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Spatial transcriptomics identifies candidate stromal drivers of benign prostatic hyperplasia


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**Introduction**

Benign prostatic hyperplasia (BPH) is the nodular proliferation of epithelial and stromal elements within the transition zone of the prostate (1). BPH leads to prostate enlargement and lower urinary tract symptoms — including urinary storage and voiding problems — that cause substantial morbidity in men over 50 years old (1). Current BPH treatments — including α-adrenoceptor antagonists and 5α-reductase inhibitors — are only partially effective and may not target the underlying pathologic processes, which are still incompletely known.

In the 1970s, Stanford pathologist John McNeal recorded detailed observations of BPH histology that informed a new hypothesis of BPH etiology (2, 3). He noted that the tangent ducts bordering BPH nodules exhibited epithelial hyperplasia and neoductal growth exclusively on the side facing into the nodule (Figure 1A). This eccentric effect suggested exposure to stromal inductive processes known to occur in prostate embryogenesis and later shown to be critical to epithelial and ductal growth by tissue recombination studies (4). Thus, McNeal proposed that BPH nodules arise by the “reawakening” of embryonic inductive activity by adult prostate stroma. Whether the diffusible factors of BPH stroma mirror those of prostate embryogenesis has remained largely unknown.

Studies of gene-knockout mice and of rat prostatic rudiments in culture have identified secreted factors that modulate prostate development and ductal branching morphogenesis (5, 6), including fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), transforming growth factors (TGFs), bone morphogenic proteins (BMPs), WNT proteins (WNTs), and Sonic Hedgehog (SHH). By extension, many of those same factors have been implicated in BPH (7). More recently, bulk tissue and single-cell transcriptome studies have enumerated the genes expressed in human BPH tissues and cells (8, 9) but have lacked histologic spatial resolution.

Here, by spatial transcriptomics of human BPH nodules, we identify secreted factors expressed in the presumptive inductive stroma adjacent to hyperplastic branching ducts. Our findings provide insight
into McNeal’s hypothesis of embryonic reawakening and suggest what we believe to be new avenues for disease-targeted therapy.

Results

Spatial transcriptomics identifies factors secreted by presumptive inductive BPH stroma. McNeal observed that tangent ducts bordering BPH nodules exhibit epithelial hyperplasia (with taller columnar epithelial cells) and neoductal growth (green arrow) exclusively on the side facing into the nodule, implying an inductive inner stroma (yellow star) and noninductive outer stroma (blue star). Histologic image from J McNeal in The Urologic Clinics of North America (3) with permission of the publisher. (B) For shorthand, we term these ductal structures “BPH hubs” for their similarity to a wagon wheel or USB hub (shown). (C–H) Six hubs (Hubs 1–6) that display the classic features noted by McNeal were selected for laser capture microdissection. Inner stroma (yellow star) and outer stroma (blue star) are indicated. Scale bar is 500 μm.

Figure 1. “BPH hubs” suggest an inductive stroma. (A) Tangent ducts at the periphery of BPH nodules display epithelial hyperplasia (with taller columnar epithelial cells) (pink arrow) and neoductal growth (green arrow) exclusively on the side facing into the nodule, implying an inductive inner stroma (yellow star) and noninductive outer stroma (blue star). Histologic image from J McNeal in The Urologic Clinics of North America (3) with permission of the publisher. (B) For shorthand, we term these ductal structures “BPH hubs” for their similarity to a wagon wheel or USB hub (shown). (C–H) Six hubs (Hubs 1–6) that display the classic features noted by McNeal were selected for laser capture microdissection. Inner stroma (yellow star) and outer stroma (blue star) are indicated. Scale bar is 500 μm.
placenta associated 9 (PLAC9). Notably, 8 of the top 10 genes (80%), and 11 of the top 20 (55%) genes, are known or predicted secreted proteins, representing a significant enrichment over the known human proteome (9.4%) \((P \leq 3.7 \times 10^{-7};\) Hypergeometric test) (12). By Ingenuity Pathway Analysis (IPA; QIAGEN), the top inner stromal genes were enriched for physiologic processes that included tissue development, tissue morphology, and organ morphology (Supplemental Table 3). Our subsequent studies focused on the 2 top genes, IGF1 and CXCL13.

By RNA in situ hybridization (RISH), we verified enriched expression of IGF1 and CXCL13 in the inner stroma adjacent to BPH hubs, including among hubs that were microdissected and transcriptionally profiled (exemplified by Hub-1 in Figure 3, A–D, and quantified in Supplemental Table 4), as well as in additional independent hubs (exemplified by Hub-7 in Figure 3, E–K). Importantly, enriched expression did not merely reflect an enrichment of prostate fibroblasts, since interstitial prostate fibroblasts — identified by expression of complement factor 7 (C7) (9) — were observed in both the inner and outer stroma (Figure 3, D, H, and K).

