Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer

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Androgen independence is responsible for most prostate cancer lethality, yet currently there are no effective clinical treatments. We have been investigating the mechanisms underlying androgen-independent prostate cancer in Nkx3.1;Pten mutant mice, which display salient features of the disease, including a requirement for wild-type androgen receptor (AR) signaling. We now demonstrate that the Akt and Erk MAP kinase signaling pathways are activated in androgen-independent lesions of these mice. Forced activation of either Akt or Erk signaling in an androgen-responsive prostate cancer cell line promotes hormone-independent but AR-dependent growth in culture. Although these pathways act additively in culture, they act synergistically in vivo to promote tumorigenicity and androgen independence in the context of the prostate microenvironment. We propose that androgen independence emerges by means of epithelial–stromal competition, in which activation of Akt and Erk promotes AR activity in the prostate epithelium while counteracting antagonistic effects of the stroma.

Akt signaling | androgen independence | MAP kinase signaling | mouse models of cancer

Androgen deprivation therapy remains the most widely used treatment for patients with advanced prostate cancer (1). However, it is not curative because tumors eventually recur, and those that emerge in the absence of androgens (androgen-independent/hormone-refractory tumors) are typically less differentiated, more aggressive, and more highly metastatic than those that initially arose in the presence of androgens (androgen-dependent tumors). Despite their ability to grow under conditions of limiting androgens, most hormone-refractory tumors remain dependent on androgen receptor (AR) signaling function (2, 3). In fact, several lines of evidence have shown that AR is up-regulated in hormone-refractory prostate cancer (e.g., ref. 4) and that AR overexpression promotes tumorigenicity in transgenic and orthotopic models (5, 6). Although sometimes amplified or mutated, the AR remains wild-type in the majority of hormone-refractory prostate tumors (2), suggesting that these tumors can maintain AR function in conditions of limiting androgens. Adding to this complexity, both the epithelial and mesenchymal compartments of the prostate require AR function (7), yet the respective roles of AR in these compartments for androgen independence have not been fully defined.

We have investigated the mechanisms underlying hormone-refractory prostate cancer using a mouse model based on the loss-of-function of Nkx3.1 and Pten (8, 9). Nkx3.1 encodes a homeodomain transcription factor located on a region of human chromosome 8p21 that is frequently deleted in prostate intraepithelial neoplasia (PIN) as well as prostate cancer (reviewed in refs. 10 and 11). Reduction or loss of NKX3.1 expression is a hallmark of prostate cancer in mice and humans (12–15), whereas its loss of function in mice leads to PIN (14, 16, 17). PTEN encodes a tumor suppressor gene that is frequently inactivated in prostate cancer through deletion, mutation, and/or down-regulation of its protein expression, resulting in activation of Akt/protein kinase B (18, 19). In mice, Pten loss of function leads to high-grade PIN and/or cancer and can collaborate in cancer progression with inactivation of other genes, including Nkx3.1 (e.g., refs. 8, 9, and 15).

Here we show that Nkx3.1;Pten compound mutant mice develop androgen-independent prostate cancer that retains wild-type AR expression and function. In this model and its derivative CASP cell lines, Akt and Erk MAP kinase signaling pathways act additively in cell culture but synergistically in vivo to promote androgen independence. Our findings suggest that combined pharmacological inhibition of Akt and Erk pathways together with androgen ablation may provide an effective therapy for hormone-refractory disease and emphasize the need for testing potential therapeutics in the context of the prostate microenvironment.

Results

Nkx3.1; Pten Mutant Mice Develop Androgen-Independent Lesions with Wild-Type AR. Compound mutant mice having germ-line deletion of one or both alleles of Nkx3.1 as well as one allele of Pten (Nkx3.1+/−; Pten wild-type) develop PIN and invasive adenocarcinoma as a consequence of aging as well as androgen independence after castration (8, 9). In particular, although castration of wild-type mice leads to regression of the prostate epithelium (Fig. 1 A and B), the castrated Nkx3.1;Pten mutant mice develop high-grade PIN and/or cancer with similar histological features as those found in noncastrated mutant mice (Fig. 1 C and D) (8, 9).

