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Multiple Requirements of the Focal Dermal Hypoplasia Gene Porcupine during Ocular Morphogenesis

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Wnt glycoproteins control key processes during development and disease by activating various downstream pathways. Wnt secretion requires post-translational modification mediated by the *O*-acyltransferase encoded by the *Drosophila* porcupine homolog gene (*PORCN*). In humans, *PORCN* mutations cause focal dermal hypoplasia (FDH, or Goltz syndrome), an X-linked dominant multisystem birth defect that is frequently accompanied by ocular abnormalities such as coloboma, microphthalmia, or even anophthalmia. Although genetic ablation of *Porcn* in mouse has provided insight into the etiology of defects caused by ectomesodermal dysplasia in FDH, the requirement for *Porcn* and the actual Wnt ligands during eye development have been unknown. In this study, *Porcn* hemizygosity occasionally caused ocular defects reminiscent of FDH. Conditional inactivation of *Porcn* in periocular mesenchyme led to defects in mid- and hindbrain and in craniofacial development, but was insufficient to cause ocular abnormalities. However, a combination of conditional *Porcn* depletion in optic vesicle neuroectoderm, lens, and neural crest–derived periocular mesenchyme induced severe eye abnormalities with high penetrance. In particular, we observed coloboma, transdifferentiation of the dorsal and ventral retinal pigment epithelium, defective optic cup periphery, and closure defects of the eyelid, as well as defective corneal morphogenesis. Thus, *Porcn* is required in both extraocular and neuroectodermal tissues to regulate distinct Wnt-dependent processes during morphogenesis of the posterior and anterior segments of the eye. *(Am J Pathol 2015, 185: 1–17; http://dx.doi.org/10.1016/j.ajpath.2014.09.002)*

Q5 Focal dermal hypoplasia (FDH) or Goltz syndrome (OMIM #305600) is an X-linked dominant syndrome resulting from defective development and interaction of ectodermal and mesodermal tissues.[1,2] FDH patients exhibit variable manifestations of skin hypoplasia, hypodontia, skeletal abnormalities (including limb and digit defects, as well as reduced bone density), and defects in ocular, kidney, and abdominal wall development. FDH is caused by mutations in the porcupine homolog (*Drosophila*) gene (*PORCN*), which encodes for a highly conserved transmembrane *O*-acyltransferase localized to the Q6 endoplasmic reticulum.[3–5] In mouse, PORCN-mediated palmitoylation is critical for trafficking and signaling activity of Wnt proteins, a family of highly conserved cysteine-rich glycoproteins.[6,7] Although Wnt-independent activity of PORCN has been reported in some cases,[8] a systematic analysis revealed that all Wnt proteins require palmitoylation by PORCN for their secretion.[9] Several human developmental disorders have been linked to mutations in Wnt pathway components.[10]

Most FDH patients are heterozygous female with mosaic PORCN function, and the variable phenotypes are possibly due to individual X-chromosome inactivation. Approximately 10% of FDH patients are male, with postzygotic mosaic mutations. Studies in mouse revealed that PORCN is strictly required during gastrulation; thus, zygotic *Porcn* mutations are most likely lethal.[11–14] Furthermore, zygotic deletion of the paternal *Porcn* allele in mouse accurately recapitulates the phenotypic mosaicism observed in female patients and results usually in perinatal lethality.[11,15] Although FDH is considered

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a rare disease, with a prevalence of 1:1,000,000 based on the number of observed live births, studies in mouse suggest that prenatal lethality may affect up to 98% of *PORCN* mutant individuals, implying a prevalence of 1:25,000.[15] Thus, FDH may affect embryonic survival much more significantly than has been acknowledged.

PORCN is expressed in the developing mouse eye and surrounding tissues, and FDH patients frequently exhibit congenital eye defects, including microphthalmia, anophthalmia, colobomata (iris, choroid, retina, and optic nerve), aniridia, and pigment abnormalities.[1,3,12,16] During normal eye development, morphogenesis of the optic cup is a critical step that involves invagination of the distal optic vesicle and overlying surface ectoderm. The resulting inner layer of the optic cup develops into the neural retina, whereas the outer layer gives rise to the retinal pigment epithelium (RPE). The ventral optic cup is connected to the forebrain by the optic stalk, and both the ventral optic cup and the stalk invaginate, resulting in formation of the optic fissure. The peripheral rim of the optic cup differentiates into the ciliary body and iris in the postnatal mouse. The surrounding periocular mesenchyme consists of multiple cell lineages, both neural crest–derived and mesoderm-derived, and its interaction with the adjacent neuroepithelium and lens ectoderm is critical for differentiation of the anterior segment, patterning of the RPE, and optic stalk. Developmental problems during these processes are likely to cause the severe congenital ocular abnormalities observed in FDH.

