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Assessing developmental roles of MKK4 and MKK7 in vitro

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In vivo gene knockout studies in mice have revealed essential roles of the mitogen-activated protein kinases (MAPKs) in embryogenesis, but due to early lethality of the knockout embryos, the underlying mechanisms and specific developmental programs regulated by the MAPK pathways have remained largely unknown. In vitro differentiation of mouse embryonic stem cells (ESCs) have opened new possibilities for understanding lineage segregation and gene function in the developmental stages that are not normally accessible in vivo. Building on this technology, in combination with gene knockout cells, we investigated the roles of MKK4 and MKK7, two upstream kinases of the MAPKs, in early lineage specification. Our results show that MKK4 and MKK7 differentially regulate the JNK and p38 MAPKs and make distinct contributions to differentiation programs. In vitro ESC differentiation is a valuable system to investigate the molecular and signaling mechanisms of early embryogenesis.

The MKK4 and MKK7 are Upstream Kinases of the MAPKs

The MAPKs are intracellular enzymes activated by extracellular cues and in turn phosphorylate effector proteins to modulate cellular activity and function. In mammalian system, there are three major MAPK subgroups, including the Jun N-terminal kinase (JNK), the p38s and the extracellular signal regulated kinases (ERK).[1] The MAPKs are activated through phosphorylation on Thr and Tyr residues within the activation loop of the kinase domain. There are six major MAP2Ks responsible for MAPK phosphorylation, but each has selective substrate specificities.[2] In principle, the MEK1 and MEK2 are MAP2Ks upstream of the ERKs, the MKK3 and MKK6 are responsible for p38 activation, while the MKK4 and MKK7 preferentially activate the JNKs, and MKK4 has also been shown to activate the p38s (**Fig. 1**).[3,4] Extracellular and intracellular stimuli, including peptide growth factors, cytokines, hormones, and various cellular stressors such as oxidative stress and endoplasmic reticulum stress, can activate the MAP2K-MAPK cascades, which in turn affect diverse cellular activities, such as proliferation, differentiation, survival and death.[5]

The Roles of MKK4 and MKK7 in Mouse Embryonic Development

Signal transduction pathways are integral components of the developmental network that guides differentiation and cell fate determination. In vivo studies show that MKK4 and MKK7 have essential roles in embryonic development. Genetic ablation of *Mkk4* in mice leads to lethality between embryonic day 10.5 (E10.5) and E12.5, and the knockout embryos display severe anemia, abnormal hepatogenesis and liver cell apoptosis.[6-8] Genetic ablation of *Mkk7* also leads to embryonic lethality, but in this case, the knockout embryos die between E11.5 and E13.5 due to disorganized liver and decreased hepatocyte proliferation.[9] Hence, MKK4 and MKK7 are both essential for embryonic survival, but they make different contributions to the developmental programs, so that MKK4 cannot compensate for loss of MKK7, and vice versa.

At an earlier developmental stage, however, MKK4 and MKK7 display some degree of redundancy for embryonic survival. While neither the *Mkk4(-/-)* nor the *Mkk7(-/-)* embryos die before E9.5, the *Mkk4(-/-)Mkk7(-/-)* double mutant mice do not survive beyond this point.[10] Based on the time of death, it is proposed that MKK4 and MKK7 are required for mammalian body plan organization. Toward this function, MKK4 makes a greater contribution than MKK7, because the *Mkk7(+/+)* and *Mkk7(+/-)* fetuses lacking MKK4 die earlier with more severe defects than the *Mkk4(+/+)* and *Mkk4(+/-)* fetuses lacking MKK7. The in vivo data together suggest that MKK4 and MKK7 have distinct and redundant roles in development, but the underlying mechanisms have remained largely unknown.

