Neuroimmune guidance cues for vascular health

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Chapter 7

Summary and General Discussion

7.1 Key findings in this thesis

This thesis aimed to provide evidence that supports a central role for NGCs in CVD by studying the expression, regulation and function of neuronal guidance cues (NGCs) in endothelial cells and monocytes, the 2 cells types that play main role in development of atherosclerosis. The findings laid the foundation for future research of NGCs as novel targets for intervention of atherosclerosis. Following a short introduction in **Chapter 1**, in **Chapter 2**, we started with an overall review of the expression profiles and known functions of semaphorins and netrins in mature vascular endothelial cells. Average expression of semaphorins and netrins were listed, based on the transcriptomic data on GPL570 platform. Roles of semaphorins and netrins in survival, renewal potential, response to vessel wall shear stress, barrier function and controlling of vascular tone were summarized. From the literature review and preliminary experiments, we spotted high expression of SEMA3F and NTN4 in almost all types of endothelial cells. However, less was known about the function of these two guidance cues in mature and quiescent endothelial cells. There was also a lack of knowledge of function of SEMA3F and NTN4 in physiological concentration range. Therefore, in **Chapter 3 and 4**, functions of SEMA3F and NTN4 in endothelial cells were investigated in a reductionist approach. In **Chapter 3**, we observed impaired barrier function, resulting from change of cytoskeleton arrangement, if expression of SEMA3F was reduced using shRNA in human primary endothelial cells. The monocytic cell line THP-1 were shown to express the SEMA3F receptors NRP2, PLXNA1 and PLXNA3. In the presence of SEMA3F, monocyte migrations were inhibited. In **Chapter 4**, we made the observation that reduced level of NTN4 led to a senescence phenotype in endothelial cells. Presence of NTN4 in extracellular matrix derived from decellularized human kidney increased ability of endothelial cell to adhere to the matrix by promoting endothelial cell survival. These studies demonstrate the necessity of the 2 NGCs in endothelial cell biology. In **Chapter 5**, we perform a gene expression meta-analysis of NGCs during the monocyte to macrophage differentiation process, which is the critical process that occurs during development of atherosclerosis. Our pathway analysis confirmed the implication of axon guidance pathway in monocyte to macrophage differentiation process. The change of NGC expression profile corresponds to the function adaptions required for classically activated macrophages. Pro-inflammatory guidance cues, like SEMA7A, are upregulated in macrophage, while anti-inflammatory guidance cues, like SEMA4D, are downregulated. NGC receptors that were responsible for direction

of cell migration were upregulated. We further investigated the regulation of NGCs by the RNA-binding protein quaking (QKI) and found a regulatory hotspot in 3'UTR of SEMA7A transcript. QKI and a cluster of microRNAs possibly antagonize each other's effect by competitive binding at this hotspot. Given the implication of NGCs in monocyte function, we went one step further to validate the relevance of monocytic NGC expression in human using a coronary artery disease patient cohort. In **Chapter 6**, we determined prediction power of NGC expression of circulating CD14-positive monocytes. Using predictive modeling methods, we were able to find models, which correctly distinguished CVD patients from healthy individual. Therefore, monocytic expression of NGCs contains sufficient information for the disease status in our cohort. This gives the first link of between monocytic NGC expression and clinical phenotype in CVD patients.

