Altered Neural Cell Fates and Medulloblastoma in Mouse patched Mutants

Lisa V. Goodrich, Lijiljana Milenković, Kay M. Higgins, Matthew P. Scott*

The PATCHED (ptc) gene encodes a Sonnie hedgehog (Shh) receptor and a tumor suppressor protein that is defective in basal cell nevus syndrome (BCNS). Functions of PTC were investigated by inactivating the mouse gene. Mice homozygous for the ptc mutation died during embryogenesis and were found to have open and overgrown neural tubes. Two Shh target genes, ptc itself and Gli, were derepressed in the ecdotum and mesoderm but not in the endoderm. Shh targets that are, under normal conditions, transcribed ventrally were aberrantly expressed in dorsal and lateral neural tube cells. Thus Ptc appears to be essential for repression of genes that are locally activated by Shh. Mice heterozygous for the ptc mutation were larger than normal, and a subset of them developed hindlimb defects or cerebellar medulloblastomas, abnormalities also seen in BCNS patients.

The human PTC gene is a tumor suppressor and developmental regulator (1). Some patients with BCNS have germline mutations in PTC and are at increased risk for developmental defects such as spina bifida and craniofacial abnormalities, basal cell carcinoma of the skin, and brain tumors (2). PTC mutations also occur in sporadic basal cell carcinomas (1), which generally have both copies of PTC inactivated.

In the fruit fly Drosophila, Ptc is a key component of the Hedgehog (Hh) signaling pathway, which controls cell fate determination during development (3). Hh protein, secreted from localized regions, antagonizes the actions of its apparent receptor, Ptc, in nearby cells (4). In the absence of an Hh signal, Ptc represses transcription of multiple target genes, including ptc itself, wingless (a Wnt gene), and the transforming growth factor β-related gene decapentaplegic (5). In flies, ptc mutations cause derepression of target genes, cell fate changes, and excessive growth in some tissues (5). Hh induces a high level of ptc transcription by inhibiting the function of Ptc protein, so paradoxically an abundance of ptc transcript is an indicator of a low level of Ptc function. Vertebrate ptc expression is also regulated by Hh proteins (6), which can bind directly to Ptc (7).

The role of the Hh-Ptc pathway in skin cancer has been established by BCNS studies and with a mouse model (8), but less is known about the brain tumors associated with BCNS. About 3% of BCNS patients develop medulloblastomas (9), cerebellar tumors that usually arise in young children and have a mortality rate of ~30% (10). ptc mutations have been detected in sporadic medulloblastomas (11), but this tumor type is rare and there are few clear animal models (12), so much remains to be learned about its origins and biology.

To study the roles of ptc in development and in tumorigenesis, we constructed mice...
lacking ptc function. By homologous recombination, part of ptc exon 1 (including the putative start codon) and all of exon 2 were replaced with lacZ and a neomycin resistance gene (Fig. 1 (13)). Protein made from any alternative ATG codon would lack the first proposed transmembrane domain, flipping the orientation of the protein in the membrane. Three independent embryonic stem cell clones were used to make chimeras that were bred to B6D2F1 animals to generate heterozygous mice on a mixed background. Interbreeding of heterozygotes produced no homozygous animals among 202 offspring examined. Analysis of embryos from timed matings suggested that ptc−/− embryos die between embryonic day (E) 9.0 and E10.5, with the first gross phenotypes appearing by E8. In ptc−/− embryos, the neural tube failed to close completely and was overgrown in the head folds, hindbrain, and spinal cord (Fig. 2, A to C). Embryonic lethality may have been due to abnormal development of the heart (Fig. 2B).

In Drosophila Ptc protein inhibits ptc transcription. By inhibiting Ptc function, Hh increases production of Ptc, which may then bind available Hh and limit the range or duration of effective Hh signal (14). Hh signaling also posttranscriptionally regulates the zinc finger protein cubitus interruptus (ci) (15). In vertebrates, Sonic hedgehog (Shh) signaling induces transcription of both ptc and a ci homolog, Gli (6, 16). Derepression of ptc and Gli in ptc−/− mice should therefore reveal where Ptc is normally active.

