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Notch signaling is required for normal prostatic epithelial cell proliferation and differentiation

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Abstract

Notch pathway is crucial for stem/progenitor cell maintenance, growth and differentiation in a variety of tissues. Using a transgenic cell ablation approach, we found in our previous study that cells expressing Notch1 are crucial for prostate early development and re-growth. Here, we further define the role of Notch signaling in regulating prostatic epithelial cell growth and differentiation using biochemical and genetic approaches in ex vivo or in vivo systems. Treatment of developing prostate grown in culture with inhibitors of gamma-secretase/presenilin, which is required for Notch cleavage and activation, caused a robust increase in proliferation of epithelial cells co-expressing cytokeratin 8 and 14, lack of luminal/basal layer segregation and dramatically reduced branching morphogenesis. Using conditional *Notch1* gene deletion mouse models, we found that inactivation of Notch1 signaling resulted in profound prostatic alterations, including increased tufting, bridging and enhanced epithelial proliferation. Cells within these lesions co-expressed both luminal and basal cell markers, a feature of prostatic epithelial cells in predifferentiation developmental stages. Microarray analysis revealed that the gene expression in a number of genetic networks was altered following Notch1 gene deletion in prostate. Furthermore, expression of Notch1 and its effector Hey-1 gene in human prostate adenocarcinomas were found significantly down-regulated compared to normal control tissues. Taken together, these data suggest that Notch signaling is critical for normal cell proliferation and differentiation in the prostate, and deregulation of this pathway may facilitate prostatic tumorigenesis.

Keywords: Notch; Prostate development; Cell proliferation; Cell differentiation; Mouse models; Prostate cancer

Introduction

Notch mediated cell–cell interaction and signaling are important for stem cell maintenance, cell fate determination, cell proliferation and differentiation in a variety of tissues (Artavanis-Tsakonas et al., 1999; Lai, 2004). In the prostate, it is believed that there is a stem cell population that gives rise to all cells in prostate epithelium during both early development and re-growth process in adult following androgen deprivation and hormone replacement (Isaacs and Coffey, 1989). However, the nature of this population remains largely elusive, albeit two functional types of stem cells, i.e., the strict-sense stem cells and so-called transit amplifying cells have been documented (to indicate this, this cell population is designated collectively as progenitor cells hereafter). In an effort to understand the role of Notch pathway in prostate development and tumorigenesis, we examined the expression pattern of *Notch1* in the prostate and found that Notch1 expression in early postnatal prostate is much higher than in adult, and that Notch1 is expressed in the basal cell compartment where putative prostate progenitor cells are believed to reside (Shou et al., 2001). Using a transgenic cell ablation approach, we further demonstrated that Notch1-expressing cells are indispensable for prostate branching morphogenesis, growth, differentiation and re-growth, suggesting that Notch1 may define the progenitor cells in the prostate

Abbreviations: BPH, benign prostatic hyperplasia; CK, cytokeratin; Cre, Cre-recombinase; DAPI, 4,6-diamidino-2-phenylindole.

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(Wang et al., 2004). However, how Notch signaling regulates cell proliferation and differentiation in the prostate remains unclear.

Notch protein requires three cleavage steps to become fully functional (Lai, 2004). After ribosomal synthesis, Notch protein is first processed by a furin-like protease in the endoplasmic reticulum membrane and then transported to the plasma membrane, where the two chains of Notch form an inactive heterodimer. Upon ligand binding to the extracellular domain, the membrane-attached intracellular subunit is successively cleaved by tumor-necrosis-factor-α-converting enzyme and γ-secretase/presenilin (De Strooper et al., 1999; Okochi et al., 2002; Ye et al., 1999). Because presenilin is responsible for maturation of amyloid-β protein from its precursor protein and accumulation of amyloid-β protein causes Alzheimer's disease, a number of inhibitors have been synthesized (Citron, 2004). The elucidation that presenilin is responsible for the last step of Notch cleavage and activation has allowed the use of its inhibitors (or γ-secretase inhibitors) in Notch signaling and functional studies (Dahlqvist et al., 2003; Geling et al., 2002; Micchelli et al., 2003; van Es et al., 2005).

In the present study, we employed two strategies of inactivating Notch signaling to determine its role in regulating prostate progenitor cells. We first took advantage of γ-secretase inhibitors to prevent the maturation of Notch protein and studied the growth of neonatal prostate in culture. The inhibitors used were WPE-III-31C (hereafter 31C) (Esler et al., 2002) and L-685,458 (hereafter L) (Li et al., 2000; Shearman et al., 2000). They have been shown to specifically inhibit the γ-secretase activity of presenilin and Notch activation (Doerfler et al., 2001; Esler et al., 2002). We also used *Notch1* conditional knockout mouse models to evaluate the effect of *Notch1* gene inactivation on prostate in vivo. Our results indicate that disruption of Notch signaling led to enhanced proliferation and impaired differentiation of prostatic epithelial cells. In addition, it appears that inactivation of Notch pathway may facilitate prostatic tumorigenesis as in *Notch1* deletion mouse model, the prostate exhibited morphological features similar to early stages of prostatic neoplasia. Microarray analysis revealed that a number of genetic networks were altered as a result of Notch1 inactivation. Examination of a microarray database also showed a very significant down-regulation of *Notch1* and its effector *Hey-1* gene expression in human prostate adenocarcinomas.

