Modifications of collagen and chromatin in ECM-related disease

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CHAPTER 1

Introduction And Aims

INTRODUCTION

The extracellular matrix (ECM) is a macromolecular network composed of various (structural) proteins, proteoglycans and glycosaminoglycans. It forms a gel-like substance and specifies, amongst others, the stiffness and elasticity of tissues (1). Several ECM components are highly conserved throughout evolution, while extensive adaptive changes to certain ECM molecules are seen in the phylogenetically more advanced species (2). This supports the notion that ECM has spurred multicellular organisms to develop into complex systems that are adaptable to their environment. Metaphorically, ECM can be seen as the cement between bricklayers that is needed to reinforce a solid structure. Without the cement, the construction would be highly instable. In multicellular organisms, the ECM forms an attachment site for cells and connects various different cell layers to form organs. However, this classical view of ECM function denoted as connective tissue is obsolete as in the last two decades many additional functions of ECM in relation to cell function have been identified, such as cell polarization, migration, proliferation, differentiation, and intercellular communication (3-6).

Collagens comprise the largest subset of ECM molecules, and throughout the human body they are estimated to cover up to 30% of total protein mass. In humans 27 different collagens types are known, with some of these containing more than one variant (e.g. α1 or α2), leading to a total of 42 different genes. All these collagen types are categorized into fibril forming collagens (collagen type I, II, II, V, XI, XXIV and XXVII), fibril-associated collagens (collagen type IX, XI, XIV, XVI, XIX, XX, XXI, XXII and XXVI), and non-fibril forming collagens (collagen type IV, VII, X, VI, VII, XIII, XVII, XXIII, XXV, XV and XVIII) (7). About 90% of the total collagen mass is represented by the fibril-forming collagens; therefore most of our understanding of collagens is based on these collagen types. The core structure of all collagens is similarly shaped as it is composed of three α-chains entangled in a triple helix. The three α-chains can originate either from one collagen type or as a combination of collagen variants. The collagen content in ECM can vary greatly depending on the specific tissue or location. For instance, in basement membranes between cell layers the largest fraction is collagen type IV and for bone or tendons it is collagen type I (1). What greatly extends the complexity of collagen molecules is that they can acquire various post-translational modifications that affect folding, secretion, but also their extracellular functions.

Disparities in collagen homeostasis have emerged as important contributors to a wide variety of diseases. Due to the abundance of collagens in the human body, one can imagine that errors in collagen expression, synthesis or degradation, would result in serious distortions of normal cell and tissue function. Indeed, heritable connective tissue disorders related to mutations in genes involved in collagen synthesis have very severe consequences for the patient, but such heritable diseases are relatively rare (8,9). On the other hand, fibroproliferative diseases, characterized by an over-accumulation of collagen, are estimated to account for more than 45% of U.S. deaths and costs U.S. healthcare more than $10 billion annually (10). This makes a firm understanding of ECM biology and the quest for new pharmaceutical treatments targeting these diseases of considerable interest to health care and patient.

COLLAGENS AND DISEASE

Heritable connective tissue disorders

A large portion of the current understanding of collagen biosynthesis has been derived from investigations related to various heritable connective tissue disorders. Interestingly, out of 42 collagen genes, mutations in 23 of them were found in clinical settings of which the largest proportion is in *COL1A1*, *COL1A2*, *COL2A1*, *COL3A1*, *COL4A5* and *COL7A1*. Also, mutations in genes coding for several collagen-modifying enzymes have been found (see Chapter 2). Ehlers-Danlos syndrome, osteogenesis imperfecta and Bruck syndrome make up the core of inherited connective tissue disorders in which fibrillar collagens are affected. Both Ehlers-Danlos syndrome and osteogenesis imperfecta are classified into various subtypes that are related to mutations in specific genes either coding for certain collagen types or various collagen-modifying enzymes (7,11). Bruck syndrome is different in that it is correlated only to mutations in two collagen-modifying enzymes (12). Bruck syndrome is a rare autosomal recessive disorder that has previously been classified as osteogenesis imperfecta type III with joint contractures, bowing or fractures of long bones and clubfeet. Bruck syndrome (BS) is subdivided into two types that are phenotypically indistinguishable, and classification is therefore based on underlying genetic mutations. BS type 1 is caused by mutations in *FKBP10* (13-16) whereas BS type 2 is caused by mutations in *PLOD2* (17-19). The molecular etiology of both types is an almost complete reduction in trifunctional cross-linking of collagen type I in bone, caused by an under-hydroxylation of lysyl residues in the collagen telopeptides (20,21). Although lysyl hydroxylase 2 (LH2), encoded by *PLOD2*, is directly responsible for telopeptide hydroxylation, it is not known how mutations of *FKBP10* can affect this collagen modification.

