Increased Wingless (*Wnt*) signaling in pituitary progenitor/stem cells gives rise to pituitary tumors in mice and humans

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Wingless (Wnt)/β-catenin signaling plays an essential role during normal development, is a critical regulator of stem cells, and has been associated with cancer in many tissues. Here we demonstrate that genetic expression of a degradation-resistant mutant form of β-catenin in early Rathke's pouch (RP) progenitors leads to pituitary hyperplasia and severe disruption of the pituitary-specific transcription factor 1-lineage differentiation resulting in extreme growth retardation and hypopituitarism. Mutant mice mostly die perinatally, but those that survive weaning develop lethal pituitary tumors, which closely resemble human adamantinomatous craniopharyngioma, an epithelial tumor associated with mutations in the human β-catenin gene. The tumorigenic effect of mutant β-catenin is observed only when expressed in undifferentiated RP progenitors, but tumors do not form when committed or differentiated cells are targeted to express this protein. Analysis of affected pituitaries indicates that expression of mutant β-catenin leads to a significant increase in the total numbers of pituitary progenitor/stem cells as well as in their proliferation potential. Our findings provide insights into the role of the Wnt pathway in normal pituitary development and demonstrate a causative role for mutated β-catenin in an undifferentiated RP progenitor in the genesis of murine and human craniopharyngioma.

The Wingless (Wnt)/β-catenin signaling pathway plays a critical role in the control of cellular proliferation and differentiation during embryonic development and organogenesis (1–3). Elegant studies in vitro and in vivo have demonstrated an essential role of this pathway in controlling the maintenance of embryonic and adult progenitor/stem cells by securing not only their cell numbers but also the balance between self-renewal and differentiation in many tissues and organs (4–7). Deregulation of this biological process leads to disease, including cancer, and mutations in components of the Wnt pathway resulting in stabilization of β-catenin have been identified as the molecular mechanism underlying a number of human tumors, including colorectal, epidermal, liver, intestinal, brain, and prostate cancer, among others (8–10). In several cases, the cellular mechanism underlying tumorigenesis caused by aberrant Wnt signaling is mediated primarily via progenitor/stem cells. These observations add support to the concept that cancer stem cells underlie many of these human tumors, a finding that has been confirmed in mouse by specifically targeting normal progenitor/stem cells (11, 12).

The anterior pituitary is a major endocrine organ controlling basic physiological functions in vertebrates including growth, metabolism, stress response, and reproduction. The presence of postnatal pituitary progenitor/stem cells able to self-renew and differentiate into hormone-producing cells has been demonstrated (13–17). Recently, by using a genetic cell lineage tracing approach, it has been shown that the adult anterior pituitary is a mosaic organ containing cells derived from embryonic and adult pituitary progenitor/stem cells. Presumptive adult pituitary progenitor/stem cells were identified first at 11.5 d post coitum (dpc) intermingled with embryonic progenitors in Rathke's pouch (the anterior pituitary primordium) and remained quiescent until birth (16). Evidence for a possible role of pituitary progenitor/stem cells in the genesis of mouse tumors has been shown recently. Conditional deletion of the retinoblastoma tumor suppressor in *Pax7* precursors was sufficient to generate nonsecreting adenomas in mice (18), and floating clonal spheres have been isolated from some human adenomas (19).

The involvement of β-catenin in the genesis of pituitary tumors is not clear. Activating mutations in the gene encoding β-catenin catenin (cadherin-associated protein β1; *CTNNB1*) have been identified in the pediatric form of human craniopharyngioma (adamantinomatous craniopharyngioma, ACP) (20–22). These tumors are rare but are the most common nonneuroepithelial intracerebral tumors in children and are potentially devastating because of their highly infiltrative behavior and tendency to recur after surgical resection (23, 24). Whether the identified mutations in *CTNNB1* are causative of human ACP remains unknown. Also uncertain is the cellular origin of human ACP and, in particular, the potential role of progenitor/stem cells in the genesis of ACP. We sought to investigate these questions by using a genetic approach to overactivate the Wnt pathway in specific pituitary cell types. Here, we demonstrate a causative role of mutated β-catenin in pituitary progenitor/stem cells in the etiology of mouse tumors that closely resemble human ACP. Our research provides further support for the cancer stem cell paradigm in the etiology of human pituitary tumors.

Results

Nuclear β-Catenin Accumulation and Activation of Wnt Signaling Occurs in a Minority of Rathke's Pouch Progenitors in Homeobox Embryonic Stem Cell expressed 1[Cre recombinase/+];Ctnnb1[+/loxp(exon3)] Embryos. We have shown previously that the Homeobox Embryonic Stem Cell Expressed 1-Cre recombinase (*Hesx1-Cre*) mouse line drives β-d-galactosidase (*lacZ*) expression in the periluminal progenitors of the developing Rathke's pouch of Homeobox Embryonic Stem Cell expressed 1[Cre recombinase/+];Gene

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trap ROSA 26, Philippe Soriano$^{lacZ/+}$ (*Hesx1$^{Cre/+}$;R26$^{lacZ/+}$*) embryos at 9.5–10.5 dpc (25). To demonstrate that descendants of these progenitors give rise to hormone-producing cells of the anterior pituitary, we analyzed their cell fate in *Hesx1$^{Cre/+}$; R26$^{Yellow\ Fluorescence\ Protein\ (YFP)/+}$* embryos at 18.5 dpc. Double immunostaining on pituitary sections of these embryos showed colocalization of YFP and specific marker expression for all hormone-producing cells in the anterior pituitary (Fig. S1). Next, we crossed the *Hesx1-Cre* mouse line with Ctnnb1loxp(exon3)/loxp (exon3) [*Ctnnb1$^{lox(ex3)/lox(ex3)}$*] animals (26) to express a degradation-resistant form of β-catenin leading to the activation of the Wnt/β-catenin pathway in early Rathke's pouch progenitors and hormone-producing cells at late gestation.

