CHAPTER 10

Summary & general discussion
The extracellular matrix (EM) is essential to many biological functions, and as such is likely to be tightly regulated from the transcriptional level to the post-translational level. When this homeostasis is disrupted, a large variety of ECM-related diseases can arise that are subdivided into heritable connective tissue diseases and acquired diseases. A large proportion of the acquired diseases are made up of fibro-proliferative pathologies. The occurrence of fibroproliferative pathologies (tissue fibrosis) is reaching levels of epidemic proportions. Although they are not in the spotlight as such, they have a serious contribution to a long list of pathologies: e.g. myelofibrosis, systemic sclerosis, Dupuytren’s contracture, hypertrophic scars, keloids, cardio-vascular diseases, lung diseases, kidney diseases, macular degeneration, and various types of solid tumors. Logically this poses an enormous burden on health-care systems worldwide. In fact, anomalies in ECM synthesis are the silent number one killer worldwide as it is estimated that around 45% of the deaths in the US are related to fibroproliferative diseases. Clearly there is a great necessity to develop (novel) therapeutic strategies that could be applied for a long list of these ECM-related diseases. With this thesis we provide a large body of work that goes full circle on ECM-related diseases by presenting essential information on collagen biosynthesis and offering insights and novel avenues for treating ECM-related diseases.

TARGETING COLLAGEN CROSS-LINKING IN FIBROPROLIFERATIVE DISEASES

Excessive collagen deposition is an integral part of fibrosis and is considered the leading cause to the resulting loss of tissue function. Collagen pyridinoline cross-linking is an important mediator in this collagen accumulation, as these cross-links reduce the proteolysis of collagens by proteinases, and enable the progression to an irreversible form of fibrosis that is currently untreatable. Therefore, the underlying steps leading to an enhanced collagen cross-linking offer promising targets to tackle fibrosis. Collagen pyridinoline cross-linking is initiated in the endoplasmic reticulum (ER) by lysyl hydroxylase 2 variant b (LH2b), encoded by \textit{PLOD2}, which specifically catalyzes lysyl 5-hydroxylation (Hyl) of the collagen telopeptides prior to assembly of the procollagen molecule. In addition the helical region of collagen a-chains are hydroxylated at specific lysine residues by LH1. The latter step, however, is not essential to form trifunctional pyridinoline cross-links as loss of LH1 still results in a specific type of pyridinoline, lysylpyridinoline (LP) (1,2), which has equal biomechanical implications as the other type seen the most in fibrosis, hydroxylysylpyridinoline (HP). However, a loss of functional LH2b due to mutations in \textit{PLOD2} as seen in Bruck syndrome type 2, results in an almost complete loss of telopeptide hydroxylation levels and subsequent LP and HP cross-links (3-6). After these and other intracellular posttranslational modifications (see \textbf{Chapter 2}), collagen is deposited in the extracellular matrix. Here the last enzymatic step in collagen cross-linking is performed by members of the lysyl oxidase family (LOX, LOXL1-4) which reduce telopeptide lysine residues (Hyl and Lys) through oxidative deamination, thereby promoting spontaneous formation of a large array of
successive intermolecular cross-links (Chapter 1, Figure 2a). Enhanced pyridinoline cross-link levels are of great concern to cure tissue fibrosis, as they negatively correlate to the reversibility of progressive fibrosis (7-9). Therefore, developing strategies that prevent the formation of these cross-links have great priority. However, currently there are no effective therapeutics available that specifically target pyridinoline cross-links in a clinical setting. In order to attain a successful anti-cross-linking therapy, the most appropriate component needs to be targeted.

Anti-fibrotic treatments by inhibiting expression or activity of LOX and LOXL2 have been glorified, as their inhibition induces strong anti-fibrotic effects of both cancer and fibrosis in pre-clinical screenings that included cellular and animal models (10-15). However, a major point of concern is that the positive effects are observed in models where fibrosis has not reached the irreversible level yet. Meaning, hardly any pyridinolines have yet been formed (7,16). As the formation of pyridinoline out of its precursors (deH-DHLNL and deH-HLNL) is a slow and spontaneous (non-enzymatic) process in vivo, most animal models in fibrosis research could be insufficient to correctly resemble the fibrotic collagen to the human situation (7). Unfortunately, the majority of reports that target LOX and LOXL2 do not include biochemical analysis of collagen cross-links, and only few evaluated this by Picrosirius red staining (10,17). However, this staining does not distinguish the different types of collagen cross-links, but rather reflects collagen fiber thickness and packing (18). Over the last few years, several Phase 2 clinical trials were started to assess the effects of the LOXL2 neutralizing antibody Simtuzumab on fibrosis and cancers, by its manufacturer Gilead Sciences. Although several studies are still ongoing and awaiting publication of the results, two recently finished trials with advanced pancreatic cancer patients and chronic liver injury patients did not show significant effects after treatment (2014 press release and (19)).

