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Wnt/ β -catenin signaling in the mouse embryonic cranial mesenchyme is required to sustain the emerging differentiated meningeal layers

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Abstract

Cranial neural crest cells (CNCC) give rise to cranial mesenchyme (CM) that differentiates into the forebrain meningeal progenitors in the basolateral and apical regions of the head. This occurs in close proximity to the other CNCC-CM-derivatives such as calvarial bone and dermal progenitors. We found active Wnt signaling transduction in the forebrain meningeal progenitors in basolateral and apical populations and in the non-meningeal CM preceding meningeal differentiation. Here, we dissect the source of Wnt ligand secretion and requirement of Wnt/ β -catenin signaling for the lineage selection and early differentiation of the forebrain meninges. We find persistent canonical Wnt/ β -catenin signal transduction in the meningeal progenitors in the absence of Wnt ligand secretion in the cranial mesenchyme or surface ectoderm, suggesting additional sources of Wnts. Conditional mutants for *Wntless* and *β -catenin* in the cranial mesenchyme showed that Wnt ligand secretion and Wnt/ β -catenin signaling were dispensable for specification and proliferation of early meningeal progenitors. In the absence β -catenin in the CM, we found diminished laminin matrix and meningeal hypoplasia, indicating a structural and trophic role of mesenchymal β -catenin signaling. This study shows that β -catenin signaling is required in the cranial mesenchyme for maintenance and organization of the differentiated meningeal layers in the basolateral and apical populations of embryonic meninges.

Keywords

cell fate selection; meningeal progenitors; skull bone

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Introduction

The mammalian cranial lineages, such as the frontal bone of the skull, overlying dermis of the skin, and meninges that enclose the brain, differentiate from cranial mesenchyme (CM) that are derived from cranial neural crest cells (CNCC). (Serbedzija, Bronner-Fraser, and Fraser 1992; Jiang et al. 2002). Between E11.5–13.5, in mouse, these three lineages differentiate from CM in close proximity to one another between the neuroepithelium of the brain and the surface ectoderm in the basolateral mesenchyme of the supraorbital arch (Angelov and Vasilev 1989; McLone and Bondareff 1975; Tran et al. 2010). The meninges are a protective, fluid-filled membranous sac that cover the brain and serve as a reservoir of trophic factors, stem cells, and extracellular matrix that affect brain and skull bone development (Richtsmeier and Flaherty 2013; Bjornsson et al. 2015). Studies suggest there are two distinct pools of meningeal progenitors, with the basolateral population differentiating earlier than the population in the apex. The basolateral meningeal progenitors begin to differentiate to the distinct pia, arachnoid, and dura layers from E13.5 (Angelov and Vasilev 1989; McLone and Bondareff 1975). Concomitantly with the invagination of the dorsal brain at E10.5, there is a second residential population of dense CM in the dorsal midline/apex that appears to expand laterally by E14.5 and then begins to differentiate into the apical meningeal layers after E14.5 (Choe et al., 2014). Meninges complete their differentiation into the three-layer structure only after E19.5 (Mercier, Kitasako, and Hatton 2002; Bjornsson et al. 2015; Bifari et al. 2015).

In the absence of lineage-specific genetic tools in the CM, several existing mutants have been used to understand the contribution of neighboring brain and calvarial bone to meningeal development (Ito et al. 2003; Kume et al. 1998; Rice et al. 2003). In the spontaneous mouse mutant, *congenital hydrocephalus (ch)*, that results in a truncated *Foxc1* protein, the apical expansion and differentiation of the basolateral meningeal mesenchyme is compromised and tracks the expansion of cranial bone anlagen, suggesting communication between the two mesenchymal populations during morphogenesis (Rice et al. 2003; Vivatbutsiri, Ichinose, Hytönen, et al. 2008). In addition, the *Foxc1* null mutants lack closure and invagination of the dorsal telencephalon, and is also devoid of meninges and skull bone in the apex, suggesting a structural or signaling role of the dorsal brain (Kume et al. 1998). Reciprocally, the embryonic meninges have been implicated in producing inductive signaling to promote calvarial bone development in the deletion of TGF- β receptor 2 (TGF β RII) in the premigratory CNCC (Ito et al. 2003). However, the cell-autonomous signals required for meningeal specification, differentiation, and apical expansion *in vivo* remains to be tested systematically.

Wnt signaling is one of the earliest pathways required in the induction, migration, and later in the differentiation of CNCC-derived CM lineages (Mani et al. 2010; Tran et al. 2010; Brault et al. 2001; Ikeya et al. 1997). Canonical Wnt signaling is transduced by β -catenin protein and has pleiotropic roles in embryonic development by regulating expression of context-specific downstream target genes (van Amerongen and Nusse 2009). Wnt signaling reporters are visible in the premigratory CNCC at E8.5–9.5, and then in the CM in the apex and in the newly invaginated cortical hem of the dorsal telencephalon at E10.5 (Choe, Zarbališ, and Pleasure 2014; Mani et al. 2010). Interestingly, mice with conditional deletion

of β -catenin with *Emx1Cre* in the mouse forebrain have CNCC-derived structures, suggesting that Wnt signaling in the forebrain is dispensable for CM differentiation (Campos, Du, and Li 2004). Conditional deletion of β -catenin/*Ctnnb1* in the premigratory CNCC with *Wnt1Cre* or *Sox10 Cre* leads to diminished forebrain and loss of craniofacial structures (Choe, Zarbalis, and Pleasure 2014; Brault et al. 2001; Ikeya et al. 1997). Furthermore, the β -catenin-mutant in premigratory CNCC fail to laterally expand at the apex and the cortical midline invagination is diminished, thereby confounding the analysis of meningeal development (Choe, Zarbalis, and Pleasure 2014).

Many questions remain about early differentiation of the meningeal progenitors and the role of canonical Wnt signaling in meningeal development in the apex and basolateral regions. For instance, what is the role of Wnt signaling in post-migratory CNCC for meningeal mesenchyme specification, differentiation to the distinct layers, expansion of the meningeal progenitors, proliferation, and survival? What is the source of Wnt ligands for Wnt signal transduction in the basolateral and apical meningeal progenitors? What is the requirement of the adjacent calvarial bone for the morphogenesis, differentiation and expansion of meningeal progenitors in the basal and apical region? We previously showed that conditional deletion of β -catenin in the post-migratory CNCC allows for the formation of CM in the supraorbital arch and closure of dorsal brain; therefore, making this model more amenable to study the role of Wnt signaling in forebrain meninges formation (Tran et al. 2010; Goodnough et al. 2012). Furthermore, deletion of β -catenin in the supraorbital arch CM leads to loss of calvarial bone and dermal fates, prompting us to investigate its role in meningeal specification and early differentiation.

In the continued absence of meningeal progenitor-restricted genetic tools, we performed this study to gain insights into these questions. We generated and examined various mouse models *in vivo* that lack Wnt ligand secretion specifically in either the ectoderm or the CM preceding specification of the various lineages. We directly tested the role of Wnt signal transduction in the meningeal vs non-meningeal mesenchyme and the requirement of cranial bone for meningeal differentiation in the basolateral population *in vivo*. Our results show that Wnt signaling in the meningeal mesenchymal progenitors is not required for the specification, but for the maintenance of the emerging meningeal layers, suggesting a trophic role.

RESULTS:

Meningeal progenitors transduce Wnt signaling

To investigate the spatiotemporal role of canonical Wnt signaling during the specification, differentiation, and expansion of meningeal progenitors, we first queried Wnt signaling activity in the cranial mesenchyme using two canonical Wnt signaling transgenic reporters: *Axin2-LacZ/+* and *TCF-LEF H2B-GFP/+*, and target genes between E11.5–14.5 in the basolateral and apical regions (Figure 1A) (Lustig et al. 2002; Ferrer-Vaquer et al. 2010). Between E11.5–14.5, expression of Wnt signaling reporters was visible in the meningeal progenitors in the basolateral (Figure 1B-G) and apical sites (Figure 1L-N, S1). A subset of FOXC1+ meningeal progenitors were Axin2LacZ expressing cells (Figure 1F). At E12.5, *Axin2-LacZ* was clearly visible in the neighboring dermal and cranial bone progenitors in

the basolateral site (Figure 1C). Between E11.5–12.5, *Axin2-LacZ* expression was visible in the cortical hem, the CM, and the surface ectoderm in the apex (Figure 1 L, M). At E13.5, *Axin2-LacZ* expression in the apical site became more restricted and remained visible in the meningeal progenitors and the dermal progenitors (Figure 1N). *Lef1* mRNA expression is dependent on high levels of Wnt signaling (Rudloff and Kemler 2012). At E12.5, LEF1 protein expression was visible in a subset of the meninges closest to the brain, in the frontal bone progenitors, dermal progenitors, and in a few cells in the cortex (Figure 1H). As a direct readout of Wnt signaling activation, we detected nuclear β -catenin expression at E12.5 in the meningeal progenitors and the neighboring non-meningeal mesenchyme (Figure 1I).

