

Fighting the Fiber: Targeting Collagen in Lung Fibrosis

Claudia A. Staab-Weijnitz, PhD*

¹Comprehensive Pneumology Center, Institute of Lung Biology and Disease, Helmholtz-Zentrum München, Munich, Germany; Member of the German Center of Lung Research (DZL)

*To whom correspondence should be addressed:

Claudia A. Staab-Weijnitz, PhD, Comprehensive Pneumology Center, Ludwig-Maximilians-Universität and Helmholtz Zentrum München, Max-Lebsche-Platz 31, 81377 München, Germany, Tel.: 0049(89)31874681; Fax: 0049(89)31874661; Email: staab-weijnitz@helmholtz-muenchen.de

ORCID-ID: <https://orcid.org/0000-0002-1211-7834>

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Abstract. Organ fibrosis is characterized by epithelial injury and aberrant tissue repair, where activated effector cells, mostly fibroblasts and myofibroblasts, excessively deposit collagen into the extracellular matrix. Fibrosis frequently results in organ failure and has been estimated to contribute to at least one third of all global deaths. Also lung fibrosis, in particular idiopathic pulmonary fibrosis (IPF), is a fatal disease with rising incidence worldwide. As current treatment options targeting fibrogenesis are insufficient, there is an urgent need for novel therapeutic strategies. During the last decade, several studies have proposed to target intra- and extracellular components of the collagen biosynthesis, maturation, and degradation machinery. This includes intra- and extracellular targets directly acting on collagen gene products, but also such that anabolize essential building blocks of collagen, in particular glycine and proline biosynthetic enzymes. Collagen, however, is a ubiquitous molecule in the body and fulfils essential functions as a macromolecular scaffold, growth factor reservoir, and receptor binding site in virtually every tissue. This review summarizes recent advances and future directions in this field. Evidence for the proposed therapeutic targets and where they currently stand in terms of clinical drug development for treatment of fibrotic disease is provided. The drug targets are furthermore discussed in light of (1) specificity for collagen biosynthesis, maturation and degradation, and (2) specificity for disease-associated collagen. As therapeutic success and safety of these drugs may largely depend on targeted delivery, different strategies for specific delivery to the main effector cells and to the extracellular matrix are discussed.

Keywords: LARP6, HSP47, FKBP10, prolyl hydroxylase, lysyl oxidase

Introduction: Collagen as a drug target in fibrosis

Fibrosis is an aberrant tissue regeneration response following injury and can affect most tissue types and organs. Estimated to contribute to at least one third of all deaths worldwide and even 45% of deaths in the developed world, fibrosis undoubtedly is a major global cause of mortality and morbidity (1, 2). Irrespective of affected tissue, fibrosis is characterized by increased extracellular matrix (ECM) deposition. The culprit effector cells are most often fibroblasts, which, upon specific activating cues, exit quiescence, proliferate, migrate, and secrete excessive amounts of ECM components. Deposition and subsequent crosslinking of the ECM lead to a drastic reduction of tissue functionality, ultimately resulting in organ failure when disease progresses (3, 4).

Constituting between 30% and 70% of ECM protein in all tissue types, collagen clearly is the main component of the ECM and its biosynthesis, deposition and crosslinking is highly upregulated in fibrotic disease (4-6). Attenuation of collagen synthesis and deposition therefore is the most common read-out when efficacy of new potential drugs is evaluated *in vitro* and *in vivo*. Many drugs targeting central fibrogenic pathways like *e.g.* transforming growth factor β (TGF- β), connective tissue growth factor (CTGF), or mammalian target of rapamycin (mTOR) signaling have a pronounced effect on collagen synthesis and maturation, but, owing to the functional pleiotropy of their targets, do not specifically act on collagen (7-9). Clearly, targeting central upstream mechanisms of fibrogenesis can be advantageous as typically various profibrotic processes are affected, but this approach may also lead to unanticipated and undesired side effects (7, 10).

During the last decade, the collagen biosynthesis and maturation pathway itself has emerged as an attractive therapeutic target for fibrotic disease. This includes targets directly acting on collagen but also such that anabolize essential building blocks of collagen, in particular glycine and proline biosynthetic enzymes. However, it is important to acknowledge that collagen is a ubiquitous molecule in the body, which fulfils essential functions as macromolecular scaffold, growth factor reservoir, and receptor binding site in virtually every tissue. The challenge therefore consists in targeting the disease-specific collagen. This review aims to provide a comprehensive overview on currently discussed drug targets in this context and to offer conceptual insight into how fibrosis-specific targeting could be achieved. First, collagen types (Table 1) and biosynthesis, maturation, and degradation of fibrillar collagens is reviewed. Then, taking into consideration all types of organ fibrosis, the current state on potential antifibrotic drug targets within the collagen biosynthesis and maturation pathway is summarized (Table 2). This is followed by an assessment of the targets' disease specificity and a discussion on potential strategies for targeted delivery. Ultimately, such considerations may be crucial for the successful implementation of anti-collagen biosynthesis drugs to treat fibrogenesis.

The collagen superfamily

In vertebrates, the collagen superfamily to date comprises 28 members, numbered with Roman numerals I to XXVIII. Collagens contain other domains, too, but the presence of at least one triple helical domain, termed collagen domain, is the most important unifying and defining feature for this superfamily. Every collagen molecule is assembled from three parallel polypeptides, the so-called α -chains, which are in a polyproline II-type helical conformation and wind around each other, forming a right-handed triple helix (11). The α -chains that make up a collagen can be

identical or different, depending on the collagen type. For instance, type III collagen is made up of three identical α_1 -chains encoded by *COL3A1*. In contrast, type I collagen is a heterotrimer made of two identical α_1 -chains and an α_2 -chain encoded by *COL1A1* and *COL1A2*, respectively, and type VI collagen is even composed of three different chains.

Collagens are classified according to their extracellular supramolecular assembly (Table 1) (12-16). Fibril-forming collagens, in particular types I, III, and V, are well established as overexpressed in fibrotic disease and most of what we know about collagen biosynthesis and maturation derives from fibril-forming collagens, above all from the archetypal type I collagen. Even though alterations of other collagen types in fibrosis have been described, including *e.g.* type IV, VI, VII, XV, XVIII, and XXVII (17-20), these have received much less attention to date and their roles in disease pathogenesis has yet to be elucidated.

Unique characteristics and requirements of the collagen triple helix

The triple-helical three-dimensional structure is a unique property of the collagen domain (Figure 1A, B, C). It provides exceptional strength and stability and confers resistance to mechanical and tensile stress. Two amino acids, namely proline and glycine, are of exceptional importance for triple helix formation, and their frequencies are much higher in collagen domains than in the average vertebrate protein (21) (Figure 1D). Overrepresentation of proline increases the propensity of the individual α -chains to adopt the above-mentioned polyproline II-type helical conformation which in turn decreases the entropic cost for triple helix folding (11). The use of proline, however, is associated with a drawback: All collagen prolines must be in *trans* conformation in order to allow for linear chains, because *cis*-prolines stereochemically induce

bends and turns in proteins. While for all other amino acids, the *trans* conformation typically is energetically far more favorable, this is not so pronounced for prolines: Up to 6% of all prolines in protein structures adopt the *cis* conformation (22); collagen folding therefore requires peptidyl prolyl isomerases (PPIs), enzymes that interconvert *cis*- and *trans*-prolines.

In addition, amino acid repeats of Gly-Xaa-Yaa are essential for the assembly of the three α -chains, because triple helix assembly positions the residue of every third amino acid towards the interior of the helix where the available space only accommodates the smallest side group possible, the single hydrogen atom of Gly (Figure 1C). This also means that all other amino acids in Xaa and Yaa positions point outwards (shown for Pro in Figure 1B). Fibrillar collagens, like type I and type III collagens, are typically composed of a single uninterrupted collagen domain, once propeptide cleavage has occurred in the extracellular space. Consequently, glycine makes up more than 30% of their amino acid sequence (Figure 1D).

All amino acids can be obtained by diet, and endogenous Pro and Gly anabolism is generally not considered sufficient to maintain collagen biosynthesis on its own (23). However, within the last years it has become increasingly clear that myofibroblast differentiation comes along with profound metabolic changes, including upregulation of glycolysis and glutaminolysis, which both feed into the equally upregulated *de novo* biosynthetic pathways for Pro and Gly (24). This metabolic reprogramming makes myofibroblasts much less dependent on dietary supplementation for collagen biosynthesis and provides interesting novel therapeutic strategies. Given the exceptional importance of Pro and Gly for collagen biosynthesis, their *de novo* biosynthetic pathways including the most important enzymes will be described in the following.

***De novo* proline (Pro) biosynthesis**

Proline (Pro) can, in principle, be synthesized from several precursors. The classical pathway of *de novo* Pro biosynthesis commences with α -ketoglutarate (α KG), an intermediate of the citric acid cycle (Figure 2). Glutamic-oxaloacetic transaminases (GOT) convert α KG into glutamate (Glu) under concomitant conversion of aspartate (Asp) to oxaloacetate (OA). Aldehyde dehydrogenase 18A1 (ALDH18A1) then catalyzes the two-step conversion from Glu to glutamate-semialdehyde which spontaneously rearranges to 1-pyrroline-5-carboxylate (P5C). Finally, pyrroline-5-carboxylate reductases (PYCR) convert P5C to proline (25).

Interestingly, TGF- β -induced collagen synthesis has been demonstrated to be independent of GOTs, suggesting that, in a profibrotic milieu, synthesis of Glu from α KG is dispensable for proline biosynthesis. Instead, glutamine (Gln) is a far more important precursor for Glu in this context (26, 27) (Figure 2). Gln is the most abundant circulating amino acid in blood and can serve to replenish the citric acid cycle and act as precursor for multiple biosynthetic pathways including Pro biosynthesis (24, 28, 29). Glutaminase (GLS) catalyzes the hydrolytic deamidation of Gln to generate Glu. GLS, all enzymes downstream of Glu in the biosynthesis of Pro, *i.e.* ALDH18A1, PYCR1, PYCR2, and PYCRL (Figure 2), are induced by TGF- β , and at least GLS and ALDH18A1 are required for collagen biosynthesis in lung fibroblasts. In contrast, Gln is not required for mitochondrial oxygen consumption in response to TGF- β , indicating that Gln does not feed into the citric acid cycle under these conditions (26).