Given the central role of AR in hormone-refractory prostate cancer in humans (2), we investigated its status in androgen-independent lesions from the castrated Nkx3.1;Pten mutant mice. We found that AR protein was expressed in the prostate epithelium (Fig. 1 E–H). Notably, we failed to detect any mutations after sequencing of the entire AR coding region from prostate tissue of castrated or intact mice (n = 9) (Fig. 6 A, which is published as supporting information on the


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Abbreviations: AR, androgen receptor; PIN, prostate intraepithelial neoplasia.

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PNAS web site). Furthermore, equivalent levels of AR were expressed in prostate tissue of castrated and noncastrated mice (n = 17) (Fig. 6B). Therefore, the androgen-independent lesions of the Nkx3.1;Pten mutant mice retain wild-type AR.

**Activation of Akt and Erk MAP Kinase Signaling Pathways in Androgen-Independent Prostate Cancer.** A prominent feature of androgen-independent lesions from Nkx3.1;Pten mutant mice is robust activation of the Akt signaling pathway as a consequence of Pten loss of function (18, 19). Akt was uniformly activated in all androgen-independent lesions in these mice (Figs. 1 I–L and 6C) and was accompanied by focal activation of its downstream effector, S6 kinase (Fig. 7 A–D, which is published as supporting information on the PNAS web site). These findings are in agreement with studies showing that Akt is activated in advanced stages of human prostate cancer (20, 21).

To gain insights into additional pathway alterations in castrated Nkx3.1;Pten mutant mice, we investigated the activity of MAP kinase signaling pathways, which have been implicated in human prostate cancer progression and androgen independence (22). Using phosphospecific antibodies, we found that the MAP kinases Erk1/Erk2 (hereafter referred to as Erk), rather than p38 or Jnk, were activated in the androgen-independent lesions of Nkx3.1;Pten mutant mice (Figs. 1 M–P and 6C). Furthermore, inspection of a human tissue microarray (23) revealed that Akt and Erk were activated in a significant percentage of tumors that had undergone androgen deprivation therapy and were frequently coexpressed with AR (Fig. 8, which is published as supporting information on the PNAS web site). Together, our findings indicate that Akt and Erk are frequently activated in androgen-independent disease in Nkx3.1;Pten mutant mice and human prostate cancer.

**Androgen Independence Requires AR Signaling.** We further investigated the relationship between Akt and Erk activation and AR signaling for androgen independence using a tissue recombinant approach. We generated tissue recombinants comprised of prostate epithelium from adult wild-type or mutant mice and mesenchyme from the urogenital sinus of embryonic rats and implanted these under the kidney capsule of nude male hosts (for details see Supporting Methods, which is published as supporting information on the PNAS web site). Whereas tissue recombinants made with epithelium from wild-type Nkx3.1;Pten mutant mice regressed after castration of the hosts (Fig. 2 A and B), those with prostate epithelium from Nkx3.1;Pten mutants grew equally well in the castrated and noncastrated hosts (n = 10 per group) (Fig. 2 E and F). These tissue recombinants contained high-grade PIN lesions that histologically resembled those in castrated Nkx3.1;Pten mutant mice (compare Figs. 1 D and 2 I and J) and expressed AR as well as activated Akt and Erk (Fig. 2 M, N, Q, R, U, and V), demonstrating sufficiency of the Nkx3.1;Pten prostate epithelium for androgen independence.

We investigated the requirement of AR signaling for androgen-independent growth using Flutamide, a pharmacological inhibitor of AR (1). Specifically, we generated tissue recombinants in castrated hosts that were implanted with silicon pellets containing flutamide. We found that flutamide abolved growth of the recombinants in the castrated hosts regardless of whether they were generated from wild-type or mutant epithelium (n = 6 per group) (Fig. 2 D and H). Furthermore, the recombinants generated with mutant epithelium and grown in flutamide-treated castrated hosts failed to develop high-grade PIN (Fig. 2L). Similar findings were observed after administration of flutamide to castrated Nkx3.1;Pten mutant mice (n = 6) (W.A.B.-P. and C.A.-S., unpublished data), indicating that the
inhibitory effects of flutamide for prostate growth in the absence of androgens are not restricted to tissue recombinants. The inhibitory consequences of flutamide are consistent with its known antagonist function when AR is expressed at normal levels, as is the case for the Nkx3.1;Pten mutant mice (see Fig. 6B), and contrast with its agonist functions when AR expression is elevated (e.g., ref. 4).

