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Molecular Profiles of Mitogen Activated Protein Kinase Signaling Pathways in Orofacial Development

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Abstract

Background—Formation of the mammalian orofacial region involves multiple signaling pathways regulating sequential expression of and interaction between molecular signals during embryogenesis. The present study examined the expression patterns of members of the MAPK family in developing murine orofacial tissue.

Methods—Total RNA was extracted from developing embryonic orofacial tissue during gestational days (GDs) 12–14 and used to prepare biotinylated cDNA probes, which were then denatured and hybridized to murine MAP kinase signaling pathways gene arrays.

Results—Expression of a number of genes involved in the (ERK1/2) cascade transiently increased in the embryonic orofacial tissue over the developmental period examined. Numerous members of the SAPK/JNK cascade were constitutively expressed in the tissue. Genes known to play a role in p38 MAPK signaling exhibited constitutive expression during orofacial development. Western blot analysis demonstrated that ERK2/1, p38 and SAPK/JNK kinases are present in embryonic orofacial tissue on each of GD 12, 13 and 14. By using phospho-specific antibodies, active ERK was shown to be temporally regulated during orofacial development. Minimal amounts of active p38 and active SAPK/JNK were detected in orofacial tissue during GDs 12–14.

Conclusions—Our study documents specific expression patterns of genes coding for proteins belonging to the ERK1/2, p38 and SAPK/JNK MAP kinase families in embryonic orofacial tissue. We also demonstrate that active, phosphorylated forms of ERK1/2 only, were detected in the embryonic tissue investigated, suggesting a more central role for members of this family in embryonic orofacial development.

Keywords

embryonic; palate; MAP kinase; orofacial; ERK

INTRODUCTION

The craniofacial region is one of the most rapidly growing and developing areas in the embryo, and thus it is highly susceptible to malformations. Normal craniofacial development depends on a series of precisely orchestrated morphological and molecular events such that an alteration in any one of these coordinated processes could lead to abnormal development. In mammalian embryos, the orofacial complex that includes palatal processes develops chiefly from the first pharyngeal arch in the form of bilateral extensions of the maxillary

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processes and contains cranial neural crest–derived mesenchyme surrounded by an epithelial layer (Johnston, 1966; Noden, 1975). The palatal processes, medial projections of the maxillary processes, first grow vertically on either side of the tongue, then undergo a series of morphogenetic movements that result in their reorientation above the tongue thereby bringing this medial edge epithelia (MEE), on the apical surface of each process, into contact. Subsequent fusion of the palatal processes results in formation of the definitive secondary palate (Greene et al., 1995).

Past morphological, biochemical and molecular analyses of normal orofacial development have provided fascinating insights regarding control of cellular growth and differentiation of orofacial tissue. Normal growth of the entire craniofacial region, via precisely regulated spatio-temporal patterns of proliferation, is known to be critical for proper development in both the human (Greene et al., 1992; Yoneda and Pratt, 1981) and laboratory animals (Fraser et al., 1956; Shah et al., 1987). In this context, the developing secondary palate has proved to be of inestimable value as a model system for gaining insight into the etiology of palatal clefts and has also provided an excellent paradigm for elucidating the interplay between myriad molecular signals governing cellular proliferation, differentiation and death during embryogenesis. Exquisite controls are placed upon these processes during morphogenesis. While basic cues and mechanisms are undoubtedly shared by the adult and developing organism, the intricacies involved in regulating these cellular processes in a spatial and temporal continuum, to result in the genesis of new tissues and organs, is likely to include mechanisms unique to the embryo or fetus. Development of the orofacial region involves integration and regulation of the sequential expression and interaction of numerous molecular signals (Greene et al., 1991; Nugent et al., 1995; Potchinsky et al., 1998; Weston et al., 1998). Among the multiple signaling pathways active in the embryogenesis of this region, the mitogen-activated protein kinase (MAPK) pathways are thought to play important roles (Seger and Krebs, 1995).

The MAP kinases comprise a family of highly conserved eukaryotic enzymes that are among the most thoroughly studied of signal transduction systems. At least 5 distinct MAPK cascades, functioning as separate modules, are known to exist in mammalian cells (Garrington and Johnson, 1999; Widmann et al., 1999). Each cascade can be triggered by a diversity of signaling molecules. The cascades include: 1) the mitogenic extracellular signal regulated kinase (ERK1/2) cascade, which preferentially regulates cell growth and differentiation; 2) the stress activated protein kinase 1 (SAPK) cascade, comprising the c-jun N-terminal kinase (JNK) family; 3) the SAPK2/3 cascade, comprising the p38 family of proteins; 4) the mitogenic extracellular signal regulated kinase (ERK5/BMK1) cascade; and 5) the ERK3 cascade. Pathways 2 and 3 function primarily in stress response, whereas pathway 4 is preferentially activated in response to growth factors and stress.

Typically, these cascades are organized into a 3-kinase pathway consisting of a MAPK, an MAPK activator (MKK or MAP kinase kinase), and an activator of the MAPK activator (MKKK or MAP kinase kinase kinase). MKKs are characteristically dual specificity kinases that phosphorylate MAPKs on both tyrosine and threonine residues, while the MKKs are themselves phosphorylated on serine or threonine residues by the MKKKs (Widmann et al., 1999). Transmission of signals is achieved by a sequential series of phosphorylation reactions wherein each downstream kinase serves as a substrate for the upstream activator. For example, in the ERK1/2 cascade, the two related mammalian MAPKs, ERK1 and ERK2 ($p44^{mapk}$ and $p42^{mapk}$), are phosphorylated (activated) by MAP kinase/ERK kinase (MEK), which in turn was phosphorylated (activated) primarily by the ser/thr protein kinase Raf-1 after having been recruited to the plasma membrane by Ras. In the ERK5/BMK1 pathway, ERK5/BMK1 are phosphorylated by MEK5, which is activated by MEKK2/MEKK3.