IGF1 and CXCL13 expression were not exclusive to the BPH hub–adjacent stroma. By examining the full prostate cross sections, we observed stromal IGF1 and CXCL13 expression elsewhere within BPH nodules, but always in association with adjacent epithelial hyperplasia and ductal branching (as observed for the BPH hubs) (examples in Supplemental Figure 1).

Since IGF1 is expressed in hub-adjacent BPH stroma, we next asked whether its receptor — insulin-like growth factor 1 receptor (IGF1R) — was expressed in the hub ductal epithelium. Indeed, by 2-color RISH, we observed epithelial IGF1R expression adjacent to IGF1+ stroma (Figure 5, A, B, and C).
and E, and quantified in Supplemental Table 4). Perhaps more surprisingly (given the studied role of CXCL13 in lymphocyte chemoattraction), we also observed epithelial expression of the CXCL13 receptor — CXC chemokine receptor 5 (CXCR5) — adjacent to CXCL13+ stroma (Figure 5, C and F).

Although expressed at lower levels than IGF1R, CXCR5 expression was clearly visible in comparison with negative control probes (Figure 5D).

IGF1 is a growth factor known to promote cell proliferation (13). Consistent with that function, we observed by immunohistochemistry increased Ki-67+ (proliferating) epithelial cells in BPH hub epithelium adjacent to inner IGF1+CXCL13+ stroma, in comparison with the outer IGF1−CXCL13− stroma (Figure 5, G and H, and Supplemental Figure 3) (P = 0.04, paired 2-sided Student’s t test). CXCL13 may have more than one function, but it has been best characterized as a lymphocyte chemoattractant (11). Perhaps surprisingly then, we observed B and T cell infiltrates within both the inner CXCL13+ stroma and the outer CXCL13− stroma (example shown in Figure 5I).

IGF1 but not CXCL13 is expressed in human embryonic prostate stroma. McNeal’s hypothesis — that BPH represents a reawakening of embryonic patterns of stromal induction — would imply that IGF1 and CXCL13 are also expressed in human embryonic prostatic stroma. To investigate this possibility,

Figure 3. RISH verifies IGF1 and CXCL13 expression in inner (inductive) stroma. (A–D) RISH exemplified for a laser-microdissected hub, Hub-1. (E–K) RISH exemplified for an independent hub, Hub-7. (A and E) H&E stain. Inner stroma (yellow star) and outer stroma (blue star) are indicated; scale bar is 500 μm, and all figure insets are an additional 2.7× magnification. (B, F, and I) IGF1 expression by RISH (brown staining). (C, G, and J) CXCL13 expression by RISH (brown staining). (D, H, and K) C7 expression (marks interstitial prostate fibroblasts) by RISH (brown staining); arrows indicate fibroblasts interspersed within the “outside” stroma. Note, RISH for IGF1, CXCL13, and C7 was conducted on 7 additional BPH hubs (Supplemental Table 4).
we carried out RISH on human fetal prostate tissues (obtained from elective terminations) of 12–15 weeks of gestation, within the time frame of embryonic ductal branching (14). We observed marked IGF1 expression within the embryonic prostate stroma encircling the urethra (Figure 6, A and B), and adjacent to new ductal budding (Figure 6, D and E) and ductal branching (Figure 6, G and H), as well
as IGF1R expression in the prostatic epithelium (Figure 6, B, E, and H). In contrast, CXCL13 expression was not apparent in the embryonic prostate stroma, although low-level expression of CXCL13 and/or CXCR5 could be observed in a small minority of epithelial cells (Figure 6, C, F, and I).

Inhibition of IGF1 signaling abolishes BPH spheroid and organoid formation. Our spatial transcriptomics identified stromal IGF1 as a candidate inducer of BPH ductal epithelial hyperplasia, proliferation (Ki-67 staining), and/or neoductal growth. To evaluate IGF1 function on glandular epithelium, we used 2 different 3D cell culture models: spheroid culture of BPH-1 cells (a prostate epithelial cell line established from human BPH tissue) (15) and organoid culture (16) of primary patient-derived BPH epithelial cells. Since IGF1 (and/or the cross-activating insulin) (17) is a component of BPH-1 cell culture media (containing fetal bovine serum), prostate organoid media (containing insulin), and Matrigel (where growth factor–depleted Matrigel retains IGF1), we evaluated the effect of IGF1R inhibition. Two small molecular IGF1R tyrosine kinase inhibitors — BMS-754807 (18) and picropodophyllin (PPP) (19) — at 1 μM thwarted BPH-1 spheroid formation and
BPH organoid formation in Matrigel ($P < 0.001$; 2-sided Student’s $t$ test) (Figure 7, A–C, E–H, and J, and Supplemental Figure 4).

