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Deciphering gene expression program of MAP3K1 in mouse eyelid morphogenesis

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ABSTRACT

Embryonic eyelid closure involves forward movement and ultimate fusion of the upper and lower eyelids, an essential step of mammalian ocular surface development. Although its underlying mechanism of action is not fully understood, a functional mitogen-activated protein kinase kinase kinase 1 (MAP3K1) is required for eyelid closure. Here we investigate the molecular signatures of MAP3K1 in eyelid morphogenesis. At mouse gestational day E15.5, the developmental stage immediately prior to eyelid closure, MAP3K1 expression is predominant in the eyelid leading edge (LE) and the inner eyelid (IE) epithelium. We used laser capture microdissection (LCM) to obtain highly enriched LE and IE cells from wild type and MAP3K1-deficient fetuses and analyzed genome-wide expression profiles. The gene expression data led to the identification of three distinct developmental features of MAP3K1. First, MAP3K1 modulated Wnt and Sonic hedgehog signals, actin reorganization, and proliferation only in LE but not in IE epithelium, illustrating the temporal-spatial specificity of MAP3K1 in embryogenesis. Second, MAP3K1 potentiated AP-2α expression and SRF and AP-1 activity, but its target genes were enriched for binding motifs of AP-2α and SRF, and not AP-1, suggesting the existence of novel MAP3K1-AP-2α/SRF modules in gene regulation. Third, MAP3K1 displayed variable effects on expression of lineage specific genes in the LE and IE epithelium, revealing potential roles of MAP3K1 in differentiation and lineage specification. Using LCM and expression array, our studies have uncovered novel molecular signatures of MAP3K1 in embryonic eyelid closure.

Introduction

MAP3K1, also known as MEK kinase 1 (MEKK1), is a serine/threonine protein kinase of the MAP3K superfamily (Davis, 1995). This family consists of at least 21 members, responsible for activation of the MAP2K–MAPK cascades and regulation of a wide variety of biological functions (Craig et al., 2008). *In vitro* studies show that MAP3K1 specifically activates the MAP2K4 and MAP2K7 kinases, which in turn phosphorylate the c-Jun N-terminal kinases (JNKs) and/or p38 MAPKs, whereas in vivo studies have shown that MAP3K1 has diverse roles in the biological systems (Xia et al., 1998; Yan et al., 1994; Yujiri et al., 1998). The *in vivo* data collectively show that MAP3K1 regulates T and B cell-mediated immune responses, erythropoiesis and cardiogenesis; in addition, MAP3K1 plays a pivotal role in ocular surface morphogenesis during embryonic development (Gallagher et al., 2007;Gao et al., 2004;Geh et al., 2011;Labuda et al., 2006; Minamino et al., 2002; Venuprasad et al., 2006).

Mammalian ocular surface development involves the transient closure and re-opening of the eyelid (Findlater et al., 1993). In mice, eyelid development begins at embryonic day 12–13 (E12–13), when the surface ectoderm adjacent to the developing cornea folds to form the lid buds. The eyelid at this stage is a simple structure consisting of surface periderm covering undifferentiated mesenchyme. From E13 onward, the eyelid grows toward the center of the ocular surface. Between E15.5 and E16, a massive morphological change occurs at the junction of the outer and inner eyelid epithelium, causing the eyelid tip cells to elongate and migrate, leading to ultimate fusion of the upper and lower eyelids. Mouse eyelid remains closed between E16.5 and postnatal day 12–14, during which time the outer layer epithelium differentiates towards epidermis, the inner layer becomes conjunctiva, and the underlying mesenchyme derived from the migrating neural crest cells begins to form muscle, tarsus and other connective tissues. Eyelid re-opening takes place two weeks after birth, when cells at the fusion junction undergo

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apoptosis separating the upper and lower eyelids. While mice with normal embryonic eyelid closure have their eyelids closed at birth, mice with defective embryonic eyelid closure exhibit an eye open-at-birth (EOB) phenotype.

In mice, deletion of either the full-length (MAP3K1-null or *Map3k1*$^{-/-}$) or the kinase domain (*Map3k1*$^{\Delta KD/\Delta KD}$) of MAP3K1 results in the EOB phenotype (Yujiri et al., 2000; Zhang et al., 2003). Immunohistochemical analyses show that the *Map3k1*$^{\Delta KD/\Delta KD}$ fetuses have decreased phosphorylation of MAP2K4 and JNK in the eyelid tip epithelial cells (Takatori et al., 2008), whereas genetic studies show that although one functional *Map3k1* allele is sufficient for normal eyelid closure, it becomes haploinsufficient for eyelid closure in *Jnk1*$^{-/-}$ and *Jnk1*$^{+/-}$*Jnk2*$^{+/-}$ mice. These observations suggest that MAP3K1 regulates at least partly embryonic eyelid closure through activation of the MAP2K4–JNK pathways.

c-Jun, a member of the AP-1 group of transcription factors, is a potential downstream effector of this pathway. Phosphorylation of c-Jun by JNK at the N-terminal serine 63 and 73 is an important mechanism of AP-1 activation, because N-terminal phosphorylation promotes c-Jun dissociation from a transcriptional repressor complex, resulting in transcriptional activation of target genes (Aguilera et al., 2011). We showed that c-Jun N-terminal phosphorylation and AP-1 activity was much lower in the eyelid tip epithelial cells of *Map3k1*$^{\Delta KD/\Delta KD}$ compared to wild type fetuses (Geh et al., 2011; Takatori et al., 2008). However, lacking c-Jun phosphorylation cannot be responsible solely for defective eyelid closure. Transgenic mice harboring a mutant c-Jun with serines 63 and 73 replaced by alanines {c-Jun (AA)} completely lack c-Jun N-terminal phosphorylation, but have normal eyelid development (Behrens et al., 1999), suggesting that phosphorylation of c-Jun is dispensable for embryonic eyelid closure. Hence, the MAP3K1–JNK axis must regulate eyelid closure through other downstream effectors whose identities are still unknown.