Activation of Akt and/or B-Raf/Erk Confers Androgen-Independent Growth in Culture. To investigate the functional consequences of Akt and Erk activation for prostate tumorigenicity and androgen independence, we used prostate epithelial cell lines that we previously generated from Nkx3.1;Pten mutant mice (24). These CASP cell lines include nine independent epithelial lines that are derived from primary prostate tumors and are moderately tumorigenic (24) (Table 1, which is published as supporting information on the PNAS web site). Two of these cell lines (CASP 2.1 and CASP 6.0) depend on androgens for growth in culture and can be paired with cells lines (CASP 1.1 and CASP 1.2) with similar growth and tumorigenic properties that are not androgen-responsive (Table 1). Importantly, sequence analyses of the entire coding region have confirmed that AR remains constitutively active (26). For activation of the Erk pathway, we expressed a mutated form of B-Raf (B-RafV600E, *B-Raf) (27), in part because B-Raf is up-regulated during cancer progression and in the androgen-independent lesions of the Nkx3.1; Pten mutant mice (Fig. 6 E–H). As expected, forced expression of *Akt and/or *B-Raf resulted in Akt and Erk activation, respectively (Fig. 3A).

We found that forced expression of either *Akt or *B-Raf alleviated the requirement for androgens for proliferation and anchorage-independent growth of CASP 2.1 cells in culture, whereas coexpression of both *Akt and *B-Raf had an additive effect (Fig. 3 B, C, and E); similar results were obtained with a second androgen-responsive line, CASP 6.0 (data not shown). The growth-promoting activities of *Akt and *B-Raf in the absence of androgens were dependent on AR signaling because they were completely blocked by flutamide (Fig. 3F). Moreover, the growth-promoting activities of *Akt in the absence of androgens were mediated by the m-TOR pathway because they were blocked by rapamycin, whereas those of *B-Raf were mediated by MAP kinase signaling because they were blocked by U0126 (Fig. 3 D and G). In addition, we found that treatment with rapamycin together with U0126 inhibited proliferation of the non-androgen-responsive cell line CASP 1.1 (Fig. 9, which is published as supporting information on the PNAS web site). Notably, rapamycin and U0126 have also been shown to inhibit the androgen-independent growth of human prostate cancer cell lines in culture (28). Taken together, these findings demonstrate that Akt and MAP kinase signaling pathways act individually as

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**Fig. 3.** Activation of Akt and B-Raf/Erk signaling pathways is additive for androgen independence in cell culture. Retroviral vectors expressing *Akt or *B-Raf or a control (vector) were introduced individually or in combination into the androgen-responsive cell line CASP 2.1, and cells were grown in conditions of androgen deprivation. (A) Western blot analyses of CASP 2.1 protein extracts using the indicated antibodies. (B and C) Proliferation (B) and anchorage independence (C) assays after growth in media depleted of androgens or with androgens (+ DHT). Shown are representative assays done in triplicate; error bars represent one standard error. (D) Western blot analyses of cells expressing *Akt plus *B-Raf and treated with 50 mM rapamycin and/or 50 mM U0126 (as indicated). (E–G) Cell-cycle analyses of CASP 2.1 cells expressing *Akt and/or *B-Raf. Shown are relative percentages of cells in G2/M phase compared with the vector or vehicle control. (Insets) FACS data and percentage of cells in G2/M for representative samples (indicated by a and b). Cells were grown in androgen-depleted media without additions (E) or with 20 mM flutamide (F) or 50 nM rapamycin and/or 50 mM U0126 (G) as indicated. Experiments were performed a minimum of three times in triplicate; the standard error is shown. **, *P < 0.01 (between a and b in each set).
well as additively to promote growth and tumorigenicity of prostate cells in culture in the absence of androgens.