Once activated, MAP kinases translocate to the nucleus and effect downstream ser/thr phosphorylation of regulatory molecules such as transcription factors thereby effecting changes in gene expression. Nuclear translocation is best understood for ERK1 and ERK2 where phosphorylated ERKs dimerize and are actively transported across the nuclear membrane (Brunet et al., 1999; Khokhlatchev et al., 1998).

While MAPK modules appear to signal independently of each other, several of these pathways converge on identical nuclear transcription factors, some of which can be regulated by >1 MAPK cascade (Garrington and Johnson, 1999). Moreover, MAPKs can also integrate signals derived from other signaling pathways such as the STAT and cAMP cascades (Vossler et al., 1997; Xia et al., 1995). Indeed, in embryonic orofacial tissue, MAPK phosphorylation at tyrosine-185 and subsequent activation can be stimulated via multiple pathways, many known to play significant roles in differentiation of orofacial tissue (Potchinsky et al., 1998). Thus, the MAPK pathways can coordinate responses to multiple diverse effectors. This is potentially of critical importance during metazoan development wherein responses to environmental and genetic cues depend on regulation and coordination of multiple signal transduction pathways.

Various growth and differentiation factors control the formation of the orofacial region. Two of the more important of these factors are epidermal growth factor (EGF) and the transforming growth factors beta (TGFβ) (Kaartinen et al., 1995; Potchinsky et al., 1998). The EGF signaling pathway is active during embryogenesis of a number of structures, such as the secondary palate whose development is dependent on epithelial–mesenchymal interactions (Doetschman, 1999). Moreover, EGF plays a crucial role in the regulation of cell proliferation and differentiation (Pisano and Greene, 1987). The binding of EGF to its receptor activates receptor tyrosine kinase (RTK) with concomitant tyrosine autophosphorylation (Greene and Pisano, 2000). Although RTK activation leads to a variety of downstream effects, the primary pathway for EGF-induced effects is via ERK1/2 phosphorylation. TGFβ is a member of a large family of multifunctional growth and differentiation factors, many of which are involved in the development of the orofacial region in mammals (Gehris and Greene, 1992). Several lines of evidence support the notion that TGFβ signaling may be mediated in part through the MAPK cascade in the developing embryo (Weston et al., 1998). Indeed, TGFβ-mediated signaling can modulate the effects of EGF on cell growth in cells derived from the embryonic orofacial region (Weston et al., 1998). Hence, a MAPK may lie downstream of TGFβ receptors in much the same way that the RAS/ERK MAPKs lie downstream of tyrosine kinase receptors.

Developmental roles of MAPKs have been demonstrated in vivo (Massague, 1998). Embryos deficient in ERK2 exhibit defects in mesoderm differentiation and placental angiogenesis. Deletion of p38α MAPK results in angiogenic defects in the placenta and peripheral vessels (Wang et al., 2004). ERK5-deficient embryos are embryonic lethal due to defects in angiogenesis and cardiovascular development (Hayashi and Lee, 2004; Kuida and Boucher, 2004; Regan et al., 2002). Because family members mediate the effects of several critical growth factors during embryonic orofacial development, we sought to define the expression profile of MAPK family members during orofacial ontogeny. We examined the expression of 96 genes associated with MAPK families during the critical period of murine orofacial growth and morphogenesis. Levels of ERK2/1, p38 and SAPK/JNK kinase proteins were also determined by immunoblotting and their active forms analyzed using phospho-specific antibodies.

MATERIALS AND METHODS

Animals

Mature male and female ICR mice (Harlan, Indianapolis, IN) were housed in a climate-controlled room with a 12-hr alternating dark-light cycle and were mated overnight. The presence of a vaginal plug the following morning (gestational day [GD 0]) was considered as evidence of mating. On GDs 12, 13, and 14, female mice were euthanized by asphyxiation, and embryos were dissected from uteri in sterile Ca^{++}- and Mg^{++}-free PBS. Extraembryonic membranes were removed, and maxillary tissue, including primary palatal tissue and secondary palatal processes, was excised, minced and stored at −20°C in RNALater solution (Qiagen, Chatsworth, CA) for subsequent extraction of total RNA. Tissue from 5 to 8 embryos (on each of the 3 days of gestation) was pooled into single samples for RNA extraction. Moreover, 3 distinct RNA samples from fetal orofacial tissue were processed for *each* day of gestation in order to prepare triplicate sets of target RNAs for hybridization to SuperArray nylon membranes (SuperArray Inc., Bethesda, MD) (9 samples and 9 chips total).

