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TGF β -1 and Wnt-3a interact to induce unique gene expression profiles in murine embryonic palate mesenchymal cells

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Abstract

Development of the secondary palate in mammals is a complex process under the control of numerous growth and differentiation factors that regulate key processes such as cell proliferation, synthesis of extracellular matrix molecules, and epithelial-mesenchymal transdifferentiation. Alterations in any one of these processes either through genetic mutation or environmental insult have the potential to lead to clefts of the secondary palate. Members of the TGF β family of cytokines are crucial mediators of these processes and emerging evidence supports a pivotal role for members of the Wnt family of secreted growth and differentiation factors. Previous work in this laboratory demonstrated cross-talk between the Wnt and TGF β signaling pathways in cultured mouse embryonic palate mesenchymal cells. In the current study we tested the hypothesis that unique gene expression profiles are induced in murine embryonic palate mesenchymal cells as a result of this cross-talk between the TGF β and Wnt signal transduction pathways.

Keywords

embryo; palate; TGF β ; Wnt; microarray; gene expression

1. INTRODUCTION

Clefts of the secondary palate in humans occur in approximately 1 in 700 live births and are one of the most common birth defects [1]. In humans and in mice, the bilateral palatal shelves originate from the maxillary processes, initially developing vertically alongside the tongue, with subsequent elevation above the tongue and fusion to form the definitive secondary palate, separating the oral and nasal cavities. Several key processes are important for normal palate formation, including mesenchymal cell proliferation, extracellular matrix synthesis, fusion of the shelves, and subsequent removal of the medial edge epithelial seam.

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Perturbations in any of these processes can lead to incomplete palate development and clefts.

TGF β and Wnt are secreted cytokines that regulate many biological processes, including cell proliferation and extracellular matrix production, each critical for normal development of the secondary palate. Both of these cytokines comprise a family of structurally, if not functionally, related proteins that signal via binding to cell surface receptors that, in turn, activate a nucleocytoplasmic protein that ultimately leads to changes in gene expression patterns. The precise nature of these gene expression changes depends upon a number of factors, including cell type and the microenvironment in which the cell exists. Currently, there are 3 known TGF β isoforms (-1, -2, and -3) each of which is expressed in specific spatio-temporal patterns in the developing murine secondary palate [2, 3]. The importance of TGF β s in palate development has been demonstrated through several approaches: exogenous application of TGF β to *in vitro* cultured palates induced precocious differentiation of the medial edge epithelium (MEE), whereas antisense oligonucleotides to TGF β -3 prevented MEE differentiation in embryonic palates grown in culture [4, 5]. Gene knockout studies of TGF β -3 in mice support a role in MEE differentiation and fusion of the embryonic palatal processes [6] and knockout of the type 2 TGF β receptor leads to a cleft palate [7].

The vertebrate Wnt gene family is currently composed of 19 members that activate multiple pathways through at least 12 membrane-bound receptors (Frizzleds). Much like the TGF β s, Wnts are morphogens that regulate early developmental processes such as axis specification, neural patterning, and tissue growth and differentiation [8]. In addition, roles for Wnts in proliferation and apoptosis have been verified experimentally in numerous cell types [9-11]. Wnts have previously been broadly classified into two groups based upon function: those that activate the β -catenin dependent (canonical) pathway and those that activate one of several non-canonical pathways. Wnt-1, -3a, -8a, and -8b are generally classified as members of the former group while Wnt-4, -5a, and -11 are generally classified as members of the latter group. More recent experiments, however, have shown that prediction of the specific pathway activated by a particular Wnt is not straightforward, and goes beyond the identity of the Wnt. For example, Wnt-5a was generally believed to activate the planar cell polarity pathway, however, it can also activate the canonical/ β -catenin pathway when co-expressed with Frizzled-4 and LRP5 [12]. Mutations in several Wnt genes, including Wnt-3a, have been found in patients with non-syndromic cleft palate [13] and mouse gene knockout models have suggested roles for Wnt-5a and Wnt-9b in the development of the secondary palate [14, 15]. We have recently characterized the expression of members of the Wnt family in the developing murine secondary palate and found that several were expressed in unique spatial and temporal patterns suggesting specific functions during palate development [16].

