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Wnt and Notch Pathways Have Interrelated Opposing Roles on Prostate Progenitor Cell Proliferation and Differentiation

Payam Shahi^{a,b}, Mamatha R. Seethammagari^a, Joseph M. Valdez^c, Li Xin^c, and David M. Spencer^{a,b}

^aDepartment of Pathology and Immunology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, USA

^bInterdepartmental Program in Cell and Molecular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, USA

^cMolecular and Cellular Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas, USA

Abstract

Tissue stem cells are capable of both self-renewal and differentiation to maintain a constant stem cell population and give rise to the plurality of cells within a tissue. Wnt signaling has been previously identified as a key mediator for the maintenance of tissue stem cells; however, possible cross-regulation with other developmentally critical signaling pathways involved in adult tissue homeostasis, such as Notch, is not well understood. By using an in vitro prostate stem cell colony (“prostatesphere”) formation assay and in vivo prostate reconstitution experiments, we demonstrate that Wnt pathway induction on Sca-1⁺ CD49f⁺ basal/stem cells (B/SCs) promotes expansion of the basal epithelial compartment with noticeable increases in “triple positive” (cytokeratin [CK] 5⁺, CK8⁺, p63⁺) prostate progenitor cells, concomitant with upregulation of known Wnt target genes involved in cell-cycle induction. Moreover, Wnt induction affects expression of epithelial-to-mesenchymal transition signature genes, suggesting a possible mechanism for priming B/SC to act as potential tumor-initiating cells. Interestingly, induction of Wnt signaling in B/SCs results in downregulation of Notch1 transcripts, consistent with its postulated antiproliferative role in prostate cells. In contrast, induction of Notch signaling in prostate progenitors inhibits their proliferation and disrupts prostasphere formation. In vivo prostate reconstitution assays further demonstrate that induction of Notch in B/SCs disrupts proper acini formation in cells expressing the activated Notch1 allele, Notch-1 intracellular domain. These data emphasize the importance of Wnt/Notch cross-regulation in adult stem cell biology and suggest that Wnt signaling controls the proliferation and/or maintenance of epithelial progenitors via modulation of Notch signaling.

Keywords

Prostate stem cell; Wnt; β -Catenin; Notch; p63

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Correspondence: David M. Spencer, Ph.D., Baylor College of Medicine, One Baylor Plaza/M929, Houston, Texas 77025, USA. Telephone: 713-798-6475; Fax: 713-798-3033; dspencer@bcm.edu.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

INTRODUCTION

Despite expanded treatment options, prostate cancer (PCa) remains the second leading cause of cancer-related death in men in western countries [1]. Investigations into the molecular basis for prostate growth have revealed novel roles for developmentally regulated genes in tumor progression. Signaling pathways such as Wnt, Notch, and Hedgehog have proven to be critical for proper embryogenesis and remain important throughout adulthood for maintaining tissue homeostasis. In particular, these signaling pathways function as key regulatory mechanisms in adult stem cell maintenance. Notably, the Wnt signaling pathway plays a recently recognized key role in development and homeostasis of the prostate [2–6]. Accordingly, mutations in this pathway are highly correlative with prostate tumor formation. Immunohistological analysis of β -catenin, an essential signal transducer for the canonical Wnt pathway, has provided early evidence for Wnt pathway involvement in prostate tumorigenesis. The data revealed Wnt pathway activation by evidence of β -catenin nuclear localization in 20% of lethal PCa cases [7]. Since these initial findings, follow-up studies have further expanded the percentage of aggressive PCa cases resulting from deregulation of Wnt/ β -catenin. High levels of Wnt1 and β -catenin have been reported in 77% of lymph node metastases and in 85% of skeletal metastases. Moreover, β -catenin can directly interact with the androgen receptor (AR) and augment its activity [8]. Thus, AR:Wnt crosstalk adds an additional layer of complexity to the Wnt pathway's role in prostate biology [9–11].

In the absence of Wnt ligand, the “destruction complex,” comprising axin, adenomatous polyposis coli, glycogen synthase kinase-3 β (GSK-3 β), and other proteins sequesters and phosphorylates cytoplasmic β -catenin. Following phosphorylation, β -catenin is ubiquitinated by the SCF ^{β -TrCP1} -complex before proteasomal degradation [12], maintaining low free cytoplasmic concentrations. However, when Wnt ligands associate with Frizzled family receptors and coreceptors, low-density lipoprotein receptor-related protein 5 (LRP5) or LRP6, a signaling cascade initiates involving activation of regulatory adapter protein Dishevelled (Dvl). Dvl activation, in turn, leads to dissociation of the destruction complex and accumulation of β -catenin, which can translocate to the nucleus to associate with promoter-bound lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) transcription complexes [13]. This association results in displacement of Groucho-like family proteins, converting LEF/TCF from a corepressor into a coactivator, allowing for Wnt-induced expression of target genes.

Wnt pathway induction also promotes proliferation and self-renewal of adult stem cells in various tissues, such as hematopoietic stem cells (HSCs), intestinal stem cells, and neuronal stem cells [14–16]. The ability of the Wnt pathway to promote adult stem cell proliferation and self-renewal is associated with its ability to influence the Notch signaling pathway, which is upregulated as a result of Wnt pathway induction [16]. Notch signaling has been shown previously to promote “stemness” in HSCs [17], but has potentially distinct outcomes in other tissues. Propagation of Notch signaling is initiated when one or more of the four Notch receptors come into contact with Delta or Jagged family ligands on adjacent cells. Notch “liganding” leads to two proteolytic cleavages of the receptor, resulting in release of the Notch intracellular domain (NICD) [18, 19]. On its release, NICD translocates to the nucleus where it converts the transcription factor RBP-Jk from a transcriptional repressor to an activator, thereby promoting expression of Notch target genes [20, 21].

Although accumulating data has underlined the importance of crosstalk between Wnt and Notch pathways [22, 23], their respective roles in prostate and prostate stem cell (PSC) biology and Wnt's ability to cross-regulate Notch signaling remains elusive. Given the above findings, it is likely that deregulation of Wnt signaling could lead to further

perturbations of other signaling pathways, leading to an imbalance in stem cell numbers and potential tumorigenesis.