Unexpectedly, immunostaining with β-catenin antibodies revealed intracellular β-catenin in only a minority of cells in *Hesx1$^{Cre/+}$; Ctnnb1$^{+/lox(ex3)}$* pituitaries, whereas the majority showed normal localization of β-catenin on the cytoplasmic membrane. β-catenin–accumulating cells formed clusters and were detectable from 9.5 dpc (Fig. 1*A* and *B* and Fig. S2 *A–C*). Analysis of *Hesx1$^{Cre/+}$; Ctnnb1$^{+/lox(ex3)}$;R26$^{YFP/+}$* triple heterozygotes demonstrated YFP expression throughout Rathke's pouch and developing pituitary but intracellular β-catenin accumulation only in cell clusters (Fig. 1*F*). Laser-capture microdissection followed by DNA quantitative PCR analysis using specific primers confirmed the excision of exon 3 in both β-catenin–accumulating and nonaccumulating cells (Fig. S2*D*).

In agreement with these findings, quantitative RT-PCR analysis of dissected pituitaries revealed a significant increase in transcription of the Wnt/β-catenin direct targets lymphoid enhancer binding factor 1 (*Lef1*), Cyclin D1, and axis inhibition protein 2 (*Axin2*) in the compound pituitaries relative to controls (Fig. 1*D*). In situ hybridization and immunostaining showed discrete foci of increased *Lef1*, *Axin2*, Cyclin D1, and Cyclin D2 expression in the anterior pituitary of the *Hesx1$^{Cre/+}$; Ctnnb1$^{+/lox(ex3)}$* embryos, which, at least for these cyclins, colocalized with β-catenin–accumulating cell clusters (Fig. 1*E*). *Lef1*, Cyclin D1, and Cyclin D2 have been shown previously to be direct Wnt/β-catenin targets in the pituitary gland, and *Axin2* is an additional universal target (27, 28). Finally, use of the β-catenin–activated transgene driving expression of nuclear β-galactosidase Wnt reporter mice *BAT-gal* (29), revealed the activation of the Wnt pathway only in cell clusters within the developing pituitary glands of *Hesx1$^{Cre/+}$; Ctnnb1$^{+/lox(ex3)}$;BAT-gal* triple mutants (Fig. 1*C*). Therefore, the accumulation and activation of the Wnt pathway is restricted to a population of Rathke's pouch progenitors, despite the expression of mutant β-catenin in the majority of the cells.

Hesx1$^{Cre/+}$;Ctnnb1$^{+/lox(ex3)}$ Mice Develop Pituitary Tumors That Resemble Human ACP.