Wnt and TGF β have many overlapping activities and recent studies have demonstrated that these two pathways functionally interact in a number of systems to regulate gene expression. Activin (a member of the TGF β superfamily) and Wnt signaling pathways interact to stimulate expression of the Wnt target gene *Siamois* in the *Xenopus* embryo [17]. Additionally, the Wnt transcription factor Lef1 coordinates signals both from Wnt and decapentaplegic (a TGF β -like ligand) in *Drosophila* [18]. In chondrocytes, TGF β can activate β -catenin translocation by promoting an interaction with Smad 3 [19]. Importantly, our own work has demonstrated a physical and functional interaction between the TGF β and Wnt pathways in mouse embryonic palate/maxillary mesenchymal cells (MEMM) [20, 21]. The goal of the current study was to determine the gene expression profile in MEMM cells stimulated with both TGF β and Wnt, compared to that in cells treated with either cytokine

alone, in order to identify unique genes that require concomitant signaling inputs from both TGF β and Wnt signaling pathways.

2. MATERIALS and METHODS

2.1 Animals

ICR mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed at an ambient temperature of 22°C with a 12 h light/dark cycle and access to food and water *ad libitum*. For timed matings, mature male and female mice were housed overnight and the presence of a vaginal plug the following morning was taken as evidence of mating and designated as embryonic day 0.5 (E0.5). Pregnant mice were euthanized on E13.5 for preparation of primary cultures of palate mesenchymal cells.

2.2 Cell culture/cytokine treatment/RNA purification

Primary cultures of mouse embryonic palate mesenchymal cells were established from secondary palatal tissue microdissected from E13.5 mouse embryos. The dissected tissue was dissociated by a 10 min digestion with 0.05% (w/v) trypsin and suspended in OptiMEMI (GIBCO Invitrogen Corp., Gaithersburg, MD) supplemented with 5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), filtered through a 70 μ m filter, and 9×10^5 cells plated in a 10 cm diameter tissue culture dish. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air. The following day, the cultures were typically 60-70% confluent. Cell cultures were rinsed two times with DME/F12 media (Sigma Chemical Co.) supplemented with glutamine and antibiotics/antimycotics (GIBCO Invitrogen Corp.) and incubated overnight in the same medium. Cell cultures were then treated for four hours with vehicle (1% [w/v] BSA in PBS), 2 ng/ml TGF β -1 (R & D Systems, Minneapolis, MN), 200 ng/ml recombinant Wnt-3a (R & D Systems), or a combination of both TGF β -1 and Wnt-3a. Following treatment, cells were washed two times, harvested in ice-cold PBS, and total RNA purified with the Qiagen RNeasy system (Qiagen, Valencia, CA). For these studies, the cells were not passaged, but treated within 48 hours of establishing primary cell cultures. Three independent sets of cell cultures were established and processed for microarray analyses as detailed below.

2.3 cRNA preparation

Biotinylated cRNA was prepared from 1-2 μ g purified RNA using the GeneChip® expression 3'-amplification one-cycle cDNA synthesis kit and *in vitro* transcription reagents (Affymetrix, Santa Clara, CA). Twenty μ g of cRNA was hydrolyzed into 35-200 nucleotide length fragments. At each step, the quality of the RNA, cDNA, or cRNA was determined by analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA).

2.4 GeneChip® hybridization

cRNAs from individual samples were hybridized to separate oligonucleotide array chips that contained 45,101 genes and expressed sequence tags (GeneChip® mouse genome 430 2.0 array, Affymetrix). All chips were from the same production lot and were processed simultaneously according to the manufacturer's instructions.

