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EXPRESSION PROFILING OF TGFß SUPERFAMILY GENES IN DEVELOPING OROFACIAL TISSUE

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Abstract

Background—Numerous signaling molecules have been shown to participate in the dynamic process of orofacial development. Amongst these signal mediators, members of the transforming growth factor ß (TGFß) superfamily have been shown to play critical roles. Developing orofacial tissue expresses TGFß and BMP mRNAs, their protein isoforms and TGFß- and BMP-specific receptors. All these molecules display unique temporo-spatial patterns of expression in embryonic orofacial tissue, suggesting functional roles in orofacial development. For example, the TGFßs and BMPs regulate maxillary mesenchymal cell proliferation and extracellular matrix synthesis. This is particularly noteworthy in that perturbation of either process results in orofacial clefting. Although the cellular and phenotypic effects of the TGFß superfamily of growth factors on embryonic orofacial tissue have been extensively studied, the specific genes that function as effectors of these cytokines in orofacial development has not been well defined.

Methods—In the present study, oligonucleotide-based microarray technology was utilized to provide a comprehensive analysis of the expression of the panoply of genes related to the TGFß superfamily, as well as those encoding diverse groups of proteins functionally associated with this superfamily, during orofacial ontogenesis.

Results—Of the ~7000 genes whose expression was detected in the developing orofacial region, 249 have been identified that encode proteins related to the TGFß superfamily. Expression of several (27) of these genes was temporally regulated. In addition, several candidate genes, whose precise role in orofacial development is still unknown, were also identified. Examples of genes constituting this cluster include: TGFß1-induced anti-apoptotic factor-1 and -2, TGFß-induced factor 2, TGFß1 induced transcript -1 and -4, TGFß inducible early growth response 1, follistatin-like 1, follistatin-like 3, Tmeff (transmembrane protein with EGF-like and two follistatin-like domains) -1 and -2, nodal modulator 1, various isoforms of Stat (signal transducers and activators of transcription), notch and growth and differentiation factors (GDFs).

Conclusions—Elucidation of the precise physiological roles of these proteins in orofacial ontogenesis should provide unique insights into the intricacies of the TGFß superfamily signal transduction pathways utilized during orofacial development.

Keywords

TGFß; orofacial; microarray; fetal; development

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INTRODUCTION

The orofacial region in mammalian embryos develops from the first pharyngeal arch and normal development of this region is contingent on proper spatio-temporal patterns of mesenchymal cell proliferation, apoptosis, tissue differentiation and the synthesis and degradation of extracellular matrix components (Ferguson 1998; Greene and Pisano, 2005; Greene and Pisano, 2004). Epithelial-mesenchymal interactions, as well as a number of molecular autocrine and paracrine interactions are also known to be critical (Ferguson 1998; Greene and Pisano, 2005; Greene and Pisano, 2004; Greene et al., 1998; Richman and Tickle, 1989). In mammals, the bilateral maxillary processes of the first arch enlarge and fuse with the medial nasal processes thereby forming the primary palate and upper lip. This fusion is dependent upon proper growth of the facial processes and localized tissue differentiative events. Such directed growth of the orofacial processes is dependent, at least in avian embryos, on epithelial-derived cues (Wedden 1987; Shuler 1995). Later in embryonic development, the secondary palate originates as bilateral extensions from the oral aspect of the maxillary processes. These extensions grow in size due to cell proliferation and the elaboration of extracellular matrix, elevate to a horizontal plane, and fuse with one another in the midline thereby separating the oral and nasal cavities. Significantly, abnormal development of the embryonic midfacial region is often associated with a variety of orofacial clefts of both the primary and/or secondary palates. These malformations can manifest themselves as isolated defects, or as part of a syndrome (such as DiGeorge, Treacher Collins and Apert). Thus, the embryonic orofacial region has proven to be an excellent model system, of human clinical significance, with which to investigate regulation of the sequential expression and interaction of molecular signals that effect morphogenesis and cellular differentiation.

The transforming growth factor ß (TGFß) superfamily represents a class of signaling molecules that plays a central role in development of the orofacial region. Members of this family, especially the TGFßs and the bone morphogenetic proteins (BMPs), are vital to development of orofacial region wherein they regulate mesenchymal and epithelial cell proliferation, differentiation, apoptosis and extracellular matrix synthesis (Greene and Pisano, 2004; Francis-West et al., 1998; Bennett et al., 1995; Zhang et al., 2002; Potchinski et al., 1997; Lu et al., 2000). The importance of this growth factor family is further emphasized by the observations that perturbation of TGFß or BMP expression results in a variety of orofacial malformations (Lu et al., 2000; Sanford et al., 1997; Kaartinen et al., 1995; Proetzel et al., 1995). Other members of the TGFß superfamily, such as activins, and their related protein, follistatin, are also important contributors to orofacial morphogenesis, as mice with targeted deletion of these genes exhibit various orofacial defects (Matzuk et al., 1995a; Matzuk et al., 1995b). Inactivation or deletion of genes encoding various TGFß or BMP type I (Alk-2, Alk-5) and type II (ActRII, TgfbrII) receptors (Dudas et al., 2004a; Matzuk et al., 1995c; Dudas et al., 2004b; Ito et al., 2003), or inactivation of genes encoding the TGFß and BMP signaling mediators (Smad2, Smad5) (Chang et al., 1999; Nomura and Li, 1998; Cacheux et al., 2001), also result in a spectrum of orofacial developmental abnormalities.

The BMP subfamily of TGFß signaling molecules was originally identified in, and isolated from, demineralized bone matrix and characterized by its ability to induce ectopic bone formation *in vivo* (Wozney, 1993; Rittenberg et al., 2005; Urist, 1965; Wozney et al., 1990; Wozney et al., 1988). Subsequently, it was demonstrated that BMPs, similar to TGFßs, are widely expressed in the vertebrate embryo and fetus (Rosen and Thies, 1992; Lyons et al., 1990; Lyons et al., 1991) where they regulate various aspects of development including patterning of mesoderm, neurogenesis, ossification, organogenesis and tissue growth (Kishigami et al., 2004; Hogan, 1996a). TGFßs and BMPs function through a Smad-

mediated signaling mechanism which regulates the level of target gene expression. The induction of TGFß and BMP target genes represents a balance between recruitment of transcriptional activators such as CBP, p300 and OAZ (Takizawa et al., 2003; Nishimura et al., 2003; Lin et al., 2002; Postigo et al., 2003; Hata et al., 2000) and transcriptional repressors such as c-Ski, SnoN, BF2 and HIPK2 (Wang et al., 2000; Baker et al., 1999; Mariani and Harland, 1998; Harada et al., 2003; Wotton and Massague, 2001). Our laboratory has recently demonstrated the presence of a Smad-containing-transcriptional complex, in embryonic orofacial tissue, which contains, among other proteins, phosphorylated-CREB, phosphorylated-Smad, the co-activator CBP, and the co-repressors c-Ski and SnoN (Warner et al., 2003).

Over the past several years significant advances have been made in our understanding of the role of the TGFß superfamily signaling pathways in embryonic orofacial morphogenesis. It remains unclear however, precisely how members of this family orchestrate the unique repertoire of expressed genes that execute normal orofacial development. To identify and comprehensively catalog genes that are members of, and/or functionally related to, the TGFß superfamily of growth factors, as a function of stages of orofacial development, a comprehensive “molecular fingerprint” of developing murine orofacial tissue has been established through utilization of an oligonucleotide-based microarray chip technology. Exploiting such an *embryogenomic* approach in the current study, the expression of a wide range of genes encoding signaling mediators belonging to, as well as numerous transcription factors and target genes functionally linked with, the TGFß superfamily of growth factors has been detected during murine orofacial development. The experimental findings from this study offer valuable information concerning the expression of genes encoding various members of this superfamily of growth factors, along with those encoding functionally associated proteins, in embryonic orofacial tissue.

