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Molecular fingerprinting of BMP2- and BMP4-treated embryonic maxillary mesenchymal cells

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Dates:
Accepted 10 August 2005

To cite this article:
Orthod Craniofacial Res **9**, 2006; 93–110
Mukhopadhyay P, Singh S, Greene RM, Pisano MM:
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Structured Abstract

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Objective – To determine the differences in gene expression between control-, bone morphogenetic protein (BMP)2- and BMP4-treated murine embryonic maxillary mesenchymal (MEMM) cells.

Design – Transcript profiles of BMP2-, BMP4- and vehicle-treated MEMM cells were compared utilizing the murine high-density GeneChip arrays from Affymetrix. The raw chip data (probe intensities) were pre-processed using robust multichip averaging with GC-content background correction and further normalized with GeneSpring v7.2 software. Cluster analysis of the microarray data was performed with the GeneSpring software. Changes in the gene expression were verified by TaqMan quantitative real-time PCR.

Results – Expression of ~50% of the 45 101 genes and expressed sequence tags examined in this study were detected in BMP2-, BMP4- and vehicle-treated MEMM cells and that of several hundred genes was significantly altered (up or downregulated) in these cells in response to BMP2 and BMP4. Expression profiles of each of the 26 mRNAs tested by TaqMan quantitative real-time PCR were found to be consistent with the microarray data. Genes whose expression was modulated following BMP2 or BMP4 treatment, could be broadly classified based on the functions of the encoded proteins such as the growth factors and signaling molecules, transcription factors, and proteins involved in epithelial–mesenchymal interactions, extracellular matrix synthesis, cell adhesion, proliferation, differentiation, and apoptosis.

Conclusion – Utilization of the Affymetrix GeneChip microarray technology has enabled us to delineate a detailed transcriptional map of BMP2 and BMP4 responsiveness in embryonic maxillary mesenchymal cells and offers revealing insights into crucial molecular regulatory mechanisms employed by these two growth factors in orchestrating embryonic orofacial cellular responses.

Key words: bone morphogenetic protein; craniofacial; embryo; gene expression; transcription growth factor β

Introduction

The development of the vertebrate face is a complex morphogenetic process that is initiated upon the formation of branchial (pharyngeal) arches. The first pair of branchial arches gives rise to the maxillary and mandibular processes which ultimately contribute to the formation of the upper and lower jaws, respectively. The bilateral maxillary processes of the first branchial arch enlarge and fuse with the medial nasal processes thereby forming the primary palate which includes the entire upper lip. Later in embryonic development, the secondary palate originates as bilateral extensions from the oral aspect of the maxillary processes. Morphogenesis, cellular proliferation and tissue differentiation of the first branchial arch and the frontonasal process are influenced by a variety of secreted signals including, among others, those derived from the BMP signaling pathway (1–10).

Expression of various BMPs (BMP2, 4, and 5) is pronounced in the embryonic orofacial tissue (11–13), BMP2 being widely expressed in the facial skeletal tissue, developing tooth germ, facial mesenchyme, and Meckel's cartilage (14). BMP2 has also been known to stimulate osteoblast maturation *in vitro* (15,16) and BMP4 has been shown to have a major role in the epithelial–mesenchymal interactions that precede tooth formation (17). Tissue distribution of the BMPs during orofacial ontogeny strongly suggests a developmental role. For example, on gestational day 9.5 in mice, *Bmp4* is expressed in the epithelium of the maxillary and mandibular processes (11), while prominent expression of *Bmp2* is observed on gestational day 13.5, throughout the development of the facial tissue, in the neural crest derived mesenchyme, floor of the mouth and the ventral tongue (11). Moreover, during the development of midface in mice (13), and chicks (18,19), *Bmp4* expression becomes restricted to epithelia in the region of pre-fusion contact between midfacial processes. Interestingly, Ashique et al. (18) noted that chick *Bmp7* was expressed in a manner similar to *Bmp4*, while *Bmp2*, was expressed predominantly in the underlying mesenchyme in the pre-fusion zone, indicating a complex interplay between the various members of BMP family in the orofacial development. The unique temporo-spatial expression pattern of *Bmp4* in the embryonic midfacial region suggests a critical role of BMP4 in mediating morphogenesis and/or fusion of the orofacial process (13). The importance of properly coordinated BMP signaling during orofacial development is further illustrated by the recent finding that spatio-temporal expression of BMP2, 4 and 5 is tightly regulated during normal palatogenesis and that a reduction in the expression of their messenger RNAs (mRNAs) may contribute to the cleft palate formation (12). Treatment of avian embryos with the BMP antagonist Noggin, resulted in reduced proliferation and outgrowth of the frontonasal mass and maxillary prominences and ultimately to the absence of the maxillary and palatine bones (18), demonstrating a requirement for endogenous BMP in the proliferation of facial mesenchyme. Functionality of the BMPs in orofacial development is further supported by the observation that expression of *Bmp4* and *Bmp2* in developing palate mesenchyme requires the expression of *Msx1* homeobox gene (20). The significance of this resides in the fact that mutations in the *Msx1* gene are associated with non-syndromic cleft palate and tooth agenesis in humans (21), and the transgenic expression of *Bmp4* in $Msx1^{-/-}$ murine embryonic palatal mesenchyme rescues the cleft palate phenotype (20). Interestingly, facial clefting and exencephaly have also been observed in transgenic mice overexpressing a BMP-target gene *Msx2*, and in embryos in which the expression of *Msx2* had been activated by BMP2 and 4. (9,22). Collectively, these experimental findings support the notion that during embryogenesis, members of the BMP subfamily play key roles in developing the orofacial processes.

Although the cellular and phenotypic effects of BMPs on embryonic craniofacial tissue have been studied in some detail, the specific genes that act as downstream mediators of BMPs, especially BMP2 and BMP4, in this tissue remain poorly defined. To identify BMP2 and BMP4 target genes, Affymetrix

GeneChip microarrays were utilized in this study to assess changes in the mRNA expression in primary cultures of murine embryonic maxillary mesenchymal (MEMM) cells in response to stimulation by exogenous BMP2 and BMP4.

Materials and methods

Cell culture

Embryos from pregnant ICR mice (Harlan, Inc.; Indianapolis, IN, USA) (date of vaginal plug detection was considered day 0 of gestation) were dissected on day 13 of gestation from uteri; maxillary tissue dissected from the embryos and primary cultures of MEMM cells were initiated. In brief, embryonic maxillary tissue was minced and dissociated with 0.25% trypsin 1:250/0.1% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline for 10 min at 37°C with constant shaking. Trypsin was inhibited by the addition of Opti-MEM containing 5% fetal bovine serum (FBS). Cells were plated at an initial density of 2.0–3.0×10^5 cells/60 mm culture dish in Opti-MEM [containing Earle's salts and 25 mM *N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer] supplemented with 2 mM glutamine, 5% FBS, 100 μg/ml streptomycin and 100 units/ml penicillin. MEMM cells were grown to subconfluence at 37°C in a 95% air/5% CO_2 atmosphere, with media replaced every other day.

BMP2 and BMP4 treatment regimen

MEMM cells were maintained in medium containing 5% FBS for 96 h. Cells were then rinsed with serum-free medium and received medium containing 0.25% FBS to effect growth arrest and cell synchrony. After 48 h, serum-deprived cells were released from growth arrest by exposure to fresh medium containing 5% serum with vehicle [4 mM HCl, 0.1% bovine serum albumin (BSA)], BMP2 or BMP4 (100 ng/ml; R & D Systems; Minneapolis, MN, USA). The kinetics of embryonic maxillary mesenchymal cell induced proliferative quiescence and release from cell growth arrest using this procedure has been described previously (23). Following treatment, cells were then incubated at 37°C in a 95% air/5% CO_2 atmosphere for an additional 4 h.

