Development 135, 2321-2329 (2008) doi:10.1242/dev.021170

β-Catenin has sequential roles in the survival and specification of ventral dermis

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The dermis promotes the development and maintains the functional components of skin, such as hair follicles, sweat glands, nerves and blood vessels. The dermis is also crucial for wound healing and homeostasis of the skin. The dermis originates from the somites, the lateral plate mesoderm and the cranial neural crest. Despite the importance of the dermis in the structural and functional integrity of the skin, genetic analysis of dermal development in different parts of the embryo is incomplete. The signaling requirements for ventral dermal cell development have not been established in either the chick or the mammalian embryo. We have shown previously that Wnt signaling specifies the dorsal dermis from the somites. In this study, we demonstrate that Wnt/β-catenin signaling is necessary for the survival of early ventral dermal progenitors. In addition, we show that, at later stages, Wnt/β-catenin signaling is sufficient for ventral dermal cell specification. Consistent with the different origins of dorsal and ventral dermal cells, our results demonstrate both conserved and divergent roles of β-catenin/Wnt signaling in dermal development.

KEY WORDS: Dermis, Cell fate, Cell survival, Skin, Sternum

INTRODUCTION

Identifying the molecular controls of dermal cell fate from diverse origins in the mammalian embryo is crucial for expanding our understanding of congenital skin defects and for advancing strategies in skin tissue engineering (Paller, 2007; Supp and Boyce, 2005). Craniofacial, dorsal trunk and ventral trunk dermis originate from different multipotential progenitor populations (Couly et al., 1992; Mauger, 1972), but is the signaling mechanism for mammalian dermal cell fate selection conserved or divergent in these different populations?

The skin consists of the epidermis, derived from the surface ectoderm, and the underlying dermis. Reciprocal interactions between the surface ectoderm and dermis induce the development of the epidermal appendages, such as hair follicles and glands of the skin (Millar, 2002). Dermal fibroblasts from different parts of the embryo have distinct inductive properties (Foley et al., 2001; Hardy, 1992; Miletich and Sharpe, 2004) and maintain positional identity in adult humans (Chang et al., 2002). Diverse origins of the dermis might be the source of variation in skin patterning, pigmentation color, and types of epidermal appendages that are found on the body (Candille et al., 2004). The genetic program to establish the distinctiveness of dermal cells in different parts of the mammalian embryo is yet to be discovered.

To address the underlying basis for differences in dorsal versus ventral skin patterning, studies have been carried out in the chick embryo, and these implicate different signaling molecules that are important for ventral dermal cell development (Fliniaux et al., 2004a; Fliniaux et al., 2004b; Sengel and Kieny, 1967). Fate-mapping studies in the chick embryo demonstrate that the proximal part of the somatopluere closest to the somites gives rise to the feather-forming dermis in the ventral trunk (Fliniaux et al., 2004a; Mauger, 1972). Suppression of bone morphogenetic protein (BMP) signaling by endogenous Noggin expression or ectopic expression of Sonic Hedgehog can induce the differentiation of ventral trunk dermal progenitors into feather-forming dermis (Fliniaux et al., 2004a). Early during lateral plate mesoderm (LPM) cell differentiation in the chick embryo, Wnts are expressed in the ectoderm overlying the somatopleure (Fliniaux et al., 2004a; Rodriguez-Niedenfuhr et al., 2003; Schubert et al., 2002). The requirement of any signaling molecule in ventral dermal cell development has not been determined in either the chick or the mammalian embryo. In this study, we examine the role of Wnt signaling in ventral dermal cell development.

The canonical Wnt signaling pathway is involved in early embryonic patterning, cell fate specification, proliferation, and the maintenance of stem cell compartments (Nelson and Nusse, 2004). β-Catenin is a key transducer of the Wnt signaling pathway (Nelson and Nusse, 2004). Embryos fail to gastrulate in the absence of β-catenin activity (Haegel et al., 1995), and unregulated β-catenin activity leads to cancer in adults (Giles et al., 2003). In the absence of Wnt signaling, the β-catenin protein is phosphorylated and marked for degradation (Nelson and Nusse, 2004). In the presence of Wnt signaling, the unphosphorylated form of β-catenin accumulates in the cytoplasm, translocates to the nucleus, binds to the TCF/Lef family of transcription factors, and promotes the transcription of Wnt target genes (Brantjes et al., 2002). Changes in downstream target gene expression mediate the diverse roles of Wnt signaling in development and disease (Nelson and Nusse, 2004; Sancho et al., 2003).

Studies in the chick embryo demonstrate the requirement of Wnt signaling in early dorsal dermal cell development from the dermamyotome (Olivera-Martinez et al., 2002; Olivera-Martinez et al., 2004). Our previous studies in the mouse embryo on dorsal dermal fate specification from the central somite indicate that Wnts, via β-catenin, provide an instructive signal for dermal fate (Atit et al., 2006). Prior to and during dorsal and ventral dermal cell specification in the mouse and chick embryo, members of the Wnt

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Accepted 10 May 2008

family are expressed in the entire dorsal surface ectoderm (Cauthen et al., 2001; Parr et al., 1993; Rodriguez-Niedenfuhr et al., 2003; Schubert et al., 2002). Using a transgenic Wnt signaling reporter, we now show that Wnt signaling is transduced in ventral subectodermal cells, which include dermal progenitors. The role of Wnt signaling in the specification and development of the ventral dermis in the mouse or chick embryo is unknown (Fuchs and Raghavan, 2002; Millar, 2002; Millar, 2005). In this study, we have used two different mouse conditional mutants to identify the role(s) of the Wnt signaling pathway in the development of the ventral trunk dermal cells.

Here, we identify multiple roles for Wnt signaling/β-catenin in ventral dermal development. First, Wnt signaling/β-catenin is required for survival of the early dermal progenitors in the LPM. Later in development, Wnt signaling/β-catenin is necessary and sufficient for the specification of ventral dermal progenitors that are derived from the flank and ventral subectodermal mesenchyme. In the conditional absence of Wnt signaling/β-catenin, the ventral dermis fails to develop. Our studies on ventral dermal development reveal a new role for Wnt signaling/β-catenin in cell survival, and a conserved role in dermal cell specification.