2.5 Data analysis

Hybridized GeneChips® were scanned and initial data collected with the GeneChip® scanner 3000 (Affymetrix) and processed using GCOS 1.2 software. The resultant CEL files containing the raw data were imported into GeneSpring ver. 7.2 (Agilent Technologies) for subsequent processing to normalize data. Filters were applied to generate a list of genes whose expression was significantly changed (>1.5-fold) in response to the various treatment

regimens. Gene mapping was performed with the `mm430mmrefseqcdf` R package followed by normalization with the `rma` function for convolution background detection, quantile normalization, and median polish. Lists of genes that were significantly different as defined by an adjusted p value of less than 0.05 are reported.

2.6 Real-time PCR (TaqMan®)

Purified RNA from each sample that was used for cRNA preparation was used to prepare cDNAs using the SuperScript first strand cDNA synthesis system (GIBCO Invitrogen Corp.). An amount equivalent to 10-20 ng of the initial input of total RNA was used as the template in real-time TaqMan® PCR assays with probe:primer pairs purchased from Applied Biosystems (Foster City, CA). PCR was performed on the ABI Prism 7000 Sequence Detection Platform (Applied Biosystems). Threshold values (Ct) were normalized to those for GAPDH, previously demonstrated to be a stable control gene in cultured MEMM cells [22]. Fold-change values were determined according to the relationship: fold-change = $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in Ct for the same probe:primer pair on cytokine treated MEMM cells vs. vehicle treated cells, and $\Delta\Delta Ct = \Delta Ct$ of the sample minus ΔCt of GAPDH [23]. The analysis was performed on 2-3 independent sets of cDNA. In some cases, the genes selected for analysis were from lists generated from combined treatment with TGFβ-1 and Wnt-3a, but not yet analyzed for genes unique to the combined treatment, and thus are not present in the included tables.

3. RESULTS

Primary cultures of murine embryonic palate mesenchymal (MEMM) cells were established and stimulated with 2 ng/ml TGFβ-1 in the presence or absence of 200 ng/ml Wnt-3a and analyzed for changes in gene expression (relative to control) using high-density oligonucleotide-based microarray technology in order to test the hypothesis that simultaneous signaling inputs from the TGFβ-1 and Wnt pathways lead to the regulation of a unique set of genes in these cells. Previous studies have established that the response of cultured MEMM cells to growth factors is similar to that observed *in vivo* with respect to cell proliferation, extracellular matrix production, and signal transduction [4, 24]. Therefore, these cells provide an excellent *in vitro* model to examine the effects of growth and differentiation factors, the results of which can be potentially translated to development of the palate *in situ*. In previous studies, the Wnt pathway was activated in primary cultures of MEMM cells with conditioned media from cultures of mouse L-cells stably transfected with the cDNA for Wnt-3a [20]. This source of Wnt-3a is efficient at stimulating the canonical Wnt pathway, however, for the current experiments, purified recombinant Wnt-3a was used to avoid potential interference from other growth factors present in serum-supplemented medium. Results from preliminary experiments demonstrated that 200 ng/ml Wnt-3a yielded the maximum response in MEMM cells transfected with the canonical Wnt reporter plasmid, TOPflash (data not shown). Subconfluent cultures of MEMM cells were stimulated for 4 hours with 2 ng/ml TGFβ-1, 200 ng/ml Wnt-3a, or a combination of both cytokines, at which time the cells were harvested and RNA was isolated and analyzed for gene expression as detailed in the **Materials and Methods** section. Only those genes with an adjusted p -value less than 0.05 and whose expression changed by 1.5-fold or greater were included in the analyses.