Another disadvantage of inhibiting lysyl oxidases to reduce pyridinoline cross-links is their involvement in the formation of all other enzymatic cross-links, whereas this is not the case when LH2b would be inhibited. For example, lysyl oxidases are important for the formation of reducible ACP, deH-HHMD and deH-LNL and the irreducible HHL cross-links that are endogenous to soft-tissues (Chapter 1, Figure 2a), and stabilize the collagen fibrils of these tissues. Therefore, effects seen in experimental settings with LOX and LOXL2 inhibitors are more likely to be related to collagen destabilization than by preventing pyridinoline cross-linking, and could potentially lead to destabilized tissues on the long run. Taking into account the current discrepancies between pre-clinical and clinical trials, a conclusive answer on effectiveness of lysyl oxidases as targets to reduce collagen cross-linking and ultimately fibrosis, is still awaited.

Cross-links derived from the hydroxyallysine route comprise the majority in fibrotic tissues, and are linked to irreducible fibrosis (20-23). Specifically inhibiting this route would markedly reduce potential side effects related to collagen instability when blocking collagen cross-linking as a whole. Due to the time that is required for the chemical ripening out of the difunctional precursors, pyridinoline cross-links are normally established after wound closure of a wound healing reaction. Therefore, pyridinolines are expected not to functionally contribute to the wound healing
reaction, but are merely an unwanted side effect related to the stimulation of LH2b expression by cytokines. From this it follows that preventing their formation would not lead to impaired wound healing reaction that is necessary to resolve tissue damage. LH2b decides de facto which collagen cross-linking pathway is initiated by performing 5-hydroxylation (Hyl) of the collagen telopeptides. However, as no LH2 specific inhibitors exist to prevent the formation of this particular cross-linking route, novel insights into the process underlying LH2b function and expression could offer potential avenues for interference. In this thesis we have deepened our understanding of collagen cross-linking, providing novel angles to therapeutically intervene in this process.

In order to intervene with LH2b at the protein level, it is necessary to understand which molecular events are crucial for LH2 to function properly. Previous reports indicated that the collagen chaperone FKBP65 is somehow related to telopeptide lysyl hydroxylation performed by LH2b, as Bruck syndrome type 1 patients that display mutations in the gene encoding for FKBP65 (FKBP10) have almost no telopeptide Hyl. We revealed in Chapter 3 that inactivation of the PPIase domain of FKBP65 by the immunosuppressant Tacrolimus, or depletion by siRNA KD of FKBP10, prevents the dimerization of LH2, resulting in inactive LH2b. We here provide the answer to a long-lasting question for Bruck syndrome type 1 patients: how can these patients suffer from reduced collagen cross-links, while having no mutations in the gene encoding LH2 splice variants? Our findings also open up the possibility for a therapeutic strategy to prevent LH2 activity and thus pyridinoline cross-linking in fibrotic tissues. Given that inhibition of FKBP65 by Tacrolimus inhibits the activation of LH2b without affecting LH1 (Chapter 3), we even provide a way to selectively inhibit the collagen cross-linking initiator LH2b. Tacrolimus is a FDA approved drug that is prescribed for various purposes. Next to topical use for eczema treatment, Tacrolimus is often part of an immunosuppressive cocktail supplied systemically to transplant recipients to prevent rejection through graft fibrosis (24-27). Anti-fibrotic effects of Tacrolimus have also been confirmed during treatments of experimental and clinical cases of lung fibrosis and liver fibrosis (28-30). Furthermore, Tacrolimus treatments were shown effective in easing keloids and (hypertrophic) scarring when applied topically (31,32). However, for all above cases timing and dosage are critical for such Tacrolimus treatments, since its anti-inflammatory effects could potentially impair this initial phase of wound healing that is essential to wound closure (33,34).

Since Tacrolimus binds a wide range of proteins (immunophilins) (35), it is impossible to relate these anti-fibrosis effects solely to FKBP65. However, a recent study by Staab-Weijnitz et al. showed, that depletion of FKBP65 by siRNA resulted in favorable anti-fibrotic effects in bleomycin-induced lung and idiopathic pulmonary fibrosis (36). In addition to collagen biosynthesis, FKBP65 modulates the assembly of elastin (37-39). Although information regarding this property of FKBP65 is limited, a disturbed elastin assembly could potentially display favorable anti-fibrosis effects as this ECM molecule is increased in fibrotic ECM as well, but obscuring the effects related to reduced collagen cross-links. Based on our findings in Chapter 3, assessing the short term and dosage response of FDA approved Tacrolimus in clinical settings, could be very rewarding to apply Tacrolimus as an anti-cross-linking drug as a solo or
combination therapy.

