2013

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Recommended Citation
Li, Aimin; Chan, Belinda; Felix, Juan C.; Xing, Yiming; Li, Min; Brody, Steven L.; Borok, Zea; Li, Changgong; and Minoo, Parviz, "Tissue-dependent consequences of Apc inactivation on proliferation and differentiation of ciliated cell progenitors via Wnt and Notch signaling." PLoS One.8,4. e62215. (2013).
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Tissue-Dependent Consequences of Apc Inactivation on Proliferation and Differentiation of Ciliated Cell Progenitors via Wnt and Notch Signaling

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Abstract

The molecular signals that control decisions regarding progenitor/stem cell proliferation versus differentiation are not fully understood. Differentiation of motile cilia from progenitor/stem cells may offer a simple tractable model to investigate this process. Wnt and Notch represent two key signaling pathways in progenitor/stem cell behavior in a number of tissues. Adenomatous Polyposis Coli, Apc is a negative regulator of the Wnt pathway and a well known multifunctional protein. Using the cre-LoxP system we inactivated the Apc locus via Foxj1-cre, which is expressed in cells committed to ciliated cell lineage. We then characterized the consequent phenotype in two select tissues that bear motile cilia, the lung and the testis. In the lung, Apc deletion induced β-catenin accumulation and Jag1 expression in ciliated cells and by lateral induction, triggered Notch signaling in adjacent Clara cells. In the bronchiolar epithelium, absence of Apc blocked the differentiation of a subpopulation of cells committed to the ciliogenesis program. In the human pulmonary adenocarcinoma cells, Apc over-expression inhibited Jag1 expression and promoted motile ciliogenic gene expression program including Foxj1, revealing the potential mechanism. In the testis, Apc inactivation induced β-catenin accumulation in the spermatogonia, but silenced Notch signaling and depleted spermatogonial stem cells, associated with reduced proliferation, resulting in male infertility. In sum, the present comparative analysis reveals the tissue-dependent consequences of Apc inactivation on proliferation and differentiation of ciliated cell progenitors by coordinating Wnt and Notch signaling.

Introduction

Motile cilia perform many vital functions both during embryonic development and in maintenance of various organs. In early development, motile cilia are essential for establishment of embryonic left-right asymmetry. They are also necessary for normal lung function and fertility. Mutations causing ciliary deficiency underlie the human syndrome Primary Ciliary Dyskinesia (PCD) [1]. Emergence of fully differentiated ciliated cells from progenitor/stem cells is a tightly orchestrated step-by-step process that is amenable to detailed genetic and biochemical analysis. As such ciliogenesis may be exploited to address questions regarding the role of specific signaling pathways and how they impact progenitor/stem cell decision-making related to proliferation and differentiation under homeostatic conditions and in the face of injury, repair or remodeling.

The tumor suppressor Adenomatous Polyposis Coli, Apc is a key component of the destruction complex in the Wnt pathway that enables the maintenance of signaling within a homeostatic range. The function of the destruction complex relies on the ability of Apc to promote phosphorylation of β-catenin (Ctnnb1) via Gsk3b. In its inactive state, Gsk3b fails to phosphorylate Ctnnb1, leading to its cytoplasmic accumulation and subsequent transport to the nucleus where it affects transcription of several Wnt-target genes including c-Myc and Axin2 [2]. Thus absence of Apc leads to stabilization of Ctnnb1 and activation of Wnt signaling. Apc has additional roles unrelated to regulation of Ctnnb1. It binds directly and indirectly to microtubules, regulating their assembly, cell migration, chromosomal stability and mitotic spindles [3]. Mutations in Apc affect cell proliferation, differentiation, apoptosis and migration. Low levels or absence of Apc is associated with the ability of cells to serve as functional tissue-embedded stem cells [4]. Previously, using the developing murine lung model, we found that Apc is expressed at high levels in airway ciliated cells [5]. The function of Apc in cells with motile cilia and whether its
aberrations have a role in such disorders as PCD remain unexplored.

Studies in species from Drosophila to humans also implicate Notch signaling in establishment of asymmetry, cell fate decision and timing of differentiation during development. In the mouse lung, Notch receptor expression is seen in cells of endodermal as well as mesodermal origins [6]. Of the five Notch ligands, Jagged 1 is known to be induced by Wnt/Ctnnb1 signaling. Both receptors and ligands are heterodimeric type I membrane proteins that require cell–cell contact for activation. Ligand-receptor interactions trigger cleavage of the Notch intracellular domain (NICD) by gamma-secretase [6]. Thus detection of NICD has been an accepted marker of Notch pathway activation. Biological integration of Wnt and Notch signaling has been observed in the establishment and maintenance of diverse tissues and organs either collaboratively [7] or antagonistically [8].

The forkhead box transcription factor Foxj1 is specifically expressed in cell types that bear motile cilia in various organs including lung and testis, and drives the motile ciliogenesis program by directly stimulating the expression of various genes [9]. In the mouse lung, Foxj1 is initially detected in airway epithelial cells at E15.5, before the appearance of cilia, and thus is an early marker of ciliated cell fate determination [10]. In the mouse testes, Foxj1 expression is associated with the appearance of haploid germ cells and confined to specific stages of spermatogenesis [11].

Zhang et al used a Foxj1-cre line to mediate genomic recombination of floxed alleles specifically in cells committed to ciliary lineage [12]. Using the same Foxj1-cre driver line we generated mice carrying a targeted disruption of Apc specifically in ciliated cell progenitors. We subsequently analyzed and compared the phenotype of lungs and testes of the mutant mice. We found that absence of Apc caused expected hyperaccumulation of Ctnnb1 in both the airway epithelium and the spermatogonia. In the mutant lungs, Foxj1-cre-mediated inactivation of Apc triggered expression of Jag1, induced Notch signaling and blocked differentiation of a select group of bronchiolar ciliated cells. In contrast to the lung, absence of Apc silenced Notch signaling associated with progressive depletion of spermatogonial stem cells. We therefore propose that Apc may be crucial to ciliate cell differentiation and spermatogonial stem cell self-renewal via coordination of Wnt–Notch cross-talk.

