Neural Crest-Derived Mesenchymal Cells Require Wnt Signaling for Their Development and Drive Invagination of the Telencephalic Midline

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Abstract

Embryonic neural crest cells contribute to the development of the craniofacial mesenchyme, forebrain meninges and perivascular cells. In this study, we investigated the function of β-catenin signaling in neural crest cells abutting the dorsal forebrain during development. In the absence of β-catenin signaling, neural crest cells failed to expand in the interhemispheric region and produced ectopic smooth muscle cells instead of generating dermal and calvarial mesenchyme. In contrast, constitutive expression of stabilized β-catenin in neural crest cells increased the number of mesenchymal lineage precursors suggesting that β-catenin signaling is necessary for the expansion of neural crest-derived mesenchymal cells. Interestingly, the loss of neural crest-derived mesenchymal stem cells (MSCs) leads to failure of telencephalic midline invagination and causes ventricular system defects. This study shows that β-catenin signaling is required for the switch of neural crest cells to MSCs and mediates the expansion of MSCs to drive the formation of mesenchymal structures of the head. Furthermore, loss of these structures causes striking defects in forebrain morphogenesis.

Introduction

A unique feature of vertebrate neurulation is the delamination of neural crest progenitors from the dorsal neuroepithelium before and during neural tube formation. In mice, rostral neural crest cells detach from the closing neural tube by embryonic day (E)9.0, one day before the dorsomedial telencephalon invaginates to form the bilateral telencephalic vesicles, the prospective cerebral cortical hemispheres [1]. At E10.5, regional specification of the dorsomedial forebrain neuroepithelium divides areas of the hippocampus, the cortical hem, and the non-neural secretory choroid plexus, which extends into the lateral ventricle [2]. The secreted signaling factor Wnt3a is first expressed by the cortical hem at E10.5 in concordance with the invagination of the dorsal telencephalon [3].

In addition to the role Wnt signaling plays during the development of the central nervous system (CNS), this pathway is also known to exert important functions during induction and migration of neural crest cells. Wnt proteins activate an array of downstream target genes by stabilizing the intracellular signal transducer β-catenin that binds Tcf family transcription factors in the nucleus and recruits co-activators. However, β-catenin also binds to cadherins localized at adherence junctions contributing to the establishment of polarized epithelial tissues [4,5]. Breakage of these junctions in epithelia outside the nervous system produces mesenchymal cells via a process termed epithelial-mesenchymal transition (EMT) [6,7,8]. Analysis of Wnt1/Wnt3a double mutants showed a profound loss of neural crest-derived structures, clearly demonstrating the critical role of Wnt signaling in the development of neural crest derivatives [9]. Interestingly, the neural crest-specific deletion of β-catenin by using Wnt1-Cre mice showed both profound defects in neural crest-derived craniofacial structures and diminished neural precursor development in the forebrain [10,11]. This raises the question of whether loss of Wnt signaling in head structures leads to separate mutant phenotypes in the cranial neural crest and forebrain, or whether there is a causal relationship between these two phenotypes.

Conditional inactivation of β-catenin during mouse forebrain development using different Cre lines has thus far produced two distinct dorsal telencephalic phenotypes. First, mice with Emx1-Cre-dependent deletion of β-catenin survive to adulthood without apparent neural crest defects while displaying diminished dorsomedial forebrain structures [12]. The dorsomedial structures properly invaginate forming bifurcated lateral ventricles. Contrarily, Foxg1-Cre-mediated deletion of β-catenin in both dorsal neuroepithelial and mesenchymal cells, results in severe loss of midline telencephalic structures, failure of midline invagination and associated craniofacial defects [13,14,15]. The marked

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difference in phenotypic alterations in these two mutant lines may stem from the loss of β-catenin signaling in mesenchymal cells in Foxg1-Cre;β-catenin mutants. Even though not clearly described in the existing literature, additional evidence for a correlation between cortical hem-mediated Wnt signaling and the failure of midline invagination through interstitial mesenchymal cells exists in several mouse mutants. For instance, the dorsomedial neuroepithelium of Emx1/Emx2 compound mutants transforms into the roof plate with a diminished cortical hem and choroid plexus [16]. Loss of Emx1 and Emx2 expression is observed in Gli3 (Xt/Xt) mutants [17], Nevin-Cxcl12-catenin mutants [18], Gdf7-DTA mutants [19], and ectopic ShhV expressing mutants [20]. The common feature in all of these mouse lines is diminished cortical hem-mediated Wnt signaling and incomplete midline invagination. From these seemingly separate mutant phenotypes, it is thus reasonable to investigate whether Wnt signaling to and from the mesenchyme and forebrain may regulate midline development.

