CHAPTER VI

General Discussion

General discussion

The Mitogen activated protein kinase (MAPK)family is engaged in complex networks of signal transduction orchestrating cellular behaviours such as proliferation, differentiation, migration and apoptosis. MAPK signalling depends highly on the cellular environment as the cascades can respond to different extracellular cues. What adds also to signalling complexity is the presence of various MAPK family members and isoforms which can be functionally redundant in one cellular context while differing in another context where they can even antagonize each other. In addition, several factors such as the presence and abundance of activated receptors, presence of scaffold or adaptor proteins, or crosstalk with other pathways, collectively fine tune the duration and magnitude of MAPK signals to direct a precise cellular response. Giving this complexity, precise elucidation of the molecular mechanisms underlying MAPK associated cellular processes or pathologies remains challenging.

The use of multiple experimental models is valuable to discover MAPK conserved functions among species. In this thesis the zebrafish model is used for further understanding the role of MAPK protein family in embryogenesis. Since most MAPK proteins are conserved within vertebrates the zebrafish is a suitable model to study functional redundancies and specialisations between different members during development. Firstly we have approached this study by applying probes to display specific tissue expression of different MAPK genes. Secondly, we have developed tools to up-regulate and down-regulate MAPK cascades by generating mutants with increased kinetic activity and by using morpholino knockdown, dominant negative mutants or chemical inhibitors.

ChapterII

Activated forms of the upstream ERK kinases RAF and RAS were used for the study of cell transformation and for discovery of anti-cancer drugs. Being part of the RAS-RAF-MEK1/2-ERK1/2 cascade, ERK2 is often hyperactivated in tumors harbouring oncogenic EGFR, RAF and RAS mutations[1]. However this cascade is a core module within a complex signalling network involving positive and negative signalling loops, isoform availability and crosstalk with other cascades. To understand isoform specific signalling differences between ERK1 and ERK2 in development and tumor formation we constructed intrinsically activated forms refractory to inhibition (chapter II). Since naturally occurring activating mutations in ERK have never been identified in cancer patients, developing artificial constitutively activated mutants was necessary. Giving its physiological functional importance and involvement in severe pathologies, ERK2 was an interesting subject for former

structural studies using crystallographic methods[2;3]. The activated (phosphorylated) structure and inactivated (unphosphorylated) ERK2 structure were solved and an overlap revealed the conformational changes, resulting from phosphorylation of the dual phosphorylation site, that facilitate kinase activity and make the protein more accessible for substrate binding. Phosphorylation of the dual phosphorylation site of ERK2 triggers a complex reorganization of hydrogen bounds between amino acids resulting i.a. in the positioning of the activesite. Increasing ERK2 kinetic activity with a simple strategy was impossible and mutational screening for constitutive active mutants were carried out to identify mutations that mimic this effect. *Brunner et al.* was successful in developing an active mutant of the Drosophila ERK2 ortholog Rolled by mutating the docking domain on ERK2 recognised by inhibiting phosphatases (D330N zebrafish)[4]. This mutation was called the sevenmaker mutation and the mutation site was conserved among various species. Furthermore, other gain-of function mutations were published from yeast genetic screens of Fus3. *Emrick et al.2001* measured the *in vitro* kinetic activity of yeast ERK2 double and triple mutant combinations relative to ERK2 basal activity and we have selected the most potent combinations for generating similar constitutively active zebrafish ERK2 mutants[5].