The existence of PSCs was first demonstrated through multiple rounds of androgen inhibition with ensuing prostate involution, followed by androgen replacement-mediated prostate regeneration [24]. Recently, more direct evidence for the existence of PSCs has been collected through identification of several cell surface markers, such as Sca-1, CD49f, c-kit (CD117), CD44, and CD133, that can be used to enrich for PSCs [25]. A recent study demonstrated that basal/stem cells (B/SCs) marked by Lin⁻Sca1⁺CD49f⁺ cells (LSCs) are highly enriched for PSC activity [26, 27]. In that study, 1 in 40 LSCs had the ability to form self-renewing spherical colonies (“prostaspheres”) in vitro. Moreover, transplantation studies using LSCs in combination with inductive murine urogenital sinus mesenchyme (UGSM) have proven that LSCs contain PSCs that can promote prostate gland regeneration. Because of the high incidence of androgen-independent PCa relapse, tumor-initiating cancer progenitor cells (CPCs) have been proposed as a possible cause of PCa development. Moreover, Lawson et al. [28] were able to fractionate various prostate cell populations and, via prostate regeneration studies, demonstrate that only the B/SC fraction is capable of regenerating prostate ducts with a neoplastic phenotype. They showed that the B/SC fraction possesses PSCs that when induced with proliferative signals, such as AKT or fibroblast growth factors (FGFs), are the target of cell transformation. In contrast, recent work by Wang et al. [29] have identified a distinct stem cell source, castration-resistant NK3.1-expressing cells as possible prostate CPCs.

Herein, we have investigated the role of Wnt signaling in LSCs. LSCs were subjected to Wnt pathway induction via recombinant Wnt3a ligand, Wnt3a-conditioned media (Wnt3a-CM), or lentivirus (LV)-mediated transduction of constitutively active β -catenin (β -cat*-LV). The Wnt signaling pathway induction in PSC-enriched LSCs promotes enlargement of prostaspheres and expansion of “triple positive” (TP) prostate progenitor cells, expressing cytokeratin (CK) 5, CK8, and p63 in vitro. Moreover, reconstituted prostate grafts expressing β -cat*-LV demonstrated expansion of p63⁺ cells concomitant with increased CK5/CK8 double positive (DP) cells. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis further indicates upregulation of proliferative and antiapoptotic genes in Wnt-activated LSCs. Furthermore, we observed changes in expression of key epithelial-to-mesenchymal transition (EMT) signature genes, suggesting that Wnt induction can potentially prime PSCs for malignant transformation.

In contrast to canonical Wnt signaling, recent findings have suggested an antiproliferative role for the Notch pathway in prostate cells [30, 31]. Consistent with this, in response to Wnt pathway induction, *Notch1* mRNA and protein were both downregulated. Moreover, Notch pathway inhibition via a γ -secretase inhibitor promoted enlargement of prostaspheres, whereas Notch1 overexpression via the constitutive NICD inhibited prostasphere formation in vitro and proper prostate duct development, in reconstitution experiments. Together, these observations indicate that Wnt signaling promotes proliferation of B/SCs via upregulation of cell cycle-stimulating genes and downregulation of Notch1. Furthermore, activation of the Wnt pathway in B/SCs induces an EMT signature, suggesting that Wnt pathway induction could potentially drive B/SCs into tumor-initiating cells.

MATERIALS AND METHODS

Animals, Cell Lines, and Plasmids

All mice were housed at the Baylor College of Medicine Transgenic Mouse Facility. Wnt3a-expressing cells were obtained from American Type Culture Collection, (ATCC, Manassas, VA, <http://www.atcc.org>; CRL-2647). Wnt3a-CM was produced according to the

manufacturer's instructions. Control-CM was obtained from L cells (ATCC; CRL-2648). Constitutively active β -catenin contains several Ser/Thr to Ala changes in the GSK-3 β phosphorylation target region of β -catenin, including S33A, S37A, T41A, and S45A [32]. β -Cat*-LV was kindly provided by T. Reya (Duke University, NC). LV-encoding the intracellular domain of Notch1 (NICD-LV) was made in two steps. Initially, a functional human ICN domain (starting at E1761) was amplified from MSCV-Notch-ICN (a generous gift from D. Scadden [33]) using *Xho*I-linked primer, 5'-caagtcctcgaggagggttcaagtgctgagg-3' and *Sal*I-linked primer, 5'-gtcaagtcgacctgaaggcctccggaatcg-3' to get the 2.4-kb Notch1-ICN(X,S) fragment, which was, in turn, subcloned into *Xho*I/*Sal*I-digested expression vector pSH1/Sn-F_vF_{VL}S-hLRP5c-FLAG to get pSH1-Sn-Notch(ICN)-FLAG ("ICN-FLAG"). Second, the NICD from ICN-FLAG was reamplified using *Eco*RI-linked primers 5'-aactgaattcgccaccatgggactcgaggaggc-3' and 5'-tcaggaattctcaagctgtcatcgctcg-3' and cloned into *Eco*RI-digested lentivector, FU-CRW [34].

LV Production

LV was generated by transfecting 7×10^6 293T cells with 15 μ g of gag/pol plasmid, 6 μ g of vesicular stomatitis virus glycoprotein, 9 μ g of LV vector via FuGENE6 transfection reagent (Roche, Indianapolis, IN, <http://www.roche-diagnostics.us/>) overnight (ON). On day 2, transfection media was replaced with 10 ml fresh media. Viral supernatant was collected 2 and 4 days post-transfection. Viral supernatant was concentrated by centrifugation for 3 h at 2.5×10^4 relative centrifugal force (RCF) using SW32Ti rotors at 4°C. Supernatant was removed and 500 μ l of viral concentrate (pellet) was saved as concentrated virus.

