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Fibrillin microfibrils and proteases, key integrators of fibrotic pathways

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ABSTRACT

Supramolecular networks composed of multi-domain ECM proteins represent intricate cellular microenvironments which are required to balance tissue homeostasis and direct remodeling. Structural deficiency in ECM proteins results in imbalances in ECM-cell communication resulting often times in fibrotic reactions. To understand how individual components of the ECM integrate communication with the cell surface by presenting growth factors or providing fine-tuned biomechanical properties is mandatory for gaining a better understanding of disease mechanisms in the quest for new therapeutic approaches. Here we provide an overview about what we can learn from inherited connective tissue disorders caused primarily by mutations in fibrillin-1 and binding partners as well as by altered ECM processing leading to defined structural changes and similar functional knock-in mouse models. We will utilize this knowledge to propose new molecular hypotheses which should be tested in future studies.

Contents

1. Introduction

1.1. The evolved repertoire of ECM ligands for building functional cellular microenvironments

Considering that the complexity of extracellular microenvironments has evolved during evolution, it is obvious that increasing molecular

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diversity reflects the different mechanical demands of individual organisms. Fibrillin proteins (fibrillin-1, -2, and -3 in humans) are among the more ancient ECM proteins emerging considerably before fibronectin (first emergence in tunicates) prior to the split of cnidaria and bilateria [1–3]. This fact is reflected by its early appearance during embryogenesis (8 cell stage, E3) [4] and significant role in the assembly of life sustaining ECM networks, as genetic ablation of fibrillin microfibrils (FMF) production results in early embryonic lethality [3]. Fibrillins are large (350 kD) cysteine-rich glycoproteins that assemble into small diameter (10–12 nm) extracellular "microfibrils" with a characteristic "beads-on-a-string"-like appearance that are ubiquitously found in the connective tissue space [5]. FMF represent the core scaffolds for elastic fiber formation, since tropoelastin deposition and maturation into cross-linked elastin is orchestrated via functional interactions among the fibrillin-1 and -2 ligands fibulin-4, fibulin-5, latent TGF-β binding protein-2 and -4 (LTBP-2, -4), and lysyloxidase (LOX) [6–13]. At the same time, FMF control access of cellular stimuli by targeting pluripotent growth factors such as TGF-β superfamily, and Wnt ligands either by binding them directly (BMPs: bone morphogenetic proteins [14,15]), or their carriers (LTBPs [12,16]), and modulators (EMILINs: Elastin-Microfibril-Interface-Located-proteins [17]). In addition, it was recently reported that the FMF ligand LTBP-2 which was shown not to interact with TGF-β [18] harbors a high affinity binding site for FGF-2 and may thereby influence its activity in fibrotic tissues [19]. In this context, it is important to note that LTBPs and EMILINs which are required to sequester and fine tune the activity of growth factors fulfil also structural functions in FMF assembly and elastic fiber formation. For instance, both LTBP-2 and LTBP-4 interact with fibulin-5 and were shown to play a crucial role in elastic fiber formation in vitro and in vivo [6,7,9,13]. EMILIN-1 was also shown to interact with elastin, fibulin-4, and fibulin-5, and *Emilin1* null mice show alterations in elastic fiber morphology [20,21]. Despite previous studies describing dysregulated TGF-β signaling in lungs, colon, and kidney [22–24] caused by genetic ablation of LTBP-4S (S: short isoform) in mice, recent findings suggest that LTBP-4 does not play a major role in TGF-β sequestration. Mutational ablation of TGF-β binding to LTBP-4 in mice resulted in no visable abnormalities suggesting that LTBP-4 has no TGF-β-dependent functions [8]. However, a recent report showed that genetic ablation of both LTBP-4 isoforms (S: short, L: long) leads to increased TGF-β activity in the lung [24] consistent with the previous observation that LTBP-4L interacts more effectively with TGF-β [25]. Interestingly, a recent report showed that LTBP-2 and -4 serve important functions in the process of FMF assembly in vitro and in vivo [26].

Overall, FMF can be viewed as dynamic architectural elements which integrate structural mechanisms required for building functional networks with defined mechanical properties, which are also crucial to exert control over signaling events from the cell surface into the nucleus [27].

The importance of FMF in organ formation and tissue homeostasis is illustrated by the various connective tissue disorders caused by mutations in the fibrillin genes, summarized as "fibrillinopathies". The fibrillinopathies represent disorders with distinctive, common, or opposing phenotypes affecting the musculoskeletal, cardiovascular, ocular, pulmonary, central nervous and dermal system [28]. The most prevalent disorder caused by *FBN1* mutation is Marfan syndrome (MFS) which is characterized by long bone overgrowth (tall stature, arachnodactyly), lack of muscle tone, hyperelastic skin, and cardiovascular complications such as aneurysm formation at the aortic root [28]. According to the Universal *FBN1* Mutation Database (http://www.umd.be/FBN1/) more than 3000 *FBN1* mutations in all exons have been identified leading to MFS while also rare *FBN1* mutations lead to long bone undergrowth (short stature, brachydactyly), hypermuscularity, thick skin, and no signs of cardiovascular involvement [29]. This suggests that FMF control tissue homeostasis probably by balancing growth factor signals. To study what control FMF and associated ligands exert over fibrotic reactions, the skin represents an informative organ. Fibrillin mutations result in skin phenotypes ranging from thick, stiff, and fibrotic skin to thin hyperelastic and fragile skin (Table 1).

Table 1
Congenital connective tissue disorders characterized by fibrotic skin conditions caused by mutations in genes involved in TGF-β ECM targeting, signal transduction, and activation.

Genetic disorder	Gene	OMIM#	Inheritance	Reference
ACMICD	*FBN1*	102370	AD	[105]
GPHYSD1	*ADAMTSL2*	231050	AR	[104]
GPHYSD2	*FBN1*	614185	AD	[105]
GPHYSD3	*LTBP3*	617809	AD	[108]
LP	Microduplications of chromosome 8q22.1	151200	AD	[119]
MFS	*FBN1*	154700	AD	http://www.umd.be/FBN1/
MONA	*MMP2*	259600	AR	[124]
MYHRS	*SMAD4*	139210	AD	[109]
SSKS	*FBN1*	184900	AD	[93]
WMS1	*ADAMTS10*	277600	AR	[191]
WMS2	*FBN1*	608328	AD	[29,101,105]
WMS3	*LTBP2*	614819	AR	[115]
WMS4	*ADAMTS17*	613195	AR	[26,117]

AD: autosomal dominant; AR: autosomal recessive.

2. The murine fibrilllin-1 tight skin mutation, a popular model for exploring new antifibrotic therapeutic avenues

An involvement of fibrillin-1 in skin fibrosis was first suggested when a large in-frame duplication in *Fbn1* was identified in the tight-skin (tsk) mouse [30]. Tsk mice develop cutaneous fibrosis and serve as a disease model for systemic sclerosis (SSc). It could be demonstrated that the expression of elongated, 418 kDa tsk fibrillin-1 leads to microfibrils with compromised ultrastructure [31,32]. Such structurally abnormal FMF were proposed to be functionally deficient and instable, however, how this translates into disease mechanisms remains unclear. To gain mechanistic insights into the pathomechanisms leading to fibrosis in tsk mice the therapeutic potential of numerous drugs has been explored in preclinical studies. An overview of drugs tested in tsk mice and identification numbers of attempted clinical trials administering them to SSc patients is provided in Table 2.