Mesenchymal stem cells (MSCs) are among the most promising candidates for future cell-based therapeutic applications [21,22]. Therapeutic MSCs are currently derived from newborn umbilical cord blood, adult bone marrow or adipose tissues. However, due to their mesodermal origin, these currently obtained MSCs may face limitations in their regenerative use for disorders of the forebrain and skull vault. Cranial neural crest cells are transient, highly migratory cells originating from the dorsal neuroepithelium before neural tube closure, migrating along the neuraxis, and contributing to a great variety of mesenchymal structures of the skull and forebrain vasculature [1]. Elements of the cranial skeleton such as the frontal bone develop by intramembranous osteogenic condensation of mesenchymal cells derived from neural crest cells, which also contribute substantially to the meninges that cover the telencephalon [23,24,25]. In addition, neural crest cells produce non-neural-cell types within the brain such as perivascular smooth muscle cells and pericytes [24]. The regenerative use of neural crest-derived MSCs may become very important for a range of disorders of the brain and skull and thus greater examination of the underlying developmental programs mediating their specification in the developing head is well warranted [26,27,28].

There are many questions remaining concerning the transition of cranial neural crest cells to mesenchymal progenitors forming the mesenchymal derivatives of head and face. Most importantly the molecules guiding conversion of neural crest cells to MSCs and their expansion along the rostromedial midline need to be further explored. We hypothesized that Wnts secreted from the cortical hem act as a proliferative signal on neural crest derived mesenchymal progenitor cells, which induces their expansion adjacent to the dorsomedial telencephalon and that this initial expansion of mesenchymal progenitor cells contributes to neural crest derived craniofacial structures and proper development of the forebrain. To address this question, we conditionally manipulated β-catenin in late premigratory neural crest cells using Sox10-Cre mice [29]. Our results clearly demonstrate that Wnt/β-catenin signaling plays a critical role in the development of neural crest-derived mesenchymal derivatives and that dorsomedial mesenchymal progenitors contribute to cortical midline invagination and lateral ventricle formation.

**Results**

Neural crest-derived mesenchymal stem cells in the dorsomedial interhemispheric region are Wnt responsive

Loss of canonical Wnt signaling in early-born neural crest cells leads to severe loss of forebrain structures, impaired neural crest lineages, and loss of neural crest-derived mesenchymal derivatives [9,10,11,30]. In the rostral neural tube, Wnt3a expression starts in the dorsal neural epithelium and the cortical hem around E10.5 as the dorsal telencephalon invaginates to form the bifurcated hemispheric forebrain [3,17]. To trace the developmental expansion of cranial neural crest-derived mesenchymal progenitors resident in the dorsal area, we used immunostaining for Pdgfrα, a well-described marker of this cell population [31]. Pdgfrα expression from E10.5–12.5 was seen widely in cells overlying the neuroepithelium but also concentrated at the dorsal midline as invasion proceeded (E10.5) and adjacent to the newly formed cortical hem (E12.5) (Fig. 1A). These Pdgfrα+ mesenchymal cells were densely distributed in the space between the telencephalon and the non-neural ectoderm. Two days later at E14.5, most of the mesenchymal cells were dispersed into the space between the epidermis and the cortex (Fig. 1A, B). Since Pdgfrα+ mesenchymal cells reach their highest density in the dorsomedial region of the midline mesenchyme, opposed to the cortical hem, a source of Wnt secretion, we examined whether the cells were also responding to Wnt signaling at this stage. X-gal staining of β-gal transgenic embryos expressing the lacZ reporter under the control of Wnt-responsive elements revealed β-galactosidase+ (β-gal+) cells scattered along the mesenchyme of the dorsal cortical midline at E10.5 confirming Wnt responsiveness of these mesenchymal cells (Fig. 1C). This result raised the possibility that Wnt activation mediates proliferation of neural crest-derived MSCs in the dorsal interhemispheric region during invagination of the dorsal telencephalic midline. To test this idea we investigated suitable Cre driver lines specifically expressing Cre recombinase in neural crest-derived mesenchymal cells near the cortical hem during the period of dorsal neural tube invagination. We examined Sox10-Cre;ROSA-lacZ reporter mice, in which late premigratory neural crest cells are specifically recombined and found that this driver might be suitable [10,29]. Indeed, X-gal staining in Sox10-Cre;ROSA-lacZ reporter mice at E9.5, confirmed β-gal expression both in interstitial neural crest cells of the rostromedial neural tube and emigrating dorsal neural crest cells (Fig. 1D).