In Vitro Sphere Cultures, Sphere Isolation, RNA Isolation, and qRT-PCR Analysis of Prostatespheres

LSCs were cultured in Matrigel (BD Bioscience, Franklin Lakes, NJ, <http://www.bd.com/>) as previously described [27]. LSCs were cultured in the presence of 40 ng/ml recombinant Wnt3a ligand (R&D Systems, Minneapolis, MN, <http://www.rndsystems.com/>), Wnt3a-CM (final 60 ng/ml Wnt3a), or control-CM mixed with prostate epithelial growth medium (Lonza, Walkersville, MD, <http://www.lonza.com/>) at 1:3 ratios. In vitro Wnt pathway inhibition by Dickkopf-related protein 1 (DKK1) was performed by DKK1 (R&D Systems) to the cultures (at 4 μ g/ml). Cells were cultured in Matrigel for 10 days, with media replaced every 3 days. To collect Matrigel-embedded prostaspheres for immunostaining analysis, spheres were removed from Matrigel by dispase (Stem Cell Technologies, Vancouver, BC, Canada, <http://www.stemcell.com/>) digestion at 37°C for 45 min. Cells were washed in ice-cold phosphate-buffered saline (PBS) and fixed for 10 min in 10% neutral phosphate-buffered formalin, washed with 70% EtOH, and placed in HistoGel (Lab Storage System Inc., St. Peters, MO, <http://www.labstore.com/>). HistoGel "plugs" were paraffin-embedded and sectioned for analysis. Similarly, Matrigel-seeded prostaspheres were isolated via dispase treatment. Spheres were washed twice with cold PBS and placed in TRIzol (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com/>) for RNA isolation, according to the manufacturer's instructions. The cDNA was generated using the Superscript III reverse transcriptase kit (Invitrogen), and qRT-PCR analysis was performed using Taq-Man Gene Expression Assay protocols using an ABI Prism 7,000 sequence detection system (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com/>).

LSC Infection and Transplants

LCS cells (1×10^5) in 200 μ l of complete Dulbecco's modified Eagle's media (DMEM) were mixed with 100 μ l LV (1×10^7 pfu/ml). Polybrene (Sigma, St. Louis, MO, <http://www.sigmaaldrich.com/>) was added to 4 μ g/ml. Cells were infected via centrifugation at 1,800 rpm for 90 min at room temperature (RT). Cells were washed 2 $\times$ with complete

DMEM media. For in vitro analysis of β -cat*-LV-infected LSCs, 1×10^4 cells were cultured in Matrigel, as described in [27]. For in vivo transplant assays, LV-infected LSCs were mixed with UGSM before implantation. UGSM cells were prepared as described in [35]. Infected LSCs were mixed with 1×10^5 UGSM cells and pelleted by centrifugation at 600 RCF. Cells were resuspended in 100 μ l Matrigel on ice. The Matrigel:cell mixture was injected subcutaneously into CD1 nu/nu mice. After 8 weeks, transplants were analyzed via IVIS Imaging System (Caliper, Hopkinton, MA, <http://www.caliperls.com/>) and grafts were harvested for further analysis.

Immunofluorescence, IHC, and Western Blot Analysis

Formalin-fixed samples were embedded in paraffin and 5 μ m sections were stained with H&E. Immunofluorescence and immunohistochemistry (IHC) were performed on similarly sectioned paraffin-embedded samples. Sections were deparaffinized and hydrated in 100% and 95% EtOH steps and rinsed in water. Sodium citrate (10 mM) antigen retrieval was performed, and sections were blocked with goat serum or M.O.M (Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>) kit for a minimum of 1 h at RT before immunofluorescence staining. Sections were incubated with 1° antibody ON at 4°C. Primary antibody dilutions were as follows: β -catenin 1:500 (Cell Signaling Technology, Danvers, MA, <http://www.cellsignal.com>), p63 1:500 (Thermo Fisher Scientific, Fremont, CA, <http://www.thermofisher.com/global/en/home.asp>), green fluorescent protein (GFP) 1:2,000 (Santa Cruz Biotechnology, Santa Cruz, CA, <http://www.scbt.com>), Notch1 1:2,000 (Santa Cruz Biotechnology), Ki-67 1:3,000 (Novocastra Ltd., Newcastle upon Tyne, U.K., <http://www.novocastra.co.uk>), CK5 1:5,000 (Covance, Emeryville, CA, <http://www.covance.com>), and CK8 1:1,000 (Developmental Studies Hybridoma Bank, Iowa City, IA, <http://www.uiowa.edu/~dshbwww>; TROMA-1). Secondary antibody incubation was performed in darkness for 1 h at RT. Secondary antibodies (Invitrogen) were goat anti-mouse Alexa Fluor 488, donkey anti-mouse Alexa Fluor 599, goat anti-rabbit Alexa Fluor 488, goat anti-rabbit R-phycoerythrin, goat anti-rabbit Alexa Fluor 350, and goat anti-rabbit Alexa Fluor 488. The slides were overlaid with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) for double-stained images or ProLong Gold antifade reagent without DAPI for triple-stained images (Invitrogen) and covered with glass coverslips.

When performing β -catenin IHC, cellular peroxidases were neutralized using 3% H_2O_2 for 15 min. Antibody blocking was performed using goat serum for 1 h at RT before addition of 1° β -catenin antibody (1:500 ON at 4°C). Slides were washed with PBS and incubated with biotinylated goat anti-rabbit (1:500 for 1 h at RT). Staining was visualized by treatment with ABC reagent for 45 min followed by 3,3'-diaminobenzidine for 3 min (Vector Laboratories) using hematoxylin counterstain.

Notch Signaling Pathway Manipulation

Inhibition of Notch signaling was mediated via γ -secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) (Sigma). DAPT was dissolved in dimethyl sulfoxide (DMSO) at 10 mM. Stock DAPT was further diluted to a 5 μ M working concentration and DMSO solvent acted as control. Media containing DAPT or DMSO was refreshed daily. Notch signaling was induced by NICD-LV. Infected cells (1×10^4) were used for in vitro prostasphere analysis. The prostaspheres were allowed to grow 7–10 days before analysis. Matrigel containing 1×10^5 infected LSCs and 1×10^5 UGSMs were used for in vivo transplants assays. The grafts were analyzed 8 weeks following transplantation.

Statistical Analysis

Statistical analysis was performed by the two-tailed *t* test. Error bars indicate standard deviation.