In addition, Pro can also be generated from arginine, *via* action of arginase 1 or 2 (ARG1, ARG2), followed by deamination of ornithine by ornithine aminotransferase (OAT), a reaction which

yields P5C and Glu (Figure 2). While this pathway appears to contribute to collagen biosynthesis and lung fibrosis in the mouse (30, 31), its significance for human fibrosis is less clear and requires further investigation.

***De novo* glycine (Gly) biosynthesis**

Endogenous glycine (Gly) is mainly produced from serine, including dietary serine and *de novo* synthesized serine, by the action of serine hydroxymethyltransferases 1 and 2 (SHMT1 and SHMT2, Figure 2) (32). Starting from the glycolysis intermediate 3-phosphoglycerate (3PG), the first reaction of *de novo* serine biosynthesis is oxidation of 3PG to 3-phosphooxypyruvate (3POP), catalyzed by the enzyme phosphoglycerate dehydrogenase (PHGDH). Then, phosphoserine aminotransferase (PSAT1) catalyzes amino group transfer from glutamate (Glu), a reaction yielding phosphoserine (PSer) and α -ketoglutarate (α KG). Finally, dephosphorylation catalyzed by phosphoserine phosphatase (PSPH) yields serine (Ser, Figure 2). Similar to *de novo* Pro biosynthetic enzymes, also enzymes of this pathway are upregulated by TGF- β and necessary for TGF- β -induced collagen synthesis in lung fibroblasts (33). Gln seems equally important as precursor for Glu, which, here, serves as a cofactor in the PSAT1-mediated amino group transfer (Figure 2). Hence, GLS1-mediated hydrolytic deamidation of Gln generates Glu as an important precursor for both *de novo* Pro and Gly biosynthesis.

Finally, dietary intake of Pro, Gly, their precursor amino acids, as well as amino acids released by degradation of exogenous and endogenous proteins, serve as additional pools for these amino acids. Interestingly, neither serine nor glycine deprivation protected mice from bleomycin-

induced lung fibrosis (34), indicating that dietary supplementation may play a subordinate role in the context of collagen biosynthesis in fibrosis.

Proposed targets in the context of Pro and Gly biosynthesis

A number of recent reports have highlighted the great potential that targeting myofibroblast-specific metabolic reprogramming holds for treatment of fibrosis. Even though this is a relatively new concept, several *in vitro* and *in vivo* studies have already put forward a number of possible drug targets in Pro and Gly biosynthesis. With multiple collective evidence at hand, **glutaminase 1 (GLS1)**, which generates Glu as important cofactor for both biosynthetic pathways, may be the most promising emerging drug target in this context. Knocking down GLS1 in normal human lung fibroblasts or treatment with the GLS inhibitors CB-839 or Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) attenuated TGF- β -induced collagen synthesis (26, 35, 36). Conditional deletion of GLS1 in mouse fibroblasts protected against bleomycin-induced lung fibrosis and CB-839 was therapeutically effective in two independent mouse models of lung fibrosis (37). Notably, the GLS inhibitor CB-839 (telaglenastat) is currently under investigation in phase 1 and 2 anticancer clinical trials and new classes of GLS inhibitors are being developed, emphasizing that this approach may be readily translated into anti-fibrotic treatment (28, 38).

Another drug target for which therapeutic proof-of-concept exists *in vivo* is the enzyme **phosphoglycerate dehydrogenase (PHGDH)** (34). Hamanaka *et al*, using a small molecule inhibitor of PHGDH (NCT-503), were able to show that blocking *de novo* Ser/Gly biosynthesis protected from the development of lung fibrosis in the mouse model of bleomycin-induced lung fibrosis (34). PHGDH is increasingly recognized as drug target in the cancer field and even though

small molecule inhibitors for PHGDH have not entered clinical trials yet, lead compounds are being optimized (39).

Finally, *in vitro* evidence in primary human lung fibroblasts suggest that additional enzymes like **serine hydroxymethyltransferase 2 (SHMT2)**, **phosphoserine aminotransferase 1 (PSAT1)**, and **aldehyde dehydrogenase 18A1 (ALDH18A1)** may qualify as drug targets. At least for SHMT2 specific preclinical small molecule inhibitors exist (40) while for PSAT1 and ALDH18A1 antisense strategies may hold the greatest potential to date.

Post-transcriptional collagen biosynthesis and maturation

After transcription of collagen genes to mRNA, protein biosynthesis and maturation of collagen is a complex multi-step process which involves regulatory events at the translational level, an elaborate ER-resident modification and folding machinery, but also extracellular processing and crosslinking (16) (Figure 3). As fibril-forming collagens, particularly types I, III, and V, are the collagens predominantly overexpressed in fibrotic disease, the following text will focus on biosynthesis of those, with type I collagen as the best-described representative for this collagen class.

Collagen chain translation

Type I collagen is a heterotrimer composed of two $\alpha 1(I)$ chains and one $\alpha 2(I)$ chain, which are encoded by *COL1A1* and *COL1A2*, respectively. Translation efficacy depends on unique conserved sequences in the 5'- and 3'-UTRs of *COL1A1* and *COL1A2* mRNAs. Firstly, both transcripts - as well as *COL3A1* mRNA which encodes the type III collagen homotrimer – are characterized by a unique 5' stem loop structure which is specifically recognized by the RNA binding protein **La-related**

protein 6 (LARP6). LARP6 plays a crucial role in acceleration of type I and III collagen biosynthesis upon profibrotic stimuli, because it facilitates the recruitment of additional translational factors and the coordinated translation of these collagen chains. Secondly, just like the majority of mammalian genes (41), also collagens are regulated by numerous microRNAs which bind to the 3'-UTRs and induce translational repression. Here, the human micro-RNA family **hsa-miR-29** stands out, because it targets many collagens in concert, acting as a master regulatory hub for collagen expression (42, 43). Even if this is in addition to regulation of other ECM components and ECM regulators, hsa-miR-29-mediated regulation of collagen types and proteins involved in collagen synthesis and crosslinking is clearly overrepresented (42, 43).

Co- and post-translational modification

During translation at the ribosome, the growing polypeptide is directly extended into the lumen of the rER where numerous modifications are inserted into the nascent unfolded chain in a co-translational fashion. These include hydroxylation of proline and lysine residues as well as glycosylation of hydroxylysines, all of which are mediated by an rER-resident procollagen-specific molecular ensemble, enzymes existing in defined multiprotein complexes with other chaperones and protein folding catalysts (Figure 3) (44). Many of these modifications are crucial for stability, triple helix folding, and secretion of procollagen, as well as for the final extracellular supramolecular structure (12, 14, 44, 45). For instance, as mentioned previously, 4-hydroxylation of proline residues in position Y (4-hydroxyproline, Figure 4C) of the Gly-X-Y repeats is known to increase thermodynamic stability of the triple helix (44, 46-50). Also, the degree of lysine hydroxylation in the collagenous and telopeptide regions of fibrillar collagens defines the extent, type, and stability of extracellular intermolecular collagen crosslinks (51). At the same time, the

functions of hydroxylysyl glycosylations (Figure 4D,E) and the comparatively rare prolyl-3-hydroxylation (3-hydroxyproline, Figure 4B) in position X are far less understood.

Numerous enzymes and chaperones are involved in that process. Collagen prolyl-4-hydroxylation is mediated by **prolyl-4-hydroxylases** which exist as $\alpha_2\beta_2$ heterotetramers composed of one of three catalytical α -subunits (encoded by *P4HA1*, *P4HA2*, and *P4HA3*) and the structural β -subunit protein disulfide isomerase (PDI, encoded by *P4HB*). The latter helps to maintain the α -subunit in soluble active form and to retain the full complex in the ER (44, 49). Collagen prolyl-3-hydroxylation is a much less frequent and only poorly understood collagen PTM introduced by collagen **prolyl-3-hydroxylases** (49, 52-59). Prolyl-3-hydroxylation is typically found on prolines in the X position of the Gly-X-Y repeat, when Y is a 4-hydroxylated proline; prolyl-3-hydroxylation is therefore thought to occur after prolyl-4-hydroxylation (44, 49). The collagen prolyl-3-hydroxylase or leprecan family comprises five members (P3H1-P3H4, cartilage-associated protein, CRTAP), of which only P3H1, P3H2, and P3H3 exert prolyl-3-hydroxylase activity. Also these enzymes form multimeric complexes with other collagen-modifying proteins and chaperones. For instance, P3H1 forms a tight trimeric complex with CRTAP and the peptidyl-prolyl isomerase cyclophilin B (PPIB). CRTAP, despite no enzymatic activity of its own, promotes P3H1 activity (53, 60). P3H3 and P3H4 (also termed SC65) form a trimeric complex with lysyl hydroxylase 1 (LH1). Here, P3H4, also without a prolyl-3-hydroxylase activity of its own, seems to adopt a role similar to CRTAP with which it is closely related (61, 62). In contrast, the enzymatic activity of P3H2 does not require CRTAP (63) and other interaction partners have not been identified so far.

