The function of mitogen activated protein kinases in zebrafish development

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Summary and General Discussion

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Mitogen activated protein kinase (MAPK) are enzymes that are conserved in plants, fungi and animals. All eukaryotic cells use multiple MAPK modules for signal transduction. MAPK signaling cascades are critical signaling pathways that mediate intracellular responses induced by extracellular signals. Pharmacological and genetic studies using *in vitro* cell culture systems have revealed that these pathways control numerous cellular processes, such as tissue morphogenesis, cell proliferation and differentiation, cell survival, immune responses and adaptation. As a result of these broad functions, perturbed MAPK signaling can lead to diseases or affect the developmental program during embryogenesis. In mammals, 14 *mapk* genes have been identified, of which the archetype MAPK pathway is the extracellular signal-regulated kinase 1 (ERK1)/ERK2 module. The main aim of the study described in this thesis was to investigate activation and function of ERK1 and ERK2 signaling in developmental processes like cell migration and differentiation. To investigate these issues we used both embryonic zebrafish cells and whole zebrafish embryos.

First we addressed the question whether Chitin Oligosaccharides (COs) are able to activate the canonical ERK pathway in embryonic zebrafish cell cultures. The MAPK/ERK pathway was chosen as biological tool because of their major role in the integration and control of multiple cellular and developmental processes. The large extracellular polysaccharide hyaluronan (HA) and its synthesizing enzymes (HAS) have been implicated in regulating the migratory potential of metastatic cancer cells. Members of the HAS family have been shown to be involved in the biosynthesis of both hyaluronan and COs. We found that chitin tetrasaccharides transiently induced phosphorylation of ERK1/2 with a maximum after 15 minutes stimulation. We also examined the signaling pathway to ERK activation by COs and concluded that a high-affinity oligosaccharide receptor system exists that transduces the signal to Raf, MEK, and ERK. Once activated, ERK phosphorylates the downstream target p90RSK kinase (chapter 2). Injection of the chitin tetrasaccharides into zebrafish embryos also elevated the level of ERK activation during gastrulation stages, detected with antibodies recognizing dual-phosphorylated ERK1/2. This result suggests that CO signaling mechanisms are functional during early developmental processes.

Initially, we planned to find specific CO targets using a morpholino antisense oligonucleotides knockdown of ERK1 and ERK2. However, these knockdown studies resulted in severe phenotypes which changed the direction of the initial research outline. Phenotypic characterization of the obtained ERK1 and ERK2 knockdown embryos indicated distinct functions of ERK1 and ERK2

during vertebrate development. To study the role of the different mapk genes in zebrafish, we first identified, cloned and characterized the zebrafish *mapk* gene family (chapter 3). The zebrafish genome encodes for members of all *mapk* subfamilies, the ERK, JNK and p38. We determined their specific spatial and temporal expression patterns during zebrafish development. All *mapk* are expressed during zebrafish development and their expression was predominantly localized in the brain structures at 24 and 48 hpf, except for *p38b*. *P38b* expression was only detected at blastula stage. The dynamic expression patterns of the mapk genes led us to conclude that their expression is tightly regulated and the spatiotemporal differences in expression indicate towards distinct functions of the different MAPKs during embryogenesis. Subsequently, we focused on specific functions for ERK1 and ERK2 during early embryogenesis using morpholino knockdown approach and micro-array based gene expression profiling of ERK1 and ERK2 knockdowns.

Our study showed distinct effects of morpholino knockdown of ERK1 and ERK2 on gastrulation cell movements. The observed cell migration effects were considered to be primary changes in gastrulation cell movements and not caused by altered cell fate specification since the expression of patterning genes was not significantly altered in the knockdown embryos. However, the question of which upstream signals coordinate ERK1 and ERK2 activity and divergent downstream responses still remains elusive.