7.2 NGCs are among the mediators of endothelial functional adaption to vessel wall shear stress

Atherosclerotic plaques profoundly develop at regions with disturbed flow or lower vascular wall shear stress [1]. This evidence has led to recognition of unfavorable hemodynamic environment as a key driver of endothelial dysfunction and subsequently atherogenesis. Vessel wall shear stress is the coplanar force applied to the vascular endothelium by the blood flow. Laminar shear stress (> 10 dyn/cm^2) provokes endothelial quiescence, which denotes the state of endothelial cells being mature and functional. Quiescence endothelial cells have a good barrier function, less coagulant activity and higher eNOS function (higher bioavailability of NO) and are anti-inflammatory. Therefore, laminar shear stress is believed to be atheroprotective, while disturbed flow (turbulence, oscillatory flow) is believed to be atherogenic. Genes that are regulated by shear stress are interesting, because they could be potential mediators of functional adaption to shear stress in endothelial cells, which may subsequently affect development of atherosclerosis. Previously it has been reported that NTN1, SEMA3A and EFNB2 expression is regulated by different flow conditions in both aortic arch of LDLR knockout mice and cell culture. With expression of NTN1 and SEMA3A being higher in atheroprotective flow condition (outer curvature of aortic arch and laminar flow), while expression of EFNB2 being higher in atherogenic flow condition (inner curvature of aortic arch and oscillatory flow) [2]. In **Chapter 3 and 4**, we found that SEMA3F and NTN4 were also upregulated by laminar shear stress, adding 2 more guidance cues that are regulated by shear stress. As reviewed in **Chapter 2**, NTN1 could serve as repellents of

monocyte migration and leukocyte adhesion, thus adding to the anti-inflammatory property of quiescent endothelial cells [3]. Passacquale et al observed downregulation of endothelial NTN1 in ApoE knockout mouse and showed that counteracting the downregulation with aspirin resulted in reduced atherosclerotic plaque progression by reducing monocyte infiltration [4]. Serum levels of NTN1 have been found to correlate with grades of atherosclerosis in human. In contrast to NTN1, EFNB2 could serve as monocyte attractant, so that low expression of EFNB2 in quiescent endothelial cells is intuitive [2]. Despite various studies confirming the involvement of SEMA3A and EFNB2 in cell types related to atherosclerosis, no in vivo evidence showing direct role in atherogenic has been published. In **Chapter 3**, we found SEMA3F was essential for stability of endothelial adherens junction and subsequently endothelial barrier function. Like SEMA3A and NTN1, SEMA3F was found to inhibit monocyte migration, although the inhibitory effect was not directional. Under baseline condition, global knockout of SEMA3F in mice do not display atherosclerotic phenotype [5]. Evidence of atheroprotective effect of SEMA3F in partial ligation mouse model is just emerging [6] (unpublished data suggesting 48% increase of lesion size in SEMA3F knockout mice compared to WT littermates following partial ligation procedure). In a Japanese population, a polymorphism in SEMA3F was associated with decreased risk of myocardial infarction. In **Chapter 4**, we demonstrated that NTN4 deficiency in endothelial cells lead to reduced survival and senescence phenotype of endothelial cells. Our observation is in line with other reports on the role of exogenous NTN4 in promoting endothelial cell survival via kinase activation or activation of integrins [7]. In ApoE knockout mouse, increased endothelial cell proliferation and turnover was observed in the aorta surface, especially in atheroprone regions [8]. The increased turnover could be viewed as a repair response to damage of endothelium. As reviewed in **Chapter 2**, proliferation of endothelial cells requires the cells to deviate from the quiescent state, one the consequences of which is impaired endothelial barrier function. It has long been known that leukocyte infiltration and endothelial cell proliferation occurs concurrently in early atherosclerosis [9]. Together, these observations provide theoretical possibility that factors, like NTN4, that promote endothelial cell survival could counteract damage to the endothelium. However, the essence of NTN4 in such role in vivo is still yet to be established. In summary, NGCs are among the mediators of functional adaption to vessel wall shear stress of endothelial cells. Insights in functional importance of NGCs are accumulating on cell level, but investigations of the in vivo functions of NGCs in relation to atherosclerosis are largely lacking, except for well-studies ones like NTN1.