MATERIALS AND METHODS

Mice and genotyping

TCF/Lef-lacZ reporter transgenic embryos were used to monitor Wnt pathway activity (Mohamed et al., 2004) (obtained from Daniel Dufort). *HoxB6Cre* (Lowe et al., 2000) and *En1Cre* mice (Kimmel et al., 2000) (obtained from Michael Keuhn and Alexandra Joyner, respectively) were crossed to *R26R* mice (purchased from Jackson Laboratories) to determine the efficiency of Cre-mediated recombination (Soriano, 1999). To temporally restrict the recombination of *R26R*, *HoxB6Cre-ER^{T1}* (obtained from Susan Mackem) and *En1Cre-ER^{T1}* (Sgaier et al., 2005) (obtained from Alexandra Joyner) mice were used. Tamoxifen was administered by intraperitoneal (IP) injection (1 mg/40 g mouse) of pregnant females carrying E7.5 *HoxB6Cre-ER^{T1}; R26R* embryos. Females carrying E10.5 *En1Cre-ER^{T1}; R26R* embryos were given 3 mg/40 g of Tamoxifen by gavage. For deletion of β-catenin in the ventral dermal progenitors, *HoxB6Cre; R26R* or *En1Cre*; *R26R* mice were crossed with mice carrying an exon 2-6 floxed allele of β-catenin (*β-cat^{lof}*) (Brault et al., 2001) (purchased from Jackson Laboratories). For activation of β-catenin in ventral dermal progenitors, *En1Cre*; *R26R* mice were crossed with mice carrying an exon 3 floxed allele of β-catenin (*β-cat^{gof}*) (Harada et al., 1999) (obtained from Makoto M. Taketo). To determine the efficiency of Wnt pathway activation and inactivation, *TCF/Lef-lacZ; En1Cre* mice were crossed with *β-cat^{lof}* or *β-cat^{gof}* mice. All the mice and embryos were genotyped as previously described. For each experiment, five to eight embryos from two to three litters were analyzed. All mouse experiments were done according to protocols approved by the Case Western Reserve University IACUC committee.

In situ hybridization, immunohistochemistry and histology

Tissue preparation, histology, immunohistochemistry, *lacZ* expression, and in situ hybridization with digoxigenin-labeled probes were performed as previously described (Atit et al., 2006). The *Dermo1* probe was a gift from Eric Olson (Li et al., 1995). Antibodies against β-catenin (mouse, 1:1000; Sigma), and BrdU (mouse, 1:8; Roche) were used. Appropriate secondary antibodies conjugated to biotin (1:250; Vector Laboratories, Jackson ImmunoResearch) were used. For β-catenin immunostaining, antigen retrieval was performed on paraffin sections by boiling for 10 minutes in citrate buffer, then applying reagents from the M.O.M. kit, and incubating overnight with the primary antibody (Vector Laboratories). M.O.M. kit reagents were used as described by the manufacturer. Brightfield images were captured using the Olympus BX60 microscope and Olympus DP70 digital camera using DC Controller software. Whole-mount embryo images were captured using a Leica MZ16F microscope, and a SPOT camera system and software. Images were processed using Adobe Photoshop and Macromedia Freehand.

Cell proliferation and survival studies

Embryos were collected and processed for proliferation and survival studies as previously described (Atit et al., 2006). To assess cell proliferation in embryos, BrdU incorporation was detected by immunohistochemistry and quantified as previously described (Atit et al., 2006). In addition, antigen retrieval was performed by boiling for 10 minutes in citrate buffer prior to the application of primary antibody. Statistical significance (P>0.05) was determined by conducting a Student's t-test. Analysis was restricted to *En1* lineage-marked cells in the flank and ventral subectodermal mesenchyme. Cell survival was assayed by brightfield TUNEL staining on cryosections, as previously described (Gavrieli et al., 1992), before counterstaining with 2% methyl green for 2 minutes at room temperature.

RESULTS

Wnt/β-catenin signaling is active in ventral dermal progenitors

To identify whether Wnt/β-catenin signaling is active during ventral dermal specification and differentiation, we analyzed *TCF/Lef-lacZ* transgenic embryos. The *TCF/Lef-lacZ* transgene is a reporter of endogenous Wnt signaling activity (Mohamed et al., 2004). *lacZ* expression was present at embryonic day 8.5 (E8.5) in the somatopleure of the LPM, and in the subectodermal mesenchyme of the flank at E9.5 and E10.5 (Fig. 1A-C). By E11.5, *TCF/Lef-lacZ* transgene expression was observed in the subectodermal mesenchyme of the ventral midline (Fig. 1D). By E14.5, *lacZ* was expressed in a significant number of dermal cells, evenly around and between hair follicles (Fig. 1E, inset). Wnt signaling reporter expression in embryonic skin suggests a role in dermal differentiation and interfollicular dermal development. In our analysis, we observed a similar expression pattern of the *TCF/Lef-lacZ* transgene expression along the anteroposterior axis until the level of the hind limb (data not shown). Prior to the onset of expression of the earliest dermal progenitor marker *Dermo1* at E11.5 (Fig. 6J), cells process Wnt signaling in the dermal progenitors between E8.5 and E11.5 (Fig. 1A-D). These results suggest that Wnt signaling may have roles in early and late ventral dermal cell development.

Mouse ventral dermal cells originate from the lateral plate mesoderm

We used *Cre/loxP* tools to conduct lineage analysis of ventral dermal cells. At the forelimb level, the *HoxB6Cre* transgene drives the expression of *Cre* recombinase early in the LPM, starting at E8.0 and later (Lowe et al., 2000). *HoxB6Cre; R26R* lineage-marked cells contributed extensively to the ventral dermis by E16.5 (Fig. 2A). When we temporally restricted the recombination of *R26R*, by using an inducible *HoxB6Cre-ER^{T1}* driver, to the LPM tissue between E7.75-E8.75, we found β-gal^{+} cells dispersed in the flank and ventral mesenchyme by E11.5, and then in the ventral dermis at E17.5 (Fig. 2B-D). Lineage-marked cells were also present in the sternum and endothelial cells in blood vessels, within muscle, and adjacent to the sternum. (Fig. 2C,E). Endothelial cells were identified by morphology and immunostaining with anti-PECAM antibody (data not shown). In the absence of Tamoxifen, we did not observe β-gal expression in these lineages (data not shown). Thus, similar to the chick embryo, the ventral dermis in the mouse embryo originates from the LPM.