Activation of β-catenin in neural crest lineages expands mesenchymal cells

To understand the role of Wnt signaling in the development of the neural crest-derived MSCs we used mice expressing a constitutively active floxed version of β-catenin (Ctnnb1(Cαf)) [32] and crossed these with Sox10-Cre mice. RNA in situ hybridization for Col2a1, a marker for chondrogenic mesenchymal cells [23,33], revealed that Col2a1+ mesenchymal progenitors were expanded dorsally and laterally in Sox10-Cre;Ctnnb1(Cαf) embryos at E16.5 (Fig. 2A, A'). At this developmental stage, Pdgfrα expression was restricted in perivascular cells while Pdgfrα expression was more ubiquitously seen in the mesenchyme (data not shown). Examining the dorsal midline in Sox10-Cre;Ctnnb1(Cαf) mice at higher resolution showed both an expanded population of Pdgfrα+ cells as well as increased number of proliferative Kit+/+ mesenchymal cells in the dorsal area between the epidermis and the cortex at E16.5 (Fig. 2B). Furthermore, the thickness of the mesenchymal tissue overlying the dorsal cortical midline was significantly increased in the mutant compared to control (Student's t-test, P<0.001) (Fig. 2B'). These results suggest that activation of β-catenin signaling in the premigratory neural crest induces the expansion of MSCs.
The initial expansion of mesenchymal cells is dependent on \( \beta \)-catenin signaling

While our results showed that activation of \( \beta \)-catenin signaling was sufficient to drive expansion of the dorsal interhemispheric mesenchyme they did not necessarily establish the requirement for Wnt signaling in this process. To further examine this question, we used the Sox10-Cre line to conditionally inactivate \( \beta \)-catenin in neural crest cells of Ctnnb1(lof)flx mice [10]. In Sox10-Cre;Ctnnb1(lof)flx/flx mutants, the telencephalon formed with an expanded, partially-invaginating, split dorsal midline at E10.5 (Fig. 3A). At E10.5 cells expressing the mesenchymal cell markers vimentin (Vim) and Pdgfr\( \beta \) [34,35], were substantially reduced in the mutants compared to littermate controls. We quantified the difference for Pdgfr\( \beta \)\(^+\) mesenchymal cells and found a loss of approximately 50\% in areas overlying the dorsomedial cortex (Student’s \( t \)-test, \( P<0.0001 \), Fig. 3A’). To examine the proliferation mesenchymal cells in the dorsal interhemispheric region, we counted Ki67+ cells at E12.5 and E14.5 in mesenchymal tissues adjacent to the cortical hem. Mutant Ki67+ cells were significantly reduced at E12.5 and recovered at E14.5 (Student’s \( t \)-test, \( P<0.05 \), Fig. 3B, B’). However, Ki67+ cells in the lateral mesenchymal tissues were reduced significantly at E14.5, perhaps resulting from the earlier loss of Ki67+ cells in the dorsal interhemispheric region (Student’s \( t \)-test, \( P<0.01 \), Fig. 3C, C’). These data, along with our analysis of the Sox10-Cre;Ctnnb1(lof)mutants, supports the idea that \( \beta \)-catenin signaling is necessary and sufficient for neural crest-derived mesenchymal cells to proliferate in the dorsal interhemispheric region.