Materials and Methods

Mice

All animals were maintained and housed in pathogen-free conditions according to a protocol approved by The University of Southern California Institutional Animal Care and Use Committee (IACUC) (Los Angeles, CA, USA). The Apc<sup>flox/flox</sup> mouse and Foxj1-cre transgenic mice were kindly provided by Dr. Raju Kucherlapati (Harvard Medical School, Boston, MA) [13] and Dr. Michael J. Holtzman (Washington University School of Medicine, St. Louis, MO) [12], respectively. Ciliated cell progenitor specific Apc conditional knockout mice, Apc<sup>Foxj1</sup> (Apc<sup>flox/flox</sup>; Foxj1-cre) were created by crossing Apc<sup>flox/flox</sup> homozygous females with Apc<sup>Foxj1</sup> (Apc<sup>flox/flox</sup>; Foxj1-cre) double heterozygous males and genotyped as previously described [14].

Asn2-Lac<sub>Z</sub> and TopGal-Lac<sub>Z</sub> Wnt activity reporter mouse lines were described previously [15]. Triple transgenic Foxj1-cre; Apc<sup>flox/flox</sup>; Asn2-Lac<sub>Z</sub> (simply Apc<sup>Foxj1</sup>; Asn2<sup>Lac</sup>) and Foxj1-cre; Apc<sup>flox/flox</sup>; TopGal (simply Apc<sup>Foxj1</sup>; TopGal) mice were generated and LacZ activity was assessed by X-gal staining or by immunohistochemistry as previously reported [16].

Naphthalene Exposure

Adult female Apc<sup>flox/flox</sup> (Control) and Apc<sup>Foxj1</sup> mice were injected with 300 mg/kg Naphthalene (Sigma) dissolved in Mazola corn oil (Cordova, TN). Animals were sacrificed at 1 day and 7 days following Naphthalene exposure.

LacZ (β-galactosidase) Staining

Activity of β-galactosidase in lungs and testis was determined by X-gal staining as described previously [15].

Western Blot

Protein extract preparation and western blot analysis were carried out as previously described [17]. Primary antibodies used are described in Table S1.

Immunohistochemistry (IHC)

Tissue slides were deparaffinized and rehydrated through an alcohol gradient series to water. Antigens were retrieved and endogenous peroxidase activity was quenched using 3% hydrogen peroxide. After normal serum blocking, the sections were incubated with primary antibodies at 4°C overnight. Immunohistochemistry (IHC) was performed using Peroxidase Substrate Kit DAB (Vector Laboratories). For immunofluorescence (IF) staining, the sections were incubated with primary antibodies overnight at 4°C and then reacted with a mixture of Cy3 anti-rabbit IgG or fluorescein antimouse or anti-goat IgG (Jackson ImmunoResearch Laboratories, Inc) for 1 hr in the dark at RT. After thorough rinses with PBS containing 0.1% Triton X-100, the sections were mounted with VECTASHIELD mounting medium containing DAPI to visualize nuclei. Primary antibodies used are described in Table S1. Stained sections were visualized on a Zeiss fluorescent microscope and images captured with a SPOT INSIGHT QE camera (Diagnostic Instruments) and analyzed using SPOT Advanced software (Diagnostic Instruments).

Morphometric Analyses

The percentage of cells immunostained for Ki67, βIV-tubulin and Ctnnb1 was determined by counting cells in lung or testis sections from control and mutant animals at 40X magnification. For each maker, ten fields or a minimum of 1000 cells were analyzed in at least three animals per group. Data were represented as mean±standard deviation (SD). Statistical analysis was performed using Student’s t-test. Differences were considered significant at P<0.05.

Real Time Polymerase Chain Reaction (PCR)

DNase-free RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. After DNase treatment, RNA was reverse-transcribed to cDNA using the Superscript III kit (Invitrogen) according to the manufacturer’s instructions. The cDNA was subjected to real-time PCR using SYBR Green PCR Master Mix with a LightCycler (Roche Applied Sciences, IN) as previously described [18]. Gene expression was normalized to β-actin or TBP define. All primers for real time PCR were designed by using the program of Universal Probe Library Assay Design Center from Roche Applied Sciences (IN).

Cell Culture and Transient Transfection Assays

Human pulmonary adenocarcinoma H441 cells (ATCC) were used for transfection experiments. Cells were maintained in RPMI
1640 containing 10% fetal bovine serum and 1% penicillin-
streptomycin (Invitrogen Life Technologies, Inc). Transfection of
Apc expression constructs to H441 cells was performed with
SuperFect (Qiagen) as described previously [14]. Twenty-four
hours after transfection, cells were harvested and analyzed for gene
expression by real time PCR.

Results

Foxj1-cre-mediated Recombination in the Lung and
Testis

In whole mount preparations, LacZ activity in Foxj1-cre; Rosa26
lungs was clearly detectable only after embryonic day E18.5 but
persisted in postnatal samples (Fig. S1, A–C and G–I). Using an
anti-β-galactosidase (β-gal) antibody was more sensitive, identify-
ing cre activity in tracheal, bronchial and bronchiolar epithelium
of E16.5 lungs (Fig. S1, D–F). Foxj1-cre activity pattern in later
stages of lung development (4–6 weeks of age) has been previously
reported (Zhang et al., 2007). In the testes, Foxj1-Cre-mediated
recombination activity in 4–6 week old mice was found primarily
in the spermatogonial stem cells (Zhang et al., 2007). In the
present study using Foxj1-cre; mT/mG double transgenic older mice
(>2 months of age) we observed green fluorescence not only in the
spermatogonia, but also in their descendents, the spermatids and
spermatozoa (Fig. S2), which is consistent with endogenous
expression pattern of Foxj1 in the testis [10].