The survival of early dermal progenitors requires β-catenin

To determine the function of Wnt signaling in mouse ventral dermal development, we used the *HoxB6Cre* driver to genetically alter Wnt signaling activity levels in the LPM, and analyzed mutants with a

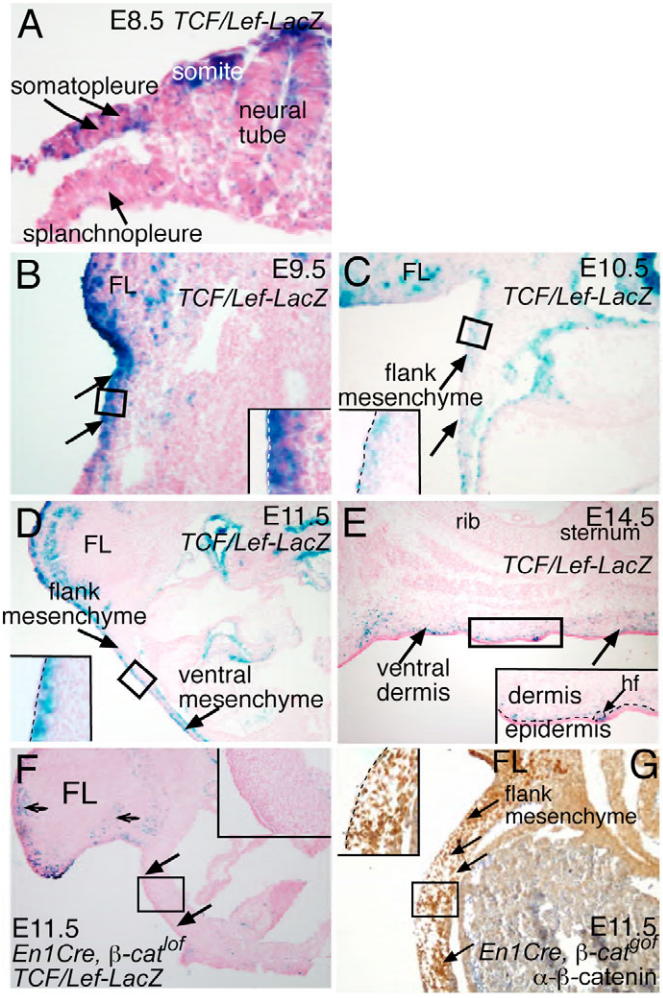

Fig. 1. Wnt signaling reporter expression during ventral dermal cell development. (**A-F**) X-gal-stained transverse sections of *TCF/Lef-lacZ* embryos at the forelimb (FL) level. (A-C) *lacZ* expression is detectable in the somatopleure at E8.5, and in the subectodermal mesenchyme cells in the flank at E9.5 and E10.5. (D) At E11.5, expression of the *TCF/Lef-lacZ* transgene expands to the midline and is visible throughout the subectodermal mesenchyme (arrows and inset). (E) At E14.5, during dermal cell differentiation, *lacZ* is expressed extensively in dermal cells (arrows and inset), and in the hair follicle placode (hf). (F) At E11.5, in the conditional absence of β-catenin in *En1*-expressing cells, *TCF/Lef-lacZ* expression is absent in the entire subectodermal flank and ventral mesenchyme (arrows, inset), but *lacZ* is expressed in cells of the limb mesoderm where *En1* is not expressed (small arrows). (**G**) When β-catenin activity is stabilized in *En1*-expressing cells, nuclear β-catenin is ectopically visible in the *En1*-lineage cells away from the ectoderm (arrows). Compare F and G with D.

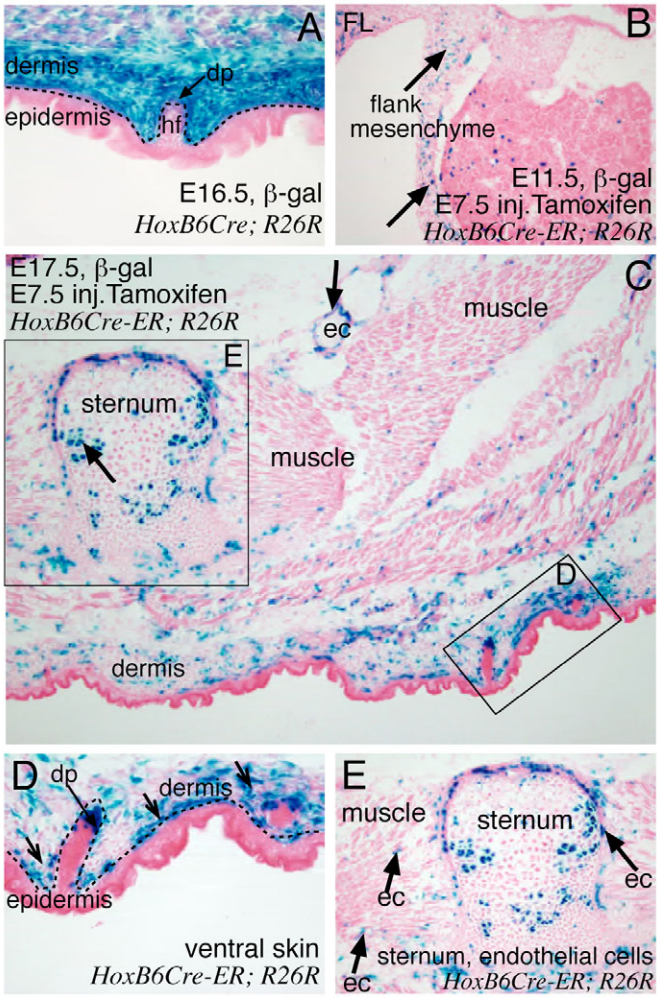

Fig. 2. Ventral dermal cells originate from the LPM. (**A-E**) X-gal-stained transverse sections. (A) At E16.5, *HoxB6Cre; R26R* lineage-marked cells comprise the ventral dermis. (B-E) *HoxB6Cre-ER^{T1}; R26R* embryos were given Tamoxifen at E7.5 and β-gal^{+} cells are found in the flank mesenchyme at E11.5 (B, arrows). (C-E) Later at E17.5, β-gal^{+} cells are found in the dermis (small arrows) and dermal papillae (dp) of the hair follicle (hf), and in the sternum and endothelial cells (ec, arrows). Dashed line demarcates the epidermis from the dermis in the embryonic skin. (D,E) High magnification images of the boxed areas in C.

conditional loss of β-catenin function in the flank and ventral subectodermal mesenchyme. By E9.5, *HoxB6Cre*-mediated recombination of *R26R* is nearly 100% in all the flank mesenchyme cells (Lowe et al., 2000). We used a loss-of-function floxed allele of β-catenin (β-cat^{lof}) to eliminate Wnt signal transduction in the *HoxB6Cre* lineage cells of the LPM (Brault et al., 2001). *HoxB6Cre; R26R; β-cat^{lof}* mutant embryos lacked a ventral body wall and died between E12.5 and E13.5 (data not shown). We examined the role of β-catenin in the cell survival of the early dermal progenitors in the flank mesenchyme. At E9.5, there was no TUNEL staining of the LPM in the control or in the *HoxB6Cre; R26R; β-cat^{lof}* mutant (data not shown). By E10.5, we found fewer *HoxB6Cre, R26R* lineage-labeled cells and a significant increase in the TUNEL staining of cells in the flank mesenchyme of the *β-cat^{lof}* mutant (Fig. 3C,D). By comparison, we did not find any TUNEL staining in the flank mesenchyme of the control *HoxB6Cre, R26R;β-cat$^{lof/+}$* lineage-labeled cells at E10.5 (Fig. 3A,B). These data demonstrate that β-catenin is required for the cell survival of early dermal progenitors and perhaps, but not necessarily, the progenitors of other tissues derived from the LPM.