METHODS

Animals

Mature male and female ICR mice (Harlan, Indianapolis, IN) were housed in a climate-controlled room with a 12-h alternating dark-light cycle and were mated overnight. The presence of a vaginal plug the following morning (day 0 of gestation) was considered as evidence of mating. On gestational days (GD) 12, 13 and 14, which represent the period of secondary palate development in the mouse, female mice were euthanized by asphyxiation and embryos were dissected from uteri in sterile calcium/magnesium-free phosphate buffered saline. Extraembryonic membranes were removed from the embryos and first branchial arch derived tissue, including primary palatal tissue and secondary palatal processes were dissected as demarcated in Fig. 1 (Gehris et al., 1991;Mukhopadhyay et al., 2004), was excised, minced and stored at −20°C in RNALater solution (Qiagen, Chatsworth, CA) for subsequent extraction of total RNA. For each day of gestation, three independent pools of 20 to 25 staged embryos were used to procure embryonic orofacial tissues for preparation of three distinct RNA samples (n=3 biological replicates). Those three distinct RNA samples were processed to prepare three separate sets of target RNAs (n=3) from fetal orofacial tissue for each day of gestation. The three biological replicate RNA samples, for *each* of the days of gestation, were then applied to individual GeneChips (i.e. nine samples and nine GeneChips total).

RNA Extraction

Total RNA from excised tissue samples was isolated using the RNeasy Protect Mini Kit (Qiagen) 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. Synthesis of poly $(A)^+$ mRNA, double stranded cDNA, biotin-labeled cRNA and GeneChip hybridization were performed as described previously (Mukhopadhyay et al., 2004).

Microarray Data Analysis and Presentation

Analysis of microarray data was accomplished as previously described (Mukhopadhyay et al., 2004). Briefly, images from the scanned murine U74Av2 GeneChip arrays were processed using Affymetrix Microarray Analysis Suite (MAS) 5.0. Independent fetal orofacial tissue samples were prepared and analyzed in triplicate for *each* day of gestation (gd-12, -13 and -14). Sample loading and variations in staining were standardized by scaling the average of the fluorescence intensities of all genes on an array to constant target intensity (250) for all GeneChip arrays utilized. All array data report files demonstrated acceptable $3^{/}/5^{/}$ signal ratios for the internal housekeeping controls: GAPDH and ß-actin, and comparable values for various quality control parameters such as “Noise”, “Scaling factor” and “Number of genes present”. Mean fold change values were determined for each gene represented on the GeneChip array. On the murine U74Av2 GeneChip, each gene is represented by the use of 16 perfectly matched (PM) and mismatched (MM) control probes. The MM probes acted as specificity controls allowing direct subtraction of both background and cross-hybridization signals. The number of instances in which the PM hybridization signal was larger than the MM signal was computed along with the average of the logarithm of the PM:MM ratio (after background subtraction) for each probe set. These values were used to make a matrix-based decision concerning the *presence or absence* of a RNA molecule. To quantitate RNA *abundance* in the experimental sample, the average of the differences representing PM minus MM for each gene-specific probe family was calculated, after discarding the maximum, the minimum and any outliers beyond three standard deviations. For analysis of the three different embryonic orofacial tissue target RNA samples (GD-12 vs GD-13 vs GD-14), the GeneChip image of the GD-12 sample was normalized to the corresponding image of the GD-13 sample and that of the GD-13 sample was normalized to the corresponding image of GD-14 sample across all probe pair sets. Difference call, fold change, average difference value, and absolute call data from each of the three embryonic orofacial samples were exported. The full dataset was obtained using Affymetrix MAS 5.0 and contained expression levels at GD 12, 13, and 14 for all 12,488 genes and ESTs as well as logarithms (base 2) of the estimated fold changes. Of the ~7000 genes found to be expressed in developing orofacial tissue on the basis of the aforementioned microarray analysis, we have delineated several hundred genes encoding various members of the TGFß superfamily of growth factors, their signaling mediators, and other proteins that are functionally associated with this superfamily. Within this latter group, genes whose expression was increased or decreased two-fold or more on successive days of gestation have also been identified. Hierarchical clustering analysis was performed using the GeneSpring v6.1 software (Silicon Genetics, Redwood City, CA) to generate dendrograms (Fig. 2) representing developmentally regulated TGFß superfamily associated genes, based on their expression profiles. A heat map (Fig. 2) was generated by dividing each measurement by the 50th 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 <0.01 were set to 0.01). The list of genes comprising the heat map is listed in Table 2. Finally, gene expression profiling results obtained by the microarray analyses were independently validated using TaqMan quantitative real-time PCR (Bustin, 2000). The experimental data obtained from this microarray analysis of developing orofacial tissue is available from the GEO gene expression database (http://www.ncbi.nlm.nih.gov/geo/info/linking.html; accession no. GSE1624).

Quantitative Real-Time PCR

Total RNA prepared from GD-12, GD-13 or GD-14 orofacial tissue was treated with DNase I in the presence of RNaseOUT (Invitrogen Life Technologies, Inc., Carlsbad, CA) to remove contaminating DNA before cDNA synthesis. cDNA was synthesized with random hexamer primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Inc.). Real-time PCR analysis was performed on a TaqMan ABI Prism 7000 Sequence Detector (Applied Biosystems; Foster City, CA). Primers and their corresponding fluorescence probes (Assays-on-Demand) were purchased from Applied Biosystems. For each gene 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 with 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. For each reaction, a parallel reaction lacking template was performed as a negative control. Raw data were acquired and processed with ABI Sequence Detector System software, version 1.0 (Applied Biosystems, UK). Each determination of mRNA amount for the 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 was utilized to define the expression profiles of genes functionally related to the TGFß superfamily of growth factors during mammalian orofacial development. Of the greater than 12,000 genes represented on each of the Affymetrix Murine GeneChip arrays, approximately 7000 genes and ESTs demonstrated a detectable level of expression in murine embryonic orofacial tissue from the three gestational ages examined (Mukhopadhyay et al., 2004). Among these ~7000 genes whose expression was detected, 249 genes belonged to, or were targets of, the TGFß superfamily. These genes, encoding members of the TGFß superfamily of growth factors, as well as other proteins functionally associated with this superfamily, are listed in Table 1. Expression of all of these genes was reproducible in triplicate biological samples from each stage of gestation. This group includes expression of a panoply of genes encoding a number of growth factors/ligands belonging to the TGFß superfamily such as the TGFßs, BMPs, growth/differentiation factors (GDFs), follistatins, inhibins and activins; a range of type I and type II receptors for TGFß superfamily ligands; signaling mediators such as the Smads; transcriptional co-activators such as CBP, p300, SKIP; corepressors such as c-ski, sno, sin-3A and -3B, NcoRs; modulators of TGFß superfamily signaling such as the latent TGFß binding proteins (Ltbps), endoglin, SARA (Smad anchor for receptor activation), gremlin-1 and -2, chordin, cerberus-1, noggin; and various target genes of TGFß superfamily signaling pathways such as genes encoding cell division cycle 20 homolog (cdc 20), CD79A antigen, procollagens type I, alpha 1 and alpha 2, type III, alpha 1, cyclin-dependent kinase inhibitor 1A (p21), 1B (p27) and 2B (p15), Id1-4, Runx-1 and -2, several Msx and Dlx family of homeobox transcription factors, and oncoproteins such as c-fos, c-jun, c-myc. These results clearly indicate that, the developing orofacial region expresses numerous genes functionally associated with the TGFß superfamily signaling pathway, encoding a variety of molecular markers, such as growth and differentiation factors, signal transduction modulators and effectors, cytoskeletal and extracellular matrix proteins and transcription factors. Moreover, expression of a number of genes linked to the TGFß superfamily was differentially regulated during orofacial growth and morphogenesis (Table 2). Heat maps (Fig. 2) generated by hierarchical clustering analysis of the data with the GeneSpring v6.1 software (Silicon Genetics) demonstrated diverse patterns of expression of those TGFß superfamily and

functionally associated genes (listed in Table 2) during murine orofacial morphogenesis. The developmentally regulated genes shown in Table 2, exhibited an average increase or reduction in expression of 2.0-fold or greater in at least one set of comparisons (e.g. either in GD-13 vs. GD-12 or in GD-14 vs. GD-13) in all three biological replicates, and hence were considered as having undergone a significant and reproducible alteration in their expression levels during orofacial ontogenesis. Examples of such differentially expressed genes include those encoding TGFß1, BMP10, BMP15, BMP receptor type II, Ltbp-1, cerberus-1, inhibin alpha, dachshund-1, Stat-1, Sox-4, cyclin-dependent kinase inhibitors 1A (p21), 1B (p27), 2B (p15) and the oncoprotein, c-fos. These findings reinforce the notion that components of the TGFß signal transduction machinery are integral to the regulation of cellular processes such as cell proliferation, tissue differentiation, and elaboration of extracellular matrix critical for orofacial morphogenesis.

