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Interaction between Smad 3 and Dishevelled in murine embryonic craniofacial mesenchymal cells

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Dates:
Accepted 25 February 2005

To cite this article:
Orthod Craniofacial Res **8**, 2005; 123–130
Warner DR, Greene RM, Pisano MM: Interaction between Smad 3 and Dishevelled in murine embryonic craniofacial mesenchymal cells

Structured Abstract

Authors – Warner DR, Greene RM, Pisano MM

Objectives – To determine the *in vivo* interaction between Smad 3 and Dishevelled-1.

Design – Cell culture transfection followed by immunoprecipitation with specific antibodies.

Setting and Sample Population – The Department of Molecular, Cellular, and Craniofacial Biology, Birth Defects Center, University of Louisville.

Experimental Variable – Overexpression of myc-Smad 3.

Outcome Measure – Western blotting of anti-Dishevelled immunoprecipitates for Smad 3.

Results – Smad 3 and Dishevelled isoforms-1, -2, and -3 all bind Smad 3 in glutathione-S-transferase (GST) pull-down assays and Smad 3 binds to Dishevelled-1 *in vivo*. Stimulation of the transforming growth factor beta (TGFβ) pathway leads to increased binding of Smad 3 and Dishevelled-1 *in vivo*.

Conclusion – Smad 3 binds all three known isoforms of Dishevelled and binds Dishevelled 1 *in vivo*. TGFβ signaling modulates the interaction between Smad 3 and Dishevelled-1.

Key words: cross-talk; Dishevelled; orofacial; Smad; TGFβ; transcription; Wnt

Introduction

Early embryonic development is controlled by surprisingly few signaling pathways. Two major pathways are those activated by the transforming growth factor beta (TGFβ) and Wnt families of secreted growth and differentiation factors. The TGFβ superfamily (TGFβs, activins/inhibins, and bone morphogenetic proteins (BMPs)] alter patterns of gene expression, leading to changes in cellular proliferation, differentiation,

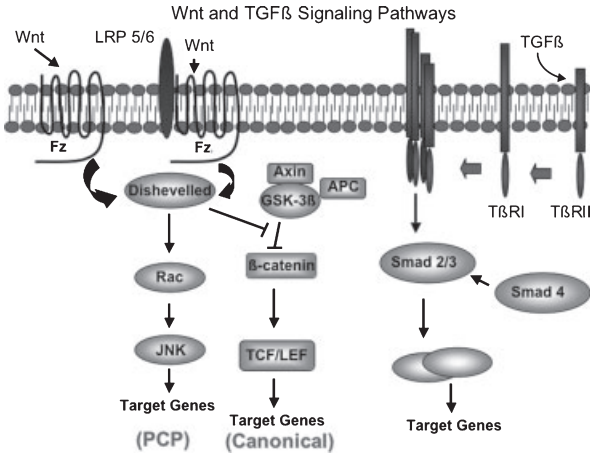

Fig. 1. Simplified schematic diagram of the Wnt and TGFβ signaling pathways. The Wnt signaling pathway has multiple signaling outcomes. The canonical pathway is activated by LRP-Frizzled-bound Wnt leading to activation of Dvl, which in turn blocks the phosphorylation of β-catenin by the GSK-3β-APC-Axin complex. β-catenin translocates to the nucleus where it interacts with TCF/LEF and activates gene transcription. The planar cell polarity pathway (PCP) is activated by Frizzled, and is mediated by Dvl leading to activation of RhoA/ROCK and JNK (Jun N-terminal kinase) with subsequent cytoskeletal rearrangements. Note the central role that Dvl plays in mediating the two pathways. The TGFβ signaling pathway is activated following binding of TGFβ to a TβRII which in turn phosphorylates TβRI leading to receptor heterodimer formation and phosphorylation of Smad 2 and Smad 3. Phospho-Smad 2/3 bind Smad 4, translocate to the nucleus, and activate or repress transcription of target genes. Adenomatous polyposis coli (APC); TβRI and TβRII, types I and II TGFβ receptors; PLC, phospholipase C.