Ventral dermal cells differentiate from flank and ventral subectodermal mesenchyme

In order to study the function of β-catenin at later stages, we used the endogenous engrailed1 (*En1*) promoter to drive the expression of *Cre* recombinase in the flank and ventral subectodermal

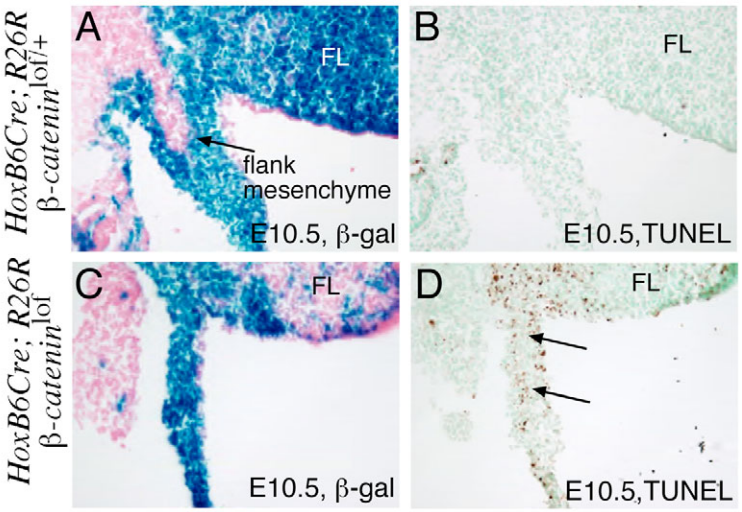

Fig. 3. β-catenin activity is required early for cell survival. (**A**,**C**) Transverse sections of an E10.5 control embryo (A) and a conditional β-catenin loss-of-function mutant (C) stained with X-gal to show the distribution of lineage-labeled cells. (**B**,**D**) Alternate sections assayed for cell survival by TUNEL (brown, arrows); nuclei are counterstained with methyl green. (D) Note the significant increase in TUNEL$^+$ cells in the *HoxB6Cre; R26R; β-cat^{lof}* mutants. (A-D) Images of forelimb (FL) level sections taken at the same magnification.

mesenchyme at E10.5 and later (Kimmel et al., 2000). *En1Cre*-mediated recombination of the *R26R* was seen in essentially all of the subectodermal cells of the flank mesenchyme starting at E10.5 (Fig. 4A,B), and in the ventral mesenchyme at the midline by E11.5 (Fig. 4C,D). *En1Cre*-mediated recombination of *R26R* at E10.5 coincides with the spatiotemporal expression of *TCF/Lef-lacZ* (Fig. 4B, Fig. 1C) and precedes the onset of *Dermo1* expression at E11.5 (Fig. 6J).

Next, we examined whether *En1Cre; R26R* cells contribute to the ventral dermis. In E16.5 *En1Cre; R26R* fetuses, β-galactosidase-labeled cells were present in the entire ventral dermis and epidermis (Fig. 4E). To determine whether the ventral epidermal and dermal cells were descendants of *En1*-expressing cells in the ventral trunk prior to E11.5, we temporally restricted the recombination of *R26R* by using a tamoxifen-inducible *En1Cre-ER^{T1}* line (Sgaier et al., 2005). To lineage-mark *En1*-expressing cells in the ventral trunk between E10.75 and E11.75, we administered Tamoxifen to pregnant females carrying E10.5 *En1Cre-ER^{T1}; R26R* embryos (Sgaier et al., 2005). At E16.5, β-galactosidase-labeled cells were found throughout the ventral dermis (open arrows, Fig. 4F). Our inducible lineage-marking experiments demonstrated that β-galactosidase-labeled cells in the ventral trunk epidermis of *En1Cre; R26R* embryos must arise as a result of *En1* expression in the ventral trunk ectoderm starting at E13.5 (Fig. 4E,F; data not shown). Therefore, the early development of mutant dermal progenitors in conditional *En1Cre;* β-catenin mutant embryos occurred next to

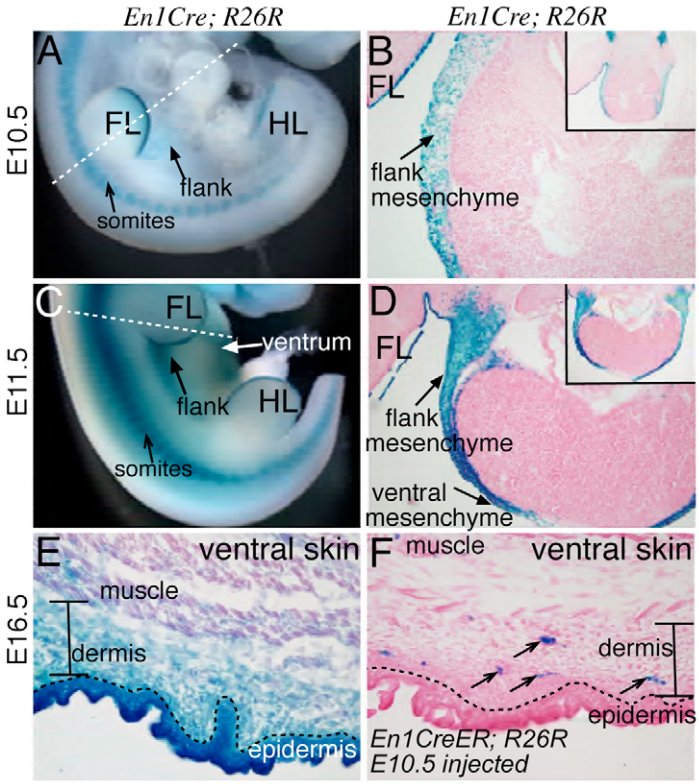

Fig. 4. Ventral dermal progenitors in the flank mesenchyme express *En1* and contribute extensively to ventral dermal cells. (**A-D**) In *En1Cre; R26R* embryos, *En1* lineage-marked (blue) cells are found in the flank mesenchyme at E10.5 (A,B) and in the midline of the ventrum by E11.5 (C,D). (A,C) Whole mounts; (B,D) sections. Insets (B,D) are lower magnification views of the sections. (**E**) *En1* lineaged-marked cells are found extensively in the epidermis and dermis of E16.5 *En1Cre; R26R* embryos. (**F**) *En1Cre-ER; R26R* embryos given Tamoxifen at E10.5; at E16.5 β-gal$^+$ cells are present only in the dermis (arrows). Black dashed line demarcates the epidermis from the dermis.