Next, we examined the source of Wnt ligand-expressing cells. *Wntless (Wls)* is required for the secretion of all Wnt ligands and we found WNTLESS protein expression in all layers of the cranial mesenchyme, including meningeal progenitors and the overlying surface ectoderm at E12.5 (Figure 1J) (Bänziger et al. 2006; Bartscherer et al. 2006; Goodman et al. 2006). We previously identified numerous Wnt ligands expressed in a spatially-restricted pattern in the cranial ectoderm, mesenchyme, and cortex at E12.5 (Goodnough et al., 2014). Of the canonical Wnt ligands detectable in the cranial mesenchyme, we reliably detected protein expression of the canonical Wnt16 ligand in the meningeal mesenchyme, calvarial bones, and in the cartilage base at E13.5, showing that meningeal fibroblast progenitors can produce a canonical Wnt ligand (Figure 1K). Together, these results show that canonical Wnt signaling transduction and Wnt ligand secretion occurs in the meningeal progenitors and neighboring cranial lineages in the basal and apical sites during early meningeal progenitor specification and differentiation (Figure 1O, P).

Cranial mesenchyme Wnt ligand secretion is dispensable for meningeal specification

Preceding the differentiation of meninges into distinct layers, numerous Wnt ligands are expressed in the CM and surface ectoderm between E11.5–12.5 (Goodnough et al., 2014). First, we examined the role of Wnt ligand secretion by CM in meninges development by conditionally deleting *Wntless (Wls)* with the tamoxifen-inducible *PDGFR α CreER^{T2}/+* and *Dermo1Cre/+* line by E9.5 (Figure 2A, S2) (Rivers et al. 2008; Carpenter et al. 2010). Relative quantity of *Wls* mRNA was markedly reduced in the CM of conditional *Wls* mutants, showing robust deletion of *Wls* (Figure 2B). The distribution of *PDGFR α CreER/+; Rosa26- β -gal* lineage-marked descendants was comparable between controls and conditional *Wls* mutants in the basolateral region (Figure 2C,D compare E,F). At E14.5, we found comparable domains of protein expression of meningeal fibroblast markers for FOXC1 (pan-meningeal), RALDH2 (subset of meningeal fibroblasts), in the basolateral domains of control and *PDGFR α CreER/+; Wls^{fl/fl}* mutants (Figure 2G, H, K, L) (Vivatbutsiri, Ichinose, Hytonen, et al. 2008; Spector et al. 2002; Zarbalis et al. 2007). Using qRT-PCR on E14.5 cranial mesenchyme, we found that relative quantity of *Raldh2*, *Foxc1*, and *Foxd1* mRNA levels were comparable in the CM of control and *Wls* mutants at E14.5 (Figure 2W). The relative quantity of *Msx2*, a marker of the dura mater layer and calvarial bone primordia (Vivatbutsiri, Ichinose, Hytönen, et al. 2008) was significantly lower in the *PDGFR α CreER/+; Wls^{fl/fl}* mutant at E14.5. In the apical meningeal progenitors at E14.5, the distribution of FOXC1⁺ and RALDH2 expressing cells was comparable between controls

and *PDGFRaCreER/+; Wls^{fl/fl}* mutants (Figure 2I, J, M, N). Previously, we showed pan-mesenchyme *Wls* mutants have diminished calvarial bone differentiation, thereby contributing to decrease in relative quantity of *Msx2* in our cranial mesenchyme preparation (Goodnough, et al., 2014). The number and volume occupied by FOXC1⁺ cells were not significantly different between control and *PDGFRaCreER/+; Wls^{fl/fl}* mutant mutants in the basolateral and apical sites (Table 1 n=4–6 embryos per genotype)

Meninges secrete matrix molecules such as laminin and heparin sulfate proteoglycans and contribute to the basement membrane (BM) of the meninges (Bifari et al. 2015; Richtsmeier and Flaherty 2013; Mercier, Kitasako, and Hatton 2002). The vasculature of the meninges with PECAM/CD31 expression in endothelial cells and gap junctions expressing CONNEXIN(CX43) in the arachnoid layer. Expression of these proteins have been used as an indicator of meningeal development (Baldwin et al. 1994; Yancey, Biswal, and Revel 1992; Vivatbutsiri, Ichinose, Hytönen, et al. 2008; Zarbalis et al. 2007). We found continuous expression of LAMININ protein medial to the layer of PECAM⁺ endothelial cells in control and *PDGFRaCreER/+; Wls^{fl/fl}* mutants at E14.5 in the basolateral and apical regions (Fig 2 O-R). Similarly, we found comparable distribution of CX43 expression in the basolateral and apical sites in the control and the *PDGFRaCreER/+; Wls^{fl/fl}* mutants (Figure 2S, T, U, V). We examined the presence of Wnt signal transduction in the meningeal mesenchyme by LEF1 protein expression and TCF-LEF H2B-GFP protein expression and found that *PDGFRaCreER/+; Wls^{fl/fl}* mutants transduced canonical Wnt signaling in the meningeal progenitors in the basolateral and apical sites, suggesting other sources of Wnts (Figure S1). We obtained similar results in the conditional *Wls* mutants with another pan-CM *Cre* line, such as the *Dermo1Cre/+; Axin2LacZ/+; Wls^{fl/fl}* mutants (Figure S2). Together, these studies suggest that Wnt ligand secretion in the cranial mesenchyme is dispensable for the specification and early differentiation of meningeal progenitors.

The ectoderm is a rich source of numerous Wnt ligands and is required for canonical Wnt signaling transduction in the underlying CM (Goodnough, et al., 2014). This led us to hypothesize that ectoderm Wnt ligands could be required for meningeal development. We used the *Crect/+* line, which is active in the cranial ectoderm by E9.5, to delete *Wls* in the the surface ectoderm (Reid et al. 2011; Goodnough, et al., 2014). *Crect/+; Wls^{fl/fl}* mutants lack dermal and calvarial bone progenitors specification and apical expansion, and instead have ectopic cartilage at the basolateral site (Goodnough, et al., 2014). At E13.5, FOXC1⁺ meninges and RALDH2 expression in a subset of meningeal fibroblast progenitors was present in the control and *Crect/+; Wls^{fl/fl}* mutants (Figure S3). In the basolateral region, distribution of FOXC1⁺ cells was shifted laterally towards the ectoderm and appeared beneath the ectoderm in the medial regions (Figure S3). We found meningeal progenitors were also able to transduce canonical Wnt signaling in the *Crect/+; Wls^{fl/fl}* mutants at E13.5, suggesting the source of Wnt ligands for meningeal Wnt signaling excludes the surface ectoderm and CM (Figure S3). Given the absence of cranial bone and atrophy of the cranial mesenchyme, we could not analyze these mutants at later time points. Thus, by E13.5, ectoderm Wnts are not required for the specification of FOXC1⁺ meninges in both the basolateral and the apical regions, but our analysis does not exclude a role for ectodermal Wnts later in meningeal differentiation.