The data from the present study are reported as genes regulated (induced or repressed by more than 1.5-fold) independently by TGFβ-1 (Tables S1-S2) or Wnt-3a (Tables S3-S4), and those genes whose expression was changed following treatment with *both* TGFβ-1 and Wnt-3a, but that were not regulated by either alone (Tables S5- S6). Stimulation of MEMM cells with 2 ng/ml TGFβ-1 for 4 hours resulted in up-regulation of the expression of 96 genes (Table S1) and down-regulation of the expression of 56 genes (Table S2), while

treatment of MEMM cells with Wnt-3a led to up-regulation of the expression of 66 genes and down-regulation of the expression of 23 genes (Tables S3 and S4, respectively). When MEMM cells were stimulated with a combination of TGF β -1 and Wnt-3a, the expression of 160 genes was induced and that of 123 genes was repressed - all representing genes that were unique to cytokine co-treatment of the cells (Tables S5 and S6, respectively). This pattern of gene expression is summarized in Figure 1. Therefore, with the current experimental design, exposure to TGF β -1/Wnt-3a together led to the establishment of a unique gene expression profile in palate mesenchymal cells that may have implications for palate development. Real-time PCR (TaqMan®) was used to verify the changes in expression of 9 genes and were compared to the values obtained by microarray analysis (Table 1). For 8 out of 9 genes examined, the fold-changes in expression were similar. These data, combined with the observation that many known TGF β -1 and Wnt-3a target genes were identified (see below), confirm the validity of the array data.

In order to determine the biological significance of the genes regulated by TGF β -1 and Wnt-3a and interpret them in the context of secondary palate development, a bioinformatic analysis of these six gene sets was performed using DAVID (the Database for Annotation, Visualization and Integrated Discovery) [25]. Clustering of functionally annotated genes regulated in MEMM cells by TGF β -1, Wnt-3a, or a combination of both, is presented in Table 2. As expected, TGF β -1 regulates genes associated with promoting differentiation. In addition, it regulates the expression of genes involved in glycosaminoglycan binding, cell adhesion, and in the regulation of its own signaling pathway, as well as the related gene family, Bone Morphogenetic Proteins (BMPs). TGF β -1 also (negatively) regulates transcription regulator activity and cell proliferation. Additionally, TGF β -1 represses genes linked to negative regulation of signal transduction pathways, possibly resulting in a net increase in signal strength. Wnt-3a regulates the expression of genes involved in chemokine signaling, cell migration, and mesenchyme differentiation. Similar to TGF β -1 regulation of its downstream signaling proteins, Wnt-3a regulates the expression of other Wnt pathway molecules, such as Dickkopf-2, Axin-2, and Frizzled-1 (Table S3). Also similar to TGF β -1, Wnt-3a inhibits the expression of genes involved in transcription regulation and cell proliferation, in addition to those involved in metal ion binding and signal transduction. The combined TGF β -1/Wnt-3a treatment of MEMM cells led to induction of genes linked to transcription regulation, apoptosis, extracellular matrix, and protein modification. The combined treatment led to the repression of genes linked to chromatin remodeling, anti-apoptosis, and development of the epithelium. This analysis suggests that TGF β -1 and Wnt-3a have overlapping functions (regulation of the expression of genes involved in cell proliferation, cell differentiation, and transcription regulation and signal transduction), but more importantly, function to regulate additional unique categories of genes when combined (apoptosis and chromatin remodeling).

4. DISCUSSION

Development of the secondary palate in mice and in humans is a complex process that requires the coordinated actions of multiple cytokines and signaling pathways to regulate cell proliferation, apoptosis, extracellular matrix synthesis, and cell differentiation. Disturbances due to gene mutation or environmental insult can disrupt these signaling networks and lead to palatal clefts, one of the most common birth defects in humans. Signaling pathways do not function in isolation but interact with each other at multiple levels and the precise nature of these interactions determines the ultimate cellular response. Members of the TGF β and Wnt families of cytokines are found in defined and overlapping temporospatial expression domains in the developing murine secondary palate. Importantly, we have previously demonstrated that the pathways activated by these two cytokine families interact in mouse palate mesenchymal cells [20]. The data presented above are the first

systematic analysis of gene expression changes as a direct consequence of co-exposure of embryonic palate mesenchymal cells with TGF β -1 and Wnt-3a, with the goal of identifying genes that require inputs from both cytokines for their expression.