In the present work, we have used global gene expression analyses to identify regulatory processes downstream of MAP3K1 critical in eyelid development. We found that MAP3K1 was abundantly expressed in the eyelid leading edge (LE) and inner eyelid (IE) epithelium of the developing fetuses. We used Laser Capture Microdissection (LCM) to isolate these cells from wild type and *Map3k1*$^{\Delta KD/\Delta KD}$ fetuses at embryonic day 15.5, a developmental stage immediately prior to eyelid closure. Gene expression signatures in LE and IE cells provide novel insights into the role that MAP3K1 plays in eyelid morphogenesis.

Materials and methods

Experimental animals, cells and reagents

Map3k1$^{+/\Delta KD}$ mice were previously described (Xia et al., 2000; Zhang et al., 2003) and were backcrossed to the C57BL/6 background for seven generations. The fetuses were collected at E15.5 and genotypes were determined by PCR. Experiments conducted with these animals have been approved by the University of Cincinnati Animal Care and Use Committee. Antibody for β-catenin was from Sigma, phospho-H3 was from Millipore, α2-actin and SK myosin were from Thermal Scientific and Abcam, respectively. The HEK293 cells were purchased from American Type Culture Collection (ATCC). The Wnt3a expression vector, TCF/LEF DNA-binding sites-driven luciferase reporter (TCF/LEF-luc) and SRE-luc plasmids were kindly provided by Drs. Xinhua Lin (Cincinnati Children's Hospital Medical Center), Yujin Zhang (University of Cincinnati), and Jinsong Zhang (University of Cincinnati), respectively (Belenkaya et al., 2008; Hu et al., 2011). The AP-1-luc, SBE-luc and the MAP3K1 mammalian expression vector were described before (Zhang et al., 2005). The wild type {MAP3K1(WT)} and kinase-inactive {MAP3K1(KM)} mammalian expression plasmids were described before (Xia et al., 1998).

Tissue and cell preparation, RNA and cDNA generation and microarray

For LCM, the heads of E15.5 fetuses were embedded in Tissue-Tek OCT medium (Sakura Finetek USA) and stored in −80 °C. Eight μm coronal sections were mounted on plain uncoated glass slides, dehydrated and stained with HistoGene LCM frozen section staining kit, and were used for LCM following the manufacturer's protocol (Molecular Devices). Cells from 4 sections were collected on one LCM cap within one hour of collection to avoid RNA degradation. The cells were lysed in 100 μl Trizol (Invitrogen, Carlsbad, CA) at room temperature for 5 min and stored at −80 °C. It was estimated that 50 sections (12 caps) were processed for each fetus, which together generated 2000 cells from the LE and 3000 cells from the IE eyelid epithelium. The lysates from each fetus were pooled and processed as one biological sample.

The lysates were mixed with 0.1% Linear Acrylamide (Ambion, Austin, TX) and RNA was extracted by two rounds of chloroform phase separation and isopropanol precipitation, followed by washing three times with 75% ethanol. Samples were air dried and dissolved in 10 μl nuclease-free water. It was estimated that 10 ng and 15 ng total RNA were obtained from LE and IE eyelid epithelium, respectively, per fetus. RNA was analyzed by Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) and samples with RNA Integrity Number (RIN) >5.5 were processed for cDNA amplification. cDNA amplification and biotinylation was done using Ovation Pico WTA System (NuGEN, San Carlos, CA) following the manufacturer's instructions. Specifically, RNA (10 ng) was processed into first strand cDNA, a DNA/RNA heteroduplex, and thereafter a linear isothermal amplified cDNA. The amplified cDNA was purified with a PCR Purification Kit (QIAGEN, Valencia, CA).

The cDNAs from each fetus were considered one biological sample and 3 samples of each genotype were used for triplicate hybridization on the Affymetrix GeneChip Mouse Gene 1.0 ST array (P/N 901168, Affymetrix, Santa Clara, CA). The arrays are hybridized with 15 μg of fragmented aRNA. The hybridization, staining, and washing are carried out using the Affymetrix GeneChip Hybridization Wash and Stain Kit (P/N 900720) following the manufacturer's protocols. The arrays are hybridized for 16 h at 45 °C using Affymetrix Hybridization Oven 640 (P/N 800139). FS450_0001 protocol is used for staining and washing the GeneChips using the Affymetrix Fluidics Station 450 (P/N 00-0079). The GeneChips are scanned with Affymetrix GeneChip Scanner 3000 7G Plus using Affymetrix GeneChip Command Console 3.2.3.1515 software and Affymetrix preset settings.

Quantitative RT-PCR

Quantitative PCR was performed using an MX3000p thermal cycler system and SYBR Green QPCR Master Mix (Stratagene), using conditions optimized for each target gene primers with efficiency greater than 85%, cycles less than 28 and sample locations on the plates been randomized. The PCR products were subjected to melting curve analysis and the relative cycle differences in qRT-PCR were determined using ΔCt, as described (Schnekenburger et al., 2007). The ΔCt value for each sample was determined using the cycle threshold (Ct) value of the specific gene normalized to that of *Gapdh*. The fold change was calculated based on the ratio between treated versus untreated (control) samples, designated as 1. Data are based on triplicate reactions of at least three biological samples.