RNA extraction

Total RNA from BMP2-, BMP4- or vehicle-treated MEMM cell samples was isolated using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's recommendations. The quality and quantity of the extracted total RNAs were assessed by formaldehyde agarose gel electrophoresis and spectrophotometric UV absorbance at 260/280 nm, respectively. Three distinct RNA samples were processed to prepare three sets of target RNAs from BMP2-, BMP4- or vehicle-treated MEMM cells and were applied to three GeneChips for each of the three types of treatments (nine samples and nine GeneChips total).

Target synthesis

To prepare double-stranded cDNA, 5 μg of total RNA was denatured and annealed to 0.1 nM of HPLC-purified, T7-tagged, oligo-dT primer (5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-3′, Genset; La Jolla, CA, USA) for 10 min at 70°C. The reaction was cooled on ice and then reverse transcription was performed with 400 U of Superscript II at 37°C for 10 min in 1X first-strand buffer, 10 mM of dithiothreitol, and 0.5 mM of each dNTP, in a total volume of 20 μl (all reagents from one cycle target labeling kit, Affymetrix; Santa Clara, CA, USA). Second-strand cDNA was synthesized by adding 40 U of DNA polymerase I, 10 U of *Escherichia coli* DNA ligase, 2 U of RNase H, 30 μl of 5X second-strand buffer, 3 μl of 10 mM each dNTP, and water to 150 μl total volume and incubating for 120 min at 16°C. Subsequently, 20 U of T4 DNA polymerase was added, and the incubation continued at 16°C for 10 min, following which second-strand cDNA synthesis was stopped by the addition of 10 μl of 0.5 M EDTA. The resulting double-stranded cDNA was purified by using the cDNA clean-up module (Affymetrix), precipitated with 0.5 volume of 7.5 M ammonium acetate containing 2 μg of glycogen as carrier and 2.5 volumes of absolute ethanol. The resulting double-stranded cDNA pellet was resuspended in RNase-free water.

Synthesis of biotin-labeled cRNA and GeneChip hybridization

One microgram double-stranded cDNA was transcribed *in vitro* using the cRNA Transcript Labeling Kit (Affymetrix) according to the manufacturer's

instructions using biotinylated CTP and UTP. Following a 16-h incubation at 37°C, the resultant biotin-labeled cRNA was purified with the cRNA clean-up module (Affymetrix) and eluted in 40 μl of RNase-free water. The concentration of biotin-labeled cRNA was determined by spectrophotometric UV absorbance at 260/280 nm. Twenty microgram of labeled cRNA was fragmented in 40 μl 1X fragmentation buffer (40 mM Tris-acetate pH 8.1, 100 mM K-acetate, 30 mM Mg-acetate) for 35 min at 94°C and assessed by agarose gel electrophoresis. The fragmented cRNA was brought to a total volume of 300 μl with 1X hybridization buffer (100 mM Morpholine Ethane Sulphonic Acid [MES], 1 M NaCl, 20 mM EDTA, and 0.01% Tween 20) resulting in final concentrations of 100 μg/ml herring sperm DNA, 500 μg/ml acetylated BSA, 50 pM biotinylated control oligonucleotide B2 and 1X eukaryotic hybridization controls (1.5 pM BioB, 5.0 pM BioC, 25 pM BioB, and 100 pM cre; Affymetrix; Santa Clara, CA, USA). Target cRNAs corresponding to BMP2-, BMP4- or vehicle-treated MEMM cells were hybridized to individual GeneChips from an identical lot of Affymetrix Murine Genome 430 2.0 GeneChip arrays for 16 h. GeneChip arrays were washed and stained using antibody-mediated signal amplification and the Affymetrix Fluidics Station's standard Eukaryotic GE Wash 5′ protocol.

Microarray data analysis and presentation

Images from the scanned chips were processed using Affymetrix GCOS 1.2 software. For analysis of the three different, treated MEMM target RNA samples (Vehicle- vs. BMP2- vs. BMP4-treated), the GeneChip image of the vehicle treated sample was normalized to the corresponding images of either the BMP2- or BMP4-treated samples across all probe pair sets. Difference call, fold change, average difference value, and absolute call data from each of the three MEMM cell samples were exported. The full dataset was obtained using Affymetrix GCOS 1.2 and contained expression levels in Vehicle-, BMP2- or BMP4-treated MEMM cells for all 45 101 genes and expressed sequence tags (ESTs). The CEL files containing individual raw chip data (probe intensities) were imported to GeneSpring 7.2 and were pre-processed using Robust Multi-chip Average, with GC-content background correction (GC-RMA). These GC-RMA normalized data were then further normalized using the 'per gene normalization' step in which all the samples were normalized against the median of the control samples (i.e. the expression value for one gene across the different conditions is centered on 1, by dividing the expression value by the median expression value for that gene across the conditions. This ensured that genes that did not change across conditions received a normalized expression value of 1, allowing for easy visual detection of differentially expressed genes). To define a set of statistically significant, differentially expressed genes, a one-way ANOVA (parametric test, assuming equal variances) was applied with 'Benjamini and Hochberg false discovery rate' as the multiple testing correction ($p = 0.05$). This restriction tested each of the 45 101 genes and generated a list of 1135 genes with statistically significant expression values. A filter on the fold change (Probes with fold differences >1.5 were considered significant) was next applied to the list of 1135 genes and two lists of genes were generated based on the treatment conditions (either BMP2 vs. vehicle or BMP4 vs. vehicle). One such list included 749 genes that were either >1.5-fold up or downregulated in BMP2-treated MEMM cells with respect to vehicle treatment. The other list contained 679 genes that exhibited >1.5-fold increase or decrease in expression in response to BMP4 treatment when compared with controls.

Hierarchical clustering analysis was performed using the GeneSpring v 7.2 software (Silicon Genetics, Inc., Redwood city, CA, USA) to generate dendrograms (Fig. 1) representing each functional category of genes based on their expression profiles. Heat maps (Fig. 1) were generated by dividing each measurement by the 50.0th percentile of all measurements in that sample, then setting the average value of expression level for each gene across the samples to 1.0, and plotting the resulting normalized signal value for each sample (values below 0.01 were set to 0.01). The list and the order of various genes in which they appear in the heat maps can be viewed in tabular form (Tables S1 and S2).