In the current study, we found that, when combined, TGF β -1 and Wnt-3a altered the expression of a significantly greater number of genes in MEMM cells than when the cells were exposed to either cytokine individually. Notable, however, was the discovery of a unique set of genes regulated by both TGF β -1 and Wnt-3a, suggesting that co-expression of these two pathways in the developing palate may likewise influence distinct developmental processes.

A previous membrane-based cDNA array study from our laboratory examined the expression of 1,178 genes following stimulation of MEMM cells with TGF β -1 [26]. The analysis presented in the current study not only increases the number of genes screened for responsiveness to TGF β -1 in palate mesenchymal cells, but also provides the first comprehensive screen of genes regulated by Wnt in this embryonic cell type. Because the role of Wnt is only now unfolding, these results are an important contribution toward an understanding of this signaling pathway in development. Although signaling through the TGF β -1 and (canonical) Wnt pathways has been extensively characterized in many cell types, the functional consequence of cross-talk between these two pathways in most tissues is not known. Recently, Labbé *et al.* performed a similar analysis of the cooperation between TGF β -1 and Wnt in regulating genes in an epithelial cell line, NMuMG [27]. Interestingly, they identified many of the same genes regulated by TGF β -1/Wnt-3a in embryonic palate mesenchymal cells. For example, they found that thrombospondin-1, inhibin β A, heparan sulfate 6-O-sulfotransferase 2, interleukin-11, and SRY-box containing gene 11 were all up-regulated in response to TGF β -1 + Wnt-3a [27]. These observations suggest that there may be common gene targets regulated by TGF β -1 + Wnt-3a in multiple cell types.

Classification of the genes regulated by simultaneous exposure of MEMM cells to TGF β -1 and Wnt-3a into functional groups with DAVID [28], a web-based data-mining tool, suggests that many of the genes are linked to apoptosis, extracellular matrix production, regulation of gene expression, protein modification, epithelial development and chromatin remodeling. The categories of genes regulated by cytokine co-treatment of the cells differs from the gene categories regulated by either cytokine alone, suggesting that there are unique gene signatures depending on which cytokine, or combination of cytokines, is expressed and functional at any given time in development of the tissue. Combined treatment of MEMM cells with TGF β -1 and Wnt-3a altered the expression of genes associated with apoptosis and extracellular matrix synthesis/breakdown. Along with its well-known role in removing damaged cells, the process of apoptosis is important during development, where it removes transient tissue and aids in remodeling or “sculpting” of existing tissue. Likewise, the extracellular matrix is indispensable for remodeling nascent tissue as the embryo grows and develops. The importance of apoptosis during secondary palate development is highlighted by a study by Okano *et al.* demonstrating that one contributing factor for retinoic-acid-induced cleft palate was due to perturbations in the rate and extent of apoptosis [29]. The synthesis and degradation of the extracellular matrix in the secondary palate has been proposed to be necessary for palatal shelf elevation [30] and although the spatio-temporal expression patterns of many extracellular constituents in the developing secondary palate have been characterized [31, 32], a complete description of the individual molecules is lacking. While the regulation of these molecules requires additional clarification, it is recognized that tight control is critical for normal palate development [33]. Coordinated regulation of both apoptosis and extracellular matrix metabolism is necessary for proper growth and elevation of the palatal shelves and the work described in the current paper

suggests that crosstalk between the TGF β and Wnt signaling pathways may be an important means of regulation for both of these processes.

Post-translational protein modifications have been largely unexplored in previous studies of palate development. While sumoylation, has been linked to cases of non-syndromic cleft lip/palate [34, 35], no studies report arginylation, palmitoylation, or ubiquitylation/deubiquitylation as playing a role in normal or abnormal development of the palate. Results from the current array analysis suggest that signaling through the TGF β and Wnt pathways may indirectly result in protein modifications that could alter the activities of proteins involved in palate development. A complete understanding of how TGF β and Wnt singularly or coordinately regulate protein modifications will yield valuable insight into the mechanisms of palate development.