Activation of Akt and B-Raf/Erk Synergizes in Androgen-Independent Growth in Vivo. To investigate the consequences of Akt and/or Erk pathway activation for tumorigenicity and androgen independence in vivo, we performed orthotopic injection of CASP 2.1 cells expressing Akt and/or B-Raf into the dorsal prostate of castrated or mock-castrated nude male hosts (Fig. 4 A–F and Fig. 10, which is published as supporting information on the PNAS web site). In the noncastrated hosts, the Akt-expressing cells developed small but discernible tumors (3–4 mg; n = 16), whereas the B-Raf-expressing cells formed tumors that were significantly larger (15–25 mg; n = 12) (Fig. 4 A and B). However, cells expressing both Akt and B-Raf formed very large tumors that encompassed the entire urogenital region (>250 mg; n = 12) (Fig. 4 C). In the castrated hosts, CASP 2.1 cells expressing either Akt or B-Raf alone formed tumors that were significantly smaller than those grown in the intact hosts (~1 mg and 5 mg, respectively; n = 16 per group) (Fig. 4 D and E). In contrast, cells expressing Akt plus B-Raf formed large tumors in the castrated hosts that were similar in size to those in the intact hosts (>250 mg; n = 12) (Fig. 4 F).

We observed similar results in a cell recombination assay, in which CASP 2.1 cells were combined with embryonic urogenital mesenchyme and implanted under the kidney capsule, followed by growth in castrated or intact nude male hosts (Figs. 10 and 11, which are published as supporting information on the PNAS web site). We found that cells expressing Akt plus B-Raf gave rise to recombinants that were larger than those expressing either Akt or B-Raf alone (n = 10 per group) (Fig. 11 A–D). Furthermore, recombinants expressing Akt plus B-Raf grew comparably well in the castrated and androgen-intact hosts (n = 10 per group), unlike those expressing either Akt or B-Raf, which were significantly smaller in the castrated hosts (Fig. 11 E–H). Notably, flutamide was ineffective at inhibiting the growth of the recombinants made with cells expressing Akt plus B-Raf, in striking contrast to its inhibitory action in culture (compare Figs. 3F and 11J–N).

The striking difference between the additive effects of Akt and B-Raf on cells in culture and their synergism in vivo is likely to be related to their behavior in response to androgen deprivation under these two conditions. Although CASP 2.1 cells depleted of androgens in culture cease to grow but do not undergo apoptosis (Fig. 4 G–J), these cells undergo apoptosis in the prostate microenvironment, an effect that is overcome by forced expression of Akt and B-Raf in combination but not alone (Fig. 4 K–N and Fig. 12, which is published as supporting information on the PNAS web site). Therefore, activation of Akt or B-Raf/Erk pathways can act separately and additively to promote androgen-independent growth in cell culture, whereas they act synergistically to promote androgen independence in the context of the prostate microenvironment in vivo.

Discussion

Following the work of Hodges and Huggins (29), the term “androgen independence” was coined to define hormone-refractory prostate cancer that arose in patients after androgen deprivation therapy. Over the years our understanding of androgen independence and its relationship to prostate tumorigenesis and AR signaling has evolved considerably. Despite initial presumptions to the contrary, the occurrence of hormone-refractory disease does not signify a lack of dependence on AR signaling (2, 3). Instead, AR is functional in most hormone-refractory tumors (2) and clearly plays a pivotal role in the emergence of androgen independence (e.g., ref. 4). The current study demonstrates central roles for the Akt and Erk MAP kinase signaling pathways in promoting androgen independence in an AR-dependent manner. We find that these signaling pathways can be activated individually or together to promote androgen independence (Fig. 5).

Although activation of Akt has been previously implicated in androgen independence, many aspects of its role and relation-
ship to AR signaling have remained unresolved (reviewed in refs. 3 and 19). Some studies performed in cell culture have suggested that Akt may enhance AR function and/or stability, possibly by direct phosphorylation, whereas others have proposed that Akt can inhibit AR function. These discrepancies may arise from the complexity of Akt function in different cell lines that vary in their AR status. More generally, these studies may be limited in their ability to extrapolate from cell culture models to the in vivo phenomenon of androgen independence.

