From microenvironment to epigenetics in endothelial cells
Maleszewska, Monika

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CHAPTER VI

General Discussion
I. MAIN FINDINGS

This Thesis investigates the mechanisms connecting particular microenvironmental cues through the signaling and epigenetics, to the changes in endothelial transcriptome and phenotype.

In Chapter II we showed that the process of EndMT, which takes place in profibrotic environment, is potentiated by the combination of inflammatory (IL-1β) and fibrotic (TGFβ2) signaling and mediated by NFκB. This study showed that the effect of the activation of specific signaling pathways depends on the context, e.g. other pathways activated in the same time. This interdependency might have synergistical effects on transcription and phenotype, as demonstrated by the enhancement of expression of mesenchymal genes in ECs exposed to the combination of inflammatory and fibrotic stimuli.

In Chapter III, using **TAGLN** as a model gene we extended the mechanism of action of IL-1β and TGFβ2 by showing that IL-1β regulates the expression EZH2 methyltrasferase, while the combination of IL-1β and TGFβ2 decreases the H3K27me3 levels and increases the expression from **TAGLN** promoter. These results showed that signaling events not only lead to activation of transcriptional machinery, but can also affect chromatin-modifying factors, to shape the accessibility of specific gene promoters.

In Chapter IV we investigated the global action of the EZH2 methyltrasferase in endothelial gene expression, in context of the mechanical force of FSS. We identified genes regulated by EZH2 or by FSS in endothelial cells, as well as candidate genes regulated by FSS through the decrease in EZH2 expression. Finally, we linked the changes in expression of a selected group of genes, the cell cycle-related genes, to the change in cellular function: we demonstrated the decrease in proliferation. This study showed that EZH2 is a global epigenetic regulator in endothelial cells, whose expression can be modified by the mechanical force of FSS, further supporting EZH2’s regulation by microenvironmental cues.

In Chapter V we used the knowledge on the behavior and function of EZH2 under FSS to help explain the anti-oxidant properties of FSS. We identified four genes, **HMOX1**, **GPX3**, **PTK2B** and **PTGS1**, that are candidate targets of both EZH2- and FSS-exerted regulation. The computational analyses of the promoters of **HMOX1**, **GPX3**, **PTK2B** and **PTGS1**, suggested that they are all regulated through repression, both by epigenetic activity of EZH2 (H3K27me3 mark) and by putative repressive transcription factors. This study shows how the epigenetic regulators such as EZH2, besides the global character of their action, constitute the part of more complex chromatin-regulating machinery at the local level of specific promoters.
In summary, we showed how microenvironment influences the endothelial cells through signaling and epigenetic events. In particular, we found the central role of EZH2 in the regulation of gene expression in endothelial cells.

II. FROM MICROENVIRONMENT THROUGH CHROMATIN TO ENDOTHELIAL PHENOTYPE

Our results showed how the microenvironment shapes the endothelial phenotype through signaling events changing the activity of transcription factors and chromatin modifying enzymes. Changes in the phenotype allow the endothelial cells to fulfill their numerous functions in a microenvironment-responsive manner.

In simple terms, the microenvironmental cues constitute the input signals. The information is transmitted by the cellular signaling, and the net force of that signaling – all the pathways activated at a certain time – needs to be weighed and resolved at the chromatin level. Here the interplay between different microenvironmental influences comes down to the molecular interplay between transcription factors and chromatin modifying factors. This interplay will determine the cell’s transcriptome. The transcriptome – the collection of all transcription-derived RNA species in the cell at a given time point – will to a great extent shape the cellular phenotype. The resulting phenotype will allow

Figure 1. The main axis of cellular information flow relevant to this Thesis. Microenvironmental stimuli constitute the input. They are received by the cell through receptors and exert signaling, which transduces the signal, and often includes integration. The signal is interpreted at the level of chromatin, through the balance and interplay of chromatin modifying factors and transcription-related factors. The outcome of this process is the transcriptome, which is also the primary output of the system. The secondary, transcriptome-based output is the cell’s phenotype. Beyond this axis, the acquisition of a new phenotype can lead to execution of a specific function, dysfunction, loss of function and/or gain of a new function.
the cell to respond and fulfill its function, which can altogether be considered the \textit{output} of the system.