Quantitative real-time PCR (TaqMan)

Total RNA prepared from BMP2-, BMP4- or vehicle-treated MEMM cells was treated with DNase I in the presence of RNaseOUT (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) to remove DNA contamination before cDNA synthesis. cDNA was synthesized with random hexamer primers and Superscript II reverse

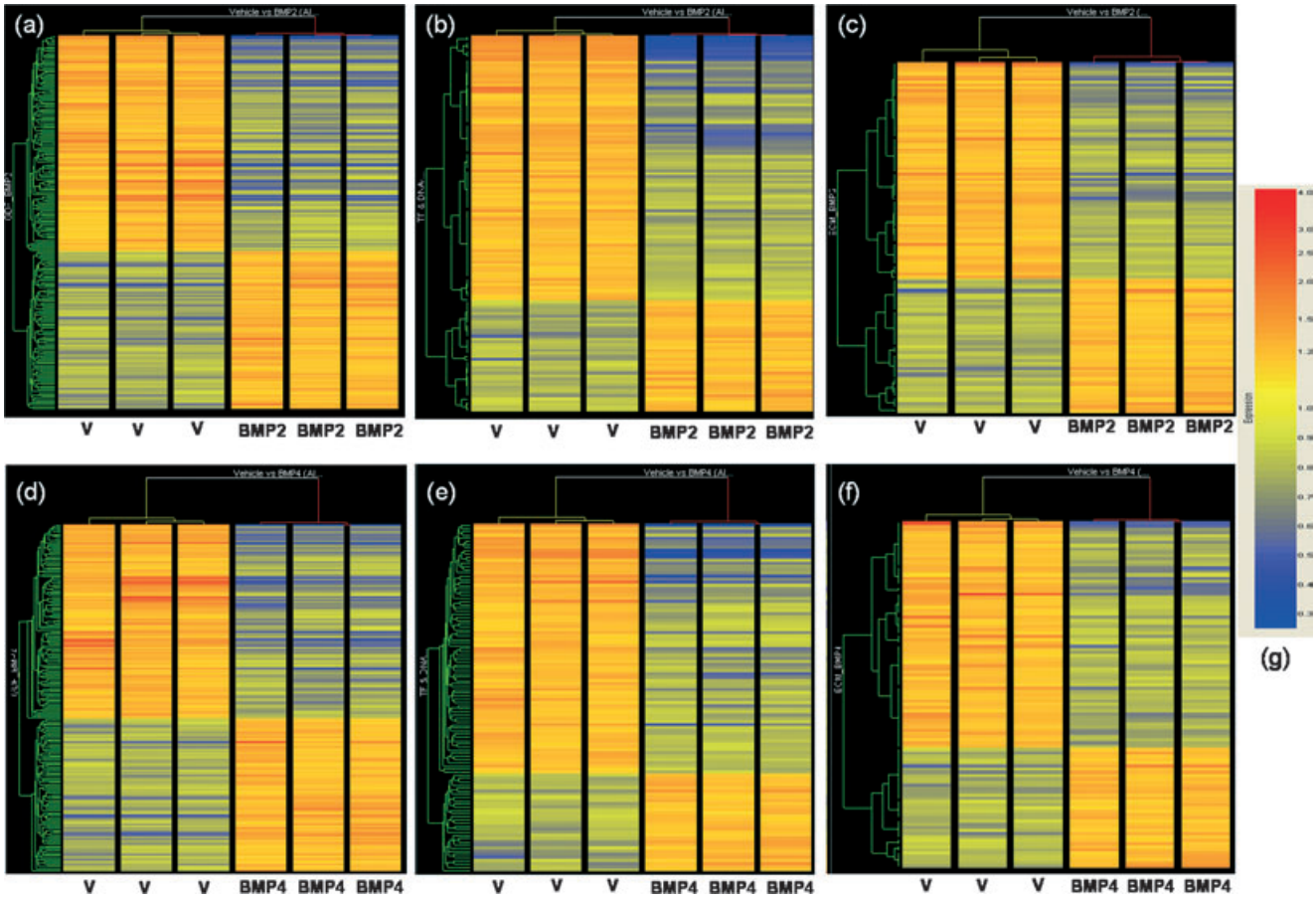

Fig. 1. Heat maps illustrating the differentially regulated genes undergoing significant alteration in expression in the murine embryonic maxillary mesenchymal (MEMM) cells following vehicle, BMP2 and BMP4 treatments. (a, d) Growth and differentiation factors and miscellaneous signaling molecules; (b, e) transcription factors and DNA-binding proteins (BMP2 vs. vehicle treatment); (c, f) extracellular matrix and matrix-associated proteins (BMP2 vs. vehicle treatment); (a, b & c): (BMP2 vs. vehicle treatment); (d, e & f): (BMP2 vs. vehicle treatment). Each row of the heat map represents a gene, and each column represents a treatment (as labeled at the bottom). The color saturation (g) represents the level of gene expression. Red indicates an increase in gene expression, whereas blue indicates a decrease. Genes whose expression demonstrated a 1.5-fold or greater increase or decrease are depicted.

transcriptase (Invitrogen Life Technologies, Inc.). Real-time PCR (TaqMan) analysis was performed on a TaqMan ABI Prism 7700 Sequence Detector (Applied Biosystems; Foster City, CA, USA). Matching primers and fluorescence probes for each of the selected genes and glyceraldehyde triphosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems. For each of the 26 genes analyzed, both forward and reverse primers were used at a concentration of 900 nM and the final fluorescent probe concentration was 200 nM. The PCR reaction was performed in a total volume of 25 μl containing 0.2 mM dATP, dCTP, and dGTP, 0.4 mM dUTP, 0.625 unit of Amplitaq Gold and 2 μl of cDNA template. Cycling parameters were: 50°C for 2 min for probe and primer activation, 95°C for 10 min for denaturation of DNA strands, followed by 40 cycles of denaturation at 95°C for 15 s, and primer extension at 60°C for 1 min. Data were acquired and processed with Sequence Detector software, version 1.0 (Applied Biosystems). For each reaction, a parallel reaction lacking template was performed as a negative control. Each determination of mRNA amount for the 26 genes analyzed was normalized to GAPDH mRNA present in each sample by using TaqMan GAPDH PCR primers and probe.

Results

A high-density oligonucleotide-based microarray technique was utilized to investigate gene expression profiles of MEMM cells treated with either BMP2 or BMP4. Double-stranded cDNA mixtures derived from three independent sets of vehicle-, BMP2- and BMP4-

treated MEMM cells (nine total samples) were transcribed into biotin-labeled cRNA and were used to probe separate Affymetrix high density GeneChip arrays containing oligonucleotide probes representing 45 101 genes and ESTs. When hybridized with cRNA derived from vehicle-, BMP2- or BMP4-treated MEMM cells, >22 000 genes and ESTs demonstrated a detectable level of expression. Steady-state levels of 448 genes (probe sets) were upregulated and those of 301 genes (probe sets) were downregulated in BMP2-treated MEMM cells in comparison with vehicle-treated MEMM cells. Similarly, steady-state levels of 409 genes (probe sets) were upregulated and those of 269 genes (probe sets) were downregulated in BMP4-treated MEMM cells in comparison with vehicle-treated MEMM cells (Tables S1 and S2). Such alterations in gene expression were reproducible in triplicate samples of MEMM cells treated with either BMP2 or BMP4 and include differential expression of a panoply of genes encoding molecular markers, such as: extracellular matrix (ECM) and matrix-associated proteins, growth and differentiation factors and miscellaneous signaling molecules, and transcription factors and DNA-binding proteins. Heat maps and dendograms (Fig. 1) generated by hierarchical clustering analysis with the GeneSpring v7.2 software (Silicon Genetics, Redwood City, CA.) provided visual demonstrations of differentially expressed genes in MEMM cells representing all the three functional categories in response to either BMP2 or BMP4 treatment.