The expression of ptc and Gli was greatly increased in ptc−/− embryos. In ptc+/− mice, expression of the lacZ gene fused to the first ptc exon during targeting accurately reported the pattern of ptc transcription (Fig. 2, C and D). In ptc−/− embryos, expression of ptc-lacZ was extensively derepressed starting at about E8.0 in the anterior neural tube and spreading posteriorly by E8.75 (Fig. 2, C and E). Derepression was germ layer-specific: both ptc-lacZ and Gli were expressed throughout the ectoderm and mesoderm, but not in the endoderm (Fig. 2, D to G). ptc expression may be excluded from the endoderm so that Shh can signal the endoderm to the mesoderm (17). A differential requirement for Ptc may distinguish the germ layers.

As revealed by ptc mutants, an early site of Ptc activity is the neural tube, where Shh and Ptc act antagonistically to determine cell fates. Shh induces the floor plate and motor neurons in the ventral neural tube (18). These cell types fail to form in Shh mutants (19). Large amounts of Shh produced by the notochord may induce floor plate by completely inactivating Ptc (18). If so, elimination of ptc function might cause floor plate differentiation throughout the neural tube. Prospective floor plate cells transcribe the forkhead transcription factor HNF3β first and then Shh itself (18). In E8.5 ptc mutants, transcription of HNF3β and Shh was expanded dorsally (Fig. 3, A to C). Ectopic Shh expression was most extensive in the anterior, where transcripts could be detected throughout the neurepithelium (Fig. 3, B and C). Cells in this region were in a single layer with basal nuclei, like floor plate cells that are normally restricted to the ventral midline (Fig. 3, D and E). Expression of the lateral neural tube marker Pax6 (20) was completely absent from ptc mutant embryos, suggesting that only ventral, not ventrolateral, cell fates are specified (Fig. 3, F and G).

In principle, dorsalizing signals from the surface ectoderm (21) could confer dorsal cell fates even in the absence of ptc function. In E8-E9 ptc homozygotes, the dorsal neural tube marker Pax3 was not expressed in the anterior neural tube but was transcribed in a very small region at the dorsalmost edge of the posterior neural tube (Fig. 3, H to J). In addition, erb-b3 transcription, which marks migratory neural crest cells (Fig. 3K) (22), was not detected in the somites of ptc mutants (Fig. 3L). We conclude that only limited dorsal fate determination occurs in the absence of ptc. Bone morphogenetic protein (BMP) signals appear to maintain dorsal gene expression (21) so either ptc is required for BMPs to work or BMP signaling is ineffective in most cells expressing Shh targets.

Ventralization of the neural tube in ptc mutants occurred without affecting cell identity along the rostrocaudal axis. In ptc−/− embryos, cells in the anterior neural tube expressed the forebrain marker Nkx2.1 (23), and cells in the spinal cord transcribed hoxb1 (24) (Fig. 3, M and N). hoxb1 was not transcribed in the fourth rhombomere of ptc mutants (Fig. 3N). This may reflect a transformation of hind-
brain cells to floor plate, because hoxb1 is excluded from the midline of wild-type embryos. Conversely, in the anterior, Nkx2.1 expression was expanded dorsally in mutants compared with wild-type embryos (Fig. 3M).

The ptc+/− mice had features in common with BCNS patients: the mice were larger than their wild-type littersmates [30.72 ± 3.83 g (average ± SD; n = 29)] compared with 26.54 ± 2.51 g (n = 39) at 2 to 3 months; P = 0.000001 by t test], a small fraction (3 of 389 mice examined) had hindlimb defects such as extra digits or syndactyly (Fig. 4A) or obvious soft tissue tumors (1 of 243), and many developed brain tumors.