Using TaqMan quantitative real-time PCR (Bustin, 2000), a specific and sensitive method permitting detection and quantification of mRNA species, the gene expression profile of TGFß superfamily members in developing orofacial tissue obtained by the microarray analyses was independently validated. Expression levels of 14 candidate mRNAs of the TGFß superfamily and functionally associated proteins, that showed diverse patterns of differential regulation during the three days of orofacial development, were quantified by TaqMan quantitative real-time PCR and compared to those levels determined by the microarray technique. The overall expression profiles of 12 of the 14 mRNAs examined were found to be in consistent agreement between the two methodologies (Table 3).

DISCUSSION

The TGFß superfamily includes nearly 30 proteins in mammals, e.g. TGFßs (three isoforms; TGFß-1,-2 and -3), activins and inhibins, nodal, bone morphogenetic proteins (BMPs), growth/differentiation factors (GDFs; includes myostatin), and anti-Mullerian hormone (AMH, also called Mullerian inhibiting substance or MIS). Earlier studies reported the expression of a number of members of this family and their receptors in the developing orofacial region (Ferguson, 1998; Greene and Pisano, 2005; Greene and Pisano, 2004; Francis-West et al., 1998; Dewulf et al., 1995; Roelen et al, 1997). Members of the TGFß superfamily of signaling molecules, as well as their transporter/effector Smad molecules, regulate growth, differentiation and tissue morphogenesis in species as diverse as worms and mammals, eliciting a variety of biological responses including morphogenesis, cell proliferation, cell differentiation, apoptosis and extracellular matrix synthesis (Kingsley et al., 1994; Hogan, 1996b; Moses and Serra, 1996). They also play a critical role in the developing mammalian orofacial region as discussed below.

While the cellular and phenotypic effects of TGFß during mammalian orofacial development have been investigated, the specific genes that function as downstream mediators of TGFß in morphogenesis of the tissue remain poorly defined. Genomic array technology has been utilized increasingly in a wide spectrum of biological systems in order to identify individual genes as well as pathways critical for embryonic development (Jochheim et al., 2003; Richards et al., 2002). In the present study, oligonucleotide-based microarray chips were utilized to profile expressed genes, directly and indirectly associated with the TGFß superfamily in the developing murine orofacial complex *in vivo*, by comparing mRNA derived from the tissue on each of GD-12, GD-13, and GD-14 of development. Three mRNA samples were prepared from each of the three days of gestation. This approach enabled delineation of genes encoding members of the TGFß superfamily and other proteins (as defined in the methods section) functionally associated with the signal transduction system of this growth factor superfamily. Murine embryonic orofacial tissues and cells have been utilized in the past to address questions of maxillary/palatal

mesenchymal and epithelial cell function. Abundant experimental evidence, especially that from gene 'loss of function' studies, indicates that the TGFß superfamily of growth factors play a critical role in morphogenesis of the embryonic orofacial region (Kaartinen et al., 1995; Proetzel et al., 1995).

Molecular genetic analyses have documented over 30 genes that, when disrupted, result in orofacial dysmorphogenesis (Francis-West et al., 1998; Schutte and Murray, 1999). Many of these genes encode members of the TGFß superfamily of growth factors and their receptors (TGF-ß2 & -ß3, BMP4, Alk2, TGFßRII, Activin ßA, ActRIIa), TGFß signaling mediators (such as, Smad2, Smad5, SMADIP1) or TGFß target genes (such as Dlx-1 and -2, Msx-1 and -2) (for a review see Francis-West et al., 1998). In the current study, gene expression profiling of the developing orofacial tissue enabled detection of expression of all of the aforementioned genes. Additionally, a number of other genes, encoding members or associated proteins of the TGFß superfamily, were detected as being expressed in the developing orofacial complex. However, the precise biological functions of many of these genes in orofacial ontogenesis are still unclear. Examples of some of these genes include TGFß1-induced anti-apoptotic factor-1 and -2, TGFß-induced factor 2, TGFß1 induced transcript -1 and -4, TGFß inducible early growth response 1, follistatin-like 1, follistatin-like 3, Tmeff (transmembrane protein with EGF-like and two follistatin-like domains) -1 and -2, Stat (signal transducers and activators of transcription) -1,-3,-4,-5A, 5B, a number of GDFs, and several isoforms of notch (Table 1). Understanding the specific functional roles of these proteins in orofacial maturation would greatly enhance our knowledge of embryonic orofacial ontogenesis and the molecular/genetic mechanisms whereby development is perturbed with a resultant orofacial cleft.

TGFß superfamily signals are initiated upon binding of the ligand to cell surface serine/threonine kinase receptors, inducing the stable assembly of heteromeric complexes of transmembrane types I and II receptors (Derynck and Feng, 1997). Embryonic orofacial tissue contains functional types I, II and III TGFß receptors (Linask et al., 1991; Cui and Shuler 2002) that, when activated, elicit changes in palatal cell proliferation (Linask et al., 1991), glycosaminoglycan synthesis (D'Angelo and Greene, 1991), collagen metabolism (D'Angelo et al., 1994) and the remodeling of the extracellular matrix via matrix metalloproteinases such as MMP2 and MMP9 (also known as gelatinases A & B, respectively) (D'Angelo et al., 1994, Brown et al., 2002). Moreover, the TGFßs exhibit unique spatio-temporal patterns of expression in the developing orofacial region (Fitzpatrick et al., 1990; Gehris et al., 1991; Jaskoll et al., 1996; Pelton et al., 1991).

Functionality of the TGFßs has been documented through genetic deletion studies. Murine embryos, in which TGFß2 or TGFß3 are deleted by homologous recombination, exhibit a cleft palate, though the penetrance and etiology of the cleft differs between the two lines of knockout mice (Sanford et al., 1997; Kaartinen et al., 1995; Proetzel et al., 1995; Doetschman, 1999). In addition, an association between TGFß3 and the incidence of cleft palate in humans has been proposed, though not confirmed, for all allelic forms of the gene (Lidral et al., 1997; Romitti et al., 1999; Sato et al., 2001). Moreover, evidence from animal studies suggests that the TGFßs play a central role in various aspects of secondary palate development including palate epithelial differentiation (Kaartinen et al., 1995; Brunet et al., 1993a; Gehris and Greene, 1992; Bhattacherjee et al., 2003; Melnick et al., 2000; Nugent and Greene, 1994) and mesenchymal (D'Angelo and Greene, 1991; D'Angelo et al., 1994) cell differentiation. These processes are critical to normal palatal ontogenesis in that perturbation of either palate medial edge epithelial (MEE) cell differentiation (Brunet et al., 1993b) or mesenchymal cell growth (Greene and Pisano, 1989) results in a cleft of the palate. An interesting finding of the current study is that, during the time period examined, genes encoding TGF-ß2 and -ß3 were constitutively expressed (Table 1) whereas, there was

a 6.5-fold increase in TGFß1 mRNA expression from GD-13 to GD-14 (Table 2). These findings confirm that the three TGFß isoforms are expressed throughout the critical period of orofacial ontogenesis, and that during this interval transcription of their genes may be differentially regulated in the developing tissue.

