

Sox2⁺ Stem/Progenitor Cells in the Adult Mouse Pituitary Support Organ Homeostasis and Have Tumor-Inducing Potential

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<http://dx.doi.org/10.1016/j.stem.2013.07.004>

SUMMARY

Sox2⁺ adult mouse pituitary cells can self-renew and terminally differentiate *in vitro*, but their physiological role *in vivo* and possible contribution to oncogenesis remain largely unknown. Using genetic lineage tracing, we show here that the Sox2⁺ cell compartment of both the embryonic and adult pituitary contains stem/progenitor cells that are able to differentiate into all hormone-producing lineages and contribute to organ homeostasis during postnatal life. In addition, we show that targeted expression of oncogenic β -catenin in Sox2⁺ cells gives rise to pituitary tumors, but, unexpectedly, the tumor mass is not derived from the Sox2⁺ mutation-sustaining cells, suggesting a paracrine role of Sox2⁺ cells in pituitary oncogenesis. Our data therefore provide *in vivo* evidence of a role for Sox2⁺ stem/progenitor cells in long-term physiological maintenance of the adult pituitary, and highlight an unexpected non-cell-autonomous role for these cells in the induction of pituitary tumors.

INTRODUCTION

Somatic stem cells have been identified in numerous organs, where they play a critical role in normal tissue homeostasis and regenerative capacity after insult (Barker et al., 2012; Oshimori and Fuchs, 2012; Reya and Clevers, 2005; Rosen and Jordan, 2009; van Amerongen et al., 2012), and are often involved in tumorigenesis (Nguyen et al., 2012; Visvader and Lindeman, 2012). Somatic stem cells specifically targeted with oncogenic genetic mutations were shown to generate tumors in a cell-

autonomous manner in several mouse models (Barker et al., 2009; Zhu et al., 2009). This finding has promoted the cancer stem cell paradigm, which holds that tumors contain cells that are able to self-renew and differentiate into other cells that populate the tumor mass (Chen et al., 2012; Driessens et al., 2012; Schepers et al., 2012). Whether somatic stem cells can contribute to oncogenesis in a different manner is not known.

The anterior pituitary (AP) serves as a major regulator of mammalian physiology by secreting endocrine factors that control many physiological processes, including growth, reproduction, metabolism, and stress. Comprising the anterior and intermediate lobes (AL and IL, respectively), the AP has a developmental origin in Rathke's pouch (RP), a region of the oral epithelium that is specified at ~9.0 days postcoitum (dpc) in the mouse embryo (Kelberman et al., 2009; Mollard et al., 2012). Proliferation of RP embryonic precursors generates a pool of undifferentiated progenitors, which upon exiting the cell cycle initiate commitment into specific cell lineages (e.g., Pit1 and Tpit, also known as Pou1f1 and Tbx19, respectively) and eventually terminally differentiate into the major AP cell types by late gestation (Bilodeau et al., 2009; Davis et al., 2011). These cell types include growth hormone (GH)-secreting somatotrophs; prolactin (PRL)-secreting lactotrophs; thyroid stimulating hormone (TSH)-secreting thyrotrophs; adrenocorticotrophic hormone (ACTH)-secreting corticotrophs; gonadotrophs, which secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH); and melanotrophs, which secrete melanocyte-stimulating hormone (MSH). Although all of the differentiated hormone-secreting cells are present before birth, the organ continues to develop postnatally, with a dramatic expansion of different cell populations at variable rates (Carbajo-Pérez and Watanabe, 1990; Taniguchi et al., 2002). Currently, it is not known whether pituitary stem cells contribute to normal organ homeostasis in postnatal life.

The transcription factor Sox2 is expressed in the AP anlage and its essential role in normal pituitary development has been

demonstrated by tissue-specific conditional deletion (Fauquier et al., 2008; Jayakody et al., 2012). Sox2 is required for the maintenance of stem cell populations in a range of tissues and is expressed in a population of cells in the adult pituitary gland that have several characteristics of stem cells (Arnold et al., 2011; Castinetti et al., 2011; Fauquier et al., 2008; Pevny and Rao, 2003). This putative stem cell population, which can self-renew and differentiate into all hormone-producing cell types in vitro, is only a subset of all pituitary Sox2⁺ cells (Andoniadou et al., 2012; Gaston-Massuet et al., 2011). Quantitative studies have shown that these cells are most abundant in the early postnatal pituitary and regress as the major postnatal expansion of the gland occurs, but a Sox2⁺ cell population with in vitro stem cell characteristics remains in the adult mouse (Gaston-Massuet et al., 2011; Gremeaux et al., 2012). Recent studies of cell ablation of differentiated cell types have suggested a potential role for these adult Sox2⁺ cells in tissue regeneration, but in the absence of lineage tracing tools, their contribution could not be defined (Fu et al., 2012; Fu and Vankelecom, 2012).

A further characteristic of the AP gland is its propensity to form benign but often locally infiltrative tumors, with evidence for pituitary adenomas in 6%–24% of human adults (Gueorguiev and Grossman, 2011; Melmed, 2011). Functional adenomas are characterized by the expansion of one or more of the hormone-producing cell types, in contrast to null-cell adenomas, which do not express any hormone. Studies of human biopsies have demonstrated the presence of stem-like cells in pituitary tumors, as well as in mice (Florio, 2011; Gleiberman et al., 2008). Recently, expression of a degradation-resistant mutant form of β -catenin (encoded by *Ctnnb1*) that leads to overactivation of the WNT pathway in embryonic pituitary precursors of *Hesx1*^{Cre/+}; *Ctnnb1*^{lox(ex3)/+} mice was shown to cause the formation of tumors resembling human adamantinomatous craniopharyngioma (ACP) (Gaston-Massuet et al., 2011). These pediatric tumors are associated with somatic overactivating mutations in *CTNNB1* and despite being histologically benign, ACPs are locally invasive, resulting in significant morbidity and mortality (Buslei et al., 2005; Müller, 2010). An embryonic expansion of the Sox2⁺ cell compartment was observed in this ACP mouse model, but whether these putative stem cells play a role in pituitary oncogenesis remains to be established. In this study, we demonstrate in vivo that Sox2⁺ cells can generate hormone-producing cells during embryonic development as well as in adulthood, and we reveal a mechanism whereby Sox2⁺ cells are able to act in a non-cell-autonomous manner to induce oncogenesis.

RESULTS

Sox2⁺ Embryonic Precursors Generate Hormone-Producing Cells during Development

Sox2 is expressed in proliferative RP progenitors during development and in regions of the postnatal pituitary that are thought to contain adult stem cells. To investigate the involvement of these cells during embryonic development and in normal adult pituitary cell turnover, we generated a mouse line (*Sox2*^{CreERT2}) by inserting a tamoxifen-inducible Cre allele (*CreERT2*) into the *Sox2* locus by homologous recombination in embryonic stem cells (ESCs) generating a null allele (Figures

1A and 1B). As expected, specific immunostaining revealed Cre expression in Sox2^{CreERT2/+} ESCs, but nuclear accumulation of Cre was observed only when tamoxifen was added to the medium (Figure S1A available online). *Sox2*-*CreERT2* mice were crossed with the *ROSA26-flox-stop-YFP* mouse reporter (Srinivas et al., 2001) to generate the *Sox2*^{CreERT2/+}; *R26*^{YFP/+} mice and embryos used in this study. In these animals, tamoxifen administration is expected to transiently activate Cre in a proportion of Sox2⁺ cells, resulting in the permanent expression of YFP in those cells and their descendants.

First, we sought to investigate the developmental potential of Sox2⁺ embryonic precursors. Pregnant females from intercrosses between *R26*^{YFP/YFP} and *Sox2*^{CreERT2/+} strains were injected with tamoxifen at 11.5 dpc and embryos were analyzed at postnatal day 1 (P1). Because tamoxifen administration has detrimental effects during gestation that can lead to premature delivery and/or death, we performed these experiments after administering a single low dose of tamoxifen (1.5 mg). YFP⁺ cells were observed in tissues that normally express Sox2 embryonically, such as the developing brain, eyes, ears, and oral and olfactory epithelia in *Sox2*^{CreERT2/+}; *R26*^{YFP/+} mice, demonstrating the utility of the genetic approach (Figure S1B). Within the pituitary gland, YFP⁺ cells were detected by immunofluorescence using specific antibodies against GFP in the IL and AL, including the dorsal region of the AL lining the cleft (the so-called marginal zone [MZ]; Figure 1C). Double immunofluorescence revealed the coexpression of yellow fluorescent protein (YFP) with markers of cell-lineage commitment (PIT1, TPIT, and SF1) and terminal differentiation (GH, PRL, TSH, ACTH, α GSU, and LH; Figures 1D and 1E). In addition, YFP⁺; SOX2⁺ cells were abundantly detected throughout the postnatal pituitary (Figure 1E). These cell-lineage tracing experiments demonstrate that Sox2⁺ embryonic progenitors generate all of the major hormone-producing cells of the AP and that a proportion remain undifferentiated as the Sox2⁺ population of the adult pituitary.