Fibroproliferative pathologies, onset and impact

Fibroproliferative pathologies such as organ fibrosis and systemic sclerosis are often defined as wound healing reactions that have gone out of control. Normally, in reaction to tissue injury, a cascade of processes controls the repair of the damaged tissue (Figure 1). One of the first steps is the accumulation of inflammatory cells at the site of injury. These cells secrete a cocktail of growth factors, cytokines and even certain ECM components. Certain secreted cytokines belonging to the Interleukin (IL) and Transforming Growth Factor Beta (TGFβ) family can activate migrating or dormant fibroblasts at the wound site, which will acquire a specialized phenotype referred to as myofibroblasts. These activated fibroblasts secrete vast amounts of ECM and are involved in wound contraction (22,23). In addition epithelial or endothelial cells can transform into a myofibroblast phenotype in reaction to similar cytokines and other stimuli, denoted respectively as epithelial-to-mesenchymal (EMT) or endothelial-to-mesenchymal (EndMT) transition, and contribute to the wound healing reaction (24-27). Normally, myofibroblasts turn quiescent or go into apoptosis after wound closure, whereby ECM deposition is halted (28-30). However, in fibroproliferative diseases, myofibroblasts remain active and continue their excessive production of ECM. This inevitably results in an undesirable buildup of ECM that distorts tissue architecture and biomechanical functions, and eventually leads to organ failure. This buildup is a

net effect from a combination of high ECM production and reduced turnover. Certain enzymes, such as those belonging to the matrix metalloproteinase (MMP) family, have proteolytic activity towards fibril-forming collagens. MMPs are secreted into the extracellular space as inactive pro-enzymes that need to be activated by cleavage of its propeptides by various ECM-resident enzymes. In turn, activated MMPs can be inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs) (31). The balance of MMPs and TIMPs highly influences the rate of collagen turnover. Another element that determines the rate of collagen turnover, and thus the course of organ fibrosis, is the presence of the trifunctional pyridinoline cross-links between fibrillar collagens. These cross-links are derived from telopeptidyl hydroxylysine (catalyzed by LH2) and negatively influence the degradability of collagen by collagenases such as MMPs (32,33), and thereby generate an irreversible type of fibrosis (34).