Many cellular responses are direct and do not involve the transcriptional step, but rather changes in e.g. protein structure or the metabolic flow. However, the axis described above and illustrated in Fig. 1, is a simple representation of the processes that involve the transcriptional level. It depicts the flow connecting the microenvironment – signaling – chromatin – transcriptome and, finally, phenotype and function. The following sections of the Discussion (A – D) will discuss the findings of this Thesis in the frame of this axis (Fig. 1), placing them within the more general mechanisms of its flow.

A. Endothelial microenvironment: complexity and combined effects

The complexity of cellular signaling derives in part from the complexity of the microenvironment itself. While single factors are often studied, as they are easier to control, cells dwell in a mixture of various stimuli that activate whole networks of pathways. The interaction between inflammatory and fibrotic signaling in the process of EndMT provides a simple example of this “interaction” effect. It is the combination of IL-1β and TGFβ2, and not the single factors, that causes a strong induction of the expression of SM22α and calponin in endothelial cells. Not only the presence, but also the timing of the stimuli is of a great influence, as the stimulation with IL-1β seems to prime the cells for an enhanced response to TGFβ2.

Our findings were recently confirmed by Nie \textit{et al.}, who also observed the synergistical interaction of IL-1β and TGFβ2 in the induction of EndMT.\textsuperscript{1} Besides us, also Rieder \textit{et al.} embarked on the investigation of the interaction between fibrotic and inflammatory microenvironmental cues.\textsuperscript{2} They modeled the environment of intestinal fibrosis by combining IL-1β, TNFα and TGFβ1, to demonstrate on one hand the enhanced transdifferentiation of cells treated with all three factors together, and on the other hand the central role of IL-1β in driving the synergy. The latter supports the “priming” effect of IL-1β in the IL-1β and TGFβ2-induced EndMT.

The combination of different microenvironmental cues will elicit many parallel signaling pathways. However, the signaling from different factors is often integrated. It is common for signaling networks to acquire a bowtie-shaped structure, indicative of a convergence at the level of an intermediate mediator, e.g. a kinase that receives signals from multiple receptors and activates multiple downstream effectors.\textsuperscript{3,4} In our study such a point of convergence for IL-1β and TGFβ2 could be NFκB (Chapter II). We showed that its expression is additively
increased by IL-1β and TGFβ2, and that its activation is necessary for the synergistical effects of the two factors. But it is also likely that the convergence occurs even higher in these signaling pathways, most likely at the level of TGFβ-activated kinase-1 (TAK-1), which was shown to act downstream of both IL-1β and TGFβ, and upstream of NFκB.\textsuperscript{5,6}

Another point where the signaling pathways of IL-1β and TGFβ2 seem to converge is at the level of chromatin modification by EZH2 (Chapter III). Also in this case, IL-1β and TGFβ2 add to one another’s effects, to result in the decrease in H3K27me3 levels at the \textit{TAGLN} promoter.

\section*{B. Chromatin regulation in endothelial cells: the emerging role of EZH2}

The complex network of cellular signaling has to be resolved at the level of chromatin to result in specific transcriptomic effects. There, next to classical transcription factors, the epigenetic mechanisms that regulate chromatin structure and accessibility play a crucial role. The binding of transcription factors and transcriptional machinery is determined by the chromatin landscape of the genes’ promoters. The actions of the epigenetic modifiers determine whether the chromatin is open or closed, and therefore whether the transcription can occur. But the chromatin regulating factors can also be regulated by signaling.

EZH2 emerged from our studies as such epigenetic integrator, whose functionality depends on the context of the signaling. IL-1β and TGFβ2 affect the expression and epigenetic output (H3K27me3 mark) of EZH2, to modulate the expression of \textit{TAGLN}/SM22α, demonstrating the local action of EZH2 at a single promoter. On the other hand, Chapter IV shows how EZH2 works in endothelial cells on a global level, regulating hundreds of genes. Also in this case, EZH2 acts in a microenvironment-responsive manner: FSS decreases the expression of EZH2, which affects a large proportion of the EZH2-dependent genes.

EZH2 is important in the differentiation of mechanosensitive Merkel cells in skin.\textsuperscript{7} However, the role of EZH2 in mechanotransduction/mechanoresponse of cells, and in particular in the endothelial mechanoresponse to FSS, has not been described before.