The role of epigenetic regulatory mechanisms in secondary palate development (DNA methylation, miRNAs, and histone modifications) is also beginning to emerge [36, 37]. An important category of genes regulated by TGF β -1 and Wnt-3a are those related to chromatin remodeling, a process that can have profound influences on spatio-temporal patterns of gene expression in the developing palate. Coordinate signaling by the TGF β -1 and Wnt-3a pathways has the potential to regulate a much broader spectrum of genes than either alone. The exact nature of this regulation, once revealed, will yield important insights into the genetic/epigenetic control of palate development. Interestingly, genes associated with epithelial differentiation were down-regulated by TGF β -1 and Wnt-3a suggesting that these two cytokines may act to maintain the mesenchymal phenotype.

Potential mechanisms by which TGF β -1 and Wnt-3a regulate distinct genes sets

The integration of multiple signaling pathways ultimately resides at the level of specific transcriptional complexes mediating regulation of gene expression. These transcriptional complexes are thought to be cell/tissue-specific and are responsible for the pleiotropic nature of many signaling pathways. Indeed, we have previously reported that the TGF β -1 and Wnt-3a signal transduction pathways exhibit cross-talk at the level of transcription factor interaction (Smad-Dishevelled) [21]. Additional factors no doubt contribute to the protein complex that mediates the alterations in gene expression mediated by these two cytokines, and identification of these will be important for characterizing the mechanisms of interaction between these two pathways. It is also likely that TGF β -1 may induce/repress the expression of Wnt signaling modifier genes, altering Wnt specificity and resulting in unique patterns of gene expression. Likewise, Wnt-3a may have a similar effect on TGF β -1.

Regulation of specific genes previously linked to palate development

Although a variety of genes have been identified as important for secondary palate development in mice, the regulation of most of these genes during development of the tissue is not fully understood. Several of these genes that have been shown to play a key role in palatogenesis were found to be regulated in embryonic palate mesenchymal cells by concomitant exposure to TGF β -1 and Wnt-3a. Interleukin-6 expression was up-regulated 3.1-fold following treatment of MEMM cells with both TGF β -1 and Wnt-3a. Baroni *et al.* demonstrated that Interleukin-6 secretion was higher in fibroblasts from patients with non-syndromic cleft palate [38]. Interleukin-6 can decrease the synthesis of collagen and glycosaminoglycans in fibroblasts. Thus, it is likely that tight control over the synthesis and secretion of Interleukin-6 is critical to maintain the proper balance of ECM components. TGF β -1 and Wnt-3a increased the expression of Sox11 by 1.5-fold. Mice deficient in Sox 11 have a complete cleft of the secondary palate [39]. Sox11 is widely expressed during embryogenesis, including the secondary palate, where it is thought to play a role in tissue remodeling [39]. Conversely, TGF β -1 and Wnt-3a combined to reduce the expression of

platelet derived growth factor receptor- α (PDGFRA) in MEMM cells by 2.3-fold. The role of PDGFRA in development of the secondary palate has been demonstrated from mouse models where it has been shown to be necessary for ECM remodeling [40]. Interestingly, Eberhart *et al.* also demonstrated that the microRNA, Mirn-140 was critical for the regulation of expression of PDGFR- α in neural crest cell-derived structures of the craniofacial skeleton [41]. Thus, it appears that the regulation of PDGFR- α is complex, and involves at least TGF β , Wnt, and Mirn-140. It will be of great interest to determine if Mirn-140 is under the regulation of TGF- β , and/or Wnt, or whether all three act in concert to regulate palatogenesis.