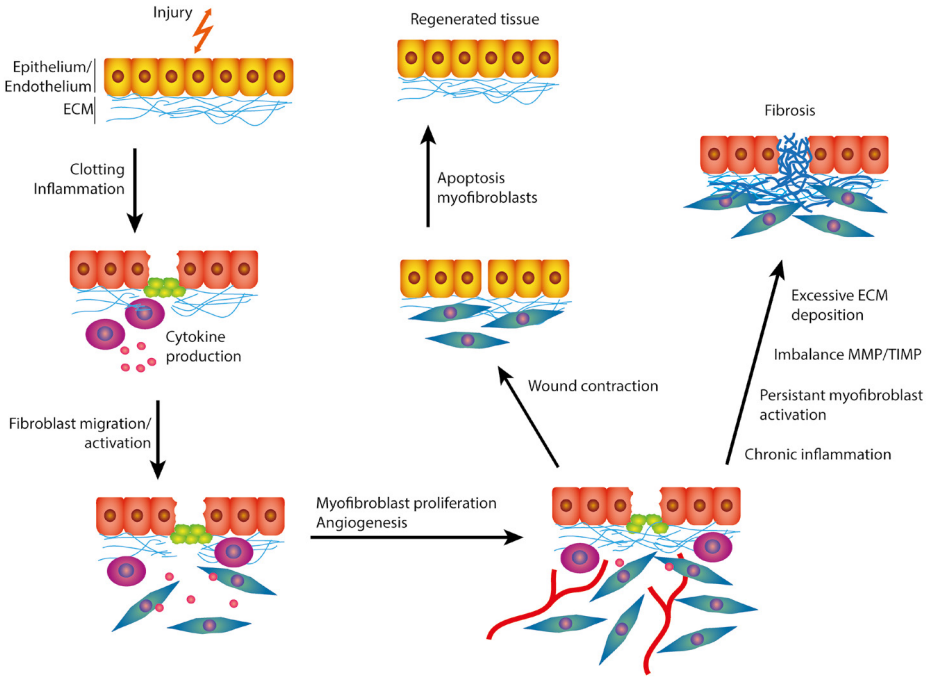

Figure 1. The wound healing process.
After tissue injury, the epithelial or endothelial layer is disrupted and quickly filled by clotting material. Inflammatory cells infiltrate the wound site and secrete cytokines that attract and activate fibroblasts. In successive events, angiogenesis of the damaged microvasculature and proliferation of myofibroblast provide regeneration of the wound site by increasing nutrients and ECM deposition. At this point a decision has to be made: either the wound is contracting while myofibroblast undergo apoptosis and a halt in ECM deposition (regenerated tissue), or myofibroblast proliferation and ECM deposition remains and result in excessive wound healing (fibrosis).

Collagen lysyl hydroxylation and cross-linking

During collagen synthesis in the endoplasmic reticulum (ER), post-translational conversion of lysine into hydroxylysine determines the biochemical type of collagen cross-link that will be formed at later stages. Three lysyl hydroxylases (LH) are known in human (LH1-3). LH1 and LH3 modify residues in the helical region of collagen (35-38), whereas LH2 splice variant b selectively modifies the C- and N-terminal telopeptides of collagen (36,39,40). LH2a, another splice-variant of LH2 that is mainly expressed in kidney and liver, fails inclusion of exon 13A. Although LH2a contains the same catalytic domain as LH2b, it has no known role in collagen lysyl hydroxylation