Figure 1. Foxj1-cre-mediated ciliate cell progenitor-specific deletion of Apc. A Deletion of Apc exon 14 was validated by PCR with genomic
DNA. Top panel shows a band of 370 bp for Foxj1-cre. Middle panel shows bands of 320 bp for Wt and 430 bp for floxed product. Bottom panel
shows a 500 bp fragment in mutant but not control DNA. B RT-PCR with primers spanning exon 14 using total RNA from control and Apc<sup>fox1</sup> lungs. Expected 528 bp (control) was detected in both control and Apc<sup>fox1</sup> lungs. A 313 bp (truncated Apc allele) was amplified only from Apc<sup>fox1</sup> lungs. C & D Western blot (C) and densitometric quantification (D) of total protein from control and Apc<sup>fox1</sup> lungs. β-actin was used as control. *P<0.01. E–J Immunostaining of Apc (red) and Ctnnb1 (green) in postnatal control (E–G) and Apc<sup>fox1</sup> (H–J) lungs. Arrows in J show selective accumulation of Ctnnb1 in cells without Apc. K & L Immunostaining of βIV-tubulin (red) and Ctnnb1 (green) in postnatal control (K) and Apc<sup>fox1</sup> (L) lungs. Arrows in L show accumulation of Ctnnb1 in βIV-tubulin<sup>+</sup> ciliated cells. Scale bar: 20 μm.

doi:10.1371/journal.pone.0062215.g001

PLOS ONE | www.plosone.org 3 April 2013 | Volume 8 | Issue 4 | e62215
Figure 2. Loss of Apc blocks ciliary differentiation in a subset of bronchiolar epithelial cells. A Representative immunostaining images of βIV-tubulin (βIV-tub, green) and Ctnnb1 (red) in control and Apc<sup>Foxj1</sup> lungs at postnatal 2 weeks are shown. Arrows indicate accumulated Ctnnb1 signals. Scale bars are 20 and 10 μm, respectively. B Three groups of ciliate cells presented in the Apc<sup>Foxj1</sup> mice airway epithelium were identified by

C

D

E

F
DNA, but not from control lungs (Fig. 1A). Furthermore, analysis of Apc transcripts by RT-PCR, using primers spanning exon 14 revealed the expected 313 bp product from the truncated Apc allele only in the ApcFoxj1 lungs (Fig. 1B). Western blot analysis showed significant increase in Ctnnb1 in ApcFoxj1 lungs (Fig. 1C&D). IHC revealed that APC is absent specifically in cells with robust Ctnnb1 accumulation (Fig. 1E–J) and Ctnnb1 accumulation occurred specifically in βIV-tubpositive ciliated epithelial cells (Fig. 1K&L).

To determine whether the accumulated Ctnnb1 in the pulmonary ciliated cells was functionally active, we generated triple transgenic Foxj1-cre; Apcflox/flox; Axin2-LacZ (simply ApcFoxj1; Axin2). In these mice LacZ activity was increased specifically in cells with Ctnnb1 accumulation (Fig. S3). IHC further validated the LacZ-based observation. Axin2 was increased in cells with accumulated Ctnnb1 and was absent from Clara cells that are CC10positive (Fig. S4). Thus the accumulated Ctnnb1 is functional. These data indicate that deletion of Apc activates the Wnt/Ctnnb1 signaling in the Foxj1-cre positive lung epithelial cells. Despite this finding, Ki67 staining revealed a lower mitotic index for cells with accumulated Ctnnb1 compared to controls (Fig. S5). Analysis of apoptosis by TUNEL revealed no significant differences (Data not shown). These data are consistent with reports that Wnt/Ctnnb1 signaling has a limited role in cell proliferation and apoptosis in airway epithelium [19].

Loss of Apc Blocks Ciliary Differentiation Only in a Subset of Bronchiolar Epithelial Cells

To elucidate the consequences of Foxj1-cre-mediated loss of Apc function on airway epithelial cell differentiation, we analyzed the expression of βIV-tubulin (βIV-tub), a ciliated cell marker by IHC on specific compartments of the conducting airway epithelium including trachea, bronchi and bronchioles. Results revealed that Apc inactivation and consequent accumulation of Ctnnb1 did not block cilia formation in the trachea and bronchi (Fig. 2A). This was in sharp contrast to the bronchiolar epithelium where clear inhibition of cilia formation was apparent (Fig. 2A). Indeed, three distinct subpopulations were identified. The first included cells distinguished by Ctnnb1neg/βIV-tubpos phenotype. These are ciliated cells in which recombination failed (Ctnnb1 neg), and ciliogenesis was normal (βIV-tubpos). The second group was Ctnnb1pos/βIV-tubpos cells in which recombination occurred, but did not block ciliogenesis. And, finally the third group, distinguished by Ctnnb1pos/βIV-tubneg profile. In these cells, commitment to ciliary differentiation occurred (Ctnnb1pos), but ciliogenesis was blocked (Fig. 2B). Manual counting of multiple samples revealed that in the proximal domain only a small percentage (1.3%) of the total number of airway epithelial cells was identified as Ctnnb1pos/βIV-tubneg. In contrast, the percentage of Ctnnb1pos/βIV-tubpos airway epithelial cells in the more distal compartments was nearly an order of magnitude higher (11.3%) (Fig. 2C). The ratio of Ctnnb1pos/βIV-tubpos cells over the total number of Ctnnb1pos cells was further analyzed which revealed 2.4% Ctnnb1pos/βIV-tubpos cells in proximal domain compared

Figure 3. Loss of Apc induced Notch1 activation in bronchiolar epithelium of ApcFoxj1 mice. Representative immunostaining of Ctnnb1 or CC10 (green) and cleaved Notch1 N–terminal intracellular domain (NICD) (red) in control (A–C and G–I) and ApcFoxj1 (D–F and J–L) lungs at postnatal 2 week-old. Empty arrows in D–F indicate co-localization of Ctnnb1 and NICDpos cells. Arrows in D–F indicate NICDpos cells in non-Ctnnb1 cells. Empty arrows in J–L indicate NICDpos cells in CC10neg cells. Arrows in J–L indicate co-localization of CC10 and NICD. Scale bar: 10 μm.

doi:10.1371/journal.pone.0062215.g003
to 32% in distal domain (Fig. 2D). We then analyzed airway epithelial cell differentiation by Western blots and real time PCR using total lung protein or mRNA from ApcFoxj1 & control mice. Western blot analysis revealed dramatic reduction of both βIV-tubulin and α-tubulin proteins in ApcFoxj1 lungs (Fig. 2E). PCR analysis revealed that βIV-tub mRNA was reduced in mutant lungs compared to controls (Fig. 2F, 0.67 ± 0.03, p < 0.01). Interestingly, CC10 & Cyp2f2 RNAs, both expressed by Clara cells were increased in mutant lungs compared to controls (Fig. 2F, 2.19 ± 0.12 vs. 1 and 1.5 ± 0.08 vs. 1, respectively, p < 0.01). Therefore, inactivation of Apc and consequent Ctnnb1 accumulation blocked cilia formation only in a distinct subpopulation of Foxj1 expressing bronchiolar epithelial cells.