Emphasizing the importance of phosphorylation as a mediator of biological function in developing tissues, genes for multiple protein kinases as well as protein phosphatases were differentially regulated in BMP2- and BMP4-treated MEMM cells (Fig. 1; Tables S1A and S2A). In this study, the modulation of expression of numerous genes encoding a wide range of transcription factors and DNA-binding proteins was also demonstrated in MEMM cells following BMP-2 and -4 treatment (Tables S1B and S2B). In support of the notion that growth factor-mediated synthesis/turnover of the ECM is essential for proper mesenchymal cell function during maxillary/palatal development, expression of a number of genes encoding multiple ECM proteins, structural proteins, and adhesion molecules were found to be differentially regulated in MEMM cells in response to both BMP2 and BMP4 treatment (Fig. 1; Tables S1C and S2C). The significance of rapid protein turnover, including turnover of the ECM, during maxillary/palatal development is also evident from differential levels of expression of genes encoding various proteases in MEMM cells treated with either BMP2 or BMP4 (Fig. 1; Tables S1C and S2C). In addition to the myriad of genes grouped into the three functional categories noted above, 75 BMP2-regulated and 60 BMP4-regulated genes with a wide variety of physiological functions were detected and categorized as 'non-classifiable' (Tables S1D and S2D). These differentially regulated genes encode enzymes, transport proteins, proteins involved in ribosome biogenesis, apoptosis, and the immune response, and proteins with several other known and unknown functions. Finally, a substantial number of differentially expressed ESTs (Tables S1D and S2D) were also detected during mammalian orofacial development. Many of these ESTs may correspond well to the novel, uncharacterized, developmentally regulated genes executing vital functions during mammalian orofacial ontogenesis.

Using the TaqMan quantitative real-time PCR technique (24), a specific and sensitive method permitting detection and quantification of mRNA species, gene expression profiling results obtained by microarray analyses were independently validated. Relative expression levels of 26 candidate genes that showed diverse patterns of differential regulation in response to either BMP2 or BMP4 treatment of embryonic maxillary mesenchymal cells were quantified by real-time PCR and compared to those levels determined by the microarray technique. Expression profiles of each of the 26 mRNAs tested were found to be consistent when comparing the two methods (Table 1).

Discussion

Genomic array technology is a powerful technique that enables analysis of genome-wide gene expression. This methodology has been utilized increasingly to identify individual genes as well as pathways critical for embryonic development (25). Numerous studies indicate that bone morphogenetic protein (BMP) signaling plays a critical role in development of the maxillary mesenchyme derived secondary palate and in the

Table 1. **Verification of GeneChip® microarray data by TaqMan™ quantitative real-time PCR***

Gene†	Concordance‡
Id-1	+/+
Id-2	+/+
Id-3	+/+
Msx1	+/+
Dlx-1	+/+
Dlx-2	+/+
Dlx-5	+/+
Smad-6	+/+
Smad-7	+/+
Smad-9	+/+
SnoN	+/+
Snail	+/+
Slug	+/+
BMP4	+/+
Noggin	+/+
BAMBI	+/+
Sox9	+/+
Sox11	+/+
Irx3	+/+
Irx5	+/+
Hey1	+/+
Osr1	+/+
MMP16	+/+
Glis2	+/+
YY1-associated factor 2	−/+
Transducin beta like 2	+/+

*Differential expression of twenty-six genes in murine embryonic maxillary mesenchymal (MEMM) cells following treatment with either BMP2 or BMP4 were compared using Affymetrix GeneChip™ arrays and TaqMan® quantitative real-time PCR as detailed in Materials and methods. All analyses were performed in triplicate.

†Target genes were selected based on results from Affymetrix GeneChip™ arrays.

‡'+/+' indicates full concordance in the pattern/level of gene expression obtained in MEMM cells following treatment with either BMP2 or BMP4 using Affymetrix GeneChip™ arrays and TaqMan® quantitative real-time PCR.

developing orofacial region in general (11–13,26). In this study, oligonucleotide-based microarray chips were utilized to profile expressed genes directly or indirectly associated with BMP2 and BMP4 signaling in murine embryonic maxillary mesenchyme (MEMM). Genes affected by BMP2 or BMP4 could be broadly categorized into three functional categories: growth and differentiation factors and miscellaneous signaling molecules, transcription factors and DNA-binding proteins, and ECM and matrix-associated proteins. The transcriptional map of BMP-responsive genes offers revealing insights into potential molecular regulatory mechanisms employed by BMP2 and BMP4 in orchestrating orofacial ontogeny.

Growth and differentiation factors and miscellaneous signaling molecules

Of the 45 101 genes and ESTs screened in this study, expression of a number of genes encoding various growth and differentiation factors was found to be significantly altered in MEMM cells by BMP2 and BMP4 treatment. Genes in this category encode a diverse group of proteins including, but not limited to, growth factors, growth factor receptors, and hormones (Tables S1A and S2A).

A class of signaling molecules central to normal development is the transcription growth factor β (TGFβ) family. Members of this family regulate growth, differentiation and tissue morphogenesis in species as diverse as worms and mammals. The TGFβs have been shown to play a critical role in the developing mammalian orofacial region (27–31). In this study, the gene encoding the type III TGFβ receptor (TβRIII) or betaglycan was downregulated 6.2-fold, the gene encoding the TGFβ type II receptor was downregulated 2.3-fold, whereas the genes encoding inhibitors of TGFβ, activin and nodal signaling, Smad-6 and -7, were upregulated following BMP-2 or -4 treatment (Tables S1A and S2A). In contrast, genes encoding several components of the BMP signaling pathways such as ActRIA (or ALK2, a type I receptor for both BMPs and activins), bone morphogenetic protein receptor, type II and Smad-9 (also known as Smad-8) were upregulated following BMP-2 or -4 treatment (Tables S1A and S2A). These results may reflect a BMP-induced bias towards the BMP signaling pathway at the expense of other competing pathways such as those initiated by TGFβs, activins, or nodal. Interestingly, expression of genes encoding a number of modulators of TGFβ superfamily signaling [noggin, BMP-binding endothelial regulator (crossveinless 2), BAMBI, follistatin, a protein related to DAN and Cerberus] was differentially altered in BMP-treated MEMM cells (Tables S1A and S2A). Downregulation of the gene encoding BMP4 (Tables S1A and S2A) may reflect an

autoregulatory capacity of the BMPs in these cells. Originally identified by their bone-inducing activities, BMP's widespread expression suggests many roles other than that in osteogenesis. BMP4 has been linked to numerous developmental processes such as epithelial–mesenchymal interactions during tooth morphogenesis (32,33), patterning and differentiation of the inner ear (34), lens (35) and mandibular cartilage (36), and programmed cell death (37), all necessary for normal craniofacial development. A number of BMPs, including BMP-2 and -4, are also expressed in discrete spatiotemporal patterns in developing orofacial tissue (12,18,19). Of particular relevance to orofacial development is the observation that Msx1-dependent expression of BMP4 and BMP2 in the mesenchyme of developing maxillary tissue is critical for normal development of the palate (20).

Members of the *Wnt* and *Frizzled* gene families have been reported to play numerous roles during embryonic development, including CNS patterning and patterning of the dorsoventral axis of the limb bud (38). At a cellular level, some members of these families have been demonstrated to modulate cell adhesion, proliferation, and communication through gap-junctions (38,39). Expression of *Wnt-5a*, *-10a*, *-10b*, and *-11* has been detected in the mesenchyme of the developing murine facial primordia (40). We have previously shown that genes encoding various Wnt family members (Wnt-3, -4, -5a, and -10b) and Frizzled-related proteins (Fzd-4, Flamigo-1, secreted frizzled related sequence protein 4 or Sfrp4) were also found to be differentially expressed in the embryonic orofacial tissue suggesting yet unknown functions of these genes in the orofacial development (41). In this study BMP-2 and -4 both upregulated the expression of genes encoding several frizzled proteins such as Fzd-4, -7 and -9 pointing towards a cooperative interaction between the BMP and Wnt signaling pathways (Tables S1A and S2A) in developing orofacial tissue.

