

Functions of MAP Kinases: Insights from Gene-Targeting Studies

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Received March 8, 2004; accepted March 8, 2004

Mitogen-activated protein kinases (MAPKs) comprise a family of well-conserved serine/threonine kinases that control a vast array of physiological functions in a number of organisms ranging from yeast to mammals. Recently gene-targeting experiments have shed light on *in vivo* functions of MAPKs. In particular, embryos deficient in extracellular signal-regulated kinase (ERK) 2 lack mesoderm differentiation and placental angiogenesis. Knockout mice for c-Jun amino-terminal kinases have revealed roles for these kinases in neural apoptosis and activation/differentiation of T cells. Deletion of p38 α MAPK results in angiogenic defects in the placenta and peripheral vessels. ERK5-deficient embryos are embryonic lethal due to defects in angiogenesis and cardiovascular development. Although these results have provided new insights for MAPK research, development and analysis of conditional knockout mice are required in order to investigate roles of MAPKs, especially, in other biological processes such as disease pathogenesis.

Key words: ERK, gene targeting, JNK, MAPK, p38.

Mitogen-activated protein kinases (MAPKs) are a family of conserved protein kinases that phosphorylate specific serine and threonine residues of target protein substrates and regulate a number of cellular activities including gene expression, mitosis, cell movement, metabolism, cell survival and apoptosis. MAPKs are one part of a three-tiered cascade composed of a MAPK kinase (MAPKK, MKK, or MEK) and a MAPKK kinase (MAPKKK or MEKK). In mammals, at least four distinct groups of MAPKs have been recognized. Two extracellular signal-regulated kinases (ERK1/2) are phosphorylated by specific MAPKKs, MKK1 and MKK2. MKK1/2 are known to be activated by upstream MAPKKKs such as RAF proteins whose functions are regulated by many growth factors and the proto-oncogene RAS. In response to stress stimuli, three JUN-amino-terminal kinases (JNK1/2/3) and four p38 protein kinases (p38 α / β / γ / δ) are phosphorylated by MKK4/7 and MKK3/6, respectively. A number of MEKKs for JNKs and p38 protein kinases have been identified, some of which activate both JNK and p38 cascades (1). In addition, mixed-lineage kinases (MLKs) have been shown to activate both cascades (2). A fourth MAPK, ERK5, is exclusively activated by MEK5, which can be phosphorylated by two MAPKKKs, MEKK2 and MEKK3. Additional protein kinases that have sequence homology to MAPKs have been identified through genome-based approaches. However, whether they constitute any distinct MAPK cascade remains to be elucidated (3). Although numerous *in vitro* studies have shed light on physiological roles of MAPKs and their signaling pathways, gene-targeting technology has contributed to our knowledge of MAPK signaling cascades *in vivo*. In particular, analysis of knockouts of MAPKs, activators of MAPKs, and substrates of MAPKs have revealed surpris-

ing and intriguing results regarding cell and development-specific functions of MAPK signaling cascades.

ERK1/2: ERK1 and ERK2 are widely expressed and involved in the regulation of proliferation and differentiation of post-mitotic cells. In *Xenopus* embryos, modulation of ERK activity affects mesoderm differentiation, *i.e.*, inhibition of ERK activation prevents animal caps from differentiating into mesodermal tissues (4–6). In order to elucidate the physiological function of ERK2, we generated mice deficient in ERK2 (7). ERK2 knockout embryos are not able to induce mesoderm. Incorporation of BrdU at normal levels and increased TUNEL-positive cells indicate that this defect is due to a problem in differentiation rather than in proliferation of ERK2-deficient embryonic cells. Analysis of embryonic stem (ES) cells deficient in ERK2 and treatment of wild-type (WT) ES cells with a specific MEK inhibitor have also revealed an indispensable role of the ERK pathway in mesoderm differentiation and ERK-independent proliferation of early embryonic cells. Additional lines of ERK2 knockout mice have been generated and exhibited embryonic lethality due to abnormal placenta development (8). The placenta defect can be rescued by tetraploid-aggregation, suggesting that the embryonic lethality of these ERK2-deficient mouse lines is secondary to abnormal development of the placenta. Although the reason for this difference between our line and the others is unclear, it is possible that differences in ES cell lines and mouse lines used for mating affect the outcome of the gene deletion; genetic modifiers may exist. As such, the BALB/C background we used may not allow embryos to have compensatory activation of ERK1, whereas ERK1 can compensate in a C57BL/6 background. MEK1 knockout embryos also show a defect in the placenta, whereas MEK2-deficient mice are viable and fertile with no obvious phenotype (9, 10). Taken together, these results illustrate indispensable roles of ERK2 and MEK1 in embryonic development. In contrast, ERK1 knockout mice are viable and have a minor defect

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Table 1. Summary of MAPK knockout phenotypes.