Loss of Apc Induces Notch Signaling in Bronchiolar Epithelium

Notch signaling plays an important role in controlling the number of ciliated versus secretory cells during airway epithelial cell differentiation [20]. Examination by IHC revealed low level of NICD in control lung epithelial cells (Fig. 3B). This contrasted with striking activation of Notch signaling as evidenced by nuclear NICD presence in a vast number of airway epithelial cells in the mutant lungs (Fig. 3E). Notch activation occurred in all luminal cells, but was particularly strong in those without accumulated Ctnnb1 (Fig. 3F) which were further identified as Clara cells (Fig. 3L).

To determine the link between Apc inactivation and Notch activation, we examined the expression of Jag1, a Notch ligand that is expressed in the lung epithelium [20]. Significantly increased Jag1 was observed in cells with accumulated Ctnnb1 (Fig. 4A). Increased Jag1 was not found in Clara cells (Fig. 4B). Western blot (Fig. 5A) analysis validated increased Jag1 and NICD. Quantitative RT-PCR revealed increased mRNA for Hes and Hey, Notch signaling-triggered target genes (Fig. 5B). Thus, inactivation of Apc and consequent Ctnnb1/Wnt signaling increased Jag1 in Foxj1-expressing Ctnnb1pos cells and activated Notch in adjacent Clara cells.

Apc Inhibits Jag1 in Human Pulmonary Adenocarcinoma Epithelial Cells

To understand the mechanism whereby Apc may regulate ciliated cell differentiation and its relationship to Notch signaling,
we used an in vitro cell culture model. Using H441 human pulmonary adenocarcinoma cell line, we found that overexpression of \( \text{Apc} \) decreased \( \text{Jag1} \) mRNA to near half of controls (Fig. 5C, 0.56±0.02 vs. 1, \( p < 0.001 \)), indicating that \( \text{Apc} \) can regulate Notch signaling via modulation of its ligand. Over-expression of \( \text{Apc} \) also increased \( \text{Foxj1} \) mRNA by 11.1-fold compared to controls (Fig. 5C). The latter data were further validated by the finding that two \( \text{Foxj1} \) downstream target genes, \( \text{Tektin} \) and \( \text{Dnahc9} \) [21] were also increased in transfected H441 cells by 16.2 and 8.6 fold respectively, while over-expression of \( \text{Apc} \) had no significant effect on expression of \( \text{Rfx3} \), another cilia transcription factor, reportedly to be essential for the differentiation of nodal monocilia [22]. Together, these data strongly indicate that \( \text{Apc} \) acts upstream of Notch to promote ciliated cell fate determination and differentiation by regulating \( \text{Foxj1} \) transcription factor in the airway epithelial cells.

**Accelerated Repair of Injured Airway Epithelium in \( \text{Apc}^{\text{Foxj1}} \) Mouse Lungs**

Exposure of the bronchiolar epithelium to Naphthalene results in severe and rapid loss of the majority of Clara cells within 24 hours [5]. A select but small subpopulation of Clara cells are resistant to naphthalene and are thought to act as facultative progenitors during regeneration of the bronchiolar epithelium [23,24]. We asked whether increased CC10 in \( \text{Apc}^{\text{Foxj1}} \) lungs (Fig. 2F) indicated an expanded pool of facultative progenitor cells? To address this, naphthalene was administered to \( \text{Apc}^{\text{Foxj1}} \) and control adult mice. Lung samples were processed on day 1 and 7. Within the first 24 hours, loss of Clara cells occurred in both the wild type and the mutant airways (Fig. 6C&D). Strong NICD was detected in the remaining cells in \( \text{Apc}^{\text{Foxj1}} \) airways while a more subtle increase occurred in the controls (Fig. 6C&D). In mutant lungs, as early as day 7, the airways showed significant signs of Clara cell regeneration (Fig. 6F). This is contrasted with control lungs in which little regeneration was evident, although there was clear evidence for activation of Notch signaling (Fig. 6E). These data suggest that \( \text{Foxj1} \)-cre-mediated inactivation of \( \text{Apc} \) and hyperactivation of \( \text{Ctnnb1/Wnt} \) signaling accelerates the ability of facultative progenitor cells to repair the naphthalene-injured airway epithelium.

**Loss of \( \text{Apc} \) Depletes Germ Cells & Blocks Spermatogenesis**

Male \( \text{Apc}^{\text{Foxj1}} \) mice are infertile, suggesting changes in the testes, another site of \( \text{Foxj1} \) expression. In the control testes, \( \text{Apc} \) and \( \text{Ctnnb1} \) were co-localized primarily to the cells adjacent to the outer membrane of the seminiferous tubule, the spermatogonia and the Sertoli cells (Fig. 7A–C). In the mutant testis, robust

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**Figure 5. Loss of \( \text{Apc} \) induced Notch signaling in \( \text{Apc}^{\text{Foxj1}} \) mice lung.** A Representative western blots analysis of total protein from adult control and \( \text{Apc}^{\text{Foxj1}} \) lungs. \( \beta\)-actin was used as control. B Real-time PCR of Notch-target genes in control and \( \text{Apc}^{\text{Foxj1}} \) lungs from 2 week-old to adult. Values are fold induction or inhibition compared to controls (arbitrarily adjusted to 1). (n = 3 for each genotype). C Real-time PCR of indicated mRNA levels in Human H441 pulmonary adenocarcinoma epithelial cells transfected with \( \text{Apc} \) expression construct (APC) or with vector alone (CMV) as a control. Values are fold induction or inhibition compared to controls (arbitrarily adjusted to 1). Transfections were done in triplicate, and the mean ± SD are shown. doi:10.1371/journal.pone.0062215.g005

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[23,24]
nuclear Ctnnb1 accumulation occurred in spermatogonia where Apc is undetectable (Fig. 7D–F). Again, significant increase of LacZ activity was seen in the testes of ApcFoxj1; Axin2 mice (Fig. 5S6A) indicating accumulated Ctnnb1 is in fact functional in triggering the Wnt pathway. Specificity of this reaction was further validated by IHC showing colocalization of Ctnnb1 and Axin2 (Fig. S6C).