Given the observed $\text{CXCR5}$ expression in BPH epithelium, we also sought to evaluate $\text{CXCL13}$ function on BPH epithelium. Since $\text{CXCL13}$ is not a known component of BPH-1 cell media or prostate organoid media, we evaluated the effect of $\text{CXCL13}$ addition on BPH-1 spheroid and BPH organoid culture. Physiologic concentrations of recombinant human $\text{CXCL13}$ (100 ng/mL) had no discernable impact on spheroid or organoid formation (Figure 7, D, E, I, and J). However, we note that expression of the $\text{CXCL13}$ receptor $\text{CXCR5}$, by quantitative reverse transcription PCR (qRT-PCR), was substantially lower compared with $\text{IGF1R}$ in BPH-1 spheroids and BPH organoids (Supplemental Figure 5).

**Discussion**

Decades ago, McNeal observed that the tangent ducts bordering BPH nodules display epithelial hyperplasia and neoductal growth exclusively on the side facing into the nodule, suggesting a response to inductive...
stroma (3). Here, by spatial transcriptomics of the inner versus outer stroma neighboring these BPH hubs, we have identified candidate stromal inductive factors, including IGF1 and CXCL13.

From mouse knockout studies, IGF1 was previously linked to prostate development and ductal branching morphogenesis (10), and indeed IGF signaling has long been implicated in BPH (20). However, much of the evidence has been circumstantial, and uncertainty has remained even over which prostate cell type(s) produce (or respond) to IGFs. In one study, primary human BPH fibroblasts were found to express $IGF2$ but not $IGF1$ (21). In another study, some but not other primary human prostate fibroblast cultures (albeit none derived from BPH tissue) showed androgen-stimulated expression of $IGF1$ (22). Other studies have implicated $IGF1$ expression from prostate epithelial cells and even macrophages (23). Thus, importantly, we believe our study is the first to identify high $IGF1$ expression in human BPH fibroblasts at the site of $IGF1R^+$ epithelial hyperplasia and neoductal growth, providing “smoking gun” evidence of the key role of stromal IGF1 in BPH nodular morphogenesis.

Our finding of high stromal $IGF1$ expression adjacent to BPH hubs is notable, but equally striking is what we did not find. Other growth factors — including FGFs, TGFs, BMPs, WNTs, and SHH — also have reported roles in fetal/neonatal prostate development (5, 6) and by extension have been implicated in BPH (1, 7). Yet among those growth factors, our studies identified only $IGF1$ and $IGF2$ as top enriched genes. Our findings singularly highlight IGF1 as a critical growth factor in BPH nodular proliferation and therefore as a persuasive target for therapy. Our results may also explain why hyperinsulinemia is a risk factor for BPH (24); high insulin levels can crossactivate the IGF1R (17). Nonetheless, other growth factors — including bone morphogenetic protein 5 (BMP5) that we previously found highly overexpressed in bulk BPH tissue (8) — likely contribute to BPH, albeit exhibiting less polarized expression in the inner stroma, or perhaps with activity at earlier stages of nodular growth.

We recently reported markedly elevated expression of $CXCL13$ in bulk BPH tissue, where it was also part of a 65-gene BPH stromal signature that correlated with BPH symptom severity (8), but it had until then never been implicated in BPH. $CXCL13$ has been characterized as a lymphocyte chemoattractant (11).
Nonetheless, in our preliminary studies, we observed lymphocyte infiltrates within both the inner and outer stroma. Notably, we observed expression of CXCR3 (the only known CXCL13 receptor) in BPH epithelium. This finding suggests the possibility that CXCL13 functions as a paracrine factor in the growth or some other characteristic of adjacent prostatic epithelium. While our studies in 3D culture did not reveal that function, future studies (including in vivo) should inform the function(s) of CXCL13 in BPH pathogenesis.