The Mechanisms of MKK4 and MKK7 in Embryogenesis

The distinct roles of MKK4 and MKK7 in embryogenesis may be attributed in part to their unique tissue distribution.[11] The MKK7 is ubiquitously expressed in the developing and adult tissues, with

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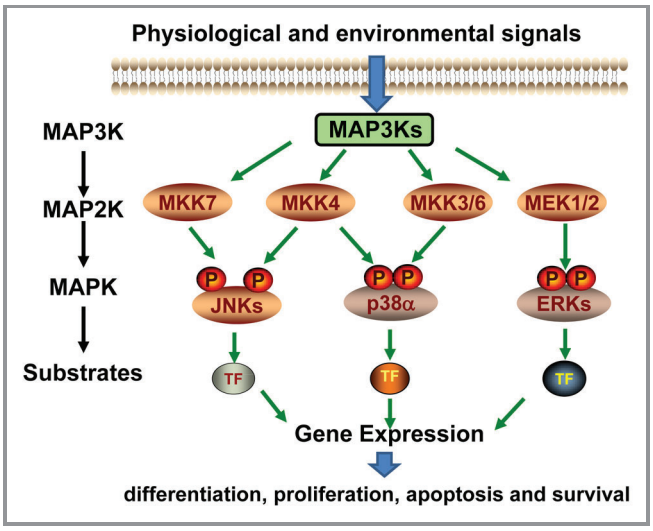

Figure 1. Schematic diagram of the MAPK signaling pathways.

a gradual increased expression in selective sites, such as hair follicle, skin and brain at a later stage of embryogenesis.[12] The MKK4, on the other hand, displays a dynamic temporal spatial expression in embryogenesis. Specifically, strong MKK4 expression is detected in the central nervous system, liver and thymus at early stages of development. While expression in fetal liver and thymus gradually decreases as embryogenesis proceeds, expression in nervous system increases over time throughout postnatal development and remains at a stable level in adult brain.[13] The expression pattern provides anatomical basis for specific roles of MKK4 in hepatogenesis and neurogenesis during development and in adult brain. Specific *Mkk4* gene ablation in the neuronal lineage, for example, causes severe neurological defects and premature death due to misalignment of the Purkinje cells in the cerebellum and radial migration in the cerebral cortex.[14]

The biological activities of MKK4 and MKK7 are likely mediated by their downstream MAPKs, specifically, JNK and p38[2,15-20] MKK4 and MKK7 share 55% amino acid identity within the kinase domains, but they have quite different properties in JNK and p38 phosphorylation. First, MKK4 phosphorylates and activates the p38 MAPKs, but MKK7 does not. Second, while MKK4 and MKK7 both phosphorylate JNK, MKK4 preferentially phosphorylates the Tyr, whereas, MKK7 phosphorylates the Thr at the Thr-X-Tyr site. As the consequence, MKK4 and MKK7 each makes distinct contribution, while both are required for optimal JNK activity.[21] Hence, MKK4 and MKK7 may exert distinct effects on embryogenesis through differential activation of JNK and p38.

The mammalian systems have three *Jnk* genes, of which *Jnk1* and *Jnk2* are expressed ubiquitously, whereas, *Jnk3* is expressed specifically in cells of the neuronal lineages.[5,22-24] Neither *Jnk1* nor *Jnk2* knockout perturbs fetal development; however, knocking out both results in death of the embryos at E11 due to defective neural tube formation and regional reduced apoptosis.[25,26] The p38 family has four members, namely, p38α, p38β, p38γ and p38δ. Among these members, only p38α.has displayed an essential role in embryogenesis.[27-29] Knockout p38α in mice causes embryonic lethality at E10.5 due to abnormal placenta development and erythropoiesis.[30-33] Since none of the MAPK knockout phenotypes resembles that of *Mkk4(-/-)* or *Mkk7(-/-)*, MKK4 and MKK7 must regulate embryogenesis through complex signaling mechanisms, not simply due to activation of JNK or p38.

In Vitro Embryonic Stem Cells (ESCs) as Surrogates to Understand the Mechanisms of Development

Embryonic development starts with the fertilization of the ovum, followed by rapid mitotic division and differentiation of embryonic stem cells (ESCs) in the inner cell mass. The ESCs first commit to ectoderm, mesoderm and endoderm lineages, followed by more restricted differentiation toward specific fate.[34] Ultimately, these processes lead to the generation of over 200 different mammalian cell types that are organized into tissues to provide all the functions required for viability and reproduction.