Genes	Summary of phenotypes
ERK1	Decreased T cell responses in the thymus (11)
ERK2	Lack of mesoderm differentiation (7) Defects in the placenta (8)
JNK1	Defects in inhibiting Th2 differentiation (21) Defects in T cell activation and apoptosis of thymocytes (18) Less susceptible to insulin resistance in diabetes models (23)
JNK2	Defects in Th1 differentiation (22) Defects in T cell activation and apoptosis of thymocytes (19)
JNK1+JNK2	Defects in neural tube closure (14, 15) Increased proliferation and IL-2 production in T cells (20) Resistance to UV-induced apoptosis in embryonic fibroblasts (16)
JNK3	Resistance to kainate-induced neural damage (24)
p38 α	Defects in placental angiogenesis (28, 29) Defects in Epo production (30)
p38 β	No obvious phenotype (KK & DB, unpublished)
p38 γ	No obvious phenotype (KK & DB, unpublished)
ERK5	Defects in angiogenesis and cardiovascular development (37–39)

in T cell development and exhibit reduced activation by anti-CD3 antibody and/or PMA (11). Although ERK2 cannot compensate for the defect in T cells, ERK1-deficient mice show a stimulus-dependent increase of ERK2 activity resulting in hypersensitivity of these mutant mice to the rewarding properties of morphine (12). Given no differences in substrate specificity between ERK1 and ERK2 and ubiquitous expression of both isoforms in multiple tissues, preferential or compensatory activation of either ERK could be explained by the existence of specific scaffold proteins, such as MP-1, that only bind to ERK1 and MEK1 (13).

JNKs: Although knockout mice deficient in any of the individual JNK isoforms can develop normally, compound mutations of JNK1 and JNK2 result in embryonic lethality at mid-gestation due to defects in neural tube closure (14, 15). In addition, embryonic fibroblasts deficient in both JNK1 and JNK2 are resistant to stress-induced apoptosis (16). These data may suggest a role of JNK1 and JNK2 in regulating apoptosis due to environmental stress. Given JNK has been implicated in T cell activation and cytokine production, several studies on T cell functions have been conducted with JNK as well as MKK4 and MKK7 gene-targeted mice. Proliferation and IL-2 production after CD28 co-stimulation are decreased in MKK4-deficient and JNK1/JNK2 double heterozygous T cells, both of which have partially reduced JNK activity (17, 18). Under some conditions, either JNK1- or JNK2-deficiency reduces activation of peripheral T cells and apoptosis of immature thymocytes (18, 19). In contrast, T cells generated from ES cells deficient in both JNK1 and JNK2 or from JNK2-deficient mice expressing a dominant negative form of JNK1 exhibit slightly increased production of IL-2 and proliferation indicating that JNK1 and JNK2 negatively regulate IL-2 production (20). MKK7-deficient T cells with profoundly low JNK activity also show a similar abnormality with increased proliferative responses upon treatment with anti-CD3 antibody in the presence or absence of anti-CD28 antibody (20). Although these results suggest that JNK activity may work as a threshold switch in T cell activation, a physiological role of JNK activity in T cell activation remains elusive. On the contrary, further studies on those knock-

out mice have revealed a significant role in induction of effector T cells, *i.e.*, T helper type-1 (Th1) and type-2 (Th2) cells. Mice deficient in JNK1 exhibit a skewed Th2 response (21). In particular, CD4 T cells lacking JNK1 can produce Th2 cytokines in the absence of CD28 co-stimulation and differentiate into Th2 cells under the condition where WT CD4 T cells become mostly Th1 cells. In Th1 cells, JNK2 accounts for most of the total JNK activity. T cells from JNK2 knockout mice exhibit impaired Th1 responses represented by decreased production of IFN γ , while Th2 responses appear to be normal (22). Thus, JNK1 and JNK2 may play non-redundant roles in induction of specific T helper cell types. The JNK signaling pathway is also activated by inflammatory cytokines and free fatty acids (FFAs) that have been implicated in the etiology of obesity-induced insulin resistance in diabetes. As such, obesity is associated with elevated JNK activity predominantly mediated by JNK1. JNK1 knockout mice are substantially less susceptible to obesity-induced insulin resistance when fed a high fat diet or crossed with ob/ob mice, whereas JNK2 knockout mice fed a high fat diet develop diabetes. Biochemical analysis of liver tissues from JNK1 knockout mice indicates that TNF α or FFA-induced phosphorylation of insulin receptor substrate (IRS)-1 at Ser 307 is mediated by JNK1 (23). Taken together, these results indicate that this modification of IRS-1 by JNK1 is, in part, responsible for insulin resistance induced by obesity and JNK1 could be a therapeutic target for insulin-independent diabetes in humans. In contrast to JNK1 and JNK2, JNK3 is mainly expressed in the nervous system. JNK3-deficient mice are resistant to kainate-induced killing of neurons in the hippocampus (24). This phenotype has been recapitulated by mice with mutations in two phosphorylation sites (Ser-63 and Ser-73) of c-Jun (25). c-Jun knockout mice, however, are embryonic lethal due to abnormal liver formation and hemorrhage (26), a phenotype that has been documented for MKK4 knockout mice (27). Although c-Jun appears to be the essential substrate for JNK3, MKK4 may regulate protein expression of c-Jun as well as phosphorylation of those two sites through activation of JNK1 and JNK2.