The top gene transcripts overexpressed in inner (presumptive inductive) stroma were enriched for secreted factors, as predicted by McNeal’s hypothesis. Notably, their known functions suggest roles not only in epithelial induction (IGF1, IGF2), but also in the proliferation, differentiation, or function of other cell types, including immune cells (CXCL13) (11), endothelial cells (PROK1) (25), and peripheral neurons (EDN3) (26). This finding underscores that BPH nodular proliferation is more than merely epithelial and fibroblast proliferation, but rather encompasses the morphogenesis of diverse new tissue elements. The function of other top-ranked inner stroma gene transcripts (e.g., PTN, PLAC9) remains to be investigated. Future studies will also evaluate the protein expression of the above factors (which is notoriously challenging for secreted proteins). Also of future interest are the gene transcripts enriched within the outer stroma of hubs, which might include factors that constrain epithelial proliferation. Single-cell spatial approaches will also refine the list and cell type source of inductive factors, though we note that current approaches would have missed CXCL13, which is not included in existing catalog gene sets.

Our studies were motivated by McNeal’s observations and hypothesis that BPH is the reawakening of embryonic inductive activity by adult prostate stroma. Though mesenchymal induction of prostatic epithelial development was elucidated in the mouse and rat, recent studies demonstrated that human urogenital sinus mesenchyme is also an inducer of prostatic epithelial development (27). It is therefore notable that our findings only partly support McNeal’s hypothesis. IGF1 is expressed in BPH inner (inductive) stroma and in the stroma of human fetal prostates undergoing ductal branching morphogenesis. However, CXCL13 expression is absent from the stroma of those fetal prostates. Thus, in the most straightforward interpretation, BPH nodular proliferation is not simply the reactivation of embryonic inductive patterns. Future studies that employ single-cell profiling of active chromatin (e.g., by ATAC-seq) (28) may reveal the processes regulating IGF1 and CXCL13 expression in BPH fibroblasts, and whether these reflect normal embryonic patterns or focal responses, for example to hormonal changes, inflammation, or aging.

Our studies also have several therapeutic implications. Most notably, 2 small molecule IGF1R inhibitors, each with different off-target profiles (18, 19), abolished BPH-1 spheroid and patient-derived BPH organoid growth in cell culture. While follow-up studies (including in vivo studies) are needed, our findings suggest the potential efficacy of IGF1R inhibitors as a disease-targeted approach in preventing or managing BPH and its associated lower urinary tract symptoms. In cancer clinical trials, IGF1R inhibitors have shown manageable toxicity (albeit with limited clinical benefit against many cancers) (29); thus, they may find utility in BPH, particularly if delivery or activity can be restricted to the prostate. Indeed, inhibition of mTOR (which functions downstream of IGF1R) was reported to reduce prostate size (30). And saw palmetto extract, an alternative BPH therapy, may modulate the IGF1 signaling pathway (31).

Also intriguing is that the BPH fibroblasts expressing IGF1 and CXCL13 also express SRD5A2 (5α-reductase) and AR. Currently, 5α-reductase inhibitors (e.g., finasteride and dutasteride) are a first-line therapy for BPH, where they inhibit the conversion of testosterone to the more potent dihydrotestosterone, leading to the involution of androgen-dependent prostatic epithelium and prostate shrinkage (26). Of note, during prostate development, androgens act primarily on embryonic mesenchyme to prompt the secretion of paracrine-acting epithelial growth factors (32). Thus, it will be of interest to determine whether 5α-reductase inhibitors also affect the AR+ BPH fibroblast expression of IGF1, CXCL13, and/or other secreted/inductive factors. If so, then these therapies might prove “more targeted” to BPH disease mechanisms than currently recognized.

**Methods**

*Prostate tissue specimens.* Human BPH tissue specimens were obtained from radical prostatectomy cases (done for prostate cancer) at the Stanford Hospital, with IRB approval and patient informed consent. LCM studies were done using archived FFPE blocks, while single-cell RNA-Seq and patient-derived organoid studies used fresh BPH tissue. Patient age, prostate size, and International Prostate Symptom Score are listed in Supplemental Table 1. Human fetal prostate specimens were obtained from elective terminations done at UCSF, with IRB approval and patient informed consent.
**LCM and RNA-Seq.** For each BPH hub, LCM was done on 3 consecutive 10 μM FFPE sections, using an Arcturus XT System. RNA was isolated from captured tissue using QIAGEN RNeasy FFPE kit, then quantified by Agilent 2100 BioAnalyzer. RNA-Seq libraries were constructed from 2 ng RNA input using Takara SMARTer Stranded Total RNA-seq kit v3 — Pico Input Mammalian. RNA-Seq libraries were evaluated by Agilent BioAnalyzer, then sequenced on an Illumina NovaSeq 6000 (Novogene) to an average 46 million read pairs per sample.