The ESCs captured from the inner cell mass of pre-implantation embryos can be expanded in vitro for extended periods of time.[35] These cells are able to self-renewal and maintain pluripotency in the presence of leukemia inhibitory factors (LIF), but will differentiate in the absence of LIF, giving rise to a broad spectrum of lineages.[36,37] The most common in vitro differentiation protocol uses the "hanging drop," in which the ESCs are forced to aggregate to form embryoid bodies (EBs), mimicking the inner cell mass of the developing embryos (**Fig. 2**).[38-40] The EBs can continue to grow and differentiate spontaneously over

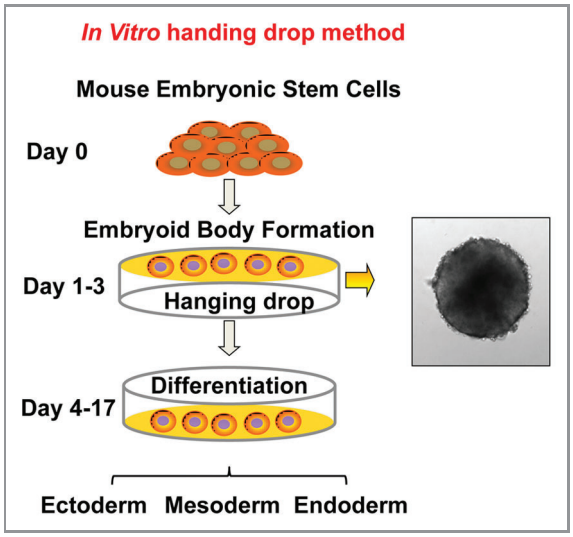

Figure 2. Schematic diagrams of embryonic stem cell differentiation. (A) Diagrammatic illustration of the early stage of fetal development and the in vivo origin of embryonic stem cells. (B) In vitro ESC differentiation using the "hanging drop" protocol. Differentiation is initiated when the cells are grown in the absence of anti-differentiation factors, such as LIF and fibroblast feeder cells. Approximately 500 cells/20 μl were applied to the lids of tissue culture plate, as hanging drops, to force the cells aggregate to form embryoid bodies (EBs). The EBs can further differentiate in vitro, leading to the generation of cell types of ectoderm, endoderm and mesoderm lineages.

time to generate a wide variety of cell types. By tracing lineage- and cell type-specific gene expression, we found that despite the potential of ESCs to produce all cell types, the EB culture produced most abundantly cells of mesodermal lineages, with limited commitment to endodermal and ectodermal lineages (**Fig. 3**). To extend the application of this system, a number of modified protocols have been developed to direct ESC differentiation along the endodermal and ectodermal lineages, so that the in vitro system can be used to generate a wide range of cell types, such as neuron, epithelium and endothelium.[41-47]

Although the spatial and temporal orchestration of early embryogenesis is missing in cell culture systems, many of the regulatory machineries affecting embryonic development also regulate ESC differentiation in vitro. In contrast to the in vivo studies limited in their ability to attain clear mechanistic insight into the effects of exogenous factors, the in vitro differentiation affords more controlled methods to present morphogens and environmental cues and directly assess differentiated cell phenotypes. An additional advantage is that the in vitro system can be coupled with genetic knockout to study gene functions in early developmental stages that are otherwise not accessible in vivo. For these reasons, the ESCs have emerged as a unique in vitro experimental system for the investigation of basic principles of mammalian cell differentiation and development.