Supporting the presence of a functional (cellular membrane to nucleus) TGFß signaling system in the developing orofacial region, expression of the following repertoire of genes has been detected in the present microarray analysis of developing orofacial tissue: genes encoding Type I TGFß receptors such as ALK-1 (activin A receptor, type II-like 1), ALK-5, Type II TGFß receptor such as TGFßRII, Type III TGFß receptor such as TGFßRIII or betaglycan, TGFß-specific Smads such as Smad-2, -3, the co-Smad Smad-4, inhibitory Smads such as Smad-6 and -7, Smad nuclear interacting protein 1 (Snip1, a corepressor), SARA (Smad anchor for receptor activation), several modulators of TGFß synthesis and signaling such as decorin, biglycan, endoglin, involucrin, latent transforming growth factor beta binding protein (Ltbp) -1,-2,-4, FK506-binding protein 1b, FK506 binding protein-like (Fkbpl), leucine aminopeptidase 3 (Lap3), thrombospondin 1 (activator of TGFß), furin (TGFß precursor cleavage and activation), α2-Macroglobulin (clearance factor) (Table 1). Furthermore, expression of the genes encoding extracellular proteins such as Ltbp-1 and decorin, was found to be differentially regulated (Table 2) in the developing tissue. A number of TGFß signaling pathway-regulated genes were also found to be expressed in GD-12, -13 and -14 orofacial tissue: MMP2, MMP9, tissue inhibitor of metalloproteinase 1 (Timp1), procollagens type I, α1 and α2, type III, α1, cyclin-dependent kinase inhibitor -1A (p21 or Cdkn1a), -2A (p16INK4a or Cdkn2a), -2B (p15 or Cdkn2b), involucrin, tissue plasminogen activator, urokinase-type plasminogen activator protein, TGIF (TG interacting factor), Goosecoid, TGFß1-induced anti-apoptotic factor-1 and -2, TGFß-induced factor 2, TGFß1 induced transcript -1 and -4, TGFß inducible early growth response 1 and oncogenes such as *c-myc*, *fos*, *fosB*, *jun*, among others (Table 1). Among these TGFß-inducible genes, those encoding MMP-2 and -9, procollagen type I, alpha 1, cyclin-dependent kinase inhibitor -1A (p21 or Cdkn1a) and -2B (p15 or Cdkn2b), fos and invoulcrin were found to be differentially regulated during the course of orofacial ontogenesis (Table 2).

It is now well documented that Smads - the TGFß and BMP signaling mediators - bind DNA with poor affinity and that high affinity binding requires additional cofactors for a robust transcriptional response (Derynck et al., 1998). Thus, induction of TGFß and BMP target genes represents a balance between recruitment of transcriptional co-activators (such as CBP, p300 and OAZ) and co-repressors (such as TGIF, c-Ski, SnoN, BF2 HIPK2, and Evi1) (Pouponnot et al., 1998; Shim et al., 2002; Stroschein et al., 1999; Xu et al., 2000; Mariani and Harland, 1998; Alliston et al., 2005; Harada et al., 2003; Attisano and Wrana, 2000). Moreover, by preferentially associating with a wide spectrum of nuclear proteins, including a number of other transcription factors, Smad proteins are capable of eliciting both positive and negative regulation of a variety of genes (Attisano and Wrana, 2000; ten Dijke et al., 2000). In the current study, expression of a number of genes encoding transcriptional co-activators and co-repressors of TGFß superfamily signaling have been detected in the developing orofacial tissue. Examples of such transcriptional regulators include CBP, p300, Cited-1 and -2, CREM (cAMP responsive element modulator), p300/CBP-associated factor (Pcaf), c-Ski, SnoN, Skip, TGIF, Dachshund-1, Evi-1, Sin-3A and -3b, HDAC-1, -2, -3, -5, -6, -7a, NCoR-1, -2, HIPK-2 (co-repressor of BMP signaling) (Table 1). Moreover, expression of the genes encoding Sin3B, Dachshund-1 and HIPK-2 was differentially regulated during the critical period of orofacial development (Table 2).

Bone morphogenetic proteins (BMPs) belong to a distinct subfamily of the TGFß superfamily. Similar to TGFßs, BMPs exert their effects on cell growth, differentiation and tissue morphogenesis by first binding to two types of serine/threonine kinase cell surface

receptors, activation of which leads to phosphorylation, and translocation into the nucleus, of intracellular signaling molecules, including Smad1, Smad5, and Smad8 ("canonical" BMP signaling pathway) (Balemans and Van Hul, 2002). BMP effects are also mediated by activation of the mitogen-activated protein (MAP) kinase pathway ("noncanonical" BMP signaling pathway) (Gallea et al., 2001; Lou et al., 2000; Monzen et al., 1999). As mentioned earlier, vertebrate orofacial development is dependent, in part, on growth of the facial primordia, which has been shown to be regulated by members of the BMP family (Francis-West et al., 1994). Following migration of craniofacial progenitor cells – the neural crest – into the branchial arches, BMPs exhibit distinct spatiotemporal patterns of expression in developing orofacial processes contributing to the development of the midface (Bennett et al., 1995; Gong and Guo 2003; Lu et al., 2000;). Further, BMP-mediated epithelial-mesenchymal interactions in this region induce a differentiation cascade leading to bone and cartilage formation (Bennett et al., 1995) and odontogenesis (Wang et al., 1999). Treatment with the BMP antagonist noggin, results in proliferation and outgrowth of the avian frontonasal mass and maxillary prominences and ultimately to the deletion of the maxillary and palatine bones (Ashique et al., 2002), thereby demonstrating a requirement for endogenous BMP in the proliferation of facial mesenchyme. Further evidence for functionality of the BMPs in orofacial development comes from the observation that expression of Bmp4 and Bmp2 in developing palate mesenchyme requires expression of the MSX1 homeobox gene (Zhang et al., 2002). 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 (van den Boogaard et al., 2000), and that transgenic expression of human BMP4 in $Msx1^{-/-}$ murine embryonic palatal mesenchyme rescues the cleft palate phenotype as well as restores Shh and BMP2 expression and normal levels of mesenchymal cell proliferation (Zhang et al., 2002). There is a growing body of evidence implicating integration of the sonic hedgehog (Shh) and BMP signal transduction pathways in morphogenesis of the craniofacial complex. Shh is essential for growth and morphogenesis of the facial processes. Underexpression of Shh results in facial clefting, holoprosencephaly and cyclopia, while overexpression of Shh leads to hyperteleorism (Hu and Helms, 1999). Moreover, mutations in the human and murine Shh genes result in holoprosencephaly and cyclopia (Muenke and Cohen, 2000; Roessler et al., 1996). Shh – expressed in embryonic palate epithelial tissue – activates BMP2 expression in the palatal mesenchyme which functions as a mitogen to stimulate mesenchymal cell proliferation (Zhang et al., 2002). In the present study, expression of Shh was detected in embryonic orofacial tissue during the course of its development (Table 1). Moreover, expression of genes encoding several members of the BMP subdivision of the TGFß superfamily was detected in developing orofacial tissue. This includes BMP-1, -2, -4, -5, -6, -7, -8a, -8b, -10 and -15 (or GDF-9b) (Table 1). Detection of genes encoding Type I BMP receptors [BMPRIa (ALK3), BMPRIb (ALK6), activin receptor Ia (ALK2; binds to BMP-6 and -7)], the Type II BMP receptors [BMPRII] and BMP-specific signaling mediators [Smad-1,-5], in embryonic orofacial tissue from GD12-14 reinforces the existence and functionality of the BMP signaling pathway during orofacial morphogenesis (Table 1). Expression of the genes encoding BMP10, BMP15 and BMPRII was differentially regulated during orofacial development (Table 2). Expression of genes encoding a range of BMP signaling modulators such as noggin, chordin, tob-1, gremlin-1 and -2, DAN, cerberus1 (inhibitor of BMP and activin signaling) and expression of several other BMP target genes including Sox-4, Stat-1,-3,-4,-5A, 5B, PIAS-1,-3, Zfhep, cyclin-dependent kinase inhibitor p27kip1, twisted gastrulation homolog 1 (Drosophila), was detected in the orofacial region of GD-12, -13 and -14 embryo. Amongst these, genes encoding Sox-4, Stat-1 and -5B were found to be developmentally regulated (Table 2). Genes encoding a series of BMP-regulated transcription factors such as Runx-1 and -2, Id-1,-2,-3.-4, Msx-1,-2,-3, Dlx-2,-3,-5,-6,-7, most of which are well-known for their vital roles in orofacial ontogenesis, were also detected in developing orofacial tissue (Table 1).

