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Regulation of Prostate Development and Benign Prostatic Hyperplasia by Autocrine Cholinergic Signaling via Maintaining the Epithelial Progenitor Cells in Proliferating Status

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SUMMARY

Regulation of prostate epithelial progenitor cells is important in prostate development and prostate diseases. Our previous study demonstrated a function of autocrine cholinergic signaling (ACS) in promoting prostate cancer growth and castration resistance. However, whether or not such ACS also plays a role in prostate development is unknown. Here, we report that ACS promoted the proliferation and inhibited the differentiation of prostate epithelial progenitor cells in organotypic cultures. These results were confirmed by ex vivo lineage tracing assays and in vivo renal capsule recombination assays. Moreover, we found that M3 cholinergic receptor (*CHRM3*) was upregulated in a large subset of benign prostatic hyperplasia (BPH) tissues compared with normal tissues. Activation of CHRM3 also promoted the proliferation of BPH cells. Together, our findings identify a role of ACS in maintaining prostate epithelial progenitor cells in the proliferating state, and blockade of ACS may have clinical implications for the management of BPH.

INTRODUCTION

Regulation of proliferation and differentiation of prostate epithelial progenitor cells is important for the prostate development process of branching morphogenesis and prostate diseases. In the normal prostate, there are three epithelial cell types: basal cells (p63$^+$ and CK5$^+$), luminal cells (CK8$^+$), and neuroendocrine cells (Synapthophysin$^+$) (Leong et al., 2008). Even though several studies have identified the multipotent potential of luminal cells under castration conditions (Wang et al., 2009), in organoid cultures or in renal capsule recombination assays (Chua et al., 2014; Karthaus et al., 2014), it is also believed that multipotent epithelial progenitor cells are located in the basal cell compartment (Goldstein et al., 2010; Lawson et al., 2007; Leong et al., 2008; Ousset et al., 2012), which can differentiate into not only basal cells but also luminal cells and neuroendocrine cells. Previous studies, including the work from our group (Shou et al., 2001; Wang et al., 2003, 2006b, 2008), have shown that various signaling pathways can regulate epithelial progenitor cell proliferation and differentiation and, in turn, affect the formation of prostate diseases (Shen and Abate-Shen, 2010). However, whether or not there are additional important molecules that regulate prostate epithelial progenitor cells is still unclear.

Muscarinic receptors belong to the family of G-protein coupled receptors. There are five members: CHRM1–CHRM5 (Spindel, 2012). Activation of muscarinic receptors by acetylcholine usually stimulates Ca^{2+} influx, glandular secretion, and smooth muscle contraction (Wessler and Kirkpatrick, 2012). It was traditionally believed that acetylcholine was predominantly synthesized in the neuronal system. However, besides the neuronal cholinergic system, there is also a widespread cholinergic system in non-neuronal tissues, which has been identified in airway epithelial cells, hematopoietic stem cells, small intestine epithelial cells, colon epithelial cells, mesenchymal stem cells, and embryonic stem cells (Wessler and Kirkpatrick, 2012). The non-neuronal cholinergic signaling functions in regulating the differentiation and proliferation of embryonic stem cells (Landgraf et al., 2010), hematopoietic stem cells (Serobyan et al., 2007), and small intestine stem cells (Takahashi et al., 2014). In particular, our previous study demonstrated a role of autocrine cholinergic signaling (ACS) in promoting prostate cancer growth and castration resistance (Wang et al., 2015b). However, whether or not such ACS also plays a role in regulating proliferation and differentiation of prostate epithelial progenitor cells is unknown.

In the present study, we discovered the existence of ACS in the developing mouse epithelium. We used an organotypic culture system, which is free of functional nerve fibers, to study the roles of ACS in regulating prostate development. We further confirmed the results by lineage tracing and renal capsule tissue recombination assay. We

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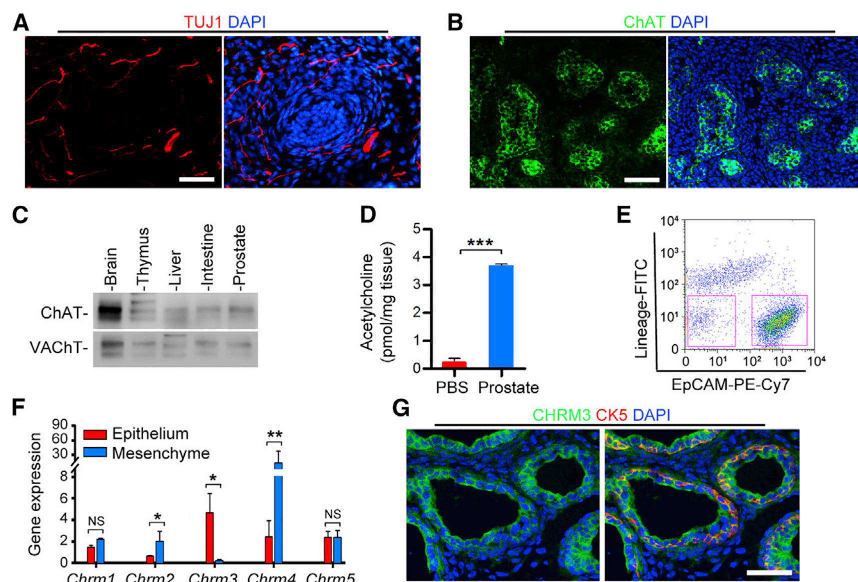