Basolateral meningeal layers are supported by Wnt/ β -catenin-transducing non-meningeal mesenchyme

Next, we tested the requirement of β -catenin in CM for meningeal differentiation between E14.5–16.5. We used two different *Cre* lines to dissect the requirement of canonical Wnt/ β -catenin signaling in meningeal (*Dermo1Cre*) and non-meningeal mesenchyme (*Engrailed1Cre*, *En1Cre*) during meningeal development in the basolateral and apical sites (Figure 3A, 4A). We use the *En1Cre* line to efficiently delete β -catenin in non-meningeal cranial mesenchyme between E10.5–11.5 as previously shown (Tran et al. 2010; Goodnough et al. 2012). The *En1Cre* lineage-marked cells do not contribute to meningeal fibroblast progenitors basolaterally and apically beneath the frontal bone (Figure 3B-G). Therefore, the *En1Cre*^{+/+}; *β -catenin*^{fl/del} mutants allow us to test the hypothesis that *β -catenin* and canonical Wnt signal transduction in the adjacent non-meningeal cranial mesenchyme is required for meningeal differentiation. At E14.5 we found the meninges appeared thinner in the basolateral region of *En1Cre*^{+/+}; *β -catenin*^{fl/del} mutants, and by E16.5 we observed marked hypoplasia of the meninges in the basolateral region (Figure 3B-E). Compared to controls at E16.5, the volume of pan-meningeal markers, such as FOXC1⁺, was thinner in the mutants. However, RALDH2 expression domains was comparable in the control and mutant in the basolateral region (Figure 3H, I, L, M). In order to determine the source of the hypoplasia, we examined expression of the LAMININ and PECAM at E14.5. Compared to the control, the expression of BM-LAMININ adjacent to the PECAM⁺ cells was markedly diminished in the mutant. The PECAM cell layer was disorganized and appeared consistently overlapping the LAMININ layer in the mutant, suggesting changes in the matrix and vasculature in basolateral region. Thus, at the basolateral site by E16.5, Wnt/ β -catenin signal transduction in the non-meningeal cranial mesenchyme is required for maintenance and organization of meningeal volume, BM, and vasculature. In the apex, we found the morphology and expression of meningeal markers, and LAMININ in the *En1Cre*^{+/+}; *R26R*^{+/+}; *β -catenin*^{fl/del} mutants were comparable to controls, showing that Wnt/ β -catenin signaling in the non-meningeal cranial mesenchyme is dispensable for meningeal development in the apical site (3J, K, N, O, R, S).

The *En1Cre*^{+/+}; *R26R*^{+/+}; *β -catenin*^{fl/del} mutants have ectopic cartilage nodules in place of calvarial bone and dermis. To test the requirement of calvarial bone primordia for specification of meningeal progenitors, we analyzed *Dermo1Cre*^{+/+}; *R26R*^{+/+}; *Twist1*^{fl/fl} mutants, which lack calvarial osteoprogenitors and *Twist1* throughout the cranial mesenchyme (Goodnough, DiNuoscio, and Atit 2016). At E12.5, we found comparable expression domains of FOXC1 expression in the controls and conditional *Twist1* mutants, suggesting the calvarial bone primordia and *Twist1* are not required to specify FOXC1⁺ meningeal progenitors (Figure S4). Due to extensive agenesis of cranial mesenchyme in the apical regions, we could not analyze the apex and meninges differentiation in later stages of development in the *Twist1* mutants.

Deletion of Wnt signal transduction in cranial and meningeal mesenchyme leads to structural changes and hypoplasia of the meninges

Finally, we queried if transduction of canonical Wnt/ β -catenin signaling in the meningeal layers is required for meningeal differentiation in the basolateral and apical regions. In the

absence of a meningeal progenitor-specific *Cre* line, we conditionally deleted β -catenin broadly in the CM by E10.5 with *Dermo1Cre* (Figure 4A)(Goodnough et al. 2012). In the control and mutant, *Dermo1Cre* lineage-marked cells were present in the meningeal fibroblast progenitors at E14.5 in both the basolateral and apical regions (Figure 4B-E). Histology of the *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* mutants showed marked hypoplasia of the meningeal layers at E14.5 in the basolateral and apical sites (Figure 4F-I). Marker analysis at E14.5 revealed the distribution of expression domains of RALDH2 in a subset of meningeal fibroblasts appeared compressed in the basolateral region and had breaches in expression in the apical region (Figure 4J-M). FOXC1 was also expressed in controls and mutants but the domain of expression appeared narrower (Figure 4N-Q). *Msx2* expressing meningeal fibroblasts form in close proximity to the calvarial bone primordia (Vivatbutsiri, Ichinose, Hytonen, et al. 2008). We found *Msx2* mRNA expression domain at E14.5 was continuous in the control and had gaps in expression between the ectopic cartilage nodules in the basolateral region of the *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* (Figure 4R, S, R' S').

Quantifying the average number of FOXC1⁺ meningeal fibroblast progenitors in a fixed field revealed that the number of cells were not significantly different between controls and *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* mutants in the basolateral region (Figure 4N-Q, Table 1). However, in the apical region, the number of FOXC1⁺ were significantly lower in the mutants. Next, we analyzed the volume of FOXC1⁺ cells in a fixed area in control and in the *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* mutant. We found significantly decreased volume in the mutant and conversely the density was increased in the basolateral region; thereby, accounting for the meningeal hypoplasia in the basolateral site. In the apical site, the FOXC1⁺ cell number and volume were significantly lower in the mutant and the density did not approach statistical significance (Table 1). Finally, we examined if meningeal hypoplasia was due to changes the meningeal-derived LAMININ or vasculature. We found continuity in the BM-LAMININ in the basolateral and apical regions at E14.5 in the controls and *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* mutant. However, the distribution of PECAM⁺ endothelial cells in the meninges was different. The PECAM⁺ layer was not juxtaposed to the BM-LAMININ, but was overlapping with the BM in the basolateral and apical regions. These results suggest there is a collapse of the matrix and vasculature layers on each other.

Next we examined other causative mechanisms that can contribute to the observed meningeal hypoplasia in the *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* by E14.5 First, we examined cell survival by activated-CASPASE 3 protein expression at E12.5 and E14.5. We did not detect an appreciable increase in cell death preceding the hypoplasia of the meninges in the basolateral region of the mutants (Figure S5). Second, the proliferation index of meningeal progenitors was comparable in controls and mutants at E12.5 (51% \pm 6% in controls and 47.4% \pm 1.8 in the mutants) and E14.5 (49.4% \pm 5.3% in controls and 43% \pm 7.2 in the mutants). Our proliferation index in E14.5 controls is consistent with a published report in embryonic rat meninges (Bifari et al. 2015). Third, we investigated the possibility of some of the meningeal progenitors convert to ectopic cartilage phenotype in the conditional β -catenin^{fl/del} mutant; thereby, leading to less meningeal tissue. The non-meningeal mesenchyme, containing cranial bone and dermal progenitors, ectopically express the key cartilage determinant marker, SOX9, by E12.5 (Tran et al. 2010; Goodnough et al. 2012). We did not find ectopic expression of SOX9 in the basolateral or apical meningeal

sites (Figure S6). SOX5, a downstream target of SOX9 in chondrocytes was excluded in the meningeal mesenchyme in the control and in the mutant, showing the meningeal progenitors do not convert to the ectopic cartilage in the conditional β -catenin^{fl/del} mutant (Figure S6) (Lefebvre et al., 1998). Fourth, meninges can also produce several growth factors that can signal through mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERK) (Bifari et al. 2015; Richtsmeier and Flaherty 2013; Mercier et al., 2002). Compared to the control, we could not detect an appreciable loss of activated pERK at E12.5 (data not shown), suggesting the meningeal hypoplasia is due to changes in the BM and vasculature.

At E16.5, we found progressive meningeal hypoplasia in *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* mutants (Figure 5). First, we examined the distribution of lineage marked cells by β -gal staining. Compared to the control, the mutants had fewer lineage-marked cells in the hypoplastic meninges and replacement by wild-type cells in the basolateral region. In the apical site, substantial thinning was consistently observed (Figure 5 B-E). At E16.5, there were breaches in RALDH2 expression domain in the *Dermo1Cre/+; R26R/+; β -catenin^{fl/del}* mutants at the basolateral site and apical sites (Figure 5F-I). Consistent with the visible hypoplasia at E14.5 and progressive hypoplasia by E16.5, the domain of FOXC1 expression pattern in the basolateral and apical sites at E16.5 was markedly diminished in the mutants (Figure 5 K-M). In the mutant, the periosteum of the ectopic cartilage nodules and cartilage expressed FOXC1 and was juxtaposed to the hypoplastic meninges in the basolateral site (Figure 5K). By E16.5 in the mutant, we found the BM became poorly organized with significant breaches in the domain of LAMININ protein expression in the basolateral and apical sites (Figure 5 P-S). These data show that in the absence of Wnt/ β -catenin signaling in the meningeal and non-meningeal cranial mesenchyme, the number of FOXC1⁺ cells are affected at the apical site, and the whole meninges become progressively hypoplastic and less organized earlier in development, most likely due to BM defects.

Taken together, these results demonstrate that the basolateral and apical regions of the meninges have differing requirements for Wnt/ β -catenin signaling to prevent meningeal hypoplasia. Wnt/ β -catenin transduction is required in the non-meningeal mesenchyme in the basolateral site and in the meningeal apical mesenchyme to sustain the emerging differentiated layers of the meninges and organization of BM-Laminin and vasculature (Figure 6).