SOX2⁺ Cells, Including S100B⁺ Folliculostellate Cells, Are Efficiently Targeted in Sox2^{CreERT2/+}; *R26*^{YFP/+} Mice

The Sox2⁺ cell compartment in the adult and embryonic pituitary has been extensively explored in various studies (Andoniadou et al., 2012; Fauquier et al., 2008; Gremeaux et al., 2012). These studies demonstrated that the vast majority of the Sox2⁺ cells are undifferentiated and do not express markers of cell-lineage commitment or differentiation. We reexamined this notion using the *Sox2*-eGFP mouse line, in which Sox2⁺ cells are marked by enhanced GFP (eGFP) expression (Ellis et al., 2004). Double immunofluorescence showed that essentially all GFP⁺ cells contained for nuclear SOX2 and none of the GFP⁺ cells were positive for any marker of cell-lineage commitment or terminal differentiation (Figures S2A–S2C). It was previously shown that there is a significant degree of overlap between SOX2 and S100B expression in cells from the adult mouse (Fauquier et al., 2008) and rat pituitary (Yoshida et al., 2011). S100B labels folliculostellate (FS) cells in the AP, a heterogeneous cell population of non-hormone-producing cells that are involved in regulating AP function by secreting diverse signaling molecules and have been proposed to contain undifferentiated progenitors (Allaerts et al., 1990; Devnath and Inoue, 2008). Expression analyses of the *S100B*-eGFP transgenic line, which expresses eGFP in S100B⁺ cells (Vives

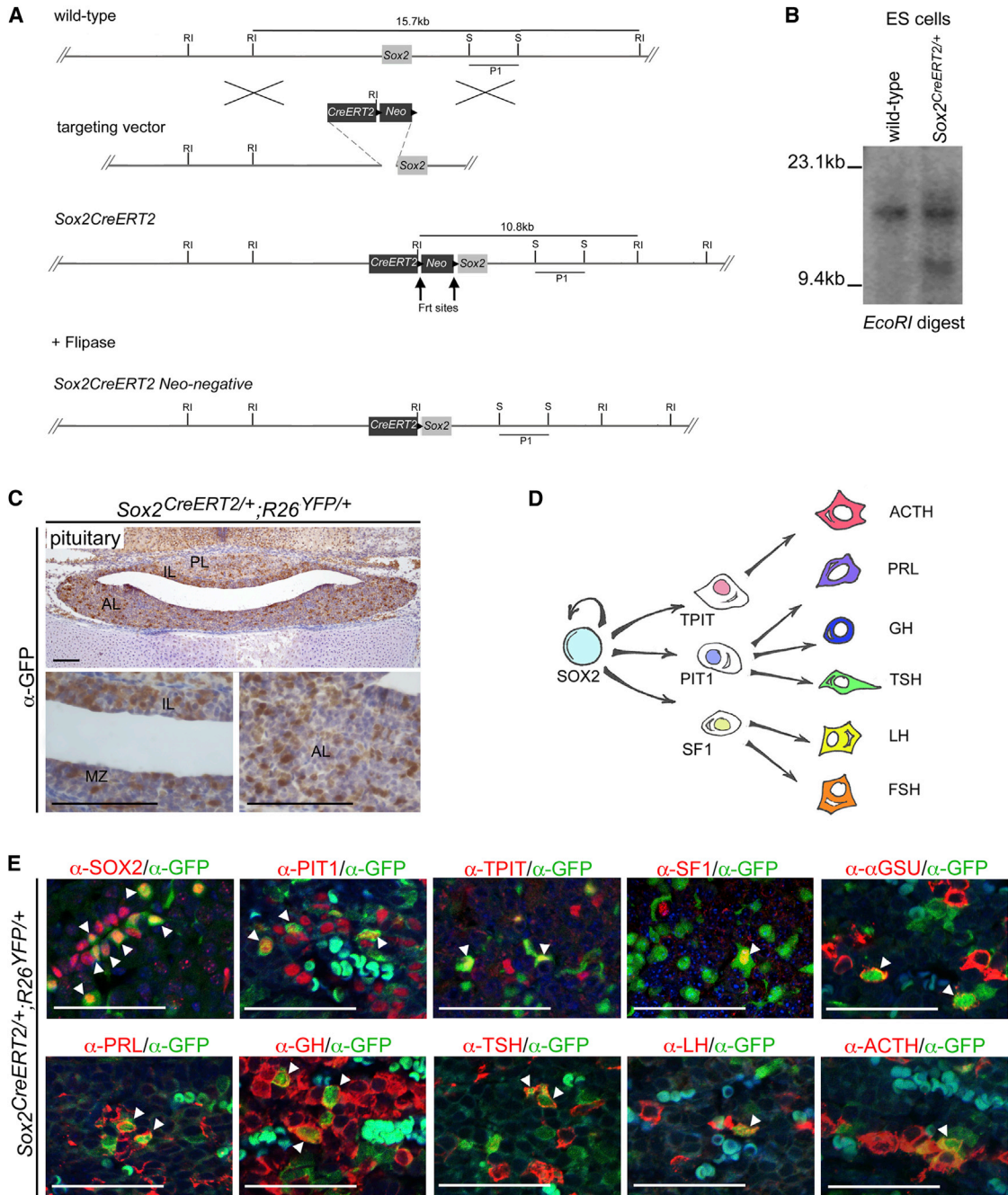


Figure 1. Sox2⁺ Embryonic Pituitary Cells Generate All Types of Hormone-Producing Cells and Sox2⁺ Cells in the Postnatal Pituitary

(A) Top to bottom: structure of the murine wild-type Sox2 locus, Sox2-CreERT2 targeting vector, and the Sox2-CreERT2 allele prior to and after flippase excision of the Neo cassette. RI, EcoRI; S, Sall.

(B) Southern blot hybridization of DNA samples from wild-type and heterozygous Sox2^{CreERT2/+} ESC clones with an external probe (P1 in A).

(C) Embryonic induction at 11.5 dpc and analysis at P1 in Sox2^{CreERT2/+};R26^{YFP/+} mice. α -GFP immunostaining on wax sections reveals YFP⁺ cells in the AL, IL, and PL of the pituitary, as well as in the region of the AL lining the cleft (the MZ).

(D) Model of lineage commitment and terminal differentiation of pituitary cell types derived from SOX2-expressing progenitor/stem cells.

(E) Double immunofluorescence reveals costaining of YFP (detected by α -GFP) and SOX2, as well as with markers of cell-lineage commitment (TPIT, PIT1, and SF1), and terminal differentiation of hormone-producing cells (α GSU, PRL, GH, TSH, LH, and ACTH).

Scale bars, 100 μ m (C) and 50 μ m (E). See also Figure S1.