Of 243 ptc+/− mice that were between the ages of 2 and 9 months and were not killed for other studies, 18 died or were killed because of sickness. No wild-type littersmates died. Ten of the affected heterozygotes were autopsied, and eight were found to have large growths in the cerebellum that resembled medulloblastomas (Fig. 4, B and C). Human medulloblastomas are believed to arise from a "primitive neuroectodermal" cell type (25). They are most common in children, can be metastatic or nonmetastatic, and can have glial and neuronal properties. The histology of tumors from ptc+/− mice was similar to that of human medulloblastoma: tumor cells were small, with dark carrot-shaped nuclei and little cytoplasm (Fig. 4, D and E), and although a subset expressed neurofilament protein and synaptophysin (Fig. 4F) (26), the majority of cells appeared undifferentiated. Of the two autopsied animals without apparent medulloblastomas, one had a large tumor growing out of its rib muscle and the other died for unknown reasons. Medulloblastomas and soft tissue tumors were also observed in ptc+/− mice maintained on an inbred 129S background: 6 of 27 had obvious medulloblastomas, 2 of 27 had soft tissue tumors, and 3 of 27 died but were not examined.

The ptc and Gli genes were strongly transcribed in the brain tumors but not in surrounding tissue (Fig. 5, A and B; n = 3 of three tumors examined). There was no detectable increase in Shh expression (Fig. 5C). To assess the incidence of medulloblastomas, brains from 47 asymptomatic ptc+/− mice were randomly collected and stained with X-Gal. Nine brains contained medulloblastomas that were easily recognized by their disorganized morphology and intense ptc-lacZ expression (Fig. 5D). Medulloblastomas were observed in 1 of 12 (8.3%) ptc+/− mice at 5 weeks of age, 1 of 12 (8.3%) mice at 9 to 10 weeks, and 7 of 23 (30.4%) mice at 12 to 25 weeks. Tumors can therefore arise as early as 5 weeks after birth but increase in severity and frequency as the animal ages.

We looked for changes in ptc-lacZ expression that might reflect early stages of tumorigenesis. At all stages examined, about half of the animals [50% at 5 to 10 weeks (n = 24), 56.5% at 12 to 25 weeks (n = 23)] exhibited regions of increased X-Gal staining on the surface of the cerebellum (Fig. 5E). These regions were usually lateral and often extended down into the fissures separating the folia (Fig. 5, E and F). The mouse medulloblastomas may arise
from these cells, which are superficial to the molecular layer of the cerebellum (Fig. 5F). During fetal development, prospective cerebellar granule cells proliferate in the external granule layer (EGL), the outermost layer of the cerebellum. Granule cells then leave and migrate past the Purkinje cells to form the internal granule cell layer of the adult animal, gradually depleting the EGL. The remnants of the fetal EGL have been proposed to be a source of human medulloblastoma progenitors, a hypothesis consistent with the higher frequency of these tumors in children (27).

The abundance of cerebellar ptc transcripts was reduced by about 50% in the ptc−/− mice compared with wild-type littermates (Fig. 5G). This reduction could lead to ectopic expression of Shh target genes and to uncontrolled cell proliferation. Brain tumors might arise from Ptc haploinsufficiency alone, from additional mutations in the second ptc allele, or from a combination of ptc mutations with mutations in other tumor suppressor loci. We have not observed basal cell carcinomas in ptc−/− mice, perhaps because somatic inactivation of the second ptc gene is required as it is in human basal cell carcinomas.

Our analysis has revealed that Ptc controls growth and pattern formation in early neural development and in the adult cerebellum. Autoregulation of ptc occurs in vertebrates as it does in Drosofila, and the balance between Hh and Ptc activities appears critical for normal development. The importance of Ptc dosage is emphasized by the phenotype of the ptc−/− mice, which develop a tumor type observed in the corresponding human cancer predisposition syndrome. Medulloblastoma is a common childhood brain tumor and the prognosis remains grim. The Hh−Ptc pathway may provide new diagnostic tools and new insights into tumorigenesis that can be directed toward potential therapies.