Analysis of testicular development and morphology in ApcFoxj1 male mice showed that at earlier stages of development, from embryonic to 4 weeks of age, the testes of wild-type and ApcFoxj1 mice were similar in size (data not shown). By 2 months of age, the ApcFoxj1 testes consistently weighed about 40% less and by 6 months, 70% less than controls (Fig. S6A). In ApcFoxj1 testes, PAS staining revealed a progressive loss of spermatogonia (Fig. 8). At 2 months of age, the testicular cross-sections of the ApcFoxj1 mice showed germ cells from all stages of spermatogenesis although frequent loss of mature flagellated spermatozoa was readily seen (Fig. 8B). By 6 months, many ApcFoxj1 testicular tubules had undergone progressive degeneration and spermatogonia, spermatocytes and spermatids were depleted significantly and mature spermatozoa were never seen (Fig. 8D). At 12 months, depletion of germ cells resulted in Sertoli cell-only seminiferous tubules (Fig. 8F).

To verify the identity of the germ cells that remained in the seminiferous tubules of the mutant mice, three known germ cell markers, namely DDX4 [25], which is expressed in differentiated germ cells from spermatogonia to round spermatids; DAZL, expressed in both differentiated and undifferentiated spermatogonia and PLZF, a spermatogonial stem cell (SSC)-specific marker [26] were analyzed by immunofluorescence (Fig. 9A). In the control testes, DDX4-positive cells were present across the width of entire seminiferous epithelium; DAZL-positive cells were arranged at the periphery and PLZF-positive cells were present as a relatively small population. In sharp contrast, in the ApcFoxj1 testes all three germ cell-specific makers were nearly undetectable. In the wild type testes Apc and PLZF were co-expressed in the gonocytes at postnatal day 3 and later in the SSCs (Fig. S7). Moreover, ApcFoxj1 testes remarkably phenocopied PLZF null testes [26,27]. Thus it appears that similar to PLZF, Apc may be necessary for self renewal of SSCs.

Germ cell depletion could result from alterations in spermatogonial proliferation or apoptosis. IHC revealed that Ki67-positive cells were arranged uniformly at the periphery of seminiferous tubules of control mice (Fig. 9B). On the contrary, Ki67-positive cells were either entirely absent or found in clusters in ApcFoxj1 mice. In addition, cells with accumulated Ctnnb1 were not Ki67 positive (Fig. 9B, empty arrows). This is consistent with the findings in the lung epithelium (Fig. S5A). Phospho-Histone-H3 staining further confirmed decreased mitotic activity in the mutant testes (Fig. 9B). Manual cell counts revealed Ki67-positive cells were significantly reduced in ApcFoxj1 testes (Fig. 9C). TUNEL assessment showed no differences (data not shown). Thus, absence of Apc blocks proliferation of SSCs and causes spermatogonial depletion.

**Loss of Apc Inhibits Notch Signaling in Testicular Epithelium**

In the control testis from young mice, NICD was clearly present in the SSC, and differentiated spermatocytes and spermatids, suggesting active Notch signaling in the testicular epithelium (Fig. 10B&G). In the mutant testis, NICD was absent in SSC with Ctnnb1 accumulation (Fig. 10E&F). In older mutant mouse testes NICD was expressed only in few remaining germ cells (Fig. 11A).
Consistent with reduced Notch signaling, western blot analysis showed decreased NICD (Fig. 11B) and quantitative RT-PCR revealed decreased transcripts of its downstream targets Hes and Hey genes (Fig. 11C). Jag1 was also undetectable in the SSCs with accumulated Ctnnb1 (Fig. S8). Thus in the testes, inactivation of Apc and consequent hyperactivation of Wnt signaling inhibit (rather than increase) Notch signaling, which is opposite to the observation in the lung. Thus inhibition of Notch signaling in the SSC may underlie their failure to maintain a reservoir population of stem cells.

Discussion

Using Foxj1-cre, we inactivated Apc by targeted deletion of exon 14 specifically in cells committed to differentiate along ciliated cell lineage in various organs, including the lungs and the testes, two tissues analyzed in detail in this study. Our results reveal that in the lung, disruption of Apc activated the Wnt/Ctnnb1 signaling which in turn induced Notch and inhibited differentiation of only a sub-population of ciliated cell progenitors in the bronchiolar epithelium. In contrast, absence of Apc in the testes silenced Notch and caused male infertility by depletion of spermatogonial stem cells (SSC).

In the lung, Apc inactivation blocked only the differentiation of approximately 30% of the bronchiolar epithelial cells with evidence of commitment to ciliated cell program (Foxj1-cre<sup>pos</sup>). This suggests that the latter cells are derived from a progenitor pool distinct from that which gives rise to all other ciliated cells along the tracheal, bronchial and parts of the bronchiolar epithelia. Ciliated cells are thought to arise from two sources.

The embryonic lung endoderm serves as a major source of progenitor cells. Alternatively, they can be generated by “trans-differentiation” from “facultative” Clara-like progenitors [28,29,30]. We propose that it is the latter pool of progenitors that fails to undergo trans-differentiation thus accounting for the approximately 30% Ctnnb1<sup>pos</sup>/βIV-tub<sup>neg</sup> cells in the bronchiolar epithelium. Although this proposed scheme remains to be experimentally validated by cell lineage analysis, it is consistent with the finding by Reynolds et al that stabilization of Ctnnb1 blocks Clara to ciliated cell differentiation in the bronchiolar epithelium, increases the number of naphthalene-resistant reparative cell pool and depletes the ciliated cell population [31].

