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Keratinocyte-Derived Chemokine Induces Prostate Epithelial Hyperplasia and Reactive Stroma in a Novel Transgenic Mouse Model

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Abstract

Background—Interleukin-8 (IL-8) is upregulated in fibrotic and malignant diseases and is a key mediator of proliferative responses. Elevated IL-8 was recently correlated with benign prostatic hyperplasia epithelium and a myofibroblast reactive stroma. Thus, we sought to determine whether overexpressed IL-8 and keratinocyte-derived chemokine (KC), the functional murine homolog of IL-8, induce prostate epithelial hyperplasia and a reactive phenotype.

Methods—Transgenic mice that overexpress KC within prostate epithelia and xenograft models with engineered human cells that overexpress IL-8 were developed.

Results—Overexpression of KC in transgenic mice produced hyperplastic prostate epithelial acini associated with a periacinar reactive stroma. KC induced an altered epithelial/stroma proliferation index ratio, increased acini diameter, epithelial infolding, and expression of prototypical reactive stroma markers. Overexpression of IL-8 in normal human prostate epithelial xenografts correlated with elevated epithelial proliferation index and altered morphology. Elevated human prostate stromal and epithelial cell proliferation, nodule-like morphology and increased xenograft survival were observed in IL-8-overexpressing orthotopic xenografts.

Conclusions—Together, these data demonstrate that overexpression of IL-8/KC results in a prostate epithelial hyperplasia with an associated reactive stroma phenotype. The novel transgenic mouse and human xenograft models described here may be useful in dissecting key mechanisms of IL-8 induced prostate hyperplasia and reactive stroma.

Introduction

Keratinocyte-derived chemokine (KC) is the functional paralog of human interleukin 8 (IL-8) in mice [1,2]. KC competitively binds the human IL-8 type B receptor and interacts specifically with a murine cognate receptor, CXC receptor 2 (mCXCR2) [3]. KC is a member of the Glu-Leu-Arg (ELR) motif CXC family of α -chemokines, associated with angiogenesis and chronic fibroproliferative disorders [4]. Parallel to IL-8 in both biological role and expression pattern [5], KC is a key mediator of murine acute inflammation and neutrophil chemotaxis [3,6–8], rapidly expressed by endothelial cells at sites of vessel injury [9] and by epithelial cells at sites of wounding [2].

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Overexpression of IL-8 in epithelium most likely relates to the barrier function of epithelium and epithelial responses to wounding. The epithelium, as a cohesive barrier layer, is exquisitely sensitive to alterations in the integrity of the epithelial layer or proximal extracellular matrix (ECM). Overexpression of IL-8 parallels alterations in epithelial barrier homeostasis. Examples include gastrointestinal mucosal dependence on epithelial expression of IL-8 for recruitment of mononuclear phagocyte progenitors and for initiating mucosal inflammation [10], progression of insult-dependent cystic fibrosis as a function of sustained IL-8 production by airway epithelium [11], IL-8 secretion by pulmonary epithelia in response to matrix metalloproteinase 12-mediated ECM degradation in a model of human pulmonary inflammation [12], and glial cell stimulation induced by release of IL-8 by retinal epithelia in ocular inflammatory disease [13]. Thus, IL-8 is a key secreted factor in epithelium during stress responses following epithelial damage or homeostatic perturbations [14,15]. Less clear is the role of IL-8 in regulating epithelial cell proliferation, repair and wound repair responses in the immediate stroma environment, particularly in hyperplastic disorders.

We have reported recently that IL-8 is significantly elevated in epithelial cells within benign prostatic hyperplasia (BPH) nodules [16]. Furthermore, we showed that elevated IL-8 in BPH epithelia correlated spatially with a periacinar reactive stroma composed of myofibroblasts and fibroblasts with expression of prototypical reactive stroma markers [16]. BPH reactive stroma phenotype was typical of a myofibroblast-based reactive stroma we reported previously associated with prostatic intraepithelial neoplasia and foci of well-differentiated cancer [17]. The myofibroblast is a critical cell type in wound repair [18], mediating fibroproliferative disorders in multiple organs, including skin [19], lungs [20], liver [21], pancreas [22], kidneys [23,24] and urogenital tissue [25].

While the origin of the myofibroblast is not yet resolved, this cell type is closely tied to inflammation, and is likely activated by α -chemokines like KC and IL-8. Supporting this premise, deletion of the cognate receptor for KC, mCXCR2, resulted in delayed wound healing and inhibition of angiogenesis [26,27]. Therefore, targeted expression of KC or IL-8 to prostate epithelium would address whether this α -chemokine directly influences epithelial proliferative status or induction of a periacinar reactive stroma phenotype. As IL-8 is overexpressed in BPH, prostate cancer and several other human adenocarcinomas associated with a reactive stroma tumor microenvironment [28–33], it is important to understand the role of IL-8 activity in regulating epithelial proliferation and the reactive stroma tumor microenvironment in cancer as well as in benign disease. To address these issues, the present study was focused on developing a novel transgenic mouse model targeting expression of KC to mouse prostate epithelium and xenograft models constructed of human prostate epithelial cells (PrEC) engineered to overexpress IL-8 recombined with human prostate stromal cells.