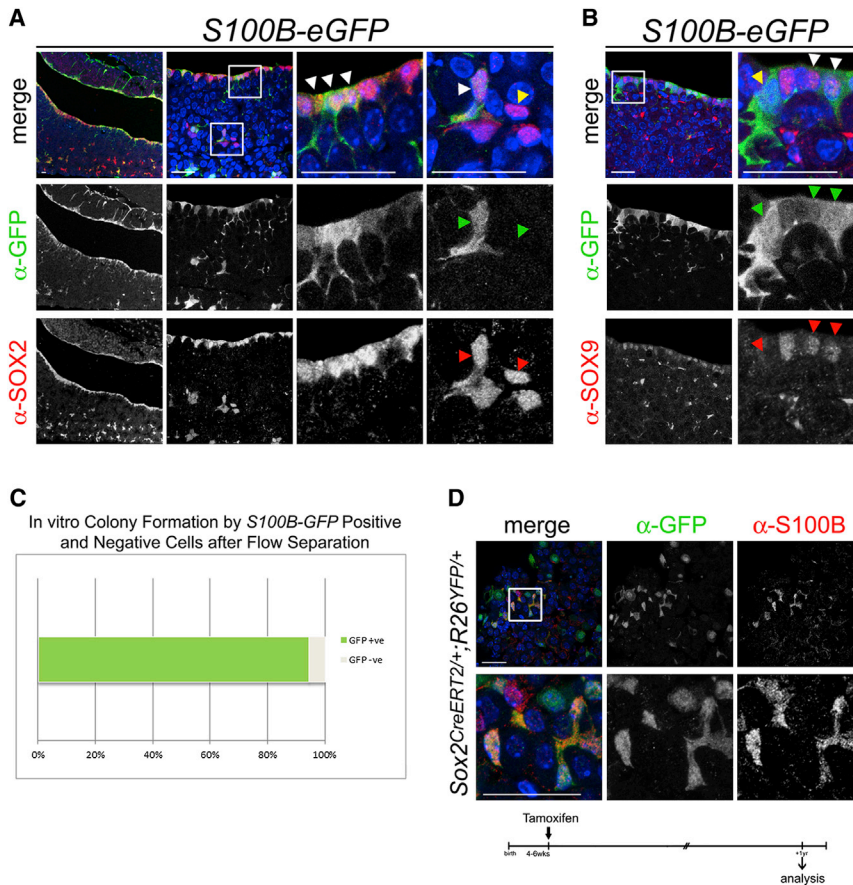


Figure 2. Sox2⁺ Cells in the Adult Pituitary Contain S100B⁺ Undifferentiated Progenitors and Do Not Express Markers of Cell-Lineage Commitment or Terminal Differentiation

(A and B) S100B⁺ FS cells are contained within SOX2⁺-expressing cells (A, arrowheads) and most are SOX9⁺ (B, white arrowheads), although a few GFP⁺ cells are not SOX9 immunoreactive (yellow arrowhead in merge).

(C) *S100B-eGFP* mouse pituitaries were dissociated into a single-cell suspension, separated into GFP⁺ and GFP⁻ populations by flow sorting and cultured in stem-cell-promoting media. The colony-forming potential of each fraction was assessed after 7 days in culture and the data are presented graphically as the percentage of colonies formed from the total. Note that the vast majority of the colonies are formed in the GFP⁺ (S100B⁺) fraction.

(D) Pituitaries from *Sox2^{CreERT2/+};R26^{YFP/+}* mice induced with tamoxifen at 4–6 weeks and traced for 1 year contain YFP⁺ cells (detected using α-GFP antibody) that colabel with S100B. Scale bars, 25 μm. See also Figure S2.

in *Sox2^{CreERT2/+};R26^{YFP/+}* mice induced at 7 months of age (data not shown). To investigate whether these populations arise through rapid differentiation of Sox2⁺ cells or coexpress SOX2, we carried out triple immunostaining against SOX2, YFP, and either hormones or PIT1. No cells were observed to be double labeled with SOX2 and commitment/differentiation markers, confirming that the observation of targeted committed/differentiated cells after 30 hr is due to rapid commitment of a proportion of targeted Sox2⁺ progenitors (Figure 3). Together, these data demonstrate that tamoxifen induction results in the activation of CreERT2 and expression of YFP in Sox2⁺ cells, including S100B⁺ FS cells, which do not express hormones. However, Sox2⁺ cells can rapidly initiate differentiation by activating cell-lineage commitment markers such as PIT1, which precede terminal differentiation of hormone-producing cells.

et al., 2003), revealed that up to 57% of Sox2⁺ cells costained for GFP, and essentially all S100B⁺ cells were SOX2⁺ and SOX9⁺ (Figures 2A and 2B). Moreover, isolation of S100B⁺ (eGFP⁺) cells by flow sorting and culture in stem-cell-promoting media demonstrated that S100B⁺ cells have clonogenic potential in vitro (Figure 2C). Together with previous findings, these results indicate that Sox2⁺ cells are undifferentiated, do not express any cell-lineage commitment or differentiation marker, and include S100B⁺ FS cells.

Next, we used the *Sox2^{CreERT2/+};R26^{YFP/+}* mice to trace the fate of Sox2⁺ cells in the adult pituitary. Initially, 4- to 6-week-old *Sox2^{CreERT2/+};R26^{YFP/+}* were injected intraperitoneally once with a single high dose of tamoxifen (0.3 mg/g) and the pituitary was analyzed 24 hr postinduction. Double immunostaining against Cre and SOX2 revealed a widespread colocalization of the two proteins, demonstrating that CreERT2 expression was restricted to the Sox2⁺ cell compartment of the pituitary gland, even when induction was carried out with a high dose of tamoxifen (Figure S3A). YFP⁺ cells were abundantly detected from 30 hr postinduction, mostly coexpressing SOX2. In these pituitaries, colocalization of YFP and hormones was very rare, as assessed using a pan-hormonal cocktail of antibodies against GH, PRL, TSH, ACTH, αGSU, LH, and FSH (0.5%, n = 4 pituitaries). Costaining of PIT1, a cell-lineage marker that is required for differentiation of adult somatotrophs, lactotrophs, and thyrotrophs (Li et al., 1990), and YFP was observed occasionally (~5% of YFP⁺ cells; Figure 3A). Similar results were obtained

in *Sox2^{CreERT2/+};R26^{YFP/+}* mice induced at 7 months of age (data not shown). To investigate whether these populations arise through rapid differentiation of Sox2⁺ cells or coexpress SOX2, we carried out triple immunostaining against SOX2, YFP, and either hormones or PIT1. No cells were observed to be double labeled with SOX2 and commitment/differentiation markers, confirming that the observation of targeted committed/differentiated cells after 30 hr is due to rapid commitment of a proportion of targeted Sox2⁺ progenitors (Figure 3). Together, these data demonstrate that tamoxifen induction results in the activation of CreERT2 and expression of YFP in Sox2⁺ cells, including S100B⁺ FS cells, which do not express hormones. However, Sox2⁺ cells can rapidly initiate differentiation by activating cell-lineage commitment markers such as PIT1, which precede terminal differentiation of hormone-producing cells.

We previously demonstrated that in vitro clonogenic potential is exclusively retained within the Sox2⁺ cell population of the AP; however, only ~2.4% of these cells are capable of self-renewal and clonal expansion when cultured in stem-cell-promoting media (Andoniadou et al., 2012). To further validate the efficiency of the genetic approach, the APs of *Sox2^{CreERT2/+};R26^{YFP/+}* tamoxifen-induced mice (n = 4) were dissected 48 hr postinduction and dissociated into single-cell suspensions. YFP⁺ cells were then isolated by flow sorting and cultured in stem-cell-promoting media at clonal density (Figure 4C). Most of the colony-forming cells (94.2%) were included in the YFP⁺, suggesting that the genetic approach and induction protocol labeled a significant proportion of the Sox2⁺ population with clonogenic potential. When these colonies were grown in differentiation conditions, in the absence of growth factors, they downregulated progenitor marker expression (*Sox2*, *Sox9*, and *Nestin*) and

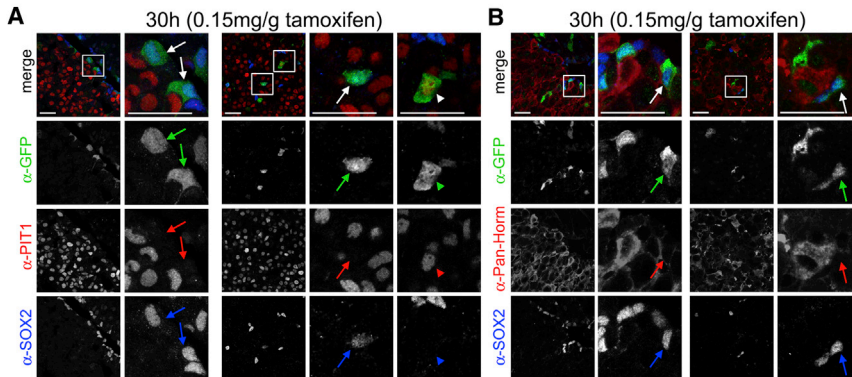
Sox2^{CreERT2/+};R26^{YFP/+}

Figure 3. The Sox2-CreERT2 Mouse Line Drives Recombination Exclusively in Sox2⁺ Cells

(A) Triple immunofluorescence against YFP (detected using α -GFP antibody), PIT1, and SOX2, confirming that the majority of the YFP⁺ cells are SOX2⁺ and PIT1⁻ (arrows). Sporadic YFP⁺;PIT1⁺ cells were identified (arrowhead).

(B) Triple immunostaining against YFP (α -GFP), a pan-hormone cocktail, and SOX2, showing that YFP⁺ cells in the AP do not express hormones 30 hr postinduction (arrows).