Discussion

In this study, we examined both loss of Wnt ligand secretion and Wnt signaling function in meningeal and non-meningeal populations during early meningeal development between E11.5–16.5. The salient findings are as follows: (1) The loss of Wnt ligand secretion in either the whole cranial mesenchyme or the surface ectoderm still allows for canonical Wnt signaling in the mesenchyme, and meningeal specification and differentiation proceeds. (2) β -catenin is dispensable or functionally redundant in meningeal mesenchyme for specification of FOXC1⁺ meningeal progenitors. (3) Wnt/ β -catenin signaling in the non-meningeal cranial mesenchyme is required to prevent meningeal hypoplasia, likely through a paracrine mechanism in the basolateral site and cell-autonomously in the apical site. (4) Wnt/ β -catenin signaling in the meningeal mesenchyme is required for supporting the cell

number and volume of meninges and BM in basolateral and apical sites. These data suggest a mode where Wnt/ β -catenin signaling in the cranial and meningeal mesenchyme supports the early differentiating meninges that produce organized and sufficient BM and prevents meningeal hypoplasia in the basolateral and apical regions.

The canonical Wnt/ β -catenin signaling pathway is one of the earliest in the cranial meningeal and non-meningeal mesenchyme. The cranial surface ectoderm, CM, and the cortical hem are major sources of Wnt ligands for the basolateral and apical meninges preceding the specification and differentiation of the meninges, dermis, and calvarial bone (Goodnough, 2014a; Choe et al., 2012). The developmental program of the meninges coincides with the specification of calvarial bone and dermal progenitors in the CM, and Wnt/ β -catenin signaling is required in the CM for the specification of those two tissues. Thus, the timing of these developmental programs prompted us to investigate the role of Wnt-mediated interactions within the meningeal and from the non-meningeal mesenchyme in post-migratory CM. Loss of Wnt ligand secretion from either the surface ectoderm or the cranial mesenchyme still allowed canonical Wnt signaling transduction in the meningeal progenitors in the basolateral and apical sites. We suspect that Wnt ligands from the brain and cortical hem or elsewhere may be transduced in the meningeal progenitors. Testing this hypothesis will be challenging because WNTLESS is required for brain development in Wnt 1 expressing cells (Fu et al. 2011). We found that conditional deletion of β -catenin in the meningeal and non-meningeal cranial mesenchyme allowed for the specification of FOXC1+ and RALDH2+ meningeal progenitors, suggesting a redundant role of another signaling pathway, or that Wnt/ β -catenin signaling is dispensable for meningeal specification. Formally testing this hypothesis awaits the development of meningeal progenitor-specific genetic tools to demonstrate if other key signaling pathways such as retinoic acid signaling, TGF β , and Bone morphogenetic protein (BMP) signaling converge with Wnt signaling to specify the meningeal progenitors.

As development proceeds, basolateral meningeal mesenchyme begins to differentiate into layers in close proximity to the ossifying cranial bone primordia. Previous studies implied that the dura and arachnoid layers fail to differentiate in the absence of calvarial bone (Kume et al. 1998; Zarbalis et al. 2007). Conditional deletion of transforming growth factor β receptor 2 (TGF β R2) in the premigratory CNCC led to agenesis of calvarial bones and defects in the dura layer, suggesting interaction or inductive signaling events between paraxial mesoderm cranial bone and CNCC-derived meningeal progenitors (Ito et al. 2003). We investigated the requirement of cranial bone primordia or ossified cranial bone in the specification and differentiation of meningeal layers using three different mutants (Figure 3, 4, S4). We found that specification of FOXC1+ meningeal fibroblast progenitors occurs in the basolateral site, suggesting that cranial bone primordia and cranial bone are not required for specifying meningeal progenitors. But, we did find changes in *Msx2* expression in the conditional Wls and *Dermo1Cre; β -catenin* mutants, suggesting that a subset of meningeal fibroblasts may be dependent on cranial bone primordia. Similarly, in the *En1Cre; β -catenin* mutants, where we preserve Wnt signaling transduction in the meningeal layers and have cartilage replacement of cranial bone primordia, the basolateral meninges had changes in the organization of BM-LAMININ and vasculature and subsequently became hypoplastic in development, suggesting that Wnt signaling is required to sustain the emerging meningeal

layers structurally after specification events. Future experiments will focus on elucidating how Wnt/ β -catenin signaling in the non-meningeal mesenchyme sustains the basolateral meninges, BM, and vasculature layers.

A clear implication of our study is that an optimal level of Wnt signaling must be maintained in the cranial mesenchyme for the normal development of the meninges and production of organized and sufficient BM. Using our different genetic lines, we found that the basolateral and apical meninges have different signaling requirements to maintain meningeal layers. β -catenin signaling in the non-meningeal mesenchyme was required for the maintenance of meningeal layer volume and BM layer as demonstrated by both β -catenin mutant mouse lines. The *En1Cre; β -catenin* mutant indicates that Wnt/ β -catenin signaling is required in the non-meningeal mesenchyme for meningeal mesenchyme development and organization of the basolateral site and not the apical site. However, as demonstrated by the *Dermo1Cre; β -catenin* mutant, cell autonomous β -catenin signaling in the meningeal progenitors in the apical site was required to support and maintain the volume of the meninges and cell number of the progenitors, suggesting a structural and trophic role. The lower number of FOXC1+ cells can disrupt the ability of the meninges to deposit laminin and maintain the BM which leads to BM defects (Zarbalis et al., 2007). We previously found that β -catenin signaling transcriptionally regulates mRNA expression of ECM genes, such as *Laminin*, proteoglycans, and other ECM modulating proteins in embryonic and adult dermal fibroblasts (Budnick et al. 2016; Hamburg-Shields et al. 2015; Mullins et al., 2017) The trophic factors originating from the non-meningeal mesenchyme in the basolateral site appears to be β -catenin-dependent. The absence of β -catenin in meningeal mesenchyme accelerates the hypoplasia of the meningeal layers showing the cell autonomous and non-cell autonomous requirement for β -catenin in the meningeal layers. The overall picture that emerges is of a finely tuned system, in which the correct balance of β -catenin-dependent mesenchyme factors and subsequent response in the meningeal progenitors is needed to allow for promoting and maintaining meningeal layers and sufficient deposition of the BM matrix proteins.

This study provides new results related to early meningeal development. Meninges, with their trophic factors, ECM chemotactic factors, matrix molecules, and stem cells, serve as key players in brain and skull bone development. Further understanding of how meninges develop and interact with neighboring tissues will inform our understanding of the etiology of congenital defects arising in the CM-derived lineages and the interplay between them during disease and fracture healing.

Materials and Methods:

Mice and Genotyping

Mouse lines used for conditional functional studies included *Dermo1/Twist2Cre* (Yu 2003), *En1Cre* (Kimmel et al. 2000) (gift of Alexandra Joyner), *PDGFR α CreER* (Jax stock# 018280), *Wls flox* (Carpenter et al. 2010)(gift of Richard Lang), *β -Catenin deleted* (Haegel et al. 1995), *β -Catenin conditional floxed* (Brault et al. 2001). These mouse lines were maintained on a mixed genetic background. Mouse Cre lines were crossed with *R62R* (Soriano 1999) for genetic lineage tracing. *Wnt signaling reporters Axin2 lacz* (Jax stock

009120) Reference and *TCF1efH2BGFP* (Jax stock 013752) were also used. Mice were backcrossed to CD1 background at least 10 generations. Mice were time-mated to yield desired crosses with vaginal plug day assigned as E0.5. Dams carrying embryos between E8.5 and E9.5 were orally gavaged with tamoxifen (25 μ g/gm body weight) to activate CreER and conditionally delete *Wls* and activate *R26R* in the cranial mesenchyme. At desired time points, embryos were harvested and processed for frozen or paraffin sections as previously described (Atit et al. 2006). Each experiment was performed on a minimum of three mutants with litter-matched controls. A minimum of three litters were used for each functional analysis. All animal handling and experimental procedures were approved by the Case Western Reserve Institutional Animal Care and Use Committee.