Next, we asked whether loss of \( \beta \)-catenin in neural crest cells not only hinders the development of MSCs but expectedly also of cell lineages derived from them, such as the meninges. We recently reported that activation of Wnt signaling induces meningeal expansion during mid-gestation [36], which prompted us to examine by RNA in situ hybridization the expression of meningeal markers Raldh2 and Cxcl12 in the dorsal midline. Loss of \( \beta \)-catenin expression in the neural crest lineages, however, did not inhibit the meningeal expression of Cxcl12 (Fig. 4), as also previously shown for the selective deletion of \( \beta \)-catenin in meningeal tissues [37]. Interestingly though, Raldh2 showed a discontinuous pattern of expression, implying that \( \beta \)-catenin signaling might be required for the expansion or development of Raldh2\(^+\) meningeal cells. Consistent with these findings, we previously showed that loss of \( \beta \)-catenin in meningeal tissues during corticogenesis affected the development of the pial layer and cells expressing Raldh2 and Bmp7 [37].

We have previously shown that the Foxc1 transcription factor is critical for the development of the meninges [38]. To better understand the development of neural crest derivatives in Sox10-Cre;Ctnnb1(lof)mutant embryos, we compared these to Sox10-Cre;Foxc1flx/flx mutant embryos, which exhibit a substantial impairment in meningeal integrity. At E12.5, the normal expansion of Pdgfr\( \beta \)\(^+\) meningeal cells was not seen in the dorsal midline of the Sox10-Cre;Ctnnb1(lof)mutant mutants. In contrast, Sox10-Cre;Foxc1flx/flx mutants exhibited a substantial number of Pdgfr\( \beta \)\(^+\) meningeal cells suggesting that the meninges are not necessary for the proliferation of neural crest-derived mesenchymal cells in the dorsal interhemispheric region (Fig. 5A). Pdgfr\( \beta \)\(^+\) meningeal

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**Figure 1. Wnt-responding mesenchymal cells expand in the dorsal interhemispheric region.**

**A** Staining of Pdgfr\( \beta \)+ neural crest-derived meningeal cells from E10 to E14. Dashed lines highlight the dorsomedial mesenchymal cells, which expand at E10 and spread laterally at later ages. At E14, perivascular cells strongly express Pdgfr\( \beta \). **B** A diagram showing mesenchymal cells at the level of dorsoventral axis in the forebrain; the dorsal (Md), lateral (Ml), and ventral (Mv) mesenchymal cells. The derivatives of the neural crest cells are listed with markers used in this study. Ep = epidermis, Ch = cortical hem, Cp = choroid plexus, P = pericytes, m = meninges. **C** 3D-a is presented in 3-b. X-gal staining of E10.5 Bat-gal transgenic embryos. A high magnification image of the boxed area in C-a is shown in C-b. X-gal\(^+\) mesenchymal cells are localized in the interhemispheric region (red dashed lines). **D** X-gal staining of E10 ROSA-lacZ Cre reporter mice crossed with Sox10-Cre, a neural crest driver. A high magnification image of the boxed area in D-a is shown in D-b. Red dashed lines mark the area with mesenchymal cells. Scale bars = 100 \( \mu \)m.

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cells covering lateral aspects of the neocortex were lost in both Sox10-Cre;Ctnnb1(lof)flx/flx and Sox10-Cre;Foxc1flx/flx mutants. This interesting result suggests that the meninges may be involved in the lateral dispersion of mesenchymal cells that originate dorsomedially, but are not required for the initial expansion of this cell population. At E14.5, when dermal mesenchymal cells become positive for CD44, a mesenchymal stem cell marker we further analyzed distribution of these cells [35]. Our analysis revealed that both mutant lines Sox10-Cre;Ctnnb1(lof)flx/flx and Sox10-Cre;Foxc1flx/flx exhibited reduced MSCs between the skin and the cortex (Fig. 5B). Since Sox10-Cre;Ctnnb1(lof)flx/flx mutants still produce the meninges, the loss of MSCs stems likely from the failure of initial transition of neural crest cells into MSCs. In summary, the differences in mesenchymal cell expansion between Foxc1 and Ctnnb1 mutants suggests that β-catenin function is critical to derive MSCs from neural crest cells while the meninges are required for the distribution of MSCs over the developing cerebral cortex.