RNA Extraction

Total RNA from excised tissue samples was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations. The quality and quantity of the extracted total RNA was assessed by spectrophotometric ultraviolet (UV) absorbance ratio at 260/280 nm and absorbance at 260 nm, respectively. Absorbance ratios measured for total RNA samples were from 1.98 to 2.10 in Tris-EDTA buffer solution (pH 7.5), indicating the quality of the samples.

cDNA Expression Array Analysis

Nonradioactive Mouse MAP Kinase Signaling Pathways Gene Array (GEArray Q Series MM-017; SuperArray Inc.) was used to analyze the gene expression profile of members of the MAP kinase signaling families during orofacial development. Procedures were carried out according to the manufacturer’s protocol. Briefly, 3 μg of total RNA was used as template for biotinylated cDNA probe synthesis. RNA was reverse-transcribed by gene-specific primers (supplied with the SuperArray kit) with biotin-16-dUTP. Biotinylated cDNA probes were denatured and hybridized to MAP kinase signaling pathway gene-specific cDNA fragments spotted on the membranes. The GEArray membranes were then washed and blocked with GE-blocking solution, and incubated with alkaline phosphatase-conjugated streptavidin. The hybridized biotinylated probes were detected by chemiluminescence using the alkaline phosphatase substrate, CDP-Star.

Images of membranes were acquired and quantitated using the Kodak 1D image analysis software on a Kodak Imaging Station, model 440 CF. Raw image data were transferred to the online GEArray Expression Analysis Suite (www.SuperArray.com). All signal intensities were corrected for background by subtracting the minimum value, defined as the numerical value of spots with least intensity. Relative expression levels of different genes were estimated by comparing their signal intensity with that of the internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The average of 3 data sets for each of the 3 days of gestation was used for results analysis. Genes were considered as not being expressed if their expression level was <20% of the control gene. Genes were considered to be constitutively expressed if the change of their relative expression levels between 2 gestational days was <1.5 fold. If the fold change was >1.5, or <0.67, then the genes were considered as having increased or decreased levels of expression, respectively.

Confirmation of gene expression data

Expression data was confirmed using the real time PCR-based pathway focused gene profiling "RT^2 profiler system" from SuperArray Inc (MAP Kinase Signaling Pathway PCR Array, APM-061, corresponds to the GEArray Q Series MM-017 used for gene expression profiling above). Procedures were carried out according to the manufacturer's protocol. Briefly, 1 μg of total RNA was used as template for reverse-transcription of cDNA. This was then diluted and added to a master mix containing the fluorescent SYBR Green dye. Aliquots from this mix were added to a 96-well plate, where each well contains predispensed gene-specific primer sets. The plates (1 for each day of gestation) were then placed in a TaqMan ABI Prism 7000 Sequence Detector System (Applied Biosystems, Foster City, CA) and real-time PCR analysis was performed.

Cycling parameters were as follows: 95°C for 10 min for activation of "HotStart DNA polymerase," followed by 40 cycles of denaturation at 95°C for 15 sec each, and finally, primer extension at 60°C for 1 min. Each plate contains a panel of housekeeping gene primers for normalization the PCR array data, as well as estimation of the linear dynamic range of the assay. Further, for each reaction, both "no reverse transcription control" and "no template" samples were included as negative controls. Raw data were acquired and processed with ABI Sequence Detector System software, version 1.0 (Applied Biosystems, UK) to calculate the threshold cycle (C_t) value and relative gene expression values subsequently determined according to standard $\Delta\Delta C_t$ method (Singh et al., 2005).

Tissue Preparation for Protein Collection

Pregnant female mice were euthanized by carbon dioxide asphyxiation, and gravid uteri were removed and placed in PBS on ice. Embryonic orofacial tissues from the midfacial region (maxillary tissue, including primary palatal tissue and secondary palatal processes) were dissected, minced, and washed twice with Ca^{++}- and Mg^{++}-free PBS. Embryonic tissues were homogenized in ground glass combination conical/cylindrical tissue grinders at 4°C in an appropriate volume of lysis buffer (250 mM NaCl, 50 mM Tris [pH 7.4], 5 mM EDTA, 0.1% [v/v] Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], 50 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and 50 μg/ml aprotinin). Adult tissues were homogenized in the above buffer using a Tekmar Tissumizer at half maximal speed for 30 to 45 sec at 4°C. All homogenates were sonicated for 30 sec at 4°C and allowed to sit on ice for 30 min with vigorous vortexing every 5 min, then cleared by centrifugation for 10 min at 13000 × *g* at 4°C. Total protein in the extracts was determined according to the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

Immunoblotting

Primary antibodies against phospho and total ERK1/2 and β-actin were obtained from Cell Signaling (Beverly, MA) while those for phospho and total SAPK/JNK and p38 were obtained from New England Biolabs (Beverly, MA). Appropriate amounts of lysate were denatured by boiling in 2× Laemmli sample buffer (Laemmli, 1970) and the proteins separated by SDS-PAGE electrophoresis at 125 volts using 8–16% polyacrylamide Tris-Glycine gels (Novex, San Diego, CA) followed by electrophoretic transfer (30 volts for 2 hrs) of the proteins to PVDF membranes. Gels were subsequently stained with Coomassie blue dye and membranes with 0.1% fast green to visualize proteins and ensure the efficiency of protein transfer. Blots were blocked by incubation in 5% nonfat dry milk in TBST buffer (50 mM Tris, pH 7.6; 150 mM NaCl; 0.1% Tween 20) for 1 hr at room temperature. Antibodies were diluted in blocking solution and blots incubated with primary antibody (1:1000) for 1–1.5 hrs at room temperature, and washed extensively. A HRP-conjugated goat anti-rabbit IgG1 (Zymed Labs, Carlsbad, CA) was used as the secondary antibody at 1:10000 dilution. Immune complexes were detected using the ECL-Plus chemiluminescent

detection system (Amersham Pharmacia Biotech, Arlington, IL) according to the manufacturer's instructions. "No primary antibody" control immunodetection was performed on each blot prior to blotting with the primary antibody in order to distinguish between specific immunoreactive bands and nonspecific protein bands due to interaction of the secondary antibody with endogenous proteins. Immunoblots were replicated with similar results on a minimum of 3 complete sets of tissue samples.