Histology, β -Galactosidase, AP staining

Embryos were drop-fixed in 4% paraformaldehyde at 4°C, sucrose dehydrated, and embedded in O.C.T. Compound (Tissue-Tek Sakura) and sectioned at 10 microns as previously described (Atit et al. 2006; Ohtola et al. 2008). H&E staining was performed by standard protocol. For β -Galactosidase staining, embryos were prepared and sectioned as above. Slides were washed in X-gal wash buffer (0.1% deoxycholate, 0.2% NP40, 2 mM MgCl₂, made up in 1X phosphate-buffered saline pH 7.4) and then incubated in X-gal staining solution (Amresco) at 1mg/mL in 5 mM ferricyanide, 5 mM ferrocyanide in wash buffer overnight at room temperature. The following day, slides were washed in PBS, counterstained with Eosin as described above, and mounted with Permount mounting medium (Fisherbrand). Alkaline phosphatase staining on sections was done as previously described (Goodnough, 2012). All microscopy was done on an Olympus BX60 and all microscopy photos were taken with a Leica DP72 camera using cellSens software.

In situ Hybridization, Immunohistochemistry

In situ hybridization on cryosections was performed as previously described (Holmes and Niswander 2001). *Msx2* in situ probe was a gift from Dr. Jill Helms. For immunofluorescence, cryosections were post-fixed for 5 minutes in 4% PFA, and were blocked in 1% BSA. Species-specific serum with 0.01% Triton was used to block and incubate overnight in primary antibody at 4°C. Appropriate species-specific secondary antibodies were incubated for 1 hour the next day at room temperature, and then slides were mounted with Fluorshield (Sigma). For immunohistochemistry, heat-citrate-mediated antigen retrieval for 10 minutes and peroxidase block was performed before incubating with primary antibody overnight at 4°C. Secondary antibody incubation for 1 hour was performed the next day, followed by amplification with ABC reagent (Vector) and visualized with DAB (Amresco) and hematoxylin (Fisher) counterstain. Primary antibodies used include: anti-LEF1 (1:100)(Cell Signaling 2286), WNT16 (1:50)(Santa Cruz Bio 20268), β -CATENIN (1:100)(BD Biosciences 610153), non-phospho β -CATENIN (1:200)(Cell Signaling 8814), KI67 (1:100)(Abcam 15580), CONNEXIN43 (1:3200)(Sigma Aldrich c6219), PECAM (1:10)(BD Biosciences 550274), FOXC1 (1:100)(Cell Signaling 8758), RALDH2(1:200) (Sigma Aldrich HPA010022), active-CASPASE3 (1:250)(Abcam 13847), LAMININ (1:500) (Sigma Aldrich L9393), SOX9 (1:2000)(Chemicon ab5535), SOX5(1:1200)(Abcam ab94396), WNTLESS (1:2000)(gift of Richard Lang), β -Galactosidase (1:100; Abcam ab 9361).

Quantification of FOXC1⁺ cell number, volume, density was calculated in a fixed field. Total number of FOXC1⁺ cells were manually quantified and meningeal volume was quantified by outlining the domain occupied by FOXC1⁺ cells using FIJI Image J software. Cell density was calculated by dividing # of FOXC1⁺ cells/meningeal volume measurements. Significance was calculated using two-tail, type 3, Student *t*-test in Microsoft Excel.

Proliferation

Sections were collected and Ki67 immunohistochemistry was performed as above (Goodnough, DiNuoscio, Ferguson, Williams, Lang, and Atit 2014a). Total meningeal cells were counted in a fixed field of similar region on two to five sections per embryo a minimum of 50 microns apart (n = 6–9). Two-tail, type 1, Student's *t*-test was used to determine statistical significance of Ki67 positive vs. negative cells.

Cell Death Assay

Sections were collected and active caspase3 immunohistochemistry was performed as above to assay for survival. Slides were mounted with Fluoroshield (Sigma). Photos were taken in aforementioned manner. Total meningeal cells were counted in a fixed field of similar region on two to five sections per embryo a minimum of 50 microns apart (n = 6–9). Two-tail, type 1, Student's *t*-test was used to determine statistical significance of active Caspase3 positive vs. negative cells.

RNA Isolation and qPCR

Cranial mesenchyme, including the meningeal layers, was mechanically isolated from embryonic mouse heads at E14.5 and total RNA was isolated using Trizol (Invitrogen) per manufacturer's instructions. cDNA was made using a Multiscribe Reverse Transcription kit (Life Technologies). qPCR was performed on a StepOne Plus (ABI) using Taqman chemistry. Probes used were Wls (Mm00509695_m1), Foxc1 (Mm01962704_m1), Raldh2 (Mm00501306_m1), Msx2 (Mm00442992_m1), and Foxd1 (Mm00843738_s1). Relative gene expression was calculated using the 2^{-Ct} method (Livak and Schmittgen 2001).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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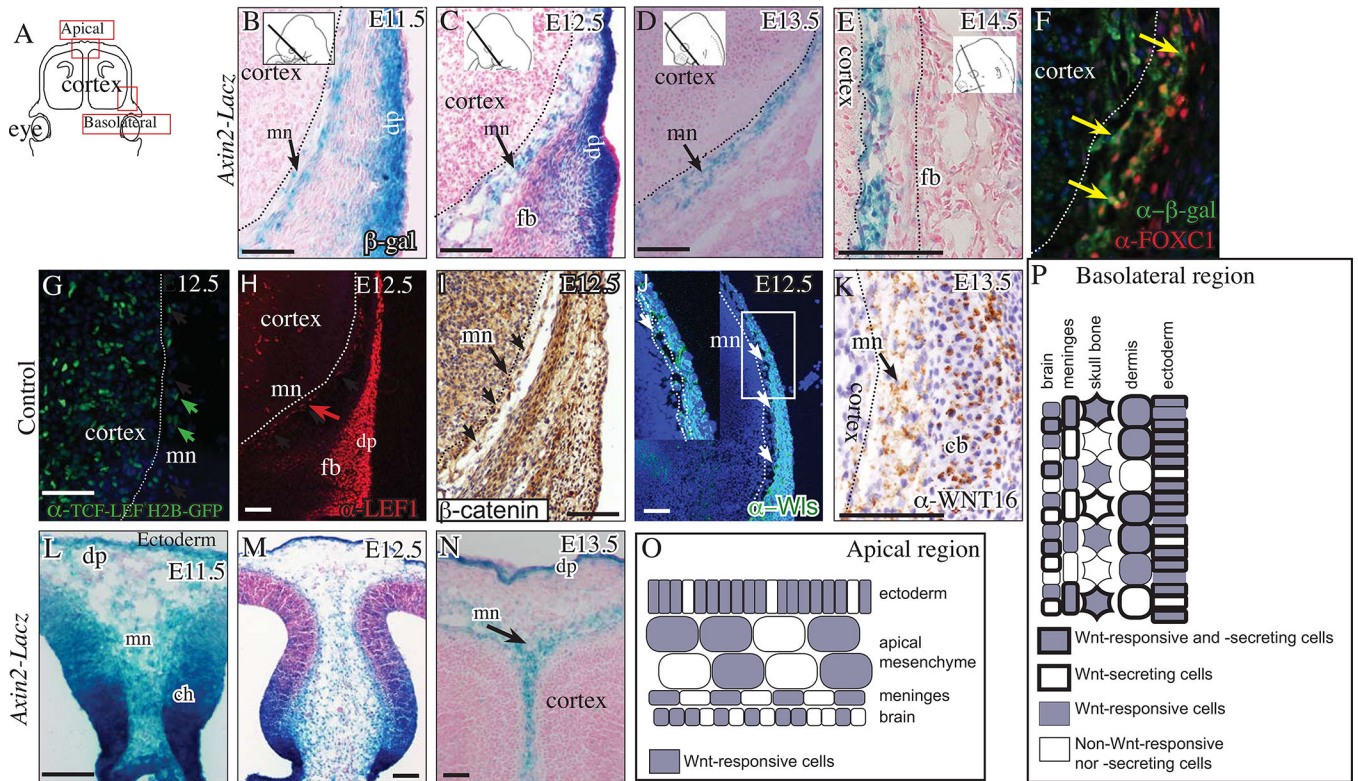


Fig. 1. Meningeal mesenchyme transduces Wnt signaling preceding differentiation of meningeal layers.

Schematic illustration of coronal embryonic mouse head sections at the eye level along the dorsoventral axis in the forebrain (A). β -galactosidase staining and counterstain with eosin Y showing canonical Wnt signaling in meningeal mesenchyme progenitors (mn) between E11.5–14.5 (basolateral, B-E; apical L-N). Indirect immunofluorescence with DAPI counterstain (F, G, H, J) and immunohistochemistry with hematoxylin counterstain (I, K) showing canonical Wnt signaling (G, H, I) and Wnt ligand secretion capacity (J, K) in the meningeal mesenchyme (F) and neighboring cranial lineages. Summary schematic illustration of the cranial mesenchyme lineages and meninges in the apical region (O) and basolateral (P) regions at E12.5. Other abbreviations: dermal progenitors (dp) mesenchyme, and frontal bone (fb) primordia, cartilage base (cb), cortical hem (ch). All arrows point to the meningeal mesenchyme. Dotted line demarcates the frontal cortex of the brain from the meningeal mesenchyme. Scale bars represent 100 μ m.