Materials and methods

Cell culture and treatment

Mouse myoblast cell line C2C12 was kindly provided by Dr. G. Weinmaster at UCLA. The cells were maintained at low density in DMEM+RPMI1640 (50/50) medium containing 20% fetal bovine serum to prevent differentiation. Gamma-secretase inhibitors were purchased from EMD Calbiochem (San Diego, CA). For brevity, inhibitor X, {1S-Benzyl-4R-[1-(1S-carbamoyl-2-phenethylcarbamoyl)-1S-3-methylbutylcarbamoyl]-2R-hydroxy-5-phenylpentyl} carbamic acid *tert*-butyl ester, also named as L-685,458, is designated as L; inhibitor XVII, WPE-III-31C, is designated as 31C. Cells at about 60% confluency in 24-well tissue culture plates were treated with γ-secretase inhibitors and harvested 16 h later for total RNA. Triplicates were included for treatment with each compound. The concentrations used for 31C and L were 2 μM and 1 μM, respectively.

TaqMan real-time quantitative RT-PCR

Total RNA was prepared from cells or tissues with RNeasy kits (Qiagen, Valencia, CA) and used for evaluation of gene expression on ABI-7700 (Applied Biosystems, Foster City, CA). *Hes-1*-specific probe and primers were 5′-CGGAACCTGCAGCGGGCG-3′, probe; 5′-CATTCTGGAAATGACTGTGAAGCA-3′, forward primer; 5′-TGCTGAGCGCGGCG-3′, reverse primer. The probe and primers for *Hey-1* were 5′-CGGAACCTGCAGCGGGCG-3′, probe; 5′-CATTCTGGAAATGACTGTGAAGCA-3′, forward primer; 5′-TGCTGAGCGCGGCG-3′, reverse primer. Those for *c-Fos* were 5′-CCCAAGCCATCCTTGGAGCCAGT-3′, probe; 5′-CCTGCCCCTTCTCAACGAC-3′, forward primer; and 5′-TCCACGTTGCTGATGCTCTT-3′, reverse primer. Those for *c-Jun* were 5′-AGCAGAAAGTCATGAACCACGTTAACAGTGG-3′, probe; 5′-TCAGGGAACAGGTGGCACA-3′, forward primer; 5′-TGCGTTAGCATGAGTTGGCA-3′, reverse primer. Those for *FGF18* were 5′-TAGGATCAGTGCCCGTGGCGAGG-3′, probe; 5′-AGCACATTCAAGTCCTGGGC-3′, forward primer; 5′-CTGGGCATACTTGTCCCCG-3′, reverse primer. Those for *PSCA* were 5′-CAGAAATGGAGCTGGGAGGTGGGTG-3′, probe; 5′-GGCTGAGATGGGATGGACTG-3′, forward primer; 5′-CTGGGACTCCTGGTGCCTC-3′, reverse primer. Housekeeping gene *GAPDH* was used for internal control. The sequences of the probe and two primers for *GAPDH* were 5′-TTCCTACCCCCAATGTGTCCGTCGT-3′, 5′-ACTGGCATGGCCTTCCG-3′, and 5′-CAGGCGGCACGTCAGATC-3′ respectively. PCR products were checked on agarose gel to ensure specific amplification. Gene expression levels were normalized to *GAPDH* level in the same sample using the ΔCt method. Data in triplicates were calculated and presented as mean ± SD.

Dissection and culture of developing prostate tissue

Both left and right lobes of ventral prostate were dissected from postnatal day 3 (P3) rat pups and placed individually onto cell culture inserts (8-μm pore size; BD Clontech, Palo Alto, CA). The culture was maintained with serum-free medium, which was Dulbecco's modified Eagle's medium/F-12 (50 v/50 v) with insulin–transferrin–sodium selenite media supplement (I-1884; Sigma, St. Louis, MO), 2 mM glutamine, 5 mg/ml glucose, 100 U/ml penicillin and 100 mg/ml streptomycin, as well as 1×10^{-8} M testosterone (Sigma) and 1:1000 epidermal growth factor (Cambrex, East Rutherford, NJ). Gamma-secretase inhibitors 31C at 2 μM or L at 1 μM were added the same day when prostate was dissected, and the medium was changed every 3 days. The concentrations of inhibitors used in our organ culture studies were slightly higher than concentrations required for 50% inhibition (IC_{50}) of cleavage by γ-secretase on substrate peptides in cell lines (300 nM for 31C and approximately 50 nM for L) due to lower penetration efficiency in whole mount tissues than monolayer cells. Images of the cultures were taken under Nikon TE300 inverted microscope. BrdU (RPN201, Amersham Biosciences, Piscataway, NJ) was added into medium 16 h prior to tissue fixation with 4% paraformaldehyde.

Mouse colony maintenance and induction of Cre expression

Colony generation, maintenance and genotyping were performed as described (Radtke et al., 1999). *Notch1*$^{lox/lox}$*MxCre*$^{+/-}$ mice were expanded from breeding of *Notch1*$^{lox/lox}$*MxCre*$^{+/-}$ mice with *Notch1*$^{lox/lox}$ *MxCre*$^{-/-}$ mice. Their male littermates negative for *Mx-Cre* allele were used as control. Postnatal day 6–8 (P6–8) male pups were injected five times intraperitoneally with 400 μg polyI–polyC (Sigma, St. Louis, MO) at 2-day intervals. Genotyping was performed in this period. Mice were sacrificed at 1 week or 3 weeks after the last injection, and the whole prostates were collected. The evaluation of Notch inactivation including expression of Cre recombinase, PCR for *Notch1* gene deletion and TaqMan real-time RT-PCR analysis for Notch downstream gene expression were performed with prostate at 1 week post-induction. For histochemical analyses, prostates harvested at 3 weeks post-induction were used, and two larger lobes, ventral and anterior, were examined.