Approximately 77% of *Hesx1$^{Cre/+}$;Ctnnb1$^{+/lox(ex3)}$* mice died by 4 wk of age, and those that survived exhibited marked hypopituitarism resulting from increased cell proliferation and altered *Pit1* cell lineage differentiation (Table S1, Fig. 2 *A* and *B*, and Fig. S3). Subsequently, all surviving mice died by 6 mo of age with a median survival time of 11 wk (Fig. 2*E*). Post mortem examination of *Hesx1$^{Cre/+}$; Ctnnb1$^{+/lox(ex3)}$* mice revealed the presence of large cystic tumors under the brain and no pituitary gland (Fig. 2 *C* and *D*). These tumors were well circumscribed and usually contained cysts filled with red blood cells and eosinophilic proteinaceous material. Tumors isolated from adult mice expressed cytokeratins 8 and 18, but cytokeratin 5 was not detectable, indicating epithelial differentiation and generally in keeping with an origin from oral epithelium (Fig. S4). Analysis of
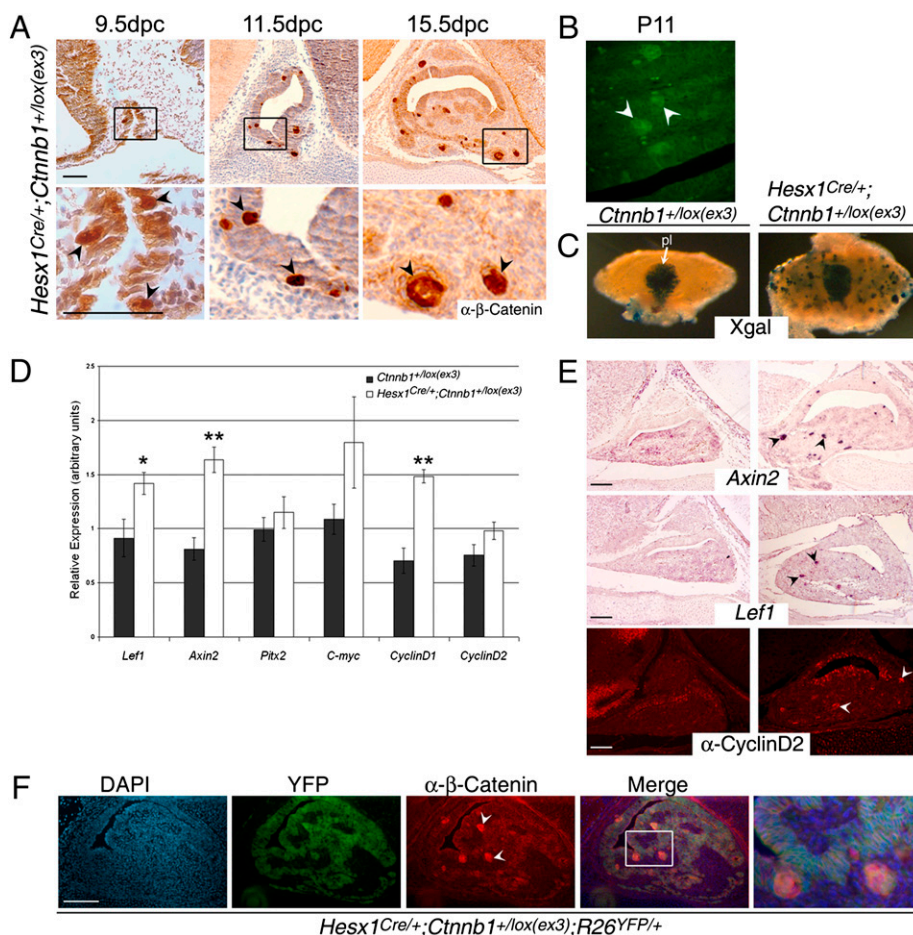