2.1. Dysregulated TGF-β and Wnt signaling contribute to the tight skin phenotype

Since LTBPs directly target TGF-β in the form of large latent complexes (LLCs) to FMF (Fig. 1), one of the most frequently proposed ideas is that deficient FMF in tsk mice are not capable anymore to maintain TGF-β sequestration leading to aberrant activation of the growth factor. Therefore, aberrantly increased TGF-β signaling is thought to be the most upstream signaling event responsible for the tsk phenotype. This notion is supported by genetic breeding experiments using *Tgfbeta1* heterozygous null mice which showed that deletion of one allele of the TGF-β-1 gene resulted in diminished dermal thickness [33]. Since the interaction repertoire of FMF is not fully known, also other dysregulated signaling events may contribute to the tsk pathogenesis. For example, it was found that EMILIN-2, a newly identified ligand of FMF [17] which modulates the bioavailability of Wnt ligands [34], was transcriptionally upregulated together with several genes involved in Wnt signaling [35]. In this context it is interesting to note that treatment of tsk mice with specific inhibitors of glycogen synthase kinase 3β (GSK-3) which regulates the phosphorylation and subsequent degradation of β-catenin, and thereby preventing aberrant activation of the canonical Wnt pathway aggravated fibrosis [36]. This illustrates the complex contributions of different signaling pathways triggered by the tsk fibrillin-1 mutation and also indicates the challenges of pharmacological intervention.

Table 2
Preclinical studies with fibrillin-1 mutant mice.

Mouse line	Drug	Mechanism	Reference	Clinical trial numbers for studies with SSc and MFS patients
Fbn1 tsk	SB216763	Inhibition of glycogen synthase kinase 3β (GSK-3)	[36]	
Fbn1 tsk	Imatinib mesylate	Inhibiting tyrosine kinase activity of c-Abl	[37,38]	NCT00555581, NCT00512902, NCT00506831, NCT01309997 NCT00613171, NCT00479934, NCT00573326
Fbn1 tsk	17-DMAG	HSP90 inhibitor	[39]	
Fbn1 tsk	Pomalidomide	Immunomodulatory, analogue of thalidomide	[40]	NCT01559129
Fbn1 tsk	TG101209	JAK2 kinase inhibitor	[41]	
Fbn1 tsk	C188-9	Inhibitor of STAT3	[42]	
Fbn1 tsk	Paquinimod	S100A9 inhibitor	[46]	NCT01487551
Fbn1 tsk	Anti-CD20 mab	CD20 blocking antibody	[55]	NCT01086540 NCT01309997
Fbn1 tsk	Anti-CD40L mAb	CD40 blocking antibody	[56]	
Fbn1 tsk	BAFF antagonist	Inhibition of potent B-cell survival factor	[57]	NCT01670565
Fbn1 tsk	Anti-IL-4 mab	IL-4 neutralizing antibody	[66]	
Fbn1 tsk	Rapamycin	mTOR pathway inhibition	[73]	NCT00241189 NCT03365869
Fbn1 tsk	T0901317	Liver X receptor agonist	[74]	
Fbn1 tsk	SUN-C8257	Mast cell chymase inhibitor	[75]	
Fbn1 tsk	BAY41-2272	sGC stimulator	[79]	
Fbn1 tsk	BAY63-2521 (riociguat)	sGC stimulator	[80]	NCT02283762
Fbn1 tsk	Edaravone	Free radical scavenger	[83]	
Fbn1 tsk	Halofuginone	Febrifugine analogue, inhibitor collagen type I synthesis	[89]	
Fbn1 W1570C	β1aAb	β1 integrin-activating antibody	[95]	
Fbn1 D1545E	β1aAb	β1 integrin-activating antibody,	[95]	
Fbn1 W1570C	TGFβNAb, 1D11	TGF-β neutralizing antibody	[95]	
Fbn1 D1545E	TGFβNAb, 1D11	TGF-β neutralizing antibody	[95]	
Fbn1 D1545E	Refametinib (RDEA119, Bay 86-9766)	MEK-1 and -2 inhibitor	[95]	
Fbn1 C1039G	TGFβNAb, 1D11	TGF-β neutralizing antibody	[111]	
Fbn1 C1039G	Losartan	Angiotensin II type I receptor inhibitor	[111]	NCT00723801, NCT01145612, NCT00763893, NCT00651235, NCT00782327, NCT00593710, NCT00429364, NCT00683124
Fbn1 mgR	Doxycycline	MMP inhibitor	[123]	NCT01949233

2.2. Treatment of tsk mice with cancer drugs

Therapeutic approaches conducted so far to reduce fibrosis in tsk mice focused on mechanisms to reduce excessive TGF-β signaling, to suppress the production of inflammatory cytokines, or to block the function of immune receptors (an overview is provided in Table 2). However, numerous available drugs designed to effectively target cancer exert their function by interference with downstream TGF-β signaling events. Testing these cancer drugs in tsk mice provided insightful information for assessing new treatment options for SSc. For instance, intraperitoneal injection of tsk mice with the cancer drug imatinib mesylate (Gleevec/Glivec; Novartis, Basel, Switzerland) which targets TGF and PDGF signaling pathways by inhibiting the tyrosine kinase activity of c-Abl and PDGF resulted in reduced dermal and hypodermal thickening, but also prevented the differentiation of resting fibroblasts into myofibroblasts [37]. However, when used in patients with diffuse scleroderma, imatinib failed to demonstrate any improvement after 6 months of treatment [38]. In addition, adverse events of imatinib were frequently observed and tolerability was poor [38]. Inhibitors of the chaperone Hsp90 are used as cancer drugs since its clients represent a variety of proteins required for survival of cancer cells. Intracutaneous injection of the Hsp90 inhibitor 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) resulted in potent antifibrotic effects in tsk mice through blockage of canonical TGF-β signaling [39]. In addition, daily oral uptake of the cancer drug pomalidomide (CC-4047), a functional analogue of thalidomide that belongs to a new class of immunomodulators, was shown to be effective to prevent skin fibrosis and reduce TGF-β target genes in tsk mice [40]. Interference with STAT-JAK signaling showed also promising results in tsk mice. Oral uptake of TG101209, a small molecule JAK2 (Janus kinase)-selective kinase inhibitor known to be effective in myeloma cells and lung cancer models, reduced skin fibrosis in tsk mice and prevented the stimulatory effects of TGF-β on fibroblasts [41]. Also pharmacological intervention with STAT3 signaling which plays a pivotal role in hepatocellular carcinoma survival, growth, angiogenesis, and metastasis decreased fibrosis in tsk mice [42]. Intraperitoneal injections of C188-9, a small molecule inhibitor of STAT3, led to a reduction of skin fibrosis, myofibroblast accumulation, pro-fibrotic gene expression and collagen deposition [42]. S100A4 and S100A9 are both calcium binding proteins with regulatory functions in cell homeostasis, proliferation and differentiation that have been described to promote cancer progression and metastasis [43]. RNAi mediated depletion of S100A4 in fibroblasts inhibited the stimulatory effects of TGF-β on collagen synthesis in fibroblasts [44]. In addition, hypodermal thickening was reduced by 66% in S100A4$^{-/-}$ mice carrying the tsk fibrillin-1 mutant allele [44]. Interestingly, the S100A9 inhibitor paquinimod belongs to a class of orally active quinoline-3-carboxamide (quinoline) derivatives with immunomodulatory properties and has shown effects in several models of autoimmune/inflammatory disorders by targeting the myeloid cell compartment [45]. Treatment of tsk mice with paquinimod in the drinking water reduced skin fibrosis as measured by reduction of skin thickness, decreased