The growth/differentiation factors (GDFs) are a subfamily of the highly conserved group of bone morphogenetic protein (BMP) signaling molecules and are known to play diverse developmental roles, especially in the musculoskeletal system (Francis-West et al., 1994; King et al., 1996; Miyazawa et al., 2002). Myostatin, also known as GDF-8, is produced by cells of skeletal muscle lineage, and inhibits their growth whereas GDF-11 is expressed in neuronal lineages and inhibits olfactory epithelium neurogenesis (Sakuma et al., 2000; Wu et al., 2003). GDF-5, also known as cartilage-derived morphogenetic protein 1 (CDMP-1), is structurally related to the BMPs, and participates in skeletal morphogenesis (Everman et al., 2002). Heterozygous mutations in GDF5, which maps to human chromosome 20, occur in individuals with autosomal dominant brachydactyly type C (Everman et al., 2002). The disorder, characterized by skeletal abnormalities restricted to the limbs, is phenotypically similar to murine brachypodism which is due to mutations in GDF5 (Everman et al., 2002). Although little is known at present regarding their role in orofacial development, expression of genes encoding a range of GDFs (e.g. GDF-1,-3,-5,-8,-9,-10,-11, and -15) were detected in GD-12, -13 and -14 embryonic orofacial tissue (Table 1).

The TGFß superfamily member nodal, plays critical roles in the induction of dorsal mesoderm, anterior patterning, and formation of left–right asymmetry (Schier, 2003). The notch signaling pathway functions upstream of the nodal gene (Raya et al., 2004). The importance of nodal signaling in craniofacial development is evident by the observation that mice trans-heterozygous for both Smad2 and nodal deletion display a range of phenotypes, including complex craniofacial abnormalities such as cyclopia (Nomura and Li, 1998). This finding indicates that Smad2 may mediate nodal signaling during craniofacial development. The Jagged2 (Jag2) gene, encodes a ligand for the Notch family of transmembrane receptors. Mice homozygous for Jag2 deletion exhibit cleft palate, fusion of the tongue with the palatal shelves and die perinatally demonstrating that Notch signaling mediated by Jag2 plays an essential role during craniofacial development in mice (Jiang et al., 1998). In the present study expression of genes encoding nodal, nodal modulator 1 (Nomo-1), and those encoding a number of notch proteins such as notch-1,-2,-3 and -4 and the notch receptor ligand, jagged2, has been detected in embryonic orofacial tissue during its development (Table 1). Demonstration, in the current study, of the expression of notch and nodal proteins suggests potential functional interactions between these two signaling pathways mediating orofacial morphogenesis.

Members of the TGFß superfamily, such as activins, inhibins and their related protein follistatin, are known mediators of orofacial development, as mice with targeted deletions of these genes demonstrate various orofacial defects (Vale et al., 2004; Lambert-Messerlian et al., 2004; Ferguson et al., 1998). Inhibins, dimers of α and ß inhibin chains, function as cytokines that inhibit the secretion of follicle-stimulating hormone (FSH) from the pituitary gland whereas activins, dimers of ß chains, stimulate the production of FSH by the same gland (Gregory and Kaiser, 2004). Activins also play crucial roles in the induction of dorsal mesoderm during early embryogenesis and act on epithelial as well as haematopoietic cells regulating their growth, differentiation and apoptosis (Fukui and Asashima, 1994; Dawid et al., 1992). Developing mammalian facial mesenchymal tissues express activin βA, (Feijen et al., 1994; Roberts and Barth, 1994) and functional deletion of this gene in mice results in cleft palate formation and loss of lower incisors (Matzuk et al., 1995a). Interestingly, such cleft palate occurs devoid of any skeletal abnormalities suggesting that the palatal cleft in activin βA knockout mice is a primary defect that arises due to aberrant formation of the orofacial primordia (Matzuk et al., 1995a). Deletion of the murine activin type 2 receptor gene, ActRIIa, also results in craniofacial defects, including hypoplasia of the mandible and cleft palate (Matzuk et al., 1995c). In the present microarray study, constitutive expression of various members of the activin/inhibin signaling pathway was apparent in the developing orofacial region including inhibin-βA,-βB,-βC,-βE and inhibin-α, Type I activin receptor

(ActRIB or ALK4), Type II activin receptors [ActRIIA and -IIB], forkhead box H1 (Fast2 or forkhead-activin signal transducer 2; mediator of TGFß/Nodal/Activin signaling), synaptogamin 2 binding protein (activin receptor interacting protein 2) (Table 1). Moreover, expression of the genes encoding inhibin-α and activin receptor IIB was found to be increased 2.25-fold and decreased 2.0-fold respectively, between GD-12 and GD-13 of orofacial development (Table 2). Developmentally regulated expression of these genes in embryonic orofacial tissue might reflect the roles of inhibin-α and activin receptor IIB, in controlling epithelial and mesenchymal cell proliferation, apoptosis and tissue differentiation during orofacial ontogenesis.

Follistatin acts as an activin antagonist *in vitro* but as an agonist *in vivo*. This observation is supported by the report that functional deletion of the follistatin gene in mice results in hard palate abnormalities comparable to those reported in activin βA knockout mice (Matzuk et al., 1995b). In addition, follistatin can modulate the activity of several other members of the TGFß family (e.g. it can act as a BMP-2 and BMP-4 antagonist). In the present study genes encoding follistatin and other related proteins such as follistatin-like 1, follistatin-like 3, Tmeff (transmembrane protein with EGF-like and two follistatin-like domains) -1 and -2, were constitutively expressed during orofacial development (Table 1).

Despite significant advances in our understanding of the contributions of the TGFß superfamily members and their functionally associated proteins during orofacial ontogenesis, the means by which perturbation of the TGFß superfamily signaling pathways, or the means by which alterations in expression of their downstream genes, results in orofacial dysmorphology remains enigmatic. The present study, offers a comprehensive “molecular fingerprint” of developing murine orofacial tissue with regard to the expression of the TGFß superfamily of growth, differentiation and morphogenesis factors. The results of the current gene expression profiling study demonstrate that genes encoding various TGFß superfamily members and functionally associated proteins are expressed in developing orofacial tissue where they are either known to play crucial roles or have as yet unidentified roles in morphogenesis, tissue growth and cellular differentiation during development. The *embryogenomic* approach utilized in the present study provides an interface between genomics and developmental biology and offers the opportunity to initiate studies investigating the complexity of cellular signal transduction mediated by the TGFß superfamily of growth factors as well as examine novel interactions between the members of this superfamily and their functionally associated proteins, during mammalian orofacial development.

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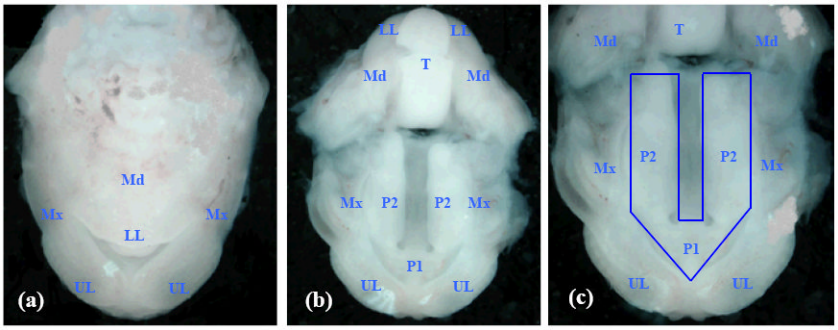

Figure 1. Photomicrographs of ventral views of the developing orofacial region of a GD-13 mouse embryo

(a) upper and lower lips and jaws (maxilla and mandible); (b) the embryonic oral cavity; the lower half of the photo contains the roof of the oral cavity with the maxillary processes, primary palate and secondary palatal processes; the upper half contains the base/floor of the oral cavity showing the tongue and the mandible; (c) a magnified view of the roof of the oral cavity: note that the upper lip and the primary palate are completely formed, and the developing secondary palatal shelves are derived from the medial aspect of each maxillary process. The region demarcated by the blue line was excised from GD-13 embryos for extraction of total RNA. Corresponding regions were dissected from the developing orofacial region of GD-12 and GD-14 embryos. (UL) upper lip; (LL) lower lip; (Mx) maxilla; (Md) mandible; (P1) primary palate; (P2) secondary palate; (T) tongue.