Scale bars, 25 μ m. See also Figure S3.

upregulated the transcription of typical markers of cell-lineage commitment and differentiation (*Pit1*, *Pomc1*, *Tsh*, and *Gh*; Figure S4A).

Sox2⁺ Cells Contribute to Pituitary Homeostasis during Adult Life

A recent genetic tracing study using an independent Sox2-CreERT2 mouse model demonstrated that Sox2⁺ cells represent stem cells in many adult tissues (Arnold et al., 2011); however, that study did not include the pituitary gland. To assess whether Sox2⁺ cells in the adult pituitary are able to generate hormone-producing cells and contribute to physiological cell turnover, we performed a series of experiments using Sox2^{CreERT2/+};R26^{YFP/+} mice that were induced at different ages and traced for variable time periods up to a year. In the first instance, we induced Sox2^{CreERT2/+};R26^{YFP/+} animals at 4–6 weeks of age (young adults) with a single dose of 0.15 mg tamoxifen per gram of body weight and analyzed the pituitaries after 48 hr and for up to 1 year. We found that at 48 hr, the majority of YFP⁺ cells coexpressed SOX2 (92%, 94%, and 96%; n = 3 pituitaries). After 9 months, we found abundant coexpression of YFP with markers of terminal differentiation, including GH, PRL, TSH, ACTH, α GSU, FSH, and LH, demonstrating that derivatives of the tamoxifen-targeted Sox2⁺ cells had contributed to all pituitary cell lineages (Figures 4A and S4B). Of note, the contribution to TSH-expressing cells was very low, perhaps suggesting a low cell turnover of thyrotrophs. In addition, YFP⁺ cells expressing SOX2, SOX9, or S100B were evident in these animals, demonstrating that the initially labeled cells were not short-lived progenitors, but rather were long-lived stem cells (Figures 2D, 4A, and S4B). However, the degree of colonization by Sox2⁺ descendants was rather limited at all stages analyzed (1-, 6-, 9-, and 12-month intervals; n = 5, 4, 3, and 3, respectively; Figure 4B), in accordance with low cell turnover of the pituitary gland under physiological conditions (Florio, 2011; Levy, 2008) especially when compared with the initial population of YFP⁺ cells observed after 48 hr and 1 week (Figure 4B). YFP⁺ cells appeared to be mostly solitary and were rarely observed in small groups after 9 and 12 months of tracing. Similar results were obtained when 3- and 6-month-old adult Sox2^{CreERT2/+};R26^{YFP/+} mice were induced with tamoxifen and traced for up to 6 months, suggesting that multipotent Sox2⁺ cells are present throughout adult life (data not shown).

Next, we sought to assess whether YFP⁺ cells coexpressing SOX2, which persisted in the pituitary of traced animals, have clonogenic potential in vitro (Figure 4C). In vitro culture of flow-sorted YFP⁺ and YFP⁻ cells from Sox2^{CreERT2/+};R26^{YFP/+} pituitaries at 6 months postinduction (n = 2) revealed that clonogenic cells were mostly included in the YFP⁺ fraction (96.4%), demonstrating the long-term persistence of SOX2⁺;YFP⁺ cells. Together, these studies demonstrate that the Sox2⁺ adult pituitary cell population includes long-lived progenitor/stem cells that are able to generate fully differentiated hormone-producing cells throughout life as well as to self-renew and clonally expand in vitro.

Targeted Expression of a Degradation-Resistant Mutant β -Catenin in Sox2⁺ Cells Leads to the Generation of Pituitary Tumors

Having shown that Sox2⁺ cells include stem cells during embryonic development and adulthood, we sought to explore the role of this cell population in pituitary oncogenesis.

We previously established that the activation of the WNT/ β -catenin pathway in Hex3⁺ RP precursors in *Hesx1*^{Cre/+}; *Ctnnb1*^{lox(ex3)/+} embryos leads to pituitary hyperplasia at late gestation followed by tumors in adult mice that are reminiscent of human ACP (Gaston-Massuet et al., 2011). The *Ctnnb1-lox(ex3)* gain-of-function allele carries loxP sites flanking exon3, the removal of which results in the expression of a degradation-resistant form of β -catenin, leading to overactivation of the WNT pathway (Harada et al., 1999). Because the *Hesx1*^{Cre/+} mouse line is not inducible and Hex3⁺ RP progenitors generate most of the AP cells, it was unclear which particular cell type, when targeted, initiated oncogenesis in *Hesx1*^{Cre/+}; *Ctnnb1*^{lox(ex3)/+} mice.

To determine whether stem cells are the mutation-sustaining cells that lead to tumor formation, we performed embryonic inductions in Sox2^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+} embryos to specifically activate the WNT pathway in Sox2⁺ cells. Pregnant females were induced at 10.5 dpc by a single low-dose tamoxifen injection (1.5 mg) and embryos were analyzed at 15.5 dpc. Sox2^{CreERT2/+}; *Ctnnb1*^{lox(ex3)/+} embryos exhibited an enlarged AP, with foci of nucleocytoplasmic accumulation of β -catenin surrounded by cells exhibiting normal β -catenin localization on the cell membrane (Figures 5A and 5B). Similar β -catenin-accumulating cell clusters are present in *Hesx1*^{Cre/+}; *Ctnnb1*^{lox(ex3)/+}

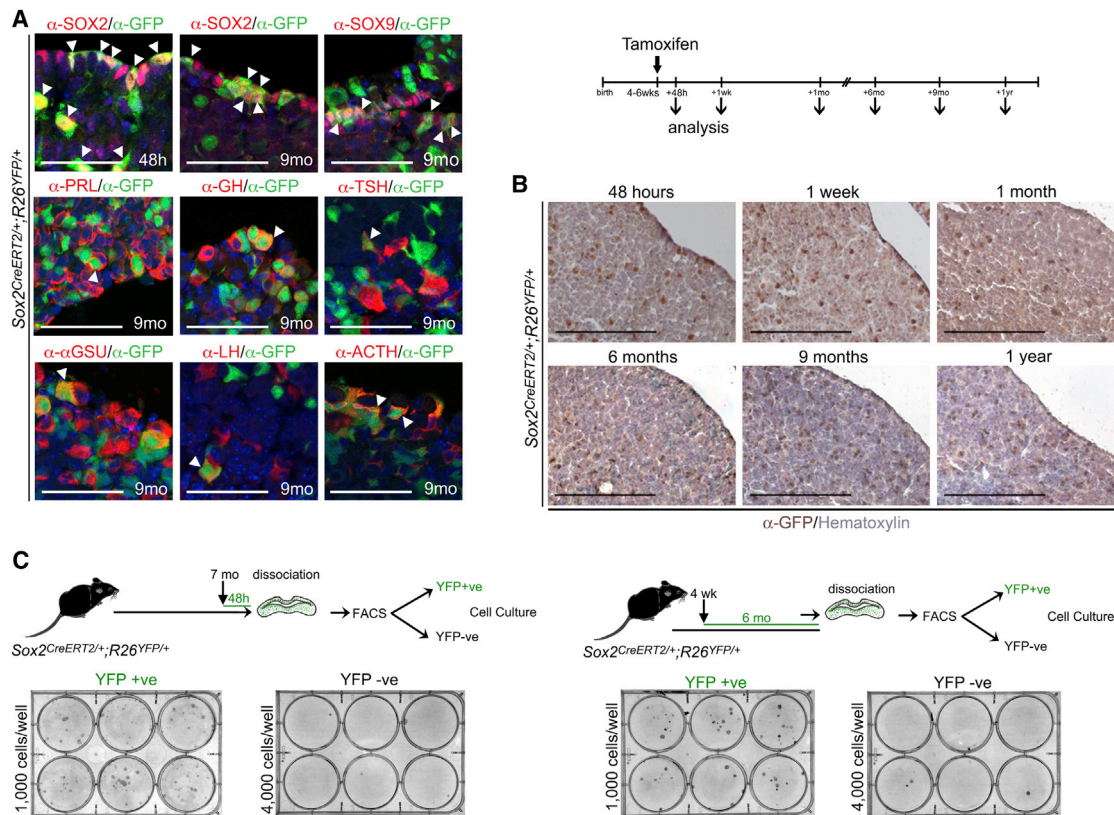


Figure 4. Sox2⁺ Pituitary Cells in Postnatal Mice Participate in Organ Homeostasis

(A) Tamoxifen induction of 4- to 6-week-old Sox2^{CreERT2/+};R26^{YFP/+} mice reveals that the majority of YFP⁺ cells coexpressed SOX2 after 48 hr. Tracing for 9 months demonstrates the persistence of SOX2 and SOX9 expression in YFP⁺ cells, as well as the coexpression of YFP with commitment and terminal differentiation markers.