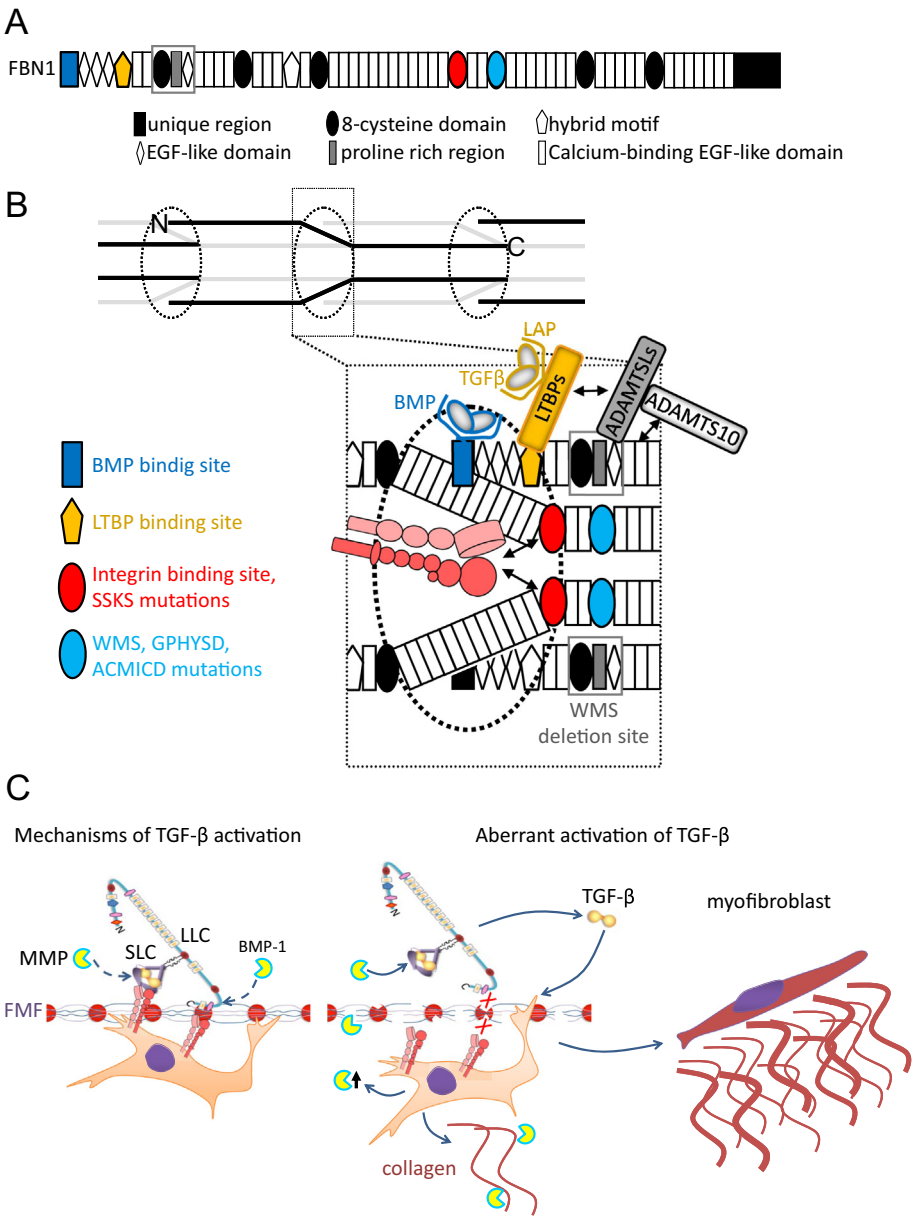

Fig. 1. The bead region of FMF provides a cellular microenvironment that is crucial for the bioavailability of TGF-β growth factors. (A) Domain structure of fibrillin-1 with marked domains indicating growth factor targeting sites for BMPs (dark blue: N-terminal unique region) and TGF-βs (yellow: LTBP binding site). Rare *FBN1* mutations found in marked 8-cysteine domains lead to SSKS (red: 4th 8-cysteine domain containing the integrin binding site) or to WMS, GPHYSD, and ACMICD (light blue: 5th 8-cysteine domain) all characterized by short stature and thick or stiff, fibrotic skin [100,105]. An additional WMS mutation was found leading to deletion of the first 8-cysteine, the proline rich region, and the 4th EGF-like domain (boxed in grey) [29]. (B) According to a model of fibrillin-1 molecules arranged as parallel, staggered molecules within the "beads-on-a-string microfibril" the N-terminal halves of fibrillin are found on the outside while the C-terminal halves are localized to the core of each microfibril polymer [114]. Beaded regions of the microfibril are represented as dashed circles. In this model, identified binding sites for ADAMTSL proteins within the identified WMS deletion region [29], for LTBP-TGF-β [12] (within the first hybrid domain: marked in yellow), and BMPs [14,15] on one molecule are very close to the integrin-binding RGD site (contained in the fourth 8-cysteine domain: marked in red) on a second molecule. Mutations in the 4th 8-cysteine domain cause SSKS, presumably by disrupting integrin binding [93]. The 5th 8-cysteine domain contains mutations in *FBN1* that result in autosomal dominant WMS, GPHYSD or ACMICD [105]. Mutations in *ADAMTSL2* also lead to recessive GPHYSD [104], and mutations in *ADAMTS10* lead to recessive WMS [102]. It is plausible that recruitment of ADAMTSL molecules to the WMS deletion site is important for ADAMTS enzymes which may be involved in growth factor activation required for connective tissue homeostasis [29]. We propose that this cluster of molecular interactions (magnified in the inset) constitutes a microenvironment controlling fibrotic reactions in the skin. (C) Model of how fibrillin deficiency may trigger fibrotic reactions. TGF-β is secreted in the form of a large latent complex (LLC) in which the TGF-β prodomain (LAP) is covalently tethered to LTBPs that target the LLC to FMF via specific interactions of their C-terminal ends and the N-terminal regions of fibrillin-1 and -2 [12,16]. Integrin interactions with FMF allow cells to gain positional and biomechanical information within the respective tissue. Mechanisms of TGF-β growth factor include engagement of integrin receptors at the RGD site within LAP [97], but also proteolytic cleavage of LAP or within the LTBP hinge region [131,153]. However, the current understanding is that fibrillin-1 deficiency results in ablation of LTBP and integrin binding leading to instability of the LLC and aberrant TGF-β activation by proteases. In turn, active TGF-β stimulates MMP production as response to remove deficient FMF and to initiate tissue repair. This feed-forward cycle results in the release of more TGF-β, increased collagen production and differentiation of fibroblasts to myofibroblasts. Alternatively, MMPs may be upregulated and released as a response of cells sensing a structurally impaired microenvironment. During the course of ECM degradation due to increased MMP activity TGF-β is aberrantly activated.

number of myofibroblasts and total hydroxyproline content [46]. Paquinimod treatment also resulted in a reduced TGF-β response in the skin and an abrogation of the increased auto-antibody production in tsk mice [46].

The administration of cancer drugs provides a new promising therapeutic avenue for the treatment of fibrotic conditions, however, a number of critical points need to be clarified. The most important is the biological impact of durable inhibition of HSP90, STAT-JAK signaling,

or members of the S100 family of proteins considering their ubiquitous role in cell homeostasis, cellular stress conditions as well as during regenerative processes such as wound healing. It may also be that there are adverse effects to be expected on other organs. For instance it is known that STAT3 activation is an absolute requirement to maintain the barrier functions of the intestinal epithelium which represents a limitation for the systemic inhibition in patients [47,48]. Paquinimod may cause at higher dosage severe muscle and joint pain and pomalidomide was shown to be antiangiogenic and teratogenic in rats and rabbits and can be neurotoxic in humans [49–51]. Another important question is the clinical safety and efficacy of TGF-β signaling inhibition in patients. While most phase I clinical trials involving TGF-β inhibitors for cancer showed that these compounds were relatively well tolerated, long-term exposure and potential side-effects have to be considered.