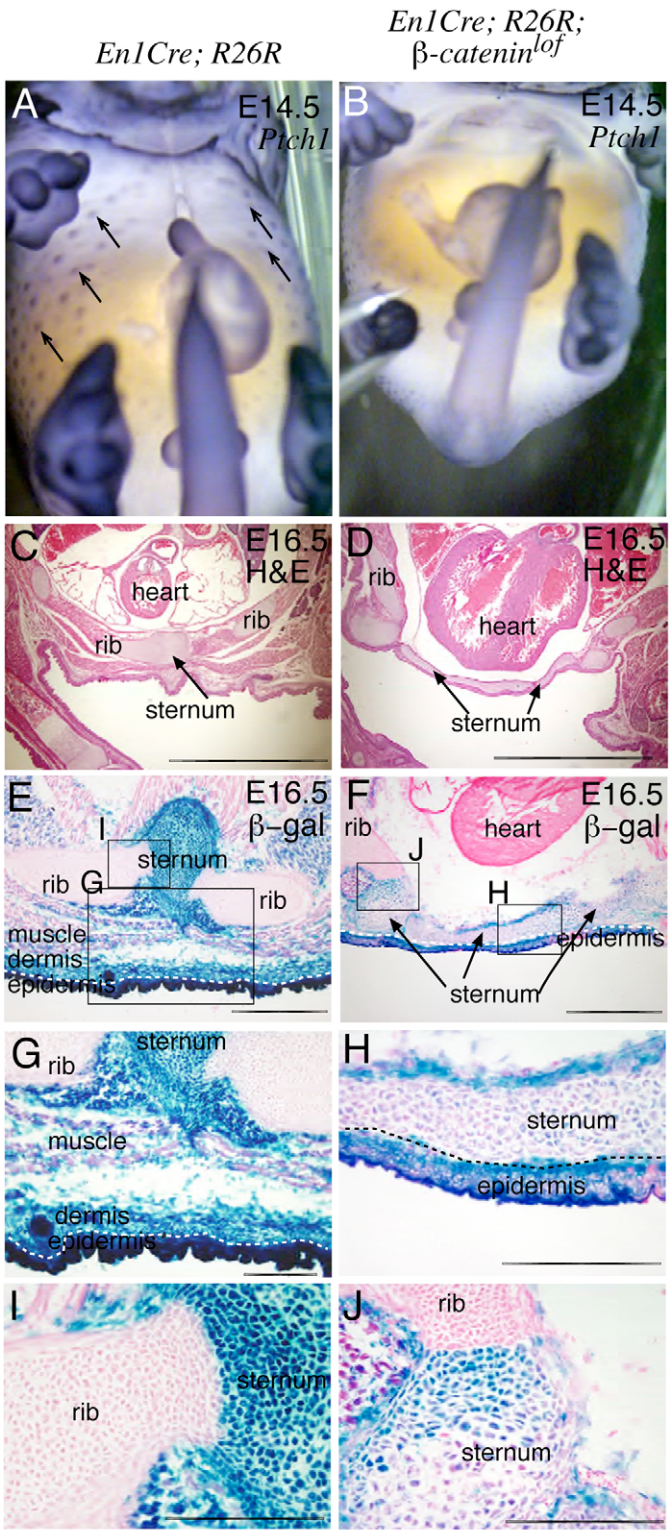

Fig. 5. Ventral hair placodes and dermis are lacking in the absence of β-catenin in the En1 lineage. (**A**) Whole-mount in situ hybridization with a *Ptch1* anti-sense mRNA probe reveals ventral hair follicle placodes at E14.5 in control embryos. (**B**) Ventral skin hair follicle placodes are absent in *En1Cre; R26R; β-cat*^{lof} mutants. (**C**,**D**) Sections of E16.5 embryos stained with Hematoxylin and Eosin (H&E). (**E-J**) X-gal-stained sections of E16.5 embryos. (E,F) The ventral dermis is present in the control (E) but is absent from the skin of mutant fetuses (F). (G-J) Higher magnification images of boxed areas in E and F. (E,G,I) In control embryos, lineage-labeled cells are visible in the sternum, dermis and epidermis, but not in the ribs. (F,H,J) In the mutants, lineage-labeled cells are present in the sternum and epidermis, and the sternum is wider. Scale bars: in C,D, 2 mm; in E,F, 500 μm; in G-J, 200 μm.

normal overlying ventral ectoderm cells up until E13.5. In addition, between E9.5 and E12.5, we did not see *En1lacZ* expression in the ventral trunk ectoderm at the forelimb level (data not shown). Taken together, these results suggest that *En1Cre* cells in the flank and ventral subectodermal mesenchyme at E10.5 include ventral dermal progenitors and contribute extensively to the ventral dermis.

TCF/Lef-lacZ expression confirms efficient Cre-mediated removal of β-catenin in the ventral dermal precursors

In subsequent studies, we used the *En1Cre* line to conditionally delete or stabilize β-catenin in the ventral subectodermal mesenchyme (Brault et al., 2001; Harada et al., 1999; Kimmel et al., 2000). To determine the efficiency of *En1Cre*-mediated recombination of two floxed alleles of β-catenin (Brault et al., 2001; Harada et al., 1999), we examined *TCF/Lef-lacZ* expression at E11.5 in mutant embryos. In E11.5 *En1Cre; β-cat*^{lof} embryos, *TCF/Lef-lacZ* was completely lost in cells of the *En1* lineage lacking β-catenin (Fig. 1F, inset). *TCF/Lef-lacZ* expression was present in normal forelimb mesoderm cells that had not expressed *En1Cre* (Fig. 1F, open arrows). Similarly, when the *En1Cre* line was used to stabilize β-catenin activity in the subectodermal mesenchyme, *TCF/Lef-lacZ* and nuclear β-catenin were ectopically expressed in cells of the *En1* lineage away from the ectoderm (Fig. 1G, inset; Fig. 1D; Fig. 4D; data not shown) (Harada et al., 1999). By E11.5, *En1Cre* can be used to efficiently eliminate or stabilize Wnt signaling/β-catenin activity in ventral dermal precursors prior to dermal specification (Fig. 6).