Data reported here show that elevated KC expression induces a hyperplastic prostatic epithelium, characterized by age-associated acini infolding and significant increases in acini diameter. Moreover, overexpression of KC was associated with a prototypical reactive stroma phenotype characterized by overexpression of tenascin-C and pro-collagen I. These results were corroborated by subcutaneous and orthotopic xenograft implants composed of human PrEC that overexpress IL-8 mixed with human prostate stromal cells. Here, IL-8 induced elevated proliferation of both epithelial and stromal cells, altered nodule-like morphology, and affected orthotopic xenograft survival.

It is anticipated that the novel transgenic mouse and human xenograft models described here will be useful in dissecting key mechanisms pertaining to how IL-8 may regulate benign and

malignant disease, and may help to facilitate development of novel therapeutics targeting IL-8-mediated tissue response.

Materials and Methods

KC Transgenic Construct Cloning

The enhanced probasin promoter, ARR₂PB (courtesy of Dr. Robert Matusik) [34,35], was cloned into the MCS of the pBSIISK subcloning vector (Stratagene, La Jolla, CA) to construct pBSIISK-ARR₂PB. Mouse KC, from the Kozak secretion signal through the penultimate amino acid, was PCR amplified from the IMAGE consortium clone BC037997 (Open BioSystems, Huntsville, AL), gel-purified (GeneClean kit, Qbiogene, Irvine, CA), shuttled through pPCRScript according to the manufacturer's protocol (Stratagene, La Jolla, CA) for digestion (EcoRI/NotI), and ligated into pGENE-V5-C (Invitrogen, Carlsbad, CA) to construct pGENE-KC-V5-C.

Intron II of the rabbit β -globin gene was PCR amplified from pSG5-KHA2M1 (Stratagene), gel-purified, shuttled through pPCRScript for digestion (BstX1), ligated into pGENE-KC-V5-C to construct pGENE-intron-KC-V5-C, and verified by sequencing with the pGENE forward primer (Stratagene). The intron-KC-V5 fragment was PCR amplified, with flanking SpeI sites, gel-purified, shuttled through pPCRScript for digestion (SpeI), ligated into pBSIISK-ARR₂PB to construct pBSIISK-ARR₂PB-intron-KC-V5, and verified by sequencing using the T7 primer (Promega, Madison, WI). The final component of the transgenic construct, the hGH poly(A) tail, was PCR amplified from pIRESneo (Clontech, Mountain View, CA), gel-purified, shuttled through pPCRScript for digestion (XbaI/SacII), and ligated into pBSIISK-ARR₂PB-intron-KC-V5 to construct pBSIISK-ARR₂PB-intron-KC-V5-poly(A). The ARR₂PB-intron-KC-V5-poly(A) fragment was PCR amplified, with flanking KpnI sites, gel-purified, shuttled through pPCRScript, and sequenced using 6 independent, designed primers to verify all 1564bp. 100 μ g of KpnI-digested, gel-purified transgenic DNA was given Dr. Franco DeMayo of the BCM Mouse Embryo Manipulation Services (MEMS) Core for microinjection into C57BL/6 mouse egg pronuclei.

Rodent Information and Screening

All experiments and animal work involving transgenic mice and wildtype littermates followed an IACUC approved animal protocol (AN-1867). Two independent transgenic lines were created; KC1 and KC2, from 2 positive C57BL/6 inbred founders (of 10 total mice received from the MEMS Core) and maintained separately in the pathogen-free BCM Transgenic Mouse Facility (TMF). All orthotopic and subcutaneous xenograft experiments involving nude mice and rats followed an approved IACUC protocol (AN-3801). Athymic NCr-nu/nu nude mice (01B74) and athymic Cr:NIH-RNU nude rats (02N01), aged ~ 5–7 weeks, were purchased from Charles River Laboratories–NCI-Frederick (Frederick, MD) and housed in sterile cage setups.

Founder mice (i.e. F0 generation) as well as transgenic DNA-bearing progeny in all subsequent generations (i.e. F1–F2 generations) via backcrossing with wildtype C57BL/6 males or females (Harlan), were identified using phenolchloroform/ethanol purified genomic DNA from tail clips with the following primers:

Forward (rabbit intron): 5'-ACCATGATCCCAGCCACCCGCTCG-3'

Reverse (V5 epitope): 5'-GAATCGAGACCGAGGAGAGGGTTAGGGA-3'

RNA Expression Analysis

After germline transmission of KC1 and KC2 were determined, prostates were harvested from only F2 mice, between 9 and 30 weeks of age, to verify productive transcription according to standard protocol. Briefly, 10–30 mg of excised mouse prostate tissue was submerged in PBS+10% RNA later (Qiagen, Valencia, CA), incubated 24 hr at RT, stored at –80°C, homogenized in TRIZOL (Qiagen, Valencia, CA) for 30 sec, incubated at RT for 5 min, and then centrifuged at 12,000 rpm for 10 min at 4°C. 200 µl of chloroform (per 1 ml of starting TRIZOL volume) was added, samples were shaken for 15 sec and incubated at RT for 3 min, then centrifuged at 12,000 rpm for 15 min at 4°C. The aqueous phase was shaken with 450 µl of isopropanol and incubated for 10 min at RT, centrifuged at 12,000 rpm for 10 min at 4°C to pellet RNA. The RNA pellet was washed with 1 ml of 75% ethanol in RNase-free water, centrifuged at 7,500 rpm for 5 min at 4°C, air dried for 5 min, placed on ice and resuspended in 100 µl of RNase-free water. The RNeasy kit (Qiagen, Valencia, CA) was used to purify RNA for RT-PCR. RNA Primers for RT-PCR screening of RNA message were designed using Primer Express v2 (Applied Biosystems, Foster City, CA):