Are facultative Clara-like cells in the mutant lung different compared to their wild type counterparts? In Apc<sup>Foxj1</sup> lungs, inactivation of Apc which potentiated Wnt signaling in ciliated cells via Ctnnb1 (Foxj1<sup>pos</sup>) also induced Jag1, which in turn uniformly induced Notch signaling in all neighboring Clara cells via juxtacrine activation. These must include also the “facultative” Clara cells (Please see below). Jag1, a known Notch ligand is a target of Wnt signaling [32] and its expression pattern in the embryonic mouse lung has been elucidated [20]. Thus, unlike the endodermal progenitors, in which Ctnnb1 and Wnt signaling predominate, in facultative Clara cells Ctnnb1 is activated in an environment that is already enriched in Notch signaling (Fig. 3).

We speculate that increased Notch signaling favors maintenance of a stem cell status in Clara facultative cells and opposes ciliated cell trans-differentiation, hence increased Clara cell genes expression and decreased βIV-tub<sup>pos</sup> cell numbers in Apc<sup>Foxj1</sup> lungs (Fig. 2). The above speculation is consistent with a number of findings.

Figure 7. Loss of Apc results in selective accumulation of Ctnnb1 in the spermatogonia of adult Apc<sup>Foxj1</sup> testis. Representative immunostaining of APC (red) and Ctnnb1 (green) in control and Apc<sup>Foxj1</sup> testis at 12 months are shown. Asterisks in D show cells with absent Apc. Arrows in E show cells lacking Apc had Ctnnb1 accumulation. Sg: Spermatogonia; Se: Sertoli cells. Scale bar: 10 μm.

doi:10.1371/journal.pone.0062215.g007
Notch is required for Clara cell regeneration and its inactivation expands ciliated cell populations, suggesting that Notch normally limits ciliated cell differentiation [18,20]. Furthermore, in human airway epithelium and *Xenopus* laevis embryonic epidermis Notch signaling must undergo miR-449 microRNA inhibition to permit ciliated cell differentiation [33]. Thus, blocked differentiation of ciliated cells in the bronchiolar epithelium may be attributed to the functional outcome of time- and likely magnitude-dependent interplay between Wnt/Ctnnb1 and Notch signaling pathways. Specific evidence that Notch is increased in all Clara cells including the “facultative” Clara or progenitor/stem cells was provided by the Naphthalene injury study. Recently, we reported that Notch1 is required for regeneration of Clara cells during repair of Naphthalene-induced airway injury [18]. Exposure of the bronchiolar epithelium to Naphthalene results in severe and rapid loss of the majority of Clara cells within 24 hours [5]. A select but small subpopulation of “facultative” Clara cells are resistant to Naphthalene and are thought to act as progenitors during regeneration of the bronchiolar epithelium [23,24]. If the population of facultative progenitor cells with high Notch1 activity is indeed increased in *Apc<sup>Foxj1</sup>* lungs, we predicted measurable differences between the mutant and control mice in the timing of Clara cell regeneration & repair after Naphthalene-induced injury. In fact a clearly more accelerated repair of the airway epithelium was detected in the mutant lungs (Fig. 4). We propose that an increase in the sub-population of Clara cell progenitors in *Apc<sup>Foxj1</sup>* lungs, due to increased Notch signaling facilitates the repair of the naphthalene-injured airways. Thus, although we found blocked trans-differentiation of the facultative Clara cells to bronchiolar ciliated cells in *Apc<sup>Foxj1</sup>* lungs, their increased pool nevertheless retained the ability to serve as progenitors for Clara cell differentiation/restoration in response to injury.

In this study, we provide the first direct evidence for a function of Apc as a key regulator of spermatogenesis. Apc inactivation caused male infertility. In wild type mice, a complex set of step-wise differentiation events leads from spermatogonia to spermatids and finally mature spermatozoa [34]. Spermatogonia serve as stem cells by undergoing asymmetrical cell division that serves to self-renew, and provide a continuous source of progenitors for production of mature sperm. In *Apc<sup>Foxj1</sup>* testes, spermatogonia are initially present at densities comparable to controls. However, there is a time-dependent progressive depletion of their pools in the mutant mice. Examination of proliferation revealed reduced mitotic index for the mutant SSCs (Fig. 9). Thus, inactivation of Apc and hyperactivation of Wnt/Ctnnb1 signaling interrupts what appears to be asymmetrical cell division that maintains a pool of SSCs throughout the life of male mice.

Importantly, and in contrast the lung, inactivation of Apc and accumulation of Ctnnb1 silenced Notch signaling in the SSC. Whether silencing of Notch is a direct impact of Apc inactivation or...
increased Wnt/Ctnnb signaling remains unknown. Nevertheless, silencing Notch may explain progressive loss of SSC as it is known to be necessary for maintenance of stem cells in many organs [35] and germ cell development in Caenorhabditis elegans [36] and Drosophila [37,38]. Apc may also directly regulate SSC asymmetrical cell division by direct impact via its role as a mitotic spindle checkpoint [39]. Apc forms complexes with other proteins including Bub1 during mitosis [40]. Inactivation of Bub1 in adult males inhibits spermatogonial proliferation [41]. In Drosophila, male germline stem cells are dependent on Apc to orient mitotic spindles perpendicular to the niche, ensuring a reliably asymmetric cell division [42]. Thus, this important function of Apc in maintenance of stem cell pools appears to be conserved over nearly five hundred million years of evolution.