Fig. 1. Nuclear accumulation of β-catenin and activation of the Wnt pathway occurs in a small subset of anterior pituitary progenitors. (*A*) Histological sections of *Hesx1$^{Cre/+}$;Ctnnb1$^{+/lox(ex3)}$* pituitary glands from 9.5–15.5 dpc immunostained with a specific β-catenin antibody (Table S2) and counterstained with hematoxylin. Magnified views of the boxed regions in the upper row are shown in the lower row. Note that accumulation of β-catenin begins in a few cells within RP epithelium (arrowheads). (*B*) β-Catenin immunofluorescent detection in the postnatal pituitary at postnatal day 11 (P11) showing the persistence of β-catenin–accumulating cell clusters (arrowheads). (*C*) The spotted X-gal staining in the *Hesx1$^{Cre/+}$;Ctnnb1$^{+/lox(ex3)}$; BAT-gal* pituitary indicates that expression of beta-galactosidase occurs only in cell clusters. The posterior lobe (pl) is strongly stained in both genotypes. (*D*) Quantitative RT-PCR analysis of dissected pituitaries at 15.5 dpc showing a significant increase in the expression of the canonical Wnt/β-catenin targets *Lef1*, *Axin2*, and Cyclin D1, but not of paired-like homeodomain transcription factor 2 (*Pitx2*), *C-myc*, and Cyclin D2 in the heterozygous pituitaries relative to controls (*n* = 5–9), (*P < 0.01, **P < 0.001, *t* test). (*E*) In situ hybridization with *Axin2* and *Lef1* and Cyclin D2 immunofluorescence on 15.5-dpc pituitary glands showing clusters of higher expression in the double-heterozygous pituitary (arrowheads, *Right*); these clusters are absent in the control (*Left*). (*F*) *Hesx1$^{Cre/+}$;Ctnnb1$^{+/lox(ex3)}$; R26$^{YFP/+}$* triple-heterozygous pituitary at 15.5 dpc showing widespread expression of YFP throughout most of the pituitary but focal accumulation of intracellular β-catenin only in cell clusters (arrowheads). A magnified view of the boxed region in the Merge image is shown on the right. (Scale bars: 100 μm.)
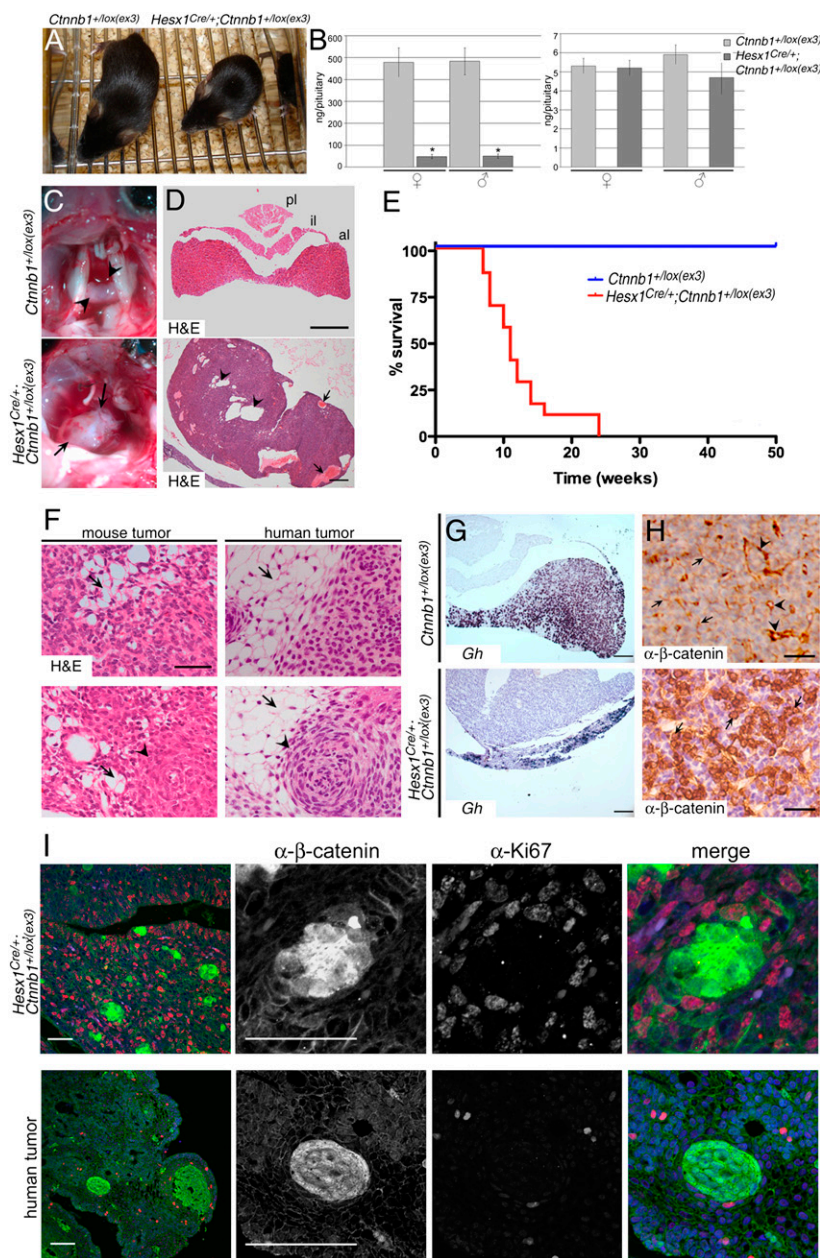
Fig. 2. $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ mice develop hypopituitarism and pituitary tumors that resemble human ACP. (*A*) A 5-wk-old heterozygous mutant exhibits marked dwarfism relative to a control same-sex littermate. (*B*) Growth hormone (GH) content in the pituitary gland is reduced significantly in $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ heterozygous mutants compared with $Ctnnb1^{+/lox(ex3)}$ control littermates (*Left*), but ACTH levels are comparable between genotypes (*Right*). Data shown are mean ± SEM of five pituitaries. ($P < 0.001$, t test). (*C*) The pituitary gland (arrowheads, *Upper*) is not distinguishable in a $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ double heterozygote, and there is a large tumor in this location (arrows, *Lower*). (*D*) Control pituitary (*Upper*) and a pituitary tumor (*Lower*) stained with H&E showing the presence of large cysts (arrowheads) and blood (arrows). al, anterior lobe; il, intermediate lobe; pl, posterior lobe. (*E*) Log-rank (Mantel–Cox) survival test of $Ctnnb1^{+/lox(ex3)}$ control and $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ heterozygous mutants that survived weaning ($n = 16$ animals per group). Median survival time is 11 wk for double-heterozygous mice ($P < 0.0001$). (*F*) H&E staining of histological sections of human ACP (*Right*) and murine tumor (*Left*) showing the presence of microcystic change (stellate reticulum; arrows) and nodular structures (cell clusters; arrowheads) in both. (*G*) In situ hybridization for *Gh* reveals no expression within the $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ tumor, but *Gh*-expressing cells are detected in what remains of normal pituitary tissue at the periphery of the tumor. (*H*) β-Catenin is detected in the cytoplasmic membrane of control pituitaries (arrows; arrowheads indicate nonspecific signal in the capillaries; *Upper*), but it is intracellular in many cells of the $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ tumor (arrows; *Lower*). (*I*) β-Catenin and Ki67 immunofluorescence detection in human ACP (*Lower*) and 18.5-dpc double-heterozygous pituitary (*Upper*) showing accumulation of β-catenin in cell clusters, which are Ki67-negative. (Scale bars: 100 μm in *D* and *G*; 500 μm in *F* and *H*; 50 μm in *I*.)

semithin histological sections revealed the presence of whorl-like clusters of epithelial cells and regions of microcystic change to some extent reminiscent of the stellate reticulum seen in human ACP (Fig. 2*F*). As in human ACP, the mouse tumors did not express pituitary hormones (Fig. 2*G*), and the synaptic vesicle glycoprotein synaptophysin, which is a diagnostic marker commonly expressed in neuroepithelial tumors and all pituitary adenomas, was not detected (Fig. S4*A*).

