Chapter 5

General discussion & summaries

General discussion

In this thesis, we embarked on a series of experimental settings in order to explore the functional role of the glucocorticoid receptor (GR) pathway. We chose the zebrafish model system that in recent years has emerged as a versatile system for conducting biological research, due to the relative ease of genetic manipulation and handling, its transparency and resemblance of many physiological systems to those of mammals, such as the stress axis and the immune response. Moreover, we opted to use microarray technology to analyze gene expression, in order to explore GR target genes at the whole transcriptome level. This way, we investigated the specificity and dynamics of transcriptional regulation by both the GR α- and β-isoform under different physiological conditions.

The validity of the zebrafish as a model organism for research on glucocorticoid (GC) action has previously been shown. Our work also supports this notion, since zGRα, upon stimulation with synthetic glucocorticoids, affects the transcriptional rate of a large number of genes. Among them, many are known to be GR targets in mammals as well (e.g. *fkbp5, pepck, il8, il1b, mmp9*). Additionally, activation of zGRα leads to immunosuppression, thereby recapitulating the well known anti-inflammatory effects of GC drug treatment.

Our microarray analysis identified many molecular pathways to be affected by zGRα activation such as metabolic, immune, and cell cycle-related signaling routes that could be used as a platform for further investigation of how exactly GCs control physiological systems in a whole organism. In our studies we also determined sets of genes to be upregulated (e.g. *hsd11b2, fkbp5, agtxb, rhcgb*) and downregulated by GCs (e.g. *il8, cyp2aa3, mmp9, mmp13a*), which could constitute marker genes in more elaborate further studies, either in order to elucidate the transcriptional mechanisms of GR action or to perform screenings for novel GC drugs using the zebrafish as a model organism.

In order to model local inflammation in zebrafish larvae at 3dpf (when only the innate immune system is active), we used a tail fin amputation assay. In this assay, it has been shown that within a few hours upon amputation a localized inflammatory response is generated, illustrated by migration of neutrophils and macrophages to the wound site. In our experiments, activation of zGRα led to a significant attenuation of this wound-induced migration of neutrophils, but not macrophages. In addition, large alterations in gene expression were found. Interestingly, it appeared that GC treatment suppressed virtually all amputation-induced gene regulation. Of all genes significantly upregulated by amputation, 84% showed an attenuated upregulation in the presence of GCs. Furthermore, 92% of the genes significantly downregulated by amputation displayed an attenuation of this downregulation after GC treatment. Interestingly, GC treatment did not entirely block the transcriptional response to amputation for the vast majority of genes, but rather resulted in a decreased response. Thus, GC treatment appears to have a very general dampening effect on alterations in gene expression induced by tail fin amputation.

Gene ontology analysis showed that among the pathways that were induced upon amputation we found mainly immune-related signaling routes. GCs, apparently, attenuate all these inflammatory signaling cascades, ruling out specificity of GCs for specific pathways or immune-enhancing effects in this model. It could therefore be argued that the GR inhibits the activity of a ‘master switch’ of immune activation. An important role in this process could be for the AP-1 transcription factor complex, which is known to be an important transcription factor in the induction of the inflammatory response. The amputation-induced induction of 3 subunits of this transcription factor complex (*fos, junb, atf3*) was attenuated by GC treatment, and that finding can be added to the already known

physical interaction of GR and specific AP-1 subunits at the protein level, which also results in inhibition of AP-1 activity.

The overall dampening effect of GC treatment on amputation-induced changes in immune-related gene expression is in contrast with specific effects observed on leukocyte migration, which show a significant suppression in neutrophils, however no effect in macrophages. It could therefore be argued that the observed effects on gene expression do not necessarily reflect the alterations at the protein level or that the amputation-induced migration of leukocytes is mediated at a non-genomic level as well.