We inserted the same mutations in ERK1 and we found the triple mutant, including the sevenmaker mutation (D352N zebrafish), to increase ERK1 phophorylation *in vitro*. However, we could not measure any differences in the phosphorylation of P90RSK or CREB which are known ERK1/2 substrates. ERK1 and ERK2 are more than 80% identical, yet when targeted for gene disruption ERK2 deficiency appears to results in dramatic effects while ERK1 seems to be dispensable for cell physiology. Two hypotheses are proposed stating i) that differences in expression levels between the low expressed ERK1 and the abundantly present ERK2 is the main and exclusive reason for the observed functional differences[6] or ii) that ERK1 signalling to the nucleus is less efficient than ERK2 and acts as a partial agonist in the ERK1/2 cascade[7]. A partial agonist binds the same upstream activators as the full agonist (ERK2), however, this is less efficient to transmit the signal further downstream of the cascade. The slow ERK1 activity becomes more significant in situations of prolonged cascade stimulation and high demand of activated ERK1/2. In support for this second hypothesis, ERK1 depletion in wild-type conditions is compensated by increased phosphorylation of ERK2 and has no effect on cell proliferation. However, proliferation of oncogenic H-Ras^{Q61L} transformed cells with hyperactivated ERK2 was inhibited by ERK1 overexpression. This suggests that ERK1, as a partial agonist of ERK2, protects cells from excessive proliferation. Differences in ERK1 and ERK2 downstream signaling can be explained by the demonstrated less efficient cytoplasmic/nuclear translocation of ERK1[8;9].

The partial agonist model may also explain the relationship between two other MAPK protein studied in this thesis, the P38α and P38β isoforms, which are 75% homologous and share upstream and downstream kinases. P38α is essential for embryonic development while mice lacking P38β survive normally and do not show any obvious phenotype[10;11]. P38α plays a key role in various developmental processes (myoblast differentiation, neural differentiation, survival, cell migration, proliferation and cytokine production) while P38β function within the Mekk3/6-P38α/β cascade seems to be insignificant[12]. Over activation of P38s is believed to be part of the cause for various inflammatory diseases, including inflammatory bowel diseases and rheumatoid arthritis, as well as for congestive heart failure and breast cancer[13-15]. It would be interesting and valuable to know if P38β acts as a partial agonist of P38α signalling during these processes especially in cytokine biogenesis. There are gain- of function mutations published for all P38 isoforms potentially useful to engineer the corresponding zebrafish proteins[16;17]. Transgenic zebrafish stably expressing such mutated P38 MAPKs will be valuable for future developmental and disease studies.

ChapterIII

All P38 MAPK isoforms are maternally expressed and, except for P38δ, the level of expression, remains constant during embryonic development. P38δ mRNA was detected for the first time at 16 cell stage. Interestingly, we found all P38 isoforms to be expressed in the brain of the zebrafish including the P38α and P38β isoforms of which expression was demonstrated previously in the adult mouse brain[18]. These results suggest that the P38 pathway may play an important role in the development and/or normal physiology of the brain. P38α/β is found to play a role in synaptic plasticity in the hippocampus[19;20]. The functions of the other isoforms in the brain are not known yet. The transcriptome data of the P38α morpholino knock-down embryos (Chapter IV) included a gene pool associated to the nervous system such as Neuropillin 2b, Fgf4, Elavl3, Actn3a, Stathmin, Ceruloplasmin, Lhx5, Ncam and Gbx-2 which were down-regulated and the upregulated Emx1, Crx, Camk4, Mxtx1, Bdnf protein, Her8a, Her11, Endothelin type A, Dishevelled 1b (dvl1b), Vega2, opcml, Isl1, Asl1b, reticulon 1, Jagged 1b, Fbln5 and zic3. This genes list includes possible direct target genes of P38α functioning in the nervous system development and neuronal physiology and when validated will give more insight into the role of P38α in this process. It would be interesting to compare with transcriptome data of other MAPK family members to reveal specificity.

In future research it would be interesting to perform quantitative PCR analysis to detect P38 isoform expression relative to each other. Once having developed reliable P38 isoform specific probes, primers and antibodies expression data can be

obtained in different *in vivo* contexts. Expression analysis can be combined with functional studies involving treatment of zebrafish with stressors such inflammatory cytokines, bacteria, wounding, irradiation etc. to determine possible changes in expression. To analyze protein translation and activation isoform specific antibodies are necessary. These are currently available for the P38α isoform. Structural alignment of the P38 isoforms revealed a mutation prone domain which can be exploited to develop specific antibodies. The P38α and P38β isoforms share the highest sequence similarity and since phospho-P38α specific antibodies have been developed it should also be possible to obtain such antibodies for the β, δ and γ isoforms.