Densitometric analysis

Densitometric analyses of total and phospho p42/44 bands were performed with NIH Image (v 1.63) software.

RESULTS

MAP kinase pathway gene expression profile

Murine orofacial tissue was excised on each of GD 12–14, the critical period of orofacial growth and morphogenesis, and total RNA was prepared from the tissues. Three distinct RNA samples for each GD were prepared and subsequently hybridized to SuperArray cDNA membranes. The nonradioactive Mouse MAP Kinase Signaling Pathways Gene Array was used to probe for expression profiles of genes known to be members of MAP kinase signaling family.

Among the 96 genes tested on the SuperArray cDNA membrane, 39 genes exhibited constitutive expression on each of GDs 12, 13 and 14 (Table 1). The relative expression level of each of these genes did not change >1.5 fold from GD 12 to 13, and from GD 13 to 14. Ten genes that were not expressed (their expression levels were <20% of the positive control genes) are listed in Table 2. Of the 47 genes that exhibited differential expression during orofacial development (Table 3), 46 genes showed at least a 1.5-fold increase from GD 12 to GD 13. However, half of these genes exhibited a diminished level of expression from GD 13 to GD 14. Moreover, no genes exhibited increased expression levels between GD 13 and GD 14. Gene expression data was confirmed by RT-PCR-based gene profiling "RT^2 profiler system" (SuperArray). Each array is a 96-well plate containing SYBR Green-optimized primer sets corresponding to a total of 96 genes, including 5 housekeeping genes and 2 negative controls. The particular array used for confirmation of gene expression data contained primer sets that paralleled those imprinted on the nylon membrane used for gene expression profiling above. Table 4 contains representative genes from the profiler array used for this study. As can be seen, there is an overwhelming concordance with the data presented in Tables 1 (constitutively expressed genes) and 3 (differentially expressed genes).

The ERK pathway is active in embryonic orofacial tissue during its growth and differentiation. *Mapk1* (ERK2), *Mapk3* (ERK1), *MEK1*, *MEK2*, *Araf* and *Raf1* as well as the Raf regulating proteins: *Hras1*, *Kras2*, and *Ksr* displayed a transient increase in expression during GD 12–14 (Table 3). The activated transcription factors: *Creb1*, *Egfr*, *Elk1*, *Ets1*, *Ets2*, *Madh4*, *Mapkapk5*, *Max*, and *Mef2c*, also displayed a transient increase in expression during GD 12–14 (Table 3), as did several genes known to be induced by MAPK pathways: *Col1α1*, *Egr1*, and *Hspa5* (Grp78). Expression of several genes encoding cell cycle proteins regulated by the Erk1/2 pathway showed constitutive levels of expression (Table 1). *Cyclin B2*, *Cyclin E2*, and *p27kip1*, however, displayed increasing levels of expression during GD12–14 of orofacial development.

MAP kinases *Mapk8* (JNK1) and *Mapk9* (JNK2), involved in the SAPK/JNK cascade, were constitutively expressed (Table 1), while expression of MAP kinase kinase *Map2k4* (MKK4, JNKK1) and *Map2k7* (JNKK2) increased transiently between GD 12 and 14 (Table 3). Expression of *Map3k1* (MEKK1) and MAPK8 interacting proteins *Mapk8ip* (JIP-2),

Mapk8ip2 (JIP-1), and *Mapk8ip3* (JIP-3) was constitutive (Table 1) while *Map3k11* (MLK3) was not expressed.

MAP kinases *p38*, *p38*β, and *p38*γ showed modestly increased expression from GD 12 to GD 13, and expression remained elevated until GD 14. Genes known to play a role in p38 MAPK pathways, such as *Rac*, *Cdc42*, *Mapkap-2*, and *Mnk-1*, exhibited constitutive expression during orofacial development (Table 1).

Activated ERK, SAPK/JNK and p38 MAP kinase protein analysis

Embryonic orofacial tissue was dissected on GDs 12, 13 and 14, and protein (total cell lysates) was extracted and analyzed for the presence of MAPK proteins by Western blotting. Figures 1 and 2 demonstrate Western blot analysis of active and total ERK, p38 and SAPK/JNK in embryonic orofacial tissue on GDs 12, 13 and 14. Western blot images were quantified by densitometric scanning using the NIH Image (version 1.62) software. MAP kinases p42/44 (ERK2/1), p38 and SAPK/JNK were expressed in embryonic orofacial tissue on each of GDs 12, 13 and 14 (Fig.1 and 2).