Forward: 5'-GCACCCAAACCGAAGTCAT-3'

Reverse: 5'-GTAGAATCGAGACCGAGGAGA-3'

KC Transgenic Mouse Husbandry and Harvesting

Mouse prostates were harvested beginning at 8 weeks of age (transgene expression begins) through 60 weeks of age. Transgene was represented at between 10–50% of the total litter, thus between 2 and 10 transgenic mice were harvested at each age-point. Prostate wet weight was not measured because of inherent variability in prostate morphology in the C57BL/6 inbred strain.

Harvesting prostate tissue was based on standard anatomical murine prostatic lobe assignments. Following overnight fixation at 4°C in 4% paraformaldehyde, prostates were processed and embedded by the BCM Pathology core, being positioned such that the urethra was parallel to the z-axis of the paraffin block, which allowed for a urethral reference point, as well as full exposure of dorsal, dorso-lateral, lateral, ventro-lateral and ventral lobes. Ventral, and ventro-lateral, lobes were selected for characterizing the KC mouse phenotype for two reasons: probasin is highly expressed in this lobe [35] and any perturbation away from the normally uniform, un-folded, cuboidal epithelial morphology, or sparse stromal compartment morphology, is more readily apparent.

IL-8 Retrovirus Cloning and Transduction

pIRESneoIL-8 (received from Dr. Ping Li, BCM) was PCR amplified over 20 cycles using the following primers:

Forward = 5'-TCTCACTGTGTGTAAACATGACTTCC-3'

Reverse = 5'-TTTATGAATTCTCAGCCCTCTTCAAAAAC-3'

The IL-8 insert was gel purified, shuttled through pPCRScript for digestion (XhoI/NotI) for ligation into pBMN-IRES-EGFP (provided by Gary Nolan) to construct pBMN-IL8-IRES-EGFP, which was transformed into MAX Efficiency Stbl2 competent cells (10268-019, Invitrogen), according to the manufacturer's protocol.

Phoenix 293T-variant A (amphotropic) cells were purchased from ATCC (Manassas, VA), transfected with pBMN-IL8-IRES-EGFP and empty pBMN-IRES-EGFP vector as a control, and utilized for retrovirus production as we have previously published [36]. Serial dilution

of retroviral supernatant was utilized to transduce normal human prostate epithelial cells (PrEC) at ~ 80% efficiency (*data not shown*).

Human and Mouse Cell Lines

Human PrEC, purchased from Lonza Bioscience (Basel, Switzerland) as intermediate basal cells [37], and PrEC transduced with IL-8 retrovirus, were maintained according to manufacturer's instructions, utilizing Lonza growth medium (PrEGM, CC-3166) and subculturing reagents (CC-5034). Human PrSC (Prostate Stromal Cells), also purchased from Lonza, were maintained in high glucose DMEM supplemented with 10% FBS, 5 µg/ml insulin (Sigma), 25 units/ml penicillin, and 25 µg/ml streptomycin. HTS-2T human prostate stromal cells were maintained according to our previous publication [17].

Nude Rat Orthotopic and Nude Mouse Subcutaneous Xenografts

Human HTS-2T, and PrEC transduced with either IL-8 or empty vector retrovirus (+/- IL-8), were thawed (25 ml warm PrEBM), centrifuged (400g, 2 min), washed (20 ml warm PrEBM), centrifuged and resuspended in PrEBM at 2×10^6 HTS-2T cells and 2×10^6 PrEC per 100 µl per rat, for 3 rats per cell type (6 rats total).

The following live surgical procedure was adapted from training with Dr. Annie Luo, BCM Scott Department of Urology. Inhaled isoflurane was utilized for intraperitoneal delivery of a combination anesthetic (42.86 mg/ml ketamine, 8.57 mg/ml xylazine, 1.43 mg/ml acepromazine) at 700 µl/kg body weight. Rats were prepped for surgery via abdominal hair removal, 70% ethanol to remove clippings, and betadine to sterilize. Skin and muscle fascia were incised anterior to the penis, and cell suspension was implanted into the ventral prostate using an ultra-thin 500 µl insulin barrel with a 31G syringe (BD Biosciences). Following injection, muscle fascia and dermis were sutured using 3-0 polydioxanone II sutures with an FS-1 3/8 cutting needle (Ethicon). Ketaprofen (5 mg/kg body weight) and ampicillin (50 mg/rat) were administered under sedation during surgery, followed by delivery once a day under isoflurane sedation, for 5 days post-operative. Orthotopic implants were developed for 14 days, harvested, fixed overnight in 4% paraformaldehyde, washed, processed and sectioned at 5µm for H&E or IHC. Every 7th slide was H&E stained in the process of determining whether an implant had developed successfully. Samples were scored as a negative incidence if no implant could be visualized by H&E after sectioning at least 80% of the tissue.