RESULTS

Wnt Pathway Induction Promotes Enlargement of PSC Spheres and Expansion of Prostate Progenitors

To enrich for basal epithelial-derived stem cells, fluorescence-activated cell sorting (FACS) was used to isolate LSCs from single cell suspensions of dissociated mouse prostates (Supporting Information Fig. 1). LSCs promote clonal outgrowth of prostaspheres when cultured in Matrigel. LSC-Matrigel cultures were supplemented with control media, recombinant Wnt3a ligand, or Wnt3a-CM for 10 days. Wnt-mediated activity in the Wnt3a-CM was demonstrated by activation of a β -catenin/TCF-responsive promoter coupled to a secreted alkaline phosphatase (SEAP) reporter (i.e., TOP-SEAP) in 293T cells (Supporting Information Fig. 2A). TOP-SEAP induction was inhibited by addition of Wnt pathway inhibitor DKK1, further supporting the functional activity of the Wnt3a ligand in Wnt3a-CM. The specificity of Wnt3a activity in Wnt3a-CM was also examined. Wnt3a-CM only induced TOP-SEAP reporter activity, and it did not alter the activity of other reporter genes such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) or AP1 reporters (Supporting Information Fig. 2B). Western blot analysis of Wnt3a-CM-induced LSCs indicated increased β -catenin staining when compared with control media, further supporting that Wnt3a-CM activates Wnt signaling (Supporting Information Fig. 2C). Activation of Wnt signaling in prostaspheres was supported by overall higher β -catenin staining accompanied by nuclear-localized β -catenin in Wnt3a-treated spheres (Fig. 1A).

Prostaspheres were analyzed for various prostate lineage markers such as CK5, CK8, and p63. Several reports suggest that p63 marks more primitive prostate cells with stem cell characteristics. Signoretti et al. [36] demonstrated that p63 is essential for normal prostate development. Xin et al. [27] also demonstrated that p63⁺ prostate cells have enhanced self-renewal ability. Moreover, others and we have shown that the majority of B/SCs are p63⁺ (Supporting Information Fig. 1B) [28]. Wnt-induced prostaspheres displayed a twofold expansion of p63⁺ cells (Fig. 1B). The p63⁺ cells were observed in all cell layers within the Wnt-induced spheres as opposed to control spheres in which basal cell-associated p63 expression was confined mainly to the outer cell layer. Interestingly, Wnt3a-treated prostaspheres were composed of a solid mass of cells and lacked the “compacted” cells typically found in the inner cell layers of control spheres. One possible explanation for the observed differences in sphere structures could be due to inhibition of differentiation signals and/or enhancement of cell survival upon Wnt pathway induction. Moreover, the majority of cells within Wnt-induced prostaspheres were CK5⁺CK8⁺p63⁺ TP; whereas, cells in the inner layers of control spheres were only CK5⁺CK8⁺ DP (Fig. 1C). Prolonged treatment of LSCs (≥ 15 days) with Wnt3a promoted formation of greatly enlarged (by approximately fourfold) prostaspheres when compared with control colonies (Fig. 1D). Moreover, DKK1, a LRP5/6 antagonist, blocked Wnt3a-mediated prostasphere expansion (Supporting Information Fig. 2D).

We were able to observe a similar phenotype using β -cat^{*}-LV. Induction of Wnt signaling via constitutively active β -catenin resulted in the formation of solid spheres (Fig. 2A). The spheres demonstrated higher β -catenin staining (Fig. 2B) and, similar to previous observation using Wnt3a, they contained increased disseminated p63⁺ cells. Therefore, our data suggests that induction of the Wnt pathway in LSCs promotes the expansion of prostaspheres. Additionally, Wnt induction disrupts cellular organization within the

organoids and results in formation of prostaspheres composed of a solid mass of cells that predominantly possess progenitor cell markers.

Constitutively Active β -Catenin Promotes Expansion of p63⁺ Prostate Epithelial Cells in PSC Grafts

To determine whether our in vitro findings extend in vivo, we used a subcutaneous (subQ) PSC transplantation assay. Six- to eight-week-old bioluminescent, EZC3-Prostate ("EZC3") cells from transgenic mice were used as an LSC source to measure transplant growth in living animals using an IVIS Imaging System. In EZC3 mice, firefly luciferase is targeted to prostate epithelial cells under the control of a composite prostate-specific antigen enhancer/promoter [37]. LSCs were FACS-isolated and infected with either β -cat*-LV or control LV. LV-infected LSCs (1×10^5) were mixed with 1×10^5 cells from embryonic day 17 (E17) rat UGSM in 100 μ l of Matrigel and injected subQ into the back of immunodeficient CD1 nu/nu mice. During the 8-week growth period, luciferase expression was analyzed regularly (Supporting Information Fig. 3A). After 8 weeks, grafts were removed and analyzed via H&E staining. LSC grafts successfully gave rise to glandular structures resembling acinar prostate tissue (Supporting Information Fig. 3B).

To correlate observed changes with transgene expression, we analyzed subQ grafts for evidence of LV infection. As both LV vectors contained enhanced GFP (eGFP), we analyzed infected acini for eGFP expression via anti-GFP immunostaining. Serial sections of infected acini were used for other immunostaining analysis (Supporting Information Fig. 3B). Immunostaining for β -catenin demonstrated strong β -catenin stabilization in grafts expressing constitutively active β -catenin when compared with control grafts (Fig. 3A). β -Catenin nuclear localization was detected by the presence of β -catenin staining overlapping with nuclear DAPI staining. Grafts that were generated via control LV-infected LSCs predominantly contained membrane-bound β -catenin. Analysis of p63 expression in serial sections indicated a more than 2.5-fold increase in p63⁺ cells in β -cat*-LV-infected grafts (Fig. 3B). Similar to the in vitro results, canonical Wnt pathway induction promotes expansion of p63⁺ cells from LSCs.

To further investigate the phenotype of the expanded p63⁺ cells in Wnt-induced LSC-derived grafts, we analyzed additional prostate epithelial cell markers as studied in the sphere assays. Serial sections from grafts were examined for basal and luminal cell markers, CK5 and CK8, respectively. Whereas control acini demonstrated clearly demarcated boundaries between CK5⁺ and CK8⁺ cells (Fig. 3C), reminiscent of wild-type lobes, β -cat*-LV-infected acini demonstrated increased numbers of CK5/CK8 DP cells (Fig. 3C). Furthermore, the boundary between basal and luminal cells was not as distinct as that seen in control acini. Overall, staining analysis indicates that induction of Wnt signaling in B/SCs promotes preferential proliferation of p63⁺ basal epithelial and CK5/CK8 DP cells in vivo. Thus, it appears that induction of Wnt signaling results in maintenance or perhaps expansion of more primitive (undifferentiated) prostate epithelial cells.

