DISEASE CONNECTIONS

Identification of Nucleus Pulposus Precursor Cells and Notochordal Remnants in the Mouse: Implications for Disk Degeneration and Chordoma Formation

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A classically identified "notochordal" cell population in the nucleus pulposus is thought to regulate disk homeostasis. However, the embryonic origin of these cells has been under dispute for >60 years. Here we provide the first direct evidence that all cell types in the adult mouse nucleus pulposus are derived from the embryonic notochord. Additionally, rare isolated embryonic notochord cells remained in the vertebral column and resembled "notochordal remnants," which in humans have been proposed to give rise to a rare type of late-onset cancer called chordoma. Previously, this cell type had not been identified in the mouse model system. The development and characterization of a mouse model that can be used to fate map nucleus pulposus precursor cells in any mutant background will be useful for uncovering the cellular and molecular mechanisms of disk degeneration. In addition, the identification of notochordal remnants in mice is the first step towards generating an in vivo model of chordoma. *Developmental Dynamics 237:3953–3958, 2008.*

Key words: nucleus pulposus; intervertebral disk; Shh; notochord; disk degeneration; back pain

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INTRODUCTION

It has been estimated that two-thirds of Americans will experience at least one episode of back pain in their lifetimes. The majority will recover within a month. However, 4.5 million people a year will become disabled from back pain at a cost of $16 billion a year (health care costs and lost work time) (Praemer et al., 1992; Pope, 1997). Both the severity and incidence of back pain increase as people age.

The most common cause of back pain is degeneration of the intervertebral disks (Hunter et al., 2003). This usually manifests itself in one of two ways; either through herniation of disk material into the vertebral column or through the reduction of disk thickness. Reduction in the thickness of the disks results in the compression of the vertebral facets, which then exert pressure on the nerve roots, leading to back pain. In a normal vertebral column, the intervertebral disks join adjacent vertebral bodies where they provide shock absorption and facilitate mobility of the spine (Urban and McMullin, 1988; Hunter et al., 2003).

Each disk has three major components: (1) the nucleus pulposus, the gelatinous inner core of the intervertebral disks; (2) the annulus fibrosus, a fibrous capsule that surrounds the nucleus pulposus and consists of concentric lamellae of collagen fibers; (3) and the superior and inferior cartilaginous end plates, which are situated at the articular surfaces of the intervertebral disk and the adjacent vertebrae (Humzah and Soames, 1988). It

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Developmental Dynamics

is the nucleus pulposus that is thought to be required for the generation and maintenance of the disk's structural integrity (Setton and Chen, 2006). Damage to or loss of the nucleus pulposus as a person ages often leads to disk disease and back pain (Hunter et al., 2003).

In humans, cells found in the adult nucleus pulposus are primarily small, chondrocyte-like cells. In juvenile and adults, a second population of cells in the nucleus pulposus has been proposed to function in disk renewal and homeostasis. These cells are much larger than the chondrocyte-like cells and, although their cell lineage is unclear, classical histological studies described them as being of "notochordal" origin (Walmsley, 1953). The coincident loss of the notochordal population of cells and the onset of disk degeneration during the life of many mammalian species suggests that this cell population may be involved in maintenance and/or repair of the disk (reviewed in Hunter et al., 2003).

Over 60 years ago, the embryonic nucleus pulposus was postulated to form from the embryonic notochord (Walmsley, 1953) and studies in rat have supported this hypothesis (Rufai et al., 1995). However, a number of more recent reports have suggested that the adult nucleus pulposus is only partially formed from the embryonic notochord or has a different origin entirely (Kim et al., 2003; Vujovic et al., 2006). The ability to identify in vivo the precursor cells of the nucleus pulposus would greatly aid in developing and characterizing mouse models of disk degeneration.