Only one other study so far has investigated the role of EZH2 in endothelial cells at a global level, similarly to us, but in static conditions. Dreger \textit{et al.} showed that short-term (72h) siRNA mediated knock-down of EZH2 affects the expression of 964 genes (more than 2-fold change).\textsuperscript{8} Of those, the genes associated with GO terms Cell adhesion and Cell communication were among the most enriched groups of genes. It corresponds with our findings that the
Cell adhesion-related genes are enriched upon knock-down of EZH2. However, in our study (Chapter IV) we achieved a long-term shRNA-mediated knock-down of EZH2, allowing for more downstream effects of EZH2 to take place. By these means, while Dreger et al. reported lack of influence on endothelial proliferation upon siRNA-mediated knock-down of EZH2, we identified the cell cycle-associated genes as one of the most important groups affected by the decrease in EZH2, and we further confirmed that EZH2-depletion results in decrease in proliferation. Large portion of these cell cycle regulating genes was also decreased by high FSS, which implies that the decrease in EZH2 upon FSS might serve to induce endothelial quiescence.

Therefore, Dreger et al. also showed that EZH2 is an important global regulator of gene expression in endothelial cells and identified its direct target genes. We extended on their work by investigating the long-term effects of the loss of EZH2 and identified perhaps more downstream targets. As such, it was not of our interest to limit these to the direct targets of H3K27me3-mediated repression: many of the genes we identified might be secondary targets, affected through changes to their upstream regulators, and yet they might be important for the functional output under FSS. Nevertheless, future chromatin immunoprecipitation followed by sequencing (ChIP-seq) analysis of H3K27me3 localization in EZH2-depleted cells and cells exposed to the microenvironmental stimuli of interest (e.g. FSS) would be an interesting endeavor. It would allow identification of the direct targets of EZH2 (through H3K27me3), relevant to the specific environmental conditions.

While Dreger et al. speculated, that EZH2 might play a role in endothelial response to inflammation and growth factors signaling, we indeed demonstrated the influence of microenvironmental cues, IL-1β and FSS, on EZH2. Therefore, we connected EZH2 in endothelial cells to relevant microenvironmental factors, providing a perspective towards its physiological and pathophysiological roles in endothelium (which will be discussed further in the “Perspectives” section of this chapter).

Finally, in Chapter V we showed how EZH2 works simultaneously with other mechanisms, including repressive transcription factors and histone modifications, to regulate specific target genes. ENCODE data used in that study implies that the promoters of at least two of these genes carry the active H3K4me3 mark besides the repressive H3K27me3 mark of Polycomb/EZH2, reminiscent of the bivalent domains. This work therefore exemplifies how EZH2, at the local level of single promoters is accompanied by other factors, which altogether shape the transcriptional responses of single genetic loci. While specific chromatin features and transcription factors may vary
greatly between different promoters, there are several general examples of how Polycomb/EZH2/H3K27me3 interact with other molecular factors to shape the chromatin accessibility landscape. An important example are the abovementioned bivalent domains: the regions that carry both the repressive H3K27me3 mark as and the active H3K4me3 mark. These domains, which harbor many developmental genes, can be regulated by adjusting the balance of H3K27me3 and H3K4me3 levels, and are therefore referred to as poised.\(^9\)\(^,\)\(^10\) They can be timely activated in response to appropriate stimuli, e.g. inducers of differentiation. The importance of the balance between H3K27me3 and H3K4me3 is further reflected by the balanced relationship between Polycomb and Trithorax epigenetic complexes, which are respectively responsible for deposition of these epigenetic marks.\(^11\) Both Polycomb and Trithorax complexes were shown to play a role in the development or function of endothelium and vascular system.\(^12\)\(-\)\(^16\) On the other hand, once the repression by H3K27me3 takes place, it can be further reinforced by additional mechanisms, e.g. DNA methylation\(^17\) or recruitment of histone deacetylases (HDACs) and histone deacetylation.\(^18\),\(^19\) Moreover, recent reports indicate that Polycomb can also participate in the establishment of long-range chromatin interactions.\(^20\),\(^21\) In addition, while no actual EZH2- or Polycomb Repressive Complex-2 (PRC2)-specific binding sites are known in mammals, there are several factors involved in its recruitment to specific genomic loci. First of all, presence of PRC2 is associated with unmethylated CG-rich regions,\(^22\),\(^23\) where it maintains the repression of untranscribed genes.\(^24\) In our study in Chapter V, the most enriched motif common to the promoters of all analyzed genes was a CG-rich sequence. Although it was highly similar to the ZNF263 binding site, it might also correspond to a putative Polycomb preferred site, but this notion requires further exploration. Polycomb binding is also mediated by other epigenetic regulators, transcription factors or long non-coding RNAs (lncRNA).\(^25\),\(^26\) Finally, some reports imply that in the process of replication PRC2 complexes are able to recruit themselves (by binding its own mark H3K27me3), which preserves the H3K27me3 mark upon cell division and therefore enables the epigenetic inheritance.\(^27\) The fact that other factors affect Polycomb’s action or recruit Polycomb to the target genes corroborates the idea that the activity of EZH2 is highly context-dependent: it depends on the composition (network) of signaling at a given time point (compare also Figure 2).
C. Endothelial phenotype