In addition to Akt, activation of MAP kinase signaling is also functionally relevant in prostate cancer progression and androgen independence (22). MAP kinase pathway activation is thought to occur by up-regulation of HER2/neu or other growth factor signaling, which confers hormone-independent growth of cells in an Akt-independent manner (30). However, roles for Ras and/or Raf for activation of MAP kinase pathway signaling in prostate cancer are less clear. Although expression of v-Ras confers hormone-independent growth of human prostate cancer cells in culture (31), mutations of neither Ras nor Raf have been reported in prostate cancer, except in limited populations (27, 32, 33). Interestingly, Raf-1 is up-regulated in androgen-independent human prostate cancer (34), and we have observed up-regulation of B-Raf as well as down-regulation of the Raf inhibitor RKIP in androgen-independent lesions of Nkx3.1;Pten mutant mice (X.O. and C.A.-S., unpublished observations), raising the possibility that the pathway may be deregulated even in the absence of B-Raf mutation.

Notably, our findings highlight the distinction between the androgen-responsive properties of prostate epithelial cells in culture and the androgen dependence of the prostate gland in vivo (35, 36). In particular, our results imply that androgen independence is a consequence of molecular events within the prostate epithelium that include activation of Akt and/or Erk, which promote AR activity in the epithelium while counteracting antagonistic effects of the prostate stroma (Fig. 5). Importantly, Cunha and colleagues (37) have shown that stromal expression of AR is necessary for epithelial apoptosis after castration, presumably via stromal expression of paracrine death-inducing factors. Thus, AR signaling is not only required in the epithelium for its survival and proliferation, it is also necessary in the stroma for normal prostate homeostasis and the apoptotic response to androgen deprivation.

Therefore, we propose that the combined activation of Akt and Erk pathways stimulates epithelial AR function and blocks apoptosis-inducing paracrine signals from the stroma, leading to androgen-independent cancer (Fig. 5). Notably, in this view, androgen independence does not simply arise through superactivation of proliferative and antiapoptotic pathways in a cell-autonomous manner within the prostate epithelium. Instead, under conditions of limiting androgens, the emergence of androgen-independent tumors reflects the outcome of a competition between the proliferative activities of the epithelium and the proapoptotic activities of the stroma, both of which require AR function. Furthermore, our findings suggest that combined pharmacological inhibition of Akt and Erk pathways simultaneous with androgen ablation may provide an effective treatment for hormone-refractory prostate cancer and emphasize the importance of assessing potential therapeutic interventions in the context of the prostate microenvironment.

Materials and Methods

Analyses of Androgen-Independent Phenotypes in Nkx3.1;Pten Mutant Mice. The Nkx3.1;Pten mutant mice have been described previously (8, 9, 17). Castration was performed by surgical removal of the testes and epididymis. Histopathological analyses of the prostate and other tissues were done as described (8, 9, 17). Criteria for designation of PIN and/or cancer have been described (8, 9, 14). Histological analyses shown are for anterior prostate; similar results were observed in other prostate lobes (H.G. and C.A.-S., unpublished data). Tissue recombinants were made by using dissociated prostate epithelium from adult wild-type or mutant mice and mesenchyme from rat embryonic urogenital sinus and grown in athymic nude male mice as described (8, 14, 17). To examine growth after androgen ablation, tissue recombinants were grown for 2 weeks followed by surgical castration of the host and growth for an additional 4 weeks. Where indicated, flutamide was administered by implantation of 100-mg pellets (Innovative Research of America, Sarasota, FL), which were surgically implanted in the upper back at the time of castration or mock surgery. Additional details are provided in Supporting Methods and Table 2, which are published as supporting information on the PNAS web site.

Gain-of-Function Studies in CASP Cells. The CASP cell lines were derived from Nkx3.1;Pten mutant mice, as reported (24). Retroviral gene transfer (24) was used to introduce myristoylated Akt (26) and/or mutated B-Raf (B-RafV600E) (27) into CASP 2.1 cells. Proliferation assays and anchorage-independent growth assays were performed as described (14) by using media containing 1% charcoal/dextran-treated FBS. For cell-cycle analysis, cells were grown in media containing charcoal/dextran-stripped FBS and analyzed by using a CytoFacs FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA). Where indicated, cells were grown in the presence of 20 μM flutamide (Sigma, St. Louis), 50 nM rapamycin (Sigma), or 50 μM U0126 (Cell Signaling Technology, Beverly, MA) for 24–48 h before harvesting.