**RNA-Seq data analysis.** Illumina sequence reads were used to quantify transcript abundance as transcripts per million (TPM) using kallisto (33). TPM data were processed sequentially by thresholding low values (to 0.01), log, transforming, mean centering genes and samples, and excluding genes with low variability in expression (SD < 1). Differentially expressed genes (inner/inductive versus outer stroma) were identified using paired-SAM (34), which estimates false discovery rates against randomly permuted samples. Pathway analysis was done using IPA (QIAGEN). Predicted secreted proteins were identified from the Human Protein Atlas (12).

**RISH and immunohistochemistry.** RISH was done by manual RNAscope Chromogenic assays, using RNAscope 2.5 HD Assay—BROWN or Duplex Assay according to the manufacturer (ACDBio). Chromogenic probes were Hs-IGF1, Hs-CXCL13, Hs-C7, Hs-CXCL13-C2, Hs-IGF1R-C2, Hs-SRD5A2-C2, and Hs-AR-C2. Semi-quantitative scoring of RNAscope assays (based on dots per cell, 0–4 scale) was done per ACDBio guidelines. Immunohistochemistry was done using Tris-EDTA antigen retrieval, with ImmunPress HRP Detection Kit and ImmunPress Duet Double Staining Polymer Kit (HRP/AP) (Vector Laboratories). Primary antibodies were anti–Ki-67 (D2H10, 1:400) (Cell Signaling Technology), anti-CD20 (L26, 1:400) (MilliporeSigma), and anti-CD3 (MRQ-39, 1:800) (MilliporeSigma).

**Single-cell RNA-Seq.** For single-cell RNA-Seq, fresh human BPH and normal prostate tissues were separately disaggregated to single cells by sequential incubation with collagenase/hyaluronidase, trypsin, and dispase, following Prostate Tissue Dissociation protocol (STEMCELL Technologies) (35). Red blood cells were lysed by Ammonium Chloride Solution (STEMCELL Technologies), and then live cells (propidium iodide negative) were flow-sorted (Stanford Shared FACS Facility). Single-cell RNA-Seq libraries were generated separately for BPH and normal prostate tissue using 10x Genomics Chromium Controller (Stanford Functional Genomics Facility), reagent kits, and protocols. Libraries were sequenced on an Illumina X Ten (Novogene). Single-cell data were processed using Cell Ranger, and BPH and normal prostate data were then aggregated (cellranger aggr command) and visualized using Loup Browser (10x Genomics).

**Prostate cell culture experiments.** BPH-1 cells (15) were obtained from the DSMZ cell culture repository, then verified by short tandem repeat profiling (ATCC). For spheroid assays, 50,000 cells (per 6-well plate well) were plated in growth factor–reduced Matrigel (Corning) using Matrigel “3D on-top” assays (36). BPH-1 cell media comprised RPMI-1640 media with 5% FBS. For patient-derived organoids, fresh human BPH tissue was disaggregated to single cells as detailed above. Cells were then plated in Matrigel as droplets (20,000–50,000 cells) (16) or “3D on-top” assays (100,000–200,000,000 cells) in a 24-well plate (36). BPH organoids were grown in Clevers media, exactly as described (16), with media changes every 2–3 days. BMS-754807 and PPP were obtained from SelleckChem and human recombinant CXCL13 from PeproTech. Spheroids and organoids were imaged using a Keyence BZ-X810 and diameters measured using ImageJ (NIH). In some experiments, spheroids were harvested from Matrigel by dilution in cold PBS with 5 mM EDTA, then fixed in 4% paraformaldehyde, and embedded in paraffin for subse-

**Statistics.** Unless otherwise indicated, a 2-sided Student’s t test was performed to assess statistical significance, using GraphPad Prism 10.0, where P < 0.05 was considered significant. Paired 2-class analysis of RNA-Seq data was done using SAM (34), which estimates false discovery rates against randomly permuted samples. Pathway analysis was done using IPA, where P values are calculated by Hypergeometric test.

**Study approval.** The study was approved by the Stanford University IRB. Written informed consent was received from participants prior to inclusion in the study.

**Data availability.** All RNA-Seq and single-cell RNA-Seq data are available from the NIH Database of Genotypes and Phenotypes (phs003477.v1.p1). Supporting Data Values associated with the manuscript figures are available as supplemental material, in the Excel file.
Author contributions
ASP, CAK, GC, LSB, RBW, JDB, and JRP conceived and planned the studies; ASP, ZS, SV, and JRP performed experiments; GRC and LSB contributed key resources; ASP, NB, and JRP analyzed data; and ASP and JRP wrote the manuscript.

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