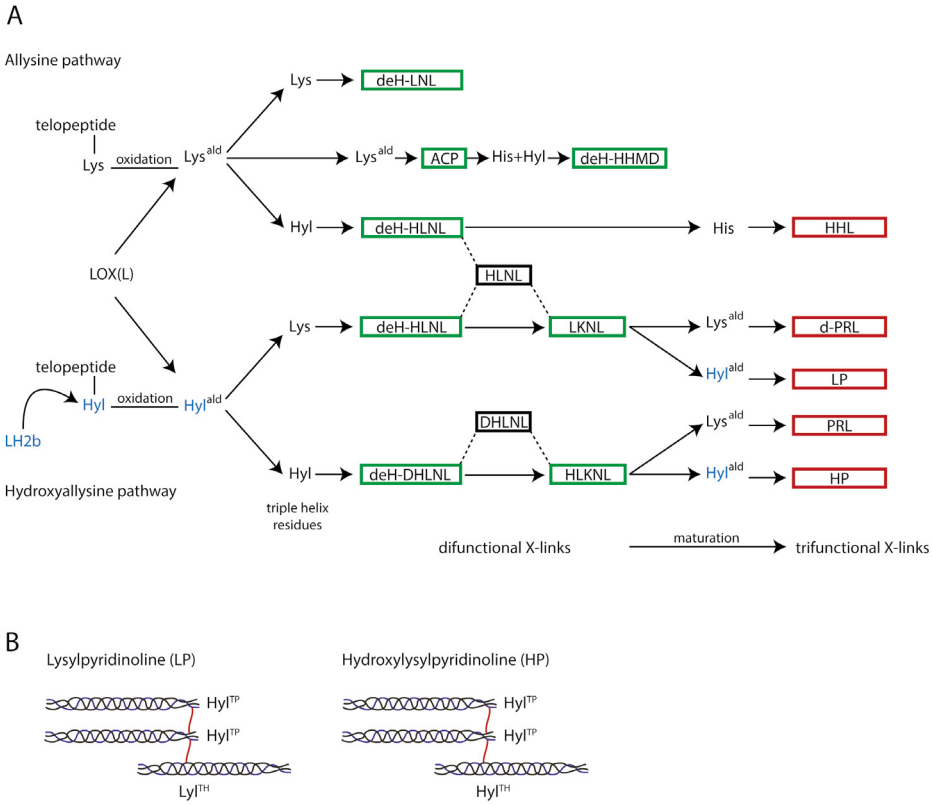

Figure 2. Pyridinoline cross-linking of collagen.
(A) Collagen cross-linking is initiated at the telopeptide. Here LH2b decides if a Lys if hydroxylated (Hyl). After collagen molecules are secreted, LOX(L) enzymes perform oxidative deamination of telopeptide Hyl or Lys thereby deriving aldehydes of hydroxallysine (Hylald) or allysine (Lysald) respectively. The allysines can condense with triple helix residues to form various reducible difunctional cross-links (green box) or with allysine from the same collagen molecule to form ACP. Several difunctional cross-links can mature with telopeptide aldehydes to form various irreducible trifunctional cross-links (red box). (B) Lysyl hydroxylase 2b performs hydroxylation of telopeptide lysine. The formation of the trifunctional lysylpyridinoline (LP) or hydroxylysylpyridinoline (HP) depends on the inclusion of a non-oxidized lysine or hydroxylysine from the collagen triple helix, respectively. Thus, in both casese the reaction product is made from three collagen molecules: LP from Hyl+Hyl+Lys, and HP from Hyl+Hyl+Hyl.

and cross-linking (19,41,42). After collagen folding, processing, and secretion into the extracellular space, the next step in collagen cross-linking comprises oxidative deamination of the ε–amino group from either telopeptide lysine (Lys) or hydroxylysine (Hyl) by lysyl oxidases (LOX and LOXL1-4) (43,44). These steps can be subdivided into hydroxyallysine or allysine derived enzymatic cross-links and depends on the presence of Hyl or Lys, respectively, in the telopeptides. The allysine route starts with the oxidative deamination of telopeptide Lys, whereas telopeptide Hyl is the starting residue for the hydroxyallysine route (45). Both hydroxyallysine and allysine form a difunctional cross-link by spontaneously reacting with either a Lys or Hyl residue in the helical region of another collagen molecule. When a difunctional cross-link derived from a hydroxyallysine reacts on its turn with another Hyl in the telopeptides, it forms respectively lysylpyridinoline (LP) or hydroxylysylpyridinoline (HP) trifunctional cross-links (Figure 2a).

The absolute amount of HP and LP is dependent on the level of lysyl hydroxylation of the telopeptides, whereas the ratio of both type of trifunctional cross-link (HP:LP) depends on the level of hydroxylysine residues in the triple helix (Figure 2b). The total amount of pyridinoline collagen cross-links has a profound effect on biomechanical endurance of tissue, whereas the ratio between LP and HP does not result in biomechanical differences. Normally, tissues such as bone and certain types of cartilage that have to withstand massive applied forces contain a higher number pyridinoline cross-links per collagen than softer tissues such as skin and internal organs (46). However, in fibroproliferative disorders of soft tissues, such as organ fibrosis and solid tumors, elevated levels of trifunctional pyridinoline cross-links are detected as well (41,47-50). The normal biomechanical benefit of these cross-links are in these disorders a disadvantage, as it impairs normal tissue function at the fibrotic lesion and is suggested to promote metastasis in the case of tumors. The successive actions of LH2 and LOX/LOXL are fundamental to trifunctional collagen cross-linking, and enhanced expression and activity of these enzymes are found in many, if not all, fibroproliferative diseases such as organ fibrosis, systemic sclerosis and solid tumors (41,48,49,51-53). Fundamental understanding of how these enzymes are regulated on a transcriptional level is of major interest for future treatments of fibrosis and cancer.

TRANSCRIPTIONAL REGULATION AND INTERFERENCE OF ECM-RELATED DISEASES

Epigenetic modifications and gene regulation

It is estimated that the cells that make up the human body can be categorized into 200 different phenotypes. However, as all these cells contain the same genome researchers were puzzled for a long time how to relate these phenotypic differences to genetics. It is now obvious that it is the regulation of gene activity that makes the difference. Indeed, our current understanding of this matter describes a highly interconnected transcriptional control system that contains complex multilayered regulatory systems to ensure correct gene regulation. A central factor in this is regulating the accessibility of

DNA for specific factors that orchestrate gene transcription. Eukaryotic DNA is packed into higher order chromatin by stretches of nucleosomes formed out of DNA winded around a histone octamer, which is hold in place by linker histone H1. The histone core consists of two copies of histone H2A, H2B, H3 and H4, which all have protein structures (tails) extruding from the core structure that can regulate attachment of the octamer to the DNA.