Figure 9. Loss of Apc causes spermatogonial stem cell (SSCs) depletion and blocks proliferation of SSCs in adult Apc<sup>Foxj1</sup> testes. A Representative immunostaining of DDX4, DAZL and PLZF in control and Apc<sup>Foxj1</sup> testis at 12 months are shown. Dotted lines indicate the basement membrane of seminiferous tubules. Arrows show PLZF<sup>pos</sup> cells on the basement membrane. Scale bar: 50 μm. B Mitotic activity of germ cells in the adult control and Apc<sup>Foxj1</sup> testis. Representative immunostaining of Phospho-Histone-H3 (green), Ki67 (red) and double staining of Ki67 (red) and Ctnnb1 (green) in control and Apc<sup>Foxj1</sup> testis at 6 or 12 month-old are shown. Note obvious decrease of Phospho-Histone-H3 or Ki67 staining in Apc<sup>Foxj1</sup> testis. Arrows show Ki67<sup>pos</sup> cells; empty arrows show cells with Ctnnb1<sup>pos</sup> are not Ki67<sup>pos</sup>. Scale bars are 50 and 20 μm, respectively. C Manual counting of Ki67<sup>pos</sup> cells in control and Apc<sup>Foxj1</sup> testis from 6 month to 12-month old (n = 4 for each genotype). doi:10.1371/journal.pone.0062215.g009
Interestingly, the effect of Apc deletion on Notch signaling is different between lung and testis. In the lung, ectopic Wnt signaling is reported to attenuate airway cell differentiation [31]. This is not what we observed in ApcFoxj1 lungs. We believe this is primarily due to the timing of Foxj1-cre activation, which occurs subsequent to cell fate commitment in the majority of endodermally derived ciliated cell progenitors. Thus, in the proximal conducting airways of the mutant lung, differentiated ciliated cell numbers were wild-type like. The exception was blocked differentiation of a subset of Foxj1pos cells in the bronchiolar epithelium. These cells had already committed to differentiate along ciliated cell phenotype, but had not proceeded to overt differentiation (expression of βIV-tubulin). We propose that such cells originate from facultative Clara cells whose molecular phenotype in the mutant lung is altered by induction of Notch signaling. In the lung and in particular the facultative Clara cells, activation of Notch which occurs via lateral induction of Jag1 temporally preceded potentiation of Wnt signaling (increased Ctnnb1) triggered by Foxj1-cre-mediated recombination. Notch is well recognized to favor maintenance of pluripotency and its interruption favors ciliated cell hyperplasia [18,20]. Compared to the lung, we found a significantly different Wnt~Notch signaling environment in the testis. We propose that this is principally due to timing of Foxj1 activation which in contrast to the lung occurs early in spermatogonial stem cells. This leads to early activation of Wnt signaling by stabilized Ctnnb1, which is a known inhibitor of Notch signaling [43]. Here gradual depletion of spermatogonial stem cell pools was observed. In addition, Jag1 whose expression triggered increased Notch signaling in the lung is not expressed in the testis [44] (Fig. S8). Thus, in the testis, Wnt inhibits Notch activity to promote a Wnt-ON/Notch-OFF output, thereby enhancing the differentiation rather than “stem-cell-ness” of spermatogonial stem cells, which eventually caused depletion of the SSC reservoir (Fig. 9). However, we cannot exclude the possibility that the multifunctional properties of Apc, disruption of its functions other than Wnt~Notch signaling may also have contributed to the observed overt phenotype in the lung and testis. Apc is well known to play a role in regulating junction structure and function in the intestine [45] and testis [46]. Although histologically we observed no gross or fundamental epithelial abnormalities in ApcFoxj1 mutant lungs, the nuclear Ctnnb1 accumulation in the spermatogonia could cause defects in sertoli-spermatogonia adhesion located in the basal compartment of blood-testis barrier, which may ultimately lead to germ cell loss. Further studies are needed to determine what role if any Notch may play in maintaining integrity of testicular epithelium.

Infertility in men is widespread yet poorly understood. Failure in spermatogenesis appears frequently as idiopathic and may be due to genetic causes [47]. Apc is expressed in some spermatogonia and interstitial (Leydig) cells of human fetal testis [48] and in germ cells and Leydig cells of the adult [49]. This is in agreement with our IHC results in mice where Apc is highly expressed in spermatogonia with lower expression found in other cells of the seminiferous tubules (Fig. 7) suggesting a role for Apc in testicular development and maintenance. Extrapolation based on the similarities between mouse and human Apc expression patterns

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**Figure 10. Loss of Apc inhibits Notch1 activation in ApcFoxj1 mouse testis**. Representative immunostaining of Ctnnb1 (green) and NICD (red) in control and ApcFoxj1 mouse testes at 2 months are shown. Arrows in B indicate NICDpos cells. Arrows in E indicate disappearance of NICDpos cells in Ctnnb1 accumulated cells. Scale bar: 20 μm.

doi:10.1371/journal.pone.0062215.g010
raises the possibility that mutations in the human Apc gene may be contributory to infertility in man.

Finally, we provide evidence that Foxj1 expression is directly activated by Apc (Fig. 5C and Fig. S9). As this work was in progress, another Wnt/Ctnnb1 signaling negative regulator, Chibby (Cby1) was identified as a regulator of ciliogenesis [50]. Of interest, while Cby1 is directly activated by Foxj1 in the latter study, we found that both genes are affected by Apc (Fig. S9). Compelling evidence has been obtained by studying a zebrafish Foxj1 protein that the principal mechanism by which Foxj1 controls motile ciliogenesis is through the transcriptional regulation of genes encoding components that are critical for the synthesis and function of motile cilia [9]. Our in vitro data demonstrate that by regulating Jag1 and consequently Notch signaling in the airway epithelium, Apc can control the fate of a select group of progenitor cells that give rise to the bronchiolar epithelium. The bronchiolar epithelium is currently thought to be the site of important facultative stem cells. For example, bronchoioalveolar stem cells (BASCs), distinguished by Spc/CC10 double expression are thought to reside within this region at the cross section between conducting airways and the respiratory compartment of the lung [51]. The observation that Foxj1-cre mediated inactivation of Apc does not block ciliated cell differentiation throughout the airway epithelium suggests functional differences between the progenitors from which the proximal versus bronchiolar ciliated cells are derived. In this regard the timing of Apc inactivation, dictated by the specific promoter that drives cre expression is an important factor in determining the outcome. For example, in Nkx2.1-cre; Apcflox/flox lungs, differentiation of both ciliated and Clara cells throughout the airway epithelium is blocked (Li et al., Submitted).