Vascular endothelial growth factor (VEGF) is one of the several cytokines and growth factors that mediate vasculogenesis and angiogenesis in the developing embryo. This multifunctional cytokine, secreted by cells at angiogenic sites, triggers an angiogenic cascade by interacting with two high-affinity tyrosine kinase receptors that are selectively expressed on vascular endothelium (42). The expression of VEGF is potentiated in MEMM cells in response to TGFβ1, implicating that TGFβ-induced VEGF contributes to vasculogenesis in embryonic craniofacial tissue (49). In this study, both BMP-2 and -4 repressed the expression of VEGFc in MEMM cells suggesting an additional level of regulation of VEGF-induced vasculogenesis (Tables S1A and S2A).

Fibroblast growth factors (FGFs) represent a large family of paracrine and autocrine factors that function in controlling cell differentiation, proliferation, survival and motility (43). Six members of this family (*FGF-1*, *-2*, *-4*, *-5*, *-8*, and *-12*) have been localized in the developing facial primordia and have been reported to regulate outgrowth of these primordia. Differentially regulated expression of two FGF-encoding genes, *Fgf-2* and *Fgf-15*, and a steady upregulation of the *FGF-BP1* gene, have also been reported in gestational days 12–14 murine orofacial tissue (41). Moreover, in humans, several craniofacial syndromes have been linked to mutations in the genes encoding FGF receptors (40). A growing body of evidence implies positive and negative interactions between members of the BMP and FGF families at various stages of embryonic, especially craniofacial development (44). For instance, FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth morphogenesis as well as regulate apoptosis (32,37). In this study *Fgf-7* and *Fgf-9* were significantly downregulated whereas the gene encoding FGF receptor 2 was considerably upregulated following BMP2 or BMP4 treatment (Tables S1A and S2A). These findings add further support to the notion of FGF/BMP cross-talk during orofacial patterning and morphogenesis.

Retinoic acid (RA) is a potent cleft palate-inducing teratogen. The effects of retinoic acid on gene expression follow mainly from its translocation to the nucleus and the activation of specific elements within the promoter/enhancer of its target genes. Both translocation and promoter activation are mediated by two classes of proteins that specifically bind RA: the nuclear retinoic acid receptors (RARs) and retinoid $\times$ receptors (RXRs), and the cytoplasmic cellular retinoic acid-binding proteins (CRABPs) (45). CRABP I and CRABP II, belong to a family of small cytosolic lipid-binding proteins that are expressed during embryogenesis in specific spatio-temporal patterns (46,47). Notably, cells derived from mammalian embryonic maxillary tissue also express CRABP I and CRABP II (48). It is likely that these proteins serve as the regulators of transport and

metabolism of retinoic acid in the developing embryo and throughout adult life. Genes encoding RXRγ, Rai-12 (a RA-induced growth regulatory protein), and RBP-4 demonstrated increased expression from gestational day 13 to gestational day 14 in developing orofacial tissue suggesting a role for endogenous RA in embryonic orofacial morphogenesis (41). TGF-β1, -β2 and -β3 have been shown to downregulate the expression of CRABP I mRNA in cells derived from embryonic maxillary tissue, while TGFβ1 stimulated a dose-dependent increase in the expression of CRABP II mRNA (48, 49). In this study, both BMP2 and BMP4 significantly downregulated ($>$2.5 to 3.0-fold) the expression of genes encoding RARβ and CRABP II suggesting that, similar to the TGFβs, BMPs also contribute to the regulation of RA signaling in MEMM cells (Tables S1A and S2A).

Transcription factors and DNA-binding proteins

Transcription factors (TFX) are key among the various functional groups of proteins that orchestrate embryonic development. Of the 45 101 arrayed genes and ESTs investigated in this study, the expression of a wide range of genes encoding TFXs was differentially regulated in response to the treatment with either BMP2 or BMP4. Recently, studies involving targeted gene mutations, micro-injection of specific mRNAs, and gene transfer experiments have identified a growing number of homeobox genes such as *Mhox*, *Dlx-1*, and *-2*, *Hoxa-2*, *Msx1*, and *Pax-9*, which play crucial roles in the development of the mammalian orofacial region (50–55). Targeted disruption or mutation of these homeobox genes results in craniofacial dysmorphogenesis. Among the homeobox genes, six *Dlx* genes (*Dlx-1*, *2*, *3*, *5*, *6*, and *7*) have been shown to be expressed in spatially restricted patterns within the embryonic craniofacial ectoderm and ectomesenchyme whereas several have been shown to be essential for normal craniofacial development (54,56). Gene targeting of *Dlx-1*, *-2*, *-3* and *-5* in the mouse germ line has revealed functions for these genes in craniofacial patterning, sensory organ morphogenesis, osteogenesis, and placental formation (57). Steady-state expression of genes encoding Dlx-3, -6 and -7 was detected in the developing orofacial region, as was considerable upregulation (4.7-fold) of expression of an EST similar to the *Dlx-1* gene (41). Expression of several *Dlx* genes has been reported to be regulated by BMP signaling (58) which is supported by considerable upregulation (2.5 to 7.0-fold) of a number of *Dlx* genes (*Dlx-1*, *Dlx-2* and *Dlx-5*) in MEMM cells following BMP-2 or -4 treatment (Tables S1B and S2B). The differential regulation of these genes during orofacial development is consistent with their importance to the proper development of first branchial arch derived structures.

Previous data from both RNA expression analyses and gene-targeting experiments have demonstrated that the paired-related homeobox genes, *prx1* and *prx2* are critical for limb and craniofacial development in mice (59). *prx1* and *prx2*, are expressed in the post-migratory cranial mesenchyme of all facial prominences and are required for the formation of proximal first arch derivatives where they coordinately regulate gene expression in cells that contribute to the distal aspects of the mandibular arch mesenchyme (60). As the first arch mesenchyme represents the contributing tissue to embryonic palate mesenchymal cells, the TGFβ-induced downregulation of *prx2* observed in an earlier study (49) as well as BMP2- and BMP4-induced upregulation of *prx2* in the current report (Tables S1B and S2B), may reflect a means by which these genes expression are coordinately regulated by the TGFβ superfamily members in the developing first branchial arch.

Members of the *Drosophila Iroquois* homeobox gene family have been implicated in the development of the peripheral nervous system and the regionalization of wing and eye imaginal discs (61). Recent studies suggest that *Xenopus Iroquois* homeobox (*Irx*) genes are also involved in neurogenesis (61). Five mouse *Irx* genes, *Irx1*, *Irx2*, *Irx3*, *Irx5* and *Irx6*, have been identified and are known to be expressed with distinct spatio-temporal patterns during neurogenesis (61,62). Beginning at E9.5, *Irx1* and *Irx6* expression is found in the epithelial component of murine branchial arches and foregut, whereas *Irx3* and *Irx5* display similar expression in these regions on E10.5 (62). In this study, BMP2 and BMP4 upregulated the expression of *Irx3* and *Irx5* in MEMM cells (Tables S1 and S2) demonstrating, for the first time, the expression of these homeobox genes in cells derived from the developing orofacial tissue and also the regulation of their expression in these cells by the BMPs (Tables S1B and S2B).