The upstream mechanisms of activation of ERK1 and ERK2 are mostly considered as highly similar, or even identical. Activation of ERK signaling is often the result of a response to growth factors or hormones, but can also be the result of a response to other compounds like neurotransmitters. Fibroblast growth factors (FGFs) are the best described activators of ERK signaling during developmental processes. Also throughout zebrafish development, activated ERK protein is localized in overlapping expression regions with various FGF ligands and other components of the FGF-pathway (chapter 4). Studies using other model systems, such as *Drosophila*, *Xenopus*, chicken, mouse and zebrafish, showed a link between FGF signaling and developmental cell migration processes. However, as previously mentioned, ERK activation is not only mediated by FGFs. Next to other growth factors (e.g. IGF, MDKB, NGF and TGF), also different compounds such as COs, that are known to activate ERK1 and ERK2, are produced during early zebrafish development. Interestingly, a previous study by Bakkers et al. showed a possible function for COs as signaling molecules in cell growth, differentiation and development of zebrafish embryo (Bakkers et al., 1997). In a follow-up study, Bakkers et al.. showed that MO-mediated knockdown of zebrafish HAS2 leads to the loss of hyaluronan, and severe migratory defects during gastrulation, somite mor-

phogenesis and primordial germ cell migration. In addition, their data provided evidence that convergence and extension are separate morphogenetic movements of gastrulation. This supports the data observed in our cell tracing experiments in ERK1 and ERK2 morphants embryos. In chapter 2 we showed that ERK1/2 can be activated *in vitro* by stimulation of embryonic zebrafish cells with COs. HAS2, the CO and hyaluronan synthesizing enzyme, has a role in convergence extension cell migration. In combination, this may suggest that HAS mediated CO signaling is involved in the direction of cell migration processes by affecting ERK signaling. Secondly, the data showed that convergence extension (CE) are separate morphogenetic movements during gastrulation. This observation is in line with the distinct effect on CE movements that we observed by comparing ERK1 with ERK2 knockdown embryos. ERK1 morphants revealed a convergence defect without a severe posterior-extension defect, whereas ERK2 morphants showed a more severe reduction in anterior-posterior extension.

A more stringent knockdown condition showed that the absence of activated ERK2 from the blastula margin blocked initiation of epiboly, a process driven by radial internalization of these cells. ERK2 morphants were arrested in embryogenesis, as the blastula stayed on top of the yolk, whereas ERK1 knockdown had only a mild delayed epiboly progression (chapter 4).

In order to identify specific distinct target genes for ERK1 and ERK2 and address which processes were affected by the depletion of ERK1 or ERK2, we compared the gene expression profiles from ERK1 and ERK2 stringent knockdown embryos using microarray technology (chapter 5). The obtained gene expression profiles of ERK1 and ERK2 morphants were analyzed using software programs, and web-based analysis tools, e.g. Rosetta, GenMAPP and eGOn (GeneTOOLS). Analysis of the obtained data revealed that ERK1MO and ERK2MO knockdown affect signature sets of common target genes, as well as signatures sets of specific genes. Surprisingly, we also identified gene sets in which the expression patterns were anti-correlated. The gene ontology (GO) analysis revealed that ERK1 and ERK2 knockdown signature sets showed significant differences in over- and under representation of the number of genes in distinct and common gene ontology clusters. For example, the commonly under-represented gene ontology cluster ‘development’ was represented by different genes. Further analysis of the obtained knockdown transcriptomes was therefore focused on the main early developmental pattering and differentiation signaling pathways Nodal, FGF, Wnt and BMP. The obtained results corroborate distinct developmental functions for ERK1 versus ERK2.

ERK1 morphants showed a possible disturbed dorsal-ventral patterning, as genes involved in the development of the ventral side of the embryo were

altered in their expression, possibly leading to a dorsalization of the embryos. However, only a small number of ventral genes were affected in ERK1 morphants and a concomitant up-regulation of dorsal genes was not observed. Therefore we can not conclude that ERK1 knockdown dorsalized the embryos. In addition, genes involved in gastrulation cell migration processes were found to be affected, in line with the previously described function for ERK1 during gastrulation cell migration processes. Still, it is important to note that the array analysis was performed before gastrula-stages. Therefore we can only hypothesize that the genes whose expression is affected by ERK1 knockdown are involved in the later gastrulation cell migration processes and are in fact responsible for the observed phenotypes.