Defective development of calvarial mesenchymal cells in neural crest lineages lacking β-catenin

We showed that β-catenin signaling in neural crest cells is critical for the initial proliferation of the interhemispheric fissure by MSCs. This finding raised the question as to whether inhibition of β-catenin in mesenchymal cell precursors would further hinder the development of mesenchymal derivatives into dermal or calvarial cells. For instance, the majority of skull bones form by ossification of condensing calvarial mesenchymal cells originating from neural crest cells [39,40]. Using RNA in situ hybridization, we examined the expression of Col2a1, a marker of condensing calvarial mesenchymal cells [23,33]. Col2a1 staining was similar to CD44 expression and was markedly reduced in Sox10-Cre;Ctnnb1(lof)flx/flx throughout the developing calvarium, presumably the result of reduced condensation of mesenchymal cells from a reduced initial pool of mesenchymal progenitor cells (Fig. 6). Interestingly, upon close examination, we found that Col2a1+ cells had infiltrated the cerebral cortex in the Sox10-Cre;Foxc1flx/flx mutants (arrow in Fig. 6). These Col2a1+ cells were found adjacent to endothelial cells, possibly the result of breaches in leptomeningeal blood vessels or ectopic expression of Col2a1 in a population

Figure 2. Expansion of mesenchymal cells by activation of β-catenin in neural crest cells. A) Mesenchymal cells of Sox10-Cre;Ctnnb1(gof) mutant at E16.5 marked by Col2a1 expression (top) and alkaline phosphatase activities (bottom, osteoblasts) obtained from adjacent sections. Higher magnification images of the boxed areas are shown in A’. B) Mesenchymal cells were labeled for Pdgfrα and Ki67 to show the proliferating mesenchymal cells. B’) A graph shows thickness of dermal mesenchymal cells in the midline at E16.5 (white lines of B, n = 3). Error bar indicates SEM. Scale bars = 100 μm.

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of cortical perivascular cells (data not shown). Strongly stained Col2a1+ mesenchymal cells were still present in medial aspects of the developing skull at the cortical midline of Sox10-Cre;Foxc1flx/flx mutants suggesting that the loss of cells laterally may be due to defective spreading of Col2a1+ cells (Fig. 6). To examine the resultant effect of reduced Col2a1+ calvarial mesenchymal cells in the mutants, we labeled osteoblast cells and calvarial bone by alkaline phosphatase reactivity and alizarin red staining respectively, which confirmed in both mutant lines a severe loss of osteoblasts and bones tissue (data not shown). Summarily, our findings identified an essential requirement of ß-catenin signaling for calvarial formation starting from the onset of neural crest specification [10]. In general, our results supported the notion that the initial expansion of mesenchymal progenitors by ß-catenin signaling affects the later condensation of calvarial mesenchymal cells.

Ectopic production of smooth muscle cells by inhibition of ß-catenin in the neural crest lineages

While considerable impairments in the number and distribution of MSCs were seen in the mesenchymal space overlying the developing forebrain, we wondered whether also mesenchymal derivatives that invade the brain and contribute to the vasculature of neuronal tissues may be similarly impaired. To further examine in Sox10-Cre;Ctnnb1(lof)flx/flx mutants the properties of residual perivascular derivatives derived from neural crest cells, we used isolecit IB4 immunofluorescence to identify blood vessels. As shown in Figure 7B, IB4+ blood vessels were present in the meninges and the dermis of the mutant with no tangible differences compared to control brains. To characterize remnant mesenchymal cells positioned between the meninges and the dermis of the Sox10-Cre;Ctnnb1(lof)flx/flx mutant, we tested markers of mesenchymal cells and found that markers for smooth muscle cells (such as aSMA and SM22a) were ectopically found in the mutants. SM22a staining showed a few dermal mesenchymal cells and smooth muscle cells in the control embryos, however, most neural crest-derived mesenchymal cells were stained for SM22a in the Sox10-Cre;Ctnnb1(lof)flx/flx mutant, while also losing expression of CD44, a mesenchymal stem cell marker (Fig. 7C). The interstitial mesenchymal cells in the lateral cortical ‘b’ area (see Fig. 7A) were presumably joined by neural crest-derived cells from the dorsal and ventral sources and were mostly absent in the Sox10-Cre;Ctnnb1(lof)flx/flx mutant. SM22a+ cells tapered off before reaching area ‘b’ likely indicating incompetent spreading of these cells (Fig. 7C, arrows). The Sox10-Cre;Ctnnb1(gof) mutant, however, showed an expanded dorsal mesenchymal layer and slightly reduced SM22a expression (Fig. 7C’). Consistent with SM22a expression, aSMA staining also showed ectopic smooth muscle cells after loss of ß-catenin in the neural crest cells (Fig. 7D). Interestingly, the ectopic SM22a+ smooth muscle cells were not stained for desmin, a marker for pericytes suggesting these cells are non-perivascular myofibroblasts. These data suggest that loss of ß-catenin in telencephalic neural crest cells diverts neural crest-derived MSCs to become smooth muscle cells.