The homeobox-containing *Msx* genes are considered to be mediators of epithelial–mesenchymal interactions and have been reported to be critical for proper craniofacial development. This notion is supported by the finding that the loss of *Msx1* function in mice results in a hypoplastic mandible (52) whereas a gain-of-function mutation of the *Msx2*/*MSX2* gene in mice and humans causes craniosynostosis (63,64). Thus, morphogens such as BMP2 and BMP4, regulating the expression of *Msx* genes, represent key regulators of orofacial development. Expression of *Bmp4* and *Bmp2* in facial primordia is associated with the expression of *Msx1* and *Msx2* (9). Additionally, ectopic application of BMP-2 and BMP-4 can activate *Msx1* and *Msx2* gene expression in the developing facial primordia suggesting that signaling by these two BMPs is essential for regulating the outgrowth and patterning of the facial primordia (9). Reciprocal interactions are supported by the observation that expression of *Bmp4* and *Bmp2* in developing palate mesenchyme requires expression of the *MSX1* homeobox gene (20). The functional importance of this resides in the fact that mutations in the *MSX1* homeobox gene are associated with non-syndromic cleft palate and tooth agenesis in humans (21), and that transgenic expression of human *Bmp4* in *Msx1*(−/−) murine embryonic palatal mesenchyme rescues the cleft palate phenotype (20). In view of these observations it is not surprising that both BMP2 and BMP4 significantly upregulated the expression of *Msx2* (and also *Msx1* as demonstrated by TaqMan QRT-PCR) in MEMM cells (Tables S1B and S2B).

Interestingly, both BMP2 and BMP4 notably upregulated the expression of a gene encoding Msx-interacting zinc finger protein or Miz1 in MEMM cells (Tables S1B and S2B). Miz1, a member of the POZ domain/zinc finger transcription factor family, acts as a sequence-specific transcriptional activator, and is expressed in the developing embryo, in a spatiotemporal pattern that overlaps with the expression of *Msx2* (65). Moreover, Miz1 interacts directly with Msx2 *in vitro*, enhancing its DNA-binding affinity and forms a complex with the Myc oncoprotein, recruiting Myc to core promoter elements and repressing transcription through Miz1-binding sites *in vivo* (65). *Miz1*$^{-/-}$ embryos are severely retarded in early embryonic development and do not undergo normal gastrulation, succumbing to massive apoptosis of ectodermal cells by day 7.5 of embryonic development (66). Elucidation of the physiological function of Miz1 in orofacial development warrants further investigation.

The Sox factors are comprised of a novel group of proteins characterized by the presence of a 79–amino acid motif, known as the 'SRY box', which forms an HMG-type DNA-binding domain. It has been proposed that the Sox family of proteins governs cell fate decisions during embryogenesis by acting both as transcription factors and architectural components of chromatin (67). Some of these Sox transcription factor encoding genes (e.g *Sox9*) are downstream targets of BMP signaling (68). During early somitogenesis, *Sox1*, *Sox2*, and *Sox3* genes are expressed in the neuroectoderm, while *Sox2* and *Sox3* are also expressed in the primitive steak ectoderm, gut endoderm, and prospective sensory placodes (69). Differentially regulated expression of four *sox* genes, *Sox1*, *Sox3*, *Sox4*, and *Sox11*, has been reported in the embryonic orofacial region (41). BMP-2 and -4 upregulated the expression of *Sox9* and *Sox11* but downregulated *Sox4* expression in MEMM cells in this study (Tables S1B and S2B). These results highlight a possible role for BMP-regulated expression of Sox factors in the growth and/or differentiation of cells during orofacial ontogenesis.

A number of studies have revealed that Id (inhibitor of differentiation or inhibitor of DNA binding) Helix Loop Helix (HLH) proteins are key targets of BMPs and it is likely that they are responsible for mediating various biological activities of this growth factor family (70). Id proteins were identified as negative regulators of bHLH transcription factors, functioning as positive regulators of cell proliferation and negative regulators of cell differentiation (71). Id2 physically interacts with the active, hypophosphorylated form of Rb family proteins, inhibiting their antiproliferative functions (72). It has been postulated that during embryonic development Id proteins are involved in coordinating the balance between cellular proliferation and differentiation (73). Although the expression of Id proteins is induced by various stimuli, BMPs are one of the most effective in increasing their synthesis (74,75). Genes encoding each of the four Id proteins are expressed in the developing craniofacial region with Id1 and Id2 being strongly expressed in the maxillary and mandibular mesenchyme (76). In this study, BMP2 and BMP4 significantly upregulated the expression of genes

encoding all three Ids (Id1, Id2 and Id3) in MEMM cells (Tables S1B and S2B). These results suggest the possibility that Ids, as downstream targets of BMP signaling, mediate various biological functions of BMP2 and BMP4 in MEMM cells, especially in synchronizing the balance between cell proliferation and differentiation indispensable for normal orofacial development.

The Notch signaling pathway has been identified as a key regulator of cell fate that is conserved from *Caenorhabditis elegans* to humans (77,78). There are four known homologs of Notch (Notch 1–4) in mammalian cells (78). Notch-dependent restriction in progenitor cell formation for a variety of cell lineages is utilized in *Drosophila* during the differentiation of neurons, muscles, the intestines, heart and other organs, and in vertebrates is specifically implicated in neurogenesis and myogenesis (77). Signaling through both the TGFβ superfamily of growth factors and Notch, plays a crucial role during the embryonic pattern formation and cell fate determination (79). Recently a functional interaction between these two signaling pathways has been described (79). Thus, not surprisingly, evidence exists for both synergy and antagonism between TGFβ and Notch signaling (79–81). These reports describe a requirement for Notch signal transducers in TGFβ- and BMP-induced expression of Notch target genes, as well as in BMP-controlled cell differentiation and migration. Moreover, they reveal a direct link between the Notch and the TGFβ superfamily signaling pathways and suggest a critical role for Notch in some of the biological responses to TGFβ family signaling. The genes *Jagged1* and *Jagged2* encode two ligands for the Notch family of transmembrane receptors. Both proteins have been implicated in craniofacial development as mutations in the corresponding genes are known to be associated with craniofacial anomalies (82–83). Results from this study demonstrated that both BMP-2 and -4 upregulated the expression of *Jagged1* (Tables S1A and S2A). Recent studies suggest that Notch signaling components are necessary for BMP4-dependent induction of Notch target genes. Many of the genes encoding the Hes/Hey family of transcription factors are the target genes of Notch signaling. Examples of such genes include *Hes1*, *Hes5*, *Hey1* and *Hey2* (84). RNA *in situ* hybridization analysis revealed specific expression patterns of *Hey1* and *Hey2* during development of the nervous system, somites, the heart and the craniofacial region (85,86). Genes encoding a number of Hes and Hey family of transcription factors and all the isoforms of Notch (Notch 1–4) have been detected in the developing orofacial region (41) and in this study, genes encoding several Hes and Hey proteins were significantly upregulated in MEMM cells following BMP-2 and -4 treatment (Tables S1B and S2B). These observations are supportive of the notion that Hes and Hey transcription factors are involved in proper development of the murine orofacial region and transcriptional control of their genes may be regulated via cross-talk between BMP-2/-4 and Notch signaling.