7.3 In search for potential therapeutic targets in atherosclerosis: where do NGCs fit in?

Different methodological approaches have been applied to discover new therapeutic targets of atherosclerosis. Mechanisms involved in pathogenesis of atherosclerosis have been extensively studied with various therapeutic targets unraveled. One of the earliest studies in atherosclerosis established the importance of blood cholesterol in development of atherosclerosis with high cholesterol diet fed rabbit being the first experimental animal models of atherosclerosis [10, 11]. From that time onward, numerous efforts have been made to tackle hypercholesterolemia, which is to date most effective strategy of atherosclerosis intervention (reviewed in [12]). There were also early efforts made in epidemiological research to access the risk factors for atherosclerosis, including alcohol consumption [13], diabetes [14], blood pressure, body mass [15] and later also smoking [16]. These traditional risk factors were later confirmed in different situations and are still primary targets of intervention. It was later found that atherosclerosis is an inflammatory condition, caused by factors including vessel wall shear stress, dyslipidemia and infection [17, 18]. In early stage of atherosclerosis, these factors cause endothelial dysfunction. In **Chapter 3 and 4** of the thesis, we discussed the role of NTN4 and SEMA3F in endothelial cell function in the context of shear stress and inflammation. In **Chapter 5**, we discussed the expression, regulation, and functional relevance of NGCs in monocyte to macrophage differentiation. Our studies are part of continuing efforts to explore mechanisms involved in pathogenesis of atherosclerosis. In modern times, advances in molecular biology, genetics, high-throughput nucleotide assays, establishment of experimental atherosclerosis animal models and development of genetic engineering tools have boosted the discoveries of new therapeutic targets. Targets are discovered both by screening in population or high-risk families and by mining in the genes (or non-coding elements) that can be possibly involved in pathogenesis of atherosclerosis. As a population screening method, genome wide association studies (GWAS, GWA studies) are normally done to screen the genomic regions that are relevant to cardiovascular disease traits [19]. Regions with best association strength are picked to study further to narrow down to a causal gene or regulatory segment. GWAS led to discoveries of genomic hot spots like chromosome 1p13 near CELSR2-PSRC1-SORT1 genes [19]. GWA studies have several advantages: discoveries are directly relevance in human situation; associations can be made regarding different outcomes; findings are not limited to coding regions; results can be interpreted with future

knowledge. The principle of GWAS dictates that genotype polymorphisms with reasonably prevalence in the studied population can be found. On the other hand, rare variants with high genetic penetrance have been found in patients with familial atherosclerosis. Early studies in familial hypercholesterolemia identified causal mutations in LDLR and ApoB100 genes [20]. With the development next generation sequencing technology, whole exome sequencing can be performed now in familial premature atherosclerosis patients to reveal the causal gene targets in a high-throughput manner [21]. An on-going research in our group makes use of exome sequencing data from familial pre-mature atherosclerosis families with normal blood cholesterol level (data not shown here because it is out of the scope of this thesis). The sequencing identified several mutations in NGCs, which could potentially explain the phenotypes in these patients [22]. Such experimental setup is also beneficial in discovering gene targets that are functioning independent of hyperlipidemia if the affected individuals suffer from atherosclerosis despite having normal lipid profiles.

7.4 How can machine learning tools aid biomedical research and its applications?

Modern omics technologies like microarrays and next-generation sequencing have made it possible to generate enormous number of features from biological samples. A traditional way to combine information from different clinical features is to have an accumulative scoring system based on the value of each measurements that can be calculated manually. However, with the dimensions given by omics data, it is no longer possible to do it. Such situation prompted the need for better tools to deal with huge and complex biological features, which in our and others' opinion can be met with machine learning methods (or predictive modeling). In **Chapter 6**, we gave the proof of concept that by using NGC expression in circulating CD14-positive monocytes, one can distinguish between healthy individuals and CVD patients. Biological omics data are by nature not necessarily independent (between features) nor linear (the features themselves). Various predictive modeling methods were developed to accommodate the complexity of data in these aspects (discussed in the book by Max Kuhn [23]). By modeling the biological data to a certain disease outcome, associations of the biological data and the disease outcome can be made, giving directions for further causality studies. Machine learning methods can iterate in ranges of different parameters, the optimal models chosen from the iterations have the possibility to come close to the maximum prediction power of a certain dataset. Such ability makes decision making easier regarding

whether the pool of the observations is relevant for the purpose of the study. Furthermore, machine learning methods can build the link between functional genes/transcripts/proteins and disease phenotypes prior to full elucidation, thus accelerating the translation of knowledge to clinical situations.