The interplay between microenvironmental factors, reflected by the complex signaling networks and finally resolved at the level of chromatin by the balanced interaction of transcription factors and chromatin modifying factors yields the product – the transcriptome – that will further shape the endothelial phenotype. The “endothelial phenotype” is itself a complex concept, as it is flexible and heterogeneous. Even without any pathological cues from the microenvironment, the endothelial cells vary greatly between different vascular beds, which seems to be dictated by the specificities of the function they need to fulfill. And so the endothelium forming the blood brain barrier consists of tightly connected cells limiting the exchange between the environments they separate. On the other extreme, the fenestrated or discontinuous endothelium of liver allows for dynamic exchange of substances. Endothelium in other beds presents a range of intermediate phenotypes.28

But besides the varying tissue-level properties of endothelium in different vascular beds, also the molecular make-up of ECs varies depending on e.g. the size of the vessels (e.g. microvessels vs. large vessels) and the type of the vessels (e.g. arteries vs. veins).29 Even a uniform endothelial monolayer within a single vessel might in fact consist of phenotypically different cells. This is exemplified by the “patchy” or mosaic pattern of expression of certain molecules, e.g. von Willebrand factor, when a molecule is expressed by some cells, while completely absent in others).30

The heterogeneity of endothelial cells can be explained with the dynamical systems modeling as proposed by Regan and Aird,30 in which all possible cellular phenotypical states form the phase space that can be represented as a landscape. The valleys of the landscape define the most stable phenotypes (or attractors), whereas ridges define unlikely states of unstable and poised phenotypes, separating the valleys. Still, the model assumes that it is (theoretically) possible for the cells to acquire all intermediate states and even “migrate” into a new valley, which is equivalent to acquisition of a different phenotype.

This system is microenvironment-responsive. On one hand, the *biological noise* can be balanced by the system with no extreme influence on the phenotype (which illustrates the robustness of the system, or of a particular attractor/phenotype). On the other hand, the landscape itself can be shaped by perturbations to the system, e.g. persistent activation of a specific pathway, resulting in the system shifting towards a new stable attractor, and hence the cell acquiring a new stable phenotype. It is therefore conceivable that in some microenvironmental conditions the endothelial cell can cross the cell-identity
borders to acquire a new phenotypical landscape minimum (valley) equivalent to another cell type (e.g. a (myo)fibroblast).

A well studied example of such a process in endothelial cells is the EndMT, which can give rise to fibroblast-like and smooth muscle-like cells.\[31-38\] Endothelial cells are also able to revert towards multipotent stem cell phenotype, enabling their further differentiation towards mesenchymal lineage cells such as adipocytes, chondrocytes and osteoblasts.\[39\] This phenomenon can play a role during heterotopic ossification, e.g. in fibrodysplasia ossificans progressiva.\[39\]

Also in the endothelial tumours, hemangiomas, endothelial cells carry stem cell markers, indicative of their immaturity and possibly differentiation potential.\[40\]

This phenomenon can play a role during heterotopic ossification, e.g. in fibrodysplasia ossificans progressiva.\[39\]

The interaction of IL-1β and TGFβ2 in EndMT, investigated in Chapter II, very well illustrates how the robustness of the control endothelial phenotype, resistant to single stimuli, can be overcome by the combination of the two stimuli. In the terms of the dynamical model, the presence of these two stimuli (IL-1β and TGFβ2) likely alters the state of the system so that the transition towards a new attractor (phenotype) becomes easier or even favorable. This notion is supported by the priming role of IL-1β in our experiments, which seems to yield the cells more sensitive to subsequent stimulation with TGFβ2. The effects of single factors might be balanced out by the system (reminiscent to biological noise) or might drive the cell towards less extreme stable states (i.e. states within the range of endothelial phenotypes, e.g. inflammatory activation).