More revealing was determination of the *active* MAPK modules during development of embryonic orofacial tissue. Phospho-specific p44/p42 MAP kinase rabbit polyclonal antibody cross-reacts with murine ERKs 1 and 2 phosphorylated at tyrosine 202 and 204, and does not cross-react with either tyr-phosphorylated SAPK/JNK or p38. Thus, this antibody allows determination of the presence of active ERK. To confirm equal loading of proteins, the blots were stripped and reprobed for β-actin, a housekeeping protein. As shown in Fig. 1 (lower panel), the abundance of β-actin in the 3 lanes of the blot was similar. Total and phospho p42/44 bands obtained using the Western blot method were also densitometrically analyzed. It was observed that phospho p42 expression increased by ~2.5 fold on both GDs 13 and 14, compared to GD 12. In the case of phospho-p44, the expression increased by ~6.5 fold on GDs 13 and 14, compared to GD 12. Further, the expression of total p42 increased ~1.4 and 2.3 on GDs 13 and 14, respectively. The increase in the case of total p44 was 1.1 and 2.0 fold on GDs 13 and 14, respectively. ERK activity thus appears to be temporally regulated with greater ERK activity on GDs 13 and 14 as compared to GD 12 (Fig.1). It can be thus concluded that expressions of total and phospho p42/44 increase in the secondary palate of the developing embryo between GDs 12 and 14. Phospho-specific p38 MAPK antibody detects p38 MAPK only when activated by phosphorylation. Phospho-specific SAPK/JNK antibody detects only active (phosphorylated) SAPK/JNK. Minimal amounts of active p38 and active SAPK/JNK were detected in embryonic orofacial tissue during GD 12–14 (Fig. 2).

DISCUSSION

Development of the embryonic orofacial region involves multiple signaling pathways regulating the sequential expression and interaction of numerous molecular effectors during embryogenesis. The present study analyzed the expression of members of the MAP kinase family of enzymes in developing embryonic orofacial tissue during GDs 12–14. The MAP kinases are represented as 5 distinctive MAPK cascades: ERK1/2, SAPK/JNK, p38/SAPK2/3, BMK1/ERK5 and ERK3. All are expressed in developing embryonic orofacial tissue during GDs 12–14 and several are differentially expressed. Expression of genes involved in the ERK1/2 cascade, such as *Mapk1* (ERK2), *Mapk3* (ERK1), *Map2k1* (MEK1), *Map2k2* (MEK2), Araf, Raf1 and Raf regulating proteins (Hras1, Kras2, and Ksr) increased from GD 12 to GD 13, and then decreased during later stages of orofacial development, that is, from GD 13 to GD 14. This change corresponds to the rapid growth of the secondary palate during this critical period of orofacial development. The epidermal growth factor receptor (*EGFR*) gene exhibited a significant increase in expression from GD 12 to 13,

suggesting enhanced EGFP-mediated signaling during this period. The EGFR is known to be involved in embryonic orofacial development (Kronmiller et al., 1991). During secondary palate development in the hamster, a correlation between MAP kinase activity and cell proliferation was observed, suggesting involvement of MAP kinase in regulation of embryonic palatal cell proliferation (Young et al., 1997). EGF signaling is known to be mediated via the ERK1/2 pathway. Our results demonstrating increased expression of a series of transcriptional activators known to promote growth and cell proliferation, (creb1, elk1, Ets1/2, madh4, max, and mef2c) is also consistent with enhanced proliferation during GD 12–14.

Fusion of the medial edge epithelial (MEE) cells of the developing secondary palate is preceded by a cessation of MEE DNA synthesis prior to the initial contact of opposing palatal shelves (Ferguson, 1988; Greene, 1989). Exogenous EGF has been shown to inhibit the cessation of MEE DNA synthesis and induce cleft palate (Abbott et al., 1998; Hassell, 1975; Tyler and Pratt, 1980). Moreover, it has been shown that EGF-induced inhibition of palatal MEE fusion is dependent on nuclear ERK1/2 activation and that this mechanism must be tightly regulated during normal palatal fusion (Tyler and Pratt, 1980). Differential expansion of ERK1/2 kinases in developing orofacial tissue is consistent with this notion. ERK1/2 can be activated by various means, all of which result in the activation of various transcriptional factors and ser/thr kinases that contribute to cellular proliferation, differentiation, cell cycle regulation and cell survival (Chen et al., 1992; Gonzalez et al., 1993; Lenormand et al., 1993). It is evident that MAP kinase cascades are involved in growth factor–regulated cell proliferation during morphogenesis of quail secondary palate, where EGF stimulated proliferation of primary cell cultures was accompanied by proliferation and activation of 42-kDa MAP kinase (Hehn et al., 1998; Yamamoto et al., 2003).

Murine embryonic maxillary mesenchymal (MEMM) cells are known to be responsive to activation of the cyclic AMP (cAMP) and the transforming growth factors beta (TGFβ) signal transduction pathways (Gehris et al., 1994). These may modulate the proliferative response of MEMM cells to EGF (Weston et al., 1998), which stimulates MAPK phosphorylation and activity in these cells (Bhattacherjee et al., 2004). Thus, it is reasonable to surmise that a convergence between the TGFβ, cAMP and/or EGF signaling pathways exists in embryonic orofacial tissue. Moreover, modulation of cellular signaling brought about by such cross-talk is likely to be mediated by changing patterns of expression or activation of ERK1/2 family members.

In contrast to the ERK1/2 cascade, genes involved in the SAPK/JNK cascade, such as MAP kinases *Mapk8* (JNK1), *Mapk9* (JNK2), and Mapk8 interacting proteins *Mapk8ip* (JIP-2), *Mapk8ip2* (JIP-1), *Mapk8ip3* (JIP-3) were constitutively expressed during GD12–14. Western blot analysis demonstrated that while SAPK/JNK kinase is present in embryonic orofacial tissue on each of GDs 12, 13 and 14, it is not phosphorylated, suggesting that SAPK/JNK kinase is not active in orofacial tissues during this developmental period.

MAP kinases p38, p38β, and p38γ also exhibited differential expression from GD 12 to GD 13. Genes known to play a role in p38 MAPK pathways such as *Rac*, *Cdc42*, *Mapkap-2*, and *Mnk1* also were constitutively expressed during orofacial development. Western blot analyses demonstrated that p38 kinases are present in embryonic orofacial tissue on each of GDs 12, 13 and 14. Minimal amounts of active p38 were detected however, suggesting that, like SAPK/JNK, p38 is also not active in the tissue during this developmental period.