2.3. Treatment of tsk mice with immune modulating drugs

SSc in human is an autoimmune connective tissue disease and a similar immunological component was suggested to be implicated in the disease pathogenesis of tsk mice [52]. An instructive experiment in this regard was the infusion of bone marrow and spleen cells from tight-skin mice into normal mice which lead to the induction of a tsk-like cutaneous phenotype and the production of autoantibodies [53]. The prominent production of autoantibodies against SSc-specific antigens (e.g. against topoisomerase I, single-stranded, and double-stranded DNA) in tsk mice suggests that B cells might play an important role in disease development. Indeed, overexpression of the B cell co-receptor CD19 in Tsk mice resulted in chronic B cell activation and increased production of IL-6 [54], which is known to stimulate collagen production in fibroblasts, while ablation of CD19 attenuated the development of skin fibrosis [54]. Interestingly, B-cell depletion in newborn tsk mediated by CD20 monoclonal antibody injection significantly suppressed the development of skin fibrosis, whereas it showed no effect in adult mice after disease establishment [55]. Similarly, anti-CD40L mAb therapy showed remarkable effectiveness in tsk mice since it significantly reduced cutaneous fibrosis and anti-topoisomerase I autoantibody production in tsk mice, but not after disease establishment [56]. Furthermore, an involvement of B-cell-activating factor belonging to the tumor necrosis factor family (BAFF), a potent B-cell survival factor, in the development of fibrosis was investigated in tsk mice [57]. Intraperitoneal injections of one week old tsk mice with a BAFF antagonist consisting of the ectodomain of BAFF receptor fused to the Fc portion of human IgG1 inhibited the development of skin fibrosis, hypergamma-globulinemia, and the autoantibody production in tsk mice [57]. These findings suggest that B cells contribute to the initiation of tsk pathogenesis but are not required for disease maintenance [55]. However, the view that B cells play a major role in the pathogenesis of tsk fibrosis is challenged by the finding that the tsk fibrillin-1 mutation can induce fibrosis in RAG-2 mice that lack mature B cells and T cells [58]. Moreover, B cell depletion using rituximab, a chimeric monoclonal antibody that targets CD20 led to conflicting results in SSc patients [59–62]. Data from these studies suggest that rituximab cannot be recommended in the routine clinical care to SSc patients, however, they provide encouraging evidence that B-cell depletion might enhance the currently restricted therapeutic options of fibrotic diseases. Hence, further evidence by large multicenter randomized controlled trials is required.

One of the long standing paradigms in SSc research is that fibrosis occurs due to an imbalance between cytokines released by T helper 1 (Th1) and Th2 cells [63]. Th2 cytokines such as interleukin-4 (IL-4), IL-6, and IL-13 stimulate the synthesis of collagen by human fibroblasts while Th1 cytokines such as interferon-γ (IFNγ) and tumor necrosis factor show the opposite effect in vitro [63]. This notion is corroborated by results from tsk rescue experiments in which different ILs or their receptors are genetically targeted. Such studies showed that skin fibrosis was prevented in tsk mice by additional ablation of IL-4 [64,65], IL-4 receptor [33], STAT6 [64], or by the administration of anti-IL-4 monoclonal antibody (mAb) to newborn tsk mice [66]. Disrupting IL-4 rescues mice homozygous for the tight-skin mutation from embryonic death and also diminishes TGF-β production by fibroblasts [65]. Consequently, to the Th1/Th2 balance hypothesis treatment with interferon-gamma therapy topically administered by nanoparticles also successfully reduced skin thickness in tsk mice [67]. Since their recognition as the third T helper cell subset Th17 cells were suggested to contribute to fibrosis via the production of IL-17A, since elevated serum IL-17A levels and elevated IL-17A expression in peripheral blood lymphocytes and lesional skin were reported in patients with SSc [68]. Breeding the tsk mutation on a IL-17A null background resulted in attenuation of hypodermal thickness suggesting a contribution of this cytokine in the tsk pathogenesis [69].

Gene transfection experiments in tsk mice also showed promising results for the treatment of skin fibrosis. Histologic analysis revealed that hepatocyte growth factor (HGF) gene transfection in tsk mice resulted in a marked reduction of hypodermal thickness, including the subcutaneous connective tissue layer due to suppression of IL-4 and TGF-β mRNA expression [70]. Also, intramuscular injection of an IL-12 encoding plasmid (pCAGGSIL-12) was tested on the disease progression of tsk mice [71]. pCAGGSIL-12 plasmid was injected intramuscularly 7 times at 3 week intervals into tsk mice resulting in a marked decrease in skin thickness [71]. The serum levels of antinuclear antibodies were diminished in pCAGGSIL-12 treated mice and IL-4 production by spleen cells from pCAGGSIL-12 plasmid treated mice was significantly lower [71]. These results indicate that pCAGGSIL-12 administration into tsk mice had beneficial effects in preventing the collagen accumulation in the skin and suppressing the autoimmunity via improvement of the Th1/Th2 balance.

In another set of experiments it was attempted to target signaling pathways important for other entities of the immune system. Genetic ablation of the toll-like receptor-4 (TLR-4) which is crucial for innate immunity attenuated hypodermal fibrosis in Tsk mice [72]. Also, intraperitoneally administration of the mTOR inhibitor rapamycin, a macrolide immunosuppressive drug, reduced skin fibrosis in tks mice [73]. Rapamycin administration to tsk mice negatively affected the production of fibrogenic cytokines, such as IL-4, IL-6, IL-17, and TGF-β-1, but also resulted in a reduced production of hypergammaglobulinemia and anti-topoisomerase I antibodies [73].

Further strategies concentrated on targeting other immunocompetent cells such as macrophages and mast cells in tsk mice. Activation of liver X receptors (LXRs), which belong to the family of nuclear receptors of transcription factors, by oral application of the agonist T0901317 inhibits spontaneous skin fibrosis in tsk mice via inhibition of macrophage infiltration and IL-6 release [74]. To test whether activation of connective-tissue-type mast cells (CTMCs) contributes to the fibrosis in tsk mice, the mast cell chymase inhibitor, SUN-C8257, was applied by intraperitoneal injections [75]. Addition of SUN-C8257 significantly decreased the thickness of the subcutaneous fibrous layer of tsk mice which was accompanied by a reduction of chymase-4 gene expression [75]. An explanation for this effect is that overexpressed chymase in connective tissues is believed to promote the destruction of the ECM including FMF to which TGF-β-1 is anchored which results in the release of excessive amounts of bioactive TGF-β-1 [76,77].

Based on the above described preclinical findings in tsk mice interference with interleukin signaling provides attractive new ways to ameliorate fibrotic reactions in SSc patients. Currently, monoclonal antibodies against IL-6 (Sirukumab), the IL-6 receptor (tocilizumab), anti-IL-17 receptor (brodalumab) are explored as new drugs against SSc. Anti-IL-6 treatment may be beneficial in SSc by not only directly inhibiting IL-6 driven fibrotic reactions in fibroblasts but also blocking inflammation through the reduction of polarisation of pathogenic Th17 cells and the upregulation of the tolerance Treg cells. First results from clinical tials using tocilizumab showed promising results on skin score improvement in SSc patients [78].