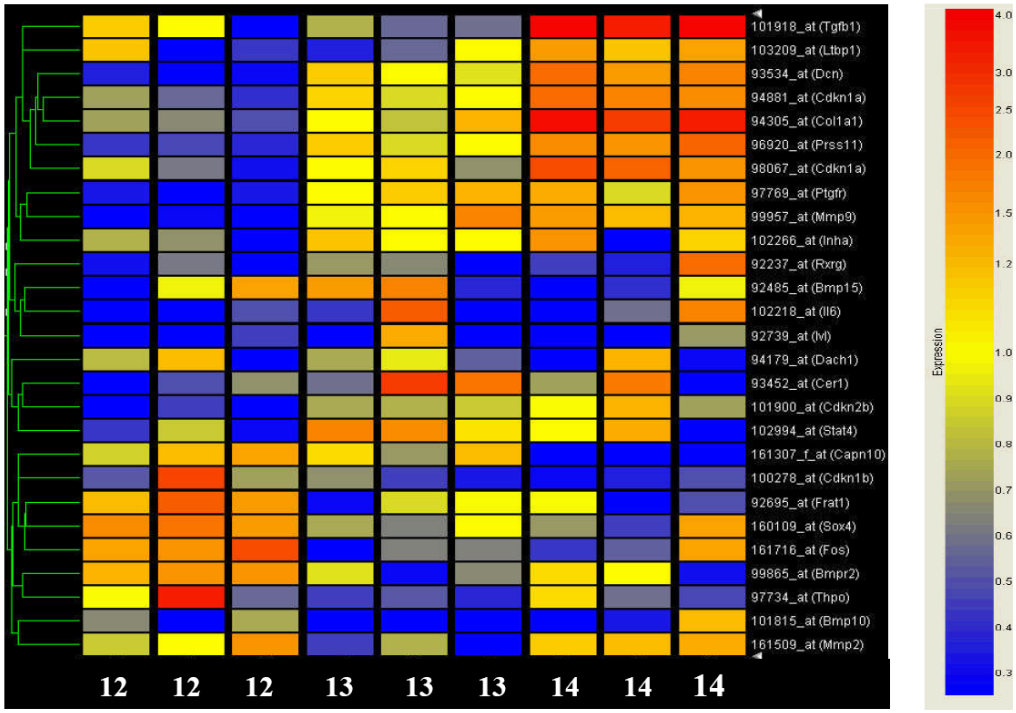

Figure 2. Heat map illustrating the statistically significant alterations in expression of the TGFß superfamily of genes during murine orofacial development

Each row of the heat map represents a gene, and each column represents a time point in development (gestational day labeled along the bottom). The color saturation represents the level of gene expression. Red indicates an increase in gene expression, whereas blue indicates a decrease. Genes whose expression demonstrated a two-fold or greater increase or decrease are depicted. The list of genes comprising the heat map is listed in Table 2.

Birth Defects Res A Clin Mol Teratol. Author manuscript; available in PMC 2010 November 7.

Table 1

Genes Expressed in Developing Orofacial Tissue Encoding Members of the TGFß Superfamily and their Associated Signaling Proteins.

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Transcription factor 1	100082_at	M57966
Jun oncogene	100130_at	X12761
T-complex protein 10c	100359_at	M22602
IQ motif containing GTPase activating protein 1	100561_at	AW209098
Calpain, small subunit 1	100610_at	AF058298
Luteinizing hormone/choriogonadotropin receptor	100705_at	M81310
Formyl peptide receptor, related sequence 2 (lipoxin A4 receptor-like protein)	101800_at	AF071180
Follicle stimulating hormone receptor	101810_at	AF095642
Mitogen activated protein kinase 3	101834_at	Z14249
Transforming growth factor, beta 1	101918_at	AJ009862
TGFß1-induced anti-apoptotic factor 1	102080_at	AF104984
TGFß1-induced anti-apoptotic factor 2 (co-activator of nuclear receptors)	102613_at	AF075717
Tumor necrosis factor alpha	102629_at	D84196
Transforming growth factor, beta receptor III / betaglycan	102637_at	AF039601
Transforming growth factor, beta 3	102751_at	M32745
E2F transcription factor 1	102963_at	L21973
MAD homolog 1 (Drosophila)	102983_at	U58992
MAD homolog 1 (Drosophila)	102984_g_at	U58992
Latent transforming growth factor beta binding protein 1	103209_at	AF022889
Budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	104097_at	AF002823
IQ motif containing GTPase activating protein 1	104300_at	AI117936
src homology 2 domain-containing transforming protein C1	104350_at	AI050321
MAD homolog 2 (Drosophila)	104536_at	U60530
Eukaryotic translation initiation factor 5	160265_at	AW123979
Thrombospondin 1 (activator of TGFß)	160469_at	M62470
Harvey rat sarcoma virus oncogene 1	160536_at	Z50013
Low density lipoprotein receptor	160832_at	Z19521

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Mitogen activated protein kinase kinase kinase 7	160854_at	D76446
Transforming growth factor, beta induced	161157_r_at	AV231282
Calpain 10	161307_f_at	AV350809
Tryptophanyl-tRNA synthetase	161337_f_at	AV121930
Transforming growth factor, beta 3	161382_at	AV246832
E2F transcription factor 1	161427_f_at	AV301683
Prostaglandin F receptor	161713_f_at	AV248803
Budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	162264_s_at	AV359014
Retinoid X receptor gamma	92237_at	X66225
Latent transforming growth factor beta binding protein 2	92335_at	AF004874
Transforming growth factor alpha	92369_at	M92420
Transforming growth factor, beta receptor I/ALK-5	92427_at	D25540
Origin recognition complex, subunit 1-like (S.cereviaiae)	92458_at	AJ00313
Frequently rearranged in advanced T-cell lymphomas	92695_at	U58974
Transforming growth factor, beta induced	92877_at	L19932
Cyclin A1	92911_at	X84311
Connective tissue growth factor	93294_at	M70642
Transforming growth factor, beta 2	93300_at	X57413
Chemokine (C-C motif) receptor 2	93397_at	U56819
MAD homolog 3 (Drosophila)	93613_at	AB008192
TGFß-induced factor 2 (TALE family homeobox)	93621_at	AA914350
src homology 2 domain-containing transforming protein C1	93713_at	U15784
Transforming growth factor beta 1 induced transcript 4	93728_at	X62940
IQ motif containing GTPase activating protein 1	93850_at	AI642553
Glial cell line derived neurotrophic factor family receptor alpha 1	93872_at	AF014117
Serine (or cysteine) proteinase inhibitor, clade E, member 1/ PAI-1 (SERPIN-1)	94147_at	M33960
TGFßRII	94702_at	D32072
WNT1 inducible signaling pathway protein 2 / connective tissue growth factor related protein WISP-2	94704_at	AF100778
Cell division cycle 20 homolog (S. cerevisiae)	96319_at	AW061324

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Oxoglutarate dehydrogenase (lipoamide)	96879_at	AI852338
RIKEN cDNA 5730427N09 gene	97229_at	AW061042
Calpain 10	97331_at	AW049679
Latent transforming growth factor beta binding protein 4	97347_at	AA838868
Eukaryotic translation elongation factor 2	97559_at	M76131
Prostaglandin F receptor	97769_at	D17433
Forkhead box H1	97789_at	AF069303
Transforming growth factor beta 1 induced transcript 1	98019_at	L22482
Nuclear factor of kappa light chain gene enhancer in B-cells 1, p105	98427_s_at	M57999
Tryptophanyl-tRNA synthetase	98606_s_at	X69656
Glycerol phosphate dehydrogenase 2, mitochondrial	98984_f_at	D50430
Fumarate hydratase 1	99148_at	AI852862
Interferon gamma	99334_at	K00083
Guanine nucleotide binding protein (G protein), gamma 12	99477_at	AI842738
TGFß inducible early growth response 1 (Kruppel-like factor 10)	99602_at	AF064088
TGFß inducible early growth response 1 (Kruppel-like factor 10)	99603_g_at	AF064088
Prostaglandin F2 receptor negative regulator	99894_at	AF006201
MAD homolog 5 (Drosophila)	102865_at	U58993
MAD homolog 1 (Drosophila)	102983_at	U58992
MAD homolog 1 (Drosophila)	102984_g_at	U58992
MAD homolog 6 (Drosophila)	104220_at	AF010133
Smad nuclear interacting protein 1	104349_at	AA870301
MAD homolog 2 (Drosophila)	104536_at	U60530
MAD homolog 4 (Drosophila)	160440_at	U79748
MAD homolog 7 (Drosophila)	92216_at	AF015260
Spectrin beta 2	93571_at	M74773
MAD homolog 3 (Drosophila)	93613_at	AB008192
Zinc finger, FYVE domain containing 16	96491_at	AI035632
Decorin (TGFb inhibitor)	93534_at	X53929