Interestingly, Runx2, the knockout of which also leads to cleft palate, regulates the expression of Osr1 [42]. Recently, it was demonstrated that Wnt and TGF β converge to regulate Runx2 expression during osteoblast differentiation [43]. Prostaglandin E₂ is also a mediator of Runx2 expression during osteoblast maturation and we found that TGF- β and Wnt-3a synergize to dramatically increase the expression of prostaglandin-endoperoxide synthase 2 (Ptgs2) (see Table 1). Because Runx2 also increases the expression of the type I TGF β receptor, it is possible that the combination of TGF β /Wnt-3a promotes a feed-forward regulatory mechanism by stimulating prostaglandin E₂ synthesis that subsequently activates Runx2 expression ultimately promoting osteoblast differentiation.

Snail2 (slug), a transcription factor implicated in epithelial/mesenchymal transformation and cell migration has been shown to be expressed throughout the mesenchyme and in the epithelium of the mouse embryonic palatal shelves [44]. While Snail1 has been implicated in the etiology of cleft palate (failure of shelf fusion), the precise role of Snail2 in palatogenesis is not known. However, *overexpression* of Snail2 in a patient with an 8q11.2→q13.3 duplication was associated with a cleft palate [45]. In cell culture models of epithelial to mesenchymal transdifferentiation, TGF β -1 and TGF β -2 up-regulate the expression of Snail1 and Snail2, that then initiate a sequence of events that culminates in up-regulation of TGF β -3 to initiate epithelial to mesenchymal transdifferentiation [46]. The results in the present study suggest that Wnt blocks this induction and promotes down-regulation, supporting the hypothesis that the activity of TGF β is strongly influenced by other signaling pathways to precisely control the cellular and morphogenetic events that comprise the developmental program governing palatogenesis.

Although the simultaneous analysis of changes in the expression of hundreds or thousands of genes is relatively straightforward, meaningful interpretation of these changes in the context of a living cell or organism is a difficult and complex process that provides, at best, only the foundation to develop additional hypotheses regarding the function of a particular gene or signaling pathway during development. Nevertheless, the data obtained from this study identify additional gene candidates that regulate embryonic palatal tissue differentiation and provide a comprehensive profile of TGF β /Wnt-regulated gene expression patterns that may impact a number of functions important for development of the secondary palate (*e.g.* extracellular matrix metabolism and apoptosis).

Our laboratory has recently defined the expression patterns of several members of the Wnt family in the developing mouse secondary palate and found each has a unique pattern of expression, both spatially and temporally [47]. Importantly, there is significant overlap between the known domains of expression for TGF β family members and these Wnts in the developing palate. Within the secondary palate, TGF β -3 is expressed primarily in the medial edge epithelial cells [2] in a domain that overlaps significantly with that for Wnt-10a and Wnt-10b [47]. Nawshad and coworkers demonstrated that TGF β down-regulates expression of E-cadherin in MEE cells through formation of a Smad-LEF complex [48]. Wnt-4 and Wnt-11 are also expressed in the secondary palate epithelium [47] [49]. In embryonic palate

mesenchyme, there is overlap between the expression domains of Wnt-2 and Wnt-16 [47] and that for TGF β -2 [2]. Although we did not detect the expression of Wnt-3a in our screen, we initially used it as a means to stimulate the canonical Wnt pathway [20]. Notably, studies in human populations have linked mutations in the Wnt-3a gene to cases of nonsyndromic cleft palate [13]. It is possible that the level of expression of Wnt-3a was below the level of detection in our previous study [47]. On E12.5, the threshold cycle (Ct) was >35 and there was a slight decrease in Ct values on E14.5, suggesting weak expression of Wnt-3a at this later stage. Due to extremely low levels of expression, no firm conclusions could be drawn regarding Wnt-3a expression changes in embryonic mouse palate tissue [47]. The importance of both canonical and noncanonical Wnt signaling for proper development of the secondary palate has been demonstrated in several previous studies. Knockout of LRP6, a specific co-receptor for canonical Wnt signaling, leads to a cleft of the secondary palate in mice [50]. Lastly, there is direct evidence for the role of the Wnt planar cell polarity pathway in palatogenesis since gene knockout of Wnt-5a leads to clefts of the secondary palate [14].