(B) Immunohistochemistry against GFP (dark brown) demonstrates the colonization of the AL by Sox2⁺ descendants in induced mice traced for up to 1 year, as indicated by the scheme. Sections are counterstained with hematoxylin, staining nuclei (purple).

(C) YFP⁺ and YFP⁻ pituitary cell fractions were separated by flow sorting and plated in pituitary stem-cell-promoting media at clonal densities at 48 hr and 6 months after tamoxifen induction. The majority of the colony-generating cells are retained within the YFP⁺ fraction, demonstrating the persistence of clonogenic Sox2⁺ cells.

Scale bars, 50 μ m (A) and 200 μ m (B). See also Figure S4.

mice, suggesting a common pathogenesis in the induced Sox2^{CreERT2/+};Cttnb1^{lox(ex3)/+} mice. These clusters are also present in human ACPs and have been proposed to be a histopathological hallmark that distinguishes human ACPs from other pituitary tumors (Hofmann et al., 2006). The cluster cells were quiescent or slowly dividing, as they did not express the proliferation-associated marker Ki67 (Figure 5C). In addition, these clusters contained undifferentiated cells, as evidenced by the lack of expression of the differentiation or commitment markers α GSU and PIT1, respectively (Figure S5C). SOX2 expression was observed in sporadic cells within the small clusters, whereas SOX9 was mostly not expressed in small clusters but was often detected in the periphery of larger ones (Figure 5C). As expected, activation of the WNT pathway occurred in β -catenin-accumulating cell clusters, as demonstrated by the expression of the WNT targets *Axin2* and *Lef1* (Figures 5B and S5A; Jho et al., 2002; Wu et al., 2012). We observed the expression of these markers in the periphery of some large cell structures concomitantly with β -catenin accumulation. Further, we identified the specific expression of the signaling molecules *Shh*, *Bmp4*,

Wnt5a, *Wnt6*, *Wnt10a*, and *Fgf3* in cells within β -catenin-accumulating clusters, as previously described in the *Hesx1^{Cre/+}; Cttnb1^{lox(ex3)/+}* mouse model and human ACP (Figure 5B; Andoniadou et al., 2012).

Next, we sought to test whether adult Sox2⁺ pituitary stem cells are also capable of contributing to tumor formation through specific activation of the WNT pathway. We injected 6-week-old Sox2^{CreERT2/+};Cttnb1^{lox(ex3)/+} mice twice with a low tamoxifen dose of 0.15 mg/g, because a higher dosage led to premature death unrelated to a pituitary phenotype. Pituitary glands were analyzed at different times postinduction by histology and immunohistochemistry. Most of the animals that were traced between 3 and 5 months developed pituitary tumors (six out of eight mice), which were obvious on dissection (Figure 5D). The pituitary glands of the remaining mice (two out of eight), as well as those of animals analyzed 2–3 months postinduction (five out of seven), appeared normal by gross morphology but contained small tumors upon histological analysis. The observed tumors were well circumscribed and contained densely packed cells and some mitotic nuclei. AL

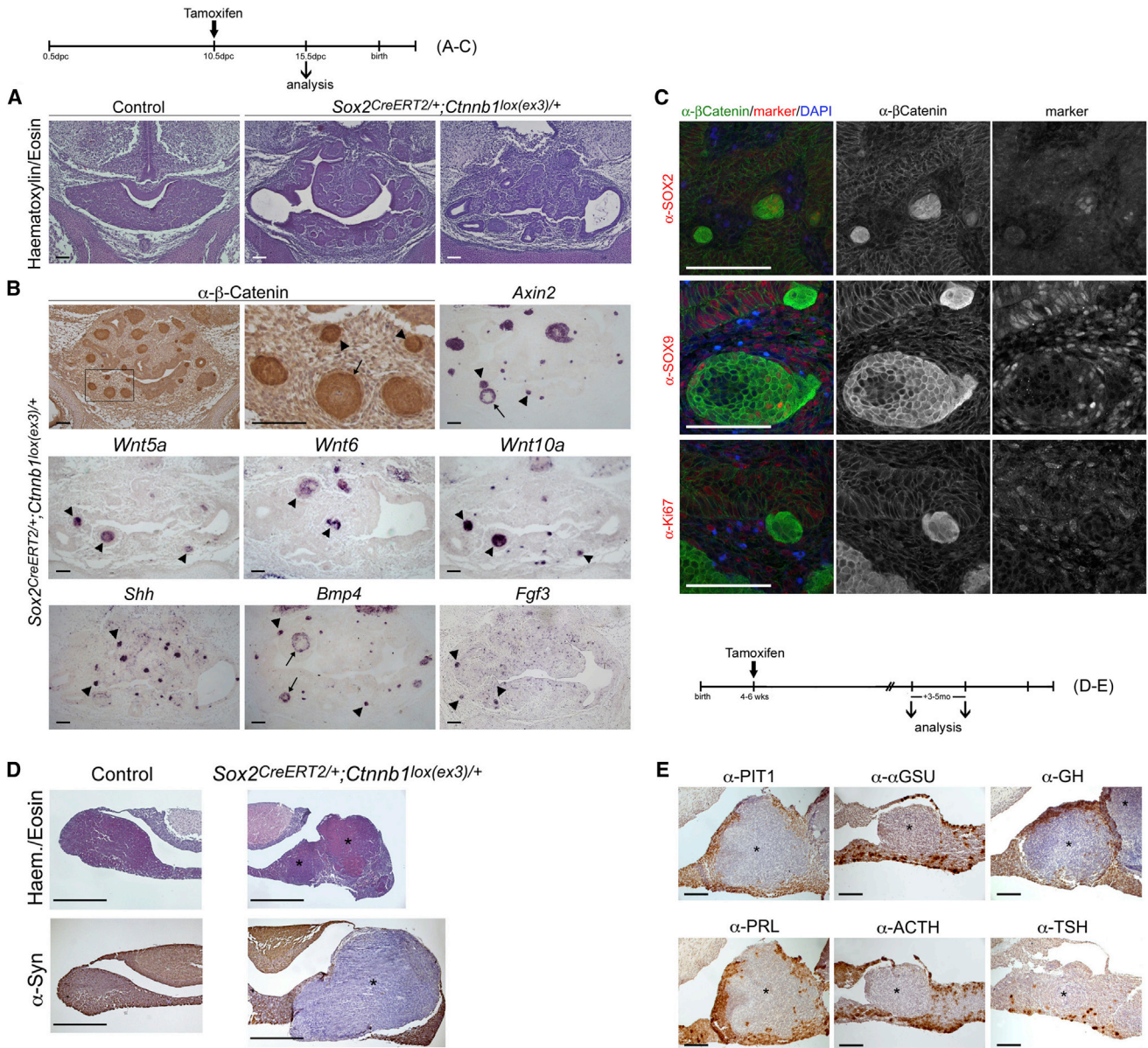


Figure 5. Overactivation of the WNT Pathway in Sox2⁺ Embryonic and Adult Cells Results in Pituitary Tumorigenesis

(A) Hematoxylin and eosin staining reveals hyperplasia and dysmorphology of the pituitary gland in two *Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+}* tamoxifen-induced mutants compared with a *Sox2^{+/+};Ctnnb1^{lox(ex3)/+}* control littermate.

(B) Immunohistochemistry demonstrates the presence of small (arrowheads) and large (arrow) clusters with nucleocytoplasmic β -catenin accumulation in *Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+}*-induced pituitaries. Note the darker staining in the small clusters and periphery of the larger ones. In situ hybridization shows that these cluster cells activate the WNT pathway, as evidenced by the activation of the WNT/ β -catenin target *Axin2*, and express *Wnt5a*, *Wnt6*, *Wnt10a*, *Shh*, *Bmp4*, and *Fgf3*, the products of which are secreted signaling molecules. Expression of these markers is also stronger in the small clusters (arrowheads) and often in the periphery of the larger ones (arrows).

(C) Immunofluorescence staining reveals that small β -catenin-accumulating clusters contain SOX2⁺ cells but do not contain SOX9⁺ or proliferative Ki67⁺ cells, in line with a quiescent phenotype. SOX9⁺ cells are occasionally included in the periphery of larger cell structures.