Chapter IV

The function of ERK2 in development was investigated in mouse, drosophila, sea urchin, xenopus and zebrafish models leading to the key finding of a conserved ERK2 cascade downstream of FGF regulating trophoblast differentiation and gastrulation cell movements in the first embryonic stages[21]. Later on the pathway is involved in formation of branchial arches, migrating neural crest cells, the midbrain/hindbrain boundary and forebrain[22]. The role of P38α in developmental processes has been predominantly studied in the relation to skeletal muscle development[23]. However, in zebrafish we have previously demonstrated the expression of all P38 isoforms, including P38α, in specific regions of the developing brain suggesting a role of this MAPK subfamily in development of the central nervous system as well. As already mentioned several genes important for neurogenesis were affected by P38α morpholino knock-down (Chapter IV). Some of these genes, Zic3, Fgf4, Elavl3, Her11, Stmn, Lhx5, Ncam, Vega2, Asl1b are also present in the ERK2 morpholino transcriptome which also includes Flh, Zic2, Hxb8a, Tcf12, Rx3, Gsc, Barhl2, Efna-L1, Cp, Irx1b, Rtn3, Dlc, Cxcr4, Dlx1a, irx7, Otx1a, Acha from the ERK2 specific pool. Neural crest cells arise from the edges of the nascent central nervous system, a domain called the neural plate border (NPB). ERK2 and P38α might be both involved in this process by stringing along in regulation of zic3 expression as Zic2 and Zic3 proteins function in specification of the NPB. Finally, both the P38 and ERK2 MAPK are expressed at the margin of the blastoderm prior and during epiboly and knock-down results in arrested embryos failing to initiate this process. We found a number of epiboly functional genes concurrent and specifically affected by P38α and ERK2 morpholino knockdown. Altogether our transcriptome data indicates that ERK2 function in development is partly redundant with the P38α MAPK which is an interesting finding for further validation using other genetic tools. It remains a question whether the specific pools of target genes deduced from P38α/ERK2 knock down comparisons contain genes that are regulated by the other MAPK family members.

Unlike other vertebrates, the zebrafish contains two copies of the P38α gene and we have found that morpholino knock-down of P38αA or P38αB results in epiboly arrested phenotypes (data not shown). As expected, reduced presence of P38α protein but not Erk2 was detected in P38αA morphants with western blotting using a gene duplicate aspecific antibody. However, a down-regulation of P38α was detected in Erk2 morphants and since the anti-P38α antibody does not distinguish between the two genes it was not clear from the biochemical analysis data which one is down-regulated in Erk2 morphants. We have performed transcriptome analysis of P38αB morphants (data not shown) and the transcriptome largely overlaps with Erk2 transcriptome data. Considering that we do identify a large pool of P38αA specific regulated genes it is likely that P38αB is down-regulated in Erk2 morphants and not P38αA. Data presented in this report and further discussion is about the P38αA gene unless otherwise indicated.

Chapter V

Using genetic and chemical tools we demonstrated that P38α is functional in zebrafish angiogenesis. With unsaturated P38 morpholino knockdown conditions we found 4 genes (Annexin1, Tnk2, Runx1, Vezf1) to be effected. Three of them are previously implicated in angiogenesis and one (Tagap) with expression enriched in the vasculature having an undescribed function in this process. The concentration of the morpholino was down scaled to circumvent severe morphological defects with the disadvantage that many P38α regulated genes were most likely not picked up by microarray transcriptomics. However, this limited number of effected genes is interesting for further validation experiments to identify P38α target genes and understand the role of this MAPK family in angiogenesis. P38α was proposed to have a tumor suppression function by activating several cell cycle checkpoint proteins including P53[24;25]. The role of P38α in tumor progression has two sides since P38α promotes tumor angiogenesis and metastasis. A detailed understanding of cell type specific P38α signalling will be valuable for designing new drug that intervene in steps in cancer development in which the P38 proteins play a role.