In humans, "notochordal remnant" cells have been proposed to transform into a rare type of tumor called a chordoma through an unknown mechanism (Yamaguchi et al., 2004, 2005). It has been proposed that notochordal remnants are derived from the embryonic notochord since notochordal remnants are similar in size to notochord cells and reside in the region of the embryo in which the embryonic notochord was present (Yamaguchi et al., 2004, 2005). In mice, notochordal remnants have not been described, which has made it very difficult to create a mouse model for chordoma.

Despite the clinical importance of nucleus pulposus cells, their embryonic origin has never been demonstrated by cell lineage analysis. In order to investigate whether the embryonic notochord gives rise to the entire nucleus pulposus, we genetically marked these cells during early mouse embryogenesis and followed their lineage into adulthood. In order to generate this fate map, we took advantage of the *Shhcre* and *ShhcreERT2* mouse alleles we created previously (Harfe et al., 2004). In mice containing the *Shhcre* or *ShhcreERT2* alleles, CRE is expressed in the notochord and activates expression of CRE-inducible reporter alleles in this tissue. Using these alleles we obtained direct evidence that, in the mouse model system, the embryonic notochord directly gives rise to all cell types present in the nucleus pulposus of the intervertebral disks. A small number of notochord cells were also found to reside in the vertebrae between the intervertebral disks. These cells are the elusive mouse notochordal remnants.

RESULTS

Shh-Expressing Cells in the Mouse Embryo Form the Nucleus Pulposus of the Intervertebral Disks

To determine if the nucleus pulposus was derived from Shh-expressing cells, we took advantage of the *Shhcre* allele we had created previously (Harfe et al., 2004) to fate map Shh-expressing cells, including those that reside in the notochord. The *Shhcre* allele was created by knocking into the *Sonic hedgehog* (*Shh*) gene the site-specific recombinase gene *cre*. Using this allele, CRE protein was expressed everywhere that *Shh* was normally expressed, including the embryonic notochord (Harfe et al., 2004). Mice containing the *Shhcre* allele were crossed to the CRE reporter lines R26R or R26R:EYFP (Soriano, 1999; Srinivas et al., 2001). Both of these reporters contain nuclear localization signals. However, both markers have been shown to leak into the cytoplasm.

In mice containing both the *Shhcre* and a reporter allele, CRE recombinase driven from the *Shh* promoter instigated activation of the reporter allele. Importantly, once reporter expression was activated, it continued to be expressed in cells in which the recombination event occurred, in this case in all Shh-expressing cells including the notochord, and in all descendants of these cells throughout the life of the animal (Fig. 1).

In embryos that contained the *Shhcre* allele and either the *LacZ* (R26R allele) or *EYFP* reporter alleles, we observed reporter gene expression in the notochord at embryonic day (E) 10.5 (Harfe et al., 2004). At E12.5, the notochord had begun to segregate along the anteroposterior axis, and bulges of labeled cells were observed at the positions where the intervertebral disks will form (Fig. 1A).

By E15.5, cells of the notochord had aggregated in areas where the nucleus pulposi were forming, and the vertebral bodies were mostly devoid of *Shhcre* descendent cells (Fig. 1B). Interestingly, at E15.5 we observed small streams of labeled cells in the developing centra (Fig. 1B). The majority of these cells were not present one day later (Fig. 1C). However, a small number of these cells remained between nucleus pulposi and could be visualized using the R26R reporter, which is more sensitive than the ROSA:eYFP reporter (see Fig. 3). These data suggest that some notochordal cells do not end up residing in the nucleus pulposi but instead remain between the intervertebral disks.

By E16.5, cells that expressed *Shhcre* had formed disk-shaped condensations between the vertebrae (Fig. 1C). Expression of the reporter in *Shhcre* cells was observed as intense staining throughout the entire nucleus pulposus in newborns (Figs. 1D, 2A). By contrast, the annulus fibrosis, which surrounds the nucleus pulposus in the intervertebral disks, was found to be composed almost entirely of cells that had never expressed *Shh* (Fig. 2A).