(D) Hematoxylin and eosin staining of tamoxifen-induced *Sox2^{+/+};Ctnnb1^{lox(ex3)/+}* (control) and *Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+}* adult pituitaries, showing the presence of tumors in the latter (asterisks). These tumors are negative for synaptophysin as detected by immunohistochemistry (brown). Sections are counterstained with hematoxylin (purple).

(E) Immunohistochemistry fails to reveal expression of cell-lineage commitment or terminal differentiation markers within the tumor mass (asterisks). Note the presence of specific signal around the tumors (dark brown).

Scale bars, 100 μ m (A–C and E) and 500 μ m (D). See also Figure S5.

pituitary tissue was evident around the tumors and the IL and PL were also apparently normal.

Immunohistochemistry revealed the absence of staining using antibodies against PIT1, GH, PRL, TSH, ACTH, and α -GSU within the tumors, but positive cells were detectable around the lesions, confirming the presence of unaffected pituitary tissue (Figure 5E). Null-cell adenomas are pituitary tumors characterized by the lack of any hormone expression but positivity for the neural and endocrine cell marker synaptophysin. However, Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+} pituitary tumors were synaptophysin negative, although staining was present in the surrounding pituitary tissue (Figure 5D). Analysis of β -catenin expression revealed a complex pattern within the tumor lesions, with some cells showing nuclear and/or cytoplasmic staining and others exhibiting normal membrane staining, such as the staining observed in unaffected pituitary cells around the lesions (Figures S5D and S5E). Finally, expression of the cell-proliferation marker Ki67 was consistently observed within the lesions (Figure S5D). Together, these analyses demonstrate that Sox2⁺ adult pituitary cells can generate tumors when targeted to express mutant degradation-resistant β -catenin. In addition, our data suggest that the pituitary tumors in Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+} mice, as in the Hesx1^{Cre/+};Ctnnb1^{lox(ex3)/+} model, share a common pathogenesis and are more similar to human ACP than to other tumors, such as adenomas.

The Cell-of-Origin of the Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+} Pituitary Tumors Is Not a Mutation-Sustaining Sox2⁺ Cell

Adult stem cells have been shown to contribute to tumor formation in a cell-autonomous manner whereby cells composing the tumor are derived from the mutation-sustaining stem cells. To investigate the mechanism by which the mutated Sox2⁺ adult pituitary stem cells drive the observed tumors, we generated Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+};R26^{YFP/+} triple heterozygous mice to enable lineage tracing of the descendants of the targeted Sox2⁺ cells through YFP expression. Mice were induced by two injections of low-dose tamoxifen at 6 weeks of age and traced for 3–5 months, when the pituitary was analyzed. As expected, these animals developed clearly identifiable, well-circumscribed tumor lesions, which were morphologically identical to those observed in Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+} mice (Figure 6A). The tumors were of variable size, possibly reflecting temporally different stages of development. Intriguingly, specific immunohistochemistry revealed that the vast majority of the cells within the tumors did not express YFP ($n = 7$ pituitaries and 23 individual tumor lesions; Figure 6). Abundant YFP⁺ cells, some of which formed clusters, were identified in histologically normal pituitary tissue adjacent to the YFP⁻ tumors, indicating that the lack of YFP detection was not due to inefficient tamoxifen induction and that an expansion of the Sox2⁺ cell lineage had occurred after Ctnnb1 activation (Figures 6A–6C).

We reasoned that the absence of YFP expression could be caused by different mechanisms, including (1) unequal Cre-mediated excision of the ROSA26 and Ctnnb1 loci, leading to recombination of only Ctnnb1-lox(ex3), but not ROSA26-flx-stop-YFP, and hence expression of mutant β -catenin but not YFP; (2) a change of fate of the cells upon expression of mutant β -catenin, resulting in low or no expression of YFP; and (3) silencing of the locus through epigenetic changes or gene dele-

tion. However, PCR analysis of DNA isolated from laser-capture microdissected tumors with specific primers to detect the different alleles revealed that neither the ROSA26 nor the Ctnnb1 loci had been excised, and both wild-type and floxed (nonrecombined) alleles were present (Figure S6). This analysis argues against any of the possibilities outlined above and demonstrates that the tumors were not derived from the β -catenin-accumulating cells.

Immunostaining against β -catenin showed a heterogeneous patterning of expression in the tumor cells, with only some cells showing strong nucleocytoplasmic accumulation (Figure 6B). This expression pattern was also observed upon in situ hybridization with *Lef1*, indicating that the accumulation of β -catenin and WNT pathway activation was restricted to some of the tumor cells (Figure 6B). Because tumor cells do not contain the recombined Ctnnb1 allele, with ensuing expression of mutant β -catenin, these results suggest that the heterogeneous nucleocytoplasmic accumulation of β -catenin and activation of the WNT pathway in the tumors is non-cell-autonomous, and may be caused by secreted WNT ligands from nearby clusters. In agreement with this notion, *Wnt5a*, *Wnt6*, and *Wnt10a* were also found to be expressed in the β -catenin-accumulating cell clusters in induced Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+} mice (Figures 5B and S5B).

YFP⁻ tumors were often observed developing in proximity to groups of YFP⁺ cells that showed strong nucleocytoplasmic β -catenin accumulation (arrowheads in Figure 6B), sometimes resembling the cell clusters observed after tamoxifen induction in Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+} embryos (Figures 5B and 5C; arrowheads in Figure 6C). Occasionally, we observed that areas of YFP⁺ and YFP⁻ cells were found adjacent to each other, mostly in small tumors, suggesting that growth of the tumor cells (YFP⁻) may require close proximity to the YFP⁺ cells at initial stages of development (Figure 6C, bottom panel). Immunostaining for the endothelial marker endomucin revealed a clear association of the clusters with blood vessels (Figure 7A). Most of the cells in these clusters were positive for S100B, suggesting that they most likely derived from Sox2⁺;S100B⁺ cells, and many cells within the clusters were also positive for p75(NTR), another marker of FS cells (Figure 7B; Borson et al., 1994). Components of the extracellular matrix (ECM), such as laminin and fibronectin, or integrin- β I associated with ECM proteins, were not enriched in or immediately surrounding the β -catenin-accumulating clusters. However, a slight enhancement of collagen type I was evident (Figure S7). Together, these results demonstrate that a proportion of the Sox2⁺ population, possibly those expressing S100B, can be stimulated to expand in vivo to form clusters, but the tumors that are induced in the Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+} mice are not derived from the Sox2⁺ cells that sustain the tumor-initiating mutation in Ctnnb1.

DISCUSSION

The possible contribution of Sox2⁺ cells to normal organ homeostasis has been controversial, since fully differentiated pituitary cells can divide postnatally. In this study, we show that Sox2⁺ embryonic precursors give rise to all cell lineages in the developing pituitary and that a remaining population in the postnatal gland acts as a reservoir of undifferentiated progenitors/stem