apoptosis, and extracellular matrix synthesis (1–5). Extracellular TGFβ binds to a receptor-serine/threonine kinase (TβRII) that heterodimerizes with a type I receptor (TβRI, also a serine/threonine kinase), resulting in transphosphorylation of TβRI by TβRII (6). Smad proteins are phosphorylated by TβRI and transduce the signal into the nucleus from the cytoplasm (Fig. 1). Receptor-regulated Smads (TGFβ-regulated Smads-2 and -3 and BMP-regulated Smads-1, -5, and -8) dimerize with Smad 4 and are imported into the nucleus (7). In the nucleus, the Smad complex binds to the promoters of TGFβ-responsive genes and either stimulates (via recruitment of CREB binding protein or p300) or represses transcription (via recruitment of c-ski and snoN) (8, 9,10). Therefore, the transcriptional outcome will vary based on cell type and availability of these coactivators and corepressors.

Wnt, the mammalian homologue of *Drosophila* Wingless, is a large gene family containing, at present 19 members encoded by separate genes. Wnts are secreted, lipid modified glycoproteins that signal via binding to receptors called Frizzled (11, 12). Much like the TGFβs, Wnts regulate early developmental processes including axis specification, neural patterning, and organ development (13–16). The importance of the Wnt pathway in adult tissues is underscored by the fact its dysregulation can lead to uncontrolled cell proliferation and cancer as demonstrated by the high frequency of mutations in the Wnt pathway in colon cancer (17). Wnts are capable of activating multiple signaling pathways, including the so-called canonical pathway mediated by β-catenin (Fig. 1) and leading to gene transcription through the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors, a planar cell polarity (PCP) pathway leading to activation of Jun N-terminal kinase (JNK) and mediating changes in tissue and cell polarity, and finally, a calcium dependent pathway. All of these pathways are dependent upon Dvl, thus positioning Dvl at a critical point in the Wnt pathways. Thus, regulation of Dvl by other factors/proteins could have profound effects on the outcome of Wnt signaling.

The interaction between the Wnt and TGFβ signaling pathways has been demonstrated on several levels. For example, Activin (a TGFβ family member) and Wnt cooperate to regulate expression of *Siamois* in the developing *Xenopus* embryo (18). Transcription of the *Xenopus* homeobox gene, *Xtwn*, requires association of Smads 2, 3, and 4 with TCF/LEF (19). Decapentaplegic and Wnt coordinately control *Drosophila* embryonic expression of *Vestigial* and *Ultrabithorax* (20, 21). Wnt and TGFβ interact in formation of Spemann's organizer in *Xenopus* (22). Conversely, the TGFβ-activated MAP kinase, TAK1, antagonizes Wnt signaling via activation of Nemo-like kinase, which down-regulates transcriptional activation generated by β-catenin/TCF/LEF in transfected HEK293 cells (23). Axin, a component of the β-catenin containing cytoplasmic E3 ubiquitin ligase complex, promotes Smad phosphorylation by activated TβRI and serves as a transcriptional cofactor to promote Smad-dependent transcriptional activation (24). Thus, multiple examples of cross-talk between the TGFβ and Wnt signaling pathways have been reported.

We have previously reported the identification of Dvl-1 as a Smad binding protein which was identified from a yeast two-hybrid screen for novel Smad binding proteins expressed in developing mouse orofacial tissue (25). In the experiments presented herein, the interaction between Smad 3 and Dvl-1 was confirmed *in vivo*. These data demonstrate several levels of

cross-talk between TGFβ and Wnt in cells derived from developing orofacial tissue.

Materials and methods

Animals and primary cell cultures

ICR mice (Harlan, Indianapolis, IN, USA), were housed in a controlled environment at a temperature of 22°C with an alternating light/dark cycle. Mature male and female mice were mated overnight and the presence of a vaginal plug the following morning was taken as evidence of mating (gestation day 0). Pregnant mice were euthanized on day 13 of gestation, a critical stage of murine orofacial development. Embryos were removed from pregnant dams and embryonic maxillofacial tissue was dissected in sterile, cold phosphate-buffered saline and cells were dispersed by gentle trypsinization, then plated at a density of 6×10^3 cells/cm^2. These cells are herein referred to as murine embryonic maxillary mesenchyme (MEMM) cells.