Although targeting LH2b, LOX, LOXL2 or FKBP65 will result in reduced collagen pyridinoline crosslinks, LH2b determines whether collagen is to be primed for pyridinoline cross-links. Interestingly, inducing a systemic loss of LH2b activity or expression would not interfere with soft tissues as these cross-links are mainly found in rigid tissues, whereas targeting the other enzymes would probably affect both tissue types. Therefore, a logical choice would be to pharmaceutically target LH2 in order to prevent the formation of these specific cross-links.

**TUMOR METASTASIS BY LH2-INDUCED COLLAGEN CROSS-LINKING: FACT OR FICTION?**

Next to causing tissue fibrosis, collagen accumulation and cross-linking in the tumor stroma are important contributors to tumor progression and metastasis of cancers (40-42). Overexpression of PLOD2 is observed in clinical samples and tumor cell lines from at least eight types of solid cancer (bladder, breast, bone, cervix, colon, glioblastoma, gastric and liver), and is suggested to shape the course of metastasis by regulating migration and invasion (43-52). Indeed, for at least bladder, breast, gastric, and liver cancer, high PLOD2 expression and protein levels correlated with poor overall survival of patients (43,47,50,52). However, linking the pro-metastatic functions of PLOD2 overexpression in the tumor cell lines to collagen cross-linking could be a misinterpretation, as it is generally accepted that the majority, if not all, of fibrosis-related collagen (type I and III) of a tumor is produced in the stroma by a type of myofibroblasts called cancer-associated fibroblasts (CAFs) and not by the (epithelial) tumor cells (53-55). Reports on collagen type I and III expression in immortalized tumor cell lines are scarce. Only when certain immortalized tumor cells are made multidrug resistant, or stimulated by exogenous stimuli such as TGFβ1 that induces EMT, a significant amount of collagen type I is observed (56-58). However, the contribution of these tumor cell-derived collagens to the total amount of stroma collagens remains to be resolved. Taking this into account, together with the fact that several functional studies of LH2 were done in a too short timeframe to generate pyridinoline cross-links, makes the current hypothesis that PLOD2 overexpression supports metastatic behavior of tumor cells through enhancing collagen cross-linking rather weak. Furthermore, Chen et al. recently reported that xenografts of lung tumor cell lines that show high PLOD2 expression displayed a stronger metastatic potential than low PLOD2 expressing cells, but that this was not related to enhanced HP cross-links (46). Instead they suggested a telopeptide Hyl independent cross-link pathway induced by high LOX expression. In conclusion, the above suggests that it is likely that LH2 overexpression might have another role in high metastasizing cells other than cross-linking.

In addition to LH2b, PLOD2 can express a shorter splice variant named LH2a, of which currently the function is unknown although it has a similar catalytic domain as LH2b. While LH2b is expressed as the dominant splice variant in almost all somatic
cells, LH2a expression is mainly seen during embryonic development, and in postnatal kidney and liver cells (59-61). Previous research on the role of LH2 in cancer did not discriminate between the two splice variants. Therefore, since LH2b is linked to cross-linking, it remains possible that LH2a has another unknown function, related to tumor cell behavior. In Chapter 4 we therefore characterized both LH2 splice variants in more detail and its potential role in cancer. Surprisingly, we found that both LH2 splice variants (LH2a and LH2b) are localized in the nucleus, next to their known residence in the ER. We characterized nuclear LH2 splice-variants as novel chromatin associated proteins that affect global epigenetic events (nucleosome occupancy and histone methylation) and likely hydroxylates several lysyl residues of histone H1. Also, LH2 was detected at promoters of various oncogenes such as \textit{TNFSF10} (TRAIL), \textit{S100A8} and \textit{JUNB}, which were transcriptionally affected in a \textit{PLOD2} knockout (KO) tumor cell line. Interestingly, these effects could be directly linked to the levels of H3K27me3 at their promoters. As these genes are all linked to tumor progression or metastasis, our findings reveal new insights in the way LH2 overexpression directs the metastatic behavior of tumor cells. Since we currently do not understand its molecular effects at the transcriptional level, further assessment of the functional roles of nuclear LH2 is needed. However, our findings mark the start of unraveling the grotesque impact of LH2 in cancer and could be of great importance for the treatment of many cancer types.