Defective midline invagination after deletion of ß-catenin in neural crest lineages

A most noticeable brain phenotype of ß-catenin inhibition in the neural crest lineages was the lack of the dorsal midline invagination (Fig. 3A) which was not previously reported in mice, which underwent Wnt1-Cre mediated deletion of ß-catenin, possibly because of even earlier loss of ß-catenin during neural crest development resulting in an abnormal forebrain and complete loss of craniofacial structures [10]. Further examination of cortical specification by RNA in situ hybridization using probes for Lhx2, Lmx1a, and Tin, markers for the neocortex, the cortical hem, and the choroid plexus respectively, confirmed the expansion of the telencephalic dorsal midline including the choroid plexus in Sox10-Cre;Ctnnb1(lof)flx/flx mutants compared to control embryos (Fig. 8A). Interestingly, the neocortex of Sox10-Cre;Ctnnb1(lof)flx/flx mutants showed a reversed, concave curvature, presumably reflecting improper dorsal midline invagination (Fig. 8A, red dashed lines). We examined the development of the cortical hem, the choroid plexus, and the cortex at E14.5 by expression analysis of Lmx1a, Tin, and Lhx2 respectively. The Lmx1a+ cortical hem was present irrespective of genotype, intriguingly though, in the Sox10-Cre;Ctnnb1(lof)flx/flx mutants the choroid plexus was exposed dorsally adjacent to the epidermis, which might cause the hypomorphic choroid plexus at this age. The massive cortical dysplasia evidenced by failure of midline invagination and lateral
ventricle formation did not alter Lhx2 expression though, which was still present throughout the neocortical neuroepithelium in the mutant (Fig. 8B). In summary, our findings suggest that ß-catenin signaling of interhemispheric mesenchymal cells is required for the development and invagination of the dorsal telencephalic midline, and formation of the lateral ventricles.

**Discussion**

In this study, we examined both loss and gain of function mutations of ß-catenin signaling in rostral neural crest-derived cells and showed that ß-catenin signaling is important for the initial expansion of MSCs in the dorsal interhemispheric region and the invagination of the dorsal telencephalic region. Cranial neural crest cells delaminate, migrate, and ensheathe the rostral cortex coinciding with dorsal telencephalic tissue growth and midline formation [1]. Wnt1 and Wnt5a are expressed in the dorsal midline of the neural tube and Wnt1;Wnt5a compound mutants show thinning of the dorsal midline in the hindbrain [9], however, the cortical hem and non-neural ectoderm are the major sources of Wnts near the dorsal forebrain [36]. The developmental period of cortical hem formation coincides with the development of craniofacial structures in the sense that Wnt signaling activates the expansion of neural crest derived mesenchymal progenitors thus enabling the supply of diverse mesenchymal cells to the developing face and head. Interestingly, the area of initial mesenchymal expansion is positioned adjacent to the cortical hem, a rich source of developmentally secreted Wnt molecules. The timing of these developmental programs led us to suspect that Wnt-mediated interactions of neuroepithelial cells and nearby neural crest-derived mesenchymal cells may occur. Consistent with this idea other mutant mouse lines with diminished cortical hems (such as Foxg1-Cre;Ctnnb1(lof), Nestin-Cre;Ctnnb1(lof), Emx1/ Emx2/ Gli3( Xt/Xt), ShhN and Gdf7-DTA mice) commonly show alterations in the positioning of the dorsal telencephalon with severe midline invagination defects [13,15,16,17,18,19,20]. Thus, it is possible that compromised invagination of the dorsal midline observed in those mutant lines stems from defective Wnt signaling in dorsal mesenchymal cells due in part to compromised cortical hem development or cell autonomous inhibition of ß-catenin function in neural crest cells. Further supporting this idea, our data here show that loss of ß-catenin expression in cranial neural crest cells leads to the failure of medial telencephalic invagination, a process required for the medial cortical area to expand and form the lateral ventricles. Moreover, previous studies have shown that removal of the roof plate causes patterning defects in the dorsal cortex [19], which lends additional support to the concept that
there are important interactions between the cortical hem and interstitial mesenchymal cells.