β-catenin is required for ventral dermal development

To determine the requirement of Wnt/β-catenin signaling later in ventral dermal development, we used *En1Cre* to conditionally delete β-catenin activity in ventral dermal precursors. We bred *En1Cre, R26R* with the floxed *β-cat*^{lof} mice to conditionally eliminate Wnt signal transduction and genetically tag the mutant cells. *En1Cre; R26R*; *β-cat*^{lof} mutants die at birth. At E16.5, *En1Cre; R26R; β-cat*^{lof} mutants are smaller in size than control embryos, and have more transparent ventral skin through which the liver can be clearly viewed (data not shown). The development of epidermally-derived hair follicles is dependent upon normal interaction with the dermis. In the absence of normal epidermal and dermal interactions, hair follicles fail to form (Atit et al., 2006; Millar, 2002). To obtain an overview of the affected area, we probed control and mutant embryos at E14.5 with antisense mRNA for patched 1 (*Ptch1*), a marker for hair follicle placodes (Oro et al., 1997). At E14.5, *Ptch1* was expressed in the hair follicle placodes of the control embryo (Fig. 5A), but was completely absent in the ventral flank and midline of *En1Cre; β-cat*^{lof} mutant embryos (Fig. 5B). In transverse sections of a E16.5 *En1Cre; R26R* control fetus, the epidermis overlaid the condensed dermis, ventral muscle and sternum (Fig. 5C,E,G). By striking contrast, the *En1Cre; β-cat*^{lof} fetuses lacked ventral dermis

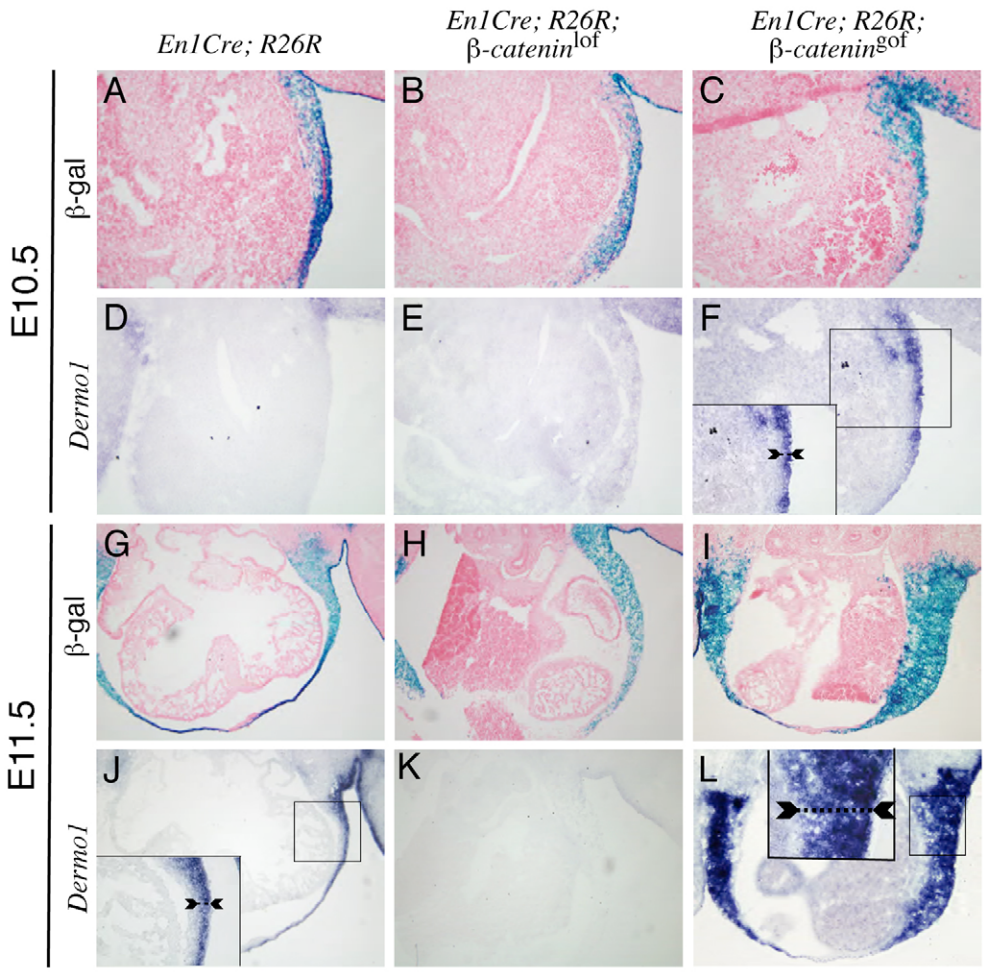

Fig. 6. *Dermo1* expression in the flank and ventral subectodermal mesenchyme is induced by β-catenin. (**A-L**) Section in situ hybridization of *Dermo1* mRNA (D-F,J-L) at E10.5 and E11.5; alternate sections are stained with X-gal to visualize *En1* lineage-marked cells (A-C;G-I). (J) *Dermo1* mRNA is first expressed in the subectodermal flank and ventral mesenchyme, starting at E11.5, in the control embryos. (E,K) In the β-catenin loss-of-function mutants, *Dermo1* mRNA is not visible at E10.5 or E11.5. (F,L) By stabilizing β-catenin in the En1 lineage, *Dermo1* mRNA is expressed earlier at E10.5, and ectopically in all the mutant cells by E11.5 (dashed line with arrows). Images are of (A-F) E10.5 and (G-L) E11.5 embryos; all insets (F,J,L) are of the same magnification. Compare E and F with D, and K and L with J.

and muscle, and the epidermis was juxtaposed next to the sternum (Fig. 5D,F,H). En1 lineage-marked cells contributed extensively to the sternum and were absent in the adjacent rib cartilage (Fig. 5E,I). In the *En1Cre; R26R*; *β-cat*^{lof} fetus, we found that the sternum, with lineage-marked cells, extended across the entire ventrum (Fig. 5F,H,J). The rib also lacked lineage-marked cells in the mutant embryos (Fig. 5F,J). We could not study dermal development in the complementary *En1Cre; β-cat*^{gof} mutants owing to embryonic lethality between E12.5 and E13.5. The late stage *En1Cre; β-cat*^{lof} phenotype illustrates that β-catenin/Wnt signaling activity is needed for ventral dermal development.