The pattern of β-catenin localization has been proposed as a diagnostic marker that distinguishes human ACP from all other pituitary tumors including the adult (papillary) form of craniopharyngioma (PCP) (30). β-catenin immunostaining of five ACP samples revealed nucleocytoplasmic accumulation in very few cells throughout the human ACP tumor, which predominantly localized in cell clusters (Fig. 2*I*). Expression of *Axin2* also has been found in the β-catenin–accumulating cell clusters (31). Indicative of activation of the Wnt pathway only in these cells, expression of *Axin2*, *Lef1* and Cyclin D1 was elevated in cell clusters (Fig. S5*B*). Despite the activation of Wnt signaling, antigen identified by monoclonal antibody Ki67 expression was not

detected in cells within the clusters in either human ACP or mouse tumors (Fig. 2*I*). Although lacking some of the differentiated features of the human counterpart (e.g., presence of wet keratin or hypothalamic infiltration), the murine tumors in the $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ mice most closely resemble human ACP and are dissimilar to other pituitary tumors, including pituitary adenomas, Rathke's cleft cysts, xanthogranulomas, posterior pituitary tumors (e.g., pituitocytomas), or even PCP.

Phenotypic Analysis of Pretumoral $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ Pituitaries Suggests That ACP Derives from Undifferentiated Progenitor Cells. Phenotypic analyses of $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ mutant pituitaries (Fig. 3*A*) showed that, in keeping with the Ki67 staining, BrdU incorporation after a 2-h pulse was detected mostly in cells outside the clusters, suggesting that cluster cells were in either a quiescent or a slow-dividing state. Numbers of growth hormone (GH)-positive cells (somatotrophs) were severely reduced, and adrenocorticotropic hormone (ACTH)-expressing cells (corticotrophs and melanotrophs) were not affected in the mutant pituitaries. Cluster cells did not express these hormones, indicating
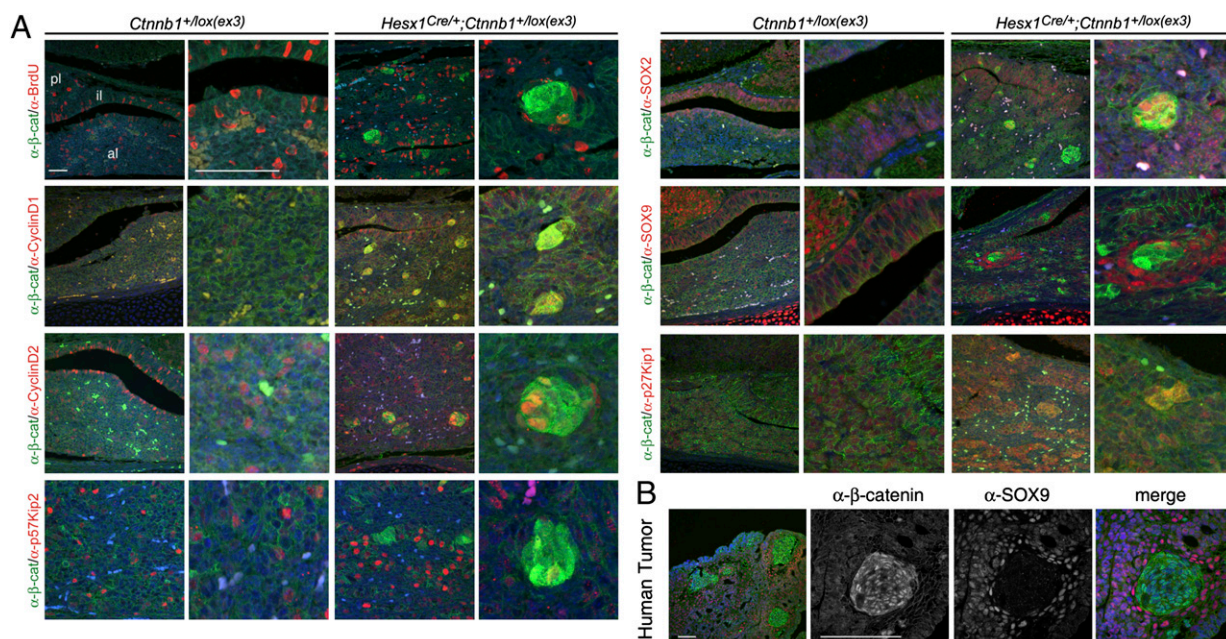
Fig. 3. β-Catenin–accumulating cells show phenotypic features of pituitary progenitor/stem cells. (*A*) Double immunostaining for β-catenin (green) and specific markers (red) in *Ctnnb1*$^{+/lox(ex3)}$ controls and *Hesx1*$^{Cre/+}$;*Ctnnb1*$^{+/lox(ex3)}$ double-heterozygous pituitaries at 18.5 dpc. BrdU incorporation short pulse reveals abundant cells in S-phase of the cell cycle throughout the gland but very few BrdU-positive cells in the clusters. Cyclin D1 and Cyclin D2, markers of undifferentiated pituitary progenitors, are expressed within the cell clusters. Overall expression of p57^{kip2}, which is transiently expressed in cells that are between the proliferation and differentiation states, is increased in the *Hesx1*$^{Cre/+}$;*Ctnnb1*$^{+/lox(ex3)}$ anterior pituitary but is not expressed in β-catenin–accumulating cells. SOX2-expressing cells concentrate around the lumen of the gland, which contains pituitary progenitor/stem cells, and this region is thickened in the *Hesx1*$^{Cre/+}$;*Ctnnb1*$^{+/lox(ex3)}$ pituitary. Note that expression of SOX2 is detected in some but not all β-catenin–accumulating cell clusters. Expression of SOX9 is rare within the cell clusters, but there are abundant SOX9-positive cells surrounding the β-catenin–accumulating cell clusters and around the lumen. Expression of the stem cell marker p27^{kip1} is up-regulated in the clusters. (*B*) In human ACP, SOX9 immunofluorescence is widespread throughout the tumor, but β-catenin–accumulating clusters are negative. al, anterior lobe; il, intermediate lobe; pl, posterior lobe. (Scale bars: 50 μm.)