ECM and matrix-associated proteins

In addition to providing mechanical support, interaction with the ECM, also supplies cells with a wealth of information necessary for the regulation of cell fate and morphology. Accordingly, cell–ECM communications are essential for mediating diverse physiological events such as lineage decisions during embryogenesis, differentiation, cell migration, cell adhesion, wound healing and apoptosis. It is thus not surprising that a wide spectrum of ECM remodeling and turnover events occurs during growth and development. Cell surface and matrix-associated proteins including various adhesion molecules and ECM proteases play central roles in these processes. Development of the orofacial region is a complex process that involves remodeling of the ECM. The period during morphogenesis of the secondary palate is marked by an increase in ECM production and mesenchymal shelf volume (87–88). Proper metabolism of this ECM in mammalian embryonic maxillary/palatal tissue is required for normal orofacial development (28) as disruption of either glycosaminoglycan (89) or collagen synthesis (90) results in cleft palate.

The cadherins are a family of calcium-dependent cell adhesion molecules that are regulated both spatially and temporally during development. Epithelial cadherin (E-cadherin) is present in both embryo and yolk sac epithelium during organogenesis (91). Expression patterns of E-cadherin suggest a vital role in normal orofacial development (92,94). Further evidence for a role of E-cadherin in orofacial development comes from the presentation of cleft lip/palate in patients with CDH1/E-cadherin mutations (93). In this study, while a gene encoding E-cadherin was detected, but not BMP-

regulated, in MEMM cells, both BMP-2 and -4 significantly upregulated the expression of a gene encoding cadherin-6 (K-cadherin) (Tables S1C and S2C). Interestingly, the molecular cascade that acts downstream of BMP4 to drive neural crest cell delamination and migration, includes two genes encoding cadherin-6B and rhoB (95). These two genes are likely to be involved in this process, as their levels are affected by modifying BMP4 activity with noggin, at stages that follow their initial expression along the neural axis (95). Clarification of the precise role of K-cadherin in orofacial ontogenesis requires further study.

Protocadherins constitute the largest subgroup within the cadherin family of calcium-dependent cell–cell adhesion molecules. Genes encoding classical cadherins and protocadherins show highly conserved features, indicating diversification from a common ancestral gene. Like cadherins, protocadherins also play a crucial role in the morphogenesis of numerous tissues (96). Similar to other protocadherins, protocadherin-8 and -9 are predominately expressed in the fetal brain and their developmentally regulated expression pattern suggests that they direct various aspects of neurogenesis (97). In this study, BMP-2 and -4 significantly downregulated the expression of the genes encoding protocadherin-8 and -9 (Tables S1C and S2C). Expression of two other genes encoding cell adhesion molecules [bystin-like protein and activated leukocyte cell adhesion molecule (ALCAM)] was also found to be BMP regulated. Differential regulation of the genes encoding cadherin-6, protocadherin-8, -9, bystin-like and ALCAM by these two BMPs in MEMM cells, points to a complex, and previously unknown, interaction between these cell adhesion molecules and the TGFβ superfamily of growth factors during orofacial development.

Hyaluronan or hyaluronic acid (HA), is a complex carbohydrate, synthesized by integral plasma membrane glycosyltransferases or hyaluronan synthases and is exported directly into the extracellular space (98). Hyaluronan interacts *in vivo*, with other ECM molecules, creating a composite matrix network of HA, link protein, and aggrecan that plays a critical role in load-bearing articular cartilage (98). Hyaluronan has also been implicated in receptor-mediated cell adhesion and intracellular signaling and plays a vital role in diverse cellular events, including cell migration, tissue remodeling, and metastasis (99). During normal palate development, increasing regional organization and progressive hydration of the palatal shelf mesenchyme correlate with the increased hyaluronan deposition (100). Significant upregulation of a gene encoding hyaluronan synthase 2, the principal source of HA during murine organogenesis (98), in MEMM cells by BMP-2 and -4, suggests that regulation of hyaluronan synthesis in developing orofacial tissue may be mediated by BMPs (Tables S1C and S2C).

The cytoskeleton is important for controlling the cell shape and migration and organizing intracellular signaling complexes during embryonic development. Filamentous actin possesses unique biophysical and biochemical properties and is required for cell locomotion, cell division, cell compartmentalization, and morphological processes. Site-specific assembly and disassembly of these structures are directed by actin-regulatory proteins (101). Formins, a group of modular proteins, containing a series of domains and functional motifs, are potent regulators of actin dynamics. The Formin homology 2 (FH2) domain binds actin filament barbed ends and moves processively as these barbed ends elongate or depolymerize. Multiple formin isoforms are found in eukaryotic cells implicating diverse cellular roles (102). *Ectodermal neural cortex 1* or *Enc1* was isolated as a gene encoding another actin-associated protein that is expressed in the neuro-ectodermal region of the epiblast during early gastrulation, and later in the nervous system (103). This gene has also been reported as a downstream target of the Wnt/β-catenin/T-cell factor signaling pathway (104). Adducins are a family of cytoskeleton proteins encoded by three genes (alpha, beta, and gamma). Alpha- and gamma-adducins are ubiquitously expressed whereas expression of beta-adducin is somewhat restricted with high level of expression in the brain and hematopoietic tissues (bone marrow in humans, and spleen in mice) (105). Adducins can function *in vitro* to bundle F-actin and control the assembly of the F-actin/spectrin cytoskeletal network. Recently, alpha-adducin has been reported to be expressed in a restricted pattern in the somitic mesoderm during *Xenopus* early development, indicating an important yet unknown role of adducins during embryogenesis (106). Genes encoding many such cytoskeletal, actin-binding proteins (e.g. formin-2, ectodermal-neural cortex 1, actin-binding LIM protein 1, and Adducin-3 among others) manifested differential expression in MEMM cells upon treatment with BMP-2 and -4 (Tables S1C and S2C). Such findings highlight

the intricate regulation of the actin cytoskeleton by the BMPs during maturation of the orofacial primordia.

Fibronectins, high-molecular weight ECM glycoproteins that promote many specialized cell adhesive events, and collagens, provide structural integrity to the ECM. Fibronectin leucine rich transmembrane (FLRT)-1, FLRT2, and FLRT3 comprise a novel gene family isolated in a screen for ECM proteins expressed in muscles (107). These genes encode putative type I transmembrane proteins, each containing 10 leucine-rich repeats flanked by N-terminal and C-terminal cysteine-rich regions, a fibronectin/collagen-like domain, and an intracellular tail (107). The structural properties of the FLRT family members suggest that they play a role in cell adhesion and/or receptor signaling. In MEMM cells, significant downregulation of a gene encoding FLRT3, by BMP-2 and -4, suggests interplay between such ECM proteins and BMP signaling during orofacial ontogenesis (Tables S1C and S2C).

Tenascins represent another family of large multimeric ECM proteins with fibronectin domains. In vertebrates, four tenascins termed tenascin-C, -R, -X and -W are expressed in the connective tissues. Expression patterns of these four tenascins are tissue-specific. Unlike many other ECM proteins, tenascins promote only weak cell adhesion and do not activate cell spreading. In fact, tenascins have been classified as anti-adhesive, adhesion-modulating or even repellent ECM proteins. Scherberich et al. (107), reported that tenascin-W (also known as tenascin-N) is expressed during palate formation, osteogenesis, and smooth muscle development. In this study, a gene encoding tenascin-N was greatly induced by BMP-2 and -4, suggesting that during orofacial growth, transcription of some of the tenascin coding genes might be under the control of the BMPs. Furthermore, α8 integrin, has been reported to act as a receptor for tenascin-W (108). A gene encoding α8 integrin was found to be expressed (data not shown) and another gene encoding a closely related protein, α9 integrin, demonstrated upregulation following BMP-2 and -4 treatment in MEMM cells (Tables S1C and S2C). These findings suggest tenascin-W activity in developing orofacial tissue.