Epigenetic modifications are covalent modifications of DNA and histones that together regulate the chromatin structure and are related to biological processes such as gene expression, DNA repair and higher order chromatin conformational changes. For DNA, cytosine methylation (5mC) in a CG order (CpG) and its oxidation products 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) as induced by TET enzymes (54) are regarded as epigenetic modifications. Histone tails, and to a lesser extend the histone core, are subjected to a wide array of posttranslational modifications such as: lysine methylation, arginine methylation, lysine acetylation, serine phosphorylation, and lysine ubiquitination. Furthermore, depending on the location of the lysine it can either support gene activation or support gene repression (55,56). For instance, trimethylation of histone 3 lysine 4 (H3K4me3) and H3K79me2 are linked to active genes, whereas H3K9me3 and H3K27me3 are linked to repressed genes. As such, these covalent modifications of histones and DNA are thought to instruct the chromatin to support or deny transcription of genes by mediating DNA access to transcription factors (TFs), transcriptional initiation complexes, and RNA polymerase (57). In addition, other factors as long non-coding RNA and antisense transcripts (58-61), and 4D positional changes of chromatin influence chromatin organization and transcription as well (62-64). However, as all these components can have a certain degree of influence on each other, it is still under debate whether epigenetic modifications by themselves are inducing the *de facto* transcriptional changes.

What makes epigenetic modifications complex with respect to functional interpretation is that certain modifications are selectively found at or around specific genomic regions, while others are widely distributed. For instance H3K4me3 is found at promoters of transcriptionally active genes (65,66), whereas DNA methylation can be found all around (67). And although DNA methylation is generally considered a transcriptional repressive epigenetic modification, this highly depends on its genomic location. As methylation of CpGs as GpG islands in promoters or enhancers is linked to gene repression, 5mC presence in the gene body is generally considered supportive of transcription (68-70). Also, *in vivo* these modifications can influence each other by performing cross talk. For example by regulating the addition of other marks as is the case for H3K4me3 that can prevent *de novo* DNA methylation (71), or as Histone H2B ubiquination supports the recruitment of H3K4me3 and H3K79me2/3 (72).

The balance of epigenetic modifications and their subsequent transcriptional effects are hold in check by proteins that are categorized into writers, readers and erasers. Writers such as histone methyltransferases (EZH2, MLL), DNA methyltransferases (DNMTs), and histone acetyltransferases (P300, CBP) catalyze modifications, while erasers such as histone demethylases (LSD1), histone deacetylases (HDACs), and 5mC hydroxylases (TETs) perform enzymatic reductions of a certain modification. Readers

are proteins that detect specific epigenetic modifications and recruit additional (epigenetic) enzymes that activate or repress components of the transcriptional (initiation) complex (73,74). This results in a highly interactive multi-protein system that translates epigenetic information into transcriptional decisions. Although a large number of writers and erasers have been described in literature, it is likely that many are still awaiting their discovery. It is also fairly conceivable that several writers and erasers act only during specific cell phases or only in specific cell types, and therefore have escaped the attention of researchers.

Epigenetic modifications are faithfully propagated throughout mitosis, and provide a blueprint for maintaining cellular identity (75). These modifications are unique features of the genome as they can encode both temporary transcriptional instructions in reactions to environmental stimuli, or even be permanently through transgenerational inheritance by offspring (76). As such, epigenetic modifications hold a treasure of transcriptional clues essential to life and potentially evolution as well. Evidently, issues regarding the homeostasis of these modifications can result in deregulation of individual genes, growth arrest or even genome instability, followed by cell death or disease. Therefore these modifications are a rich source of possible therapeutic targets.

Transcriptional regulation of fibroproliferative diseases

TGFβ signaling is one of the main signaling routes that stimulates fibroblasts to obtain an activated or myofibroblast phenotype, thereby establishing fibrosis. The TGFβ family consists of three types (TGFβ1-3), where TGFβ1 and TGFβ2 are known for their pro-fibrotic effects and TGFβ3 has more anti-fibrotic properties (77). TGFβ proteins are secreted as latent (inactive) complexes (78), and remain as such until released from this fixed inhibitory structure by e.g. proteolytic degradation or adhesion-mediated forces (79,80). Once released, TGFβ binds to a complex of TGFβ receptors (TGFBR) of which the composition depends on the type of TGFβ. In canonical TGFβ signaling, TGFβ1 binds to a receptor complex consisting of two TGFBR1 molecules and two TGFBR2 molecules. Binding initiates activation of the TFGBR1 kinase domain, which subsequently phosphorylates SMAD2/3 proteins. In reaction to their phosphorylation, SMAD2/3 proteins actively translocate to the nucleus and bind to other transcription factors and the transcriptional co-activators P300/CBP to affect target gene expression. In addition, several other epigenetic modifiers have been found to bind SMAD2/3 (81-83). The TGFβ1 signaling pathway can have overlap or conjunction with several other signaling pathways (84), which further complicates the prediction of effects at the downstream level.