Another property of the dynamical systems model described above is the memory of the system, which contributes to the multistability – the ability of (endothelial) cells to manifest different phenotypes in the same microenvironment. The memory is derived from signaling feedback mechanisms, epigenetic changes and long-lived molecular species (e.g. stored within the cells). The epigenetic changes, which can be mitotically-stable and preserve certain cellular features regardless of external cues, provide a mechanism that can seemingly counteract the microenvironmental influence. However, this thesis shows that the epigenetic regulator EZH2 can be itself regulated by extracellular cues, e.g. IL-1β or FSS. Moreover, the decrease in EZH2 levels upon FSS leads to global changes in gene expression of hundreds of genes, as shown in Chapter IV. The resulting quiescent endothelial phenotype seems to be distinct from the control, proliferative phenotype. It seems that the decrease in EZH2 under FSS might contribute to the reshaping of the landscape of the phase space of
the endothelial cells, which would then drive the cells towards a new landscape minimum (attractor) equivalent to a quiescent phenotype. This work hence provides an example of how molecules acting at the epigenetic level can also be influenced by the microenvironment, which likely creates a new epigenetic memory and facilitates acquisition of new phenotypical states.

**D. Beyond the phenotype: Function, dysfunction and change of function**

The immediate changes in the phenotype of the single endothelial cells allow those cells to adapt to and respond to their environment. All these individual responses cumulate into the response of the whole endothelial layer. This net response allows the endothelium to fulfill its functions. In a similar manner the pathological microenvironment might alter endothelial phenotype leading to endothelial dysfunction – the state of impaired endothelial functionality. The pathological microenvironment can also lead to a complete loss of endothelial function, if the cell becomes damaged and necrotic, dies through apoptosis, or transdifferentiates into another cell type. While equivalent to the loss of the endothelial function, the latter also indicates that the cell gains a new function.

**Summary**

Endothelial cells dynamically respond to and integrate microenvironmental cues that comprise of biochemical as well as mechanical triggers. Our data show how biochemical (IL-1β and TGFβ2) and mechanical (FSS) microenvironmental cues affect endothelial phenotype. In particular, the study of the interaction of IL-1β and TGFβ2 demonstrates the benefits of an integrative rather than reductionist approach to understanding the effects of complex microenvironments on shaping cellular phenotypes. At the level of signaling the information can also be integrated by a common molecule, which in the case of IL-1β and TGFβ2 interaction can be NFκB and TAK1. To result in transcriptomic changes, the incoming signaling has to be interpreted and resolved at the level of chromatin, through the interplay of various regulatory elements, including transcription factors and chromatin regulating factors. EZH2 emerges from our studies as such an epigenetic regulator, crucial in the regulation of gene expression in endothelial cells. Although it acts in a global way, its local effects depend on the interaction with other chromatin modifiers and transcription factors. Moreover, EZH2 itself is regulated by
microenvironmental signals, e.g. IL-1β or FSS. They both decrease EZH2’s expression, suggesting that they allow for release of certain sets of genes from the Polycomb repression. Nevertheless, it means that EZH2 is involved in the epigenetic control of those genes (e.g. in the absence of these stimuli).

The global epigenetic activity along with the responsiveness to microenvironmental stimuli yield EZH2 as one of the molecules providing a link between the microenvironment and the epigenetics in endothelial cells. It might help to explain how microenvironmental cues can shape endothelial cell’s epigenetic memory to facilitate its interphenotypical transitions.

Hence, the microenvironmental cues, through the signaling, the regulation of chromatin and the regulation of transcription, shape endothelial phenotype. This allows for dynamic adaptation and execution of endothelial function. Sometimes, however, it may lead to transdifferentiation from endothelial to other cell type, equivalent to the loss of endothelial function, but likely indicative of gain of a new function.

III. PERSPECTIVES

EndMT as a physiological process

The process of EndMT investigated in this Thesis is often regarded as pathological. The only case of physiological EndMT occurs during embryonic development, during heart valves formation. In the adult organism, EndMT is mostly associated with pathophysiology, in particular fibrosis. EndMT-derived cells are believed to contribute to the pool of fibroblasts in fibrotic tissue and thereby exacerbate the disease. However, as we pointed out in Chapter II, EndMT could also be part of a physiological response. The striking synergistical effect of inflammatory IL-1β and fibrotic TGFβ2, which strongly induces EndMT in endothelial cells, allows us to speculate that the same phenomenon would occur in vivo, at the verge between inflammation and tissue remodeling. In the process of wound healing, the first reaction of the tissue is the inflammation. Interestingly, one of the first papers describing EndMT-like phenomenon reported it as an inflammation-driven process occurring in wound healing. Also our work presented in Chapter II, as well as the study of Rieder et al., underscores the importance of the inflammatory component in the induction of EndMT. Inflammation is normally resolved and the pro-fibrotic factors take over to create the fibrotic tissue. The macrophages at this stage express high levels of TGFβ2, which in combination with IL-1β, could serve to rapidly induce EndMT.
Fibrogenesis is a repair-oriented process aimed at the restoration of the mechanical functionality and integrity of a tissue. A scar formation after a heart attack is in fact beneficial, because it allows the organism to preserve the mechanical cardiac function. However, excessive scarring that replaces the functional tissue, e.g. cardiac muscle, will at some point cause the organ to fail. In some organs, on the other hand, the chronically occurring damage to the tissue (e.g. alcohol-induced damage in liver) results in chronic repair processes which turn the whole organ into scar tissue. However, besides these pathological examples, the normal destiny of wound fibrosis is to preserve the functionality of the tissue, and to be resolved and replaced by functional tissue when possible.