Subcutaneous Xenografts with Normal Human PrEC and PrSC

Subcutaneous xenografts were produced according to procedures we have reported previously [38]. Briefly, 8×10^6 IL-8 or control-transduced PrEC were combined with 8×10^6 PrSC (PrEC+PrSC) or not combined (PrEC-only) prior to mixing with Matrigel (BD Biosciences) (800 µl total volume) for subcutaneous injection to form each xenograft site (100 µl, 6 sites, 2 sites/mouse/experiment) for engraftments totaling 1×10^6 PrEC alone or combined with 1×10^6 PrSC per site. Xenografts developed for 21 days prior to resection, and were then weighed, measured, fixed overnight in 4% paraformaldehyde, washed, processed and sectioned (5µm) for H&E or IHC.

Immunohistochemistry and Quantitation

Qualitative IHC—All IHC staining utilized previously published protocols and reagents following secondary antibody [16,17]. To verify protein expression by the transgene in ventral prostate epithelia via the V5 epitope tag, sections were blocked with BlockHen II (BH-1001, Aves Labs, Tigard, OR) for 30 min at 37°C, followed by chicken anti-V5 (NB600-379, Novus Biologicals, Littleton, CO) diluted in 2% BlockHen II at 1:300 and

incubated overnight at 4°C. This was followed by a biotin-conjugated goat-anti-chicken secondary antibody (103-065-155) from Jackson ImmunoResearch Laboratories (West Grove, PA) diluted in 2% BlockHen II at 1:125 and incubated for 1 hr at 37°C. To confirm overexpression of native or recombinant KC protein, sections were incubated in rabbit anti-KC (5221-100, BioVision Research Products, Mountain View, CA) at 1:400 overnight at 4°C followed by biotin-conjugated goat-anti-rabbit (D-20695, Molecular Probes, Eugene, OR) at 1:500 for 45 min at 37°C.

To assess tenascin-C, IHC was performed on representative samples incubated in rabbit anti-mouse tenascin-C (AB19013, Chemicon, Temecula, CA) at 1:50 for 1 hr at 37°C followed by biotin-conjugated goat-anti-rabbit (D-20695, Molecular Probes, Eugene, OR) at 1:500 for 1 hr at 37°C. To assess transgenic pro-collagen I stromal immunoreactivity, IHC was performed on representative samples, treated with 0.15% trypsin (1:9 dilution, ab970, Abcam) for 20 min at 37°C, using goat anti-mouse pro-collagen I (sc-8787, Santa Cruz Biotech, Santa Cruz, CA) at 1:350 overnight at 4°C, followed by biotin-conjugated donkey-anti-goat (sc-2042, Santa Cruz Biotech) at 1:400 for 45 min at 37°C.

To detect GFP-expressing (IL-8) PrEC in nude rat ventral prostate orthotopic xenografts, IHC was performed using rabbit anti-GFP (A-11122, Molecular Probes, Eugene, OR) at 1:200 overnight at 4°C followed by biotin-conjugated goat-anti-rabbit (D-20695, Molecular Probes) at 1:500 for 45 min at 37°C. The following protocols were used to detect stromal markers (HTS-2T cells) in Nude rat ventral prostate orthotopic xenografts: mouse anti- α -SMA (1A4, Sigma-Aldrich) at 1:200 for 8 min at 50°C, followed by biotin-conjugated goat-anti-mouse (D-20691, Molecular Probes) at 1:500 for 4 min at 50°C, rat anti-pro collagen 1 (MAB1912, Chemicon, Temecula, CA) at 1:500 1 hr at 37°C following incubation in 0.25% trypsin (00-3006, Zymed) for 10 min at 37°C, followed by biotin-conjugated goat-anti-rat (D-20697, Molecular Probes) at 1:500 for 45 min at 37°C, mouse anti-tenascin (T2551, Sigma-Aldrich) at 1:2000 1 hr at 37°C following incubation in 0.1% pronase (Calbiochem) in 10 mM Tris-HCl, 1 mM EDTA, pH7.5 for 3 min at 50°C, followed by biotin-conjugated goat-anti-mouse (D-20691, Molecular Probes) at 1:500 for 45 min at 37°C.

Quantitative IHC—To assess Ki67 and TUNEL immunoreactivity in a quantitative manner, IHC was performed on all samples, steamed with Citrate buffer (pH 6.0) for 20 min and cooled to RT for 20 min, using rabbit anti-mouse Ki67 SP6 (RM-9106-S0, Thermo Scientific, Fremont, CA) at 1:50 overnight at 4°C, followed by biotin-conjugated goat-anti-rabbit (D-20695, Molecular Probes, Eugene, OR) at 1:500 for 45 min at 37°C. To assess TUNEL immunoreactivity, enzymatic labeling was performed on all samples steamed with Citrate buffer (pH 6.0) for 20 min and cooled to RT for 20 min, using the In Situ Cell Death Detection Kit, Fluorescein (11-684-795-910, Roche, Indianapolis, IN) according to the manufacturer's suggested protocol, including negative (Label solution only) and positive (DNase-I treated [E78311, GE, Piscataway, NJ] using 0.3U/200ml/slide pair in 50mM Tris-HCl, 10mM MgCl₂, 1mg/ml BSA, pH 7.5) controls for each round of staining.

All images were captured using a Spot Flex 64MPx camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Nikon Eclipse TE300 microscope equipped for epi-fluorescence. Visualization and noise correction was performed using Spot Advanced v4.5.9.9 software on an Apple Mac Pro (Cupertino, CA).