As development proceeds, the derivatives of the roof plate such as the cortical hem and the choroid plexus invaginate and neural crest cell-derived mesenchymal cells fill the space between the ectoderm and the neural epithelium, which is required for the establishment of the ventricles. Formation of the lateral ventricles is essential to proper cortical development and function [41] and as our data show can be compromised by the loss of β-catenin signaling in neural crest-derived dorsal mesenchymal cells without loss of meningeal tissues. Even though it remains to be further elucidated how β-catenin signaling in mesenchymal cells triggers the invagination of the dorsal telencephalon, mesenchymal Wnt/β-catenin activation appears essential.

Cells of the cranial neural crest contribute to diverse tissues of the head and face making neural crest-derived MSCs valuable for prospective stem cell therapies of neural crest-centered pathologi-cal conditions, such as the neurocristopathies [1,21,22]. These disorders, not only manifest themselves with craniofacial defects but can also lead to defects in the brain vasculature stemming from dysfunctional pericytes and smooth muscle cells [24]. Consequently, the list of known neurocristopathies is likely to expand over coming years with the further elucidation of disease mechanisms caused by deficiencies in the blood-brain barrier function of neural crest-derived pericytes [42]. However, the future demand for neural crest stem cell-based therapies will be severely limited by the small amount of available neural crest cell-derived adult stem cells. Consequently, understanding the molecular events integral to neural crest specification and differentiation of derived tissues is paramount to the development of any stem cell-based therapies. In this study, inhibition of β-catenin expression in neural crest cells ectopically induced smooth muscles cells but hindered the formation of mesenchymal derivatives, such as dermal and calvarial mesenchymal cells, without severe defects in the formation of cortical pericytes and meningeal cells. Meningeal layers supply trophic and homing molecules for mesenchymal cells such as Cxcl12, retinoic acid and Bmps and thus the malformation of the meninges in Foxc1 mutants could lead to the inhibition of the mesenchymal derivatives [36,38,43,44,45]. The decrease of mesenchymal cells in the cortex of Foxc1 mutants is very likely secondary to the meningeal defects. The initial expansion of mesenchymal progenitors adjacent to the cortical hem in Foxc1 mutants was not affected but instead spreading or targeting of Col2α1+/PdgfrB+ mesenchymal cells seems to be compromised. Future studies focusing on the interaction of meninges and non-meningeal mesenchymal cells will likely reveal meningeal factors controlling expansion and migration of mesenchymal cells and development of craniofacial tissues.

In summary, we show that β-catenin signaling of neural crest cells is crucial for the neural crest-derived mesenchymal cells to expand and induces invagination of the dorsal telencephalon and formation of the lateral ventricles.

**Experimental Procedures**

**Animals**

All animal experiments were done with approval of the IACUC at UCSF. Mouse lines used in this study were previously described [Bat-gal [46], Sox10-Cre [47], Ctnnb1(lof) [10], Ctnnb1(gof) [32], Foxc1(flx) [48]]. ROSA-lacZ reporter mice were purchased from Jackson Laboratory. The day of vaginal plug was considered to be embryonic day (E)0.5. Mouse colonies were housed at the University of California, San Francisco, in accordance with UCSF IACUC guidelines.

**Immunostaining and in situ hybridization**

Embryos were collected at noon of given developmental days, decapitated, and immersion-fixed overnight in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Harvested tissues were cyroprotected in 15% sucrose/PBS for 4–8 hours, embedded

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**Figure 6. Defective development of calvarial mesenchymal cells by loss of β-catenin in neural crest cells.** In situ hybridization of Col2α1 was conducted to show the condensing calvarial mesenchymal cells at E14.5 (a). Bottom panels show mediadorsal Col2α1+ mesenchymal cells (b). An arrow indicates infiltrating Col2α1+ cells in the Sox10-Cre;Foxc1(flx/flx) mutants. Scale bars = 100 μm.