β-catenin is required for ventral dermal cell specification

To identify the molecular mechanism of Wnt/β-catenin signaling in ventral dermal development, we examined dermal specification in the *En1Cre* conditional loss- and gain-of-β-catenin function mutants. We bred *En1Cre, R26R* with the floxed *β-cat*^{lof} mice to conditionally eliminate Wnt signal transduction and genetically tag the mutant cells prior to ventral dermal specification. Between E10.5 and E11.5, we assayed dermal specification by the onset of *Dermo1* (also known as *Twist2*) mRNA expression in ventral trunk dermal precursors (Atit et al., 2006; Li et al., 1995). At E10.5, *Dermo1* was not expressed in the control flank subectodermal mesenchyme (Fig. 6A,D). *Dermo1* was normally detectable starting at E11.5 in the *En1* lineage-marked flank and ventral subectodermal mesenchyme (Fig. 6G,J). In the conditional absence of Wnt signaling/β-catenin activity in the *En1* lineage, *Dermo1* was not expressed at E10.5 or E11.5 in the flank and ventral subectodermal mesenchyme (Fig. 6B,E,H,K). By contrast, conditionally stabilizing β-catenin activity in the *En1* lineage led to the induction of *Dermo1* mRNA expression earlier at E10.5 (Fig. 6C,F) and to ectopic expression in most of the *En1Cre; R26R; β-cat*^{gof} mutant cells at E11.5 (Fig. 6I,L). *Dermo1* expression in the two different β-catenin activity mutants clearly demonstrates that β-catenin activity is required for the onset of ventral dermal progenitor marker expression and dermal specification.

β-catenin regulates cell proliferation of ventral dermal progenitors

Next, we examined the role of β-catenin in the cell proliferation and cell survival of ventral dermal progenitor cells at these later stages. Because the specification defect phenotype is evident by E11.5 in both the conditional mutants, we analyzed embryos for alterations in cell proliferation and cell survival at E10.5 and E11.5 (Fig. 7, Table 1). We examined cell proliferation by BrdU incorporation. There was no statistically significant difference ($P<0.05$) in the cell proliferation index between the control and the *En1Cre*; *R26R; β-cat*^{lof} or *β-cat*^{gof} at E10.5 (Table 1). At E11.5, we found a statistically significant difference in cell proliferation between control and *En1Cre*; *β-cat*^{lof} embryos only, and not with *β-cat*^{gof} mutants (Fig. 7A-C, Table 1). We assayed cell survival by TUNEL and could not find any significant changes in cell survival between control and *En1Cre*; *β-cat*^{lof} or *β*-cat^{gof} at E10.5 and E11.5 (Fig. 7D-F; data not shown).

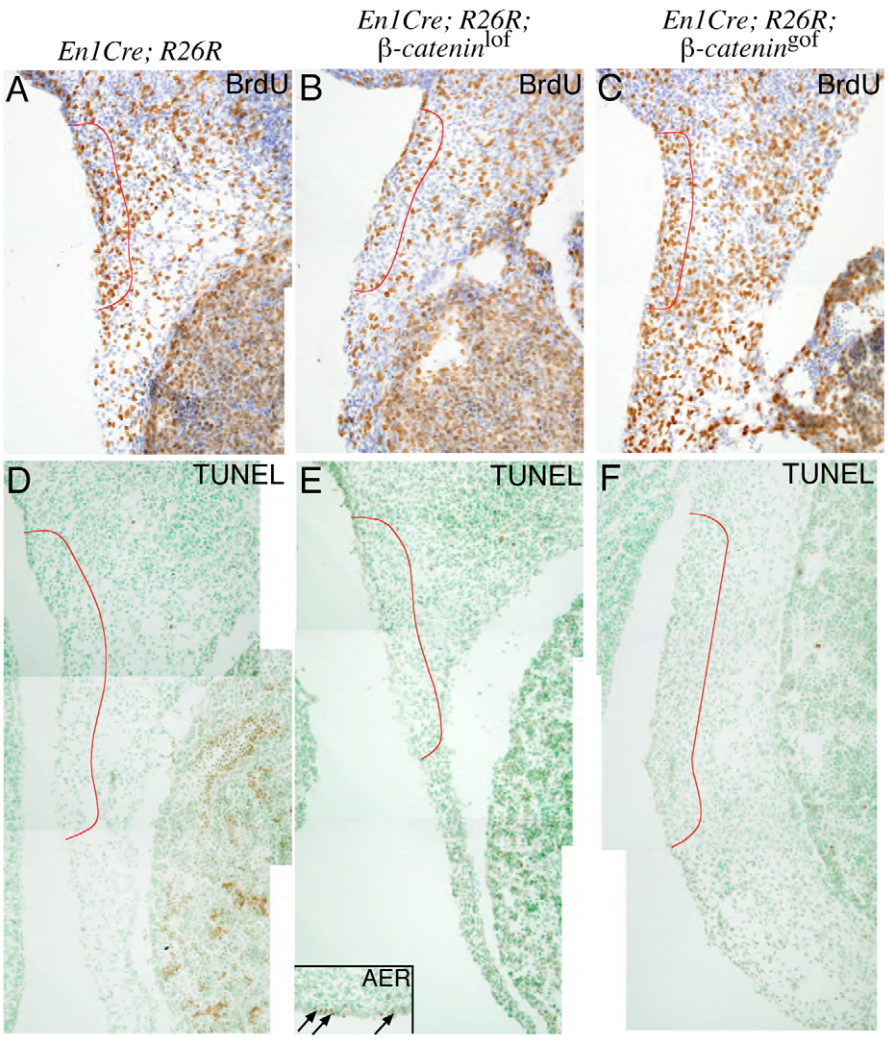

Fig. 7. Small decrease in cell proliferation in the absence of β-catenin without altering cell survival. (**A-C**) Cell proliferation at E11.5 assayed by BrdU incorporation (brown-stained cells). (A,B) There is a small decrease in cell proliferation in the *En1Cre; R26R*; *β-cat*^{lof} mutants but no significant difference in *β-cat*^{gof} mutants (see Table 1). (**D-F**) Apoptosis at E11.5 as assayed by TUNEL (brown-stained cells); sections are counterstained with methyl green. There is no detectable difference in cell death in the flank and ventral mesenchyme between the mutants and control embryos. (E) TUNEL^{+} cells are detectable in the apical ectodermal ridge of the limb (inset). (A-F) *En1* lineage-marked domains are outlined in red. Composites for each panel were created from two or three images taken at the same magnification.