Quantitative evaluation and analysis—Ki67 and TUNEL immunoreactive intensity was quantified using 5 × 200 fields from serial sections stained for each marker for all mice harvested at all time points. For both Ki67 and TUNEL, fields were captured in single-blinded fashion, at random from regions that showed successful immunoreactivity, and counted in a double-blinded fashion for each marker. Comparisons of indices of proliferation

and apoptosis between indices and groups (transgenic vs. wildtype and between transgenic lines) were performed using Mann-Whitney non-parametric tests. Regression was used to determine if age affected these relationships. Testing of the Spearman's correlation coefficients was performed in order to determine whether there were any correlations between indices measured within stroma and epithelia for the entire set, and also within groups.

The average of all available measurements on an evaluated area was used for the above analyses. Difference in indices of proliferation and apoptosis between epithelium and stroma was defined as the average of matched-area differences. One-sample t-tests were used to determine whether a difference was present and significant for different groups. The scatterplot and box-and-whiskers plot were constructed for illustration of significant findings. *P* values < 0.05 were considered statistically significant. All analyses were performed (courtesy of Anna Frolov Schroeder, M.S.) using the SPSS 16.0 software package (SPSS Inc., Chicago, IL).

Xenograft Ki67 and TUNEL Quantitation and Evaluation—To quantitatively evaluate the immunoreactive intensity of Ki67 and TUNEL, 3 fields from serial sections stained for each marker were captured at $\times 200$ for all xenografts. For both Ki67 and TUNEL, fields were captured in single-blinded fashion, at random, from regions that showed successful immunoreactivity, using equipment as listed above, and counted in a double-blinded fashion for each marker. Marker scores were grouped and averaged according to implant/xenograft type. Statistical analysis consisted of using unpaired t-tests with Welch's correction (to account for non-equal variance) to compare all Control vs. IL-8 implants/xenografts, or using Kruskal-Wallis 1-way ANOVA to compare Control vs. IL-8 trends for multiple samples. Only *p*-values < 0.05 were considered significant. All analyses were performed using Prism 5.0a for Mac (GraphPad Software, La Jolla, CA).

Quantitation of Mean Acini Diameter—H&E sections from each mouse harvested were imaged at $\times 40$ in order to quantitate acini diameter. Calibrated measuring lines were drawn on the 3 largest acini within each field, using the distal edge of the basal lamina on opposite acinus sides as the line edge. Prostatic acinar diameter (μm) was compared, as a function of age (weeks) for both wildtype littermates and transgenics. Statistical analysis consisted of unpaired t-tests with Welch's correction to compare wildtype versus transgenic at individual age-points.

Analysis of variance between wildtype and transgenic groups was calculated using 1-way ANOVA, Bartlett's ANOVA and Kruskal-Wallis ANOVA. *P* values < 0.05 were considered statistically significant. All analyses were performed using Prism 5.0a for Mac (GraphPad Software, La Jolla, CA). Data represent the following *N* values of acini measurements, where WT represents wildtype and TG represents KC transgenic: 12 8wk WT, 10 8wk TG, 9 10wk WT, 9 10wk TG, 9 14wk WT, 9 14wk TG, 15 16wk WT, 6 16wk TG, 15 20wk KC, 12 20wk TG, 24 30wk WT, 33 30wk TG, 9 40wk WT, 18 40wk TG, 15 52wk WT, 19 52wk TG.

Results

KC Targeted to the Murine Prostate Epithelium

KC transgene expression was driven using the enhanced probasin promoter (ARR₂PB), which targets high expression to mouse prostate epithelium [34,35]. Expression of transgene was tracked by inserting a carboxy-terminal V5 epitope tag. Germline transmission of the KC transgene and mRNA expression in the prostate gland was verified in 2 independent mouse lines, KC1 and KC2 (Figure 1A and B). Both lines exhibited similar phenotypic

changes in epithelium and stroma, however KC1 mice displayed a somewhat lower level mRNA expression than KC2 mice (Figure 1B). Accordingly, tissues from both KC1 and KC2 lines were paired in all analyses comparing transgenic to wildtype littermates.

There were no detectable alterations in reproductive and developmental processes in KC transgenic mice as compared with normal C57BL/6 inbred strains. To analyze prostate-specific phenotype, all lobes of the prostate gland was harvested from transgenic mice and wild-type littermates at age-points ranging from 8 to 60 weeks of age (Table 1).

As determined by immunohistochemistry (IHC) for native epitopes and the V5 epitope tag, wildtype littermates displayed basal epithelial native KC expression in the ventral prostate lobe (Figure 2A) and little to no immunoreactivity for the V5 epitope tag (Figure 2C). In contrast, ventral prostate epithelium in KC transgenic mice displayed a more intense immunoreactivity for KC (native epitope) (Figure 2B) and was positive for the V5 epitope tag (Figure 2D), associated spatially with regions of infolded or thickened epithelium. V5 epitope immunoreactivity was initially observed at 16 weeks of age, and maintained at all subsequent age-points. Elevated V5 epitope immunoreactivity in KC transgenic ventral prostate gland epithelium was evident as a uniform staining pattern at the apical surface of epithelia (Figure 2D).