The Tamoxifen-Inducible Allele ShhcreERT2 Identifies the Embryonic Notochord as the Source of Nucleus Pulposus Cells

Since *Shh* is expressed in both the notochord and the nucleus pulposus

until birth (DiPaola et al., 2005), the *Shhcre* allele has the potential to activate reporter genes at early stages in the notochord, and at later stages in nucleus pulposus cells, irrespective of their embryonic origin. Thus, using the *Shhcre* allele, we could not exclude the possibility that notochord cells marked in E11.5 embryos may be eliminated by cell death, and that CRE is then re-expressed in the fully formed nucleus pulposus. To test directly whether the notochord gives rise to all cell types in the nucleus pulposus, we used a temporally controlled *Cre*, the tamoxifen-inducible *ShhcreERT2* allele (Harfe et al., 2004), to pulse-label cells residing in the notochord but not the *Shh*-expressing cells found during later development in the nucleus pulposus. The *ShhcreERT2* allele is identical to the *Shhcre* allele used in our initial fate map studies, except that CRE protein can be activated at discrete stages of embryonic development by injecting pregnant mothers with a single does of tamoxifen. Pregnant mothers carrying E8.0 pups were injected intraperitoneally with tamoxifen and the pups were examined at E13.5 to determine the fate of the notochord. At E13.5, all cells of the nucleus pulposus were labeled (Fig. 2B and data not shown), indicating that the entire nucleus pulposus is descended from the notochord.

To verify that the injected tamoxifen was cleared from the embryo by E13.5, which is after notochord formation but prior to the formation of intervertebral disks, we analyzed reporter expression in external genitalia, in which the preputial glands are known to activate *Shh* at E13.5 (Perriton et al., 2002; Seifert et al., 2008). Tamoxifen injections at E8.0 did not label the preputial glands, indicating that the tamoxifen was cleared from the mouse prior to E13.5, which is consistent with published reports that Cre activity is undetectable ~48 hr after tamoxifen injection (Hayashi and McMahon, 2002) (Fig. 2C and D). These findings exclude the possibility that reporter gene expression is re-activated in intervertebral disks after E13.5.

The Adult Nuclei Pulposi Is Composed Entirely Of Shh Descendent Cells

To determine if the adult nucleus pulposus was composed entirely of cells that had at one time expressed *Shh*, we examined adult disks from animals containing the *Shhcre* and *R26R* LacZ reporter alleles. In these ≥19-month-old animals, all cells of the nucleus pulposus appeared to be labeled, suggesting that this tissue is derived entirely from cells that have expressed *Shh* (Fig. 3A and B). The nucleus pulposus appeared to be a homogeneous population of *Shhcre* descendent cells (i.e., non-labeled cells could not be detected).

Conversely, the annulus fibrosus, cartilaginous end plates, and the adjacent vertebrae were largely devoid of *Shhcre* descendant cells (see below for exception to this finding). Taken together with the finding that the majority of cells residing in the vertebral column and annulus fibrosus have never expressed *Shh* (they do not activate the *cre*-inducible *R26R* reporter construct nor has *Shh* expression ever been reported in these tissues (DiPaola et al., 2005; our unpublished data), these results suggest that that cells originating in the vertebral column and/or the annulus fibrosus most likely do not contribute to the mouse nucleus pulposus.

Notochord Cells That Do Not End Up Residing in the Nucleus Pulposus Form Notochordal Remnants in the Vertebral Column

Although the majority of notochord cells ended up within the nucleus pulposus, a small number of cells were found to reside in the vertebral column, either in the vertebrae or, very rarely, in the annulus fibrosus (Figs. 2A, 3C). The location in which these cells were found was characteristic of the "notochordal remnants" that have been postulated to be present in humans but have never before been observed in mice. Notochordal remnants were found in all animals examined (n=12). These cells were first observed during embryonic nucleus pulposus formation and persisted throughout life, suggesting that notochordal remnants observed in adults arise during formation of the intervertebral disks. At all stages, notochordal remnants resided along the middle of each vertebra and were enriched on the ventral surface. Notochordal remnants were found along the entire length of the vertebral column.

