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CXCL12γ Induces Human Prostate and Mammary Gland Development

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Abstract

Background: Epithelial stem cells (ESCs) demonstrate a capacity to maintain normal tissues homeostasis and ESCs with a deregulated behavior can contribute to cancer development. The ability to reprogram normal tissue epithelial cells into prostate or mammary stem-like cells holds great promise to help understand cell of origin and lineage plasticity in prostate and breast cancers in addition to understanding normal gland development. We previously showed that an intracellular chemokine, CXCL12γ, induced cancer stem cells (CSCs) and neuroendocrine characteristics in both prostate and breast adenocarcinoma cell lines. However, its role in normal prostate or mammary epithelial cell fate and development remains unknown. Therefore, we sought to elucidate the functional role of CXCL12γ in the regulation of ESCs and tissue development.

Methods: Prostate epithelial cells (PNT2) or mammary epithelial cells (MCF10A) with overexpressed CXCL12γ was characterized by qRT-PCR, Western blots, and immunofluorescence

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AUTHOR CONTRIBUTIONS

Y.J., J.K.K., and R.S.T. designed experiments. Y.J., J.K.K., E.L., F.C.C., and A.M.D. performed experiments and analyzed the data. P.H.K. discussed the results and gave valuable critique on the paper. Y.J., F.C.C., and R.S.T. wrote the manuscript.

CONFLICT OF INTERESTS/DISCOLSURES

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for lineage marker expression, and FACS analyses, and sphere formation assays to examine stem cell surface phenotype and function. Xenotransplantation animal models were used to evaluate gland or acini formation *in vivo*.

Results: Overexpression of CXCL12γ promotes the reprograming of cells with a differentiated luminal phenotype to a non-luminal phenotype in both prostate (PNT2) and mammary (MCF10A) epithelial cells. The CXCL12γ-mediated non-luminal type cells results in an increase of epithelial stem-like phenotype including the subpopulation of $EPCAM^{Lo}/CD49f^{Hi}/CD24^{Lo}/CD44^{Hi}$ cells capable of sphere formation. Critically, overexpression of CXCL12γ promotes the generation of robust gland-like structures from both prostate and mammary epithelial cells in *in vivo* xenograft animal models.

Conclusions: CXCL12γ supports the reprogramming of epithelial cells into non-luminal cell-derived stem cells, which facilitates gland development.

Keywords

CXCL12γ; non-luminal phenotype; cellular reprograming; prostate stem cells; mammary stem cells; tissue development

1 INTRODUCTION

In epithelial tissues, the reprogramming ability of epithelial stem cells (ESCs) from committed tissue cells and the plasticity of epithelial lineage commitment may have important implications in the tissue homeostasis or regeneration due to their self-renewing and differentiating properties.[1] Importantly, during the tissue regenerative process, the genetic and/or epigenetic alterations occurring in epithelial stem/progenitor cells associated with local microenvironment stimulation or stress lead to their acquisition of aberrant functions and malignant transformation, resulting in benign hypertrophy, hyperplasia, and cancer.[2]

ESCs have been described as being able to differentiate into all epithelial cell lineages in epithelial glandular tissue.[1, 3, 4] The epithelium of the prostate gland is composed of luminal, basal, and neuroendocrine cells.[5, 6] The luminal cells localize at the apical region of epithelium, and express cytokeratin 8 (KRT8/CK8), cytokeratin 18 (KRT18/CK18), prostate specific antigen (KLK3/PSA), and high levels of androgen receptor (AR). The basal cells line the basement membrane below luminal layer, and express cytokeratin 5 (KRT5/CK5), cytokeratin 14 (KRT14/ CK14), p63 (TP63), and low or undetectable levels of AR. The prostate epithelium also has a third cell type - neuroendocrine cells, which expresses secreted neuropeptides and other hormones and have contact with the glandular lumen.[5] The epithelium of mammary glands has many similarities with prostate epithelium and is comprised of luminal and basal/myoepithelial cells. The luminal cells express CK8 and CK18 and the basal/myoepithelial cells express CK5 and CK14.[7, 8] Although the origin of prostate or mammary epithelial cells remains controversial, growing evidence suggests that basal stem cells and/or progenitors are multipotent, giving rise to different types of epithelial cells.[8-15] Furthermore, the basal cell-derived prostate stem cells (ProSCs) and/or progenitors may contribute to repair of the prostate luminal epithelium[16] and the basal cell-derived

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mammary stem cells (MaSCs) have a significant ability to regenerate multi-lineage mammary glands.[8, 17-19]

Growing evidence has supported the hypothesis that normal ECSs are the cell of origin for cancer stem cells (CSCs), given their ability to self-renew although relationship of origin and lineage plasticity between normal ECSs and CSCs have been poorly understood.[20-22] Understanding the mechanisms of self-renewal and differentiation of ProSCs or MaSCs during the tissue regeneration may be crucial to elucidate the functions of CSCs in tumor progression[23]. Prostate CSCs have been defined with varying sets of markers, which often show some similarity to basal type cells.[24, 25] However, luminal type cells can also lead to prostate tumors and have been proposed to cause more aggressive disease.[22] Dysregulation of self-renewal in normal ESCs may result in the generation of CSCs, which play crucial roles in the tumor initiation, tumor heterogeneity, and recurrence after chemotherapy[26]. These aberrant stem cells may provide targets for the development of cancer prevention strategies. Interestingly, CSCs share with normal ESCs in the similar molecular mechanisms and functional capabilities.[22, 24, 27, 28] Therefore, understanding the relationship between normal ESCs and CSCs and their progeny could lead to improved cancer treatments.