2.4. Treatment of tsk mice with antioxidative drugs

Moreover, treatment strategies have been tried to scavenge free oxides or free radicals in tsk mice. Stimulation of soluble guanylate cyclase (sGC) which catalyzes the production of cyclic guanosine monophosphate (cGMP) upon binding of nitric oxide (NO) also represents a therapeutic strategy for the tsk mediated fibrosis. The sGC stimulators BAY 41-2272 and BAY 63-2521 (riociguat) were shown to be highly effective in preventing progressive hypodermal thickening in tsk mice in a dose-dependent manner [79,80] which is suggested to be achieved by blockage of the non-canonical TGF-β signaling cascade [81]. These data paved the way for a currently ongoing randomized, double-blinded, placebo-controlled phase II study to investigate the efficacy and safety of riociguat in SSc patients (NCT02283762). However, due to its known cardiovascular effects such as decrease in systemic arterial diastolic pressure [82] it remains to be seen whether there will be any limitations on the use of sGC stimulators in the treatment of fibrosis. Intravenous administration of edaravone, a free radical scavenger, into the tail vein reduced fibrosis in Tsk mice [83]. Production of fibrogenic cytokines such as interleukin-6 and TGF-β1, production of anti-topoisomerase I antibody, and the degree of hypergammaglobulinemia were reduced by edaravone [83]. However, due to its content of sodium bisulfite, allergic type reactions were reported as side effects of edaravone injections including respiratory failure, dermatitis and eczema (FDA New drug application number: 209176). Moreover, halofuginone (7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone) one of the febrifugine analogues known to selectively inhibit collagen type I synthesis in vitro [84] by inhibiting TGF-β signaling through prevention of Smad3 phosphorylation [85] was also shown to be effective in tsk fibroblasts [86] and tsk mice [87,88]. Halofuginone administration to tsk mice eliminated the increase in skin collagen, prevented the thickening of the dermis and the loss of the subdermal fat in a dose-dependent manner independent of the route of administration (intraperitoneally, administered locally in a cream, or given orally) [87,88]. The effect of halofuginone treatment was correlated with a decrease in the levels of autoantibodies against topoisomerase I and fibrillin-1 antibodies [89,90]. However, using halofuginone topically on 12 patients with SSc demonstrated limited efficacy, five patients responded by demonstrating a significant reduction in skin score whereas seven did not respond [91].

3. Stiff skin syndrome as model disorder for systemic sclerosis

Stiff skin syndrome (SSKS) represents a rare congenital connective tissue disorder leading to childhood onset of diffuse skin fibrosis with autosomal dominant inheritance and complete penetrance [92]. SSKS patients suffer from hard, thick skin, usually over the entire body, which limits joint mobility and causes flexion contractures. Other occasional findings include lipodystrophy and muscle weakness [93]. Recent genetic findings gave additional insight into the pathogenetic pathways triggered by fibrillin-1 deficiency. SSKS patients with a heterozygous W1570C missense mutation within the fourth 8-cys domain of fibrillin-1 harboring the only canonical RGD integrin binding site were identified [93]. Electron microscopy analysis showed abnormal aggregates of FMF with a sparse and patchy deposition of elastin [93]. Dermal biopsies revealed increased TGF-β activity as shown by accumulation of LTBP-4, increased nuclear accumulation of phosphorylated Smad2 (pSmad2) and expression of connective tissue growth factor (CTGF), a direct response gene of TGF-β signaling [93]. From these data it was hypothesized that FMF accumulation as a result of failed fibrillin-1-integrin interaction leads to local concentration of TGF-β which is then chronically activated by integrin-TGF-β prodomain (latency associated peptide: LAP) interactions [93]. This aberrant TGF-β activation may be a consequence of an additional upregulation of integrins. It was shown that integrin αvβ3 is upregulated in dermal fibroblasts from SSc patients, and that its inhibition prevents collagen expression and reverses the myofibroblastic phenotype in a TGF-β dependent manner [94]. In order to determine whether failed interaction between integrins and fibrillin-1 is sufficient to initiate skin fibrosis, two *Fbn1*-targeted knock-in mouse models were generated: one with the mouse equivalent identified SSKS causing mutation (W1570C) and the other with an RGD to RGE substitution (D1545E) to abolish integrin binding to fibrillin-1 [95]. Isolated cells from mutant dermis showed increased surface levels of integrin α5β1 and integrin αvβ3 in its active conformation [95]. Twelve week intraperitoneal injection every five days of β1aAb, a β1 integrin-activating antibody, normalized integrin expression, skin stiffness, distensibility and skin architecture in both SSKS mouse models [95]. In addition, targeted introduction of haploinsufficiency or complete deficiency for β3 integrin in SSKS mice normalized skin stiffness, collagen deposition and subcutaneous fat by three months of age [95]. The contribution of aberrantly activated TGF-β signaling was investigated by intraperitoneal injection of a panspecific TGF-β neutralizing antibody (TGFβNAb, 1D11) [95]. Clinical and histological findings confirmed full reversal of skin stiffness and restoration of skin architecture in NAb-treated animals [95]. As seen in SSc, both SSKS mouse models show increased circulating anti-nuclear and anti-topoisomerase I antibodies, and an elevated immune cell infiltration/activation which were normalized upon treatment of mutant mice with β1aAb [95]. Similarly, treatment with TGFβNAb also normalized the immune cell response in SSKS mouse models [95]. It is known that TGF-β can also initiate so-called non-canonical cascades, via extracellular signal regulated kinase (ERK1/2) signaling facilitated by kinase cascades involving mitogen-activated protein kinase (MAPK). Inhibition of MEK-1 and -2, the two enzymes which phosphorylate and activate MAPK, by oral gavage of refametinib (RDEA119, Bay 86-9766) prevented skin stiffness, dermal collagen accumulation, and loss of subcutaneous fat in *Fbn1*$^{D1545E/+}$ mice [95]. Therefore, ERK antagonism represents an interesting treatment option in SSKS, although it is thought to occur downstream of TGF-β and integrin signaling.

Overall, the studies with SSKS mouse models offered new treatment strategies for SSKS and SSc patients including β1 integrin activation, blockade of β3 integrin, and antagonism of TGF-β or ERK signaling. However, the exact mechanisms by which the SSKS mutations in fibrillin-1 lead to fibrosis remain unclear. It was shown that in contrast to many *FBN1* mutations causing MFS, SSKS *FBN1* mutations do not affect fibrillin-1 secretion [96] suggesting that the SSKS mutations do not affect overall intracellular protein folding. However, extracellularly increased accumulation of structurally impaired FMF in SSKS due to failed fibrillin-1 integrin interactions may lead to increased ECM deposition of TGF-β which is then aberrantly activated by integrin-LAP interaction. However, it has been shown before that the precise presentation of TGF-β LLC is crucial for integrin interaction [97]. For instance, disturbances in the fibronectin or FMF network can lead to a loss of TGF-β activation [97]. This would be otherwise assumed given the large FMF aggregations observed in SSKS [93]. How integrins may access LAP within such dense ECM aggregates remains unclear. Also it remains obscure how β1 integrin activation could rescue the SSKS phenotype, since it was probably not able to resolve FMF aggregations as integrin fibrillin-1 interactions are ablated due to the mutation.

Recently, in SSKS patients new treatment options were described. It was reported that mycophenolate mofetil (MMF), an immunosuppressive agent, which serves as an inosine monophosphate dehydrogenase (IMPDH) inhibitor, was effective in two patients with segmental SSKS characterized by asymmetric and unilateral skin thickening [98]. Upon MMF treatment patients showed improvement of skin induration and demonstrated objective responses as measured by physician's global assessment. In addition, both patients experienced increased joint mobility while on therapy. Notably, one patient stopped MMF for several months and progressive skin tightening developed, however, upon reinitiating treatment with MMF resulted in improvement [98]. Furthermore, mild improvement in skin induration was noted during a

two-year systemic treatment of one patient with segmental SSKS with losartan [99].