Lysyl hydroxylases, which hydroxylate lysines in the C5-position (Figure 4A), comprise three enzymes termed LH1 - LH3 and are encoded by *PLOD1* - *PLOD3*, respectively (44). LH1 and LH2 display domain-specific activities: LH1 preferentially acts on lysines in triple-helical regions of fibrillar collagens (64) and the resulting 5-hydroxylysine residues are often subject to O-glycosylation by glycosyl transferases (Figure 3, Figure 4D, E) (44, 64, 65). LH2, on the contrary, acts on lysines in the non-collagenous telopeptide regions of fibrillar collagens (64, 66), where the resulting 5-hydroxylysine residues are often subject to extracellular lysyl oxidase (LOX)-mediated crosslinking. While both unhydroxylated and hydroxylated telopeptide lysines can be crosslinked by LOX, the hydroxylation status of the involved lysines affects the crosslink in terms of chemical nature and stability (44, 51, 65, 66). Also LH1 and LH2 have been demonstrated to exist in multimeric complexes, LH1, as already mentioned above, in a complex with P3H3 and P3H4 (61, 62) and LH2 in a complex with FKBP10 (66). LH3 represents a special case in that context: It is considered the evolutionary ancestor of the LH family and capable not only of lysine hydroxylation, but also of two consecutive O-glycosylation reactions, activities which the other LHs appear to have lost (44, 64, 65, 67). Finally, also two collagen **glycosyl transferases**, GLT25D1 and GLT25D2, mediate O-glycosylation of collagen 5-hydroxylysines. In general, hydroxylysines can be O-glycosylated with a monosaccharide (β -d-galactopyranose, Gal) or a disaccharide (α -d-glucopyranosyl-(1 \rightarrow 2)- β -d-galactopyranose, GlcGal), resulting in galactosylhydroxylysine (GHyl) or glucosylgalactosylhydroxylysine (GGHyl), respectively (Figure 4D, E) (44, 64, 65).

Triple helix formation

After the introduction of collagen PTMs, the N- and C-terminal propeptides fold into globular domains, and the C-propeptides of three α -chains assemble close to the ER membrane, forming

the nucleus for triple helix formation (44, 68-70). Many general ER-folding folding catalysts including Grp78 (BiP), Grp94, PDI, calreticulin, calnexin and CypB support this process (44). Once triple helix formation is initiated, it proceeds in a zipper-like fashion from the C- to the N-terminus. One of the rate-limiting steps is the *cis-trans* isomerization of proline residues, which - as detailed above - are overrepresented in the collagenous domains and must be in the *trans* state to allow for the linear continuation of the triple helix. Many ER-resident **peptidyl-prolyl isomerases** (PPIases) including FKBP10, FKBP11, FKBP14, and PPIB are involved in collagen folding, but to what extent they act as *cis-trans* isomerases or primarily as chaperones preventing premature fibril formation has not been fully elucidated (44, 70-75).

Heat shock protein 47 (HSP47) is required for multiple steps

The collagen-specific chaperone **heat shock protein 47 (HSP47)** is considered essential for all ER-resident collagen biosynthetic processes, including collagen modifications and triple helix formation. HSP47 has been demonstrated to directly interact with several of the above-mentioned proteins including PPIB, FKBP10, GRP78, and LH2 (44, 70, 75-82). In addition, HSP47 is also required for the following step, the transfer from the ER to the Golgi, where it binds to transport and Golgi organization protein 1 (TANGO1) and thus helps recruit procollagen molecules to the ER-Golgi intermediate structures. Finally, in the ER-Golgi intermediate compartment or the cis-Golgi HSP47 dissociates from procollagen in a pH-dependent manner and returns to the ER (82, 83).

Trafficking to the extracellular space

Secretion of procollagen was previously thought to involve transport of procollagen molecules via specialized, enlarged, coat protein complex II (COPII) vesicles from the rER to the Golgi. However, a recent study where the authors used live-cell imaging to track GFP-tagged procollagen molecules in the cell did not support the existence of such large COPII vesicles between the ER and the Golgi but instead rather argued for direct interconnections between organelles or less well-characterized intermediate carriers (84, 85). Either way, even if the exact mechanisms have not been fully clarified, it is well established that procollagen secretion is dependent on transport and Golgi organization protein 1 (TANGO1), cutaneous T-cell lymphoma-associated antigen 5 (cTAGE5) at the ER exit site, and COPII coat proteins such as SEC13, SEC23, SEC24, and SEC31. TANGO1 recruits procollagen molecules to COPII proteins by binding to HSP47 on the one hand and COPII coat proteins on the other (86-89). Second, recruitment of Sedlin by TANGO1 stabilizes the inner layer of the ER-Golgi intermediate (90) and ubiquitination of the outer COPII coat protein SEC31 by the ubiquitin ligase CUL3-KLHL12 is also required for ER-Golgi trafficking of procollagen (91).

Propeptide cleavage, fibril formation and crosslinking

After secretion of fibrillar-type procollagens, the N- and C-terminal propeptides are cleaved off by specific procollagen proteinases. While bone morphogenetic protein 1 (BMP1) or the tolloid-like proteins 1 or 2 (TLL1, TLL2) cleave off the C-terminal propeptide, a member of the A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family, *e.g.* ADAMTS2, ADAMTS3, or ADAMTS14 cleaves off the N-terminal propeptides; the more recently discovered meprins are capable of cleaving off both propeptides (13, 14, 45, 92). Propeptide cleavage

triggers the spontaneous quarter-staggered assembly of the triple-helical collagen molecules into fibrils (93, 94), which are then stabilized by intra- and intermolecular crosslinks.

Collagen crosslinking is mediated by enzymes of the lysyl oxidase (LOX) and transglutaminase (TGM) family (95-98). While TGM-mediated crosslinking fulfills pleiotropic functions in numerous physiological processes like blood clotting, skin cornification, and extracellular matrix remodeling (99), LOX enzymes preferentially crosslink collagens and elastin. There are five LOX family members, LOX and LOXL1-LOXL4. Among these, LOX and LOX-like 2 (LOXL2) are the best-established crosslinking enzymes for fibrillar collagens, introducing both intra- and intermolecular crosslinks (98, 100), which increase tissue stiffness and are critical for tissue formation during development and maintenance (65). In addition, antioxidant proteins like extracellular superoxide dismutase (SOD2) or glutathione peroxidase 3 (GPX3) associate with collagens and may protect them from oxidative damage (101-104).

Collagen turnover

Mature collagen is subject to a typically slow but constant turnover, the rate of which is highly tissue-dependent (105-107). Collagen can be degraded by extracellular proteases or taken up into the cell and degraded by lysosomal proteases. In general, only members of two protease families, namely **matrix metalloproteinases (MMPs)** and **cathepsins (CTSs)**, are effective in collagen degradation (16, 108-111). Interestingly, these processes often release biologically active peptides, so-called matricryptins or matrikines (112, 113).

Extracellular collagen degradation is primarily exerted by MMPs and cathepsin K. MMPs are extracellular zinc-dependent endopeptidases many of which display collagenolytic activity (108,

109). MMPs recognize specific cleavage sites consisting of distinct amino acid sequences in the collagen molecule and need to unwind the collagen structure in order to access the cleavage site (114-116). In contrast, the cysteine protease cathepsin K (CatK) can also target triple-helical collagen directly, without the need for unwinding of the helix (117) and therefore probably is the most effective protease for the extracellular degradation of fibrillar collagen (117, 118). The intracellular collagen degradation pathway is initiated by uptake of collagen into the cell via three alternative pathways. (1) Intact collagen fibrils are recognized by specific integrins and internalized by phagocytosis (119, 120). Fragmented collagen, *e.g.* following MMP- or CTSK-mediated cleavage, can be internalized by (2) macropinocytosis or (3) receptor-mediated endosomal uptake (16). While macropinocytosis is actin cytoskeleton-driven and independent of membrane receptors, receptor-mediated endosomal uptake requires cell type-specific receptors, *e.g.* the urokinase plasminogen activator receptor-associated protein (uPARAP/Endo180 or C-type mannose receptor 2, MRC2) on fibroblasts (121-123) and macrophage mannose receptor 1 (MRC1) and lactadherin (MFGE8) on macrophages (124, 125). Collagen-containing phagosomes, macropinosomes, and endosomes all merge with lysosomes which contain a range of cathepsins. Of these, cathepsins B, D, K and L are known to cleave collagen into low-molecular-weight peptides (126-128).

Proposed targets for antifibrotic therapy within the collagen biosynthesis and maturation pathway

Several drug targets within the collagen biosynthesis and maturation process, including representatives from many of the above described regulatory levels, have been suggested for

the treatment of fibrosis (summarized in Table 2 and Figure 3) and the relevant evidence will be briefly presented below.

La-related protein 6 (LARP6). Upon profibrotic stimuli, the RNA binding protein La-related protein 6 (LARP6) specifically binds to a unique 5' stem loop structure of Col1a1, Col1a2, and Col3a1 transcripts and facilitates the recruitment of additional translational factors and the coordinated translation of these collagen chains. Mutation of the 5' stem loop in Col1a1 attenuates liver fibrosis in mice (129) and lead compounds have been identified that block binding of LARP6 to the stem loop and inhibit collagen biosynthesis *in vitro* and fibrogenesis *in vivo* (130, 131).

hsa-miR-29. MicroRNAs (miRNAs) are short endogenous RNA molecules that act by repressing translation of their target genes. In the context of collagen biosynthesis, the human micro-RNA family hsa-miR-29 acts as a master regulatory hub for collagen expression (42, 43). The hsa-miR-29 family consists of four members (hsa-miR-29a, hsa-miR-29b-1, hsa-miR-29b-2, and hsa-miR-29c). They are co-transcribed as polycistronic transcripts from two separate gene clusters on chromosome 7 (hsa-miR-29a, hsa-miR-29b-1) and chromosome 1 (hsa-miR-29b-2, hsa-miR-29c). The mature sequences of hsa-miR-29b-1 and hsa-miR-29b-2 are identical and therefore collectively referred to as hsa-miR-29b. All mature miR-29 family members share identical seed regions, which determine the protein-coding targets by complementarity to the 3'UTR in their transcript sequence. Hence, predicted targets and functions of all miR-29s largely overlap, even though evidence exists for isoform- and tissue-specific effects (42). Here, as the aim of this review is to provide a short overview, no further distinction is made between the different miR-29 isoforms.

Using hsa-miR-29 (mimics) to target fibrogenesis has been put forward for essentially every organ or tissue type that can develop fibrosis (see Table 2). Several lines of evidence, both *in vitro* and *in vivo*, in different cell types and organs, argue for its suitability to control fibrosis-associated overexpression of collagen (132-138). The first-generation miR-29b mimic MRG-201 (remlarsen, miRagen Therapeutics, now Viridian Therapeutics) for treatment of skin fibrotic disorders has already successfully passed a phase 1 clinical trial (NCT02603224) and just completed phase 2 (NCT03601052) (139). The same company developed a second-generation miR-29b mimic MRG-229 which is anticipated to enter clinical trials for IPF soon (140).

