Summary

The role of galectins in the vasculature

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Deregulation of angiogenesis has been implicated in many diseases and is in fact a hallmark of cancer. When the limited diffusion distance of oxygen causes tumor cells to become hypoxic the angiogenic switch will occur. Tumor cells will secrete growth factors that can activate endothelial cells and initiate the angiogenesis cascade. We previously showed that an increase of galectin-1 in endothelial cells in response to these growth factors is essential for this process and that galectin-9 levels are also modulated in these cells. Galectin-1 is a prototype galectins, i.e. it contains one CRD and can form dimers, while galectin-9 is a tandem-repeat galectin that contains 2 CRDs linked by a linker region. Of these two, galectin-1 has been studied the most and has been implicated in intracellular processes like pre-mRNA splicing and ras signaling and in extracellular processes like VEGFR signaling and cell adhesion and migration. In the latter two processes galectin-9 is also thought to play a role. Conversely, very little is known about the intracellular role of galectin-9. A complicating factor when studying these galectins is that alternative transcripts appear to be transcribed from their respective genes and not much is known about the functional role of these yet. Both galectins, derived from tumor cells or stromal cells like endothelial cells, have also been shown or suggested to play a role in other hallmarks of cancer, which makes them attractive targets for therapy. This is further corroborated by studies who assign a prognostic role to galectin-1 and galectin-9 in cancer. In this thesis, we set out to assess the function of endothelial galectin-9 in tumor biology and to explore the function of alternative transcripts of galectin-1 and galectin-9 as well as evaluate them as prognostic markers.

In **chapter 5**, we reviewed all literature on the role of galectin-9 in tumor biology. Several studies support a role for galectin-9 in tumor cell apoptosis and adhesion and metastasis. In addition, data suggest a role for galectin-9 in tumor immune escape since it has potent immune-suppressive properties and in angiogenesis, considering its expression changes upon endothelial cell activation. In general, galectin-9 levels appear to decrease in tumors as the progress towards a more aggressive phenotype. To provide an answer to the seemingly paradox that occurs when trying to reconcile this decrease of galectin-9 levels with a role in tumor immune escape, we propose a model in which galectin-9 expression indeed decreased in tumor cells, but increases in endothelial cells which then form an immune-suppressive barrier between the circulating immune cells and the tumor cells.

We indeed provide evidence to support this model in **chapter 4**. In 4 types of cancer, i.e. breast, lung, liver and kidney cancer, we show that endothelial levels of galectin-9 are greatly increased in tumors compared to healthy tissue in a large set of patients. We could only find a limited role for galectin-9 in angiogenesis in the in vivo CAM model, which led us to explore the immune-suppressive functions of this protein. When administered to PBMCs, we show that galectin-9 specifically induces apoptosis in CD4+ T_h cells and CD8+ T_c cells but not in CD4+CD25^{hi}+ T_{reg} cells. The next step in this research is exploring whether endothelial galectin-9 levels indeed correlate with the infiltration of immune cells in tumor masses and whether endothelial knockout of galectin-9 increases tumor rejection. These studies are currently ongoing.

As mentioned, the galectin-9 transcript is subject to extensive post-transcriptional splicing and varies in the exclusion of exons 5 and 6, which encode for the linker region and exon 10, which encodes for the C-terminal CRD. At this point only recombinant gal-9Δ5, the most abundant isoform, is available commercially limiting the functional study of these isoforms.

However, by qPCR we can specifically detect all galectin-9 splice variants and found that endothelial cells express 5 of them which are differentially regulated upon endothelial cell activation. In addition, the development of this technique yielded collaboration with a group that studies the cellular changes that occur during pathologies associated with pregnancies, which we described in **chapter 3**. In this study we found that in a model for normal pregnancy galectin-9 expression in decidual cells increases and that this change was not as clear in a model for spontaneous abortion. We propose this abbarant regulation of galectin-9 expression likely affects the T_h1/T_h2 balance as well as the activation of NK cells, resulting in a deregulation of angiogenesis in the abortion model. Further studies to confirm this hypothesis are needed. In addition showed that changes in galectin-9 expression in these models are due to changes in the expression of specific galectin-9 splice variants and our data suggest that the expression of one splice variant, i.e. gal-9Δ5/10, might serve as a prognostic marker for adverse pregnancy outcome in humans. Specific galectin-9 splice variant expression by qPCR was also applied in a profiling study in tissue of NSCLC patients, as described in **chapter 2**. Here, we showed that gal-9Δ5 expression levels have prognostic value in NSCLC. In fact, both a gal-$9\Delta5^{Lo}$ and a combined gal-$9\Delta5^{Lo}$ /gal-1^{Hi} signature are associated with poor prognosis.

Aside several galectin-9 transcripts, there was also one study published in 2001 providing evidence for the existence of 2 distinct mouse galectin-1 transcripts. Rather than being the product of alternative pre-mRNA splicing, the occurrence of these transcripts depended on where transcription was initiated in the galectin-1 gene. Thus, a long transcript, i.e. GAL-1L, has 31 additional bases at the 5' end of the transcript, compared to a short transcript, i.e. GAL-1S. Three promotor elements and transcriptional control sites were described in this study and we confirmed these are conserved in human. Next, we showed that GAL-1S and GAL-1L are indeed expressed in human endothelial cells and that their expression is regulated differently upon endothelial cell activation, i.e. while GAL-1S levels increase, GAL-1L levels decrease in activated endothelial cells. When exploring the functional consequence of the addition of these 31 bases we found that the 5'UTR of GAL-1L folds into a stable loop structure that resembles a pri-miRNA. As described in **chapter 7**, this prompted us to review several profiling studies assessing the expression or miRNAs in endothelial cells as no exhaustive review on the subject was available at that point. Although we could not detect any miRNAs originating from the loop structure found in the 5'UTR of GAL-1L, we did find a processing site just downstream of the loop structure and designated the 5' RNA fragment yielded by this processing event ce-G1L, as shown in **chapter 6**. The description of so-called ceRNAs by Pandolfi and coworkers provided us with an alternative hypothesis for ce-G1L function, i.e. we hypothesized it could scavenge a miRNA that is important for endothelial cell function, thereby regulating the levels of other targets of this miRNA. In preliminary experiments we indeed obtained data supporting this hypothesis since miR-296, a miRNA with a known function in endothelial cells, is an excellent candidate to bind to ce-G1L, although additional experiments are much needed. We propose a model in which GAL-1S is the protein-coding transcript while GAL-1L has another role. The decreased expression of GAL-1L and consequently ce-G1L in activated versus quiescent endothelial cells results in less miR-296 being scavenged by ce-G1L. As a result more miR-296 is available to bind to mRNA targets like p21 and hgs, proteins which are implicated in a quiescent endothelial state. Thus, ce-G1L possibly acts as a master switch to preserve quiescence in endothelial cells.

In this thesis, we further increased the knowledge on the role of galectin-9 in tumor biology and corroborate the importance of detecting specific transcripts of both galectin-1 and galectin-9, as their expression can be differently regulated. In addition, data suggests that these transcripts can exert widely different functional roles. This new knowledge increases our understanding of the biology and expression patterns of galectin-1 and galectin-9, 2 galectins which are promising targets for anti-cancer therapy due to their multi-faceted role in tumor progression.