7.5 NGCs as targets of atherosclerosis intervention: the challenges and future perspectives

Despite a much better understandings of the functions of NGCs in cardiovascular system, several challenges remain before successful interventions can be developed targeting NGCs. The broad spectrum of functions of NGCs means targeting NGCs could lead to many undesirable or unexpected effect. The problem is further complicated by the multi-to-multi binding patterns of NGC ligands and receptors. Despite the descent amount of knowledge on the functional importance of netrins and semaphorins in endothelial cell and monocyte biology, insights in the downstream signaling pathways are relative scarce. Future work should aim at comprehensive mapping of the downstream signaling pathways to pinpoint more specific druggable targets. Moreover, it is sometimes clear that a simple agonist/antagonist approach is not feasible. For example, endothelial derived NTN1 is atheroprotective owning to its ability to work as repellent for monocytes, whereas macrophage derived NTN1 decrease inhibits macrophage emigration from atherosclerotic lesions and is therefore atherogenic [4, 24]. Tissue specific drug delivery strategy is necessary in such situation. The effects of NGCs are often dose dependent. There are sometimes optimal concentrations for benefit effect of NGCs. For example, repellent effect of NTN1 and SEMA3A on monocyte migration has optimal concentration [2]. High concentration of exogenous SEMA3F was shown to cause collapse of endothelial cytoskeleton, whereas we showed in **Chapter 3** SEMA3F deficiency in endothelial cells impaired stability of adherens junction via excessive activation of F-actin [25]. If NGCs were developed as drug targets, it is important to know the optimal dose of NGCs for the beneficial effect. It is, however, promising for NGCs to be biomarkers for CVD patient stratification. Based on functional importance of NGCs in monocytes as described in **Chapter 5**, we speculated that peripheral blood monocytes could carry information of patients' disease status. We confirmed the speculation by modeling the predictive power of monocytic expression of NGCs in **Chapter 6** in a preliminary setting. To further establish NGC expression as features for patient stratification, a larger prospective cohort would be needed. In addition, a better choice of the cell type to be profiled could theoretically im-

prove the prediction power further. In summary, to prove therapeutic value of NGCs, deeper understanding of the signaling pathways and the dosing is needed, among other challenges.

7.6 Conclusion

In the first half of my thesis, I started with reviewing the recent research regarding functions of semaphrorins and netrins in mature endothelial cell biology. In the review, I identified SEMA3F and NTN4 as two highly expressed NGCs, for which there was a lack of knowledge of their function in mature endothelial cells. In the following chapters, their roles in maintaining endothelial barrier function, inhibition of monocyte migration, prevention of endothelial cell senescence and promotion of endothelial cell survival were revealed. In the second half of my thesis, I focused on comprehensive analysis of regulation of NGC expression in monocytes and macrophages. I then took a step forward found that regulation of NGC expression in monocytes could be informative for prediction whether patients were currently affected by CVD. Findings in the thesis add to the growing knowledge that NGCs are widely involved in pathological processes underlying atherosclerosis.

References

1. *Baeyens, N., C. Bandyopadhyay, B.G. Coon, et al., Endothelial fluid shear stress sensing in vascular health and disease. J Clin Invest, 2016. 126(3): p. 821-8.*

2. *van Gils, J.M., B. Ramkhelawon, L. Fernandes, et al., Endothelial expression of guidance cues in vessel wall homeostasis dysregulation under proatherosclerotic conditions. Arterioscler Thromb Vasc Biol, 2013. 33(5): p. 911-9.*

3. *Zhang, H., D. Vreeken, C.S. Bruikman, et al., Understanding netrins and semaphorins in mature endothelial cell biology. Pharmacol Res, 2018. 137: p. 1-10.*

4. *Passacquale, G., A. Phinikaridou, C. Warboys, et al., Aspirin-induced histone acetylation in endothelial cells enhances synthesis of the secreted isoform of netrin-1 thus inhibiting monocyte vascular infiltration. Br J Pharmacol, 2015. 172(14): p. 3548-64.*

5. *Matsuda, I., H. Shoji, N. Yamasaki, et al., Comprehensive behavioral phenotyping of a new Semaphorin 3 F mutant mouse. Mol Brain, 2016. 9: p. 15.*