In addition to \textit{PLOD2}, overexpression of \textit{FKBP10} expression is associated with colon and lung cancers (62,63). The molecular function of high FKBP65 levels in these cancers is not entirely understood. However, since we show in Chapter 3 that FKBP65 enables the active form of LH2 by stimulating dimer formation, the oncogenic effects of FKBP65 in these two cancers could potentially be related to its role in activating LH2 by dimerization. Another option could be that FKBP65 mediates the transport of LH2 into the nucleus, since FKBP65 also localizes to the nucleus in addition to the ER (36). Currently, however, we do not know whether LH2 dimerization is even necessary for its nuclear activities. Therefore, dissecting these possibilities through FKBP65 depletion or inhibition should give a clear answer to the molecular function of FKBP65 in colon and lung cancer.

Next to the extracellular space, LOX and LOXL2 are also localizing in the nucleus where they directly affect NOTCH1 and SNAIL signaling, and promote EMT and fibroblast activation through FAK signaling in the tumor stroma (64-66). Indeed, LOXL2 has been shown to directly affect protein levels of E-cadherin and cytokines as TNFα and ANG-1 (67). A valid question remains whether the anti-fibrotic effects seen for LOX and LOXL2 inhibition are thus related to collagen cross-linking or altered signaling networks which normally result in activated fibroblasts and enhanced collagen expression. Interestingly, LOXL2 was found to deaminate H3K4me3 (68,69). The question arises whether nuclear LOXL2 also deaminates Hyl deposited at histone H1 subtypes by LH2 to create a similar hydroxyllysine as in collagen telopeptides. As such, nuclear cross-links could potentially arise that enable histone fixation to chromatin. Although this is pure speculation at this point, it deserves a closer look.

Our findings reveal the need for re-interpretation of current reports on LH2 and its related pathologies including fibrosis. Although we have not verified
LH2b nuclear localization in fibroblasts, based on the findings in Chapter 4, LH2b overexpression in fibrotic tissues or myofibroblasts could potentially have additional functions next to collagen lysyl hydroxylation. Particularly, the observation that JUNB is regulated by LH2 in cancer cells points in that direction, since JUNB is implicated in promoting fibrosis by inducing EMT (70).

TRANSCRIPTIONAL REGULATION OF MYOFIBROBLASTS AND COLLAGEN BIOSYNTHESIS

Another level to control fibrosis is by modulating the transcription of pro-fibrotic mediator genes. In Part II we therefore investigated the underlying transcriptional pathways and regulators for genes related to fibrosis and collagen biosynthesis. Prior to development of tissue fibrosis, fibroblasts but also epithelial and endothelial cells (trans)differentiate into myofibroblast under the influence of cytokines such as the TGFβ family and IL1β. For epithelial and endothelial cells this is respectively called epithelial-to-mesenchymal transition (EMT) and endothelial-to-mesenchymal transition (EndMT). These myofibroblasts are responsible for secreting vast amounts of ECM, predominantly consisting of fibrillar collagens such as collagen type I. Since all irreversible types of fibrosis are linked to myofibroblast differentiation, collagen synthesis, and collagen cross-linking, they are the prime targets to prevent the accumulation of fibrotic tissue in general. Being able to target these elements simultaneously at the transcriptional level would enable a broad and effective treatment of many different fibrotic tissues. All three elements are highly regulated on the transcriptional level through elements as transcriptional factors (TFs) and epigenetic modifications of DNA and histones (71-75). Therefore, providing thorough information on the underlying transcriptional regulators, as is done in this thesis, offers various novel avenues for therapeutic interventions.

The TGFβ1 pathway is considered as the most prominent pathway for the development and progression of fibrosis. As the majority of research on the TGFβ1 pathway has been performed in cancer cells and stem cells, there is a possibility that differences exist between the usage of pathway components of those cells and somatic cells such as fibroblasts. Indeed, in Chapter 5 we found that SMAD3, being one of the most important transcription factor (TF) of the TGFβ1 pathway, regulates the expression of PLOD2 by other histone acetyltransferases (HATs) than the currently known P300 and CBP reported for cancer cells and stem cells. Although these two HATs are considered the core of activating TGFβ1 downstream target genes, our data indicate that alternative activators are to be found in somatic cells. As the TGFβ1 pathway seems still far from being completely mapped, proteomic screening of pathway complexes in fibrotic tissues or cells could therefore facilitate the discovery of novel fibrosis-related pathway components, resulting in new targets for fibrosis treatment.