We have isolated GFP+ cells of pharmacological treated Tg(fli:EGFP) embryos by FACS for transcriptional profiling. This method would be valuable to determine cell type specific effects after pharmacological treatment. Zebrafish xeno-transplants are being used for high-throughput chemical screens for anti-cancer drugs. With the growing repertoire of zebrafish transgenic lines, that mark specific cell types or stages of immature differentiating cells based on identified marker genes, the treatment effects can be analyzed in detail. We treated Tg(fli:EGFP) embryos with Birb796, an P38 inhibitor severely impeding angiogenesis, in the trunk and head.

cDNA samples of sorted GFP+ cells were hybridised on a 180k Agilent array for transcriptome analysis. We also hybridised samples from GFP+ cells against GFP- cells and obtained a pool of genes of which expression is enriched in pharyngeal arch, hematopoietic or endothelial cells. These comparisons indentified a high number of genes with high fold changes compared to control samples representing tissues from the entire organism which could be validated with Q-pcr. Q-pcr analysis of Fli, Flt4 and Tbx6 indicates that after FACS sorting different ratios of endothelial and blood cell populations were obtained. The ratio differences could have been the result of a sampling error that presumably overshadows the treatment effect but did not interfere with the detection of transcripts with significantly high fold changes. Optimalization of sampling conditions to obtain samples with constant ratios of cell populations is therefore necessary for this experiment.

Conclusion

The capacity to predict treatment outcomes is highly depending on understanding molecular mechanisms of disease associated cellular processes. Tools to manipulate activity, or study expression and activity of one particular protein allow researchers to draw a precise map of the molecular events. Giving the existence of multiple MAPK variants and the divers cellular processes that are regulated, extensive input and different approaches of research is necessary to tackle the yet unknown facts regarding this protein family. How is MAPK signalling translated into different cellular behaviours such as proliferation, differentiation and migration? What are the mechanisms that control MAPK signalling localization, duration and amplitude in such a way to favour and sustain a particular cellular state. Finally how does deregulation of these mechanisms lead to disease.

This thesis was produced within the scope of the research interest of the Molecular Cell Biology department of Leiden University in the Netherlands which has a background investigating the MAPK pathway in development and cancer using the zebrafish. With the current study we provided new tools to mimic uncontrolled ERK2 activity found in tumours and to investigate the exact role of this MAPK family member in cell transformation. We have completed cloning and expression analysis of the P38 MAPK subfamily in zebrafish and provided a basis for research on isoform specific functions. Both P38α and ERK2 are essential for zebrafish epiboly and by comparing the transcriptome profiles of knock-down embryos we identified epiboly associated genes of which are commonly or specifically regulated by the two MAPK. Finally we have demonstrated that P38α is also functional in migration of endothelial cells during angiogenesis and we identified candidate target genes that are possible involved in this process. Using different approaches we aimed to

make use of the many advantages of the zebrafish and combine the fields of developmental and tumour biology by focusing on the MAPK pathway.

Reference List

1. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene 2007;26:3291-3310.

2. Canagarajah BJ, Khokhlatchev A, Cobb MH, Goldsmith EJ. Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell 1997;90:859-869.

3. Zhang F, Strand A, Robbins D, Cobb MH, Goldsmith EJ. Atomic structure of the MAP kinase ERK2 at 2.3 A resolution. Nature 1994;367:704-711.

4. Brunner D, Oellers N, Szabad J et al. A gain-of-function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell 1994;76:875-888.