Glutathione-S-transferase pull-down assay

In vitro interaction between the MH2 domain of Smad 3 and Dvl-1, -2, and -3 was determined using glutathione-S-transferase (GST) pull-down assays. GST-Smad 3 MH2 was expressed in and purified from *E. coli* by glutathione-Sepharose batch chromatography and 1 μg mixed with 5 μl of *in vitro* translated, [^{35}S]methionine-labeled, Dvl-1, -2, or -3, each prepared using the TNT® T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA). The mixture was incubated for 1 h at 4°C in GST pull-down buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM dithiothreitol, 10% glycerol, 1 mM EDTA, 2.5 mM $MgCl_2$, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) with gentle rotation. The sepharose:protein complex was washed three times with GST pull-down buffer and bound material eluted by the addition of 2X Laemmli sample loading buffer (26), followed by boiling for 5 min and separation on an 8–16% polyacrylamide gel under reducing/denaturing conditions. Gels were fixed for 30 min in 50% methanol:10% acetic acid, dried under vacuum, and exposed to Kodak X-Omat AR film (Eastman Kodak Company, Rochester, NY, USA) for 2 days at −80°C. Following autoradiography, dried gels were stained with Coomassie Brilliant Blue (Fisher Scientific, Pittsburgh, PA, USA) to compare the loading efficiency for each sample.

Co-immunoprecipitation of Smad 3 and Dvl-1

In vivo interaction between Smad 3 and Dvl was determined by co-immunoprecipitation. Murine embryonic maxillary mesenchyme cells were seeded into 60 mm tissue culture dishes at a density of 6.0×10^3 cells/cm^2 with transfection of plasmid DNAs occurring the following day. Cells were transfected using the lipophilic agent, Effectene (Qiagen Corp., Valencia, CA, USA) with 0.5 μg each of pCMV-Smad 2 and pCMV-Smad 3 (each with an amino-terminal myc-epitope tag), 1 μg V5-tagged Dvl-1 (in pCDNA3), and 1 μg of CA-TβRI, a constitutively-active mutant of the type I TGFβ receptor. Forty-eight hours post-transfection, cells were washed twice with ice-cold phosphate buffered saline (PBS), scraped from the dish, lysed by brief sonication in RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 μg/ml aprotinin) and centrifuged at $10\,000 \times g$ for 30 min. Supernatants were pre-adsorbed with 15 μl of Protein A/G PLUS-agarose (50% slurry in PBS; Santa Cruz Biotech, Santa Cruz, CA, USA) for 1 h at 4°C. Agarose beads were removed by centrifugation and V5-Dvl-1 was immunoprecipitated by the addition of 4 μg/ml anti-V5 monoclonal antibody (Invitrogen Corp., Carlsbad, CA, USA) and incubation for 1 h at 4°C. This was followed by the addition of 15 μl protein A/G PLUS-agarose (50% slurry in PBS) with subsequent overnight incubation at 4°C with gentle rotation. Agarose beads were collected by centrifugation, washed four times with RIPA buffer and bound protein eluted by addition of 2X SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (26) and heating at 90°C for 5 min. The eluted material was analyzed by Western blotting with antibodies specific for the myc amino terminal tag of Smads 2 and 3 (BD Biosciences Clontech, Palo Alto, CA, USA). Western blotting was performed as described previously (27). Blots were reprobed with anti-V5 antibodies to confirm immunoprecipitation of V5-Dvl-1.

Preparation of Wnt-3a conditioned medium

Mouse L-cells stably transfected with the cDNA for Wnt-3a were obtained from ATCC (Manassas, VA, USA) and control, non-transfected L-cells were obtained from Dr Roel Nusse (Stanford University, Stanford, CA,

USA). Wnt-3a- and control-conditioned media were prepared according to the method of Shibamoto and can be stored for at least 1 year without appreciable loss of activity (28).