Although much is known about the myriad of cellular responses regulated by the major signaling pathways, including those activated by the TGF β and Wnt families, a multitude of questions still remain unanswered, including how cell/tissue-specific responses are controlled and what the effect is of cross-talk among these different signaling systems. A comprehensive understanding of interaction between these two cytokines, as well as with other signaling pathways, is essential for dissecting the complex processes involved in the proper formation of the secondary palate with the ultimate goal the prevention of palatal clefts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Venn diagram illustrating the number genes whose expression changed in MEMM cells a result of TGFβ-1, Wnt-3a, or a combination of TGFβ-1 and Wnt-3a treatment

MEMM cells were treated with TGFβ-1, Wnt-3a, or a combination of TGFβ-1 and Wnt-3a for 4 hours and gene expression changes determined by microarray analysis. *Panel A.* Number of genes up-regulated by TGFβ-1 (96), Wnt-3a (66), and a combination of TGFβ-1 + Wnt-3a (252) [red, green, and blue circles, respectively]. The points of intersection denote common genes. Note that 160 genes in MEMM cells were up-regulated by simultaneous exposure to TGFβ-1 and Wnt-3a, but not following exposure to either cytokine alone. *Panel B.* Summary of genes down-regulated following cytokine treatment of MEMM cells. The expression of 56 genes was down-regulated by TGFβ-1, 23 by Wnt-3a, and 157 following treatment with both TGFβ-1 and Wnt-3a. Note that the expression of 123 genes was uniquely down-regulated following treatment with both cytokines.

Table 1

Verification of Microarray by Semiquantitative Real-Time PCR of Selected Genes

Gene	Microarray	PCR
<i>(Fold-change)</i>		
Smad 3	−2.3	−2.0
Wnt-9a	+2.2	+1.9
Dkk-2	+3.2	+3.0
Osr-2	nc	−1.5
Ptgs2	+29.0	+22.0
Shox2	−1.5	−1.6
Edn1	+4.5	+4.0
Ahr	+2.2	+2.1
Cadherin 6	+2.2	+2.1

A sample of the RNA used for the microarray analysis was used to synthesize first-strand cDNA which was then analyzed by real-time PCR (TaqMan®) with specific primers for each gene listed. The data are reported as fold-change following treatment with a combination of both TGFβ1 and rWnt-3a, compared to the vehicle control. The microarray results are after data reduction and normalization of the three replicates and the PCR data is a representative of 2-3 analyses. (+) indicates fold-increase and (−) fold-decrease.

TABLE 2

DAVID Analysis of Genes Regulated by TGF β -1 and Wnt-3a^a

Gene Set	GO Term	Enrichment Score
TGF β -1 induced	Glycosaminoglycan binding	4.02
	Cell differentiation	2.75
	TGF β /BMP signaling regulation	2.62
	Cell adhesion	2.37
TGF β -1 repressed	Transcription regulator activity	2.65
	Cell proliferation	2.30
	Epithelium development	2.16
	Negative regulation of signal transduction	2.28
Wnt-3a induced	Chemokine activity	3.86
	Regulation of Wnt signaling	3.00
	Cell migration	2.71
	Mesenchyme cell differentiation	1.87
Wnt-3a repressed	Transcription regulation/RNA metabolism	2.08
	Cell proliferation	1.34
	Metal ion binding	0.44
	Cell surface-linked signal transduction	0.28
TGF β -1/ Wnt-3a induced	Regulation of gene expression	3.48
	Apoptosis	2.89
	Extracellular matrix	2.34
	Regulation of protein modification	2.01
TGF β -1/ Wnt-3a repressed	Chromatin remodeling	1.31
	Negative regulation of apoptosis	1.26
	Epithelium development	0.77
	Cellular response to DNA damage	0.50

^aGene sets listed in Tables S1-S6 were categorized with the DAVID algorithm in order to identify relevant functional groups of genes regulated by TGF β -1 and Wnt-3a. Shown are four of the main functional clusters of genes based upon gene ontology (GO).