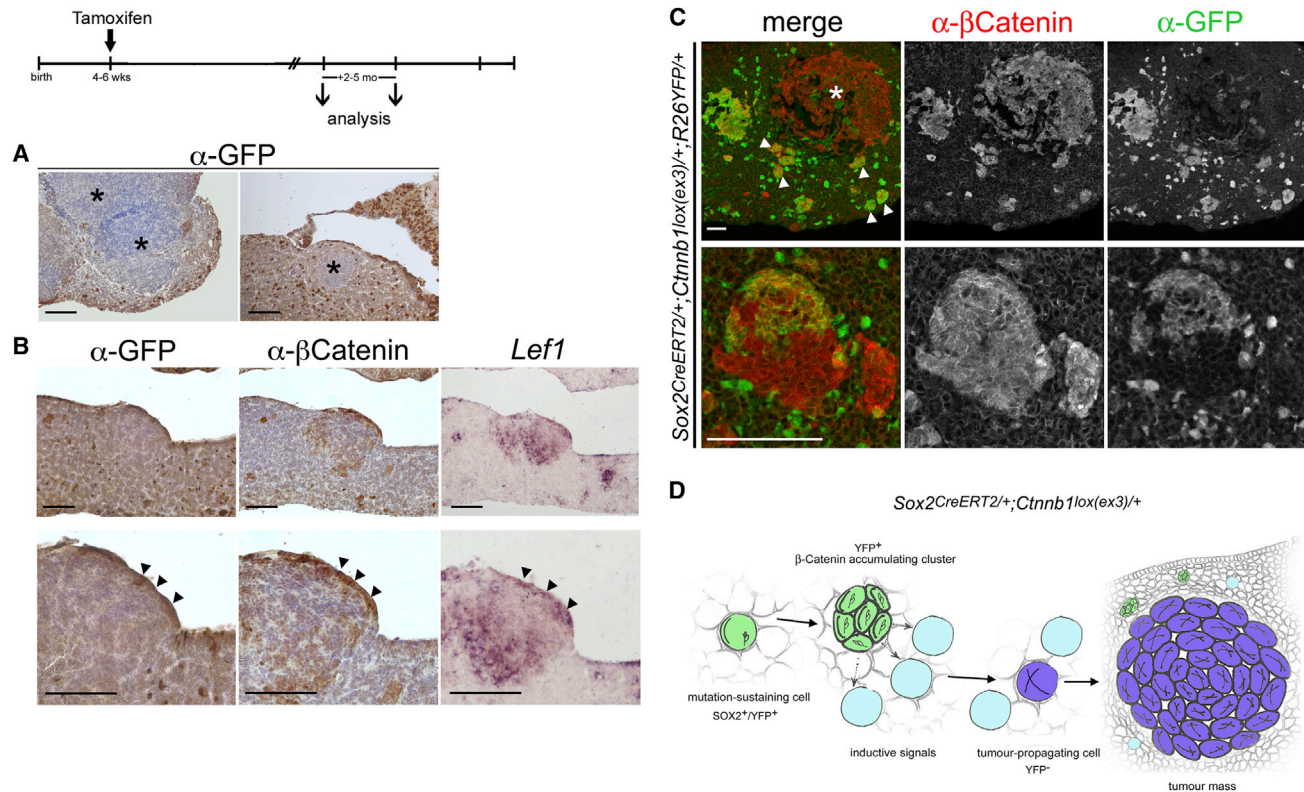


Figure 6. Sox2⁺ Cells Are Not the Cell-of-Origin of the Pituitary Tumors in *Sox2^{CreERT2}^{+/+};Ctnnb1^{lox(ex3)}^{+/+};R26^{YFP}^{+/+} Mice*

(A) Immunohistochemistry against GFP fails to detect YFP⁺ cells within the tumors (asterisks) 3 months postinduction, but YFP⁺ cells (dark brown punctate staining) are detected in the tissue around the lesions.

(B) Immunohistochemistry against GFP and β -catenin, and in situ hybridization to detect *Lef1* expression. The tumor (asterisk) does not contain YFP⁺ cells and displays a heterogeneous pattern of β -catenin expression. Note the nucleocytoplasmic accumulation in the epithelium lining the cleft. *Lef1* is expressed in the tumor cells and shows a heterogeneous pattern mimicking that of β -catenin.

(C) Double immunofluorescence staining reveals the presence of a β -catenin-accumulating lesion (asterisk) that is YFP⁻ and has developed in proximity to YFP⁺ clusters with nucleocytoplasmic β -catenin accumulation (arrowheads). Note the presence of a small developing YFP⁻ tumor that is adjacent to YFP⁺ cells and the presence of numerous single YFP⁺ cells.

(D) Proposed model for a non-cell-autonomous role for mutation-sustaining Sox2⁺ cells in pituitary tumor formation. Following a burst of proliferation, descendants of Sox2⁺ targeted progenitors/stem cells form β -catenin-accumulating clusters that are YFP⁺. Cluster cells secrete factors leading to the trans-formation and proliferation of neighboring cells that generate a tumor that is not derived from the initial targeted Sox2⁺ cells.

Scale bars, 100 μ m. See also [Figure S6](#).

cells. These postnatal Sox2⁺ cells contribute to pituitary homeostasis and persist even 1 year later in the pituitary of induced mice, suggesting that they are not short-lived precursors. Our strategy does not allow us to distinguish whether single Sox2⁺ cells are multi- or unipotent progenitors, or whether they self-renew in vivo. However, a recent study using a similar mouse model demonstrated that the Sox2⁺ compartment contains stem cells in several organs, including the stomach, testes, and lens, and that these cells derive from Sox2⁺ embryonic precursors (Arnold et al., 2011; Sarkar and Hochedlinger, 2013). In addition, Sox2⁺ cells are multipotent and can self-renew in vitro (Figure 2C; Andoniadou et al., 2012; Fauquier et al., 2008; Gaston-Massuet et al., 2011; Gremeaux et al., 2012).

The degree of Sox2⁺ stem cells' contribution to normal cell turnover appears to be limited, and even after year-long tracing, the majority of cells contained in these pituitaries were not derived from targeted Sox2⁺ cells. Genetic tracing of Lgr5⁺ or Prom1⁺ intestinal crypt stem cells has demonstrated a much

higher degree of colonization of the gut villi by descendants of these cells (Barker et al., 2009; Zhu et al., 2009). However, gut epithelial cells renew every 4–5 days, whereas the pituitary gland requires up to 70 days to replace the constituent cells under normal physiological conditions (Florio, 2011; Levy, 2002; van der Flier and Clevers, 2009). As our data demonstrate the efficient targeting of a large proportion of the Sox2⁺ cells, these results suggest that normal pituitary homeostasis results from a combination of Sox2⁺ progenitor/stem cells and the proliferation of fully differentiated cells and/or other progenitor populations (e.g., expressing Nestin or GFR α 2) as previously proposed (Carbajo-Pérez and Watanabe, 1990; Garcia-Lavandeira et al., 2009; Gleiberman et al., 2008; Taniguchi et al., 2002). The expansion of Sox2⁺-derived cells after activation of the WNT pathway, however, suggests that this Sox2⁺ cell population may be responsive to homeostatic signals within the pituitary (e.g., in repopulation after pathological challenge), with important implications for their therapeutic use (Castinetti et al., 2011).

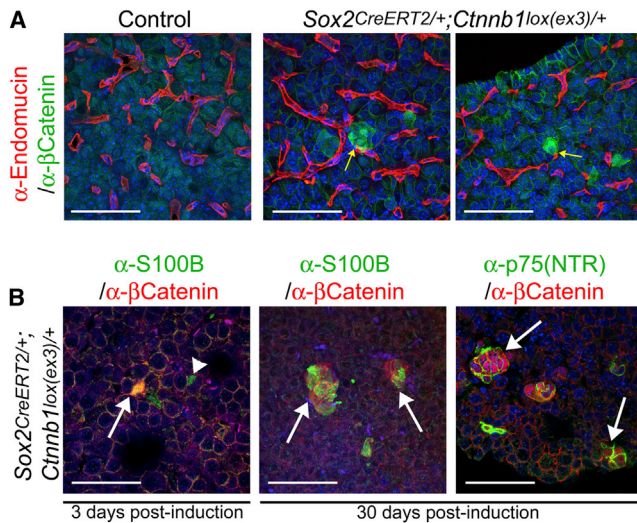


Figure 7. Analysis of β -Catenin-Accumulating Clusters in $Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+}$ -Induced Mice with Respect to Markers of Endothelial Cells and Folliculostellate Cells

(A) Double immunofluorescence against the endothelial marker endomucin and β -catenin, showing the close association of clusters with blood vessels of the AP (arrows).

(B) β -catenin accumulation is observed in single S100B⁺ FS cells as early as 3 days following induction. After 30 days, typical β -catenin-accumulating cell clusters are identifiable, and these are positive for S100B and p75(NTR), both markers of FS cells in the AP.

Scale bars, 50 μ m. See also Figure S7.

We previously showed that the genetic expression of mutant β -catenin in committed or differentiated cells in the embryonic or adult pituitary using specific Cre lines (i.e., *Gh-Cre*, *Prl-Cre*, and *Pit1-Cre*) does not give rise to tumors, and proposed that progenitor/stem cells might have to be targeted to initiate the oncogenic process (Gaston-Massuet et al., 2011). An important finding of this study is that both embryonic and adult Sox2⁺ cells play a critical role in initiating pituitary tumorigenesis. The expression of mutant β -catenin in Sox2⁺ cells results in the formation of typical ACP pretumoral β -catenin-accumulating cell clusters and tumor formation. These data establish Sox2⁺ progenitor/stem cells as target cells for tumor-initiating mutations in mouse and human ACPs. In agreement with this notion, SOX2 heterozygous mutations resulting in overactivation of the WNT pathway have been identified in patients showing pituitary tumors that may be consistent with craniopharyngioma (Alatzoglou et al., 2011). Whether other somatic mutations in Sox2⁺ cells could also lead to pituitary tumors such as adenomas is an important question that requires further research.