Fluorescence microscopy

Murine embryonic maxillary mesenchyme cells were transfected with pEGFP-Dvl-1, which encodes a fusion protein between EGFP and full-length Dvl-1 as described above, except that 1 μg of plasmid was used per 35 mm tissue culture dish. Forty-eight hours after transfection, cells were visualized under epi-fluorescent optics.

Antibodies and Western blotting

Antibodies to the V5 epitope were purchased from Invitrogen (GIBCO Invitrogen Corp., Gaithersburg, MD, USA), and the anti c-myc antibody was from BD Biosciences Clontech (Palo Alto, CA, USA). Rabbit polyclonal antibodies directed against mouse Dvl-1 (amino acids 399–695) were a generous gift from Dr Lin Mei (University of Alabama, Birmingham, AL, USA). Palates from gd 12–14 embryos were dissected from 1–2 litters, suspended in 50 mM Tris–Cl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM sodium fluoride, 0.1% Triton-X100, 50 g/ml aprotinin, 10 g/ml leupeptin, 1 mM sodium orthovanadate, and 1.25 mM phenylmethane sulfonyl fluoride and homogenized in a combination conical–cylindrical tissue grinder, followed by brief sonication. Protein concentrations in each sample were determined by the method of Bradford (29) using bovine serum albumin as the standard. Forty μg of cleared lysates were separated on 8–16% polyacrylamide gels (InVitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% (w/v) non-fat dry milk in TBST buffer [50 mM Tris (pH 7.6), 150 mM NaCl, and 0.1% Tween-20 for 1 h at room temperature]. Antibodies were diluted in the same blocking buffer and incubated with the membranes for 1 h at room temperature, washed, and then incubated with the appropriate horseradish peroxidase–conjugated secondary antibody for 30 min at room temperature. Immune complexes were visualized with the ECL-Plus™ chemiluminescent detection system (Amersham Pharmacia Biotech, Arlington, IL, USA) according to the manufacturer's instructions.

Results

Previously, a yeast two-hybrid screen was performed to identify unique Smad binding proteins expressed in developing mouse orofacial tissue (25). From this screen, a number of unique Smad binding proteins were identified (25, 30, 31). One of the previously reported proteins, Dishevelled-1 (Dvl-1), is a protein that mediates the Wnt signal transduction pathway (Fig. 1) and was found to bind to a greater extent to the MH2 domain of Smad than to the full-length protein (25).

In vitro interaction of Smad 3 with Dvl

Three isoforms of Dvl have been identified and all share approximately 80% amino acid identity, with the highest degree of identity in the PDZ domain (32–34). To determine if the TGFβ-responsive Smad 3 could bind Dvl-2 and Dvl-3, each was synthesized by *in vitro* translation with a rabbit reticulocyte lysate in the presence of [^{35}S]methionine and mixed with purified GST-Smad 3 MH2 in a GST pull-down assay. Data presented in Fig. 2 demonstrate that in addition to Dvl-1, Smad 3 also binds to Dvl-2 and Dvl-3. No interaction with GST alone was observed.

In vivo interaction of Smads 2 and 3 with Dvl-1

To determine if the TGFβ-regulated Smads 2 and 3 bind Dvl-1 *in vivo*, a co-immunoprecipitation assay was