P38: Of the four isoforms, p38 α is the best characterized and is expressed in most cell types. Identified as the molecular target of anti-inflammatory compounds, p38 regulates expression of many cytokines and is activated by stress, hormones and inflammatory cytokines. p38 α -deficient mice are embryonic lethal mainly due to a defect in placental angiogenesis (28, 29). In a mixed background between 129 and C57BL/6, some mice survive up to embryonic day (E) 16 and exhibit anemia due to decreased erythropoietin (Epo) production (30). Given that erythropoiesis is induced by hypoxia, hypoxic stress may activate p38 α to stabilize Epo mRNA, as shown in human hepatoma cells. However, this abnormality does not seem to appear when the placental defect is rescued with normal extra-embryonic tissues (tetraploid rescue) (29). In contrast to p38 α -deficient mice, neither p38 β nor p38 γ knockout mice show any obvious phenotype and can live a normal life span without any overt pathological change (Kuida, K. and Boucher, D.M., unpublished observation). These results may suggest a unique role of p38 α in placental angiogenesis, and p38 β and p38 γ may be dispensable for physiological functions in mice. p38 kinases are known to be activated by two MAPKKs, MKK3 and MKK6. Mice deficient in MKK3 are viable. However, macrophages from these mutant mice are defective in IL-12 production, resulting in decreased Th1 type responses associated with reduced p38 kinase activity (31). In addition, embryonic fibroblasts from MKK3-deficient mice do not produce inflammatory cytokines upon TNF- α stimulation whereas other stress stimuli such as UV and osmotic shock can induce p38 kinase activity in MKK3 mutant fibroblasts (32). In T cells, the deletion of MKK3 results in a slight reduction in activation-induced cell death, presumably due to lack of augmented p38 kinase activity by activation of T cell receptors (TCRs) (33). MKK6 knockout mice are also viable and develop normally with decreased p38 kinase activity in thymocytes. As a result, thymocytes from the mutant mice are resistant to apoptosis induced by activation of TCRs. Conversely, peripheral T cells are not affected by the deletion of MKK6, indicating differential regulation of p38 kinases by MKK3 and MKK6 in functions of T cells (33).

ERK5: ERK5 constitutes the fourth MAPK pathway and can be activated by serum, growth factors and extracellular stresses such as osmotic shock and oxidative stress. ERK5 has a catalytic domain homologous to ERK1/2 with a unique carboxy terminal domain which can interact with a class of transcription factors termed myocyte enhancer factors 2 (MEF2) (34, 35). In addition, biochemical studies have revealed a linear activation pathway of ERK5 mediated by MEK5 and its activator MEKK3 (36). Mice deficient in ERK5 die around E10 due to defects in angiogenesis and cardiovascular development (37–39). The angiogenic defects are prominent in the placenta, yolk sac and brain, where complexity and branching of blood vessels are compromised. In the hearts of knockout embryos, the myocardial walls are underdeveloped and trabeculae are disorganized. Although our knockout embryos did not undergo rightward looping (37), others indicated that deficiency of ERK5 affects heart development at a later stage (38). Consistent with biochemical studies on the ERK5 pathway, the phenotype of ERK5-deficient embryos resembles

that of knockout embryos of MEKK3 and MEF2C, a substrate of ERK5 (40–42). MEKK3 can also activate p38 α , and both p38 α and ERK5 can phosphorylate MEF2C, but at distinct sites. In addition, the phenotype of p38 α knockout mice is similar to that of ERK5 regarding defects in angiogenesis. Lack of heart defects in p38 α knockout embryos, however, suggests that ERK5 is able to modulate MEF2C differently than p38 α and thus play a role in cardiovascular development. Alternatively, ERK5 may regulate a distinct set of substrates during early development. Of note, the vascular defects in ERK5 knockout embryos take place when the embryonic vascular system is being exposed to increases in vessel flow and shear stress. Given that shear stress has been implicated in activation of ERK5 (43), it is plausible that a role of the ERK5 signaling pathway in early cardiovascular development is to sense mechanical stress and induce necessary physiological responses. Taken together, these data from ERK5 knockout mice have revealed novel functions of ERK5.

MAPKs and their signaling pathways are well conserved during evolution from unicellular organisms such as yeast, where a blueprint of the MAPK pathway can be identified to complex, multi-cellular creatures including human. Thus, most of the gene-targeted mice discussed here exhibit embryonic lethal phenotypes. Moreover, given the importance of MAPK pathways in maintaining homeostasis and performing complicated bio-functions, higher organisms have additional isoforms of MAPKs that often compensate for each other as exemplified by JNK1 and JNK2. In order to further investigate roles of MAPKs in disease pathogenesis, for example, it is necessary to generate conditional knockout mice where deletion of genes can be regulated in a tissue and/or developmental stage-specific manner.

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