In further studies, the modulatory effects of GCs could be further explored using more physiological and cellular readouts (e.g. tissue regeneration, cell proliferation, apoptosis, phagocytosis), also in combination with specific GR knockdown or overexpression in particular tissues or cell types. Additionally, other types of immune challenges could be employed such as systemic bacterial infections as well as assessments at later stages of development when the adaptive immune system is present as well, so that GR action could be studied in more detail.

Using morpholino knockdown of zGRα, it was observed that this receptor regulates the transcription of two distinct subsets of genes. We found that knockdown of zGRα under basal conditions resulted in the alteration of the transcription rate of a different cluster of genes than when zGRα was activated by treatment with the synthetic GC dexamethasone. In general, the first cluster of genes contains mainly genes involved in metabolism, whereas the second cluster of genes mainly involves cell-cycle-related genes. For example, we identified the p53 pathway to be regulated by decreased levels of zGRα under no exogenous stimulation with GCs. A very interesting interplay between GR and p53 has been reported before, and the zebrafish could become a powerful *in vivo* model for studying this interaction at the whole organism level, for instance via looking at alterations in the level of apoptotic cell death during development. The finding of two distinct clusters of GR-regulated genes could open up new research lines concerning the biological significance of this receptor and its function under specific physiological conditions. It could be hypothesized that under resting conditions, the GR controls basal functions like the cell cycle to maintain homeostasis, whereas upon activation by increased GC levels (e.g. those resulting from the response to a stressful stimulus), the GR starts to regulate metabolic pathways in order to restore homeostasis. Further examination of the architecture of the gene promoters (e.g. the GRE structure) in these two clusters could explain their differential response to zGRα signaling and therefore contribute to a better understanding of the molecular mechanism of GRα-mediated transcription under various physiological conditions.

Finally, we were also interested in the biological significance of the GR β-isoform. In humans this splice variant has been shown to act as a dominant-negative inhibitor of GRα and it has been suggested to be involved in GC resistance in asthma patients. However, there is a lot of controversy regarding its low expression level and its effects on transcription. The zebrafish also expresses a GR β-isoform and hence, it could be an interesting model to elucidate the role of this receptor isoform *in vivo*.

In the present work, using overexpression of zGRβ through injection of zGRβ mRNA, and through the generation of a transgenic fish, we aimed to unravel its function with respect to the regulation of gene expression. First we studied whether zGRβ acts as a dominant-negative inhibitor of zGRα. Our data do not support such a role, since only 1 gene (*si:ch211-234p6.12*, encoding an unknown protein) could be determined as a target for zGRβ's dominant negative activity, which most likely represents a false-positive result. Second, we studied whether zGRβ has intrinsic transcriptional activity, i.e. activity

independent of zGRα activation. Both the results obtained using zGRβ mRNA injection and those obtained using the zGRβ overexpressing transgenic fish did not show any evidence for an intrinsic transcriptional activity of zGRβ. This apparent lack of transcriptional activity of zGRβ suggests that the function of splicing of the zGR pre-mRNA into zGRβ mRNA could merely be a physiological way to lower the expression of the canonical GR α-isoform in order to render a cell less responsive to GCs.

Surprisingly however, upon injection of a morpholino altering the splice pattern of zGR pre-mRNA towards increased zGRβ production, we found more genes of which the expression was altered than after treatment with a morpholino that only resulted in decreased zGRα expression. Although a large overlap existed between the gene sets regulated by the two morpholinos there was a large cluster of genes that was exclusively regulated by the morpholino that increased zGRβ expression, and this cluster contained genes involved in pathways not regulated by the other morpholino. This gene cluster could be explained as evidence for an intrinsic transcriptional activity of zGRβ, but might also be a result of a more efficient knockdown of zGRα by the morpholino.

In conclusion, the zebrafish model system with its opportunities for genetic manipulation constitutes a versatile *in vivo* system, in order to further explore GR gene function and signaling. Our exploratory studies provided us a set of GR regulated genes to be used for future investigation of the mechanisms of GR action, interaction and biological significance, regarding GC regulation of various biological processes (e.g. development, inflammation, cancer, stress response).