Wnt Induction in Prostate Progenitor Cells Leads to Upregulation of Proliferation Markers and EMT Signature Genes—

To determine whether Wnt induction via recombinant Wnt3a protein in prostasphere colonies leads to induction of canonical Wnt signaling, qRT-PCR analysis was performed on several known canonical Wnt target genes (Fig. 4A). *Cyclin D1*, *c-myc*, and *survivin* were all upregulated in response to recombinant Wnt3a. However, we did not observe changes in *axin2* transcript levels relative to control transcripts. Overall, this data supports that Wnt induction promotes transcription of multiple pro-proliferation and survival genes within prostaspheres, consistent with the observed phenotype of Wnt3a-treated cells.

As Wnt signaling plays a reported role in tumorigenesis and progression, at least partly via EMT [38–41], we also examined the transcriptional status of several EMT-associated candidate genes. qRT-PCR analysis indicated downregulation of epithelial marker, *E-cadherin*, whereas mesenchyma-associated genes such as *vimentin* and *snail1* were upregulated (Fig. 4B). Additionally, qRT-PCR analysis of β -cat*-LV-transduced prostaspheres demonstrated similar transcriptional changes as observed with recombinant Wnt3a-treated samples (Fig. 4C). To further examine the effect of Wnt induction on proliferation and prostasphere colony formation of LSCs in vitro, LSCs were cultured in the presence or absence of recombinant Wnt3a at two different densities in Matrigel. At both seeding densities (i.e., 6×10^3 and 1×10^4), induction of Wnt signaling resulted in increased colony potentiation by LSCs by twofold (Fig. 4D). Moreover, Ki-67 analysis of LSC grafts revealed an over fivefold increase in Ki-67⁺ cells in acini formed by β -cat*-LV-infected LSCs when compared with control-transduced cells (Fig. 4E). Overall, our data suggest that induction of Wnt signaling via recombinant Wnt3a or constitutively active β -catenin in prostate progenitors leads to increased expression of cell cycle-associated Wnt target genes, resulting in proliferation of B/SCs in vitro and in vivo.

Wnt Signaling Downregulates Notch1 in Prostate Spheres

In addition to Wnt pathway, proper prostate development is dependent on Notch1 signaling, which has been shown to be restricted to prostate basal cells during prostate development [42]. Interestingly, inhibition of the Notch pathway results in expansion of p63⁺ cells and reduces luminal cell differentiation [43, 44], a phenotype resembling Wnt pathway induction. Notch pathway induction also inhibits proliferation of prostate tumor cells [31], and this antitumor function is reportedly mediated by direct induction of phosphatase and tensin homolog (PTEN) in prostate epithelial cells [31]. Given the importance of Notch signaling in prostate development and homeostasis, we examined the effects of Wnt pathway induction on Notch1 expression in prostaspheres.

LSCs were cultured in the presence of recombinant Wnt3a or control media in Matrigel. After 10 days, prostaspheres were harvested and qRT-PCR analysis was performed using *Notch1* and *Jagged1* (Jag1) probes (Fig. 5A). *Notch1* was downregulated moderately (1.5-fold), yet reproducibly ($p = .012$) in response to Wnt pathway induction. *Jag1* was also downregulated 1.5-fold ($p = .024$). Furthermore, qRT-PCR analysis of other Notch receptors and Notch ligands indicated a reduction in the transcriptional levels of other Notch components upon Wnt pathway induction (Supporting Information Fig. 4A, 4B).

To further investigate this correlation, we analyzed Notch1 protein levels. When compared with control samples, Wnt-induced LSCs had lower levels of Notch1 (Fig. 5B). Activation of the Notch signaling pathway leads to proteolytic cleavage of the Notch receptor with membrane release of the intracellular domain. Western blot analysis via an antibody that recognizes activated Notch1, cleaved at Val1744, also indicated a reduction of NICD1 levels upon Wnt pathway induction or administration of Notch pathway inhibitor, DAPT (Supporting Information Fig. 4C) [45]. Notch1 protein expression was also analyzed via immunostaining. When compared with control cultures, Wnt induction resulted in overall lower Notch1 levels (Fig. 5C).

To further support our initial observation, prostate epithelial-derived RWPE1 cells were transfected with Notch pathway reporter, 12xRBP-Jk-luciferase, and cultured in the presence of control media, recombinant Wnt3a, or DAPT. Consistent with our expression analysis in prostasphere assays, Notch reporter activity in RWPE1 cells was reduced in the presence of both Wnt3a and DAPT (Supporting Information Fig. 5A).

Notch1 Activity Inhibits Prostate Progenitor Function

As demonstrated above, the Wnt pathway promotes enlargement of PSC-derived prostaspheres and expansion of prostate B/SCs, which correlates with Notch pathway downregulation. Therefore, using the constitutive *Notch1*-containing vector, NICD-LV, we examined the outcome of Notch pathway overexpression on prostasphere development. LSCs were infected with either control LV or NICD-LV, both of which coexpressed red fluorescence protein (RFP). Infected cells were cultured in Matrigel. LSCs infected with control LV were able to form prostaspheres 7 days after initial seeding (Fig. 5D), and there was a mixture of RFP⁺ and RFP⁻ (noninfected) spheres in these control cultures. As predicted, however, NICD-LV-infected LSCs had reduced capacity for sphere colony formation. Seeded NICD-expressing LSCs did not expand beyond the single-cell stage, even in the presence of Wnt3a-CM (Supporting Information Fig. 5B). However, noninfected cells (lacking RFP reporter) formed prostaspheres successfully. In contrast, inhibition of Notch signaling in B/SCs using DAPT promoted expansion of prostaspheres (Fig. 5E). However, these DAPT-treated prostaspheres displayed a different morphology from Wnt3a-induced spheres. Rather than a single large sphere, DAPT prostaspheres contained smaller nodule-like spheres “budding” from a central larger sphere (Fig. 5E). Furthermore, unlike Wnt3a-treated spheres comprising a dense mass of cells that were almost homogeneously p63⁺ DAPT treatment only resulted in increased sphere size (Fig. 5F). The p63⁺ cells were localized to the outermost cell layer while the inner cells were p63⁻, suggesting that inhibition of Notch signaling results in cell proliferation without having a major effect on lineage commitment.