Collagen prolyl-4-hydroxylase (P4HA). Collagen prolyl-4-hydroxylases (P4H) catalyze the hydroxylation of proline at C4 (4-hydroxyproline, 4-Hyp, Figure 4C) in the Y position of G-X-Y repeats in collagen chains (44). 4-Hyp is the most frequent collagen post-translational modification (PTM) and known to contribute to the thermostability of collagen molecules. Care must be taken to distinguish collagen P4H enzymes from collagen prolyl-3-hydroxylases (P3H) and prolyl hydroxylase domain proteins (PHD): All three prolyl hydroxylase types catalyze hydroxylation of proline, but collagen P3Hs do so at the C3 position, generating 3-hydroxyproline (3-Hyp, Figure 4B), a comparably rare collagen PTM. PHDs, in contrast, hydroxylate two highly conserved proline residues of hypoxia inducible factor 1 α (HIF-1 α) which leads to proteasomal degradation in normoxia. Decrease of PHD activity under hypoxic conditions induces HIF-mediated gene expression; PHDs hence act as molecular sensors for oxygen (168). Notably, PHDs are also potential pharmacological targets for fibrotic disease (169), but not discussed here, as they are not part of the collagen biosynthetic machinery.

Three different isoforms of human collagen P4HAs exist, namely the α (I), α (II) and α (III) isoforms encoded by *P4HA1*, *P4HA2*, and *P4HA3*, respectively. All three isoforms have been mentioned in the context of fibrotic disease (149, 151, 170, 171), but the reported evidence is mostly circumstantial (149, 170, 171). Some studies have used the α -ketoglutarate analogue pyridine-2,4-dicarboxylate (24PDC) or an analogue thereof as P4H inhibitor and observed antifibrotic effects (148, 150). These studies, however, need to be interpreted with caution, as 24PDC similarly inhibits other enzymes that use α -ketoglutarate as a cofactor (172, 173). Probably to date the strongest evidence for a possible therapeutic benefit of P4HA as a drug target has been provided by Luo *et al* (151): Here, the authors used pyridine-2,5-dicarboxylate (25PDC) which is considered selective for P4HA enzymes (172, 173) and demonstrated that 25PDC protected from bleomycin-induced lung fibrosis (151). Nevertheless, 25PDC has a relatively high inhibition constant in the micromolar range, and may still not be specific considering the plethora of enzymes that use α -ketoglutarate as a cofactor, in particular when used at relatively high concentrations (151, 173). Other P4HA inhibitors are being developed for therapeutic application (172).

FK506-binding protein 10 (FKBP10). FK506-binding protein 10 (FKBP10), also termed FKBP65, is a procollagen chaperone (44, 74) expression of which is developmentally regulated in many tissues, limited to bone and ligaments postnatally, but reactivated also in visceral tissues upon injury (72, 152, 174). *FKBP10* mutations cause recessive *osteogenesis imperfecta* and Bruck syndrome (175), both characterized by bone fragility and low bone mass. Mechanistically, FKBP10 deficiency leads to decreased collagen secretion and deposition as well as reduced lysyl hydroxylation of collagen telopeptides and crosslinking (72, 175, 176), the latter through

mediating dimerization of lysyl hydroxylase 2 (66). To date, FKBP10 has been suggested as drug target for idiopathic pulmonary fibrosis (IPF) and studies in patient-derived primary human lung fibroblasts have shown that downregulating FKBP10 inhibits collagen synthesis and secretion, attenuates myofibroblast differentiation, and reduces fibroblast migration, all representing important hallmarks of lung fibrogenesis (71, 72). In summary, targeting FKBP10 has the potential to act on multiple regulatory levels relevant for disease. Specifically targeting FKBP10 with small molecular inhibitors is, to date, not possible. FK506 (tacrolimus) only weakly binds to one of the four FK506-binding domains of FKBP10 and has little effect on its collagen chaperone activity (74), hence not providing a promising lead structure. In addition, the three-dimensional structure of FKBP10 is not resolved preventing structure-based inhibitor design. The establishment of an FKBP10 activity screening assay is hampered by our current lack of understanding of the molecular determinants of the FKBP10/pro-collagen interaction. Hence, antisense oligonucleotide strategies which do not require any knowledge of the target except for the transcript sequence (177) may provide the only current option to specifically target FKBP10.

Heat shock protein 47 (HSP47). The potential of targeting heat shock protein 47 (HSP47, encoded by *SERPINH1*) for treatment of fibrotic disease has been comprehensively summarized very recently and the interested reader is referred to this excellent review and references therein (154). Owing to its central role in collagen triple helix formation as a procollagen-specific chaperone, many studies have explored HSP47 as a target for the treatment of fibrotic conditions. In contrast to FKBP10, the three-dimensional structure of HSP47 has been resolved and the interaction with procollagen elucidated in molecular detail (178). *In vitro* and *in silico* screening assays have been established by several groups and a number of promising small-

molecule HSP47 inhibitors have been identified (154). However, to date, none of the suggested small-molecule HSP47 inhibitors has been tested *in vivo*. Instead, efforts to target HSP47 have mostly relied on antisense strategies and all clinical trials, closed and ongoing, have applied vitamin-A coupled lipid nanoparticles containing anti-HSP47 siRNA (154). This targeting strategy will be described and briefly discussed in a broader context below. To date, no results of these clinical trials have been reported in detail – a brief poster presentation abstract on the use of BMS-98626 in patients with advanced hepatic fibrosis indicated target engagement and good tolerance of the drug, but moderate effects at best (155).

Transport and Golgi organization protein 1 (TANGO1). TANGO1 is ascribed a central role at the ER exit site, where procollagen destined for secretion is recruited for trafficking via the Golgi. Initially identified as facilitating secretion of type VII procollagen (86), subsequent studies have established a broader role for procollagen secretion involving many more types (156, 157), possibly via HSP47 as adapter protein (83). To the best of my knowledge, only one published study has addressed TANGO1 as potential antifibrotic target: Using TANGO1 depletion in hepatic stellate cells and TANGO1 heterozygous knockout mice, the authors showed that TANGO1 deficiency attenuated procollagen secretion, and that TANGO1 heterozygosity led to a decreased extent of hepatic fibrosis in two independent mouse models (157).

Lysyl oxidases (LOX/LOXL2). Lysyl oxidase (LOX) family members are secreted copper-dependent enzymes that catalyze the oxidative deamination of specific collagen lysines and hydroxylysines. The resulting highly reactive allysine and hydroxyallysine residues, respectively, trigger a number of succeeding condensation reactions resulting in divalent and trivalent crosslinks between collagen molecules (16). Increases in collagen crosslinking cause elevated tissue stiffness in

fibrosis (179) and may impede collagen degradation leading to an accumulation of extracellular collagen (180, 181). Of the five LOX family members (LOX, LOXL1-LOXL4), most studies have addressed the role of LOX and LOXL2 in collagen crosslinking and fibrogenesis (182) and, to date, there is little evidence for other LOXL members to directly act on collagen (98). Some reports, including a systematic comparison of all LOX family members in the context of pulmonary fibrosis, point towards an outstanding role of LOXL2 in fibrosis (100, 183). Therefore, this review focusses on LOX and LOXL2, even though there is evidence for contributions of other LOXL isoforms to fibrotic disease (182).

While studies in various animal models of fibrosis showed great potential for LOXL2 as a drug target in antifibrotic therapy (100, 159-162), results of clinical trials using a LOXL2-specific antibody (sintuzumab) for therapy of liver, lung and bone marrow fibrosis were disappointing, showing lack of efficacy (165-167). Investigators currently discuss whether this may be a result of limited potency and insufficient target engagement of the antibody in fibrotic areas, hence not arguing against inhibition of LOXL2 *per se* (184). In addition, comparative studies *in vitro* and *ex vivo* indicate that a pan-LOX inhibitor may be more effective than specifically targeting LOXL2 alone (164, 183). Hence, novel small molecule inhibitors are being developed and, interestingly, two pan-LOX inhibitors have already shown efficacy in an animal model of myelofibrosis (160). The pan-LOX inhibitor PXS-5505 is currently being tested in a clinical trial (NCT04676529) and interim reports state an excellent safety profile (185).

Matrix metalloproteinases (MMPs). The human matrix metalloproteinases (MMPs) comprise 23 extracellular zinc-dependent endopeptidases, which initially were thought to mainly degrade ECM components (111). Collagenolytic activity has been described for many MMPs (108, 111),

but enzymological evidence for MMP-mediated degradation of interstitial triple-helical collagens, the collagens considered most relevant for fibrosis, has been reported for the following members of the MMP family: MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and two membrane type (MT) MMPs, MMP-14 (MT1-MMP), and MMP-16 (MT3-MMP) (186).

While one would anticipate collagenolytic activity to be beneficial in fibrotic disease, multiple studies in animal models, on the contrary, have demonstrated that some MMPs rather have pro-fibrotic functions (108, 187, 188). This is probably a reflection of the fact that MMPs display moonlighting functions, cleaving a variety of non-ECM substrates, including intra- and extracellular representatives (108, 188). Hence, modulation of MMPs in animal models of fibrosis cannot be attributed to effects on collagen turnover only and tissue-specific effects complicate the matter even more (187). For example, MMP-8 deficiency protects mice from bleomycin-induced lung fibrosis and the proposed mechanisms are largely independent of ECM degradation (108, 189). In liver fibrosis, however, MMP-8 appears to serve an antifibrotic function, as overexpression of MMP-8 ameliorates CCl₄- and bile duct-ligation-induced liver cirrhosis (190). For some excellent and more comprehensive reviews on the complexity of this matter, the interested reader is referred to reviews by Craig *et al* (108), Giannandrea *et al* (187), and Roderfeld (188).

Specificity of proposed drug targets for disease-associated collagen and potential adverse effects

The brief chapter on MMPs above illustrates the importance of specificity for collagen as a substrate, if the aim is to reduce deposited extracellular collagen in fibrosis. With evidence for

many more moonlighting proteins accumulating in the literature (191), it is probably naïve to assume 100% substrate specificity for any given chaperone or enzyme. Accordingly, also for most of the potential drug targets given in Table 1, there is evidence for other substrates than collagen.