Epigenetic modifications have been correlated to transcription of pro-fibrotic mediators that prompt development and progression of fibroproliferative diseases (85,86). These processes can be related to deregulated expression of epigenetic enzymes, their genomic repositioning at pro-fibrotic genes followed by transcriptional regulation of these sites, or indirect effects by affecting expression of mediator genes. DNA methylation perturbations are associated with fibrotic phenotypes in several organ systems. For instance, *de novo* DNA methylation is initiating transcriptional repression of the pro-fibrotic mediator *RASAL1* in kidney, liver and cardiac fibrosis (87-89).

Interestingly, administration of anti-fibrotic BMP7 induced active DNA demethylation of *RASAL1,* by TET3 dependent hydroxylation of CpGs (5hmC), diminishing both kidney and cardiac fibrosis (89,90). Furthermore, the 5mC binding protein MeCP2 is increased in fibrosis and controls expression of pro-fibrotic mediators as *PPARY* and *RASAL1* in liver and pulmonary fibrosis (88,91,92). Several other pro-fibrotic mediators are found methylated in fibrosis: examples are *PTGER2* and *THY1* in pulmonary fibrosis (93,94), collagen suppressor *FLI1* in systemic sclerosis (95), and transcription factor *KLF4* in kidney fibrosis (96). Next to individual pro-fibrotic mediator genes, genome-wide alterations in 5mC and 5hmC levels have been characterized in both kidney and liver fibrosis (97-99).

In addition to DNA methylation, histone methylation and acetylation changes have been linked to fibrosis as well. For instance, H3K9 and H3K4 methylation at the fibrotic mediators *COL1A1*, *CTGF* and *PAI-I* are altered in kidney cells stimulated with TGFβ1 (100). Also, H3K4me3 methyltransferase ASH1L is upregulated in myofibroblasts derived from hepatic stellate cells and in fibrotic livers. Here, ASH1L binds to promoters of *TIMP1*, *TGFB1*, *ACTA2* and *COL1A1*, and increases H3K4me3 and subsequent gene expression (101). Also, following *de novo* DNA methylation of *PPARG*, H3K27me3 methyltransferase EZH2 is recruited to the gene body of *PPARG* and advances gene repression (91). Histone acetyltransferases P300 and CBP are part of many transcriptional complexes that include transcription factors such as SMAD2/3, NF-kB, HIF1α and SP1, and are as such responsible for chromatin decondensation and activation of many pro-fibrotic genes that are TGFβ1 responsive (102-105). Also, HDACs as part of repression complexes are known contributors to repress various genes that lead to fibrosis (106,107). The full picture of the fibrotic epigenome is far from complete. Therefore a thorough understanding how the myofibroblast epigenome supports these pro-fibrotic actions would identify potent therapeutic targets for attenuating or even reversal of fibrosis.

The epigenome as a therapeutic target

For epigenetic aberrations to be a determining factor in development of disease, pharmaceutical targeting of its components offers great possibilities to affect the resulting deregulated gene expression. Several inhibitors of epigenetic enzymes have already been FDA approved for use into the clinic. For instance, HDAC inhibitors Vorinostat and Belinostat that reduce the deacetylation of histones have been used in clinical trials of advanced-staged cancers (108,109). Furthermore, inhibitors of DNA methyltransferases Decitabine and Azacytidine that reduce DNA methylation have been used in combination with other pharmaceuticals in the treatment of myelodysplastic syndromes, ovarian and prostate cancer (110-113). In preclinical studies of fibrosis, inhibitors of HDACs, HATs and DNMTs have shown promising anti-fibrotic effects (87,114-120).

In addition, the recent spur in epigenetic research has offered a large repertoire of possible new targets. As a result, industry is investing heavily to develop inhibitors to more epigenetic enzymes and/or enhance their specificity. However, a concern that still remains is the long-term effects of inhibiting the genome-wide actions of these epigenetic enzymes (121). Furthermore, because many histone-modifying enzymes

can also modify other non-histone proteins (122), unexpected side effects are likely to occur.