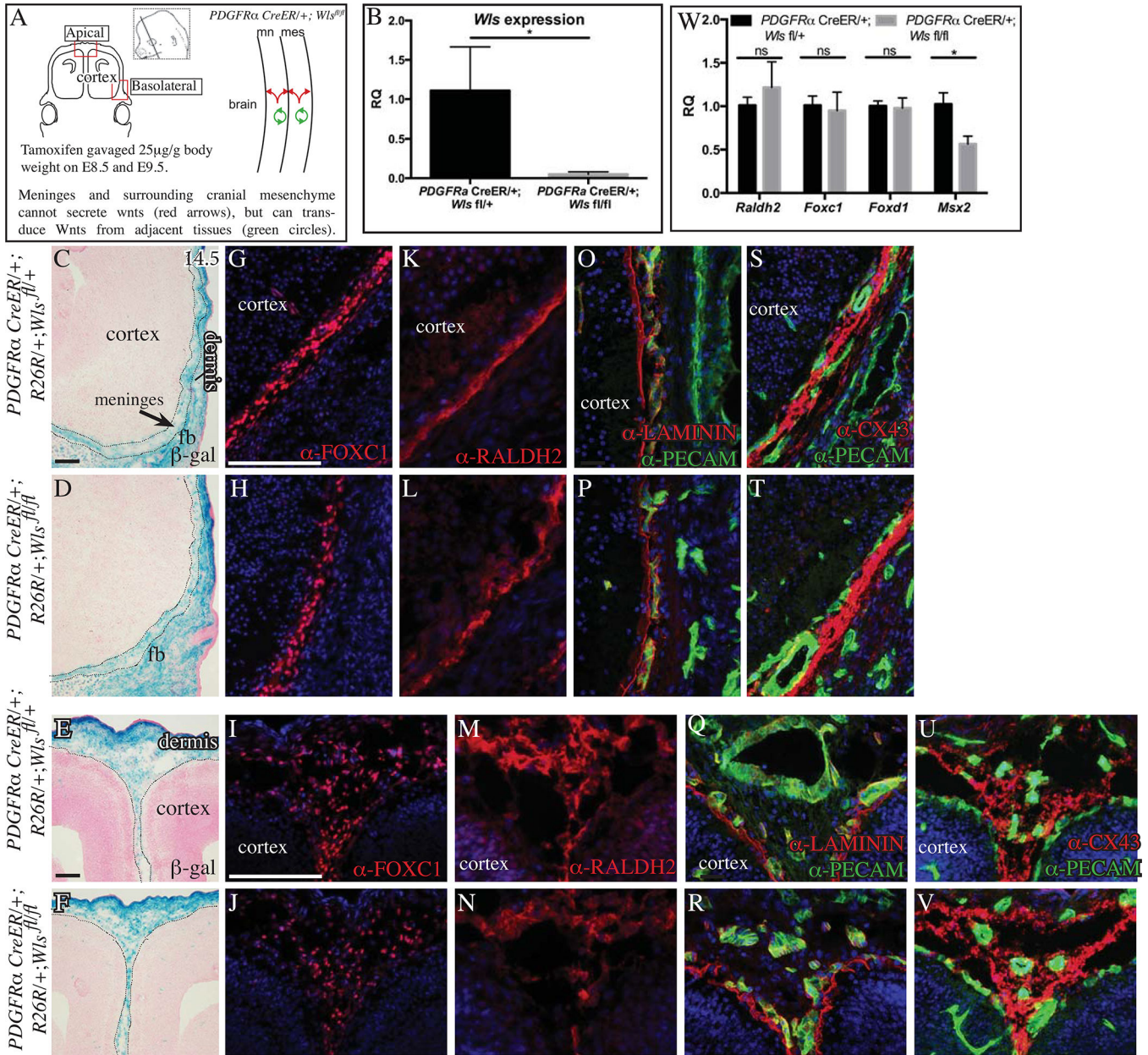


Fig.2. Conditional deletion of *Wntless* (*Wls*) in cranial mesenchyme.

Schematic illustration of the coronal plane, tamoxifen induction regimen, lateral view of the embryonic head in the region of interest, and simplified schematic of genetic model (A). Relative quantity (RQ) of mRNA in E14.5 cranial mesenchyme of control and conditional *Wls* mutant cranial mesenchyme (n=4) (B, W). β-galactosidase staining with eosin counterstaining and black dotted lines outline meningeal mesenchyme (C-F). Indirect immunofluorescence for markers of meninges (G-N), basement membrane LAMININ (red, O-R), CD31/PECAM⁺ endothelial cells (green, O-V), Connexin43 in gap junctions (red, S-V) with DAPI-stained (blue) nuclei (G-V). Scale bars represent 100µm. * indicates statistical significance with p value between 0.01 and 0.05 (B, W). White and black dotted line demarcate the meningeal mesenchyme from adjacent cranial mesenchyme and cortex.

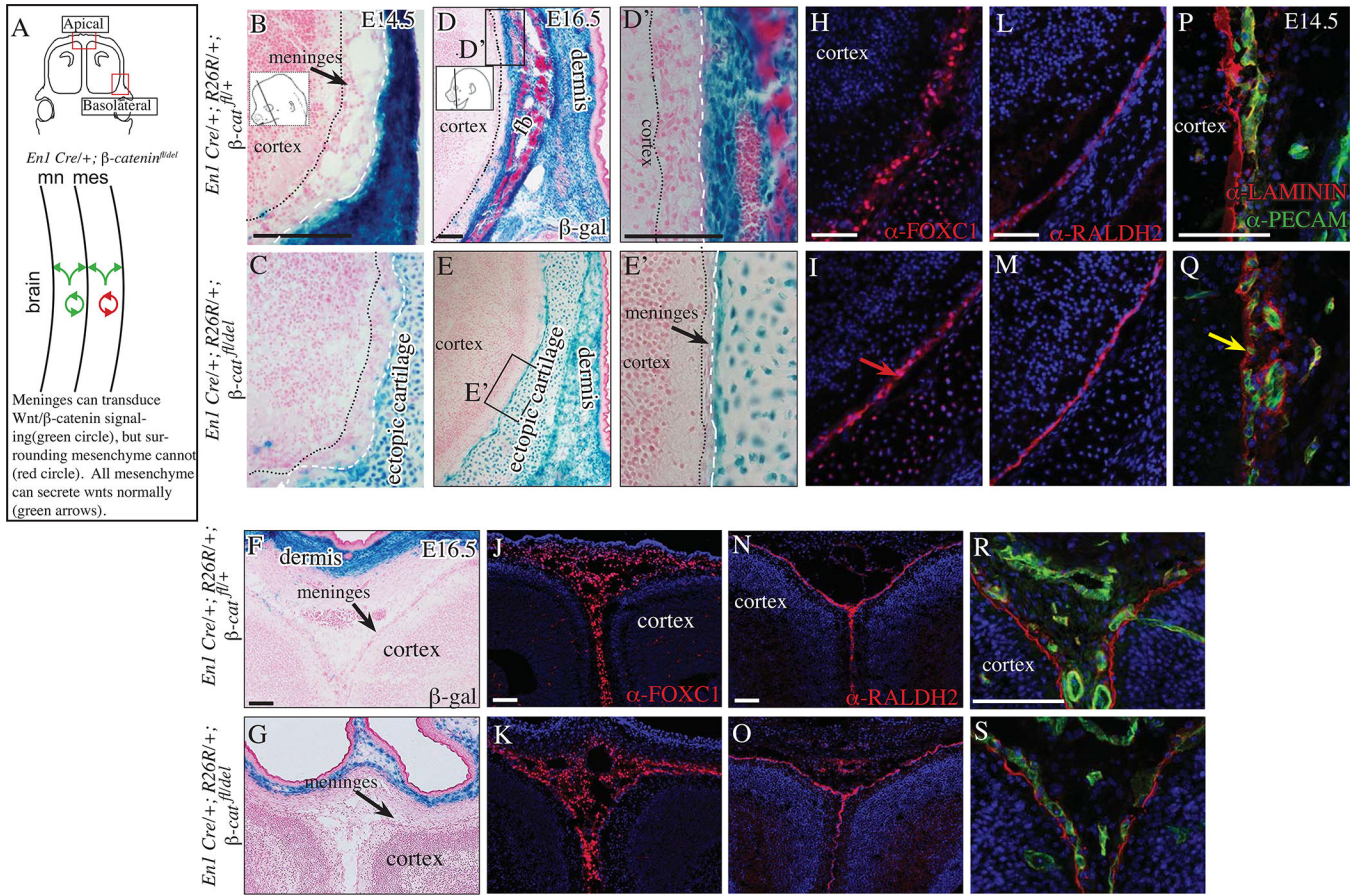


Fig 3. Basolateral meninges are diminished in the absence of β -Catenin in the non-meningeal cranial mesenchyme.