LARP6 may be the most specific representative of the described drug targets in this review. To date, no high-affinity substrates other than the COL1A1, COL1A2, and COL3A1 transcripts have been identified. Furthermore, homo- and heterozygous mice with a mutation in the 5'-UTR stem loop of Col1A1, the LARP6 binding site, display a normal phenotype, indicating that LARP6-independent baseline collagen synthesis is sufficient to maintain normal type I collagen synthesis.

In contrast, **miR-29**, as is in the nature of regulation by microRNAs, has multiple substrates. Even if it can be argued that collagens are overrepresented within that list, and that it may be beneficial to downregulate multiple profibrotic targets with miR-29 as a single regulatory hub, unintended side effects are not unlikely. Indeed, some studies cast some doubt on the applicability of hsa-miR-29 mimics as drugs for treatment of fibrosis. For instance, a comprehensive study using genetic deficiency models, pharmacological inhibition, and a cell-specific gene therapy approach has shown that cardiomyocyte-specific deletion of miR-29 may be beneficial in the context of cardiac remodeling and fibrosis (144). This is in sharp contrast to most other studies reporting antifibrotic effects of miR-29 and emphasizes the need for studies addressing the relative contribution of the different isoforms and cell types to fibrogenesis (143, 144). Moreover, overexpression of miR-29 leads to muscular dystrophy in mice, indicating that systemic application of miR-29 mimics may lead to muscular abnormalities (192). Hence, organ-targeted delivery seems to be key for successful translation of miR-29 into the clinics.

Collagen **P4HA** enzymes, even if they, to the best of my knowledge, can be considered as specifically acting on collagens, modulate cofactor availability for other α -ketoglutarate-dependent enzymes, as *e.g.* for HIF-1 α (193). Furthermore, as prolyl-4-hydroxylation is essential for collagen thermodynamic stability, it is highly likely that targeting P4HA affects baseline collagen expression, potentially leading to adverse connective tissue alterations, if not delivered in a highly specific manner to fibrotic areas only.

For **FKBP10**, primarily interactions with collagens and effects on collagen expression have been described, but several reports equally support a chaperone activity for tropoelastin (152, 194). Given its multi-domain structure, additional functions are likely, in particular during development and organogenesis, where FKBP10 is expressed in many different tissues (152, 174). A recent report on the function of FKBP10 in lung cancer even reports a more general role in lung cancer cells, as a ribosome-binding protein, which regulates translation (195). In healthy adult tissues, however, it seems that FKBP10 expression is restricted to bone under normal conditions, but reactivated in other tissues upon injury or in disease (72, 152, 195). In addition, FKBP10 has recently been put forward as a potential drug target in lung cancer, further justifying the development of FKBP10-targeting drugs (195). Given that FKBP10 deficiency results in connective tissue alterations and *osteogenesis imperfecta* (174, 175), again, organ-targeted delivery may be key for successful translation.

Similarly, also mutations in *SERPINH1*, encoding **HSP47**, cause *osteogenesis imperfecta* (196). However, even though no detailed report of the clinical trials targeting HSP47 is publicly available, a conference abstract states no changes in bone parameters upon application of a vitamin A-coupled lipid nanoparticle containing anti-HSP47 siRNA for treatment of hepatic

fibrosis (155). As preliminary as this is, it is an encouraging finding because it indicates the feasibility of targeting collagen-modifying proteins in fibrosis, even though some of these proteins are crucial for normal bone development. Furthermore, in contrast to other heat shock proteins, HSP47 displays remarkable specificity for collagen (154) and to date, only three other interacting proteins have been described. Among these, FKBP10 and TANGO equally play important roles in collagen folding and Golgi trafficking/secretion, respectively.

Interestingly, HSP47 has also been described to interact with inositol-requiring enzyme 1 α (IRE1 α), an important regulator of the unfolded protein response (UPR) (82) as a result of **ER stress**. The aspect of ER stress requires special attention, because, in general, targeting chaperone proteins like FKBP10 and HSP47 for treatment of fibrotic disease raises the concern whether such an approach would lead to the accumulation of misfolded proteins in the ER and thus cause ER stress. Importantly, ER stress-induced apoptosis of alveolar epithelial cells may be a key profibrotic event in IPF etiology (197-199) and ER stress is known to contribute to fibroblast to myofibroblast differentiation (200-202). Therapeutic strategies targeting the ER-resident collagen folding and modification machinery therefore must be assessed in terms of (1) whether they induce ER stress in alveolar epithelial cells and (2) whether they lead to intracellular procollagen accumulation, which, by inducing ER stress, may either counterbalance the initial antifibrotic effects or, in fact a potentially beneficial scenario, lead to apoptosis of myofibroblasts. Indeed, as a result of accumulating misfolded procollagen in the ER, the UPR is activated in fibroblasts isolated from *osteogenesis imperfecta* patients with loss-of-function mutations in *SERPINH1* (encoding HSP47) (82, 203). Furthermore, silencing of HSP47 in cancer cells activates ER stress via accumulation of oxidative stress-altered proteins other than collagens

(204), and HSP47 indeed also localizes to alveolar type II epithelial cells where induction of ER stress may trigger the development of fibrosis (205). These aspects argue against the suitability of targeting HSP47 for antifibrotic therapy. In contrast, FKBP10 is little expressed in alveolar epithelial cells and knockdown of FKBP10 in primary human lung fibroblasts including such from IPF patients did not result in intracellular accumulation of procollagen but even led to downregulation of FKBP10 transcription, by so far incompletely understood mechanisms that must go beyond a mere collagen chaperone function (72). Hence, considering the aspect of induction of ER stress in alveolar epithelial cells, FKBP10 may be a more suitable drug target than HSP47. For myofibroblasts, however, induction of apoptosis may be desirable, if it sensitizes myofibroblasts to apoptosis.

Many studies support the idea of **TANGO1** as a specific cargo receptor for procollagen in mammalian cells (86, 156, 157) and specificity for procollagen may in part be mediated via binding to HSP47 (83). Nonetheless, it has been observed that TANGO1 also facilitates secretion of large lipid particles like pre-chylomicrons or pre-very low-density lipoproteins (206), actually supporting a more general function in the secretion of bulky cargos. Interestingly, deficiency of TANGO1 has been shown to lead to accumulation of non-secreted intracellular protein and induce ER stress and apoptosis of fibroblasts (207), which may, under fibrotic conditions support restoration of normal tissue structure. These interesting observations clearly warrant more studies on TANGO1 as an antifibrotic target.

In addition to collagen, **LOX** enzymes also catalyze the first step in elastin crosslinking (98). Arguing against its suitability as a drug target for fibrosis in general, all members of the LOX family are comparatively strongly expressed in lung, kidney, and other tissues, maybe except for LOXL4

and except for liver, where expression of LOX enzymes is generally low, but specifically upregulated in fibrosis (184). It is therefore likely that LOX enzymes fulfill important functions under healthy conditions in extra-hepatic tissues. In addition, LOX functions in cellular proliferation and differentiation independent of ECM crosslinking have been described, but the direct substrates remain poorly understood (208).

Future directions: Strategies for specific targeting of fibrotic areas

With collagen being essential for tissue integrity, targeted delivery of collagen biosynthesis blocking agents to fibrotic areas may hold the key for therapeutic success. Unique for lung fibrosis, inhalation therapy provides a simple way of targeting specifically the lung. Whether the inhalative route is sufficient to engage the target in likely less ventilated fibrotic areas is not fully explored, but recent reports, which demonstrate *in vivo* engagement and efficacy of an inhaled small molecule $\alpha\beta6$ integrin inhibitor for the treatment of pulmonary fibrosis, are very encouraging (209, 210). Notably, inhalation has also been successfully applied to administer antisense therapeutics into the lung for treatment of animal models of cystic fibrosis (211). Inhalative antisense strategies hence hold great promise for those drug targets for which no safe and effective small molecule inhibitors exist.

However, also the inhalative route poses challenges for drug delivery to fibrotic regions in the lung. Cellular and non-cellular barriers like mucus, surfactant, the airway epithelium, and the basement membrane must be overcome and at the same time remain intact to avoid exacerbation of disease. Also for other types of organ fibrosis, different application routes need to be devised. Depending on whether the drug target is located in the intra- or extracellular

compartment, targeting the culprit cells via surface receptors or targeting the fibrotic ECM via disease-specific ECM binding tools are plausible strategies. Several membrane surface proteins of myofibroblast have been proposed that may allow for specific drug uptake in ECM-secreting fibroblasts (212). One such strategy that has already entered clinical trials takes advantage of a unique property of hepatic stellate cells (HSCs), the progenitors for the main effector cells in liver fibrosis: HSCs are responsible for storing up to 80% of total body vitamin A (213) – conjugating drugs to vitamin A therefore represents a promising strategy to specifically target drugs to HSCs. As mentioned above, clinical trials targeting HSP47 already make use of vitamin-A coupled lipid nanoparticles containing HSP47 siRNA (154). The same strategy has been reported to be effective in an animal model of lung fibrosis (214) and recently entered a clinical trial for IPF (NCT03538301, (139)). Even though, to date, there is little evidence for the concept that lung myofibroblasts, similar to their hepatic counterparts derived from HSCs, are equally targeted by vitamin A-coupled liposomes, preclinical results are encouraging and emphasize the feasibility of taking advantage of specific functions of effector cells for targeted drug delivery. But other strategies along these lines are plausible and could be explored in future work; for instance, it may be possible to exploit the metabolic reprogramming of myofibroblasts and take advantage of the increased glutamine uptake for specific targeting (26). Finally, also targeting the platelet-derived growth factor receptor (PDGFR) using PDGFR-recognizing peptides, has been demonstrated to be effective in delivering the antifibrotic cytokine IFN- γ to fibrotic areas in liver and kidney fibrosis and limit fibrogenesis (215, 216). Additional surface receptors being explored in that context are the mannose-6-phosphate/insulin-like growth factor-II receptor (M6P/IGF-IIR)

and fibroblast activation protein (FAP) (212) and currently emerging single cell studies have the potential to identify additional specific surface receptors.