Investigation of the Developmental Roles of MAPKs and MAP2Ks In Vitro

In vitro studies lead to the findings that the MAPKs are important for survival and apoptosis of ESCs in response to various extracellular signals, similar to their well-established functions in stress responses in other cells.[48-54] It is also shown that each

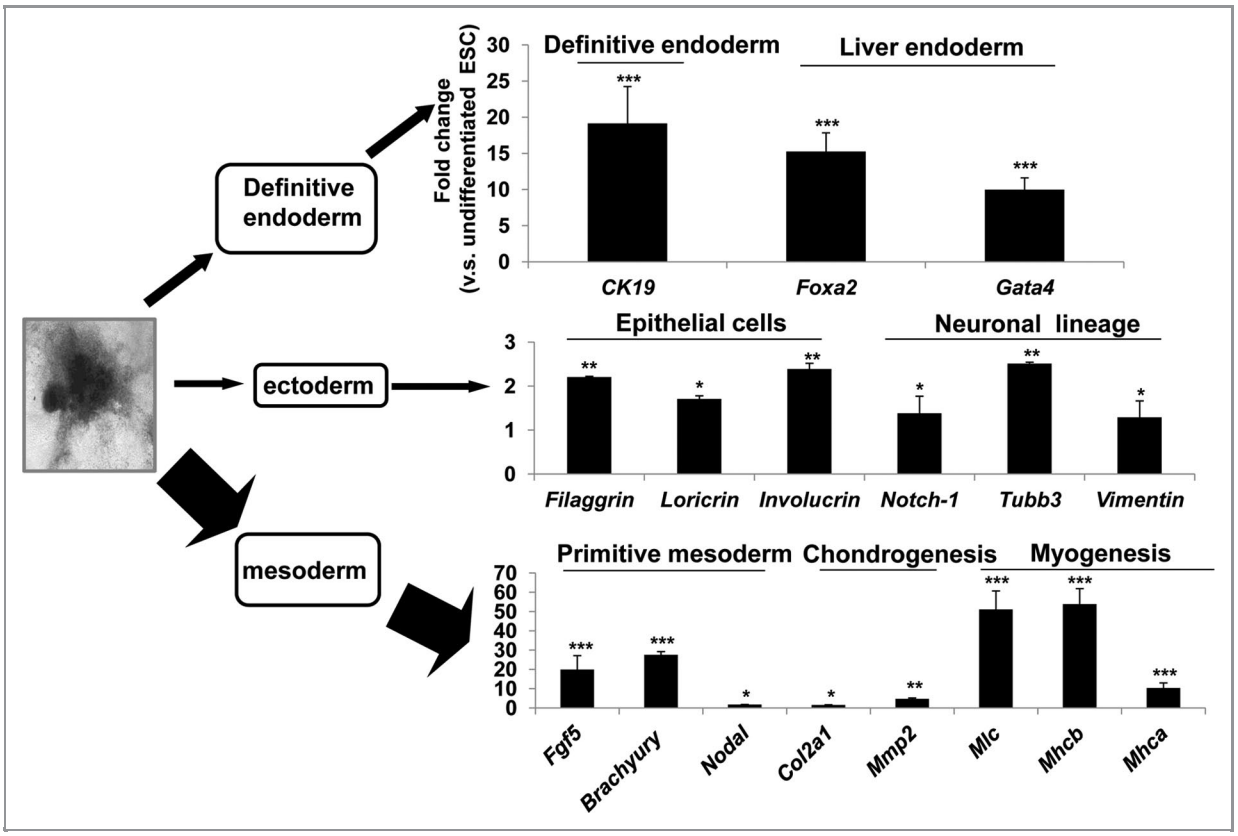

Figure 3. The differentiation profiles of EB. Wild type mouse ESCs were subjected to in vitro differentiation, following the "hanging drop" protocol and the EBs were transferred to a 10 cm^2 bacterial plate at approximately 100 EBs per plate. Under this condition, the ESCs spontaneously differentiate into mesoderm, ectoderm and endoderm lineages. RNA was isolated at day 13 of differentiation and lineage specific markers were examined by real time RT-PCR. The most abundantly expressed genes in the EB culture were those for the mesodermal lineage, including genes specific for primitive mesoderm, *Fibroblast growth factor 5* (*Fgf5*), *Brachyury* and *Nodal*, for chondrocytes, *Collagen 2a2* (*Col2a2*) and *Metalloproteinases 2* (*Mmp2*), and for myocytes, *Myosin light chain* (*Mlc*) and *Myosin heavy chain a* (*Mhca*) and *Mhcb*. Less abundantly expressed were genes for the endodermal lineage, including markers for definitive endoderm *Cytokeratin 19* (*Ck19*) and liver endoderm, such as *Forkhead box A2* (*Foxa2*) and *GATA binding protein 4* (*Gata4*). The least expressed were genes for the ectodermal lineage, including markers for epidermis, such as *Fillagrin*, *Loricrin* and *Involucrin*, for neuronal lineages, such as *Notch-1*, *Tubulin β 3* (Tubb3) and *Vimentin*. The sequences of forward primers used in PCR are: *Gapdh*, 5′-AACGACCCCTTC ATTGACC-3′, *Ck19*, 5′-TCAATGATCGTCTCGCCTCCTACT-3′, *Foxa2*, 5′-AAGTATGCTGG GAGCCGGAAGAT-3′, *Gata4*, 5′-CTG TCA TCT CAC TAT GGG CAC-3′, *Filaggrin*, 5′-GAAACG-ATATACCTG GAGATGC3′, *Loricrin*, 5′TGAGGAGACACTAGAATTGGG3′, *Involucrin*, 5′-GAGAAGCAGCATCAGAAGCC-3′, *Notch-1*, 5′-TCTGCTTATGCCTCAAGG-GAACCA-3′, *Tubb3*, 5′-TTCTGGTGGACTTGGAACCTG GAA-3′, *Vimentin*, 5′-CTTTACTCAACTTTCCAGAGCC-3′, *Fgf5*, 5′-AGAGTGGGCATCGGTTTC-3′, *Brachyury*, 5′-TGCTGCAGTCCCATGATAACTGGT-3′, *Nodal* 5′-CCAACCATGCCTACATCCAG-3′, *Col2a1*, 5′-ATCTGCACTGAATGGCTGACCTGA-3′, *Mmp2*, 5′- AACCAGCCTTCT-CCTTCAC-3′, *Mlc*, 5′- CTCCAAGAACAAGGACACTG-3′, *Mhca*, 5′-CTGCTGGAGAGGTTATTCCTC G-3′, *Mhcb*, 5′-TGCAAAGGCTCCAGGTCTGAGGGC-3′