DISCUSSION

At least two distinct cell types have been demonstrated to reside in the nucleus pulposus in humans, chondrocyte-like cells and larger cells that have been referred to classically as "notochordal cells." In addition to being larger than chondrocyte-like cells, notochordal cells have been reported to contain large vacuoles and express a number of proteins that are not found in chondrocyte cells (Maldonado and Oegema, 1992). In a number of species, notochordal cells have been observed to gradually disappear during adult life, and depletion of this cell population correlates temporally with the onset of disk degeneration (Maldonado and Oegema, 1992; Stevens et al., 2000; Hunter et al., 2003). These data have led to the proposal that notochordal cells may serve as nucleus pulposus stem cells.

The developmental origin of the two cell types found in the nucleus pulposus is currently unclear. For example, it has been proposed that both notochordal and chondrocyte cells are derived from the notochord (Walmsley, 1953; Hunter et al., 2003), that only notochordal cells come from the notochord (Kim et al., 2003), or that neither of these cells types are notochord-derived (Vujovic et al., 2006). The above conclusions were derived primarily from histological examinations of intervertebral disks. In the experiments reported here, we used novel mouse alleles to fate map the embryonic notochord. Our experiments provide the first direct evidence that the notochord is the sole source of cells that form the entire nucleus pulposus of the mouse intervertebral disks.

In mice, it is unclear what the ratio of notochordal and chondrocyte-like cells is in the mature nucleus pulposus. An electron microscopic study suggested that in 4-month-old mice, the nucleus pulposus was composed of at least some notochordal cells (Higu-

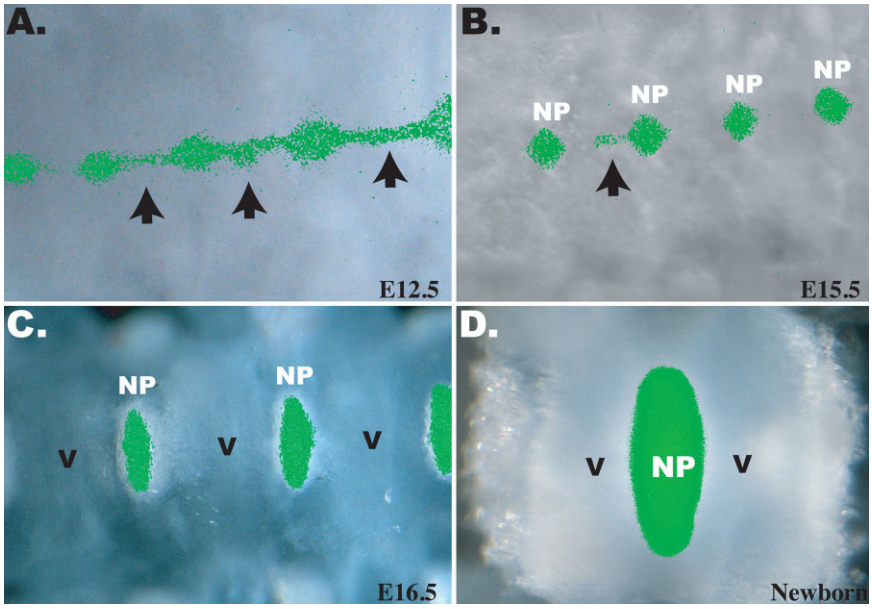

Fig. 1.