We also performed cell count analysis on prostasphere cross sections. Both Wnt3a and DAPT treatment resulted in twofold increases in prostasphere cell numbers when compared with control colonies (Supporting Information Fig. 5C). One reported mechanism describing the inhibitory role of Notch signaling toward proliferation of prostate epithelial cells is via PTEN induction [32]. Therefore, we aimed to examine the effect of Notch induction on PTEN levels in prostate epithelial cells or B/SCs. First, transduction of RWPE1 cells with NICD-LV or seeding on plates coated with Notch ligand, Jag1Fc, increased the stabilized form of PTEN, indicated by phosphorylation of S380 (Supporting Information Fig. 6A). Second, transduction of B/SCs with NICD-LV also resulted in increased phospho-PTEN (pPTEN) (S380) staining when compared with control-treated cells (Supporting Information Fig. 6A). In contrast, inhibition of the Notch signaling axis via soluble Jag1Fc reduced pPTEN (S380) levels (Supporting Information Fig. 6B). Next, we performed cell cycle analysis using RWPE1 cells. NICD-expressing cells were inhibited at the G2/M phase of cell cycle progression when compared with control cells (Supporting Information Fig. 6C). However, Wnt3a or soluble Jag1Fc treatment increased the number of cells at G2/M phase (Supporting Information Fig. 6C), consistent with a Notch-mediated G2/M block. Furthermore, qRT-PCR analysis of DAPT-treated LSCs demonstrated an increase in several cell cycle regulatory genes, including *cyclin D1* and *c-myc* (Supporting Information Fig. 7). Interestingly, we also observed an increase in steady state RNA levels of several EMT-associated genes, including *vimentin*, *snail*, and *fibronectin* in DAPT-treated LSCs.

Finally, to further evaluate the effect of Notch pathway induction in prostate progenitors, prostate regeneration experiments using control LV or NICD-LV-infected LSCs were performed. Analysis of LSC grafts demonstrated that NICD-LV-infected B/SCs formed under-developed acini (50 μ m or smaller in diameter), when compared with control acini (Fig. 5G). Notch1 staining analysis of NICD-LV-infected acini demonstrated enhanced Notch1 staining and clear nuclear Notch1 localization that over-lapped with nuclear DAPI staining (Fig. 5H). The majority of the cells in the under-develop acini still retained p63 expression although we observed a minority of p63⁻ cells within the acini (Supporting

Information Fig. 8). Altogether, the data suggest that the Notch signaling pathway inhibits proliferation of prostate epithelial/progenitor cells.

DISCUSSION

Despite the complexities of embryogenesis, a relatively modest number of signaling pathways appear to control the growth, development, and homeostasis of most tissues. Among these, we have focused on the roles of Wnt and Notch signaling in PSC proliferation and identified a striking cross-regulation between them. Previous studies have inferred a role for Wnt and Notch signaling on prostate epithelial cell growth, but these studies typically involved more mature or transformed cells. For example, Wang et al. identified the importance of Wnt signaling in prostate development and the establishment of the acinar architecture. Furthermore, they demonstrated that Wnt pathway induction promotes expansion of p63⁺ cells [5]. Although these studies establish a key role for Wnt signaling in prostate development, its impact on prostate progenitor cell biology is less clear. In addition, Bisson et al. [46] demonstrated the importance of Wnt signaling in the self-renewal and growth of stem-like cells within PCa cell lines, like LNCaP, but the origin and physiological relevance of highly passaged cell lines are still unclear.

Considering the prior correlations of Wnt pathway changes during prostate development and cancer progression, our study demonstrates a consistent and unambiguous effect of the canonical Wnt signaling pathway on basal-layer-associated B/SCs. We focused on LSCs for several reasons, including (a) LSCs are highly enriched for PSC activity, (b) LSCs can form self-renewing prostasphere colonies, and (c) LSC transplants, in the presence of inductive UGSM cells, give rise to ectopic, secretory prostate acini [26, 27].

Previous studies demonstrated that prostaspheres contain a stratified pattern of lineage markers that roughly phenocopy the normal prostate architecture. Although peripheral cells are generally p63⁺CK5⁺CK8⁺ TP, inner layer cells reflect a more differentiated state, as indicated by CK5 and CK8 coexpression without p63 [27]. Interestingly, we observed that the majority of cells throughout Wnt3a-treated spheres are p63⁺ and coexpress CK5 and CK8. Therefore, Wnt induction appears to trigger proliferation of B/SCs without significant accompanying differentiation.

Furthermore, we found that prolonged induction of Wnt signaling in LSCs embedded in Matrigel results in an approximately fourfold enlargement in prostasphere diameter relative to control colonies. In control cultures, LSCs formed 100–150 µm diameter prostaspheres within 7–10 days that did not continue to grow with prolonged culturing. Moreover, these control organoids began to stratify during enlargement and became double-layered in appearance under light microscopy [27]. In contrast, Wnt3a-treated prostaspheres continued to expand without stratification, reaching approximately 300–400 µm in diameter with extended culturing time. This expansion was accompanied by the upregulation of pro-proliferation and pro-survival Wnt-target genes, *cyclin D1*, *c-myc*, and *survivin*, respectively. In contrast, inhibition of Wnt signaling via the LRP5/6 antagonist, DKK1, blocked prostasphere expansion.

As a second method to evaluate β-catenin signaling in LSCs, cells were transduced with β-cat^{*}-LV, which also promoted expansion of p63⁺ cells in vitro and in vivo. Using prostate reconstitution experiments, we observed a minimum 2.5-fold increase in p63⁺ cells in β-cat^{*}-LV-infected LSC grafts when compared with control LSCs. Interestingly, expression of constitutively active β-catenin also resulted in an increase in the nuclear proliferation marker, Ki-67, along with the number of graft cells that stained for both CK5 and CK8

epithelial markers, suggesting that Wnt induction either maintains or promotes proliferation of more primitive epithelial cells.

Manipulation of LSCs via various mitogenic and tumor-promoting factors has recently identified them as the major cell of origin for prostate tumor initiation in mouse models [28, 47]. Induction of pro-survival Akt or receptor tyrosine kinases by ligands, such as FGF, promoted these progenitors to recapitulate many of the histological and molecular features of PCa. Interestingly, these stimulations resulted in luminal epithelial cell dominant disease, a phenotype that is dramatically different from our observation of a strong bias toward the expansion of progenitor and basal cell populations as observed in vitro and in vivo. One possibility is that this bias could be due to suppression of differentiation in combination with promotion of proliferation of the prostate progenitor cells.