MAPK plays unique roles in differentiation and lineage specification. The JNKs, for example, are most important for mesodermal differentiation and less important for ectodermal and endodermal differentiation.[55] In addition, while JNK is required for induction of neuronal lineage, but inhibition of epithelial lineage by retinoic acid, a function likely mediated through suppression of the Wnt-4/Wnt-6 and BMP signaling pathways,[56-58] it can also be activated by Wnt, leading to accelerated cardiac myocyte differentiation.[59] The p38, on the other hand, is essential for commitment of mouse ESCs into mesodermal lineages that lead to cardiac, endothelial, smooth muscle, and skeletal muscle differentiation, but inhibits early neural differentiation.[60-65] In contrast to p38, which is dispensable for epithelial differentiation,[66] the ERKs are crucial for epithelial differentiation, in addition to neuronal differentiation.[67,68]

Relatively little is known about the roles MKK4 and MKK7 play in ESC differentiation. Available in vitro data suggest that neither MKK4 nor MKK7 is essential for differentiation of ESC to mast cells[69] and that MKK4 is not required for hepatic maturation at the late stage of liver development.[70] We have recently investigated the signaling and functional properties of MKK4 and MKK7 in differentiation using single or combined gene knockout ESCs (**Fig. 4**).[71] Our results show that although MKK4 and MKK7 are dispensable for embryonic stem cell self-renewal and maintenance of pluripotency; they have complementary roles in supporting the survival of differentiated cells, likely through synergistic activation of the JNK-c-Jun cascades. We also show that MKK4, but not MKK7, is essential for activation of p38, leading to ATF2 activation and cardiomyocyte differentiation. This set of in vitro data has illustrated a MKK4-p38 cascade in cardiogenesis and a MKK4/MKK7-JNK cascade in cell survival during differentiation. Hence, MKK4 and MKK7 can differentially regulate the downstream MAPKs and make distinct contributions to the differentiation programs.