6. *Rattanasopa, C.P., C.K.Y; Liehn, E.; Castano, D.; Singaraja, R.R.; Ghosh, S., Semaphorin 3F Inhibits Transendothelial Monocyte Migration and Protects Against Atherosclerosis. Circulation, 2019. 140:A11736.*

7. *Larrieu-Lahargue, F., A.L. Welm, K.R. Thomas, et al., Netrin-4 activates endothelial integrin alpha6beta1. Circ Res, 2011. 109(7): p. 770-4.*

8. *Foteinos, G., Y. Hu, Q. Xiao, et al., Rapid endothelial turnover in atherosclerosis-prone areas coincides with stem cell repair in apolipoprotein E-deficient mice. Circulation, 2008. 117(14): p. 1856-63.*

9. *Jerome, W.G., J.C. Lewis, R.G. Taylor, et al., Concurrent endothelial cell turnover and leukocyte margination in early atherosclerosis. Scan Electron Microsc, 1983(Pt 3): p. 1453-9.*

10. *Clarkson, S. and L.H. Newburgh, THE RELATION BETWEEN ATHEROSCLEROSIS AND INGESTED CHOLESTEROL IN THE RABBIT. J Exp Med, 1926. 43(5): p. 595-612.*

11. *Morrison, L.M., L. Hall, and A.L. Chaney, Cholesterol metabolism and its relationship to atherosclerosis, coronary artery disease, and arteriosclerosis. Am J Med, 1948. 4(4): p. 616.*

12. *Hartz, J. and S. Clauss, Treatment Strategies for Hypercholesterolemia. Curr Pediatr Rev, 2017. 13(4): p. 243-254.*

13. *Wilens, S.L., The relationship of chronic alcoholism to atherosclerosis. J Am Med Assoc, 1947. 135(17): p. 1136-9.*

14. *Goodman, J.I., S. Wasserman, L.J. Marcus, et al., A study of atherosclerosis in a group of diabetic patients. Am J Med Sci, 1950. 220(1): p. 30-45.*

15. *Lund, F. and M. Faber, The influence of blood pressure and body weight on the atherosclerosis of aorta. Acta Med Scand Suppl, 1950. 239: p. 401-4.*

16. *Auerbach, O., E.C. Hammond, and L. Garfinkel, Smoking in relation to atherosclerosis of the coronary arteries. N Engl J Med, 1965. 273(15): p. 775-9.*

17. *Hemmat, N., A. Ebadi, R. Badalzadeh, et al., Viral infection and atherosclerosis. Eur J Clin Microbiol Infect Dis, 2018. 37(12): p. 2225-2233.*

18. *Taleb, S., Inflammation in atherosclerosis. Arch Cardiovasc Dis, 2016. 109(12): p. 708-715.*

19. *Manolio, T.A., Genomewide association studies and assessment of the risk of disease. N Engl J Med, 2010. 363(2): p. 166-76.*

20. *Hobbs, H.H., M.S. Brown, and J.L. Goldstein, Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. Hum Mutat, 1992. 1(6): p. 445-66.*

21. *Braenne, I., B. Reiz, A. Medack, et al., Whole-exome sequencing in an extended family with myocardial infarction unmasks familial hypercholesterolemia. BMC Cardiovasc Disord, 2014. 14: p. 108.*

22. *Bruikman, C.S., D. Vreeken, H. Zhang, et al., The identification and function of a Netrin-1 mutation in a pedigree with premature atherosclerosis. Atherosclerosis, 2020. 301: p. 84-92.*

23. *Kuhn, M. and K. Johnson, Applied Predictive Modeling. 2013: Springer.*

24. *van Gils, J.M., M.C. Derby, L.R. Fernandes, et al., The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques. Nat Immunol, 2012. 13(2): p. 136-43.*

25. *Bielenberg, D.R., A. Shimizu, and M. Klagsbrun, Semaphorin-induced cytoskeletal collapse and repulsion of endothelial cells. Methods Enzymol, 2008. 443: p. 299-314.*