The p38 pathway controls and enhances the activity of many transcription factors, including ATF1/2, CREB, ELK-1, Ets-1, MEF2A, MEF2C, and SAP-1 (Delghandi et al., 2005;

Goedert et al., 1997; Tanaka et al., 1998; Yang et al., 1999). ATF-2 phosphorylation, for example, is necessary for dimerization with Jun (phosphorylated by JNK) to form activating protein-1 (AP-1), which regulates the expression of many genes (Jochum et al., 2001). Phosphorylation of MEF2C leads to increased c-jun transcription providing additional linkage with p38 and JNK pathways.

Although much less is known about the ERK3 and ERK5 signaling pathways, they also may be involved in orofacial development. ERK3, constitutively expressed in orofacial tissue during GDs 12–14 (Turgeon et al., 2000) has been linked to MAPK-activated protein kinase-5 (MK5), identifying MK5 as a downstream target of ERK3 (Schumacher et al., 2004). Expression of ERK5 and MEK5, the upstream kinase of ERK5, was transiently increased from GD 12 to GD 14, suggesting their involvement in orofacial growth and/or differentiation. ERK5 is known to be essential for early embryonic development and is required for normal development of the vascular system and cell survival (Liu et al., 2003). ERK5 knockout mice die at ~GD 10.5 (Yan et al., 2003). In situ hybridization of ERK5 revealed strong expression in the head and trunk of the embryo during this stage of development.

In summary, our results show that constituents of 5 MAP kinase pathways are expressed in developing orofacial tissue. Further, active (phosphorylated) forms of ERK1/2 were also detected in this embryonic tissue during the critical stages of its growth and morphogenesis, suggesting an important role for members of this MAP kinase family during orofacial development. The exact relationship between ERK1/2 and its involvement in development of the embryonic orofacial region requires further investigation.

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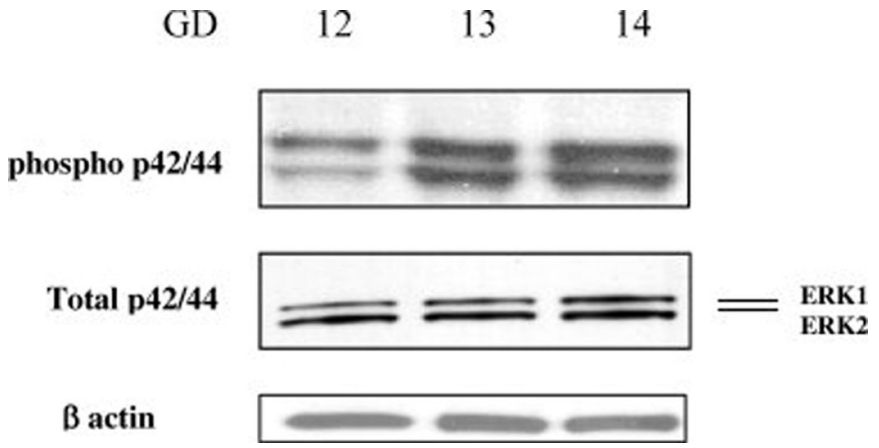

Figure 1.

Immunoblot analysis of p42/44 (ERK1/2) in embryonic orofacial tissue on GDs 12, 13 and 14. A total of 60 μg of total protein lysate per lane was separated by SDS-PAGE electrophoresis on 8–16% polyacrylamide Tris-Glycine gels. Western blot analysis was carried out using antibodies specific for phospho-p42/44 (upper panel) and total p42/44 (middle panel). Note that ERKs are temporally regulated with higher activity on GD 13 and 14 as compared to GD 12 (upper panel). Lower panel shows the western blot of the loading control β-actin.

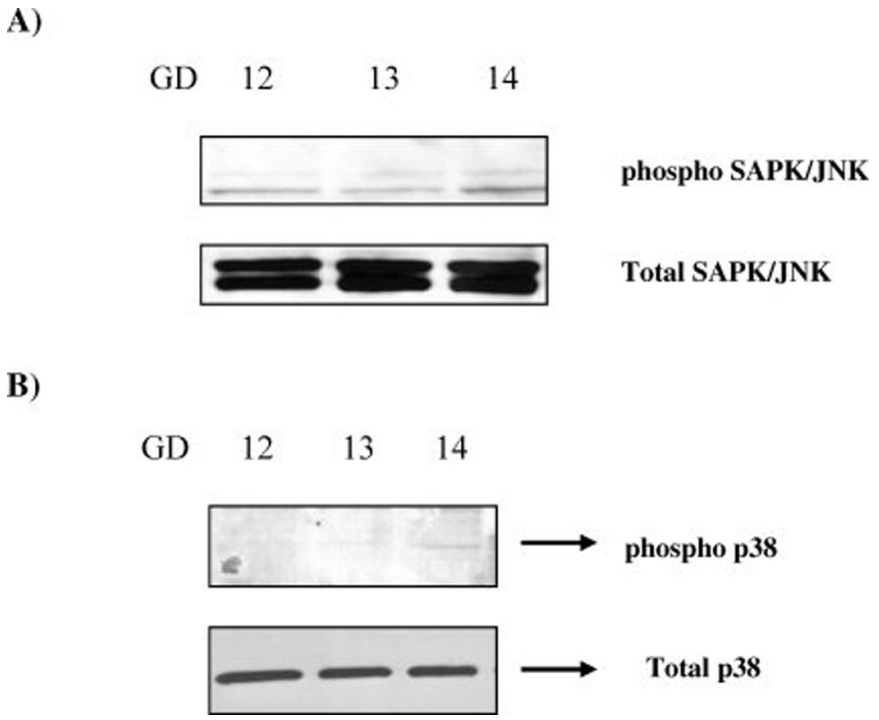

Figure 2.