During myofibroblasts differentiation, EMT, and EndMT, the cytoskeleton is reorganized to gain highly contractile features needed for wound closure during
wound healing (76). Amongst others, alpha smooth muscle actin (αSMA) and transgelin (SM22α), encoded by ACTA2 and TAGLN respectively, become highly expressed and are incorporated into the cytoskeleton, where they regulate both the contractile and migratory behavior of these cells (77-79). Based on their dominant expression in myofibroblasts, both αSMA and SM22α are seen as important fibrosis markers. In Chapter 6 we investigated into more detail the transcriptional regulation of TAGLN during EndMT, especially the mechanism that is responsible for the synergistic effects observed after co-stimulation with TGFβ2 and IL1β (80). Since the polycomb repressive complex 2 (PRC2) H3K27me3 methyltransferase EZH2 is an important regulator of normal endothelial cell behavior (81), we wondered whether the enhanced SM22α levels during EndMT could be related to a loss of EZH2 function. Indeed, we found that EZH2 depletion, and inhibition of its H3K27me3 activity, upregulated expression of TAGLN, but also ACTA2, in healthy endothelial cells. This suggests that EZH2 maintains the endothelial phenotype by suppressing genes related to the EndMT process. We further assessed the role of EZH2 during EndMT induced by co-stimulation with TGFβ2 and IL1β. During these conditions, EZH2 was downregulated and resulted in a strong increase of TAGLN expression that we could link to a decrease of repressive H3K27me3 levels at its promoter. Taken together these results show that reduced EZH2 expression or activity is a driver of EndMT. Since myofibroblasts are differentiated cells derived from various cell sources but with a similar phenotype, its regulation is thought to share a fair degree of similarity. However, while we show that EZH2 downregulation and reduction of H3K27me3 corresponds to profibrotic behavior during EndMT, inhibition of EZH2 expression or activity was previously linked to attenuate fibrosis of the liver, lung and kidney (82-84), while a heart conditional EZH2 KO showed enhanced cardiac fibrosis (85). Although the contribution of EndMT to organ fibrosis in general is still surrounded by controversies, the similarities of our finding with cardiac fibrosis could be related to a higher rate of EndMT during cardiac fibrosis than for the other organs. Therefore, this further endorses the above-mentioned notion that fibrotic signaling pathways of different cell types can use various components. Further understanding of these issues is needed to develop general anti-fibrosis compounds.

Another important component of transcriptional regulation is DNA methylation, also known as 5-methylcytosine (5mC). Several genes that regulate myofibroblast differentiation are transcriptionally regulated through 5mC in various types of fibrosis (86-90). Furthermore, inhibition of DNA methyltransferases that catalyze 5mC, show promising anti-fibrotic effects (86,91,92). Therefore, 5mC is considered an important target to regulate the induction of fibroproliferative diseases. The balance of 5mC levels is mediated by DNA methyltransferases (DNMTs), passive dilution during DNA replication, and active demethylation by TET methylcytosine dioxygenases. Active DNA demethylation starts by oxidizing 5mC into 5-hydroxymethylcytosine (5hmC), from here 5hmC remains as a stable modification or is further oxidized to 5-formylcytosine or 5-carboxylycytosine that can be removed through base excision repair processes to reintroduce an unmodified cytosine (93). In addition to its 5mC oxidation activity, TET enzymes can recruit other enzymes such as O-linked N-acetylglucosamine transferase (OGT) and Histone deacetylase 2 (HDAC2) to the DNA and thereby induce additional positive and repressive transcriptional
Summary & general discussion