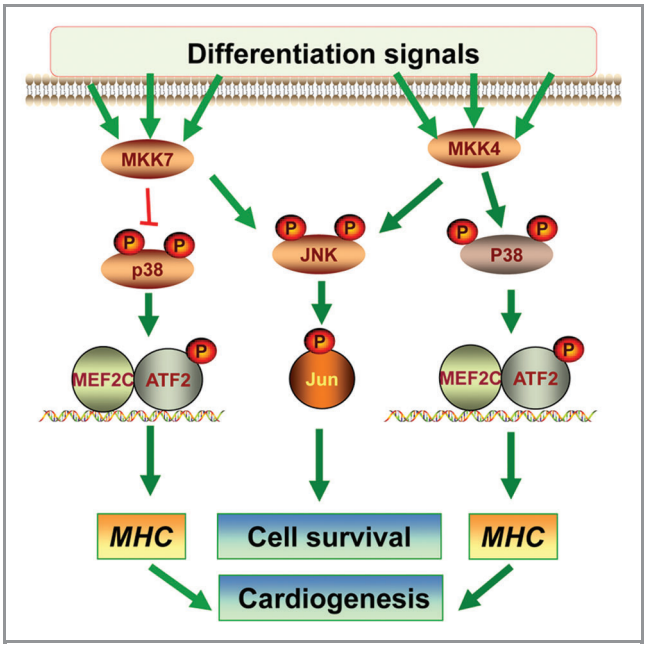

Figure 4. The roles of MKK4 and MKK7 in differentiation. A Schematic diagram summarizes the roles of MKK4 and MKK7 in vitro ESC differentiation. The MKK4 and MKK7 have complementary roles in activation of the JNK-c-Jun cascades that may be required for differentiated cell survival. Hence, loss of both MKK4 and MKK7 leads to senescence of the differentiated cells. On the other hand, MKK4 is essential for activation of the p38-ATF2/MEF2C cascades, while MKK7 may attenuate p38 activation. Consequently, the *Mkk4(-/-) ESCs* are defective in myosin heavy chain (MHC) induction and cardiomyocyte differentiation, but *Mkk7(-/-)* ESCs have enhanced MHC expression and cardiogenesis.

Conclusion and Perspectives

The in vitro ESC culture system, originally developed as a potential technique for cell replacement therapy, has become a powerful tool to understand the basic principles of development. This system offers a relatively inexpensive and easily manipulatable platform to identify extrinsic signals and intracellular factors that control the differentiation programs. Over the past few years, in vitro studies have considerably advanced our understanding of the MAPK signaling mechanisms in lineage specification; however, different experimental settings sometimes lead to opposite conclusions, partly due to variable effects of the MAPK inhibitors used in each given system.[72] One approach to overcome this problem is to take advantage of gene knockout mouse ESCs. Using this approach, we are able to obtain a clearer view of the signaling mechanisms and functions of MKK4 and MKK7 in lineage commitment. A major challenge of the in vitro system is the heterogeneity of the differentiated cell populations that not only complicate directed differentiation and propagation, but also make it difficult to assess the differentiation outcomes. Genetically manipulate ESCs have recently been developed to express green fluorescence protein (GFP) and/or selection markers under the control of a lineage-specific gene promoter. This will allow the specific cell types to be traced and purified during differentiation.[73] Incorporation of emerging new technologies holds great promises to extend the application of the in vitro system. For example, a recent study shows that chromatin immunoprecipitation and sequencing (ChiP-seq) technique has been applied to the in vitro ESC system, leading to discovery of a novel chromatin-modifying function of JNK in histone H3 Ser10 (H3S10) phosphorylation during neuronal differentiation.[74] While the in vitro findings still need to be validated in vivo, the rapid evolution of the in vitro system will undoubtedly make it extremely useful to illuminate the complex gene-gene and gene-environmental interaction mechanisms underlying development and diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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