1172/JCI39845 (2009).

218 Flanigan, K. M. *et al.* LTBP4 genotype predicts age of ambulatory loss in duchenne muscular dystrophy. *Annals of neurology*, doi:10.1002/ana.23819 (2012).