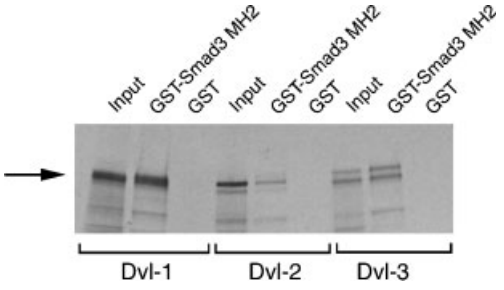

Fig. 2. Smad 3 binds to Dvl-1, -2, and 3 *in vitro*. GST-Smad 3 MH2 was expressed in and purified from *E. coli* by immobilization on glutathione-Sepharose. One μg of immobilized GST-Smad 3 MH2 was mixed with 5 μl of *in vitro* translated, [^{35}S]methionine-labeled, Dvl-1, -2, or -3, unbound material removed, and bound material analyzed by SDS–PAGE and autoradiography. The autoradiogram demonstrates specific binding of GST-Smad 3 MH2 to all three isoforms of Dvl. GST-bound glutathione-Sepharose did not interact with Dvl. The lanes marked as 'Input' demonstrate the amount of [^{35}S]methionine-labeled Dvl in 10% of the *in vitro* translation product added to each sample. The arrow indicates the position of full-length Dvl with lower molecular weight products representing truncated translation products.

performed (Fig. 3). Epitope-tagged versions of Dvl-1 (V5-tagged) and Smads 2 and 3 (both myc-tagged) were co-transfected into MEMM cells and whole-cell lysates prepared and immunoprecipitated with an anti-V5 antibody. Immunoprecipitates were analyzed by Western blotting with an anti-myc antibody (Fig. 3, bottom panel). Myc-Smad 3, and to a lesser extent, myc-Smad 2, were found in the immunoprecipitate, but only in the presence of V5-Dvl-1. No Smads were detected when V5-Dvl-1 was omitted. The small amount of Smad 2 found in the immunoprecipitate suggests that Dvl-1 has a greater capacity to bind to Smad 3 than Smad 2, *in vivo*. Smad 2 was also not expressed at the same level as Smad 3, possibly contributing to the weaker signals (Fig. 3, compare 'Input' lanes). Since it was previously demonstrated that the MH2 domain of several Smads had a greater capacity to interact with Dvl-1, it was proposed that activation of Smads via phosphorylation (which exposes the MH2 domain to downstream effectors) enhances the interaction between these two proteins (25). To directly test this hypothesis, the TGFβ pathway was stimulated by co-transfection of a constitutively-active mutant of the type I TGFβ receptor (CA-TβRI, which has a Thr to Asp mutation of amino acid 204) with myc-Smad 2 and 3 and V5-Dvl-1. Results presented in Fig. 3 demonstrate an increase in the level of myc-Smad 3 co-immunoprecipitated with V5-Dvl-1 in the presence of CA-TβRI (1.9–2.6-fold). Little change in the amount of myc-Smad 2 was observed in the immunoprecipitate. The increased amount of myc-Smad 3 co-precipitated with V5-Dvl-1 was not because of increases in the amount of V5-Dvl-1 immunoprecipitated, since Western blotting revealed similar amounts of V5-Dvl-1 immunoprecipitated with the anti-V5 antibody in the presence or absence of CA-TβRI (Fig. 2, top panel). Thus, TGFβ signaling modulates the interaction between Dvl-1 and Smad 3.

The TGFβ signaling pathway has been the subject of intense research in developing orofacial tissue, exemplified by the discovery that TGFβ3 is essential for proper palate fusion (35). In addition, knockout of the type II TGFβ receptor, which blocks all TGFβ signaling, leads to profound craniofacial defects in a mouse model (36). In contrast, the role of Wnt signaling, either through the canonical or non-canonical pathways, in orofacial development is largely unknown. To begin to dissect the function of Wnt in palatogenesis, a survey of all known Wnt signaling pathway components has been performed (manuscript in preparation). The results presented in Fig. 4 reveals that Dvl-1 is expressed during all three stages of palate development (gd12, growth;