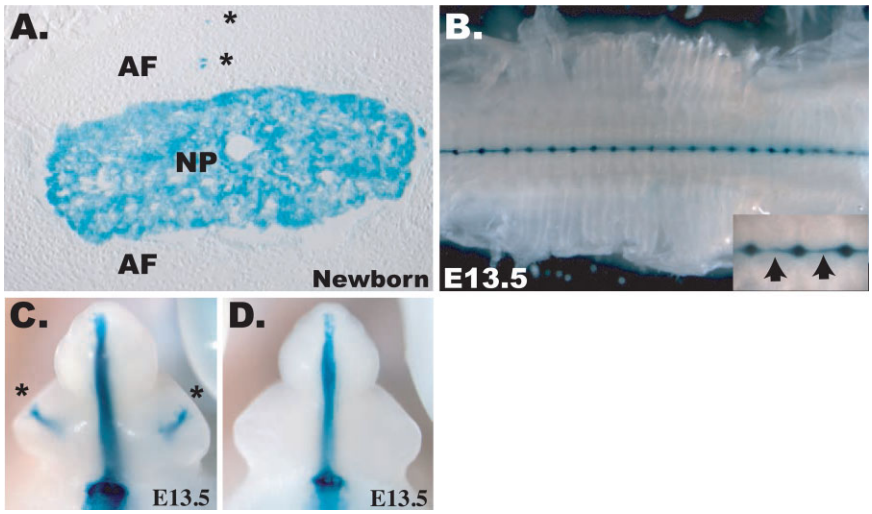

Fig. 2.

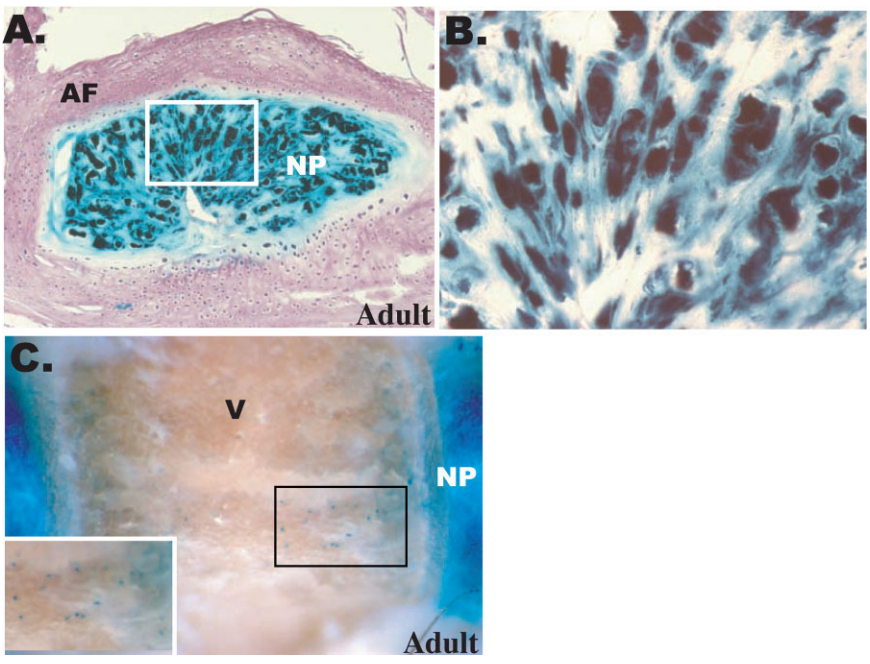

Fig. 3.

chi et al., 1982). Based on the presence of numerous matrix proteins in the adult nucleus puplosus, chondrocyte-like cells are also likely present. Our finding that all cells examined in the adult nucleus pulposus are derived from Shh-expressing cells indicates that presumptive chondrocyte-like cells of the nucleus pulposus are de-

Fig. 1. Fate mapping *Shh*-expressing cells in the axial skeleton using the *Shhcre* allele. The *Shhcre* allele (Harfe et al., 2004) was used to constitutively activate R26R::*EYFP* in the notochord. EYFP is observed as green. A–D: Merged fluorescent and bright-field images of the vertebral column. In all samples, a ventral view of the spinal column is shown. Embryos were harvested and all gut tissue was dissected away to visualize the underlying vertebral column. All images are of unfixed tissue. **A:** At E12.5, the notochord begins to form "bulges" in locations where the nucleus pulposus of the intervertebral disk will form (the arrows denote the notochord). **B:** By E15.5, clearly demarcated nuclei pulposi have formed from *Shhcre*-expressing cells. Part of the notochord is still observed between the disks (arrow). **C, D:** Cells that have expressed *Shhcre* are restricted to the nucleus pulposus and are mostly excluded from the vertebrae (v) at E16.5 (C) and at P0 (D). See Figure 2a and Figure 3b for exceptions. NP, nucleus pulposus.