Immunoblot analysis of total and active p38 MAPK and SAPK/JNK in embryonic orofacial tissue on GDs 12, 13 and 14. A total of 60 μg of total protein lysate was separated by SDS-PAGE electrophoresis on 8–16% Tris-Glycine gels. A) Active, phosphorylated (upper panel) and total (lower panel) SAPK/JNK were analyzed by immunoblotting with specific antibodies. B) Active, phosphorylated (upper panel) and total (lower panel) p38 MAPK was analyzed by immunoblotting with specific antibodies. Minimal amounts of active, phosphorylated p38 were detected in embryonic orofacial tissue during GDs 12–14.

Table 1

Genes constitutively expressed during GD 12–14 of orofacial development.

Relative expression levels of different genes were estimated by comparing their signal intensity with that of the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Genes were considered to be constitutively expressed if the change in their relative expression levels between two gestational days was less than 1.5 fold.

			Fold Change in Expression	
Gene Symbol	**Unigene**	**Genbank No.**	**d13_vs_d12**	**d14_vs_d13**
Atf2	Mm.209903	NM_009715	1.476	0.849
Ccna2	Mm.4189	NM_009828	1.125	0.828
Ccnb1	Mm.260114	NM_172301	1.003	1.010
Ccnd1	Mm.273049	NM_007631	1.211	0.889
Ccnd2	Mm.333406	NM_009829	1.369	0.743
Ccnd3	Mm.246520	NM_007632	1.201	0.944
Ccne1	Mm.16110	NM_007633	0.979	1.015
Cdc42	Mm.1022	NM_009861	1.224	0.712
Cdk4	Mm.6839	NM_009870	1.024	1.002
Cdk6	Mm.278464	NM_009873	1.285	0.914
Cdkn1a	Mm.195663	NM_007669	1.157	0.922
Cdkn1c	Mm.168789	NM_009876	0.964	1.020
Cdkn2a	Mm.4733	NM_009877	1.072	0.912
Cdkn2b	Mm.269426	NM_007670	0.990	1.017
Cdkn2c	Mm.1912	NM_007671	1.174	0.898
Crebbp	Mm.132238	XM_148699	1.275	0.867
Dlk1	Mm.157069	NM_010052	0.987	1.034
E2f1	Mm.18036	NM_007891	1.099	0.870
Elk4	Mm.254233	NM_007923	1.169	0.967
Enpp2	Mm.250256	NM_015744	0.939	1.108
Kcnn1	Mm.32074	NM_032397	1.182	0.944
Map2k3	Mm.18494	NM_008928	1.008	1.018
Map3k1	Mm.15918	NM_011945	1.380	0.933
Map3k14	Mm.158981	NM_016896	1.298	0.793
Map3k3	Mm.27041	NM_011947	1.080	0.904
Map3k7	Mm.258589	NM_172688	1.455	0.887
Mapk13	Mm.27970	NM_011950	1.264	0.909
Mapk8	Mm.21495	NM_016700	1.147	0.811
Mapk8ip	Mm.2720	NM_011162	1.275	0.786
Mapk8ip3	Mm.43081	NM_013931	1.034	0.845
Mapkapk2	Mm.221235	NM_008551	1.051	0.884
Mknk1	Mm.209327	NM_021461	1.006	1.006
Sfn	Mm.44482	NM_018754	1.398	0.737
Mos	Mm.317339	NM_020021	1.356	0.780

			Fold Change in Expression	
Gene Symbol	**Unigene**	**Genbank No.**	**d13_vs_d12**	**d14_vs_d13**
Nfatc4	Mm.27908	NM_023699	1.357	1.076
Nras	Mm.256975	NM_010937	1.008	0.986
Rac1	Mm.292510	XM_357522	1.023	0.938
Rac2	Mm.1972	NM_009008	1.363	0.849
Stk4	Mm.234472	NM_021420	0.995	0.908
Control genes				
Gapd	Mm.333399	NM_008084	0.994	0.999
Gapd	Mm.333399	NM_008084	1.006	1.001
Ppia	Mm.5246	NM_008907	1.105	0.993
Ppia	Mm.5246	NM_008907	1.038	1.003
Ppia	Mm.5246	NM_008907	1.025	0.976
Ppia	Mm.5246	NM_008907	1.006	0.990
Rpl13a	Mm.180458	NM_009438	0.996	0.972
Rpl13a	Mm.180458	NM_009438	1.013	0.958
Actb	Mm.297	NM_007393	1.004	1.003
Actb	Mm.297	NM_007393	0.989	1.015

Table 2

Genes “not expressed” during GDs 12–14 of orofacial development.*

Gene Symbol	Unigene	Genbank No.
Ccna1	Mm.4815	NM_007628
Cdk2	Mm.111326	NM_016756
Cdkn2d	Mm.29020	NM_009878
Jun	Mm.275071	NM_010591
Map3k11	Mm.185026	NM_022012
Map4k2	Mm.25860	NM_009006
Mapk10	Mm.39253	NM_009158
AI874665	Mm.222612	NM_178907
Myc	Mm.2444	NM_010849
Trp53	Mm.222	NM_011640

* Genes were considered to be “not expressed” if their expression level during the developmental time period was <20% of that of the control (GAPDH) gene.

