Homeobox genes in neuroblastoma
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Summarizing discussion
Discussion

In this thesis, we have investigated the role of three transcription factor genes in neuroblastoma and ganglioneuroma. These three transcription factors, PHOX2B, MSX1, and MEIS1, are well documented to play important roles in normal embryonal development of the neural crest and its derivatives. Expression manipulation of each of these three transcription factors identified a large amount of downstream regulated genes. We focused on important players in the Notch and the Wnt pathways. The resulting data are simple and clear when considered as isolated experiments. However, when attempting to generalize the observations and come to an integrated model for their function in normal and malignant development of neuroblasts, the findings often seem paradoxical. At the department of Human Genetics, we have not only studied these three homeobox genes, but also by similar inducible transgene expression or shRNA knockdown experiments other key players in neuroblastoma, like MYCN, ALK, DKK1, DKK3, NOTCH3, HEY1, HEY2, DLK1, CDK2, CDK4, CDK6, among others. As a result, we know hundreds of genes that are regulated by these genes. In addition, we created genome-wide expression profiles of over 110 neuroblastic tumors. Many of the genes in the identified downstream pathways are regulated by not just one, but several manipulated genes concurrently. Therefore, integration of these data into one regulatory model is extremely challenging, despite sublime bio-informatic tools like the R2 platform created to handle and analyze these data.

Each cell, whether resting or proliferating, expresses thousands of genes, which together form a dazzling regulatory network. A central principle of such a biological network is that it must be extremely stable. The cellular machinery maintains its own equilibrium, to which thousands of interactions contribute. In dividing cells, this stable system must also be highly dynamic, as it changes heavily when progressing through the cell cycle. Nevertheless, each cycle produces the same stable network in the next cell generation. In addition, the stable networks also have to be able to transit into other stable phases: during embryogenesis, precursor cells have to switch to subsequent differentiation steps, in which other stable networks of gene and protein interactions are in equilibrium. The stability of these networks is the basis for the extremely reliable performance of cells in the many different tissues and organs in adult multicellular organisms. Assuming that humans have 25,000 genes, 75,000 transcripts and 200,000 proteins (including modifications), we have as a very rough estimate 300,000 actors. With a conservative guess that each actor exerts or is subjected to 10 specific interactions with other players, at least three million interactions are actually possible. A more realistic calculation might be several orders of magnitudes higher, e.g. when metabolic products are included. In theory therefore, billions of different networks can be formed between all elements, depending on which genes are expressed in which combinations. However, humans only consist of a limited number of cell types. Of the billions of theoretically possible ‘states’ of the network, as a rough estimate, only a thousand exist in reality. Among the biggest challenges in modern biology are 1) understanding the principles how networks in actual cell types can be stable, and 2) understanding how one stable
Figure 1: Summary of the relations between the studied genes.
(Panel A) Schematic representation of the interactions between the studied genes in neuroblastic tumours. If genes indicated with a star are overexpressed the equilibrium of the network will shift towards the neuroblastoma phenotype. (Panel B) Correlations of gene expression in the NB110 series by Affymetrix micro-array analysis. Tumours are ranked on the horizontal axis from left to right according to the gene indicated in blue. Tumour histology is depicted below the graph (12 ganglioneuromas in green, 10 ganglioneuroblastomas in yellow and 88 NBs in red)
‘state’ of a network can convert into another stable state and give rise to another cell type.

Ganglioneuroma and neuroblastoma represent an intriguing model of two highly related tumor types, both derived of the sympathetic lineage of the peripheral neural system. Although very different in histology and clinical progression, both cell types can transdifferentiate into each other. An intermediate form exists, called ganglioneuroblastoma, where cells with neuroblastoma and ganglioneuroma morphology are intermixed. Both neuroblastoma and ganglioneuromas are found to transdifferentiate into ganglioneuroblastoma, and vice versa, albeit only in a small minority of patients. Furthermore, a special type of neuroblastomas exists, that initially metastasizes aggressively, but subsequently completely regresses, without any therapeutic intervention (stage 4S neuroblastoma). Insight in the molecular pathways and networks underlying the transition between ganglioneuroma and neuroblastoma is completely lacking. Ganglioneuroma cells have thus far been refractory to culturing in vitro. Of the neuroblastomas, only aggressive stages occasionally give rise to stable cell lines. The tools to study the difference between both tumor types are therefore limited to the analysis of tumor material and manipulation of neuroblastoma cell lines.

PHOX2B is a master-regulator of the development of the adrenergic lineage of the peripheral sympathetic system. PHOX2B null-mutant mice cannot form an adrenal medulla, which normally consist of differentiated, adrenalin-producing chromaffin cells. Chromaffin cells form one of the end-points in the adrenergic differentiation lineage. About half of the neuroblastomas arise in the adrenal medulla and neuroblastomas typically produce precursors of adrenalin, indicating that they stem from adrenal lineage precursor cells that are blocked in differentiation. Moreover, PHOX2B induces expression of dopamine β-hydroxylase (DBH), a key player in the adrenalin synthesis route that is highly expressed in neuroblastoma and normal chromaffin cells [1].