DISCUSSION

In this study, we investigate the origin of mammalian ventral dermis and the signaling cues necessary for the specification and survival of the ventral dermal lineage. Using four different genetic fate-mapping tools, we show conclusively that LPM cells contribute to the flank and ventral body wall mesenchyme, and that subectodermal mesenchymal cells differentiate into dermal cells. Using a Wnt signaling reporter transgenic line, we show that Wnt/β-catenin signaling is active in the somatopleure of the LPM at E8.5, and later in ventral dermal progenitors. We generated conditional β-catenin mutants early in the LPM, and later in the flank and ventral mesenchyme. When β-catenin is eliminated early in the LPM between E8.0 and E9.0, dermal progenitors die by E10.5. However, when β-catenin is removed in the subectodermal mesenchyme between E10.0 and E11.5, embryos have a dramatic loss of ventral dermis and an expanded sternum by E16.5. We show that the loss of dermis phenotype occurs as a result of loss in cell fate specification and a small decrease in proliferation. By contrast, when β-catenin

Table 1. Proliferation of β-catenin mutant cells

		En1 domain four cells adjacent to ectoderm		
β-Catenin genotype	Number of embryos analyzed	BrdU-positive cells (%)	SD	*P*
E10.5				
Control	7	48.1	13.6	–
Loss of function	6	52.9	14.1	0.685
Control (AR)	4	54.2	8.4	–
Gain of function (AR)	6	55.0	8.0	0.126
E11.5				
Control (AR)	5	46.1	6.8	–
Loss of function (AR)	6	35.0	11.0	0.011
Gain of function (AR)	5	45.6	10.5	0.887

AR, antigen retrieval protocol.

activity is stabilized in the flank and ventral mesenchyme, there is ectopic expression of the dermal progenitor marker *Dermo1* in the lineage-labeled cells. Our results in the mouse embryo conclusively demonstrate that Wnt signaling/β-catenin plays multiple roles during mammalian ventral dermal development.

Lineage mapping the ventral dermis in the mouse embryo

We use genetic tools to lineage map the mammaliam ventral dermis from the LPM. We have taken advantage of two different *Cre* and inducible *Cre-ER* alleles to improve the accuracy of our lineage analysis. First, we use the *HoxB6Cre* transgenic line that uses the LPM and limb enhancer region of the *HoxB6* promoter to drive *Cre* or *Cre-ER* expression in the lateral LPM starting at E8.0 (Lowe et al., 2000). Consistent with the fate maps from the chick embryo, we find lineage-labeled cells in known derivatives of the LPM in ventral trunk tissues, such as the body wall mesenchyme, at E11.5, and later in the dermis, sternum, and endothelial cells by E16.5. As expected, we do not see lineage-labeled cells in the ribs derived from the somites or in the epidermis, which differentiates from the surface ectoderm. We confirmed our ventral dermis fate map by using the *En1Cre* and *En1Cre-ER^{T1}* lines, which drive expression later at E10.0 in the flank and ventral mesenchyme. We find β-gal^{+} cells extensively in the ventral dermis, the dermal papillae of the hair follicles, and the sternum. Our results from multiple genetic lines and strategies demonstrate clearly that LPM cells contribute to the body wall mesenchyme by E11.5, and then later to ventral dermis in the mouse embryo. Our fate mapping results have aided our analysis of the β-catenin conditional mutant phenotypes.

Multiple roles for Wnt signaling/β-catenin in ventral dermal cell development

Wnt signaling reporter activity is evident in the somatopleure of the LPM starting at E8.5, and then in the subectodermal mesenchyme of the flank and ventrum. The Wnt signaling reporter activity is consistent with the known expression of multiple Wnt ligands in the surface ectoderm of the mouse embryo (Cauthen et al., 2001; Parr et al., 1993), and with signaling to the mesenchyme cells directly below. These subectodermal mesenchyme cells process the Wnt signal and differentiate to ventral dermis. The additional lineage-marked cells located away from the surface ectoderm must differentiate into other cell types. Our complementary results from mutants with conditional loss- and gain-of-β-catenin function, clearly demonstrate that β-catenin has a role early in cell survival and later in the cell specification of ventral dermal progenitors. In this study, by eliminating β-catenin early in the LPM, we found a role for β-catenin in early cell survival before E10.5. When we eliminated β-catenin after E10.0, the subectodermal cells survived but failed to be specified to the ventral dermal cell fate by E11.5. In our previous studies on dorsal dermis, we eliminated β-catenin early in the multipotential progenitors of the somite and demonstrated that β-catenin has an instructive role in dermal cell fate from the somite, but we did not see a role in the cell survival of dermal progenitors (Atit et al., 2006). In this study, we found that β-catenin has a new role in ventral dermal progenitor cell survival while maintaining a functionally conserved role in dorsal and ventral dermal cell fate selection.

Our fate mapping results demonstrate that we have marked cells that originate in the LPM and that contribute to sternal cartilage, ventral dermal and endothelial cell lineages. The *En1Cre; R26R; β-cat^{lof}* mutant shows an absence of ventral dermal specification and a significant expansion of the sternum. The sternum, containing lineage-labeled cells, traverses the width of the embryo. It is not clear whether the mutant ventral dermal progenitors are respecified to sternum fate. The small decrease in cell proliferation could account for a decrease in dermal progenitor population. Taken together with the absence of cell death, some of the dermal progenitors may also be respecified to the sternum cell fate.

It still remains to be determined how Wnt signal transduction instructs subectodermal mesenchyme in the flank and trunk to progress towards the ventral dermal cell fate. Wnt signaling/β-catenin activity is crucial for *Dermo1* gene expression in dorsal and ventral dermal progenitors, and *Dermo1* may be the direct target gene to promote dermal cell fate (Atit et al., 2006) (Fig. 6). Identifying the additional downstream target genes and defining the genetic program for dermal cell development are the next steps in understanding dermal cell differentiation.

We thank Makoto M. Taketo, Ozimba Anyangawe, Hilary Michel, Andrew Jarrell and Sema Sagier for their contributions. We thank present members of the lab, and Ron Conlon and Kathleen Molyneaux for critically reading this manuscript. This work was supported by Startup funds from the Case Western Reserve University (R.A.), the Basil O'Connor Junior Investigator Award from the March of Dimes Foundations (R.A.), and the Case Western Reserve University SOURCE Program (J.O. and P.S.).

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