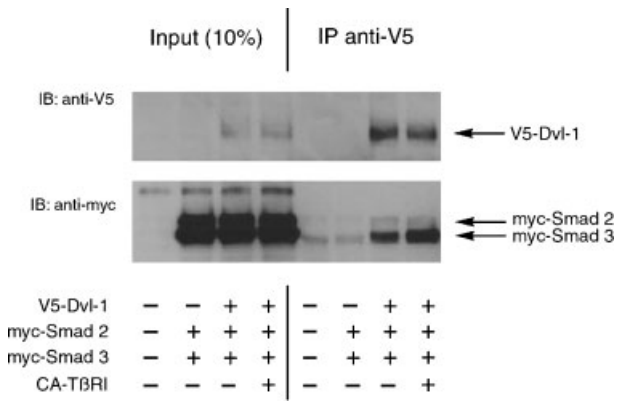

Fig. 3. Smad 3 and Dvl-1 interact *in vivo*. Myc-Smad 2, myc-Smad 3, and V5-Dvl-1 were co-expressed in MEMM cells by transfection of plasmid cDNAs. After 48 h, whole-cell lysates were prepared and V5-Dvl-1 was immunoprecipitated with antibodies specific to the V5 epitope. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting using anti-V5 (top panel) and anti-myc antibodies (bottom panel). To determine the effect of Smad activation on the interaction with Dvl-1, a constitutively-active mutant of the type I TGFβ receptor (CA-TβRI) was co-expressed with myc-Smad 2 and 3 and V5-Dvl-1. Myc-Smad 2 and, to a greater extent, myc-Smad 3 co-immunoprecipitated with V5-Dvl-1. Neither myc-Smad 2 nor myc-Smad 3 was found in immunoprecipitates when V5-Dvl-1 was omitted. Smad activation via stimulation of the TGFβ pathway with CA-TβRI led to an increase (1.9–2.6-fold) in the amount of myc-Smad 3 co-immunoprecipitated with anti-V5 compared with unstimulated cells.

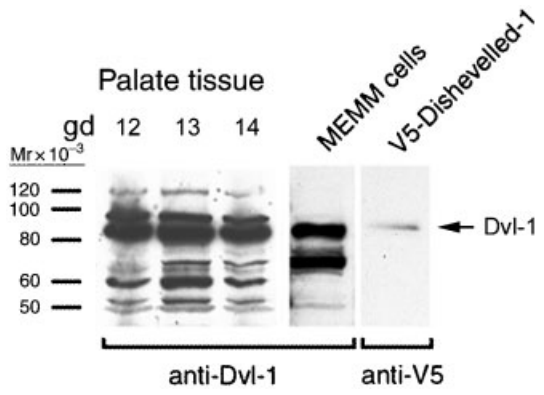

Fig. 4. Expression of Dvl-1 in mouse embryonic palate tissue and in primary cultures of MEMM cells. Palate tissue from gestation day 12–14 mice were dissected, lysates prepared, and analyzed by SDS-PAGE and Western blotting with anti-Dvl-1 antibodies. The expression in cultured cells from palate mesenchyme (MEMM) was also analyzed. Since the anti-Dvl-1 antibody also bound several other non-specific proteins, the position of Dvl-1 was determined by overexpression of V5 epitope-tagged Dvl-1 in MEMM cells followed by Western blotting with anti-V5 antibodies. Dvl-1 is expressed in secondary palates on the three days critical for palatogenesis, with no obvious temporal regulation. Cultured palate mesenchyme cells also expressed Dvl-1, demonstrating that this may be a good model for examining the role of the Wnt signaling pathway.