Figure 1. Prostate Epithelial Cells Express Cholinergic Markers and Release Non-neuronal Acetylcholine

(A) Immunostaining of TUJ-1 (red) in P5 mouse VP sections showing lack of nerve fibers in epithelial tissues. Scale bar, 50 μm.

(B) Immunofluorescent images showing epithelium-specific expressing of ChAT (green) in P5 mouse VP tissue sections. Scale bar, 100 μm.

(C) Western blotting analysis of ChAT and VAChT proteins in various mouse tissues.

(D) Fluorometric detection of acetylcholine in P5 mouse VPs after 2 days culture ex vivo (n = 3 experiments).

(E) Fluorescent-activated cell sorting of Lin^-EpCAM^+ epithelial cells and Lin^-EpCAM^- mesenchymal cells.

(F) Real-time PCR analysis of *Chrm1*-*Chrm5* levels in P5 mouse epithelial cells and mesenchymal cells (n = 3 experiments).

(G) Immunofluorescent staining of CHRM3 (green) and CK5 (red) in developing mouse VPs. Scale bar, 50 μm.

Data above were analyzed with Student's t test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Error bars indicate SEM.

found that activation of ACS promoted prostate development by enhancing the proliferation of epithelial progenitor cells and preventing these progenitor cells from differentiation. In addition, we demonstrated that these effects were achieved through Ca^{2+}/calmodulin signaling, which could be blocked or reversed by a specific calmodulin inhibitor, W-7. More importantly, we found that *CHRM3* was upregulated in a large subset of BPH tissues compared with normal tissues. ACS promoted BPH cell proliferation through Ca^{2+}/calmodulin-signaling-mediated phosphorylation of AKT. Taken together, our findings identify ACS as another important component that keeps prostate epithelial progenitor cells in the proliferating state, and blockade of ACS may have clinical implications for the management of BPH.

RESULTS

Existence of ACS in the Developing Mouse Prostate Epithelium

Our previous study demonstrated the existence of functional ACS in regulating prostate cancer growth and castration resistance (Wang et al., 2015b). However, whether there is also an ACS in developing prostate epithelium and how this ACS regulates prostate development has not been determined. To examine the expression of cholinergic components in developing prostates, we performed immunofluorescent staining of TUJ-1 (a specific neuronal lineage marker) and ChAT (choline acetyltransferase, a key enzyme for the synthesis of acetylcholine) in P5 mouse ventral prostate (VP) sections. While a substantial number of TUJ-1 immunoreactive nerve fibers were observed in the mesenchyme, no nerve fiber was seen inside the epithelium (Figure 1A). In sharp contrast, epithelial cells were strongly immunoreactive for ChAT, a key enzyme responsible for the synthesis of acetylcholine (Figure 1B). In addition, western blotting analysis confirmed the expression of ChAT and vesicular acetylcholine transporter (VAChT) in postnatal mouse VPs (Figure 1C). Furthermore, we performed a fluorometric analysis to measure the synthesis of acetylcholine in isolated mouse VPs. We found that the isolated VPs could secrete acetylcholine after 2 days in cultures (Figure 1D). Since the parasympathetic nerve fibers were cut off during the dissection of VPs, most of the nerve fibers had degenerated and lost their functions after 2 days in culture (Figures S1A and S1B). Therefore, the acetylcholine was synthesized and secreted by prostate epithelial cells rather than from the nerve endings.

Activation of ACS needs not only the non-neuronal acetylcholine, but also the expression of muscarinic receptors in prostate epithelial cells. To examine the expression of muscarinic receptors in developing mouse prostate, we sorted prostate epithelial cells ($lineage^-EpCAM^+$) from mesenchymal cells ($lineage^-EpCAM^-$) by fluorescence-activated cell sorting (FACS) (Figure 1E) and measured the expression of muscarinic receptors, *Chrm1*-*Chrm5*. Real-time PCR analysis indicated that all five subtypes of muscarinic receptors are expressed in the prostate epithelial cells. While *Chrm2* and *Chrm4* were expressed at higher levels in the mesenchymal cells than in the epithelial cells (Figure 1F), expression levels of *Chrm1* and *Chrm5* did not

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