Fig. 2. Fate map of Shh-expressing cells using the *Shhcre* and the tamoxifen-inducible *ShhcreERT2* alleles. **A:** Ten-micrometer transverse frozen section of the intervertebral disk of a *Shhcre;R26R* newborn mouse. Tissue was stained for the presence of β-galactosidase (blue). Note the entire nucleus pulposus is stained. Rare notochord descendants, denoted with an "*", are found in the annulus fibrosus (AF). B–D: Distribution of *Shh* descendent cells in *ShhcreERT2;R26R* mice pulse-labeled with tamoxifen at E8.0. Progeny were stained for β-galactosidase at E13.5. **B:** At E13.5, the nuclei pulposi of the intervertebral disks are forming. Ventral whole mount view is shown. All gut tissue has been dissected away so that the vertebral column is visible. The inset in B shows three intervertebral disks (ventral view). Note that some cells of the notochord (arrows) are still present between the forming disks. **C, D:** Whole-mount images of genital tubercles at E13.5. At this stage of development, male and female external genitalia are indistinguishable. The preputial glands (asterisks) are labeled in embryos constitutively expressing CRE in all *Shh*-producing cells (C). The absence of β-galactosidase in preputial glands of *ShhcreERT2;R26R* embryos exposed to tamoxifen at E8.0 (D) indicates that the tamoxifen has been cleared from these embryos prior to E13.5, when *Shh* expression is initiated in these glands (Perriton et al., 2002). The line of staining down the middle of the external genitalia in C and D is the urethra, which expresses *Shh* beginning at E9.75 (Perriton et al., 2002).

rived from Shh-expressing notochord cells and, in contrast to previous suggestions, not from cells located in the surrounding Shh-negative mesenchyme (Kim et al., 2003). It is important to note that we cannot rule out the possibility that in organisms other then the mouse, the nucleus pulposus may be derived, at least in part from non-Shh-expressing tissues.

In our experiments, we used both the CRE-inducible LacZ and EYFP reporters to mark the notochord and cells derived from this tissue. A number of mouse lines have been reported to undergo disk degeneration and/or premature aging (Kuro-o et al., 1997; Alini et al., 2008; Pignolo et al., 2008). However, the molecular defects underlying many of the abnormalities in these "aging" strains remain unclear. Using the *Shhcre* and *ShhcrERT2* alleles described in this report, it is now possible to conclusively determine the fate and function of the notochord, and during later development the nucleus pulposus, during disk degeneration in these mouse lines.

In addition to using the reagents described in this report to characterize disk degeneration in vivo, the ability to label notochord and intervertebral disk cells at any stage of mouse development will allow for the purification of these cells (for example using fluorescence activated cell sorting). Purified cells could then be cultured in vitro and reintroduced into degenerating disks or used in microarray experiments to identify novel genes expressed at different stages of nucleus pulposus formation.

In humans, intraosseous benign notochordal cell tumors have been identified in 20% of a random sample of 100 vertebral columns examined during autopsy. These benign tumors have been proposed to form from the embryonic notochord (Yamaguchi et al., 2004). In this study, notochordal cell tumors were identified through microscopic examination and the smallest tumors identified were 1 mm^2. The high incidence of notochordal cell tumors identified without the use of molecular markers suggests that the occurrence of these types of tumors in humans may be even higher. Our finding that notochordal remnants were present throughout the vertebral column in all samples analyzed supports this hypothesis.