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Biglycan (inhibitor)	96049_at	X53928
alpha 2-Macroglobulin (Clearance factor)	104486_at	AI850558
Endoglin (signal modulator of TGFßs, Activins, BMPs etc.)	100134_at	X77952
Nodal	99801_at	X70514
Nodal Modulator 1 (Nomo 1)	104140_s_at	AI846989
SARA (Smad anchor for receptor activation)	96491_at	AI035632
Apolipoprotein J / Clusterin (role in TGFß signaling &/or processing)	95286_at	D14077
Furin (Proprotein convertase subtilisin/kexin type 3) (TGFß precursor cleavage and activation)	100515_at	X54056
Dachshund-1	94179_at	AJ005669
SKIP	104158_at	AW046671
SnoN	94752_s_at	U10531
Lefty-1 (Leftb)	102345_at	D83921
Fkbpl; FK506 binding protein-like	102206_at	AF030001
Notch-1	97497_at	Z11886
Notch-2	104188_at	AI853703
Notch-3	92956_at	X74760
Notch-4	92652_at	AF030001
Evi-1	100754_at	X54989
Evi-1	99391_at	M21829
Jagged2	92510_at	Y14331
Sin3A	103011_at	L36831
Sin3B	93790_at	L38622
Cited-2	101973_at	Y15163
Cited-1	160705_at	U65091
CREM (cAMP responsive element modulator)	100533_s_at	M60285
CREM (cAMP responsive element modulator)	160526_s_at	M60285
CREBBP/EP300 inhibitory protein 1 or Cri1	99191_at	AI844939
CD79A antigen (immunoglobulin-associated alpha; TGFß family receptor)	102778_at	X13450
Integrin beta-5	100601_at	AF022110

Birth Defects Res A Clin Mol Teratol. Author manuscript; available in PMC 2010 November 7.

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Integrin beta 7	100906_at	M68903
Nuclear receptor subfamily 0, group B, member 1 (NrOb1)	93141_at	U41568
Cyclin-dependent kinase inhibitor 1A (P21 or Cdkn1a)	94881_at	AW048937
Cyclin-dependent kinase inhibitor 1A (P21 or Cdkn1a)	98067_at	U09507
Cyclin dependent kinase inhibitor 2B (P15 or Cdkn2b)	101900_at	AF059567
Cyclin dependent kinase inhibitor p16INK4a (P16 or Cdkn2a)	98789_at	AF044336
Procollagen type I, alpha 1	94305_at	U03419
Procollagen, type I, alpha 2	101130_at	X58251
Procollagen, type III, alpha 1	102990_at	AA655199
Procollagen, type III, alpha 1	98331_at	X52046
Fos (FBJ osteosarcoma oncogene)	160901_at	V00727
Fos (FBJ osteosarcoma oncogene)	161716_at	AV252296
FosB (FBJ osteosarcoma oncogene B)	103990_at	X14897
Goosecoid	94187_at	M85271
Involucrin (keratinocyte differentiation)	92739_at	L28819
Jun oncogene	100130_at	X12761
Plasminogen activator, tissue	93981_at	J03520
Urokinase-type plasminogen activator protein gene	97772_at	M17922
TGIF (TG interacting factor)	101502_at	X89749
Tissue inhibitor of metalloproteinase 1 (Timp1)	101464_at	V00755
c-myc	104712_at	L00039
FK506-binding protein 1b (protein folding)	103248_at	AF060872
Leucine aminopeptidase 3; Lap3; (proteolysis and peptidolysis)	98112_r_at	AI839225
HDAC-1	97706_at	AA474655
HDAC-1	96046_at	X98207
HDAC-2	160643_at	U31758
HDAC-3	103655_at	AF074882
HDAC-5	104376_at	AF006602
HDAC-6	104471_at	AF006603

Birth Defects Res A Clin Mol Teratol. Author manuscript; available in PMC 2010 November 7.

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
HDAC-7A	97550_at	AW047228
CBP	95886_g_at	AA177826
p300/CBP-associated factor (Pcaf)	104070_at	AW047728
NCoR-1	101536_at	U35312
NCoR-2	95129_at	AW121057
MMP2 (gelatinase A)	161509_at	AV145762
MMP9 (gelatinase B)	99957_at	X72795
Bone morphogenetic protein 8b	101657_at	U39545
Gremlin 1	101758_at	AF045801
Bone morphogenetic protein 10	101815_at	AF101440
Twisted gastrulation homolog 1 (Drosophila)	102032_at	AW060819
Bone morphogenetic protein 2	102559_at	L25602
Bone morphogenetic protein 6	161028_at	AI850533
Solute carrier family 29 (nucleoside transporters), member 1	161687_r_at	AV217246
Bone morphogenetic protein 6	92372_at	X80992
Bone morphogenetic protein 15 or GDF-9B	92485_at	AJ010259
Bone morphogenetic protein 1	92701_at	AA518586
Bone morphogenetic protein receptor, type 1A or ALK-3	92767_at	D16250
Solute carrier family 29 (nucleoside transporters), member 2	92950_at	X86682
Bone morphogenetic protein 8a	92982_at	M97017
Bone morphogenetic protein 7	93243_at	X56906
BMP2 inducible kinase	93376_at	AA673486
Bone morphogenetic protein 4	93455_s_at	X56848
Bone morphogenetic protein 4	93456_r_at	L47480
Bone morphogenetic protein 1	95557_at	L24755
Solute carrier family 29 (nucleoside transporters), member 1	95733_at	AI838274
Protease, serine, 11 (Igf binding)	96920_at	AW125478
Bone morphogenetic protein receptor, type 1B or ALK-6	97725_at	Z23143
Noggin	97727_at	U79163

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Bone morphogenetic protein 5	99393_at	L41145
Transducer of ErbB-2.1	99532_at	D78382
Bone morphogenetic protein receptor, type II (serine/threonine kinase)	99865_at	AF003942
Runx1 (Core binding factor alpha 2)	92399_at	D26532
Runx2 (Core binding factor alpha 1)	92676_at	D14636
Runx2	92677_s_at	AF010284
Runx2	161378_r_at	AV245229
Chordin (inhibitor of BMP signaling/ ligand-receptor interaction)	103249_at	AF069501
Cerberus 1(inhibitor of BMP & activin signaling/ ligand-receptor interaction)	93452_at	AF031896
DAN or NBL1 (inhibitor of BMP signaling; like Gremlin);	101969_at	D50263
Gremlin 2 or PRDC	103975_at	AB011030
Bone gamma-carboxyglutamate protein 2	102801_at	L24430
Idb-1	100050_at	M31885
Idb-2	93013_at	AF077861
Idb-3	92614_at	M60523
Idb-4	96144_at	AJ001972
Sox-4	160109_at	X70298
Signal transducer and activator of transcription 1 (Stat1)	101465_at	U06924
Signal transducer and activator of transcription 4 (Stat4)	102994_at	U06923
Signal transducer and activator of transcription 3 (Stat3)	99099_at	U08378
PIAS1 (protein inhibitor of activated STAT protein 1)	99029_at	AF077950
PIAS3 (protein inhibitor of activated STAT protein 3)	93708_at	AF034080
Signal transducer and activator of transcription 5B (Stat 5b)	92199_at	U21110
Signal transducer and activator of transcription 5A (Stat 5a)	100422_i_at	AJ237939
Signal transducer and activator of transcription 5A (Stat 5a)	100423_f_at	AJ237939
Zinc finger homeobox 1a (Zfhx1a or Zfhep) or Transcription factor 8	99052_at	D76432
Cyclin-dependent kinase inhibitor p27kip1 (Cdkn1b)	100278_at	U09968
Msx1	101526_at	X14759
Msx2	102956_at	X59252