Dysregulation of signaling pathways that perturb proper stem cell activity could potentially manifest in an increase in CPCs, an increasingly attractive target for overcoming tumor relapse. As mentioned previously, regulatory pathways such as the FGF signaling axis and Akt signaling have been shown to drive PSCs into acquiring a CPC phenotype [29, 47]. Interestingly, we also observed that activation of Wnt signaling in prostate progenitor cells leads to changes in the expression of EMT signature genes, such as *snail*, *vimentin*, and *E-cadherin*. Collectively, Wnt activation leads to upregulation of cell cycle genes, proliferation of B/SCs, and induction of EMT genes, indicative of the ability of the Wnt signaling pathway to prime prostate progenitors into acquiring a CPC fate. Thus, modulation of Wnt signaling and downstream target genes in PSCs could provide a potentially potent therapeutic target in eradicating or reducing CPCs.

It has also become increasingly apparent that targeting a single developmentally relevant pathway for control of neoplasia is often insufficient for therapeutic effects [48, 49]. Although Wnt signaling plays an integral role in tissue stem cell maintenance, recent studies have mainly focused on the pro-proliferative effect of Wnt signaling on adult stem cells [16, 17, 50]. Therefore, it is also important to consider other developmental regulatory pathways, like Notch, which could work in concert with Wnt signaling to promote stemness. However, the role of Notch in tissue stem cells is context-specific. For example, in HSCs, Notch signaling appears to maintain stemness and to inhibit differentiation [17]. Conversely, Notch pathway induction plays an instructive role in the differentiation of neural stem cells into glial cells [43, 51]. Moreover, Notch signaling promotes differentiation of keratinocytes [52], but the role of Notch in PSC biology is less clear. Finally, recent studies support that Wnt signaling could potentially modulate the Notch pathway [17, 53].

Our transcriptional analysis demonstrates that all four Notch family receptors and associated ligands, including *Notch1* and *Jagged1*, are downregulated in Wnt-activated prostaspheres. Additionally, Western blot analysis and immunostaining reveals that both full-length Notch proteins and NICD1 levels are also downregulated in response to Wnt3a treatment. Moreover, Wnt pathway induction results in decreased Notch reporter activity in prostate epithelial cells. Independent of Wnt, inhibition of the Notch pathway using the γ -secretase inhibitor, DAPT, also resulted in increased cell number with corresponding prostasphere enlargement. However, the morphology of DAPT-treated prostaspheres is different than Wnt3a-treated spheres. DAPT treatment results in the formation of enlarged central spheroids that are surrounded by smaller spheres budding from the surface. Furthermore, DAPT treatment of LSCs does not alter the cellular organization of treated spheres, at least, with regards to p63 expression. In this case, p63 is localized within the outer cell layer, reminiscent of untreated control spheres. In contrast, p63⁺ cells are dispersed throughout all cellular layers in Wnt3a-treated spheres. The above observations support that inhibition of Notch signaling results in proliferation of prostate cells without a significant change to their

cell lineage commitment, which is presumably directed by other targets of Wnt signaling activation.

Moreover, Notch induction via NICD-LV results in inhibition of prostasphere formation, with LSCs inhibited at the single-cell stage. As Wnt3a induction fails to affect prostasphere growth in the presence of NICD, this would place the Notch pathway downstream of Wnt signaling and in opposition to Wnt activity. Furthermore, NICD-LV-infected prostate progenitors fail to develop into full-sized acini in our in vivo transplant assays. Immunostaining of under-developed acini shows nuclear localization of NICD, which is also consistent with an inhibitory effect of Notch within prostate progenitor cells.

One potentially confounding observation is that NICD-expressing LSCs in subQ grafts form small, underdeveloped acini, suggestive of modest proliferation, whereas in culture, NICD-transduced LSCs do not grow. One possibility is that in vivo grafts require UGSM cells that could have a transient, inductive, pro-proliferative effect on infected LSCs before functional expression of inhibitory NICD, thereby eliciting the observed phenotype. As UGSM cells are typically not added to in vitro prostasphere assays, these cultured progenitor cells might fail to receive pro-proliferative instructions, and hence, antiproliferative effects of NICD dominate.

It has been suggested that Notch signaling promotes PSC differentiation, and inhibition of Notch would therefore be expected to promote expansion of less differentiated p63⁺ cells [43, 44]. Cross-regulation of the Notch pathway and p63 has been described previously, as Notch inhibits p63 transcriptional activity in keratinocytes [52]. It is believed that p63 inhibition is due partially to Notch-mediated activation of NF- κ B signaling. p63 may also oppose Notch signaling by suppressing activity of the transcription factor, Hairy and Enhancer of split (HES). However, the Wnt-mediated downregulation of *Notch1* mRNA and protein that we observed appears to be p63-independent, because p63 affects HES activity without affecting Notch transcript levels [52]. However, we cannot disregard the potential role of p63 in Notch downregulation in our assays.

As previously reported, Notch could also play a key role as an inhibitor of proliferation in prostate epithelial cells by directly activating the tumor suppressor phosphatase, PTEN [31]. However, this hypothesis had not been tested in prostate progenitor cells. Consistent with previous results, we were able to demonstrate that induction of Notch in LSCs results in increased PTEN stabilization. Furthermore, Notch induction in prostate epithelial cells results in inhibition of cell cycle progression before the G2/M phase; whereas, inhibition of Notch results in upregulation of cell cycle-associated genes, such as *cyclin D1* and *c-myc*. Dalrymple et al. [54] demonstrated that transient amplifying prostate cells require Notch signaling for their survival. Our data also indicates that the Notch pathway is functional in prostate progenitor cells. Our observations suggest that as prostaspheres develop, the presence of Notch signaling is required to exit the cell cycle, permitting proper differentiation and development of spheres. As Wnt signaling results in proliferation of immature prostate progenitors within prostaspheres, the inhibition of differentiation within Wnt-activated prostaspheres is likely due to downregulation of Notch signaling. Therefore, despite a stimulatory role for Notch in some tissues [55, 56], we report that Notch expression inhibits prostate progenitor proliferation and prostasphere formation. Taken together, Notch downregulation in response to Wnt pathway induction is one mechanism that modulates PSC potential. Furthermore, transcriptional changes in response to Wnt signaling or Notch signaling inhibition are consistent with an instructive mechanism redirecting PSC fate to CPC development. It is important to keep these complex signaling relationships in mind when considering therapeutic manipulation of signaling pathways, like Wnt and Notch, which elicit multiple pleiotropic effects.