The analysis of the gene expression profiles of ERK2 morphants showed that key pathways involved in mesoderm and endoderm differentiation were affected. This resulted in the reduced expression of mesoderm and endoderm marker genes. Analysis of signaling pathway, using the software program GenMAPP, showed that Nodal signaling (mesoderm initiation) was positively affected, whereas FGF and WNT signaling (mesoderm maintenance) were negatively affected. Combined, this indicates that mesendoderm was still initiated in ERK2 morphants, but its maintenance was perturbed. These predictions based on pathway analysis were confirmed by in situ hybridization with mesoderm and endoderm marker genes. In addition, also dorsal-ventral patterning was possibly affected, since ventrally expressed genes as well as dorsal (organizer) genes were differentially regulated, indicating that ERK2 is also of importance for the patterning of the mesoderm.

Many early mesoderm genes, which are crucial for developmental cell migration processes at later stages, were affected by ERK2 knockdown. Again here it is important to note that the ERK2 knockdown gene expression profile was obtained at blastula stages, before the gastrulation cell migration processes take place. However, stringent ERK2 knockdown depleted the active ERK signal from the margin and subsequently prevented the initiation of epiboly. In addition, the main cell movement to drive epiboly is radial intercalation. In a simplified way, it can be said that blastula cells from deeper layers start outward and intercalate with cells already at the surface, pushing the blastula cells over the yolk. To undergo these cell migration processes, the blastula cells need to recognize a signal or chemo-attractant gradient, polarize, organize their adhesiveness and rearrange their cytoskeleton. The ERK2 knockdown gene expression signature set showed changes in expression of genes involved in all these processes. Still little is known about the molecular basis of the complex movements occurring during epiboly. Follow-up experiments, using different knockdown conditions, timepoints during development

and active forms of both ERKs will help us to further understand the differential roles of ERK1 and ERK2 during theses processes. Importantly, this will also help to determine specific roles and target genes for ERK1 and ERK2 during vertebrate embryogenesis and link towards different functions of ERK1 and ERK2 in oncogenesis.

Future perspective

The work in this thesis provides evidence for distinct functions for ERK1 and ERK2 in cell migration processes and development. Morpholino knockdown technology was used to determine specific functions and downstream target genes for ERK1 and ERK2. To elucidate their individual roles at a detailed molecular level, cellular analysis and transcriptomal profiling after constitutive activation of ERKs by upstream signals or the introduction of constitutively active mutant ERK isoforms will be necessary tools.

Constitutive activation of ERK-signaling is found in several human malignancies and is likely to be involved in the development of tumors. The multiple roles for the ERK-pathway in the acquisitioning of a complex malignant phenotype of potential oncogenic cells suggests that specific blocking of the ERK pathway will result in anti-proliferative, anti-metastatic and anti-angiogenic effects in tumor cells. To further study the distinct roles of ERK1 and ERK2 as potential oncogenes in the formation and initiation of cancer and metastasis the zebrafish can be used as a novel model organism. The generation of transgenic fish that can be induced to express oncogenic forms of proteins involved in MAPK signaling will be useful to address this question. The transplantation of inducible oncogenic zebrafish cells into a transgenic zebrafish background provides an *in vivo* system to monitor progression of cancer and to conduct large scale anti-cancer drugs screenings.

In addition to the work concerning ERK1 and ERK2, this thesis also opens the possibilities to study the developmental role of other MAPKs for which no developmental studies have been reported yet. Improvement of gene annotation and ontology, and tools for proteomics and kinomics, will help to further identify molecular targets and identify new responsive downstream target genes.