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Msx3	92912_at	X96518
Msx-interacting-zinc finger protein 1 (Miz1) (Transcription Factor)	100941_at	AF039567
Dlx2	92332_at	M80540
Dlx3	99328_at	U79738
Dlx5	92930_at	U67840
Dlx6	98866_at	U67841
Homeodomain-interacting protein kinase 2 (HIPK-2) [BMP Co-repressor]	103833_at	AF077659
Homeodomain-interacting protein kinase 3 (HIPK-3)	103233_at	AF077660
Inhibin beta-A	100277_at	X69619
Activin A receptor, type II-like 1 (ALK-1)	100448_at	Z31664
Activin A receptor, type II-like 1	100449_g_at	Z31664
Activin A receptor, type II-like 1	100450_r_at	L48015
Activin A receptor, type 1B or ALK-4 (binds Activins & Nodal)	101177_at	Z31663
Synaptojanin 2 binding protein	101548_at	AI846345
Inhibin beta-C	103986_at	X90819
RIKEN cDNA 1810020G14 gene	160556_at	AW048976
Inhibin beta-B	160828_at	X69620
Activin A receptor, type 1 / TGFb type I receptor (ALK-2, ALK-8)	93460_at	L15436
Activin receptor IIB	93903_at	M84120
Synaptojanin 2 binding protein	96734_at	AA674143
Inhibin beta E	97153_at	U96386
Forkhead box H1 (Fast2; forkhead-activin signal transducer 2)	97789_at	AF069303
Activin receptor IIA	98841_at	M65287
Beta-microseminoprotein (beta-inhibin; prostatic inhibin protein)	100715_at	U89840
Inhibin alpha	102266_at	X55957
Transmembrane protein with EGF-like and two follistatin-like domains 2	103536_at	AB017270
Follistatin-like 3	104452_at	AI845896
Transmembrane protein with EGF-like and two follistatin-like domains 1	160472_r_at	AI837838
Follistatin-like 1	94833_at	M91380

Birth Defects Res A Clin Mol Teratol. Author manuscript; available in PMC 2010 November 7.

[1]Gene	Affymetrix Probe ID#	Gen Bank Accession Number
Follistatin	98817_at	Z29532
Growth differentiation factor 11	101814_at	AF100906
Growth differentiation factor 15	104646_at	AJ011967
Growth differentiation factor 9	160567_at	L06444
Growth differentiation factor 10	160860_at	AI853332
Growth differentiation factor 3	161144_r_at	AV217223
Growth differentiation factor 3	92476_at	L06443
Growth differentiation factor 1 /// longevity assurance homolog 1 (S. cerevisiae)	93642_at	M62301
Growth differentiation factor 8 / Myostatin	97155_at	U84005
Thrombopoietin	97734_at	L34169
Hepatoma-derived growth factor, related protein 2	98599_at	D63850
Growth differentiation factor 5	98793_at	U08337
Anti-Mullerian hormone	101765_at	X63240
IGF-1	95545_at	X04480
IGFBP-3	95083_at	X81581
Interleukin 6	102218_at	X54542
PDGFs	99890_at	M64849

[1]All constitutively- and differentially-expressed genes encoding members of the TGFß superfamily and their functionally associated signaling proteins from GD-12, GD-13 and GD-14 developing orofacial tissue (n=3 for each gestational day). Only those genes which demonstrated statistically significant expression in the tissue on *at least* one of the three days of gestation in *all three biological replicates (n=3)* are included in this table.

Table 2

Genes Encoding Members of the TGFß Superfamily and their Associated Signaling Proteins which are Differentially Expressed During Murine Orofacial Ontogenesis.

Probe Set ID	Gene	[1]Fold Change GD-13 vs. GD-12	[1]Fold Change GD-14 vs. GD-13
101918_at	TGF beta1	-1.12	6.50
103209_at	Latent transforming growth factor beta binding protein 1	1.10	2.14
161307_f_at	Calpain 10	1.05	-5.04
92237_at	Retinoid X receptor gamma	-1.05	2.10
92695_at	Frequently rearranged in advanced T-cell lymphomas (Frat-1) (Wnt signaling; cell growth & maintenance)	-2.20	-1.20
97769_at	Prostaglandin F receptor	2.83	1.07
93534_at	Decorin	3.33	1.62
94179_at	Dachshund-1	1.35	-2.30
101815_at	BMP10	-2.52	1.63
92485_at	BMP15 or GDF-9B	1.41	-2.70
96920_at	Protease, serine, 11 (Prss11) (Igf binding) [Negative regulation of BMP signaling]	2.20	2.25
99865_at	BMP receptor, type II	-2.14	1.18
93452_at	Cerberus 1	3.18	-2.14
102266_at	Inhibin alpha	2.25	-1.55
97734_at	Thrombopoietin	-2.41	1.18
160109_at	Sox-4	-2.05	-1.023
102994_at	Signal transducer and activator of transcription 4 (Stat4)	2.56	-2.00
100278_at	Cyclin-dependent kinase inhibitor p27kip1 (Cdkn1b)	-1.91	-1.15
98067_at	Cyclin-dependent kinase inhibitor 1A (p21)	1.78	2.20
101900_at	Cyclin dependent kinase inhibitor 2B (Cdkn2b or p15)	3.18	1.18
94881_at	Cyclin-dependent kinase inhibitor 1A (p21)	1.90	2.00
94305_at	Alpha 1 type I procollagen	1.80	3.03
161716_at	Fos (FBJ osteosarcoma oncogene)	-3.33	1.50
92739_at	Involucrin (keratinocyte differentiation)	1.55	-2.10
102218_at	Interleukin 6	2.50	-1.45
161509_at	MMP2 (gelatinase A)	-2.41	2.35
99957_at	MMP9 (gelatinase B)	6.96	1.12

[1]Triplicate gene expression data sets from orofacial tissue from each of GD-12, GD-13 and GD-14 embryos were filtered and the average fold change for each gene was calculated from three different comparisons of the gene expression patterns of GD-13 vs. GD-12 and GD-14 vs. GD-13 orofacial tissue. Only those genes which demonstrated a statistically significant ($p<0.005$) increase or decrease in expression of at least two-fold in *all three biological replicates* for either the GD-13 vs. GD-12 expression comparison or the GD-14 vs. GD-13 expression comparison were included in this table. Note that GD-13 vs. GD-12 means that expression on gestation day 12 was utilized as the baseline, and that GD-14 vs. GD-13 means that expression on gestation day 13 was utilized as the baseline. Therefore, negative numbers indicate a decrease in expression, whereas positive numbers indicate an increase in expression.

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Table 3

Verification of GeneChip Microarray Data by TaqMan Quantitative Real-Time PCR[1]

Gene[2]	[3]Fold Change GD-13 vs. GD-12	[3]Fold Change GD-14 vs. GD-13	[4]Concordance
TGFß1	-1.32	2.05	+/+
Latent transforming growth factor beta binding protein 1	1.21	2.03	+/+
Dachshund-1	1.23	-2.10	+/+
BMP10	-2.70	2.30	+/+
BMP15 or GDF-9B	2.30	-2.00	+/+
BMP receptor, type II	-2.15	1.05	+/+
Cerberus 1	2.06	1.73	+/-
Inhibin alpha	2.03	-1.36	+/+
Sox-4	-2.01	-1.41	+/+
Signal transducer and activator of transcription 4 (Stat4)	2.15	2.05	+/-
Cyclin-dependent kinase inhibitor 1A (p21)	1.70	3.00	+/+
Cyclin-dependent kinase inhibitor 1B(p27)	-2.15	-1.30	+/+
Cyclin dependent kinase inhibitor 2B (p15)	2.01	1.36	+/+
MMP9	3.03	1.19	+/+

[1]Developmental expression patterns of 14 genes in embryonic orofacial tissue were compared using Affymetrix GeneChip Arrays and TaqMan Quantitative Real-Time PCR as detailed in Materials and Methods. All analyses were performed in triplicate.

[2]Target genes were selected based on results from Affymetrix GeneChip Arrays.

[3]Note that GD-13 vs. GD-12 means that expression on gestation day 12 was utilized as the baseline, and that GD-14 vs. GD-13 means that expression on gestation day 13 was utilized as the baseline. Therefore, negative numbers indicate a decrease in expression, whereas positive numbers indicate an increase in expression.

[4]+/+ indicates full concordance in the pattern/level of gene expression obtained during both the GD-12 to GD-13 developmental interval and the GD-13 to GD-14 developmental interval using Affymetrix GeneChip Arrays and TaqMan Quantitative Real-Time PCR. “+/-” indicates a comparable pattern of gene expression was detected in the gd-12 to gd-13 developmental interval and a differing pattern of gene expression was detected in the gd-13 to gd-14 developmental interval using Affymetrix GeneChip Arrays and TaqMan Quantitative Real-Time PCR.