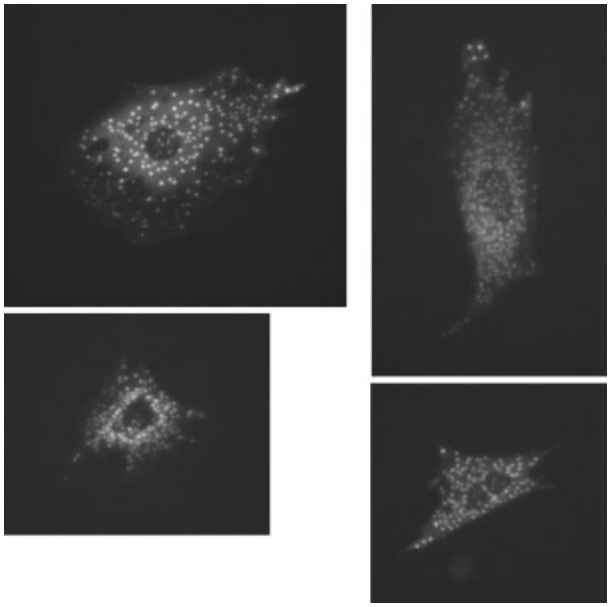

Fig. 5. Subcellular distribution of EGFP-Dvl-1 in MEMM cells. Primary cultures of MEMM cells were transfected with pEGFP-Dvl-1, and 48 hours later, examined under fluorescent optics to determine the distribution of Dvl-1 in MEMM cells. The results presented demonstrate that EGFP-Dvl-1 displays a characteristic punctate distribution throughout the cell, with higher concentrations observed in the cytoplasm, and in particular, surrounding the nucleus. These observations are consistent with the role of Dvl-1 as a cytoplasmic mediator of Wnt signaling.

gd13, shelf elevation; and gd14, shelf fusion) as determined by Western blotting with antibodies specific for Dvl-1. Not surprisingly, given the central role of Dvl-1 in mediating the disparate effects of Wnt, no developmental regulation was observed. Cultured cells derived from palate mesenchyme also expressed Dvl-1, demonstrating that this may be a good model for examining in detail the molecular mechanisms of Wnt signaling in the developing secondary palate.

Dvl-1 has been reported to exists primarily in the nucleus, although some investigators have reported Dvl-1 as capable of existing in the nucleus (37). Expression of a fusion protein between EGFP and Dvl-1 revealed a pattern similar to the primarily cytosolic, punctate expression pattern observed by others (37). No effect on the subcellular location of EGFP-Dvl-1 following stimulation with TGFβ or Wnt-3a was observed (data not shown).

Discussion

The TGFβ signaling pathway is important for early embryonic development and from a number of studies has been demonstrated to be crucial for proper craniofacial morphogenesis in (35, 36). In our attempts to further elucidate the role of TGFβ in orofacial, and specifically palate, development, a yeast two hybrid assay was performed using Smad 3 as the 'bait' protein (25). Many unique Smad binding proteins were identified, including the protein Dvl-1, a protein crucial for Wnt signaling. Smad 3 was found not only to interact with Dvl-1 (25), but also with the two other known isoforms, Dvl-2 and Dvl-3 (Fig. 2). All three isoforms share ~80 amino acid identity within the conserved DIX, PDZ, and DEP domains, with much less conservation in the remainder of the protein. Although some functional redundancy among Dvl isoforms has been described, emerging evidence exists for isoform-specific functions [e.g. see (38)]. The significance of the observed interaction between Smad 3 and all three isoforms of Dvl is unclear. Dvl-1 is present in developing palate tissue (Fig. 4), but the expression is not developmentally regulated. Considering the unique position of Dvl at the branch point of multiple Wnt signaling pathways (Fig. 1), it is likely that constitutive expression of Dvl will be found in other tissues as well.

Although the experiments demonstrating that Smad 3 binds Dvl-1 in yeast two-hybrid assays and in GST pull-down studies are unequivocal, true biological significance requires that they interact *in vivo*. The available antibodies are sufficient for Western blotting, they were not effective in immunoprecipitation experiments (not shown), therefore, epitope-tagged versions of both Dvl-1 and Smads 2 and 3 were used for transfection into MEMM cells. As revealed from the results of the experiment shown in Fig. 2, myc-Smad 3, and to a lesser extent, myc-Smad 2, interacted with V5-Dvl-1, thus demonstrating for the first time *in vivo* that Smad and Dvl-1 interact. Importantly, activation of the TGFβ pathway led to an increase in the amount of myc-Smad 3 associated with V5-Dvl-1. These data support the hypothesis that activation of Smad via phosphorylation exposes the MH2 domain of the protein creating a more optimal binding surface, and suggests that, in the basal state, Smads have poor affinity for Dvl. Thus, receptor-dependent phosphorylation and subsequent release of Smads into the cytosol leads to the formation of complexes between Smads and Dvl. The interaction between Smad 3 and Dvl-1 also occurred in a mink lung epithelial cell line (Mv1Lu, data not shown), sug-

gesting that this interaction may occur in other cell types. The observation that Smad 2 was not efficiently immunoprecipitated with Dvl-1, *in vivo*, may be because of the lower levels of myc-Smad 2 expressed in MEMM cells (compared with myc-Smad 3). In addition, Smad 2 may simply have a lower affinity for Dvl-1.