Table 3

Genes “not expressed” during GD 12–14 of orofacial development.*

			Fold Change in Expression	
Gene Symbol	**Unigene**	**Genbank No.**	**d13 vs d12**	**d14 vs d13**
Araf	Mm.220946	NM_009703	1.752	0.713
Ccnb2	Mm.22592	NM_007630	1.526	0.639
Ccne2	Mm.35867	NM_009830	1.607	0.688
Cdkn1b	Mm.2958	NM_009875	1.665	0.690
Chuk	Mm.3996	NM_007700	1.769	0.629
Col1a1	Mm.277735	NM_007742	2.340	0.809
Creb1	Mm.1376	NM_133828	2.389	0.324
Egfr	Mm.8534	NM_007912	2.360	0.408
Egr1	Mm.181959	NM_007913	1.757	0.836
Elk1	Mm.347420	NM_007922	1.903	0.771
Ets1	Mm.292415	NM_011808	1.838	0.370
Ets2	Mm.290207	NM_011809	2.716	0.251
Fos	Mm.246513	NM_010234	1.833	0.667
Grb2	Mm.6900	NM_008163	1.619	0.728
Hras1	Mm.334313	NM_008284	1.816	0.858
Hspb1	Mm.13849	NM_013560	2.008	0.737
Hspa5	Mm.330160	NM_022310	2.785	0.670
Kras2	Mm.207540	NM_021284	1.808	0.412
Ksr	Mm.4745	NM_013571	1.704	0.595
Smad4	Mm.100399	NM_008540	2.130	0.474
Map2k1	Mm.248907	NM_008927	1.685	0.822
Map2k1ip1	Mm.331392	NM_019920	1.934	0.754
Map2k2	Mm.275436	NM_023138	2.087	0.777
Map2k4	Mm.27491	NM_009157	1.744	0.715
Map2k5	Mm.192651	NM_011840	1.891	0.522
Map2k6	Mm.14487	NM_011943	1.596	0.645
Map2k7	Mm.3906	NM_011944	2.954	0.392
Map3k2	Mm.211762	NM_011946	2.146	0.599
Map3k4	Mm.28587	NM_011948	2.066	0.498
Map3k5	Mm.6595	NM_008580	2.399	0.386
Map4k1	Mm.148278	NM_008279	1.559	0.879
Mapk1	Mm.196581	NM_011949	2.210	0.625
Mapk11	Mm.91969	NM_011161	1.627	0.807
Mapk12	Mm.38343	NM_013871	1.666	0.791
Mapk14	Mm.246203	NM_011951	1.619	0.675
Mapk3	Mm.8385	NM_011952	1.754	0.587
Mapk6	Mm.18856	NM_015806	1.477	0.653
Mapk7	Mm.38172	NM_011841	2.035	0.682

Gene Symbol	Unigene	Genbank No.	Fold Change in Expression	
			d13 vs d12	d14 vs d13
Mapk8ip2	Mm.173337	NM_021921	1.533	0.707
Mapk9	Mm.68933	NM_016961	1.553	0.663
Mapkapk5	Mm.272206	NM_010765	2.271	0.740
Max	Mm.268548	NM_008558	2.716	0.363
Mef2c	Mm.24001	NM_025282	2.223	0.712
Pak1	Mm.260227	NM_011035	1.537	0.841
Pla2g4a	Mm.4186	NM_008869	1.970	1.160
Raf1	Mm.184163	NM_029780	1.569	0.747
Rb1	Mm.273862	NM_009029	1.995	0.557

* Genes were considered to be “not expressed” if their expression level during the developmental time period was <20% of that of the control *(GAPDH)* gene.

Table 4

Real-Time PCR verification of genes expressed constitutively (A) and differentially (B) expression during GDs 12–14 of orofacial development using the RT^2 profiler system.

A:				
			Concordance in gene expression	
Gene Symbol	**Unigene**	**Genbank No.**	**GD 13_vs_GD12**	**GD 14_vs_GD 12**
Atf2	Mm.209903	NM_009715	+	+
Ccnd2	Mm.333406	NM_009829	+	+
Cdc42	Mm.1022	NM_009861	+	+
Cdnk2c	Mm.1912	NM_007671	+	+
Crebbp	Mm.132238	XM_148699	+	+
E2f1	Mm.18036	NM_007891	+	+
Map3k1	Mm.15918	NM_011945	+	+
Mapk13	Mm.27970	NM_011950	+	+
Sfn	Mm.44482	NM_018754	−	−
Nfatc4	Mm.27908	NM_023699	+	+
Rac2	Mm.1972	NM_009008	+	+

B:				
			Concordance in gene expression	
Gene Symbol	**Unigene**	**Genbank No**	**d 13_vs_d 12**	**d 14_vs_d 12**
Araf	Mm.220946	NM_009703	+	+
Col1a1	Mm.277735	NM_007742	+	+
Hspb1	Mm.13849	NM_013560	+	+
Kras2	Mm.207540	NM_021284	+	+
Smad4	Mm.100399	NM_008540	+	+
Map2k2	Mm.275436	NM_023138	+	+
Map3k5	Mm.6595	NM_008580	+	+
Max	Mm.268548	NM_008558	+	+
Pak1	Mm.260227	NM_011035	+	+
Rb1	Mm.273862	NM_009029	+	+