Diagram of coronal embryonic mouse head sections at the eye level in the forebrain showing the regions of interest and simplified schematic of genetic model (A). β -galactosidase staining counterstained with eosin Y (basolateral B-E; apical F, G. Higher mag basolateral D', E'; apical F', G'). Indirect immunofluorescence for markers of meninges (H-O), basement membrane (BM) LAMININ (red, P-S), CD31/PECAM⁺ endothelial cells (green, P-S) with DAPI-stained (blue) nuclei (H-S). Black arrow points to meningeal hypoplasia (E, E') and red arrow shows compaction of FOXC1⁺ expressing cells in the basolateral site of the mutant at E16.5 (I). Yellow arrow points to diminished BM-LAMININ in the mutant (Q). Scale bars represent 100 μ m. Abbreviations: fb: frontal bone, hf: hair follicle. Dotted black line demarcates the cortex and white hatched line demarcates the frontal bone from the meninges (B-E).

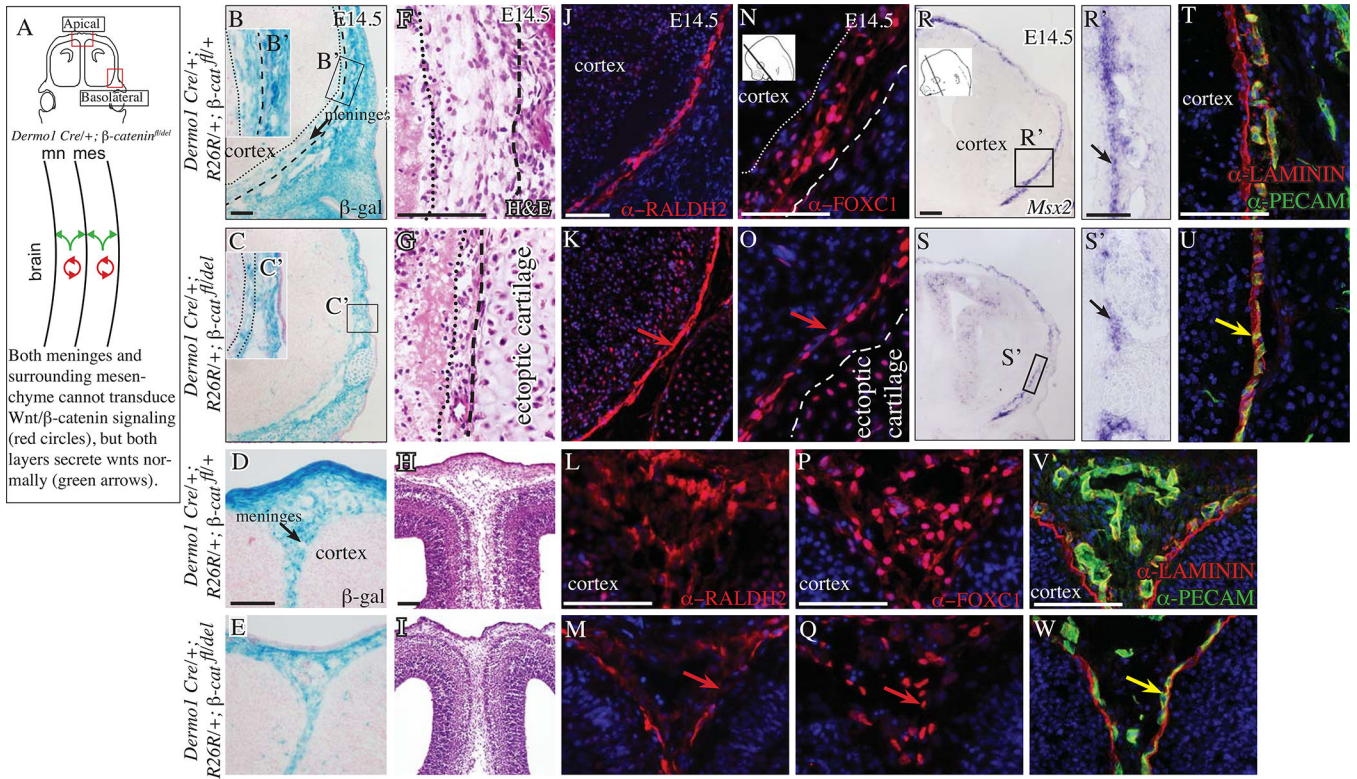


Fig 4. Conditional deletion of cranial and meningeal mesenchyme β -catenin results in thinner meninges.

Diagram of coronal embryonic mouse head sections at the eye level in the forebrain showing the regions of interest and simplified schematic of genetic model (A) Coronal mouse embryonic sections at eye level (B-W) in the basolateral region (top two rows) and apical region (bottom two rows). β -galactosidase expression counterstained with eosin Y (B-E), hematoxylin and eosin stain (F-I). Indirect immunofluorescence for meningeal markers (J-Q), basement membrane (BM) LAMININ (red, T-W), CD31/PECAM⁺ endothelial cells (green, T-W). Red arrow points to compaction of meninges marker expressing cells (K, O) and diminished domain of expression in the apical site in the mutant (M, Q). Yellow arrow points to overlap of BM LAMININ and PECAM layers in the mutant (red and green, U, W). Scale bars represent 100 μ m.