Overexpression of KC Induces Ventral Prostate Epithelial Hyperplasia

KC transgenic mice exhibited an increase in epithelial infolding, expanded cell numbers and focal epithelial tufting in secretory epithelium of the ventral prostate gland (Figure 2F). In comparison, control wildtype littermates exhibited typical ventral prostate secretory acini composed of simple cuboidal epithelial cells with no apparent thickening, infolding, or tufting (Figure 2E). To quantitate alterations in epithelial acini morphology, the diameter of ventral prostatic acini was measured as a function of age across all age-points (Figure 3A). At sexual maturity (8 weeks), acini diameter was equivalent between wildtype and transgenic littermates. Interestingly, beginning at 10 weeks of age wildtype and transgenic acini diameter fluctuated at equivalent rates, expanding and contracting to yield an eventual gain in acini diameter. This pattern was observed up to 20 weeks of age. Subsequent to 20 weeks, the elevation in mean acini diameter increased at a higher rate in KC transgenic mice as compared with wildtype littermates (Figure 3A). Significant increases in mean acini diameter at 30, 40 and 52 weeks of age were observed in KC transgenic mice (Figure 3A, $p < 0.05$). Analysis of variance between wildtype littermate and transgenic acini diameter growth patterns supported individual age-point differences, revealing a significant difference using Bartlett's ($p < 0.05$) and Kruskal-Wallis ($p < 0.0001$) ANOVA calculations. These data indicate that elevated KC expression in mouse ventral prostate gland epithelia induces a significant expansion in mean acini diameter in an age-dependent manner.

Overexpression of KC Alters Epithelial/Stromal Proliferation Index Ratios

Proliferation index analyses, as assessed by quantitative Ki67 IHC, showed that the epithelial compartment exhibited a higher proliferation index as compared with the stromal compartment in both KC transgenics and age-matched wildtype littermates (Figure 3B&C, $p < 0.05$). Of interest, the epithelial/stromal proliferation index ratios in KC transgenic mice were significantly higher than these ratios in wildtype littermates. Comparing stromal or epithelial proliferation rates directly between wildtype littermates and transgenic mice, at individual age-points, pointed towards a trend of increase in the transgenic ventral prostate, although statistical significance was not attained. The significant change observed in transgenic mouse ventral prostate epithelial/stromal proliferation index ratio was consistent with the significantly elevated acini diameter observed in transgenic mice.

Although proliferation ratio index was altered, the apoptotic index, as assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) immunofluorescence, did not change when comparing wildtype littermates to KC transgenics across all age-points in either the epithelial or stromal compartments (*data not shown*). Accordingly, the increase in acini diameter observed in KC transgenic mice likely resulted from elevated proliferative rates and not from altered apoptosis.

Overexpression of KC Induces a Periacinar Reactive Stroma

To determine the effects of elevated KC in the ventral epithelium on the immediate surrounding stroma, two prototypical markers of reactive stroma were evaluated at all age-points. Pro-collagen type 1 is a cytoplasmic precursor to mature collagen 1 ECM fibers and is associated with wound healing and reactive stroma [17,39]. The periacinar stroma in KC transgenic mice exhibited an apparent increase in immunoreactive staining for procollagen type 1 associated with regions of epithelial infolding and thickening at 40 (Figure 4B) and 52 (Figure 4D) weeks of age, relative to basal immunoreactivity in age-matched wildtype littermates (Figure 4A and 4C). These data were representative of patterns observed across all age-points.

Similar to pro-collagen I, tenascin-C, an ECM glycoprotein associated with reactive stroma [16,17,40], also exhibited an apparent increase in immunoreactive staining within the immediate periacinar stroma of KC transgenic mice. This was evident near regions of epithelial infolding and thickening at 30 (Figure 4F) and 52 (Figure 4H) weeks of age, relative to basal immunoreactivity in age-matched wildtype littermates (Figure 4E and 4G). These data were also representative of patterns observed across all age-points.

Together, these alterations in KC mice are consistent with the hyperplastic epithelial phenotype and the reactive stroma phenotype which was correlated with foci of overexpressed IL-8 observed in human BPH epithelium, as we have reported previously [16].

IL-8 Induced Proliferation of Human Prostate Epithelial Cells is Stromal Dependent

To address the role of IL-8 expressed by human prostate epithelial cells, normal human PrEC, transduced with control or IL-8 constructs, were implanted as subcutaneous xenografts either with normal human prostate stromal cells (PrEC+PrSC) or without stromal cells (PrEC-only) in athymic (nude) mice following protocols we have published previously [17,36]. Analysis of PrEC Ki67 indices revealed no difference between control and IL-8-expressing PrEC-only xenografts (Figure 5A). Importantly, a significant increase in IL-8-expressing PrEC Ki67 index was observed in PrEC+PrSC xenografts, relative to controls ($p < 0.05$, Figure 5A). Epithelial TUNEL index remained constant for all xenograft types (Figure 5B). These data suggest that epithelial proliferation, but not apoptotic rate, was dependent on stromal response to IL-8, a finding consistent with the altered epithelial proliferative phenotype associated spatially with reactive stroma observed in KC transgenic prostate.