that they were not terminally differentiated (Fig. S6 *D* and *E*). Numbers of cyclin-dependent kinase inhibitor (p57^{kip2})-positive cells, which correspond to a population of noncycling precursors confined between proliferating and differentiated states during normal pituitary development (32), were clearly elevated in the mutant pituitaries, but β-catenin–accumulating cell clusters were mainly negative. A similar result was observed for Cyclin E expression (Fig. S6*A*). The layer of sex-determining region Y (SRY) box-containing gene 2 (SOX2)-expressing cells around the lumen, which is thought to contain pituitary progenitor/stem cells (14), appeared thickened in the pituitary of double heterozygotes, and the total number of these cells was elevated (Fig. S6*G*). SOX2-positive cells often were identified in β-catenin–accumulating clusters, but not all clusters contained SOX2-expressing cells (14). Expression of SRY-box containing gene 9 (SOX9), a marker of transit-amplifying progenitors (14), was rarely observed in the clusters, and its expression appeared up-regulated in cells around the β-catenin–accumulating clusters. Cyclin D1 and Cyclin D2, which normally are expressed in undifferentiated Rathke's pouch progenitors (32), were expressed in the clusters, and cyclin-dependent kinase inhibitor 1B (p27^{Kip1}), a marker of intestinal stem cells with oncogenic potential (33), appeared elevated in the cell clusters relative to the nearby periluminal cells (Fig. S6 *B* and *C*).

Next, we explored whether the expression of some of these progenitor markers may be conserved in human ACP (Fig. 3*B*). Abundant SOX9 immunoreactivity was widely identified within the human tumor, often surrounding the β-catenin–accumulating foci, but SOX9-expressing cells were not seen to colocalize within the cell clusters. Cyclin D1 expression was detected mostly in the cell clusters and in some positive cells scattered through the whole of the ACP section (Fig. S5*A*). As observed in a proportion of clusters in the mouse pituitaries, we did not detect SOX2 expression in the human cell clusters using three different antibodies. Together, these data reinforce the *Hesx1*$^{Cre/+}$;*Ctnnb1*$^{+/lox(ex3)}$

mouse line as a valid model to study the pathogenesis of ACP and suggest that in both humans and mice mutations in *Ctnnb1* may primarily affect a small number of cells with phenotypic features of pituitary progenitor/stem cells.

***Hesx1*$^{Cre/+}$;*Ctnnb1*$^{+/lox(ex3)}$ Pituitaries Contain Higher Numbers of Progenitor/Stem Cells with Increased Proliferation Rate Relative to Control Pituitaries.** We used an in vitro approach to explore whether the progenitor/stem cell pool may be altered in *Hesx1*$^{Cre/+}$; *Ctnnb1*$^{+/lox(ex3)}$ pituitaries relative to controls. Adherent colonies arising from single progenitor/stem cells can be cultured from embryonic, postnatal, and adult pituitaries, fit an undifferentiated profile, and are capable of self-renewal and differentiation (Fig. 4*B* and Fig. S7) (16, 17). First, we investigated the percentage of colony-forming cells, as indicative of the numbers of progenitor/stem cells, in double-heterozygous and control pituitaries. At late gestation and adulthood, control pituitaries contained very low proportions of progenitor/stem cells, which increased in the first 2–3 wk of life. Double-heterozygous pituitaries showed a similar trend, but proportions were approximately threefold higher at all stages analyzed relative to controls (Fig. 4*A*). Next, we assessed the proliferation potential of these cells isolated from *Hesx1*$^{Cre/+}$; *Ctnnb1*$^{+/lox(ex3)}$ and control pituitaries. Time-lapse microscopy revealed that the proliferation rate of *Hesx1*$^{Cre/+}$;*Ctnnb1*$^{+/lox(ex3)}$ progenitors was elevated 1.7-fold compared with progenitors isolated from control pituitaries ($P = 0.0001$) (Fig. 4 *C–E*). Taken together, our data indicate that mutant β-catenin leads both to an enlargement of the progenitor/stem cell pool and to an increase in their proliferation rate.