REFERENCES AND NOTES

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13. DNA from the ptc genomic locus was isolated from a 129SV genomic phage library (Stratagene). Exons 1 through 15 of human PTC (1) were mapped by polymerase chain reaction (PCR) and sequencing. The 3‘ arm of homology was a 2.5-kb EcoRI–BamHI fragment from the second intron that gained a BamHI site from pBBSI (Stratagene) and was cloned into the BamHI site of pPNT 7 [V. L. Mylovyksff, C. E. Crawford, por. P. K. Jackson, R. T. Bronson, R. C. Mulligan, Cell 65, 1153 (1991)]. A cassette containing the gene for nuclear localized β-galactosidase (lacZ) followed by the miP intron and polycystic kidney disease was excised from pNLAC [E. H. Mercer, G. W. Hoyle, R. P. Knap, P. L. Birnstol, P. D. Palmer, Neuron 7, 703 (1991)] and cloned into the XhoI site of pPNT by using XhoI and Sal I linkers. The 5‘ arm of homology was a 6.5-kb XhoI–NruI fragment that was cloned into the XhoI site upstream of lacZ with a Sal I linker.
Epidermal Cell Differentiation in Arabidopsis Determined by a Myb Homolog, CPC

Takuji Wada,* Tatsuhiko Tachibana, Yoshio Shimura,* Kiyotaka Okada†

The roots of plants normally carry small hairs arranged in a regular pattern. Transfer DNA-tagged lines of Arabidopsis thaliana included a mutant with few, randomly distributed root hairs. The mutated gene CAPRICE (CPC) encoded a protein with a Myb-like DNA binding domain typical of transcription factors involved in animal and plant development. Analysis in combination with other root hair mutations showed that CPC may work together with the TTG gene and upstream of the GL2 gene. Transgenic plants overexpressing CPC had more root hairs and fewer trichomes than normal. Thus, the CPC gene determines the fate of epidermal cell differentiation in Arabidopsis.

The cellular organization of the primary root of Arabidopsis thaliana is relatively simple and invariant (1). During the maturation of the root epidermis in A. thaliana, each cell ultimately becomes either a root hair (trichoblast; which we shall hereafter term a root hair cell) or a hairless cell (atrioblast) (2, 3). This choice may be determined by the position of the cell relative to the underlying cortical cell layer. Epidermal cell files that make contact with two cortical cell files by lying over the junction between the two cortical cell files are root hair cells. Epidermal cells that contact only one cortical cell file are hairless cells. Primary roots in wild-type Arabidopsis normally have eight files of cortical cells (Fig. 1F). Root hairs are tip-growing, tubular-shaped outgrowths that help to anchor roots, interact with soil microorganisms, and assist in the uptake of water and nutrients. TRANSPARENT TESTA GLABRA (TTG) and GLABRA2 (GL2) are genes that determine whether epidermal cells differentiate into root hair cells or hairless cells (3, 4). In ttg and gl2 mutants, all of the epidermal cell files differentiate into root hair cells independent of their position relative to the underlying cortical cells. The GL2 gene encodes a homeodomain protein that is expressed preferentially in the differentiating hairless epidermal cells (4, 5). Although the TTT gene has not been cloned yet, it is believed to encode a protein with a Myc-like domain or a protein positively regulating a Myc-like gene, because the phenotype of the ttg mutant can be complemented by introducing a maize Myc gene, R, into the mutant. When the R gene is overexpressed in a wild-type plant, all of the root epidermal cells differentiate into hairless cells (3, 6). Thus, TTG and GL2 may inhibit the differentiation of root epidermal cells into root hair cells.

From a T3 population of transfer DNA (T-DNA)–tagged lines (7), we isolated a mutant with fewer than normal root hairs, which we named caprice (cpc) for the irregular distribution of root hairs (Fig. 1B). cpc is a nuclear mutation, not allelic to other known mutations. Heterozygous plants show the wild-type phenotype. From a cross between heterozygotes, about one-fourth (67/324) of the offspring had few root hairs, which indicated that cpc is a single, recessive mutation. The number of root hairs in the primary root of the cpc mutant was about one-fourth of that of the wild type (Table 1). The morphology and size of the root hairs produced by the cpc mutant were indistinguishable from those of wild-type hairs. The addition of 1-aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, at 5 × 10⁻⁴ M induced root hair production in cpc seedlings; however, the number of hairs was about 30% of that of the ACC-treated wild type, indicating that ethylene cannot rescue the phenotypic deficiency of the cpc mutant (8).

To examine how the CPC gene works in combination with the GL2 and TTT genes, we analyzed the phenotype of double mutants (Table 1). The cpc gl2 double mutant had about the same number of root hairs as the gl2 mutant, showing the gl2 mutation to...