A remarkable and unexpected result reported here is the discovery that mutagenized Sox2⁺ progenitor/stem cells drive tumor formation in a paracrine fashion. In previous studies, the *Ctnnb1-lox(ex3)* and *ROSA26-flox-stop-YFP* alleles were used in combination with specific inducible Cre lines to investigate the role of adult somatic stem cells in tumors of the brain (Alcantara Llaguno et al., 2009; Jacques et al., 2010; Sutter et al., 2010), gut (Barker et al., 2009; Zhu et al., 2009), pancreas (Gidekel Friedlander et al., 2009), prostate (Mulholland et al., 2009), and skin (Youssef et al., 2010). These studies demonstrated that

mutation-sustaining stem cells are the cell-of-origin of these tumors (i.e., the tumor mass derives from the mutagenized stem cells), thus providing support for the notion that somatic stem cells can act in a cell-autonomous manner according to the cancer stem cell paradigm. Reciprocal cell signaling communication between tumor and normal cells is also important for oncogenesis, suggesting that somatic stem cells may be able to influence tumorigenesis in a non-cell-autonomous manner (Hanahan and Weinberg, 2011; Lathia et al., 2011; Nguyen et al., 2012; Visvader and Lindeman, 2012). In this study, we show that Sox2⁺ cells need to be targeted to initiate tumorigenesis, but tumors are not derived from the initially targeted cells, as demonstrated by the lack of YFP expression in the pituitary tumors of $Sox2^{CreERT2/+};Ctnnb1^{lox(ex3)/+};R26^{YFP/+}$ triple heterozygous mice (Figure 6). Moreover, we show that tumors contain cells in which the *ROSA26* and *Ctnnb1* loci have not been recombined (Figure S6), which rules out the possibility that they are descendants of the β -catenin-accumulating cluster cells. Recently, a non-cell-autonomous effect of p53 was shown in a model of hepatocellular carcinoma, where p53-ablated hepatic stellate cells were able to induce tumors in a paracrine fashion (Lujambio et al., 2013). Our data indicate that mutant β -catenin exerts a transient burst of proliferation in a proportion of Sox2⁺ cells that generate daughter cells, which form β -catenin-accumulating cell clusters. These clusters become quiescent and activate the expression of several secreted factors, such as *Shh*, *Bmp4*, *Wnt5a*, *Wnt6*, *Wnt10a*, and *Fgf3*, that have important roles in tumorigenesis in both mice and humans (Labeur et al., 2010; Wesche et al., 2011; Yauch et al., 2008). It is likely that cluster cells express other mitogenic secreted signals, including interleukins, chemokines, and growth factors, as we recently revealed from a global gene profiling analysis of such cluster cells in $Hesx1^{Cre/+};Ctnnb1^{lox(ex3)/+}$ pituitaries (Andoniadou et al., 2012). The effect of these signals induces the transformation of surrounding cells that form tumors that are not derived from the targeted Sox2⁺ cells. A model for the proposed oncogenic process is depicted in Figure 6D.

Pituitary adenomas are tumors with a significant prevalence, and although they are benign, they are associated with high morbidity due to both compression of nearby structures (i.e., the hypothalamus and visual pathways) and/or abnormal secretion of hormones. A minority of adenomas are syndromic/familial and the result of germline mutations for genes such as *MEN1*, *AIP*, and *PRKAR1A* or somatic mutations such as *GNAS*. However, the vast majority of pituitary adenomas are sporadic and the initial mutation that drives tumor formation remains generally elusive. Alterations in genes that are involved in syndromic adenomas, or in well-recognized tumor-suppressor genes or oncogenes that are commonly involved in other tumor types, have not been identified in the majority of sporadic adenomas (Dworakowska and Grossman, 2012; Melmed, 2011). Our model may explain these results, as it predicts that the initial mutation that drives tumorigenesis occurs in a cell type that does not contribute cell autonomously to the tumor. This model establishes a framework for elucidating pituitary tumorigenesis and demonstrates a mechanism underlying pituitary tumorigenesis.

In summary, the results presented here exemplify the complex pathophysiology that governs pituitary oncogenesis in vivo, and extend our understanding of the role of somatic stem cells in

tumor formation by providing evidence for a non-cell-autonomous function specifically in mutated cells with progenitor/stem cell properties. Sox2⁺ pituitary cells are target cells for tumor-initiating mutations and are responsible for driving tumorigenesis, but are not the cell-of-origin of the pituitary tumors. Whether this mechanism is more generic and applicable to other tissues that contain somatic stem cells requires further investigation, with the exciting possibility of generating novel therapeutic targets in the treatment of primary and metastatic tumors.

EXPERIMENTAL PROCEDURES

Mice

A detailed description of the generation of Sox2^{CreERT2/+} mice is included in the Supplemental Experimental Procedures. The *Ctnnb1-lox(ex3)*, *Hesx1-Cre*, *ROSA26-flox-stop-YFP*, *Sox2-eGFP*, and *S100B-eGFP* mice have been previously described (Andoniadou et al., 2007; Ellis et al., 2004; Harada et al., 1999; Srinivas et al., 2001; Vives et al., 2003). Generally, Cre induction in adult mice was carried out by two to four single injections of tamoxifen (Sigma) at a dose of 0.15 mg per gram of body weight on consecutive days (two in Sox2^{CreERT2/+}; *Ctnnb1^{lox(ex3)/+}*, and two to four in Sox2^{CreERT2/+}; *R26^{YFP/+}*). For 24 hr tracing, a single dose of 0.3 mg per gram of body weight was used. For induction in embryos, pregnant dams received a single injection of tamoxifen totaling 1.5 mg and simultaneous injection with 0.75 mg progesterone (Sigma) to reduce the risk of spontaneous abortion.

Flow Sorting and Cell Culture

Flow sorting and cell culture were carried out as previously described (Andoniadou et al., 2012; see Supplemental Experimental Procedures). YFP⁺ and YFP⁻ cells were flow sorted from Sox2^{CreERT2/+}; *R26^{YFP/+}* tamoxifen-induced mice and plated separately in pituitary stem cell medium in six-well plates at a density of 1,000 cells per well for YFP⁺ and 4,000 cells per well for YFP⁻ fractions. The number of colonies from the YFP⁺ and YFP⁻ fractions is expressed as a percentage of the total number of colonies. To assess differentiation potential, colonies were established in normal growth media for 5 days, followed by culture in differentiation conditions in the absence of growth factors and addition of 10% fibroblast-conditioned media for 2 weeks. *S100B-eGFP* APs from P9 animals were dissociated, flow sorted, and cultured as previously described (Gaston-Massuet et al., 2011).

Histology, In Situ Hybridization, Immunostaining, and Microscopy

Histology, in situ hybridization, immunostaining, and microscopy procedures were carried out as previously described (Andoniadou et al., 2012; Gaston-Massuet et al., 2011). For a detailed list of the methods and antibody dilutions used, please see the Supplemental Experimental Procedures. For estimation of cell numbers, pituitaries (n = 3) were subjected to double immunofluorescence staining and five randomly selected fields were captured at 20× magnification. Between 500 and 1,000 YFP⁺ cells were counted for each marker, including only those cells with visible nuclei.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, lists of primary and secondary antibodies, and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.stem.2013.07.004>.

ACKNOWLEDGMENTS

This work was carried out with the support of the UCL Institute of Child Health and Great Ormond Street Hospital Flow Cytometry Core Facility, the Montpellier RIO Imaging Cytometry Facility, UCL Genomics, the ICH Embryonic Stem Cell/Chimera Production Facility, UCL Biological Services Unit, and UCL Cancer Institute and WIBR Scientific Support Services. We thank Dr. JrGang Cheng and the BAC Recombineering Core Facility at UNC Chapel Hill, C. Legraverend for providing the *S100beta-eGFP* transgenic mice, and the

Developmental Studies Hybridoma Bank (University of Iowa) and the National Hormone and Peptide Program (Harbor-UCLA Medical Center) for providing some of the antibodies used in this study. We thank Gabriella Carreno and Mario Gonzalez-Meljem for technical assistance; Prof. Jacques Drouin and Prof. Simon Rhodes for TPIT and PIT1 antibodies, respectively; and Prof. Sally Camper, Prof. Steve Jones, and Prof. Gregory Shackleford for *Wnt6*, *Wnt5a*, and *Wnt10a* probes, respectively. This work was supported by grants 086545 and 084361 from The Wellcome Trust. M.T.D. is funded by the Great Ormond Street Children's Hospital Charity.

Received: December 10, 2012

Revised: June 3, 2013

Accepted: July 3, 2013

Published: October 3, 2013

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