4. Acromelic dysplasias as congenital model disorders to explore fibrotic mechanisms

SSKS is clinically related to a group of rare connective tissue disorders which were termed the acromelic dysplasias since they are characterized by short stature, short hands, stiff joints, and thickened skin [100]. Elucidating the molecular mechanisms causative for fibrotic conditions in acromelic dysplasia patients may pave the way for new therapeutic avenues for fibrosis patients. The acromelic dysplasia group includes Weill-Marchesani syndrome (WMS), geleophysic dysplasia (GPHYSD), acromicric dysplasia (ACMICD), and Myhre syndrome (MYHRS) (Table 1). WMS was originally described with features "opposite to MFS" and its autosomal dominant inherited form is caused by mutations reported within the fifth 8-cysteine domain of *FBN1* (WMS2) [101]. However, the recessive form of WMS (WMS1) was found to be caused by mutations in the catalytic domain of *ADAMTS10* (A disintegrin and metalloproteinase with thrombospondin type 1 motif 10) [102]. This implies a genetic and physical interaction of ADAMTS-10 and fibrillin-1, and indeed it could be shown that ADAMTS-10 is able to bind [29,103] and proteolytically cleave fibrillin-1 [103], but also facilitates FMF assembly by fibroblasts in culture [103]. A similar interaction with FMF could be demonstrated for ADAMTSL-2 (ADAMTS-like-2). Mutations in *ADAMTSL2* were shown to lead to intracellular retention of the protein causing recessive GPHYSD [104] (GPHYSD1), while dominant GPHYSD2 is caused by mutations also within the fifth 8-cystein domain of fibrillin-1 [105]. Interestingly, ADAMTSL-2 was also found to interact with fibrillin-1 [29,106], and with LTBP-1 [104] and thus suggested to contribute to the overall stability of the TGF-β LLC. In agreement with this hypothesis, it was found that fibroblasts from GPHYSD1 patients showed elevated levels of active TGF-β [104]. In addition to a stiff and thickened skin GPHYSD is also characterized by progressive tracheal-bronchial and cardiac valve fibrosis contributing to the observed early lethality [107]. *Adamtsl2* null mice which serve as a mouse model of GPHYSD1 die at birth due to severe bronchial epithelial dysplasia which was associated with increased bronchial epithelial TGF-β signaling at E17.5 [106]. However, this condition could not be reversed by treatment of *Adamtsl2* null mice with TGF-β neutralizing antibody 1D11 by intraperitoneal injection in utero at E13.5 and E17.5 pointing to other initiating mechanisms [106]. Nevertheless an involvement of ADAMTSL-2 in mechanisms of balancing TGF-β bioavailability seems very likely since also mutations in LTBP-3 were recently found to be causative for GPHYSD and ACMICD [108]. Currently it is unclear whether the common clinical features within the acromelic dysplasia group are caused by aberrant increase of TGF-β activity or loss of proper TGF-β activation and signal transduction. For instance, in fibroblasts from a family with WMS2 and the corresponding mouse model no increase of TGF-β activity could be found [29]. In addition, mutations causing MYHRS were found in the SH2 domain of the co-Smad Smad4 [109], that is required for Smad oligomerization and translocation of TGF-β (psmad2/3) and BMP (psmad1/5/8) signal transduction from the cell surface to the nucleus. In fibroblasts of MYHRS patients defective Smad4 ubiquitination and TGF-β signal transduction was found accompanied by decreased transcriptional expression of TGF-β and BMP downstream elements that play an important role in ECM homeostasis [109]. MYHRS fibroblasts showed also defects in ECM deposition and decreased matrix metalloproteinase (MMP) and related inhibitor expression [110]. Similar to the reversion of aortic aneurysm formation as detrimental consequences of dysregulated TGF-β signaling in MFS mice [111], treatment of MYHRS fibroblasts with Losartan, a TGF-β antagonist and angiotensin-II type 1 receptor blocker, corrected defects in expression and deposition of ECM molecules thereby offering a therapeutic option to be considered also for MYHRS [110]. However it remains unclear, why some fibrillin-1 mutations lead to MFS while others lead to acromelic features. The most plausible explanation is that the three-dimensional ultrastructure of FMF provides a cellular microenvironment which is important for TGF-β and BMP growth factor sequestration and bioavailability (one model of FMF organization shown in Fig. 1B). Any structural perturbance of this microenvironment results in growth factor dysregulation manifesting in either aberrantly increased growth factor activity or loss of growth factor activation. Some mechanistic insight into the workings of this microenvironment was provided by the discovery of a familial deletion mutation within the N-terminal region of fibrillin-1 leading to WMS2 [29]. Biochemical analysis showed that this mutation resulted in deletion of the first 8-cysteine domain, the proline-rich region, and the fourth EGF-like domain [29]. Protein interaction studies showed that the deleted region harbors a universal binding sites for ADAMTSL proteins which may be utilized to recruit ADAMTS enzymes in close proximity to the LTBP and BMP binding sites on fibrillin-1 [12,14,15,29]. It is curious that all described *FBN1* mutations leading to WMS, GPHYSD, and ACMICD are found within the fifth 8-cysteine domain of fibrillin-1 [105]. This domain lies also in close proximity to the fourth 8-cysteine containing the classic integrin RGD binding site. To understand the impact of individual *FBN1* mutations on FMF ultrastructure, better knowledge about the arrangement of fibrillin-1 monomers within the FMF ultrastructure is required. For this purpose three models of FMF organization have been developed based on structural investigations of recombinant proteins spanning full length fibrillin-1 or extracted FMF from tissues. The pleated model suggests that the bead results from back folding of the monomer, especially in the region around domains TB4 and TB5 [112]. The two other models suggest an extended conformation with each monomer spanning two or three interbead regions [113,114].

However, within such an extended three-dimensional conformation of beads-on-a-string FMF in which fibrillin-1 molecules are arranged as parallel, staggered molecules [114], the N-terminal WMS2 deletion site, the LTBP and BMP binding sites, and the fourth and fifth 8 cysteine domain lie in close proximity and are required in perfect positional arrangement to each other to guarantee proper growth factor sequestration and bioavailability [29] (Fig. 1B). In this model it is unclear which ADAMTSL molecule may partner with which ADAMTS enzyme and whether some of these complexes only exist in a tissue specific manner. However, the recruitment of ADAMTS enzymes in close proximity to the LTBP binding site on FMF may allow the activation of physiologically relevant amounts of growth factors required for proper tissue growth by either proteolytic cleavage of fibrillin-1 or LTBPs. Future investigations will test and revise this concept by adding new or additional factors to the model. For instance, human WMS mutations were also found in LTBP-2 [115] which is known to bind the N-terminus of fibrillin-1 [116] and fibulin-5 [13] and is thereby involved in the formation of stable FMF [26] and elastic fibers [13]. WMS is also caused by mutations in ADAMTS-17 [117] which was shown to bind recombinant fibrillin-2 but not fibrillin-1 and thereby suppressing fibrillin-2 incorporation into FMF [118]. Recently it was also reported that Leri's Pleonosteosis (LP) shares clinical features with the acromelic dysplasia group and SSKS [119]. Microduplications of chromosome 8q22.1 in two affected families were identified as the cause of LP [119]. Dermal fibroblasts from affected individuals showed overexpression of two genes, GDF6 (growth and differentiation factor-6) and SDC2 (syndecan-2). GDF-6 belongs to the TGF-β superfamily of growth factors while syndecan-2 is a heparansulfate proteoglycan [119]. This implies an involvement of other growth factors and glycosaminoglycans (GAGs) in the underlying pathomechanisms. Another link for an involvement of GAGs in the acromelic displasias was provided by the finding that WMS, GPHYSD, and ACMICD mutations in the fifth 8-cysteine of fibrillin-1 lead to the abolishment of heparin binding to this domain [120]. Recruitment of heparin to the sensitive microenvironment within

the FMF may be crucial to fine tune growth factor activity or their presentation to cell surface receptors.