Strategies to specifically target fibrotic ECM have been less explored, but ECM-targeting approaches have gained considerable attention in the cancer field, where the tumor ECM microenvironment affects invasiveness of tumors and penetration of drugs. Investigated drug delivery strategies range from ECM-antibody-drug conjugates to drug carriers composed of liposomes and nanoparticles coated with ECM-binding peptides. Currently explored ECM targets in the context of tumor ECM are tenascin-C, fibronectin, and collagens, but also less abundant ECM components (217). Clearly, such strategies can be translated into the fibrosis field, given that composition and molecular properties of the fibrotic ECM relative to healthy ECM are known. Several studies in the past, using model systems of fibrosis and patient material, have given valuable insight into the composition of the fibrotic ECM (52, 218-220), providing critical knowledge to devise similar strategies to treat fibrogenesis. Notably, this includes small molecular changes like PTMs or crosslinks. For instance, allysine, the initial reactive aldehyde product of LOX-mediated crosslinking, is increased in fibrosis. This property has been used to generate gadolinium-coupled probes that react with allysine, which allows for imaging of areas of active fibrogenesis by magnetic resonance tomography (221). Other fibrosis-specific collagen PTMs may include prolyl-3-hydroxylations, glycosylations, or other types of crosslinks, and technology is emerging that enables the site-specific and comprehensive determination of those (16, 52, 222).

Further future directions

Some regulatory levels within the collagen biosynthesis and maturation pathway have remained largely unexplored in the quest for antifibrotic drug targets (Figure 3). For instance, collagen prolyl-3- and lysyl hydroxylases as well as collagen glycosylating enzymes may qualify as drug targets, too. Among these, targeting LH2 may be particularly promising as it, just as FKBP10, acts directly on procollagen in the ER as well as indirectly on the level and stability of extracellular collagen crosslinking (223). Furthermore, recent reports on 3D structures of LH enzymes can guide the development of small molecule inhibitors (67, 224). For reasons detailed above in the discussion about activating the UPR, it is likely not advisable to target general ER foldases and to date, to the best of my knowledge, no collagen-specific chaperones for C- or N-terminal propeptide folding or formation of the triple helix nucleus have been identified. Nevertheless, given that these processes have not been elucidated in full molecular detail, future studies may still reveal novel targets in that context.

Most approaches listed above aim to attenuate collagen deposition. While this should be effective to stop excessive extracellular collagen accumulation in fibrosis, abolish the profibrotic feedback loop exerted by fibrotic ECM (225), and stop disease progression, the challenge of restoring normal tissue architecture remains. Resolution of fibrosis in the context of normally controlled tissue repair is critically dependent on matrix degradation (226, 227). Unfortunately, activation of collagenolytic enzymes in the extracellular space may overshoot and cause tissue destruction, as has been demonstrated for induction of MMPs and cathepsins (228, 229). As to the intracellular collagen degradation pathway, there is strong evidence that its disruption promotes fibrosis *in vivo* (124, 230, 231). However, the underlying mechanisms are not fully

understood, and targets to activate intracellular collagen degradation have so far not been identified. Clearly, future work is warranted to unravel strategies that enhance intracellular collagen degradation for antifibrotic therapy. Ultimately, combination therapies may be required that collectively result in cessation of excessive ECM production on the one hand and ECM degradation and tissue restoration on the other. Given that the current standard-of-care drugs nintedanib and pirfenidone do not allow for tissue restoration, and actually also directly act on the level of collagen fibril formation (232), it can be anticipated that combination therapy with those – anti-collagen drugs with nintedanib and/or pirfenidone - may not provide a significant benefit. Instead, future work must consider more targeted strategies to cease ECM deposition in combination with novel regeneration approaches.

All processes described above and the thereof derived therapeutic strategies have focused on fibrillar collagen and the concept of excessive collagen deposition as the major cause of organ dysfunction in fibrosis. It should be mentioned in this context that this, however, clearly is an oversimplification. While it remains true that the fibrillar collagen types I and III are consistently increased in fibrosis, levels of other collagen types remain unchanged, are variably regulated dependent on disease stage or anatomic location, or are even downregulated during fibrogenesis (17). This is already evident in an *in vitro* model of lung fibrosis, where the profibrotic cytokine TGF- β 1 specifically increases and decreases different types of collagens simultaneously, thus clearly affecting the relative composition of the deposited collagen far beyond a simple increase in overall collagen (52). Some of these collagens may be indispensable for physiological tissue regeneration. For instance, loss of basement membrane integrity marks a point of no return for physiological tissue repair (116) – blocking expression of type IV collagen as major basement

membrane constituent may just perpetuate tissue fibrosis instead of halting it. Ultimately, it again boils down to the concept that we need to target the pathological collagen only. This requires a deeper knowledge on collagen-type specific biosynthetic mechanisms or, as detailed above already, specific strategies to target the culprit cells, which secrete the “bad” collagen or to directly target the “bad” collagen in the extracellular space.

Summary and conclusions

In summary, targeting collagen biosynthesis and maturation holds great promise for novel future therapies for lung and other types of organ fibrosis. Numerous drug targets have been identified and drugs targeting some of these (miR-29 mimic, HSP47 siRNA, LOXL2 antibody, pan-LOX inhibitor) have entered clinical trials already. While the LOXL2 antibody simtuzumab showed lack of efficiency, results of other clinical trials are eagerly awaited. Targeting molecules with specific expression in fibrosis and/or strategies for targeted delivery to myofibroblasts or to disease-associated collagen in combination with approaches activating resolution of fibrosis may hold the key for future therapeutic success.

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Figure legends

Figure 1: Glycine (Gly) and proline (Pro) are overrepresented in collagen sequences and crucial for formation of the triple helical structure. (A, B, C) Schematic representations of the collagen triple helical structure, given as (A) polypeptide chain backbones, (B) in ball-and-stick representation, showing the orientation of the pyrrolidine rings of proline residues, (C) in cross-section, demonstrating the unique spatial restrictions inside the triple helix, which strictly require Gly as every third amino acid. Structures are retrieved from pdb file 1K6F (233) and created with BioRender.com. (D) Amino acid frequencies in three human mature collagen chains (COL1A1, COL1A2, COL3A1), representative for fibrillar collagens overexpressed in fibrosis, compared with the average observed amino acid distribution in vertebrate proteins (21). Collagen sequences without propeptides were extracted from the UniProt database (COL1A1: P02452, 162-1218; COL1A2: P08123, 80-1119; COL3A1: P02461, 154-1221).

Figure 2. *De novo* proline (Pro) and glycine (Gly) biosynthesis. **Left box.** *De novo* proline biosynthesis starting from the citric acid cycle (CAC) intermediate α -ketoglutarate (α KG, blue filled circle in the schematic representation of the CAC). Glutamic-oxaloacetic transaminases 1 and 2 (GOT1 and GOT2) convert α KG into glutamate (Glu) under concomitant conversion of aspartate (Asp) to oxaloacetate (OA). Aldehyde dehydrogenase 18A1 (ALDH18A1) catalyzes the two-step conversion from Glu to glutamate-semialdehyde which spontaneously rearranges to 1-pyrroline-5-carboxylate (P5C). Finally, pyrroline-5-carboxylate reductases 1 and 2 convert P5C to proline. **Right box.** *De novo* glycine (Gly) biosynthesis starting from the glycolysis intermediate 3-phosphoglycerate (3PG, blue filled circle in the schematic representation of glycolysis pathway). 3PG is oxidized to 3-phosphoohypyruvate (3POP) by phosphoglycerate dehydrogenase (PHGDH),

followed by amino group transfer from glutamate (Glu) yielding phosphoserine (PSer) and α -ketoglutarate (α KG), catalyzed by phosphoserine aminotransferase (PSAT1). Dephosphorylation catalyzed by phosphoserine phosphatase (PSPH) yields serine (Ser). Finally, serine hydroxymethyltransferases 1 and 2 (SHMT1 and SHMT2) catalyze the conversion of Ser to Gly. NAD⁺, NADH; oxidized and reduced form of the coenzyme nicotinamide adenine dinucleotide (NAD); P_i, inorganic phosphate; THF, tetrahydrofolate; 5,10-CH₂-THF, 5,10-methylene-tetrahydrofolate. NAD(P)⁺, NAD(P)H; oxidized and reduced form of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP); P_i, inorganic phosphate. For both *de novo* proline and glycine biosynthesis, glutamate can be derived from glutamine (Gln) via the action of glutaminase 1 (GLS1).

Figure 3: Collagen biosynthesis, processing and maturation (adapted from (16)). **Inner box:** Overview on intra- and extracellular steps of collagen biosynthesis and maturation. **Outer box:** Individual steps given in more detail including key regulators, enzymes, and chaperones. Potential drug targets (see also Table 2) are given outside of the boxes at the level of the relevant step. **(1)** At the level of translation, amino acids (Aa) proline (Pro) and glycine (Gly) are particularly important as overrepresented in triple-helical collagen sequences. LARP6 binds to a specific 5' stem loop structure in COL1A1, COL1A2, COL3A1 mRNA and activates translation while microRNAs like has-miR-29 repress translation. **(2)** During co-translational collagen modification, prolyl-4- and lysyl hydroxylation is exerted by specific ER-resident multiprotein complexes. **(3)** Further enzymes and enzyme complexes are involved in collagen prolyl-3-hydroxylation and glycosylation. **(4)** A set of general rER folding catalysts mediate collagen propeptide folding. **(5)** Chain selection and formation of the triple helix nucleus involves protein disulfide isomerase

(PDI). **(6)** Triple helix formation proceeds in a zipper-like fashion from the C- to the N-terminus and is supported by a set of collagen-specific and general PPIs and chaperones (*e.g.* FKBP10, HSP47, PPIB). **(7)** Trafficking from the ER via the Golgi to the extracellular space is dependent on TANGO1 and cTAGE5 at the ER exit site, COPII coat proteins such as SEC13, SEC23, SEC24, and SEC31, and the ubiquitin ligase CUL3-KLHL12. **(8)** Propeptide cleavage involves BMP1 or TLL1 or TLL2 at the C-terminus side and ADAMTS family members at the N-terminus, or the more recently discovered meprins, which are capable of cleavage at both sides. Propeptide cleavage triggers fibril formation. **(9)** Fibrils are further stabilized by LOX-mediated crosslinking. **(10)** Antioxidant proteins like GPX3 and SOD2 may protect from oxidative damage. Collagen turnover is exerted by MMPS and CTSs.