Genes encoding some of the small ECM proteoglycans of the decorin family (e.g. fibromodulin and betaglycan) also displayed altered expression in MEMM cells following BMP-2 and -4 treatment. These two proteoglycans can bind activated TGFβ and modulate its activity. Moreover, fibromodulin and betaglycan can regulate collagen fibril formation and tensile strength. In this study, at least five probes representing the *fibromodulin* gene demonstrated upregulated expression and the gene encoding betaglycan was substantially downregulated in MEMM cells following BMP-2 and -4 treatment (Tables S1C and S2C). Such differential regulation by BMP-2 and -4, is likely to be indicative of BMP-mediated control of the dual activities (modulations of ECM synthesis and TGFβ signaling) of these proteoglycans in MEMM cells.

A panoply of molecules with protease activity is associated with proteolytic processes in the ECM. Such proteases can be divided into several protein families based on their distinct domain structures. One group consists of serine proteases such as thrombin, tissue plasminogen activator, urokinase and plasmin (109). Another group consisting of the matrix metalloproteinases (MMPs), represents a large family of highly conserved Zn-dependent endopeptidases (110). The serine proteases and the MMPs usually operate as broad-spectrum proteases for key ECM degradation events (109,110). The third group, comprising the bone morphogenetic protein 1/tolloid family of metalloproteinases, has been implicated in cellular differentiation and pattern formation via activation of latent growth factors of the TGFβ superfamily (111). Finally, the ADAMs (for a disintegrin and metalloprotease) or the MDC (for metalloprotease/disintegrin/cysteine-rich) proteins are a family of transmembrane glycoproteins with varied roles in cell–cell adhesion and proteolysis (112).

It has been argued that MMPs play important roles in cellular differentiation by degrading components of the ECM. The activity of the MMPs must be precisely regulated by their endogenous protein inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). Several MMPs and TIMPs are expressed in discrete spatial and temporal patterns in developing orofacial tissue (113–116), and mediate matrix degradation during craniofacial osteogenesis (117). Analogous to an earlier demonstration of upregulation of TIMP3 by TGFβ1 in MEMM cells (49), data in the present report, representing an increase in TIMP3 expression in maxillary mesenchymal cells in response to BMP-2 and -4, suggest a common means by which activity of the MMPs and ADAMTSs (as discussed below) may be regulated

in embryonic orofacial tissue by the members of TGFβ superfamily of growth factors (49). Support for this notion comes from data demonstrating that TGFβ3-deficient mice, which display clefting of the secondary palate, exhibit complete absence of TIMP-2 in palatal tissue and express significantly lower levels of MMP-2 and MMP-13 (113). The absence of orofacial clefting in numerous matrix metalloproteinase (MMP) 'knock-out' mice (118,119), does not minimize their developmental importance; rather, the complex nature of this family of proteins suggests redundant functionality.

The ADAMs (for a disintegrin and metalloprotease) have been implicated as playing a functional role during cellular adhesion and proteolytic processing of important cell surface molecules. Recently, a new family of ADAM-related proteins, collectively known as ADAMTS (A Disintegrin-like And Metalloprotease domain with ThromboSpondin type I motifs) has been identified (120). Members of this family have the characteristic ADAM-like protease domain, but unlike the ADAMs, these proteins lack transmembrane domains and are secreted into the ECM. The ADAMTS proteins have several substrates within the ECM such as procollagens and proteoglycans. Notably, in common with MMPs, some ADAMTS family members (ADAMTS-4 and -5) show potent inhibition by TIMP-3 (121). ADAMTS4 and ADAMTS5 are aggrecanases implicated in the degradation of cartilage aggrecan in arthritic diseases and as having roles during embryonic development and angiogenesis (122). ADAMTS-like 3 (also known as ADAMTSL-3/punctin-2) is a novel glycoprotein in ECM, related to the ADAMTS family of metalloproteases. Its domain structure and matrix localization suggest that it may play a role in cell–matrix interactions or in assembly of specific extracellular matrices during embryogenesis (123). In MEMM cells, BMP-2 and -4 differentially regulated three ADAMTS proteases (ADAMTS-1, -5 and ADAMTS-like 3) (Tables S1C and S2C). Further studies on this emerging group of ECM proteases may provide valuable insights into developmental or pathological processes involving ADAMTSs and ECM remodeling during orofacial ontogenesis.

Data from this study report indicate that BMP2 and 4 significantly altered the expression of a number of proteases and protease inhibitors in MEMM cells. For example, genes encoding MMP-16, tissue plasminogen activator, ADAMTS-1 and -5 were notably downregulated whereas, those encoding ADAMTS-like 3, ecto-dermal-neural cortex 1 (an actin-binding cysteine-type endopeptidase), TIMP3 and Serpine-1 (or plasminogen activator inhibitor-1) were significantly upregulated (Tables S1C and S2C). These findings lend further support to the emerging concept that members of the TGFβ superfamily of growth factors (especially, TGFβs and BMPs) mediate local balance between various proteases (such as MMPs and ADAMTSs), and protease inhibitors (such as TIMPs and Serpines) and may play a major role in ECM remodeling during orofacial development.

All the aforementioned genes have been arbitrarily classified into the three functional categories noted above and are presented in Tables S1A–S1C and S2A–S2C. Tables S1D and S2D include a series of non-classified differentially expressed genes of potentially interesting expression and function that are not addressed in the present report. Moreover, several ESTs that were found to be differentially expressed in MEMM cells as a result of either BMP2 or BMP4 treatment are listed in Tables S1D and S2D.

The oligonucleotide-based microarray technology employed in the present study has allowed us to identify and categorize extensive changes in gene expression in the developing embryonic maxillary tissue in response to BMP2 and BMP4. The data reported, and our analysis of its significance, are based on current knowledge regarding orofacial morphogenesis. The experimental findings obtained from this study offer additional clarity of insight into the potential molecular regulatory mechanisms employed by BMP2 and BMP4 in directing their phenotypic effects during craniofacial ontogenesis.

Acknowledgements: This research was supported in part by NIH grants DE05550, DE12858 and ES11775, CDC grant RO6CCR420170, The Commonwealth of Kentucky Research Challenge Trust Fund, and P20 RR017702 from the COBRE program of the National Center for Research Resources.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1A. List of differentially regulated genes encoding growth and differentiation factors and miscellaneous signalling molecules in murine embryonic maxillary mesenchymal cells following treatment with BMP2.

Table S1B. List of differentially regulated genes encoding transcription factors and DNA-binding proteins in murine embryonic maxillary mesenchymal cells following treatment with BMP2.

Table S1C. List of differentially regulated genes encoding ECM and matrix-associated proteins in murine embryonic maxillary mesenchymal cells following treatment with BMP2.

Table S1D. List of differentially regulated non-classified genes and expressed sequence tags (ESTs) in murine maxillary mesenchymal cells following treatment with BMP2.

Table S2A. List of differentially regulated genes encoding growth and differentiation factors and miscellaneous signalling molecules in murine embryonic maxillary mesenchymal cells following treatment with BMP4.

Table S2B. List of differentially regulated genes encoding transcription factors and DNA-binding proteins in murine embryonic maxillary mesenchymal cells following treatment with BMP4.

Table S2C. List of differentially regulated genes encoding ECM and matrix-associated protiens in murine embryonic maxillary mesenchymal cells following treatment with BMP4.

Table S2D. List of differentially regulated non-classified genes and expressed sequence tags (ESTs) in murine embryonic maxillary mesenchymal cells following treatment with BMP4.

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