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in OCT compound (TissueTek, Sakura), and frozen on dry ice. The frozen tissue was cut in a cryostat, mounted on glass slides, and sections of 12 μm were processed for immunostaining according to standard protocols while sections of 20 μm were processed for RNA in situ hybridization as previously described [36]. Primary antibodies used for the immunostainings were rat anti-Pdgfrα (Millipore, 1:300), mouse anti-Vimentin (Millipore, 1:1000), rabbit anti-Cav1 (Abcam, 1:500), rat anti-CD44 (eBioscience, 1:1000), rabbit anti-Ki67 (LabVision, 1:200) and rabbit anti-Pdgfrß (Cell Signaling Technology). Isolectin IB4 Alexa Fluor conjugates were obtained from Invitrogen. Templates for RNA probes (Lmx1a, Lhx2, Ttr, Raldh2, Cxcl12, and Col2a1) used for in situ hybridization were designed according to the Allen Developing Mouse Brain Atlas. All comparisons in one experiment between control and mutant sections were performed on tissues stained on the same slides to account for variations between staining processes. X-gal staining in ROSA-lacZ or Bat-gal compound carriers was conducted as described (Siegenthaler et al., 2009). To stain alkaline phosphatase activities in osteoblast cells, 20 μm sections were applied to nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate solutions (Roche) for 10 seconds. Confocal images were taken at the Nikon Imaging Center at UCSF using an upright Nikon C1 spectral confocal microscope and bright-field images were obtained using a microscope equipped with a QImaging CCD camera and QCapture Pro Software.

**Measurement of the mesenchymal cell number and thickness**

For the counting of Pdgfrß+ cells in the dorsal hemispheric region, three littermates were collected in 4% PFA for 1 hour

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**Figure 7. Ectopic generation of smooth muscle cells by loss of β-catenin in neural crest cells.**

A) A schematic drawing shows the three regions (a, b, and c) used to stain markers for smooth muscle cells. B) Isolectin IB4 staining shows distribution of blood vessels in the epidermis and meninges. Fewer mesenchymal cells were seen in the space between the blood vessels of Sox10-Cre;Ctnnb1(lof)/fix/fix mutant embryos. C) SM22a, a marker for the smooth muscle cells, and CD44, a marker for MSCs, were used to characterize the mesenchymal cells in the regions a-c. C') Sox10-Cre;Ctnnb1(gof) mutant embryos double-stained for SM22a and CD44. Yellow bars indicate the thickness of the mesenchyme. D) αSMA, a marker for smooth muscle cells, and Desmin, a marker for pericytes, were used to reveal the ectopic generation of smooth muscle cells from neural crest cells of Sox10-Cre;Ctnnb1(lof)/fix/fix mutant embryos at E14.5. Arrows indicate the incompetent spreading of mesenchymal cells into the 'b' region of Sox10-Cre;Ctnnb1(lof)/fix/fix mutants. Scale bars = 100 μm.

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followed by incubation of samples in 10% sucrose/1× PBS for additional 2 hours to prevent shrinkage of mesenchymal tissues. Twenty μm sections were stained for Pdgfrβ and Vimentin and one section at the level of the eyes was chosen to count cells based on the Pdgfrβ+ individual cells counterstained for DAPI (nucleus) (n = 3). Mutant sections were matched according to the level of the control telencephalon. To select the region for counting, a control section containing approximately four hundred cells in the dorsal hemispheric region was selected and marked the boundary as outlined in dotted line (Fig. 3A). Sections of mutant embryos were accordingly marked based on the littermate control and cells were counted, presented as a graph. Ki67+ cells were counted from the dorsal telencephalic hemisphere adjacent to the cortical hem (170 μm×220 μm) and the cells of dorso-lateral mesenchymal areas (350 μm×250 μm) were counted at the level of the cortical hem (n = 3). To show the thickness of the mesenchymal layer, tissues were fixed less than 4 hours and incubated in 10% sucrose for 2 hours to prevent the shrinkage of tissues. One representative image from three experiments was presented in each figure (n = 3).

Statistics
Student's t-test was used for the pair-wise analysis of samples (SigmaPlot program, Systat Software Inc.). Error bars depict SEM.

Author Contributions
Conceived and designed the experiments: YC KZ SP. Performed the experiments: YC. Analyzed the data: YC KZ SP. Contributed reagents/materials/analysis tools: YC. Wrote the paper: YC KZ SP.

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