Protein abbreviations (alphabetical order): ADAMTS, A disintegrin and metalloproteinase with thrombospondin motifs; BMP1, bone morphogenetic protein 1; CALN, calnexin; CALR, calreticulin; CRTAP, cartilage-associated protein; cTAGE5, cutaneous T-cell lymphoma-associated antigen 5 or melanoma inhibitory activity protein 2; CTSs, cathepsins; FKBP10, FK506-binding protein 10; GLT25, glycosyl transferase 25; GPX3, glutathione peroxidase 3; GRP, glucose regulated protein; HSP47, heat shock protein 47; La-related protein 6 (LARP6); LH, lysyl hydroxylase; LOX(L), lysyl oxidase(-like); MMP, Matrix metalloproteinase; P3H, prolyl-3-hydroxylase; P4HA, prolyl-4-hydroxylase; PDI, protein disulfide isomerase; PPIB, peptidyl prolyl isomerase B or cyclophilin B; Sec23, 24, 13, 31, protein transport protein Sec23, 24, 13, 31; SOD2, superoxide dismutase 2; TANGO1, Transport and Golgi organization protein 1; TLL, Tolloid-like protein.

Figure 4: Frequent collagen post-translational modifications mediated by ER-resident enzymes.

Structures of (A) 5-hydroxylysine, (B) 3-hydroxyproline, (C) 4-hydroxyproline, (D) galactosyl hydroxylysine, and (E) glucosylgalactosyl hydroxylysine. Structures were generated using ACD/chemsketch freeware.

Table 1: Classification of collagens according to supramolecular assembly.

Class	Collagen types
Fibril-forming collagens	I, II, III, V, XI, XXIV, XXVII
Fibril-associated collagens with interrupted triple helices (FACITs)	IX, XII, XIV, XVI, XIX, XX, XXI, XXII
Network-forming collagens	IV, VIII, X
Transmembrane collagens	XIII, XVII, XXIII, XXV
Endostatin-producing collagens (multiplexins)	XV, XVIII
Anchoring fibrils	VII
Beaded-filament-forming collagens	VI
Others	XXVI, XXVIII

Table 2: Suggested drug targets in the context of collagen biosynthesis and maturation and status of clinical development.

Potential drug target	Collagen types affected	Organ fibrosis types assessed	Status of validation	Clinical trials (139)
GLS1	All (affects <i>de novo</i> Gly and Pro biosynthesis)	Lung fibrosis (26)	Therapeutic approach <i>in vitro</i> (human lung fibroblasts) (26, 35) Therapeutic approach <i>in vivo</i> (two independent mouse models of lung fibrosis) (37)	None
PHGDH	All (affects <i>de novo</i> Gly biosynthesis)	Lung fibrosis (34)	Therapeutic approach <i>in vitro</i> (human lung fibroblasts) (33) Therapeutic approach <i>in vivo</i> (mouse model of lung fibrosis) (34)	None
SHMT2	All (affects <i>de novo</i> Gly biosynthesis)	Lung fibrosis (33, 34)	Therapeutic approach <i>in vitro</i> (human lung fibroblasts) (33)	None
PSAT1	All (affects <i>de novo</i> Gly biosynthesis)	Lung fibrosis (26)	Therapeutic approach <i>in vitro</i> (human lung fibroblasts) (26)	None
ALDH18A1	All (affects <i>de novo</i> Pro biosynthesis)	Lung fibrosis (26)	Therapeutic approach <i>in vitro</i> (human lung fibroblasts) (26)	None
LARP6	I, III (129)	Liver fibrosis (130) Lung fibrosis (131)	Therapeutic approach <i>in vitro</i> (human lung fibroblasts, hepatic stellate cells) (130, 131) Therapeutic approach <i>in vivo</i> (rat models of liver fibrosis) (130)	None

<p>miR-29a miR-29b miR-29c</p>	<p>I, II, III, IV, V, VII, VIII, XI, XV, XVI (141, 142)</p>	<p>Cardiac fibrosis (143, 144) Kidney fibrosis (145) Liver fibrosis (132, 134) Lung fibrosis (142) Muscle fibrosis (137) Skin fibrosis (136)</p>	<p>Therapeutic approach <i>in vitro</i> (rat cardiac fibroblasts, mouse cardiac myocytes, mouse lung fibroblasts, hepatic stellate cells, human skin fibroblasts) (132, 133, 138, 146) Therapeutic approach <i>in vivo</i> (mouse model of liver, lung, muscle, skin fibrosis) (134, 136-138)</p>	<p>1st generation miR-29b mimic (MRG-201, remlarsen) for treatment of skin fibrosis: Phase 1: NCT02603224 Phase 2: NCT03601052 (136)</p>
<p>P4HA1 P4HA2 P4HA3</p>	<p>all collagens are considered substrates (147)</p>	<p>Bladder fibrosis (148) Cardiac fibrosis (149) Liver fibrosis (150) Lung fibrosis (151)</p>	<p>Therapeutic approach <i>in vitro</i> (human lung fibroblasts, hepatic stellate cells) Therapeutic approach <i>in vivo</i> (rat model of bladder and liver fibrosis, mouse model of lung fibrosis) (148, 150, 151)</p>	<p>None</p>
<p>FKBP10</p>	<p>I, III, V, VI (71, 72)</p>	<p>Lung fibrosis (71, 72, 152)</p>	<p>Therapeutic approach <i>in vitro</i> (human lung fibroblasts) (71, 72)</p>	<p>None</p>
<p>HSP47</p>	<p>I, II, III, IV, V, XI (153, 154)</p>	<p>Intestinal fibrosis Kidney fibrosis Liver fibrosis</p>	<p>Proof-of concept <i>in vitro</i> and <i>vivo</i> linking HSP47 to collagen levels</p>	<p>Vitamin-A coupled lipid nanoparticle containing anti-HSP47 siRNA (ND-L02-s0201, BMS-</p>

		<p>Lung fibrosis</p> <p>Pancreatic fibrosis</p> <p>Peritoneal fibrosis</p> <p>Skin fibrosis (154) (and references therein)</p>	<p>Therapeutic approach <i>in vitro</i> (keloid fibroblasts, hepatic stellate cells)</p> <p>Therapeutic approach <i>in vivo</i> (animal models of skin fibrosis, rat models of pulmonary fibrosis, mouse model of kidney fibrosis, mouse model of peritoneal fibrosis, rat model of liver fibrosis, rat model of pancreatic fibrosis) (154) (and references therein)</p>	<p>986263) for treatment of liver fibrosis/idiopathic pulmonary fibrosis: Phase 1: NCT01858935 NCT03241264 NCT03142165</p> <p>Phase 1b/2: NCT02227459</p> <p>Phase 2: NCT03538301 NCT04267393 NCT03420768 (139, 154, 155)</p>
TANGO1	I, II, III, IV, VII, IX (86, 156, 157)	Liver fibrosis (157)	<p>Therapeutic approach <i>in vitro</i> (hepatic stellate cells)</p> <p>Therapeutic approach <i>in vivo</i> (mouse models of liver fibrosis) (157)</p>	None
LOX LOXL2	I, II, III, IV, V, IX, XI (98)	<p>Cardiac fibrosis (158, 159)</p> <p>Liver fibrosis (100)</p> <p>Lung fibrosis (100)</p>	Therapeutic approach <i>in vitro</i> and <i>ex vivo</i> (collagen hydrogels, decellularized human lung scaffolds) (164)	LOXL2-specific antibody (simtuzumab) for treatment of liver fibrosis, idiopathic

		<p>Myelofibrosis (160, 161)</p> <p>Peritoneal fibrosis (162)</p> <p>Skin fibrosis (163)</p>	<p>Therapeutic approach <i>in vivo</i> (mouse models of cardiac, liver, lung, bone marrow, and peritoneal fibrosis) (100, 159-162)</p>	<p>pulmonary fibrosis, and myelofibrosis:</p> <p>Phase 2: NCT01452308 NCT01759511 NCT01672853 NCT01769196 NCT01672879 NCT01672866 NCT01707472 NCT01369498 (165-167)</p> <p>pan-LOX Inhibitor (PXS-5505) for treatment of myelofibrosis: NCT04676529</p>
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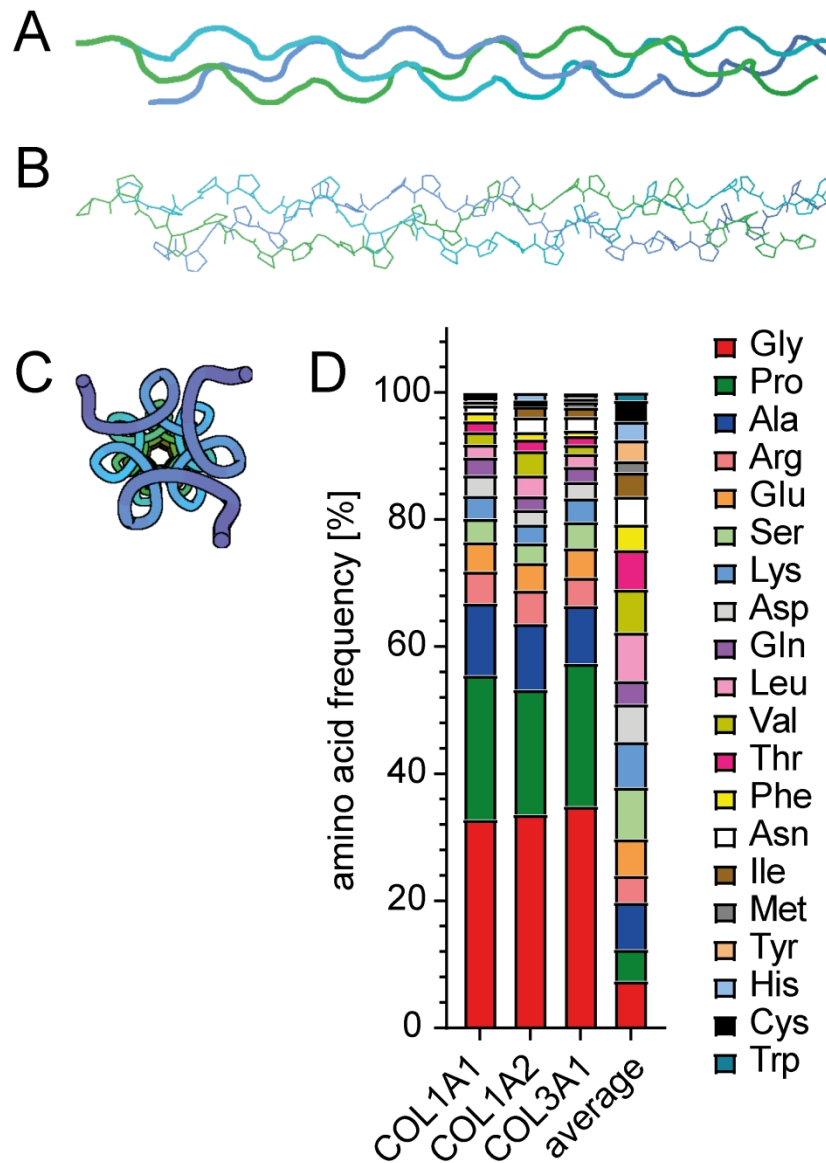