Effects respectively (94-96). Since DNA methylation is linked to profibrotic processes, we wondered whether TET enzymes have a role in regulating genes that are related to myofibroblasts differentiation and collagen biosynthesis (Chapter 7). Indeed, using a loss-of-function approach we found that all family members (TET1-3) regulate various genes related to these processes. Most notably, TET2, and to a lesser extend TET1, showed broad anti-fibrotic effects after depletion. Not only did they strongly reduce COL1A1, ACTA2 and TAGLN, also a variety of collagen biosynthesis enzymes (including LOX, LOXL2 and PLOD1) were downregulated when TET1 or TET2 was depleted in TFGb1 stimulated fibroblasts. This suggests that endogenously TET1 and TET2 are pro-fibrotic regulators that cover a wide spectrum of regulator and effector proteins. Interestingly, PLOD2 was not regulated by any of the TETs, probably because its promoter is unmethylated in fibroblasts (Chapter 5) and as a result does not recruit any of the TETs. TET2 was previously discovered as a master regulator of contractility genes in smooth muscle cells (97) that resemble myofibroblast to a certain extent by their contractile phenotype and marker gene expression. Since we show a similar finding for TET2 in myofibroblasts, TET2 might be a universal regulator of contractility by cells. Also TET3 has been implicated before in the BMP7-induced attenuation of fibrosis in renal and cardiac fibrosis models (98,99). In our experimental settings, however, TET3 depletion did not induce noteworthy changes regarding myofibroblast differentiation as measured by qRT-PCR of ACTA2, COL1A1 and TAGLN. However, the low-density RNA arrays revealed that TET3 depletion positively affected the expression of several collagen biosynthesis genes such as PLOD1, LOXL2, and MMPs, while enhancing the expression of fibrosis-related TFs such as SNAI1 and SOX9. Together this indeed suggests that TET3 is a repressor of certain fibrotic processes, although not having effects on the expression of COL1A1 and ACTA2. Genome-wide screening of 5hmC showed that myofibroblast differentiation was accompanied with an increase of 5hmC. Therefore, our results show that in addition to DNA demethylation by DNMTs, oxidation of 5mC through TET enzymes is a pro-fibrotic event as well.

TARGETING THE EPIGENOME: REWRITING A POTENT THERAPEUTIC LANDSCAPE

Epigenetic modifications provide a blueprint of heritable information that can govern the fate of various transcriptional cues and pathways. Their remodeling due to profibrotic signaling pathways could therefore potentially result in an irreversible epigenetic memory that supports the myofibroblast identity. When being able to modulate their memory, the myofibroblast identity would be lost, including and their fibrotic effects. As such the epigenome holds a treasure of possible novel pharmaceutical targets to modulate the transcriptional state of disease-related genes. Several inhibitors of histone and DNA modifying enzymes (epigenetic drugs) have already been developed with proven success in pre-clinical and even clinical settings to prevent the activity of certain epigenetic enzymes in a disease context. For instance, inhibitors of HDACs and DNMTs induce anti-tumor effects when used in mono or combination therapy
for treating various hematological malignancies (100,101). Based on these promising results the use of epigenetic drugs would therefore be theoretically applicable to a wide range of other diseases. Continuous efforts are being made to develop inhibitors against a wide spectrum of epigenetic enzymes, or to improve their specificity. In Part II of this thesis we provide new insights regarding the transcriptional regulation of various genes related to pro-fibrotic events, thereby providing novel targets for epigenetic drugs to attenuate fibrosis.

In Chapter 5 we assessed in more detail the TGFβ1-related changes to histone modifications at PLOD2. Interestingly, H3K4me3 that is found at active promoter was already highly enriched and did not change by TGFβ1. Other modifications, such as gene expression-associated modifications H3K79me2, and histone H3 and H4 acetylation (H3ac and H4ac) were enhanced, while the repression-associated modification H4K20me3 was reduced at the PLOD2 promoter upon TGFβ1 stimulation. As such this pattern followed the course of PLOD2 expression. Applying inhibitors against the enzymes that catalyze these modifications would therefore offer a potential novel way to prevent PLOD2 activation during fibrotic conditions. Various inhibitors against the H3K79 methyltransferase DOT1L have been developed to target various forms of leukemia, and are currently in clinical trials (102). Therefore, depending on the outcome, these DOT1L inhibitors could open the door for treatment of other diseases related to DOT1L activity, such as fibrosis and potentially solid cancers by interfering with PLOD2 expression. Targeting H3 and H4 acetylation to attenuate PLOD2 expression would be difficult since we could not identify the HAT responsible for the expression-related PLOD2 histone acetylation. Also, H4K20me3 was identified as a potential target, but this modification is essential to genome integrity (103), and disrupting H4K20me3 methyltransferases might lead to genomic instability that in turn could result in cell death or potentially cancer (104).

In our search to find a potential role for TET enzymes in myofibroblast differentiation and collagen biosynthesis, we observed strong anti-fibrotic effects when predominantly TET2 but also TET1 expression was depleted by siRNA (Chapter 7). Genome-wide profiling indeed indicated that TGFβ1 strongly induced 5hmC deposition at gene bodies and promoters of various fibrosis-related genes that we linked to the observed alterations in transcriptional activity. Although ACTA2 and TAGLN, several collagens, and expression of several collagen biosynthesis genes were altered in such a way that they supported an anti-fibrotic expression profile, PLOD2 expression was not affected. Interestingly, expression of LOX and LOXL2 enzymes was attenuated by TET1 and TET2 depletion. This suggests that targeting either TET could also attenuate collagen pyridinoline cross-links. Since so many fibrosis-related genes are regulated by TET1 and TET2, we feel confident that targeting TET1 or TET2 activity with inhibitors would be a highly effective anti-fibrotic therapy.