In Chapter 2 we describe that induced expression of PHOX2B in neuroblastoma cell line SJ-NB-8 results in strong down-regulation of MSX1. Expression profiling showed that PHOX2B expression is very high in almost all neuroblastomas, but only weak in ganglioneuroma, while MSX1 showed the opposite expression pattern (Chapter 2, Figures 1 and 2). In Chapter 5, we describe that inducible expression of an MSX1 transgene in neuroblastoma cell line IMR-32 down-regulates PHOX2B expression. The down-regulation of PHOX2B mRNA is only moderate, but PHOX2B protein levels are strongly reduced (Chapter 5, Figure 8). If we add up the findings in IMR-32 and SJ-NB-8, we end up with an or/or relation in the network: cells can either express PHOX2B, or MSX1, as both transcription factors silence each other (Figure 1 of this Chapter). This exactly reflects the situation in ganglioneuromas versus neuroblastomas: MSX1 and PHOX2B expression are inversely correlated.

MSX1 has not been described in literature as a master-gene in ganglioneuromas. MSX1 up-regulates the expression of 91 genes in cell line SJ-NB-8 (2logfold induction of > 0.6). Of these, 66 are significantly higher expressed in ganglioneuromas than in neuroblastomas (P < 0.01; data not shown, Chapter 2). MSX1 also down-
regulates 74 genes in SJ-NB-8, of which 48 are significantly lower expressed in ganglioneuroma than in neuroblastoma. The IMR-32 cell line experiments in Chapter 5, using the same cut-offs, show 390 genes up-regulated by MSX1, of which 286 have higher expression in ganglioneuroma than in neuroblastoma, and 152 down-regulated genes of which 106 have lower expression in ganglioneuroma than in neuroblastoma (data not shown). Together, this suggests that MSX1, if not a master-gene for ganglioneuroma, at least controls expression of important regulatory genes characteristic for this tumor type.

In Chapter 2 we describe that MSX1 up-regulates expression of NOTCH3 in cell line SJ-NB-8 (Chapter 2, Figure 6). In Chapter 5 we describe that NOTCH3 up-regulates expression of MSX1 in IMR-32 (Chapter 5, Figure 1). NOTCH3 is indeed more highly expressed in ganglioneuroma than in neuroblastoma (Chapter 5, Figure 1). If we can add-up these data, MSX1 and NOTCH3 have an and/and relation: if one is expressed, automatically both genes become expressed. Here we have, closely linked to the or/or relation of PHOX2B and MSX1 that enforces different cell types, a relation that enhances or stabilizes the expression of two important genes within one cell type. MSX1 and NOTCH3 are both highly expressed in ganglioneuroma, and relatively weakly in neuroblastoma.

In Chapter 3 we describe that MSX1 also induces high expression of several negative regulators of the Wnt pathway, although many questions remain on the downstream consequences in the Wnt signaling pathway. DKK1, DKK2, DKK3 and SFRP1 are strongly up-regulated (Chapter 3, Figure 1). Of these, DKK3 is indeed over-expressed in ganglioneuroma versus neuroblastoma ([2] and Chapter 3, Figure 2). In Chapter 4 we describe that silencing of MEIS1 in IMR-32 cells resulted in the strong down-regulation of DKK3 (Chapter 4, Figure 6). We also describe that MEIS1 is probably an important transcription factor that induces expression of many neuroblastoma-specific genes, which are significantly lower expressed in ganglioneuromas. In addition, Koppen et al. have published that MYCN, which is amplified in 20% of neuroblastomas and moderately over-expressed in a larger subset, strongly down-regulates DKK3 and DKK1 in neuroblastoma cell lines [2,3]. This suggests that the major neuroblastoma genes MYCN and MEIS1 both suppress DKK3, while the major ganglioneuroblastoma gene MSX1 up-regulates DKK3. DKK3 is known as an important negative regulator of the Wnt pathway. Elucidation of the functional consequences of DKK3 regulation on Wnt signaling will therefore be important.

Figure 1 of this Chapter summarizes some of the relations between the studied genes. It offers a first seminal model of how key-regulatory genes in neuroblastic tumors might interact to contribute to either the ganglioneuroma or neuroblastoma ‘state’. If the transcription factors MSX1 and NOTCH3 are highly expressed, a ganglioneuroma phenotype is stimulated. If PHOX2B, MEIS1 and MYCN are over-expressed, this drives the equilibrium in the network towards the neuroblastoma phenotype. This model is at best only a fraction of the real situation. We speculate that many more of such or/or relations exist between genes on opposite sides of the
demarcation line between ganglioneuroma and neuroblastoma, while the functional networks in the two opposite states will be internally stabilized by and/and relations. Such relations can now be searched for in the hundreds of identified genes regulated by MSX1, MEIS1 and NOTCH3 in their manipulated neuroblastoma cell lines. These cancer cell lines probably carry many mutations in key regulatory genes. PHOX2B and MYCN for instance, can be mutated and amplified, respectively. Not all in vitro gene manipulations might therefore be easy to interpret, as unknown, accidental, mutations might interrupt regulatory systems. The expression profiles of the ‘real life’ neuroblastomas, ganglioneuroma and ganglioneuroblastomas are therefore a powerful tool to select relations that are both supported by in vitro and in vivo data and that might explain the extremes in neuroblastoma biology: spontaneous differentiation and regression, or progressive disease with a fatal outcome.

References