Figure 1: Glycine (Gly) and proline (Pro) are overrepresented in collagen sequences and crucial for formation of the triple helical structure. (A, B, C) Schematic representations of the collagen triple helical structure, given as (A) polypeptide chain backbones, (B) in ball-and-stick representation, showing the orientation of the pyrrolidine rings of proline residues, (C) in cross-section, demonstrating the unique spatial restrictions inside the triple helix, which strictly require Gly as every third amino acid. Structures are retrieved from pdb file 1K6F (233) and created with BioRender.com. (D) Amino acid frequencies in three human mature collagen chains (COL1A1, COL1A2, COL3A1), representative for fibrillar collagens overexpressed in fibrosis, compared with the average observed amino acid distribution in vertebrate proteins (21). Collagen sequences without propeptides were extracted from the UniProt database (COL1A1: P02452, 162-1218; COL1A2: P08123, 80-1119; COL3A1: P02461, 154-1221).

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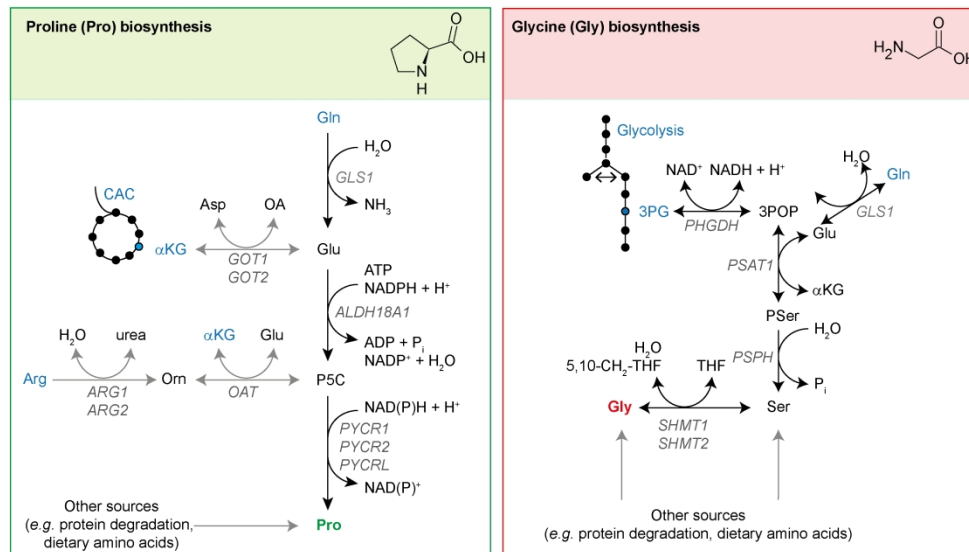


Figure 2. De novo proline (Pro) and glycine (Gly) biosynthesis. Left box. De novo proline biosynthesis starting from the citric acid cycle (CAC) intermediate α -ketoglutarate (α KG, blue filled circle in the schematic representation of the CAC). Glutamic-oxaloacetic transaminases 1 and 2 (GOT1 and GOT2) convert α KG into glutamate (Glu) under concomitant conversion of aspartate (Asp) to oxaloacetate (OA). Aldehyde dehydrogenase 18A1 (ALDH18A1) catalyzes the two-step conversion from Glu to glutamate-semialdehyde which spontaneously rearranges to 1-pyrroline-5-carboxylate (P5C). Finally, pyrroline-5-carboxylate reductases 1 and 2 convert P5C to proline. Right box. De novo glycine (Gly) biosynthesis starting from the glycolysis intermediate 3-phosphoglycerate (3PG, blue filled circle in the schematic representation of the glycolysis pathway). 3PG is oxidized to 3-phosphoxypropionate (3POP) by phosphoglycerate dehydrogenase (PHGDH), followed by amino group transfer from glutamate (Glu) yielding phosphoserine (PSer) and α -ketoglutarate (α KG), catalyzed by phosphoserine aminotransferase (PSAT1). Dephosphorylation catalyzed by phosphoserine phosphatase (PSPH) yields serine (Ser). Finally, serine hydroxymethyltransferases 1 and 2 (SHMT1 and SHMT2) catalyze the conversion of Ser to Gly. NAD⁺, NADH; oxidized and reduced form of the coenzyme nicotinamide adenine dinucleotide (NAD); Pi, inorganic phosphate; THF, tetrahydrofolate; 5,10-CH₂-THF, 5,10-methylene-tetrahydrofolate. NAD(P)⁺, NAD(P)H; oxidized and reduced form of the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP); Pi, inorganic phosphate. For both de novo proline and glycine biosynthesis, glutamate can be derived from glutamine (Gln) via the action of glutaminase 1 (GLS1).

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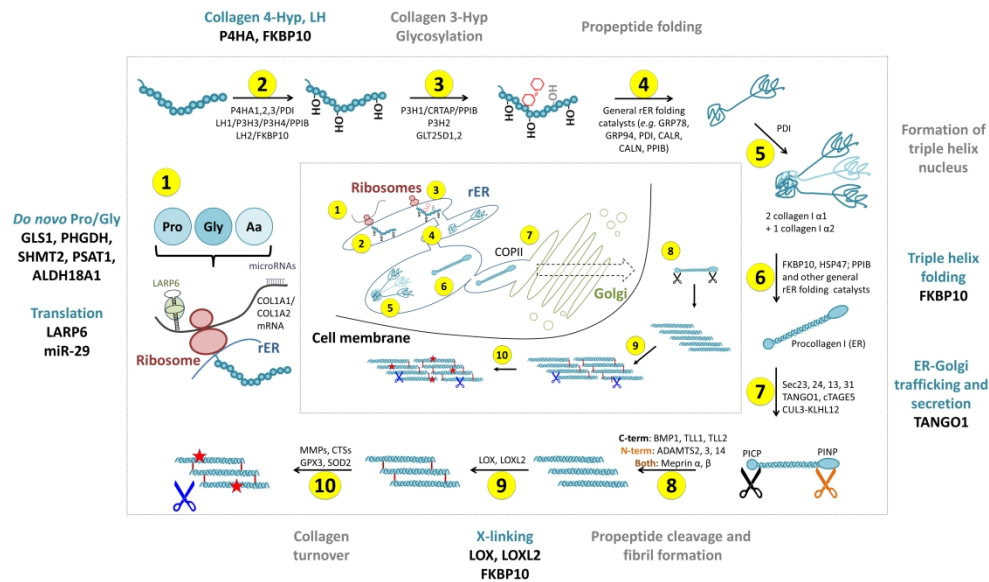


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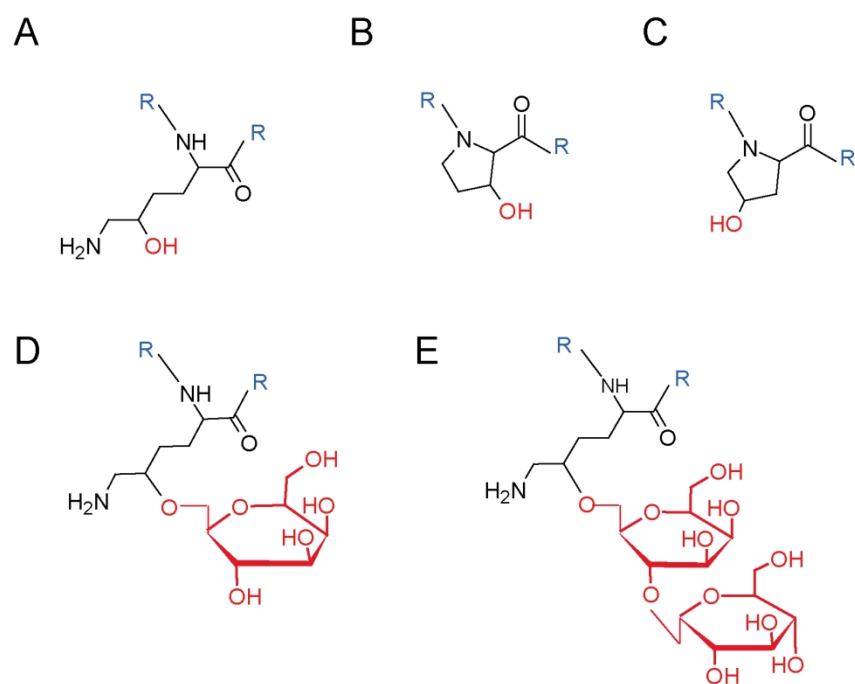


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