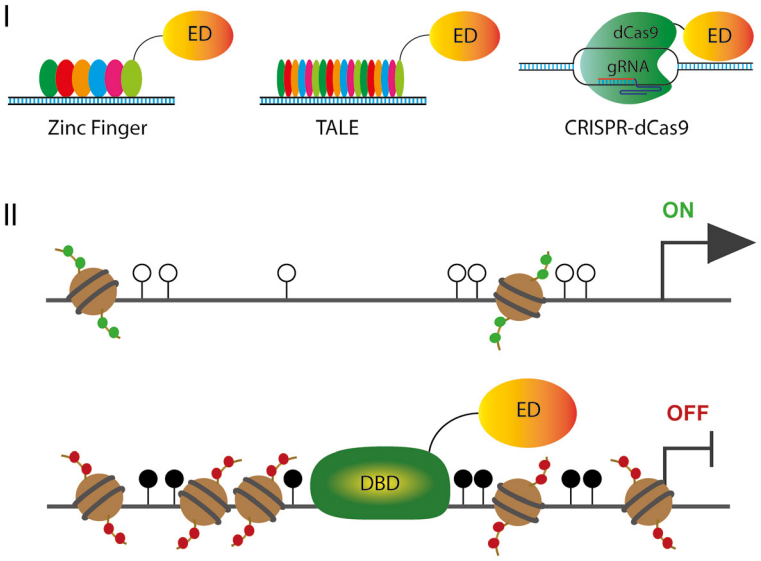

Figure 3. Epigenome editing of endogenous loci.
Epigenome editing tools (epi-editors) comprise of synthetic DNA binding domains (DBDs) tethered to effector domains (EDs). A zinc finger DBD contains six individual fingers that each recognizes 3 bps, TALEs contain 18 individual TALE domains that each recognize 1 bp, CRISPR-dCas9 is an inactivated prokaryotic nuclease that targets to genomic sites by guide-RNA (gRNA)-DNA interactions (I). When epi-editors bind to their pre-designed genomic location they are able to rewrite the local chromatin and affect subsequent transcription (II).

Epigenome editing is a recent biotechnological innovation that sidesteps the genome-wide effects of chemical inhibitors that treat aberrant transcription. This technology is essentially composed of two items: a synthetic DNA binding domain (DBD) that can recognize very specific binding sites in the genome, and secondly an epigenetic enzyme or catalytic domain that has an epigenetic reactivity. When tethered together the result is a targetable epigenetic editor that is able to modulate the epigenome at predetermined target locations, hence epigenome editing (Figure 3). Three synthetic DBD systems that have been used extensively are: Zinc fingers, TALEs and CRISPR-Cas9 (123). Initially these DBDs were used to facilitate locus-specific cleavage of DNA when tethered to nucleases, but all platforms have formed the basis for epigenome editing as well. In the last years, several pioneering reports with ZFs and TALEs based epigenetic editors have laid the fundament for a solid proof-of principle regarding epigenome editing (124-128). The recent spur in CRISPR-Cas9 tools has progressed the field exponentially by providing easy access to epigenome editing tools both for answering complex biological questions as well as therapeutic intervention.

The power of epigenome editing as a therapeutic intervention is its potential to induce stable transcriptional modulation of target genes through epigenetic modifications. Unfortunately, most of the present reports only have addressed short-term effects, with only few discussing long-term stability effects (129,130). Deciphering the rules underlying stable epigenome modulation is of considerable interest for its potential use as a one-hit strategy of disease-related genes. In turn, the development of epigenetic editors that can disable disease progression by a single administration is expected to revolutionize the pharmaceutical industry and medicine as a whole.

AIM AND OUTLINE OF THIS THESIS

Current treatments of fibroproliferative diseases have proven insufficient in reducing their prevalence. Therefore, a fundamental understanding how fibroproliferative processes are mediated on the molecular level is urgently needed in order to develop novel and more effective therapeutic strategies. The aim of this thesis is to further apprehend the understanding of enzymes that function in post-translational modification and cross-linking of collagen (**Part I**). Furthermore, we questioned how certain post-translational modifications of chromatin could influence transcriptional regulation of pro-fibrotic processes (**Part II**). Finally, we developed innovative biotechnological tools that can be exploited to interfere with given transcriptional states of disease-related genes by permanent modulation of the local epigenome (**Part II**).