5. Fibrotic mechanisms caused by dysregulation of ECM degradation

5.1. MMPs, regulators of collagen catabolism

Until now, research on MFS and the acromelic dysplasias identified growth factor dysregulation and altered matrix metalloproteinase activity as the two major common initiation causes. Interestingly, metalloproteinases which are known to activate growth factors of the TGF-β superfamily are also involved in degradation processes of the FMF and the collagen network. In addition, released TGF-β growth factors are able to stimulate the expression of metalloproteinases resulting in a bi-directional regulatory loop (Fig. 1C). Interestingly, increased metalloproteinase activity accompanied by increased TGF-β activity correlates with degradative processes such as in MFS while loss of function of metalloproteinases results in increased ECM deposition leading to fibrotic conditions. For instance, MMP-2 and -9, the two known gelatinolytic enzymes, were reported to cleave fibrillin-1 [121] and to be upregulated during aortic aneurysm formation in MFS [122]. Application of doxycycline, a non-specific MMP inhibitor, in the drinking water significantly prolonged the life span of Marfan mice which was accompanied by reduced MMP-2 and -9 expression levels [123]. Human disorders linked to mutations in MMP-2 and to reduced enzyme activity, display multicentric osteolysis, nodulosis, and arthropathy (MONA) [124] which can also cause a scleroderma-like skin thickening [125]. These phenotypes are detected, even though not as severe, in MMP-2 and partly in MMP-14 knockout mice [126,127]. In the Winchester syndrome (WNCHRS), an osteolysis and arthritis disorder, mutations in MMP-2 and MMP-14 have been associated to the onset of disease [128,129]. These data provide genetic evidence that links similar to the fibrillinopathies ECM proteolysis with human growth and skeletal development [130]. In addition to TGF-β1 release by proteolytic cleavage of LTBP-1 [131] MMP-14 has been implicated in several other processes that may indirectly contribute to fibrosis, e.g. activation of proMMP-13 and processing of fibronectin [132] which also serves as an additional ECM anchor for FMF [133] and LTBPs [134]. Studies from Sounni et al. [135] using mice with complete deletion of MMP-14 detected decreased levels of total TGF-β-1. However, in an additional model of MMP-14 deficiency [136] and in the fibroblast-deleted MMP-14 model [137] this reduction was not detected. Thus, the role of MMP-14 in TGF-β1 release in vivo is not clearly defined and requires further study. In the three described MMP-14 deficient mouse models, proMMP-2 activation was reduced, suggesting a functional role to the observed phenotypes. Since in the MMP-2 null mice fibrosis of skin or additional organs did not develop until adulthood [138], reduced activation of MMP-2 in vivo, may also contribute to, but not drive the collagen-homeostatic defect observed in the absence of MMP-14. However, MMP-14 collagenolysis seems to play a major role after birth, when fibropositors have been released and collagen fibers start to grow in length and diameter [139]. In skin, when MMP-14 deletion was specifically induced in fibroblasts of adult mice at 3 months of age, a phenotype similar to the collagenase resistant transgenic mouse was observed [137]. In these mice the collagenase cleavage site within the alpha 1 (I) chain for proper release of the typical 3/4 and 1/4 fragments was abolished by mutational inactivation ($Col1\alpha\text{-}1^{r/r}$, so-called "collagenase-resistant mice") [140,141]. Similar to collagenase-resistant mice, fibroblast-specific MMP-14 mice displayed thickened dermis, collagen accumulation, enhanced tensile strength and skin stiffness [137]. In agreement with a postulated postnatal collagenolytic activity of MMP-14 in tendons, ablation of MMP-14 in fibroblasts also resulted in enlarged tendons [137]. Accumulation of collagen in skin was not caused by alterations in de novo collagen synthesis, thus independent of TGF-β activation, but was due to an almost complete loss of collagenolytic activity and thus of reduced turnover [137]. In these mice no other characteristic features of fibrotic skin disease such as altered pattern/type of collagen crosslinks, enhanced infiltration of inflammatory cells or vascular defects were detected [141,142]. The data rather indicate a pathogenic mechanism of fibrotic disease mediated by altered collagenolysis by MMP-14.

5.2. BMP-1 and meprins, proteases required for collagen maturation

Extracellular maturation of the collagen and elastic fiber networks relies on the identical set of key enzymes which represent a vital link for the understanding how deficiencies in the FMF/elastic fiber network may impact collagen homeostasis and vice versa.

Once secreted into the extracellular space, the procollagen molecules undergo extracellular modifications including the cleavage of the N- and C-terminal propeptides and conversion of the lysine- and hydroxylysine residues into reactive aldehydes [143]. Lysyl oxidase (LOX) mediates the formation of inter-molecular cross-links between collagen triple helices thus providing mechanical strength and protection from proteolysis [143]. On the other hand, LOX also catalyzes the final enzymatic step of tropoelastin cross-linking giving rise to stretchable and resilient elastic fibers [144]. LOX is secreted as inactive proform together with its N-terminal pro-peptide and becomes activated by pro-peptide removal via proteolytic cleavage at the correct physiological site [145]. This proteolytic activation of pro-LOX is facilitated by BMP-1 which was also reported as the first collagen C-terminal propeptidase [145,146]. Interestingly, the removal of the N-terminal prodomain of pro-collagen I is catalyzed by ADAMTS-2, -3, and -14 enzymes [147] that belong to the same metalloproteinase family involved in FMF assembly [103,118]. In order to facilitate elastic fiber maturation pro-LOX is recruited to FMF via interaction of its propeptide with fibulin-4 prior to BMP-1 cleavage [148]. Interestingly, fibulin-4 is targeted to residues within EGF3/Hyb1 domains of fibrillin-1 where it competes for binding with LTBP-1 [11,12] providing another cue of how elastic fiber formation may influence TGF-β activity and thereby collagen production.

The *Bmp1* gene gives rise upon alternative splicing to the mammalian Tolloid (*mTld*) or BMP1-3 family which are all known for their capacity to process non-collagenous ECM components and ligands of the FMF network such as perlecan, and small leucine-rich proteoglycans, but also LTBPs and prodomains of TGF-β (TGFβ) superfamily ligands resulting in their activation [149–152]. Ge et al. showed that BMP-1 can activate TGF-β by cleaving LTBP-1 at its N- and C-terminal sites, resulting in the release of the large latent TGF-β complex (LLC) from the ECM [153]. Their data also demonstrate that the released latent TGF-β complex is further processed via MMP-2-mediated proteolysis of the TGF-β-1 prodomain [153]. Human *BMP1* mutations revealed *BMP1* as a highly relevant gene for osteogenesis imperfecta (OI), as affected patients show increased bone mineral density and multiple recurrent fractures [154]. Also human mutations in the COL1 C-propeptide cleavage site lead to OI, and a similar phenotype was observed in BMP1 null and BMP-1; tolloid like (TLL) double null mice [155,156]. The relevance of BMP-1 and TLL in collagen maturation is not only limited to bone, since it was recently reported that ubiquitous ablation of BMP-1 and TLL expression at 4 and 5 weeks of age resulted in skin thickness reduction and skin fragility accompanied by increased collagen fibril density and a delay in wound healing [157]. BMP-1 was also found to be upregulated during scarring of murine and human cornea [158], and moreover, BMP-1-3 were identified as therapeutic target for chronic kidney disease (CKD) [134]. BMP-1-3 were found to circulate as active enzymes in the blood of patients with chronic kidney disease (CKD) [159]. Weekly intravenous injection of BMP1-3-neutralizing antibody reduced renal fibrosis in rats with CKD, leading to retained renal function, and increased survival which was associated with low plasma levels of TGF-β-1 and connective tissue growth factor [159].

Lately, meprins (astacin, metalloproteases) were shown to process pro-collagen molecules both at C- and N-terminus which implicates

them in fibrotic pathways [160,161]. In agreement with a suggested pro-fibrotic function, the expression of meprins and BMP-1 was found to be increased in dermis from patients with fibrotic skin (keloids) [162]. Loss of meprin α and β in mice leads to reduced deposition of collagen in skin, however, not in bone or dentin [160,163]. The skin of meprin α and β mice displayed altered mechanical properties most likely due to decreased collagen and MMP-1 inactivation [160]. Experimental lung fibrosis (bleomycin treatment) in meprin α, β and α/β KO mice failed to indicate an overall difference in lung function and inflammation [164]. Only in meprin β null mice reduced collagen accumulation was detected in treated lungs as compared to the other genotypes including controls [164]. Interestingly, in bleomycin treated mice and in human idiopathic pulmonary fibrosis, meprin α and β were localized to epithelial cells where they are suggested to have a pro-collagenase independent activity [164]. It was shown that meprins cleave E-cadherin [165] and the tight-junction protein occludin [166] leading to destabilization of epithelial structures of vessels, and thereby possibly contributing to the onset of fibrosis. However, absence of meprins did not prevent E-cadherin loss in bleomycin challenged mice suggesting a possible compensation by other proteases [164]. The activity of BMP-1 and meprins is regulated by calcium concentrations which induce structural changes that modify the protease domain stability and molecular opening. In particular, it was demonstrated that high calcium concentrations favor BMP-1 activity as compared to meprins [167]. This would suggest that compartmentalization of enzyme activity, with major activity of BMP-1 in bone and dentin and meprins in skin, is driven by local calcium concentrations [167].