Although the specific role of *Porcn* during eye development is unknown, interference with downstream components of Wnt pathways in mouse, zebrafish, chick, and frog has revealed that Wnt signaling is critical for diverse processes during eye development. Wnt proteins bind to several surface receptors, including the Frizzled family of transmembrane proteins, and activate several different pathways. In mice and humans, 19 Wnt ligands and 10 Frizzled receptors have been identified. The best characterized is the canonical Wnt–β-catenin pathway, which functions through stabilization of β–catenin, its translocation into the nucleus, and activation of TCF/LEF transcription factors. The role of the Wnt–β-catenin pathway during eye development in vertebrates is often context- and species-dependent, with functions in coordinating retinal progenitor proliferation and differentiation; development of the RPE, lens, ciliary body, and iris; and ocular angiogenesis.[17–38] For example, we and others have shown that Wnt–β-catenin signaling is required for differentiation of the RPE in the mouse optic cup, most likely by direct interaction of TCF/LEF with enhancers of the key regulatory genes *Mitf* and *Otx2*.[20,21,25]

In noncanonical Wnt signaling, activation of Frizzled receptors leads to an increase in intracellular calcium and activation of PKC and CaMKII (Wnt/Ca^{2+} pathway) or activation of small GTPases (RHO, RAC1, CDC42) and JNK, with participation of VANGL and DAAM (PCP pathway).[39] In addition, noncanonical Wnt proteins such as WNT4, WNT5A, and WNT11 can activate receptors other than Frizzled (eg, ROR, RYK).[40] Studies in frog and zebrafish indicate that noncanonical Wnt signaling is essential for formation and/or maintenance of the eye field.[38,41–44] In mouse, disruption of PCP effectors encoded by the *Fuz*, *Wdpcp*, and *Int* genes cause anophthalmia (*Fuz, Wdpcp*) and coloboma (*Int*), respectively; however, the underlying cellular defects are unknown.[45–47]

Several Wnt proteins are robustly expressed in ocular and periocular tissues, such as WNT2B, WNT3, WNT4, WNT5A, WNT5B, WNT7B, and WNT11 in the optic cup, lens, or surface ectoderm and in the periocular mesenchyme.[48,49] Recent observations in chick, frog, and zebrafish demonstrate that WNT2B, WNT3A, WNT4, and WNT11 regulate eye field formation and development of the RPE and the lens.[37,41,42,44,50,51] Interestingly, TGF-β signaling can act cooperatively or adversely with Wnt proteins to regulate different processes of eye development, as recently shown in chick, suggesting that interference with Wnt signaling may also affect other pathways.[37,50,51] To date, however, no ocular defects resulting from deficiency of particular Wnt proteins have been described in mammals.

Redundancy among Wnt ligands, crossregulation between noncanonical and canonical Wnt pathways, and overlap of downstream components with other pathways (and the lack of appropriate tools) have made it difficult to analyze the role of Wnt proteins, particularly those acting via the noncanonical pathway in the developing mouse eye.[52] To gain insight into how ocular development is affected in FDH and to understand the role of *Porcn* during eye development in mammals, we disrupted *Porcn* in ocular and extraocular tissues in mouse. Our results demonstrate that PORCN is expressed in neuroectoderm, lens, and neural crest–derived periocular mesenchyme and that it regulates closure of the optic fissure and eyelid, RPE differentiation, and corneal morphogenesis.

Materials and Methods

Mice

The generation of mice carrying the floxed *Porcn* allele [$Porcn^{lox/lox}$; kindly provided by L. Charles Murtaugh (University of Utah, Salt Lake City, UT)] has been described recently.[13] For the purpose of distinction, male mice harboring the floxed *Porcn* allele are referred to as $Porcn^{lox}/Y$. In all crosses, we maintained a mixed genetic background with C57BL/6 and CD-1 mice (Charles River Laboratories International, Hollister, CA). $Porcn^{lox/lox}$ mice were crossed with *Six3-Cre* mice (kindly provided by Yasuhide Furuta, Riken Center for Developmental Biology, Kobe, Japan),[53] *Wnt1-Cre* mice (Jackson Laboratory, Bar Harbor, ME),[54] *Rx3-Cre* mice [kindly provided by Milan Jamrich (Baylor College of Medicine, Houston, TX)],[55] $ROSA26R^{LacZ}$ mice (Jackson Laboratory),[56] and $Axin2^{lacZ}$ mice (Jackson Laboratory).[57] Except as otherwise indicated, wild-type (WT) littermates without a *Cre* allele were used as controls.

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