This fact allows us to further speculate that the response of endothelial cells to the combination of inflammatory and fibrotic stimuli, which is likely to occur in the last stages of the resolution of inflammation during wound healing, might as well be a physiological phenomenon, which supplies (myo)fibroblasts that then participate in the formation of scar tissue and the contractive wound closure. Moreover, the fact that endothelial cells may not only differentiate into fibroblast-like, but also smooth muscle-like cells, further implies the potential of EndMT as a regenerative process. Future studies are required to shed more light on the possible physiological and/or regenerative capacity of EndMT-derived cells.

**Fibrosis**

While EndMT might be a physiological phenomenon, as we postulated above, it is so far mostly regarded as part of pathophysiology of fibrosis. The myofibroblast-like cells derived through EndMT contribute to the pool of fibroblasts in the fibrotic tissue and likely aggravate the disease.

In this Thesis we showed the importance of the interaction of factors present in fibrotic environment. Our results underscore the role of inflammation. Importantly though, our findings should be interpreted in the context of endothelial phenotype and function. E.g. in fibroblasts the stimulation with IL-1β has inhibitory effect on TGFβ1-induced pro-fibrotic responses. Our results are therefore specific to endothelial cells, and important for the understanding of the endothelial contribution to fibrosis through EndMT. Indeed, the synergistical interaction between IL-1β and TGFβ2 in EndMT was recently confirmed by others. Our findings are further corroborated by Rieder et al., who also demonstrated the enhancing effect of IL-1β in TGFβ-driven EndMT. In fibrosis, the TGFβ1 isoform is intensively investigated and seems to have
more profound effects on fibroblasts. However, it is TGFβ2 isoform that is crucial for the physiological embryonic EndMT. TGFβ2 is also relevant to the fibrotic microenvironment, as it is expressed by macrophages in the resolution phase of inflammation, and it was upregulated in cardiac fibrotic tissue in our experiments (Chapter II). We demonstrated that the combination of IL-1β and TGFβ2 has stronger effect on EndMT than the combination of IL-1β and TGFβ1, further corroborating the importance of TGFβ2 isoform in EndMT. Importantly, we also discovered that pro-inflammatory IL-1β can influence the expression levels of EZH2 in endothelial cells. Furthermore, combination of IL-1β and TGFβ2 seemed to further modify the action of EZH2, leading to decrease in the levels of H3K27me3 at the promoter of TAGLN, and hence contributing to the increase in expression of SM22α. This suggests a role for EZH2 and Polycomb, among other chromatin regulating factors, in the endothelial response to fibrotic microenvironment.

Our study therefore hints towards a role of EZH2 and other epigenetic factors in the tissue-remodeling processes, such as fibrosis. Future exploration of the role of EZH2 under pro-fibrotic conditions would help to elucidate the role of EZH2 in fibrosis, perhaps resulting in an interesting target for pharmacological modulation. Overall better understanding of epigenetic mechanisms contributing to fibrosis is needed to provide new targets for therapy. Given the “memory”-providing nature of epigenetic modifications, targeting epigenetic enzymes might prove useful to revert or change cellular phenotypes, and therefore to model the tissue towards less fibrotic and more regenerative.