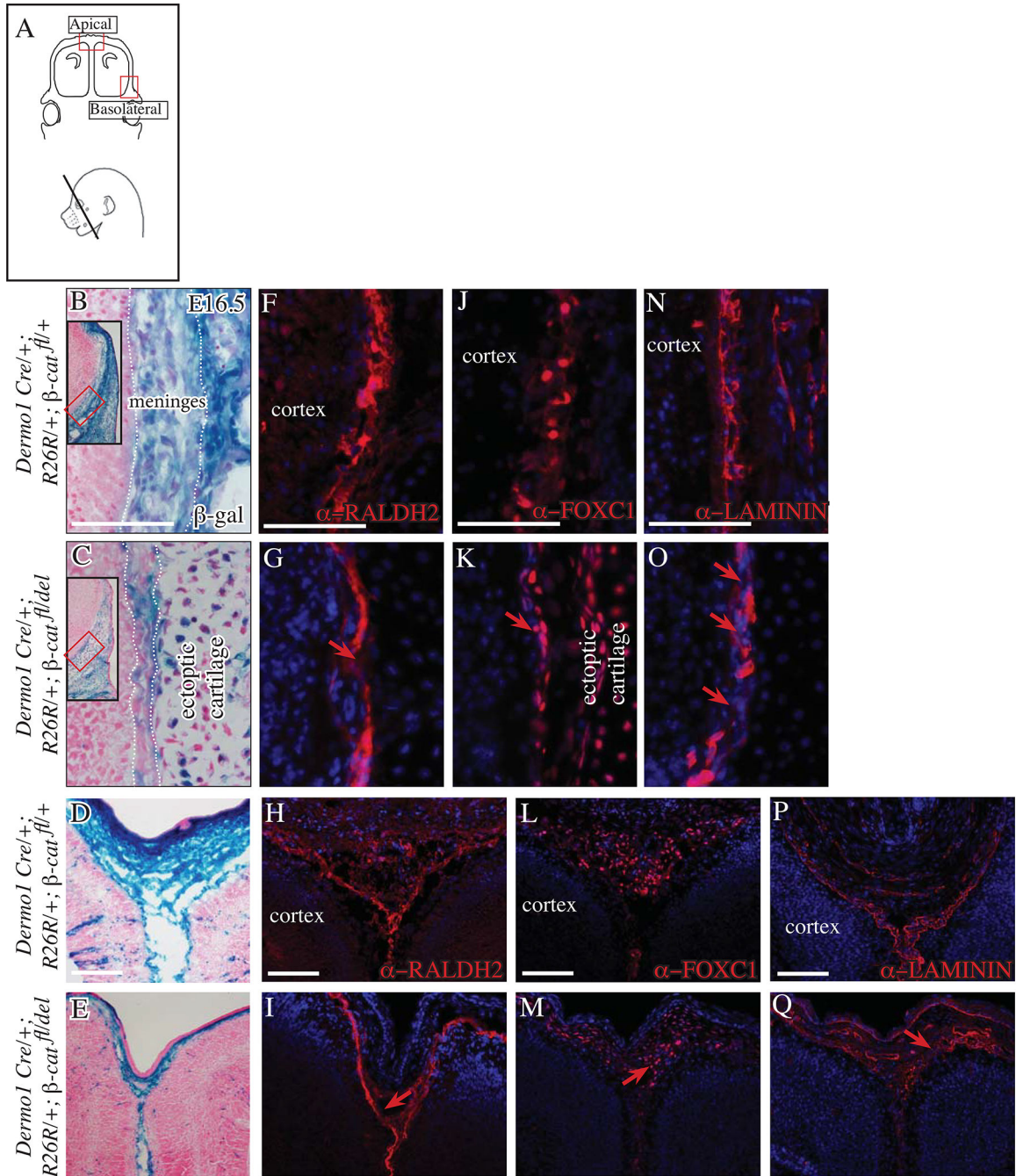


Fig 5. Conditional deletion of cranial and meningeal mesenchyme β -catenin results in meningeal hypoplasia in the basolateral and apical regions.

Diagram of coronal embryonic mouse head sections at the eye level in the forebrain showing the regions of interest (A) in basolateral region (top two rows) and apical region (bottom two rows). β -galactosidase expression counterstained with eosin Y (B-E) showing replacement with non-lineage marked wild-type cells (pink). Indirect immunofluorescence for meningeal markers (F-M), basement membrane (BM) laminin (red, N-Q) with DAPI-stained nuclei. Red arrow points to breach in RALDH2 expression (G, I), diminished domain of FOXC1

expression (K, M), breach and disorganized BM (O, Q) in the mutants. Scale bars represent 100 μ m.

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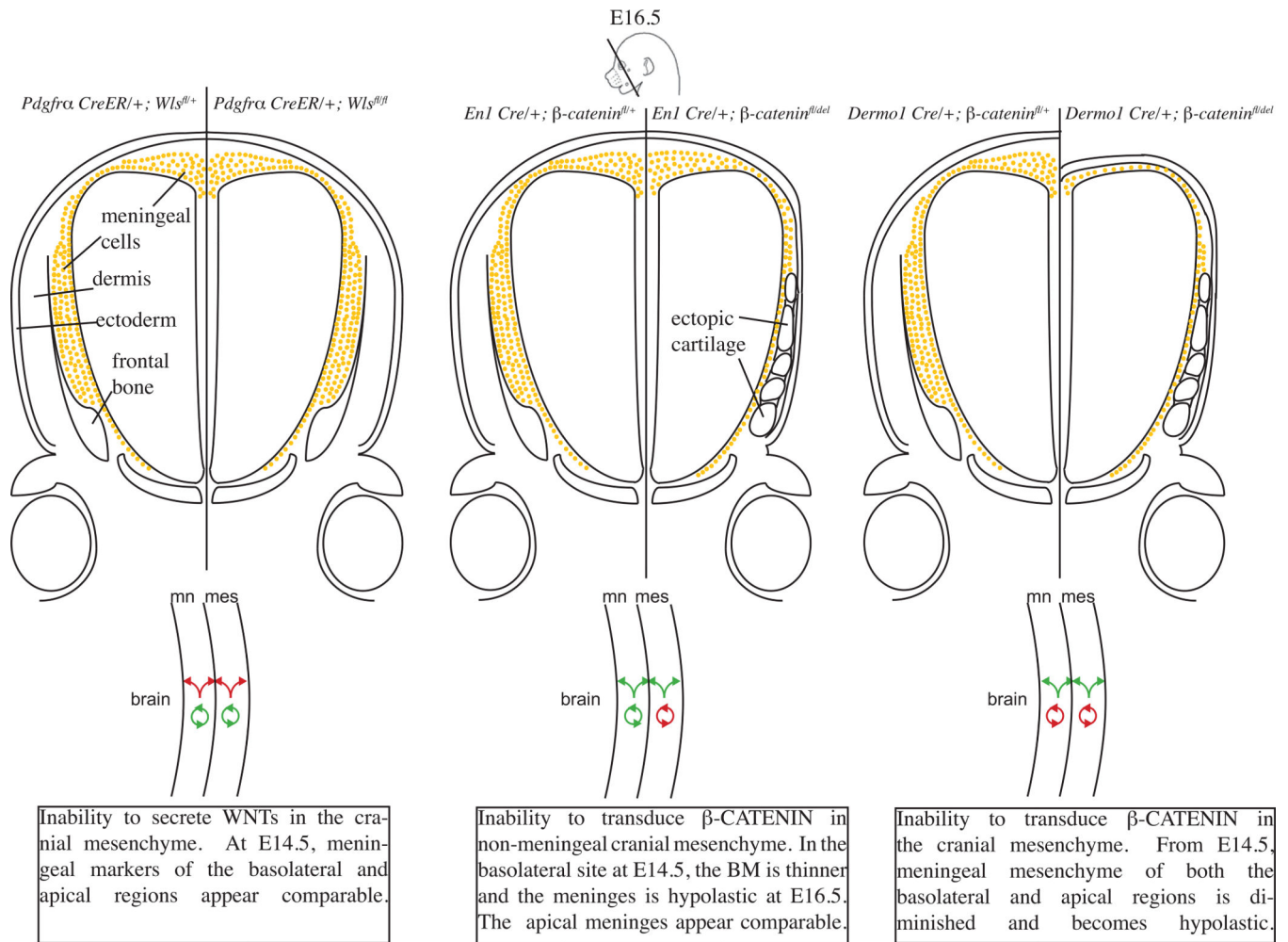


Fig. 6. Summary schematic of the genetic mutants used throughout the study and their effects on meningeal development.

Wnt/ β -catenin signaling is required in meningeal and non-meningeal cranial mesenchyme for proper development of meninges.

Table 1:
Quantification of FOXC1+ cell number, volume and density of basolateral and apical meninges.

Statistical significance was calculated by comparing Cre positive heterozygotes controls with *Wls^{fl/fl}* or *β -catenin^{fl/fl}* mutants using two-tailed Student *t*-test.

Genotype	Site	Average Cell Number per field	P Value	Average Meningeal Volume per field (pixels)	P Value	Average Meningeal Cell Density (cells per pixel)	P Value
<i>PDGFRa Cre/+; Wls^{fl/+}</i> control	Basolateral	85 ± 19.16	0.071	115368.67 ± 8854.83	0.48	7.3×10 ⁻⁴ ± 1.3×10 ⁻⁴	0.104
<i>PDGFRa Cre/+; Wls^{fl/fl}</i> mutant	Basolateral	118.1 ± 17.1		128030.48 ± 35153.08		9.6×10 ⁻⁴ ± 2.05×10 ⁻⁵	
<i>PDGFRa Cre/+; Wls^{fl/+}</i>	Apical	130.44 ± 35.77	0.95	324072.11 ± 69119.16	0.81	4.02×10 ⁻⁴ ± ×10 ⁻⁴	0.43
<i>PDGFRa Cre/+; Wls^{fl/fl}</i>	Apical	131.75 ± 35.70		311985.5 ± 81737.22		4.23×10 ⁻⁴ ± ×10 ⁻⁵	
<i>Dermo1 Cre/+; β-catenin^{fl/+}</i> control	Basolateral	119.59 ± 28.28	0.081	190945.9 ± 13511.96	0.0017**	6.607×10 ⁻⁴ ± 1.4×10 ⁻⁴	0.0063**
<i>Dermo1 Cre/+; β-catenin^{fl/fl}</i> mutant	Basolateral	92.66 ± 12.06		111161.2 ± 35280.88		8.423×10 ⁻⁴ ± 8.73×10 ⁻⁵	
<i>Dermo1 Cre/+; β-catenin^{fl/+}</i>	Apical	160.53 ± 41	0.002**	270108.6 ± 39129.62	0.0024**	6.0×10 ⁻⁴ ± 1.46×10 ⁻⁴	0.059
<i>Dermo1 Cre/+; β-catenin^{fl/fl}</i>	Apical	72.47 ± 17.94		168065.78 ± 47057.22		4.45×10 ⁻⁴ ± 9.62×10 ⁻⁵	