CONCLUSION

We show that induction of the Wnt pathway promotes the proliferation of B/SCs. Furthermore, induction of Wnt signaling leads to downregulation of Notch1 transcript and protein levels in these progenitor cells. We also demonstrate that induction of Notch signaling interferes with prostasphere formation and proper prostate reconstitution in prostate graft models, whereas inhibition of Notch leads to prostasphere growth.

Cross-regulation of signaling pathways, such as Wnt and Notch, plays an important role in regulating adult stem cell biology. Maintenance of stem cell homeostasis is paramount, and imbalances in the number of stem cells could lead to the development of hyperproliferative diseases, such as cancer. Molecular crosstalk, such as Wnt and Notch interactions described in this study, is likely to be cell context-specific and may vary among stem cell models. Ultimately, a better understanding of the cross-regulation of key regulatory pathways may lead to the development of more efficient strategies to suppress these pathway deviations, thus providing better therapies for diseases such as PCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Lin⁻Sca1⁺CD49f⁺ cells (LSCs) cultured in Matrigel. (A): Induction of the Wnt pathway via Wnt3a-conditioned media promotes stabilization and nuclear localization of β -catenin. Arrow, nuclear β -catenin. Scale bar = 25 μ m. (B): LSCs cultured in Matrigel for 10 days. Wnt induction leads to increase in the number of p63⁺ cells. Scale bar = 100 μ m. (C): p63, red; cytokeratin 5 (CK5), blue; and CK8, green staining of prostaspheres. Arrowheads, triple positive cells. Scale bar = 50 μ m. (D): Prolonged Wnt pathway induction (15 days) promotes enlargement of prostaspheres. Scale bar = 100 μ m. Abbreviations: CK, cytokeratin; DAPI, 4',6-diamidino-2-phenylindole; L-CM, L cell-conditioned media; Wnt3a-CM, Wnt3a-conditioned media.

Figure 2.

$\text{Lin}^- \text{Sca1}^+ \text{CD49f}^+$ cells cultured in Matrigel. **(A):** Bright-field image of prostatesphere infected with either control lentivirus (LV) or LV-mediated transduction of constitutively active β -catenin. Scale bar = 100 μ m. **(B):** Analysis of β -catenin expression via immunostaining. Scale bar, 25 μ m. **(C):** β -Catenin, green; p63, red; DAPI, blue. Scale bar = 25 μ m. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; LV, lentivirus; β -Cat*-LV, LV-mediated transduction of constitutively active β -catenin.

Figure 3.

Analysis of Lin⁻Sca1⁺CD49f⁺cell (LSC) grafts for β-catenin and prostate epithelial markers. **(A)**: β-Catenin immunostaining. Arrow, nuclear β-catenin. Scale bar = 50 μm. **(B)**: p63 immunostaining. Arrows, p63⁺ cells in control acini. Graph: average of four fields. Scale bar = 100 μm. **(C)**: LSC grafts stained with cytokeratin 5 (CK5) and CK8 antibodies. Arrows, CK5/CK8 double positive cells. Scale bar = 50 μm. Abbreviations: CK, cytokeratin; DAPI, 4',6-diamidino-2-phenylindole; LV, lentivirus; β-Cat*-LV, LV-mediated transduction of constitutively active β-catenin.

Figure 4. qRT-PCR analysis of Wnt3a-induced Lin⁻Sca1⁺CD49f⁺ cells (LSCs). Proliferation analysis of control and lentivirus-mediated transduction of constitutively active β -catenin (β -cat*-LV)-infected LSC grafts. **(A)**: qRT-PCR analysis of selected Wnt target genes. **(B)**: qRT analysis of epithelial-to-mesenchymal transition (EMT)-associated candidate genes. **(C)**: qRT analysis of Wnt target genes and candidate genes involved in EMT in LSCs expressing β -Cat*-LV. **(D)**: Prostatesphere colony formation analysis. LSCs were cultured in two separate experiments each containing four replicates. **(E)**: Immunostaining of LSC grafts with Ki-67 and analysis of Ki-67⁺ cells in control and β -cat*-LV-infected grafts. Scale bar = 100 μ m. Abbreviations: β -cat*-LV, lentivirus-mediated transduction of constitutively active β -catenin; LSCs, Lin⁻Sca1⁺CD49f⁺ cells.

Figure 5.

Analysis of Notch1 mRNA and protein expression in Lin⁻Sca1⁺CD49f⁺ cell (LSC)-derived prostaspheres. Manipulation of the Notch pathway in LSCs. **(A)**: Real-time analysis of Notch1 and Jagged1. Real-time data was obtained from three separate in vitro Matrigel experiments. Individual qRT-PCR experiments were performed in triplicate. **(B)**: Western blot analysis of control and Wnt-induced LSC protein fractions via anti-Notch1 antibody. Antibody detects cleaved Notch1 fragment. **(C)**: Notch1 immunostaining in prostaspheres. Scale bar = 50 μ m. **(D)**: LSCs infected with control lentivirus (LV) or LV-encoding the intracellular domain of Notch1 (NICD-LV) cultured in Matrigel. Arrow, NICD-LV-infected cells. Two separate experiments, each repeated in triplicates were performed to accumulate Notch1 induction data. Scale bar = 100 μ m. **(E)**: Inhibition of Notch signaling in LSCs using 5 μ M DAPT. Notch pathway inhibition was repeated in three separate experiments, and each experiment was performed in triplicates. **(F)**: Immunostaining of control and DAPT-treated prostaspheres. DAPI, blue; p63, red. Scale bar = 100 μ m. **(G)**: H&E stain of LSC transplants using control LV or NICD-LV-infected LSCs. Scale bar = 100 μ m. **(H)**: Immunostaining of LSC transplants using control LV or NICD-LV-infected LSCs. Arrows, nuclear staining of NICD. Scale bar = 50 μ m. Abbreviations: DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester; DAPI, 4',6-diamidino-2-phenylindole; NICD-LV: LV-encoding the intracellular domain of Notch1; RFP: red fluorescence protein; Wnt3a-CM, Wnt3a-conditioned media.