PART I: NOVEL FEATURES OF COLLAGEN MODIFYING ENZYMES

Many enzymes and chaperones function in post-translational modification of collagen, where they work together in an assembly-line fashion. Mutations in any of the genes coding for these enzymes can result in an array of rare and severe heritable connective tissue diseases (**Chapter 2**). The rare brittle bone disease Bruck syndrome is characterized at the molecular level by a reduced collagen telopeptide lysine hydroxylation that is connected to mutations in either *FKBP10* or *PLOD2*. A long-standing question has been the connection of *FKBP10* to Bruck syndrome, as the derived protein FKBP65 does not display any lysyl hydroxylase activity. We revealed the missing link between these two enzymes and how they can influence collagen pyridinoline cross-linking (**Chapter 3**). LH2 is overexpressed in many types of cancer where it is correlated to poor overall survival. Although several reports have claimed this is related to altered collagen cross-linking, biochemical evidence for this is lacking. We assessed the possibility that either of the two LH2 splice variants (LH2a or LH2b) has a role outside the scope of collagen cross-linking that could clarify its dominant role in cancer (**Chapter 4**).

PART II: TRANSCRIPTIONAL REGULATION OF PRO-FIBROTIC PROCESSES

Changes in transcriptional regulation are fundamental in shaping differentiation and disease initiation and progression. Already various epigenetic processes underlying the onset and progression of fibrosis have been characterized. However, the full spectrum is far from being complete and needs to be resolved in more detail in order to fully understand the influence of the fibrotic epigenome. The transcriptional regulation of *PLOD2* is only poorly known. As collagen cross-linking is a fundamental process in fibrosis, a detailed description might benefit potential anti-fibrotic therapies. We addressed the transcriptional principles how TGFβ1 enhances *PLOD2* expression (**Chapter 5**). EndoMT is a transdifferentiation process of the endothelial layer that contributes to development of various fibroproliferative diseases. One of the genes that is upregulated in this process is *TAGLN* that codes for SM22α. SM22α is an F-actin binding protein that regulates the organization of the cytoskeleton and contractility, but also activates NF-kB signaling cascades. As both are essential processes in myofibroblast

biology, understanding of how *TAGLN* is regulated is of major importance. We here assessed whether expression of *TAGLN* in a pro-fibrotic model of endothelial cells is functionally related to actions of histone H3K27 methyltransferase EZH2 (**Chapter 6**). Transcriptional repression of certain fibrotic mediators through DNA methylation is fundamental to the establishment of fibrosis. In recent years it was found that 5hmC, a 5mC oxidation product that is catalyzed by TET enzymes, has profound effects on transcription as well. In order to decipher whether TET enzymes and 5hmC have an underlying role in myofibroblast differentiation and ECM biology, we investigated the influence of the individual TET family members on these process (**Chapter 7**).

PART III: TARGETED REWRITING OF THE EPIGENOME TO MODULATE TRANSCRIPTION

An innovative way to modulate the transcriptional state of a gene is to target synthetic DNA binding domains (DBDs) tethered to epigenetic enzymes, also known as epigenome editing. As epigenetic modifications are faithfully propagated during cell division, epigenome editing has potentially the power to stably modulate target (disease) genes after a one-time hit. Here we assessed the possibility to induce long-term repression by rewriting the chromatin of our model gene *PLOD2* with designer DBDs tethered to either a KRAB repressor domain or a DNA methyltransferase (M.SssI) in fibrotic fibroblasts and cancer cells. Here we essentially compared the repressive power of epigenome editing, by targeted DNA methylation, to a non-catalytic repressor (**Chapter 8**). Repressed genes are deprived of the gene-expression related modification H3K4me3. The intrinsic power of this modification on gene activation is still under debate. Understanding this process in more depth would therefore open up possibilities to for instance re-express silenced tumor-suppressor or other disease genes. Therefore, we assessed whether actively rewriting of H3K4me3 by epigenome editing could re-express silenced genes, and if long-term effects were dependent on the micro-chromatin (**Chapter 9**). In **Chapter 10** the main findings are summarized and discussed within a larger context.

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PART I

NOVEL FEATURES OF COLLAGEN MODIFYING ENZYMES