5. Emrick MA, Hoofnagle AN, Miller AS, Ten Eyck LF, Ahn NG. Constitutive activation of extracellular signal-regulated kinase 2 by synergistic point mutations. J.Biol Chem. 2001;276:46469-46479.

6. Lefloch R, Pouyssegur J, Lenormand P. Single and combined silencing of ERK1 and ERK2 reveals their positive contribution to growth signaling depending on their expression levels. Mol.Cell Biol 2008;28:511-527.

7. Vantaggiato C, Formentini I, Bondanza A et al. ERK1 and ERK2 mitogen-activated protein kinases affect Ras-dependent cell signaling differentially. J.Biol 2006;5:14.

8. Harrington HA, Komorowski M, Beguerisse-Diaz M, Ratto GM, Stumpf MP. Mathematical modeling reveals the functional implications of the different nuclear shuttling rates of Erk1 and Erk2. Phys.Biol 2012;9:036001.

9. Shankaran H, Ippolito DL, Chrisler WB et al. Rapid and sustained nuclear-cytoplasmic ERK oscillations induced by epidermal growth factor. Mol.Syst.Biol 2009;5:332.

10. Kuida K, Boucher DM. Functions of MAP kinases: insights from gene-targeting studies. J.Biochem. 2004;135:653-656.

11. Mudgett JS, Ding J, Guh-Siesel L et al. Essential role for p38alpha mitogen-activated protein kinase in placental angiogenesis. Proc.Natl.Acad.Sci.U.S.A 2000;97:10454-10459.

12. Aouadi M, Binetruy B, Caron L, Le Marchand-Brustel Y, Bost F. Role of MAPKs in development and differentiation: lessons from knockout mice. Biochimie 2006;88:1091-1098.

13. Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem.J. 2010;429:403-417.

14. Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev. 2012;92:689-737.

15. Tourian L, Jr., Zhao H, Srikant CB. p38alpha, but not p38beta, inhibits the phosphorylation and presence of c-FLIPS in DISC to potentiate Fas-mediated caspase-8 activation and type I apoptotic signaling. J.Cell Sci. 2004;117:6459-6471.

16. Askari N, Diskin R, Avitzour M et al. Hyperactive variants of p38alpha induce, whereas hyperactive variants of p38gamma suppress, activating protein 1-mediated transcription. J.Biol Chem. 2007;282:91-99.

17. Avitzour M, Diskin R, Raboy B et al. Intrinsically active variants of all human p38 isoforms. FEBS J. 2007;274:963-975.

18. Maruyama M, Sudo T, Kasuya Y et al. Immunolocalization of p38 MAP kinase in mouse brain. Brain Res. 2000;887:350-358.

19. Guan Z, Kim JH, Lomvardas S et al. p38 MAP kinase mediates both short-term and long-term synaptic depression in aplysia. J.Neurosci. 2003;23:7317-7325.

20. Aouadi M, Bost F, Caron L et al. p38 mitogen-activated protein kinase activity commits embryonic stem cells to either neurogenesis or cardiomyogenesis. Stem Cells 2006;24:1399-1406.

21. Krens SF, Spaink HP, Snaar-Jagalska BE. Functions of the MAPK family in vertebrate-development. FEBS Lett. 2006;580:4984-4990.

22. Samuels IS, Saitta SC, Landreth GE. MAP'ing CNS development and cognition: an ERKsome process. Neuron 2009;61:160-167.

23. Keren A, Bengal E, Frank D. p38 MAP kinase regulates the expression of XMyf5 and affects distinct myogenic programs during Xenopus development. Dev.Biol 2005;288:73-86.

24. Hui L, Bakiri L, Mairhorfer A et al. p38alpha suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. Nat.Genet. 2007;39:741-749.

25. Hui L, Bakiri L, Stepniak E, Wagner EF. p38alpha: a suppressor of cell proliferation and tumorigenesis. Cell Cycle 2007;6:2429-2433.