Orthotopic tumor and cell recombination and assays were performed as described (24). Briefly, for orthotopic assays, 5 × 105 of the *Akt- and/or *B-Raf-expressing CASP 2.1 cells were injected unilaterally into the left dorsal prostate of athymic nude male mice; an equal number of cells expressing vector were injected into the right lobe. Cell recombinants were made by mixing 1.8 × 106 CASP 2.1 cells expressing *Akt and/or *B-Raf with 2.5 × 105 rat embryonic mesenchymal cells and grafted under the kidney capsule of nude male hosts. After growth for 2 weeks, the hosts were castrated or mock-castrated followed by growth for an additional 4 weeks. Wet weights of orthotopic tumors were determined by comparing the experimentally injected and control-injected sides. Histological analyses of the orthotopic tumors and cell recombinants are provided in Fig. 10.

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Corrections

MEDICAL SCIENCES. For the article “Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer,” by Hui Gao, Xuesong Ouyang, Whitney A. Banach-Petrosky, William L. Gerald, Michael M. Shen, and Cory Abate-Shen, which appeared in issue 39, September 26, 2006, of Proc Natl Acad Sci USA (103:14477–14482; first published September 14, 2006; 10.1073/pnas.0606836103), the authors note that the images in Fig. 4 H and I, and in Fig. 4 G and J, were inadvertently duplicated. The corrected figure and its legend appear below. These errors do not affect the conclusions of the article.

Fig. 4. Activation of Akt and B-Raf/Erk signaling pathways is synergistic for androgen independence in vivo. (A–F) Orthotopic tumor assays. CASP 2.1 cells expressing *Akt and/or *B-Raf (experimental) or a control vector (vector) were grown orthotopically in the dorsal prostates of nude male mice that were castrated or mock-castrated. Shown are representative images of dorsal prostate lobes showing the experimental or vector-injected sides. Note that the prostates infected with *Akt and *B-Raf completely overgrew the urogenital system, and thus the vector control is not evident. (G–N) Analyses of apoptosis in culture and in vivo. CASP 2.1 cells expressing the vector or *Akt plus *B-Raf were grown in culture (G and H) or orthotopically in the prostate (K–N). TUNEL assays were performed 2 days after androgen deprivation of the cells in culture or 2 days after castration of the host.

MEDICAL SCIENCES. For the article “Cyclophilin D inactivation protects axons in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis,” by Michael Forte, Bruce G. Gold, Gail Marracci, Priya Chaudhary, Emy Basso, Dustin Johnsen, Xiaolin Yu, Jonathan Fowlkes, Paolo Bernardi, and Dennis Bourdette, which appeared in issue 18, May 1, 2007, of Proc Natl Acad Sci USA (104:7558–7563; first published April 26, 2007; 10.1073/pnas.0702228104), the authors request that Micha Rahder and Katie Stem be added to the author list, between Jonathan Fowlkes and Paolo Bernardi, and be credited with performing research. The online version has been corrected. The corrected author and affiliation lines and author contributions appear below.

Michael Forte*, Bruce G. Gold†, Gail Marracci‡§, Priya Chaudhary¶, Emy Basso‡, Dustin Johnsen*, Xiaolin Yu‡, Jonathan Fowlkes*, Micha Rahder*, Katie Stem‡, Paolo Bernardi¶, and Dennis Bourdette‡*

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DEVELOPMENTAL BIOLOGY. For the article “The mouse homeobox gene Noto regulates node morphogenesis, notochordal ciliogenesis, and left–right patterning,” by Anja Beckers, Leonie Alten, Christoph Viebahn, Philipp Andre, and Achim Gossler, which appeared in issue 40, October 2, 2007, of Proc Natl Acad Sci USA (104:15765–15770; first published September 20, 2007; 10.1073/pnas.0704344104), the authors note that, due to a printer’s error, an incorrect definition was given for the abbreviation PNC in the first paragraph of the Introduction and in the Abbreviations footnote. The correct definition is “posterior notochord.” The online version has been corrected. The corrected Abbreviations footnote appears below.

Abbreviations: E(n), embryonic day n; PNC, posterior notochord.

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