Although epigenetic drugs offer a plethora of new ways to interfere with disease-related genes or complete transcriptional networks, their use is not without risk. Since epigenetic enzymes perform their activity on a genome-wide scale, but also can modify targets outside the epigenetic scope (105-107), systemic treatments with inhibitors of epigenetic enzymes in less severe diseases have the potential to trigger unintended side effects throughout the body by interfering with multiple
Summary & general discussion

gene regulatory networks (108). However, since common pharmaceuticals also show epigenetic side effects (109), it remains the question whether epigenetic drugs indeed induce the etiology of severe physiological side effects. Therefore these issues should be assessed in more depth in preclinical and clinical trials and preferably in a long-term follow-up.

Tools that harness the power to modulate the epigenome with more specificity, or even truly at specific loci, could certainly outperform the genome-wide acting inhibitors. Epigenome editing is an innovative technology that harnesses the power of targeting engineered DNA binding domains (DBDs), such as zinc fingers or CRISPR/Cas9, tethered to effector domains of epigenetic enzymes to modulate the local epigenome at any genomic locus of choice (110). When the designer DNA binding domain recognizes and binds its intended genomic location, the subsequent modulation of the local epigenetic modifications offers a way to bypass at large unintended genome-wide effects seen for epigenetic drugs. Furthermore, its potential to provide a one-hit strategy whereby the cell itself faithfully propagates the induced epigenetic remodeling, offers a unique therapeutic strategy that could potentially outpace other epigenetic and non-epigenetic treatments. The potential of epigenome editing as a transcriptional regulatory tool has been established by various pioneering reports by us and others (111-115). However, information about long-term stability of epigenetic editing is lacking. In Part III of this thesis we assessed the capability of these targetable rewriters to induce heritable (long-term) rewriting of the epigenome, accompanied with stable changes to transcription.

As a potential one-hit strategy to reduce collagen cross-linking and metastatic effects induced by lysyl hydroxylase 2, we assessed the ability to induce heritable PLOD2 repression by targeting its promoter with either a DNA methyltransferase (M.SssI) or the non-catalytic KRAB repressor (Chapter 8). Indeed, we were successful in inducing PLOD2 repression with both effector domains. Moreover, the induced repressive effects were stable in both fibrotic fibroblasts and breast cancer cells over a prolonged period of time and reflected the occurrence of local reinforcing repressive chromatin modifications. In a previous report, targeted DNA methylation of the VEGF-A promoter was not stably maintained even though they were able to realize up to 54% DNA methylation compared to 1% in untargeted cells (116). This suggests that the targeted DNA methylation at this locus is either lost passively during cell division or actively by iterative oxidation of TET enzymes, once the targeting tools have become absent. Since we show that, at least in fibroblasts, PLOD2 is not a target of TET enzymes (Chapter 7), it is likely that the targeted DNA methylation levels at PLOD2 persist because TET enzymes are not recruited. However, this needs to be addressed in more detail.

Transcriptional re-activation of endogenous genomic loci can be realized by targeting (multiple) VP16 activating domains (VP64) tethered to DBDs. Although these tools are able to re-express a wide variety of epigenetically silenced target genes, we found that the VP64-induced transcriptional effects act transient, since re-expression is not sustained (Chapter 9). Using VP64 as a model, we went on to assess what additional modifications could realize a sustained re-expression of our target genes. We found that one essential component was lacking to reprogram the epigenetic
signature in a sustained manner, namely H3K79 methylation. When we, instead of VP64, co-targeted an H3K4me3 writing enzyme (PRDM9) with DOT1L, we were able to established sustained re-expression of our DNA hypomethylated model gene PLOD2. Interestingly, this result also gives more insight why we do not see a further increase of H3K4me3 for PLOD2 during TGFβ1 stimulations, but instead see enhanced H3K79me2 that seems to support expression (Chapter 5). When we next targeted the same complexes to a DNA hypermethylated model gene (EPCAM), this only resulted in short-lived re-expression of EPCAM. Interestingly, when DNA methylation was first removed from EPCAM using a DNMT inhibitor, the effects after targeting both complexes were sustained for a prolonged period of time. Together, these results show intriguing new insights into the cross-talk between various epigenetic modifications, and also offers the first evidence in literature that targeted rewriting with multiple effector domains is possible to achieve the desired epigenetic landscape and to maintain the intended transcriptional effects.