Genetic Expression of Mutant β-Catenin in Committed or Differentiated Pituitary Cells Does Not Result in Tumor Formation. We sought to explore whether expression of mutant β-catenin in com-
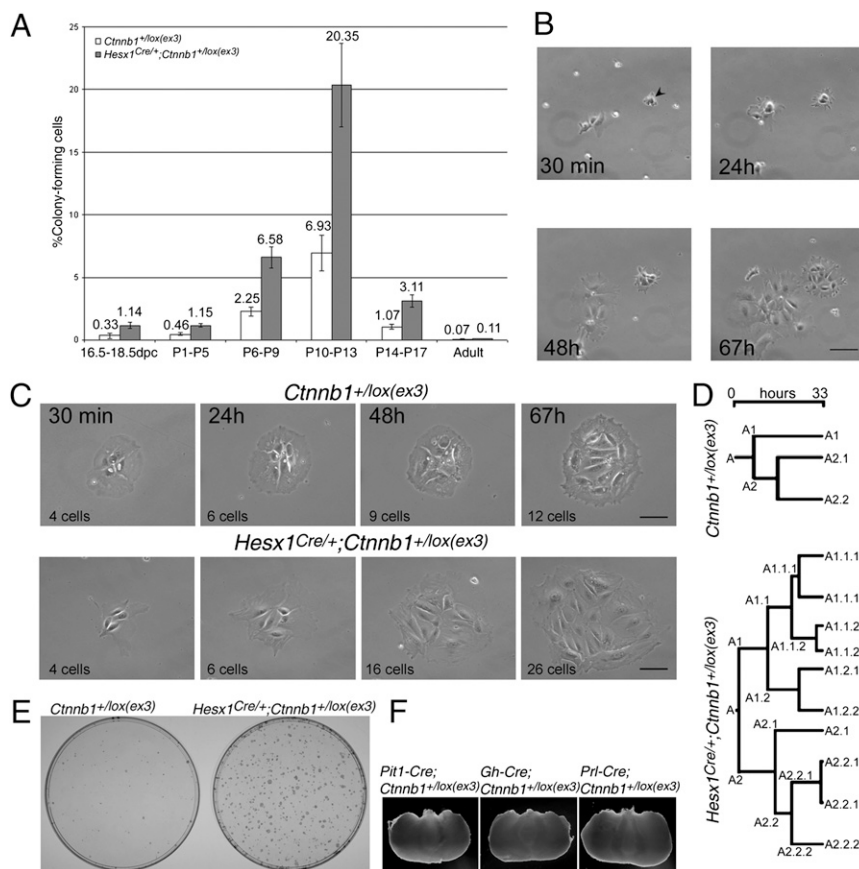
Fig. 4. Increased numbers of pituitary progenitor/stem cells and an elevated proliferation rate underlie the formation of the murine tumors. (*A*) Total numbers of progenitor/stem cells (colony-forming cells) are significantly elevated in the $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ pituitaries relative to controls from late gestation to adulthood (n = 5–8 pituitaries per stage). (*B*) Time-lapse microscopy frames showing the formation of a colony from a single progenitor/stem cell (arrowhead, *Upper left*). (*C*) Time-lapse microscopy showing that the proliferation rate of pituitary progenitor/stem cells is increased in $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ mutant (*Lower row*) relative to $Ctnnb1^{+/lox(ex3)}$ control pituitaries (*Upper row*). (*D*) Representative examples of lineage trees indicating faster division times in the double heterozygotes. (*E*) Photographs of tissue culture plates containing fixed and hematoxylin-stained colonies demonstrate the presence of higher numbers and overall larger colonies in the $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ pituitaries (*Right*) relative to $Ctnnb1^{+/lox(ex3)}$ controls (*Left*). (*F*) Expression of mutant β-catenin in committed progenitors (*Pit1-Cre; Ctnnb1^{+/lox(ex3)}*) (*Left*) or terminally differentiated hormone-producing cells (*Gh-Cre;Ctnnb1^{+/lox(ex3)}* (*Center*) and *Prl-Cre;Ctnnb1^{+/lox(ex3)}*) (*Right*) is not tumorigenic, and pituitaries from adult mice are normal. (Scale bars: 100 μm.)

mitted or differentiated cells also may lead to tumor formation. These cells continue dividing during early postnatal life, and therefore they may be expected to respond to the proliferative effect of the mutant β-catenin. Transgenic mice expressing Cre recombinase under the control of the *Pit1*, *Gh*, and Prolactin (*Prl*) promoters were crossed with the $Ctnnb1^{lox(ex3)/lox(ex3)}$ mice to drive expression of mutant β-catenin in *Pit1*-committed progenitors and differentiated hormone-producing cells (Fig. S8) (34). $Gh\text{-}Cre;Ctnnb1^{+/lox(ex3)}$, $Prl\text{-}Cre;Ctnnb1^{+/lox(ex3)}$, and $Pit1\text{-}Cre;\ Ctnnb1^{+/lox(ex3)}$ mice had normal pituitaries and did not develop tumors (n = 57, 42, and 39, respectively) (Fig. 4*F*). These analyses indicate that tumor formation requires the expression of mutant β-catenin in Rathke's pouch progenitors.