Endothelial dysfunction and atherosclerosis

In Chapter IV and V of this Thesis we focused on the FSS – the mechanical force, sensed and responded to by endothelial cells. High FSS is atheroprotective: it preserves endothelial phenotype and correlates with lower atherosclerotic capacity. Vascular regions with low FSS, often associated with disturbed blood flow, are atheropron: they correlate with the localization of atherosclerotic plaques, as well as dysfunction of endothelial cells. Our studies in Chapters IV and V suggest that the decrease in EZH2 under high FSS is a beneficial phenomenon, which could help to preserve the healthy endothelial phenotype. This seems in line with our main finding that the network of EZH2-dependent cell cycle-related genes becomes downregulated upon FSS, resulting in decrease in proliferation and, likely, quiescence. Healthy endothelium exposed to high FSS in vivo is indeed quiescent. These findings suggest that under low FSS, EZH2 should act as a factor that drives endothelial
proliferation and expression of genes associated with endothelial dysfunction. Indeed, in many types of cancer EZH2 is known to drive proliferation.\textsuperscript{54, 55} On the other hand, its decrease is crucial for quiescence of B-cells.\textsuperscript{56} All this suggests that high expression of EZH2 in endothelium might contribute to endothelial dysfunction, and thereby to atherosclerosis. Interestingly though, while the FSS-exerted decrease in EZH2 levels seems beneficial, our results demonstrated that EZH2 levels are also decreased by IL-1β. However, IL-1β combined with TGFβ2 not only decreases EZH2 levels, but also the levels of H3K27me3 at \textit{TAGLN} promoter, which results in increase of SM22α levels, reminiscent of EndMT.

This seemingly paradox of EZH2, whose decrease can lead to both beneficial and detrimental results for endothelial phenotype, is easily resolved taken into account the nature of EZH2’s action. EZH2 does not belong to transcription factors (TFs), which are usually attracted to specific binding sites and present at specific promoters. Instead, EZH2 acts globally, as a part of the Polycomb complex. Its recruitment to specific genomic loci depends on the interaction with other factors, such as other chromatin modifiers or transcription factors. All these factors can be subject to regulation by microenvironmental cues, resulting in a different molecular make-up in which EZH2 may act, affecting the outcome of its activity. Different sets of signaling pathways activated by IL-1β or FSS, will result in different factors recruiting EZH2 to different loci, removing EZH2 from other loci, or acting along and modulating EZH2’s activity (Fig. 2).

In other words, the effects of EZH2 will depend on the context in which it acts. This context consists of the already present chromatin states (memory) as well

\textbf{Figure 2. The effect of the modulation of EZH2 expression or activity depends on its molecular environment.} Both IL-1β and FSS lead to decrease in EZH2 expression. This can result in the decrease in the H3K27me3 abundance at the promoters of genes. However, IL-1β or FSS might induce different sets of transcriptional activators and repressors, e.g. transcription factors or other epigenetic modifiers. Therefore, besides the decrease in H3K27me3, all the additional factors will also shape the accessibility of the promoters to the transcription machinery. This is schematically illustrated here by Gene A and Gene B. It is possible that, although EZH2 levels decrease under both IL-1β and FSS, Gene A will be transcribed and Gene B will remain repressed under IL-1β treatment, while Gene A will be repressed and Gene B will be transcribed under FSS. Nevertheless, in both cases the decrease in EZH2 and H3K27me3 will have created the permissive chromatin state, which allowed binding of these factors in the promoter regions. Finally, Gene C represents genes that are co-repressed by other mechanisms, e.g. DNA methylation, or H3K9 methylation in heterochromatin, which might not be affected by the decrease in EZH2 levels. It is likely that if the chromatin is very inaccessible, the H3K27me3 mark will also be preserved despite the decrease in EZH2 expression. These could be for example genes that drive differentiation towards other lineages and other cell types, which are blocked when the “endothelial” programme is active in the cell, and do not become activated upon normal microenvironmental stimuli.
as of the microenvironment-induced chromatin states (through other epigenetic enzymes or transcription factors). The composition of the microenvironment and signaling will therefore direct the action of EZH2 and modify the outcome. Nevertheless, our results suggest that EZH2 could be an interesting candidate target molecule for pharmacological intervention, to prevent endothelial dysfunction. However, due to the context-dependent nature of its action, the validation of its therapeutical potential requires a system level approach. Therefore, the best to assess the appropriateness of EZH2 as a pharmacological target in preventing endothelial dysfunction would be an in vivo approach, as currently it is the only way in which we can reproduce the true complexity of the atherosclerotic microenvironment, as well as assess the outcome of treatment at a high level of an organ or an organism. While investigation of single or categorized gene promoters regulated by EZH2 provides important mechanistic insights, the role of EZH2 in the disease as a whole needs to be assessed at a higher level.