5.3. Protease targeting approaches for fibrosis

Despite available pro-collagen maturation data suggesting that meprin inhibition represents a viable therapeutic approach for fibrotic diseases, specific meprin inhibitors are not yet available. Sulfonate-moieties have shown potency in meprin inhibition, but due to moderate inhibition specificity their use was suggested only for local application [168]. This also applies for actinonin (peptidomimetic/hydroxamate) a natural compound produced by Actinomyces with antibacterial activity, which showed potency in the inhibition of meprin α and meprin β [169]. Interestingly, chemical modification of hydroxamate proved to be useful for the inhibition of pro-collagen C-proteinase activity. Usage of an azolyl-hydroxamic (US Patent WO0147901, 2004) led to reduction in the deposition of mature collagen fibers and thereby to reduced scar hypertrophy in a rabbit model of cutaneous scarring [170]. Additional developed inhibitors include N-substituted aryl sulfonamide hydroxamates, which demonstrated to be more efficient inhibitors of procollagen C-proteinases and also showed good selectivity for some MMPs [171]. However, as for actinonin these drugs could be used topically as anti-scarring therapeutic, but are not yet specific enough for systemic application.

More than 10 years ago, initial efforts to develop first generation MMP inhibitors to be effective in cancer development failed due to several reasons including missing knowledge about the variety of their functions and lack of inhibition specificity leading to cross-inhibition of various metalloproteinases including the ADAMs (A Disintegrin And Metalloproteinase) [172].

For targeting of MMPs in fibrotic disease, it has to be considered that in the initial stages MMP mediated proteolytic degradation generates a pro-fibrotic inflammatory environment, while in later stages participation of MMPs in collagen degradation serves an anti-fibrotic function [173]. Inhibition strategies of MMPs include chemical compounds interacting with specificity pockets within the catalytic domain [174], substrate peptides [175], and antibodies against the catalytically active site [176] or secondary collagen binding sites (exosites) [177]. The review from Field [90] provides more details on the MMPs inhibition strategies currently available.

A general limitation in the use of antibodies for protease inhibition is their rapid removal from circulation. However, a new generation of cell-specific antibodies conjugated to drugs may offer improved inhibition potential. For example, Quark Pharmaceuticals has generated a physiologically internalized antibody against the mannose receptor Endo180 which can be tethered to si or shRNAs aiming at intracellular depletion of target molecules (US-Patent CA2753388C).

Therapeutical benefit could also come from increasing protease activity as shown in Dupuytren's disease, a fibro-proliferative disorder characterized by enhanced deposition of collagen leading to thickening of the palmar fascia. Collagenase (*C. histolyticum*) injections were effective in reducing muscle contractures in Dupuytren's patients, a treatment that is now approved by the FDA [178,179]. Similarly, injections of *C.histolyticum* collagenase were used to treat uterine fibroids [180]. In addition, an approach to deliver MMP-1 has been undertaken using adenoviral delivery resulting in reduction of fibrosis with only moderate additional tissue damage (e.g. enhanced cell proliferation) in models of liver, heart and muscles fibrosis [181–183]. Furthermore, using adenoviral delivery of MMP-8 in a model of liver fibrosis lead to a significant enhancement of collagen degradation and a reduction of fibrotic tissue [184].

6. Conclusions

FMF with associated ligands represent an important scaffold for integrating biomechanical properties and the bioavailability of growth factors of the TGF-β superfamily in tissues. Any disturbances in the FMF architecture leads to imbalances in tissue homeostasis initiating often times fibrotic reactions. Here we provide an overview about the current knowledge and concepts derived from studying inherited connective tissue disorders and corresponding mouse models pertaining the FMF network with associated ligands. Furthermore, we provide evidences pointing at dysregulation of growth factors and metalloproteinase activity as molecular determinants in the development of fibrotic diseases resulting in collagen accumulation and FMF disturbances.

This gained information is crucial for the development of new therapeutic avenues for fibrosis. In the past the tsk skin mouse model has been proven to be insightful to dissect the underlying pathomechanisms for scleroderma which are thought to be mostly downstream of TGF-β activation. This appears to be intuitive since TGF-β is known to be directly targeted to the ECM by LTBP-FMF interactions. More recently, new evidence arose that the activation of TGF-β triggered by fibrillin-1 deficiency in MFS is a general consequence of tissue remodeling initiated by cells sensing a structurally compromised microenvironment rather than the primary disease cause. For instance it was shown that genetic ablation of TGF-β signaling by knocking-out the type II TGF-β receptor did not rescue but exacerbate aortic aneurysm formation in Marfan mice [185] suggesting that intact TGF-β signaling fulfils a protective role during aneurysm formation in MFS. This finding is consistent with the observation that postnatal loss of TGF-β signaling due to TGF-β type II receptor ablation leads to severe aortic disease [186]. However, previous reports showed that blunting TGF-β activity by application of TGF-β neutralizing antibodies to Marfan mice inhibits aneurysm formation [111]. Similar effects were seen by treatment of losartan, an angiotensin II type I receptor blocker which also inhibits TGF-β signaling [111]. While losartan was shown to be effective in mice, it did not show any major effect in a clinical trial with adolescent MFS patients [187] (clinical trial numbers of this and other losartan trails with MFS patients are listed in Table 2). These examples illustrate the challenges researchers face in designing anti-TGF-β therapies, since the levels of TGF-β signaling may vary during different stages of disease. Therefore, the precise dosage and time window of application must be determined in advance in order to avoid adverse effects of the treatment. The involvement of increased TGF-β signaling as one of the main drivers of aortic disease in MFS was corroborated by the finding that mutations in type I and II TGF-β receptors [188,189], the

TGF-β ligands -2 [190], and -3 [191], or the TGF-β signaling intermediate Smad3 [192] lead to clinical features similar to MFS such as Loeys-Dietz syndrome (LDS1-5), which was initially even described as the second locus to MFS [193,194]. Interestingly, despite the presence of loss of function TGF-β receptor mutations, investigators found paradoxically upregulation of TGF-β signaling [188]. This shows the need for the development of sophisticated treatment regimen to tackle disease mechanisms triggered by altered TGF-β bioavailability caused by structural changes in ECM architecture.

However, FMF are also important as assembly scaffold for the formation of elastic fibers. Recently it was shown that genetic interference with elastic fiber formation by knocking-out fibulin-5 proved to be effective to blunt fibrotic mechanisms in the skin [195]. This interesting finding may explain why *FBN1* MFS mutations that typically affect FMF ultrastructure and thereby compromise elastic fiber integrity appear to be protective against fibrosis, while moderate structural alterations induced by other rare *FBN1* mutations that do not affect elastic fibers but TGF-β bioavailability result in strong fibrotic reactions. Another interesting finding in this context is that LTBP-4, a known carrier of TGF-β, actively participates in elastogenesis by mediating the linear deposition of fibulin-4 and -5 onto the FMF scaffold [6–9]. Moreover, it was shown that LTBPs compete with fibulins for binding to fibrillin-1 [12] suggesting an integrative mechanism for LTBP and fibulin ECM deposition mediated by FMF. Future studies will help to expand our knowledge about how elastic fibers are involved in fibrotic mechanisms and whether this information can be utilized to develop new therapeutic avenues against fibrosis.

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