In addition to its potential therapeutic applications, epigenetic editing can assist in answering complex questions regarding epigenetic modifications and their biological responses or consequences. In relation to this, epigenome editing tools have already been used to dissect the regulatory logics of epigenetic regulators or modifications and genomic elements (117-119). Although a lot of work has been done on characterizing individual modifications and their crosstalk, experimental biologists were largely limited to functional in vivo confirmation. As such Chapter 9 is a good example how epigenetic editing could assist in dissecting crosstalk of the epigenome and its coding ability for transcriptional choices by modulating any epigenetic modification of choice at genomic locations. In Chapter 9 we addressed a long-lasting question whether promoter localized H3Kme3 has the intrinsic power to induce transcriptional activation, or merely assists other factors that do the actual job. We indeed show that by targeting a repressed gene with solely an H3K4me3 writing enzyme is enough to enable re-expression. These results stimulate follow-up work to assess the dynamics and timing of the subsequent events, e.g. chromatin remodeling, binding of transcriptional initiation complex and RNA polymerase elongation. Furthermore, in Chapter 8 we observed that targeted DNA methylation at the PLOD2 promoter resulted in subsequent changes to histone modifications (H3K4me3, H3K9me3, H3K27me3 and H3ac) in fibrotic fibroblasts. To our surprise, targeted methylation of the same region in breast cancer cells only affected H3K4me3 while in both situations PLOD2 expression was reduced. As such this revealed chromatin-dependent cross-talk differences of the same gene in different cells. Another surprising finding was that the high levels of repressive histone modifications at PLOD2 promoter induced after targeting with KRAB, was far more effective in supporting transcriptional repression than high levels of DNA methylation after targeting M.SsSI. This suggests that, at least for this locus, higher levels of repressive histone modifications are more effective for transcriptional repression. By uncovering the ability to stably repress and re-express genomic loci by (re)-introducing certain epigenetic modifications, we opened up the possibility to activate any repressed locus of choice. Therefore our findings could mark the start of a revolution in medicine and the way we have to look at future treatments. For long, gene therapy has been thought of as a promising way
to re-express silenced loci. Later, this was followed by epigenetic drugs to re-express genes. With the coming era of epigenome editing, we are nearing perfection whereby individual genes can be modulated to follow a desired transcriptional outcome and as such provide an unimaginable amount of applications.

CONCLUDING REMARKS

With this thesis we aimed to provide a better understanding of the processes that determine fibroproliferative diseases such as myofibroblast differentiation, collagen biosynthesis, and collagen cross-linking. Our approach was essentially divided over three areas: First, dissecting the molecular principles leading to collagen pyridinoline cross-linking, and assess its possible alternative function in cancer. Second, find transcriptional regulators, including epigenetic enzymes and modifications, which could pose novel targets in the quest to treat fibrosis by tackling either or all three of the processes. Third, exploit the transcriptional informative power of epigenetic modifications to enforce stable transcriptional states on model genes related to fibrosis and cancer.

Reducing collagen cross-links remains a challenging endeavor for progressive fibrosis and cancer. Not only because there have been misconceptions on its role in either disease, but also how it should be targeted most effectively. In this thesis we discuss several flaws of the current approaches and hypotheses, and urge for a focus at the initiation of cross-linking by LH2. With our findings we deliver several new angles that could be of great interest as potential anti-cross-linking therapy such as a novel way to inhibit LH2 activity by Tacrolimus, or its transcription by targeting its epigenetic landscape by inhibitors or ultimately epigenetic editing. In addition, for cancer, we uncovered that the role of the cross-linking initiator LH2 is more diverse than previously thought, and reshapes the enigma of the way it plays a dominant role in many cancers.

Myofibroblasts are at the heart of fibrosis, as they are responsible in a large extend to excessive collagen biosynthesis, cross-linking and many other pro-fibrotic processes as well. A promising strategy to block progression and possibly resolution of fibrosis would be to target the transcriptional processes leading to myofibroblast differentiation. In this thesis we uncovered several powerful transcriptional mediators that aid to this process. Especially TET enzymes seem to function in a broad manner during differentiation. It also shows that targeting the fibrotic epigenome is a great source for anti-fibrotic treatments.

Together, the findings discussed in this thesis not only provide a better understanding of fibrotic processes in general, but also offer several novel avenues that, upon thorough evaluation, could aid tremendously to finally overcome fibroproliferative diseases.
REFERENCES


Summary & general discussion

collagen crosslinking is responsible for fibrosis-enhanced metastasis. Cancer Res, 73, 1721-1732.


Chapter 10


Summary & general discussion