To model endothelial dysfunction in atheroprone conditions, one of the established mouse models could be used, e.g. the ApoE-/- mice fed high-fat diet. Administration of an EZH2 inhibitor before or during the atherogenic stage, with an appropriate control of solvent-treated animals, would allow the evaluation of the inhibitory effects of the EZH2 inhibitor on the endothelial dysfunction and onset/progression of atherosclerosis. To further evaluate the interplay between EZH2 and FSS in the development of atherosclerotic plaques, the cast model could be employed.\textsuperscript{57, 58} In this model, a restrictive cast is placed around a carotid vessel of a mouse, creating a gradient in the levels of FSS. The presence of the cast also creates a low FSS region proximal to the cast, where an atherosclerotic plaque next develops, when applied in ApoE-/- mice. The combination of the cast model with the use of the ApoE-/- mice and the treatment with an EZH2 inhibitor, would allow us to monitor the progression of atherosclerosis at a specific, known site (the site of cast placement), which would facilitate the comparison of atherogenesis between the control and treated animals.

The feasibility of such a study is increased by the fact that EZH2 has been intensely researched in the cancer field, generating knowledge and methodology, including EZH2 inhibitors. High expression of EZH2 correlates with rapid growth and poor prognosis of many types of cancer.\textsuperscript{59-61} Interestingly, also tumour-related endothelial cells depend on EZH2, which drives their angiogenic activity.\textsuperscript{16, 62, 63} Therefore, the research towards discovery of EZH2 inhibitors has received great attention and led to the development of several efficient molecules. Some of the best known examples are 3-Deazaneplanocin...
A (DZNep), GSK126 and EPZ6438. EZH2 catalyzes the methylation of H3K27 with use of the cellular universal methyl group donor, S-adeosyl-L-methionine (SAM), and the reaction yields the methylated histone lysine and S-adenosyl-L-homocysteine (SAH) as the products. DZNep interferes with the metabolism of SAM and SAH (by inhibiting the SAH hydrolase), and thereby indirectly inhibits EZH2 and decreases H3K27me3 levels.\textsuperscript{19} Due to its mechanism of action, however, DZNep is not specific for EZH2. GSK126 on the other hand belongs to the SAM-competitive inhibitors, and so far is the most specific and potent EZH2 inhibitor.\textsuperscript{60} GSK126 is about 1000-fold more selective for EZH2 than for 20 other methyltransferases, and even 150-fold more selective for EZH2 than for its close homolog EZH1.\textsuperscript{19} Finally, EPZ6438 is also a SAM-competing molecule,\textsuperscript{64} and the first EZH2 inhibitor to enter the phase I clinical trials, for use against solid tumours and B-cell lymphoma.\textsuperscript{55, 66}

The availability of these (and other) EZH2 inhibitors, along with growing body of data on their function and efficiency, should enable us and others to adapt them for use in CVD models, such as the ApoE-/- atherosclerosis model mentioned above. Furthermore, if the results are promising, the results of the clinical trials in cancer patients should one day facilitate the trials and the introduction of the EZH2 inhibitors in the treatment of CVD.

\textbf{IV. GENERAL CONCLUSIONS}

The endothelial cell phenotype is dynamic and microenvironment-responsive. The complexity of the microenvironment is transposed onto the complexity of the signaling, which then shapes the chromatin landscape, by influencing both transcription factors and chromatin modifying factors. The interaction of IL-1β and TGFβ2 studied here illustrates the need to investigate endothelial microenvironments with appreciation of their complexity, hence in an integrative rather than reductionist manner. Only then we will be able to observe and understand their true effects on endothelial phenotypes. It is also essential when studying the pathological microenvironments, to correctly dissect the mechanisms of the disease.

The plasticity of endothelial phenotype allows the endothelial cells in different beds to fulfill their function in site-specific manner. The EndMT, during which endothelial cells acquire a mesenchymal phenotype, might therefore be a manifest of their plasticity and besides its role in disease, it might serve yet undetermined physiological functions.

While the area of chromatin regulation is still developing in endothelial biology,
it might bring many important discoveries that will advance both our mechanistic insights and therapeutical approaches. EZH2 is an important epigenetic factor affecting the endothelial phenotype, and therefore an interesting candidate for pharmacological intervention. EZH2 is regulated by microenvironmental cues relevant to endothelial cells, such as IL-1β, FSS, and possibly others. On the other hand, EZH2 regulates the expression of many groups of genes shaping endothelial phenotype and function. This yields EZH2 as an important central molecule linking the microenvironment, epigenetics and phenotype of endothelial cells.

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