In summary, the current study reveals the important role of Apc as a key player in the processes of ciliogenesis and spermatogenesis in vivo. To date, no mutations in the human Apc have been identified that result in male sterility. However, given the cilia and flagella phenotypes uncovered in ApcFoxj1 mice by the present study, elucidating the function of Apc may contribute greatly to the understanding of the molecular mechanisms perturbed in

**Figure 11. Loss of Apc inhibits Notch signaling in ApcFoxj1 mouse testis.**

A Representative immunostaining of Ctnnb1 (green) and NICD (red) in control and ApcFoxj1 testes at 12 months are shown. Arrows in control mouse testis indicate NICDpos spermatogonia stem cells. Arrows in ApcFoxj1 mouse testis indicate disappearance of NICDpos signals in Ctnnb1 accumulating spermatogonia. Scale bar: 30 μm. B Representative western blots analysis of total protein from adult control and ApcFoxj1 testes. β-actin was used as control. C Real-time PCR of Notch-target genes in control and ApcFoxj1 testes at 6 month to 12-month-old. Values are fold induction or inhibition compared to controls (arbitrarily adjusted to 1) (n = 3 for each genotype). doi:10.1371/journal.pone.0062215.g011
human cilia-related disorders including PCD, and may aid in their diagnosis and treatment.

**Supporting Information**

**Figure S1** Foxj1-cre expression pattern during murine lung development. A–C LacZ staining of cre-induced β-galactosidase (β-gal) activity in whole mount lungs of Foxj1-cre; Rosa26R mouse. (A) Embryonic day 16 (E16) lungs. No LacZ staining was seen. (B) E18 lungs, Foxj1-cre mediated recombination was visible. (C) Postnatal day 3 (PN3) lungs, LacZ is similar to E18. D–F IHC for Foxj1-cre; Rosa26R E16 lungs, counterstained with methyl green. β-gal signaling (brown) was localized to epithelial cells (arrows) of trachea (D), bronchi (E) and bronchioles (F). G–I LacZ staining of Foxj1-cre;Rosa26R PN3 lungs. LacZ staining (blue) was localized to epithelial cells (arrows) of trachea (D), bronchi (E) and bronchioles (F). G–I LacZ staining of Foxj1-cre;Rosa26R PN3 lungs. LacZ staining (blue) was localized to epithelial cells (arrows) of trachea (G), bronchi (H) and bronchioles (I). Scale bar: 2 mm for Panels A–C; 40 μm for Panels D–I. (TIF)

**Figure S2** Foxj1-cre expression pattern in the adult Foxj1-cre; mTmG mouse testis. A Whole mount testis in red fluorescence showing absent recombination. B Green fluorescence showing Foxj1-cre positive recombination. C Testis section showing strong green fluorescence signal located in spermagonia, spermatids and spermatozoa (arrows) of semiferous epithelium. Scale bars: 2 mm for A&B 100 μm for C. (TIF)

**Figure S3** Wnt/Ctnnb1 signaling is activated in Ctnnb1 accumulated spermatogonia. A&E X-gal staining of control (Apcfox/flx; Axin2-LacZ and Apcflox/fox; Foxj1-cre; Apcfox/flx; Axin2-LacZ) lungs at postnatal 2-week-old mice. Arrows indicate two epithelial cells with strong LacZ activity. B–H Immunostaining of β-gal (red) and Ctnnb1 (green) on the X-gal stained sections. B–D Control lung, μm. E–H Mutant lung. Arrows in F, G & H show X-gal stained epithelial cells are both β-galpos and Ctnnb1pos. Scale bar: 40 μm. (TIF)

**Figure S4** Wnt/Ctnnb1 signaling is active only in Ctnnb1pos cells. Immunostaining of Ctnnb1 with CC10 (green) and Axin2 (red) in control (A & C) and Apcflox/fox (B & D) lungs. Arrows in B show co-localization of Ctnnb1 with the Wnt-target gene, Axin2. Arrows in D show absence of co-localization of CC10 with Axin2. Scale bar: 10 μm. (TIF)

**Figure S5** Loss of Apc does not affect cell proliferation. A Representative immunostaining of Ctnnb1 (green) and Ki67 (red) in control and Apcflox/fox lungs. Arrows in F show Ki67pos cells; asterisks show cells with accumulated Ctnnb1. Note: the cells with accumulated Ctnnb1 are not Ki67pos. Scale bar: 20 μm. B Quantification of Ki67pos cells by manual counting in control and Apcflox/fox lung from 2-weeks to adult (n = 3 for each genotype). (TIF)

**Figure S6** Wnt/Ctnnb1 signaling is active in the Ctnnb1 accumulated spermatogonia. A Whole mount X-gal staining of control (Apcfox/flx; Axin2-LacZ) and Apcflox/fox testis (right) and Apcflox/fox; Axin2-LacZ (left) testes. Note robust dark staining of β-gal (LacZ) in the Apcflox/fox testis (right) although the staining is too dark to see the detail. Scale bar: 2 mm. B & C Immunostaining of Axin2 (red) and Ctnnb1 (green) in control (B) and mutant (C) testes. Arrows in C show co-localization of Ctnnb1 with Axin2. Scale bar: 10 μm. (TIF)

**Figure S7** Co-localization of Apc and PLZF in wild-type mouse testes. Immunostaining of Apc (green) and PLZF (red) in postnatal 3 days and 2 month testes of wild type mice. Arrows indicate co-localization of Apc and PLZF signals. Dotted lines indicate the basement membrane of semiferous tubules. Scale bar: 20 μm. (TIF)

**Figure S8** Inactivation of Notch pathway in the Apcfox/fox mouse testis. Immunostaining of Ctnnb1 (green) and Jag1 (red) in control (A) and Apcflox/fox testes (B). Arrows in B indicate Ctnnb1pos spermatogonia are Jag1pos. Scale bar: 30 μm. (TIF)

**Figure S9** Loss of Apc decreased motile ciliogenic gene expression in the Apcflox/fox lung and testis. A Real-time PCR of Foxj1 mRNA level in control and Apcflox/fox lungs and testes. B Real-time PCR of Cby1 mRNA level in control and Apcflox/fox lungs and testes. Values are fold inhibition compared to controls (arbitrarily adjusted to 1) and mean ± SD are shown (n = 3 for each genotype). (TIF)

**Table S1** Primary antibodies used in western blots or immunohistochemistry.

**Acknowledgments**

The authors thank Dr. Raju Kucherlapati for providing Apcfox/flx mice, Dr. Michael J. Holtzman for providing Foxj1-cre mice and Lingyan Hu for excellent technical support.

**Author Contributions**

Conceived and designed the experiments: PM AL. Performed the experiments: AL BC ML YX. Analyzed the data: PM AL CL ZB SLB JCF. Wrote the paper: PM AL.