Altered proliferation was also associated with changes in xenograft phenotype. Control PrEC-only xenografts were composed of clusters of PrEC with occasional lumen-like structures (Figure 5C). Control PrEC+PrSC xenografts developed similarly, displaying a modest increase in apparent cluster size (Figure 5E). IL-8-expressing PrEC-only xenografts exhibited an altered morphology, with a more complex, fused acinar morphology (Figure 5D). However, an evident shift in complexity and relative size was observed in IL-8-expressing PrEC+PrSC xenografts (Figure 5F) relative to all other xenograft types. These

data suggest that stromal-epithelial interaction, as well as IL-8 overexpression, affect PrEC morphology.

The effect of IL-8 overexpression in normal human PrEC was also evaluated as an orthotopic implantation in an athymic (nude) rat model. PrEC, transduced to overexpress IL-8 or control constructs, were co-implanted with normal human prostate stromal cells derived from the transition zone (HTS-2T cells) into the nude rat ventral prostate. Interestingly, at day 14, a majority of implants (8 of 12 implants; 67%) constructed with IL-8-expressing PrEC were viable and could be located in prostate tissue. In contrast, only 2 of 12 implants (17%) with control PrEC remained viable and were detectable upon serial sectioning through the ventral prostate. In general, the IL-8 expressing implants exhibited a central focus of IL-8-expressing PrEC (Figure 6A, *IHC for GFP*) with a collar of HTS-2T cells immunoreactive for human-specific smooth muscle α -actin (α -SMA), pro-collagen I, and tenascin-C, (Figure 6B,C&D, *respectively*).

Consistent with observations from subcutaneous xenograft experiments, quantification of Ki67 proliferative index within orthotopic xenograft implants (Figure 6E) revealed a significant increase in both human epithelial and stromal Ki67-positive nuclei within implants of IL-8-expressing PrEC combined with HTS-2T, as compared to control PrEC with HTS-2T. Conversely, no significant changes were observed with quantification of TUNEL apoptotic index (Figure 6F).

Discussion

Data presented here reveal that targeted overexpression of KC in murine ventral prostate epithelial cells results in a hyperplastic, proliferative epithelial layer typified by infolding that is associated spatially with a periacinar reactive stroma phenotype. Moreover, elevated KC expression induced an increase in the mean diameter of ventral prostate secretory glands associated with an altered histological phenotype. The proliferative status of KC-expressing epithelium relative to stroma was increased significantly, while apoptotic index remained unchanged. Thus, the hyperplastic ventral prostate epithelial acini phenotype observed in KC transgenic mice was likely facilitated by proliferation, rather than by altered cell turnover, a conclusion consistent with observations regarding the proliferative status of human BPH epithelium [41]. Also consistent with observations in human BPH, hyperplastic acini were associated spatially with a reactive stroma in the immediate stromal microenvironment in KC transgenic mice. As in human BPH, this reactive stroma was typified by overexpression of α -SMA associated with altered deposition of tenascin-C and a myofibroblast-like histology.

Data from the nude mouse subcutaneous xenografts and the nude rat orthotopic xenografts where IL-8 is overexpressed in human prostate epithelial cells was fully consistent with data derived from the KC transgenic mice. Overexpression of IL-8 in human prostate epithelial cells correlated with a significant increase in proliferation index. Moreover, orthotopic implants that overexpressed IL-8 exhibited not only a survival advantage, but also a concurrent increase in human stromal cell proliferative status. It is therefore likely that survival of both cell types, as well as the proliferative increase observed in both compartments, was due to IL-8 expression by epithelial cells.

Together, data obtained from all three models described here parallel our recent publication correlating elevated IL-8 within nodular BPH epithelium to a periacinar reactive stroma [16]. In that study, overexpression of IL-8 in BPH epithelium correlated significantly with a myofibroblast reactive stroma phenotype. Data presented here suggest that both the epithelial hyperplasia and associated myofibroblast reactive stroma observed in human BPH

were likely an outcome mediated by the overexpression of IL-8 in human BPH epithelia. However, regulation exerted by other factors downstream of IL-8, or by additional factors not associated directly with IL-8 action, cannot be ruled out.

In conclusion, these studies indicate that IL-8 is a key factor in regulating prostate epithelial-stromal interactions within the context of proliferative prostate diseases. The models reported here extend our understanding of the effect of IL-8 on normal epithelial and stromal cells, indicating that proliferation, as well as the resultant growth pattern and histological phenotype, are influenced by this α -chemokine. To our knowledge, the KC transgenic mouse represents the first transgenic model to express an α -chemokine targeted to the prostate gland that exhibits a murine prostatic phenotype consistent with several histopathological parameters observed in human BPH. It should be pointed out, however, that the KC transgenic mouse phenotype reported here does not recapitulate the complete clinical manifestation of human BPH that includes an enlarged prostate gland with lower urinary tract symptoms. Accordingly, the KC transgenic mouse is not considered a complete model of human BPH, but rather a model that recapitulates the elevated proliferative status and reactive stroma that is associated spatially with overexpression of an α -chemokine (IL-8), observed in BPH. The utility of this mouse model includes induction of a reactive stroma within the context of normal, non-transformed glandular tissue. Therefore, KC transgenic mice may also be useful as a cross-genetic carrier of induced reactive stroma when crossed with other transgenic prostate mouse models.