Discussion

In this study, we show that continuous activation of the Wnt pathway in the murine pituitary leads to the development of pituitary tumors that resemble the pediatric form of human craniopharyngioma, ACP. Our research suggests that mutations in human *CTNNB1* exon 3 that have been identified in patients with ACP are likely to play an essential role in the etiology of these tumors rather than representing a second hit targeted during tumor progression. In the mouse, tumorigenesis occurs only when mutant β-catenin is expressed in embryonic pituitary progenitors but not when expression is targeted to committed or differentiated pituitary cells, even though the latter cell types also are mitotically active. Using an in vitro culture approach, we demonstrate that Wnt signaling plays an essential role in the specification of the progenitor/stem cell pool in the pituitary gland and that its overactivation leads to an elevation in total numbers and proliferation rate of these cells. In other progenitor/stem cell-rich tissues (i.e., intestinal epithelium, bone marrow, hair follicle), both factors can contribute to the tumorigenic effect of β-catenin (1, 4, 8, 35). Our research argues

that aberrant expression of mutant β-catenin in pituitary progenitors underlies formation of ACP, a human tumor of hitherto unknown molecular or cellular etiology.

This conclusion is supported further by the finding that the murine tumors and human ACP contain cells with phenotypic characteristics of progenitor/stem cells: They are slow-dividing, undifferentiated, and express markers such as SOX2, SOX9, Cyclin D1, Cyclin D2, and p27^{Kip1}, which have been associated with cell stemness in the pituitary gland and other tissues. Recently, the existence of a small population of SOX2$^+$;SOX9$^-$ cells has been suggested to correspond to progenitor/stem cells located around the lumen of the postnatal pituitary (14). Likewise, β-catenin–accumulating cell clusters in the $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ pretumoral pituitaries contain mainly SOX2$^+$;SOX9$^-$ cells and usually are associated with the lumen. In the human ACP samples, the expression of SOX2 in the clusters appears not to be conserved. It presently is unknown whether another member of the SOX family is expressed in the human clusters, but advanced tumors in $Hesx1^{Cre/+};Ctnnb1^{+/lox(ex3)}$ mice also are negative for SOX2 staining, possibly indicating a transient function of SOX2 only at the initial phases of ACP formation. However, our results suggest a more permanent role of SOX9 in tumorigenesis, because it is highly expressed throughout the human ACP tumors and in the pretumoral murine pituitaries and often is intimately associated with the cluster cells. Recent evidence has identified expression of SOX9 in a progenitor cell population that contributes to the self-renewal and repair of the adult intestine, liver, and exocrine pancreas (36). The precise cellular and molecular interaction between the β-catenin–accumulating clusters and SOX9-expressing cells remains an issue for future research; nevertheless our data argue strongly for a critical involvement of pituitary progenitor/stem cells in the genesis of human ACP.

Our genetic data also have shed light on the role of β-catenin during normal pituitary development. Previously, it was shown that lack of β-catenin has no effect during the early stages

of pituitary development, and defects are first observable at 15.5 dpc, with a reduction of *Pit1* expression leading to perturbed differentiation of this cell lineage. Gain-of-function experiments did not support a role of β-catenin in promoting cell-cycle progression, because Paired-like homeodomain transcription factor 1 [*Pitx1-Cre;Ctnnb1*$^{+/lox(ex3)}$] mutants exhibit severe and early developmental arrest and absence of pituitary tissue by 13.5 dpc (37). Our study not only suggests an essential role of β-catenin for normal differentiation of the *Pit1* lineage but also reveals a critical role in the control of cell proliferation of embryonic and postnatal pituitary progenitors.

Our data support the concept that human ACP arises from pituitary progenitors, and this concept has clinical implications. ACP often behaves aggressively with invasion of the hypothalamus and visual pathways and/or intracranial metastases. Therefore, total resection of the tumor is not always possible without damage to vital surrounding structures such as the hypothalamus and optic chiasm. In these children, radical excision is associated with unacceptable morbidity and mortality, but subtotal resection (without further adjuvant radiotherapy) predisposes them to a high (>60%) 3-y recurrence risk and further hypothalamic and visual compromise (23, 24). Although a conservative surgical approach leaving residual tumor with adjuvant radiotherapy has been adopted recently, tumor recurrence is still high. The presence of tumorigenic progenitors in human ACP may explain the significant propensity of human ACP to recur and emphasizes the need to identify novel therapies. In summary, using genetic approaches in mice, we have revealed a critical function of the Wnt pathway in the control of the progenitor/stem cell pool in the pituitary and, in particular, of mutant β-catenin in the formation of mouse and human ACP. A better understanding of the molecular and cellular mechanisms underlying ACP will lead to more refined, efficient, and safer treatments.

Materials and Methods

Mice. *Ctnnb1*$^{+/lox(ex3)}$, *Hesx1-Cre*, *Prl-Cre*, *R26-YFP*, and *BAT-gal* mice have been described previously (25, 26, 29, 34, 38). The *Pit1-Cre* and *Gh-Cre* transgenic lines were generated by M. Treier [European Molecular Biology Laboratory (Heidelberg)].

H&E and X-Gal Staining, Section in Situ Hybridization, Cell Proliferation Analysis, and Immunostaining. H&E and X-Gal staining, immunostaining, and in situ hybridization were performed as previously described (25, 39). Samples of human ACP were obtained from the Department of Histopathology at Great Ormond Street Hospital for Children (London).

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