In humans, intraosseous benign notochordal cell tumors formed from notochordal remnants are postulated to very rarely transform into malignant tumors called chordomas (Mendenhall et al., 2005). In humans, it is rare for this type of tumor to occur in patients <40 years old (Enomoto et al., 1986). Interestingly, chordomas have been reported to occur at a much lower rate then intraosseous benign notochordal cell tumors have been observed to occur (McMaster et al., 2001; Yamaguchi et al., 2004). The low occurrence of chordomas suggests that notochordal remnants lie dormant in most cases but may become malignant when stimulated, although the signals that initiate chordoma formation are unknown. Interestingly, the most prevalent DNA alteration in human chordomas has been reported to be an amplification of 7q36, which occurs in 69% of these types of tumors (Scheil et al., 2001). A similar chromosomal region has been proposed to contain a dominant oncogene in a family with familial chordomas (Kelley et al., 2001). In light of our findings that the *Shh*-expressing notochord forms notochordal remnants and all cell types in the mature nucleus pulposus, it is interesting that the region of chromosome 7 implicated in chordoma formation contains the gene *Shh* (Marigo et al., 1995).

Since the occurrence of notochordal remnants in humans is much higher than the reported incidence of chordomas, a second event, possibly a mutation or environmental insult during later life, must occur to cause notochordal cells to form tumors. Mutations resulting in constitutive activation of the *Shh* signaling pathway have been shown to result in the formation of numerous types of cancers in humans (McMahon et al., 2003) and in mice, artificial overexpression of SHH in skin using a transgenic allele resulted in the development of a basal cell carcinoma-like tumor (Fan et al., 1997).

An enhancer element responsible for notochord expression has been identified in mice (Jeong and Epstein, 2003). If a similar enhancer element exists in humans, very rare activating mutations in this enhancer may result in overexpression (or sustained) expression of SHH in notochordal remnants in older patients. These findings raise the possibility that ectopic expression of SHH in notochordal remnants may cause chordomas by inducing these cells to behave like nucleus pulposus stem cells. In addition, *Brachyury* (Vujovic et al., 2006), *Tsc1/2* (Lee-Jones et al., 2004), and *p16/CDKN2A* (Hallor et al., 2008) have been indirectly implicated in chordoma formation. The identification of mouse notochordal remnants raises the possibility of creating a mouse model of chordoma by altering expression of these genes in mouse notochordal remnants.

EXPERIMENTAL PROCEDURES

Strain Construction and Genotyping

The creation and genotyping of *Shhcre, ShhcreERT2, R26R*, and *R26R:EYFP* alleles have been described previously (Soriano, 1999; Srinivas et al., 2001; Harfe et al., 2004). *Shhcre* or *ShhcreERT2* mice were mated to mice containing reporter alleles to create double heterozygous male animals. These animals were either mated to wild type females or analyzed directly.

Fig. 3. The nucleus pulposus and notochordal remnants in adult mice are composed of cells that have expressed *Shh*. **A:** In a 19-month-old *Shhcre;R26R* mouse, the nucleus pulposus is labeled and the annulus fibrosus is negative. A 10-μm transverse paraffin section of an intervertebral disk stained for LacZ is shown. Cells were counterstained with fast red, which clearly marked the nuclei of cells. **B:** Magnification (40×) of the boxed region in A. In all samples examined (four disks from adult mice), all cells of the nucleus pulposus were stained blue indicating that the nucleus pulposus is composed of cells that have expressed *Shh*. **C:** Notochordal remnants were found in adult vertebrae between each intervertebral disk in the *Shhgfpcre;R26R* mouse. Inset in C shows notochord remnants. Ventral view of the vertebral column is shown. Adult animals were stained in whole-mount for LacZ and then dissected. Whole-mount picture is shown. V, vertebrae between the disks; NP, nucleus pulposus; AF, annulus fibrosus.

Detection of Reporter Activity

A single injection of tamoxifen (6 mg/40 g body weight) was intraperitoneally (IP) injected into pregnant dams. This dose has been shown to produce complete recombination of floxed genes (Hayashi and McMahon, 2002). LacZ staining was performed as described previously (Harfe et al., 2004). EYFP was detected using a Lecia MZ16 microscope and DFC300FX camera.

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