The majority of studies that address IL-8 action point to its role in regulating immune function. It is becoming clearer that this α -chemokine also functions to regulate innate host response mechanisms, which includes the formation of reactive stroma at many sites of benign and neoplastic diseases in addition to wound repair processes facilitated by myofibroblasts. Elucidating the role and specific mechanisms of IL-8 action in regulating reactive stroma, myofibroblast recruitment and differentiation, as well as cell proliferation is important to the overall understanding of host responses in proliferative and inflammatory diseases. It is anticipated that the use of KC transgenic mice will aid in the elucidation of key mechanisms and the development of novel therapeutic approaches.

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Figure 1. Germline transmission

Representative PCR (**A**) of transgene germline transmission from founder to F1 offspring, with β -actin as a control for template integrity. Representative RT-PCR (**B**) showing KC transgene transcript and β -actin amplification from both transgenic lines.

Figure 2. KC transgene expression and hyperplastic phenotype

Data are representative of all ventral prostates evaluated. Micrographs represent 14 (**C, D**), 16 (**A, B**) and 20 (**A, B**) week old mice. KC IHC with wildtype (**A**) and KC transgenic (**B**) mice. V5 epitope IHC with wildtype (**C**) and KC transgenic (**D**) mice. Masson's trichrome stain with wildtype (**E**) and KC transgenic (**F**) mice. All images captured at $\times 400$, all scale bars = $50\mu\text{m}$, and A–D are counterstained with hematoxylin.

Figure 3. KC transgenic mice display increased mean acini diameter in the ventral prostate gland with age, and elevated Ki67 index ratios

(A) Prostatic acini diameter as a function of age for both wildtype littermates (WT, *grey bars*) and KC transgenics (KC, *black bars*). A significant increase in acini diameter was observed at 30, 40 and 52 weeks ($* = p < 0.05$) in KC transgenic mice. Quantitative Ki67 data represented as a scatterplot distribution (B) and a box-and-whiskers distribution (C). In the scatterplot (B), the dashed line represents $\rho=1$ (no change). All wildtype mice (*grey regression line, circles*), where $\rho=0.6$ and $p<0.05$, display an elevated epithelia/stroma proliferation index ratio (*). All KC mice (*black regression line, plus signs*), where $\rho=0.48$ and $p<0.05$, display an epithelia/stroma proliferation index ratio elevated over wildtype

(**). In the boxplot (**C**) the grey line at $y=0$ indicates no change. All wildtype (**grey with black median line**) and KC (**black with white median line**) mice display an increased epithelial/stromal proliferation index ratio, with KC mice exhibiting an even greater increase over wildtype ($p < 0.05$). **B&C** represent N=54 wildtype and N=63 KC mice.

Figure 4. Pro-collagen 1 and tenascin-C immunoreactivity is increased in the periacinar stroma of KC transgenic mice

Data are representative of all ventral prostates evaluated. Micrographs represent 30 (**E, F**), 40 (**A, B**) and 52 (**C, D, G, H**), week old mice. Murine pro-collagen 1 IHC with WT (**A, C**) and KC (**B, D**) mice. Murine tenascin-C IHC with WT (**E, G**) and KC (**F, H**) mice. All images $\times 400$, scale bars = $50\mu\text{m}$, counterstained with hematoxylin.

Figure 5. Ki67 proliferation index and histopathology of PrEC-only and PrEC+PrSC subcutaneous xenografts

Ki67 (A) and TUNEL (B) index quantitation of control PrEC (grey) and IL-8-expressing PrEC (black) xenografts. (A) No difference was observed when comparing control and IL-8-expressing PrEC-only xenografts, while IL-8-expressing PrEC+PrSC xenografts displayed a significant increase in Ki67 counts relative to controls (* $p < 0.05$). (B) Epithelial TUNEL index remained constant for all xenograft types. Data reflect N=11 control PrEC-only, N=7 IL-8-expressing PrEC-only, N=9 control PrEC+PrSC, and N=9 IL-8-expressing PrEC+PrSC xenografts, within 3 $\times$ 200 fields counted per sample. Representative H&E-stained micrographs of: (C) control PrEC-only, (D) IL-8-expressing

PrEC-only, (**E**) control PrEC+PrSC, and (**F**) IL-8-expressing PrEC+PrSC xenografts. Imaged at $\times 200$, *scale bars* = $50\mu\text{m}$.

Figure 6. IL-8-expressing PrEC+HTS-2T orthotopic xenografts display increased proliferation and altered histopathology

Representative IHC micrographs from orthotopic implants (day 14) immunostained for: (A) EGFP (IL-8), and human-specific (B) α -SMA, (C) pro-collagen type 1 and (D) tenascin-C. Imaged at $\times 100$, scale bars = $50\mu\text{m}$, and counterstained with hematoxylin. Ki67 (E) and TUNEL (F) index quantitation, with implants of control PrEC (grey) and IL-8-expressing PrEC (black) plus HTS-2T. Orthotopic implants of IL-8-expressing PrEC+HTS-2T displayed a significant increase in both epithelial and stromal positive nuclei, relative to control PrEC+HTS-2T implants (E, $p < 0.05$). No significant changes were observed with quantification of TUNEL apoptotic index (F). See *Methods* for N values.

Table 1

Tissue evaluation schedule. (Indicates the number of KC transgenic mice and wildtype littermates at each age-point)

Age (weeks)	N = Wildtype Littermates	N = KC Transgenic Mice
8	4	5
10	3	3
12	6	3
14	3	3
16	3	2
20	7	7
30	9	10
35	2	4
40	3	6
45	7	10
52	5	7
60	2	4