Dishevelled has been reported to colocalize with actin fibers and cytosolic vesicles (37). The expression pattern of EGFP-Dvl-1 in cultured MEMM cells is consistent with these reports. The amino terminal DIX domain is required for the vesicular localization, since a mutant Dvl-1 missing the DIX domain showed a more diffuse cytoplasmic localization with the absence of the punctate distribution (data not shown).

The initial hypothesis was that the interaction between Smads and Dvl would alter the signaling outcome through the canonical pathway. Although reporter assays with p3TP-lux and TOPflash, which measure TGFβ- and Wnt-transcriptional activity, respectively, demonstrated a positive effect by TGFβ on Wnt-mediated transcriptional activation through the canonical pathway, the effect is mediated downstream of Wnt-Dvl-1, as LiCl was able to mediate the positive interaction between TGFβ and Wnt (manuscript submitted for publication). LiCl inhibits glycogen synthase kinase-3β (GSK-3β) and stimulates β-catenin nuclear translocation. It is still a possibility that the interaction between Smads and Dvl modulates other Wnt signaling pathways, such as the PCP pathway (Fig. 1) which is involved in establishing both cell (e.g. epithelial) and tissue polarity. Interestingly, from the same yeast two-hybrid assay that identified Dvl-1 as a Smad binding protein, also identified Erbin and Par-3, which are two proteins that have been demonstrated to be critical for establishing polarity complexes (39, 40). Thus, the possibility remains that Smads can alter the signaling outcome of the Wnt pathway by either diverting the signal to, or away from, the canonical pathway.

The importance of cell polarity in palate development is underscored by the observation that mutations in the gene for *discs large*, which codes for a protein critical for cell polarity, lead to craniofacial dysmorphogenesis, including clefts of the secondary palate (41). During fusion of the palatal shelves at gd14–15, the medial edge epithelial cells are lost, in part, through epithelial to mesenchymal transdifferentiation, a process characterized by the loss of epithelial cell polarity and the assumption of a mesenchyme phenotype (42). Thus, cell polarity is critical for proper palate development and is likely to be regulated by multiple signaling pathways, including TGFβ (43). In addition, gene knockout mouse models for Dvl demonstrate that these proteins are crucial for convergent extension movements (which utilize polarity pathways) in neural tube closure (44).

Along with ongoing studies in this laboratory, these data provide a framework and foundation to examine in more detail the functional consequence of the Smad-Dvl interaction and the characterization of the Wnt signaling pathway in developing orofacial tissue with the goal of understanding and ultimately preventing orofacial defects.

Acknowledgements: The authors wish to thank Dr Anthony Wynshaw-Boris (University of California, San Diego, CA, USA) for Dishevelled-1, -2, and -3 cDNAs; Dr Paul J. Lombroso (Yale University, CT, USA) for V5-Dvl-1 and pEGFP-N2-Dvl-1 cDNAs; Dr Roel Nusse (Stanford University, CA, USA) for mouse L-cells; Dr Lilliana Attisano (University of Toronto, Canada) for CA-TβRI (TβRI-T204D); and Dr Lin Mei (University of Alabama, Birmingham, AL, USA) for anti-Dvl-1 antibodies. This work was supported, in part, by NIH grants DE12363 (to M.M.P.), DE12858, DE05550, and P20 RR017702 from the COBRE Program of the National Center for Research Resources (all to R.M.G.), and the Commonwealth of Kentucky Research Challenge Trust Fund.

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