Stromal derived factor-1α (SDF-1α or CXCL12α) plays crucial roles in the recruitment of endogenous tissue-specific stem cells to areas of tissue injury and engraftment of tissue stem cells, which contribute to the tissue repair and regeneration of multiple organ systems such as blood vessels, bone, cartilage, skeletal muscle, skin, and myocardium.[29-38] However, little is known about its role in the prostate or mammary epithelial cell fate and tissue regeneration. Recent studies have identified an alternatively spliced isoform of CXCL12, CXCL12γ, which functions as an intracellular chemokine in normal tissues.[39-4243, 44] In development, CXCL12γ expression is abundant in brain and heart tissues and increases throughout fetal development - reaching a maximum at birth [43]. In cancer, recent studies have demonstrated that CXCL12γ expression in breast, colon, and bladder cancer is linked to more aggressive behavior [40, 41, 45, 46]. Most recently, we have demonstrated that the role of the intracellular chemokine CXCL12γ in induction of CSCs and small cell neuroendocrine phenotypes and its impact on metastatic castration-resistant prostate cancer (m-CRPC).[47]

Here we show that overexpression of CXCL12γ promotes the cellular reprograming of differentiated luminal phenotype to a non-luminal phenotype in both prostate (PNT2) and mammary (MCF10A) epithelial cells. Importantly, the CXCL12γ-mediated non-luminal phenotype results in an increase of epithelial stem-like phenotype including the subpopulation of $EPCAM^{Lo}/CD49f^{Hi}/CD24^{Lo}/CD44^{Hi}$ cells with capable of sphere formation. Critically, overexpression of CXCL12γ promotes the generation of robust gland-like structures from both prostate and mammary epithelial cells in *in vivo* xenograft animal models. Together, our results suggest that CXCL12γ supports the reprogramming of epithelial cells into non-luminal cell-derived stem cells, which facilitate gland formation.

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2 MATERIALS AND METHODS

2.1 Animals

Five to seven week-old male (for PNT2 cells) or female (for MCF10A cells) SCID mice (CB17 SCID; Taconic, Germantown, NY) were used as transplant recipients. All animal procedures were performed in compliance with the institutional ethical requirements and approved by the University of Michigan Institutional Committee for the Use and Care of Animals (ICUCA).

2.2 Cell cultures

Normal human prostate epithelial PNT2 cells (cat no. 95012613, Sigma, St. Louis, MO) were cultured in RPMI 1640, 2 mM glutamine (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GEMINI Bio-Products, Sacramento, CA), 1% penicillin-streptomycin (P/S, Thermo Fisher Scientific, Waltham, MA). The human mammary breast epithelial cell line MCF10A was kindly provided by Dr. Max Wicha (University of Michigan). MCF10A cells were cultured in DMEM /F12 Ham's mixture supplemented with 5% horse serum (Thermo Fisher Scientific), EGF 20 ng/ml (Sigma), insulin 10 μg/ml (Sigma), hydrocortisone 0.5 mg/ml (Sigma), cholera toxin 100 ng/ml (Sigma), 1% P/S (Thermo Fisher Scientific). The cells were maintained at 37°C, 5% CO_2, and 100% humidity.

2.3 Overexpression of *CXCL12γ*

The control vector, pLV and *CXCL12γ* overexpression plasmid vector, pLV-CXCL12γ were kindly provided by Dr. Ramirez (Viral Vector Facility, Technical Unit of Gene Targeting, Fundacion CNIC (National Centre for Cardiovascular Research), Madrid, Spain).[43] pLV or pLV-CXCL12γ was packaged with lentivirus at the University of Michigan viral core. Lenti viral pLV or pLV-CXCL12γ was infected into normal human prostate epithelial cells (PNT2) and normal human mammary epithelial cells (MCF10A). Infected cells were selected for 7 days in media containing 1 μg/ml puromycin and analyzed by real-time qPCR or immunofluorescence staining.

2.4 Quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy mini or micro kit (Qiagen, Valencia, CA) and converted into cDNA using a First-Strand Synthesis Kit (Thermo Fisher Scientific). Quantitative PCR was performed on an ABI 7700 sequence detector (Thermo Fisher Scientific) using TaqMan Universal PCR Master Mix Kit (Thermo Fisher Scientific) according to the directions of manufacturer. TaqMan MGB probes (Thermo Fisher Scientific) were as follows: *CXCL12γ* (AIRR9QT), *EPCAM* (Hs00901885_m1), *PSA* (Hs02576345_m1), *AR* (Hs00171172_m1), *MUC1* (Hs00159357_m1), *ESR1* (Hs01046816_m1), *CD24* (Hs04405695_m1), *KRT8* (Hs01595539_m1), *KRT18* (Hs02827483_g1), *COL4A1* (Hs00266237), *KRT5* (Hs00361185_m1), *CD44* (Hs01075861_m1), and *SYN* (Hs00300531_m1), and *TP63* (Hs00978340_m1). *β-ACTIN* (Hs99999903_m1) was used as internal control for the normalization of target gene expression.

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2.5 Western blots

Whole cell lysates were separated on 4-20% Tris-Glycine gel and transferred to PVDF membranes. The membranes were incubated with 5% milk for 1 hour and incubated with primary antibodies overnight at 4°C. Primary antibodies were as follows: anti-EPCAM (cat. 2626, Cell Signaling, Danvers, MA), anti-CK18 (cat. 4548, Cell Signaling), anti-CD24 (cat. ab179821, Abcam, Cambridge, MA), anti-CD44 (cat. 3578, Cell Signaling), anti-CK5 (cat. 25807, Cell Signaling), and anti-Synaptophysin (SYN, cat. 4329, Cell Signaling). Blots were incubated with peroxidase-coupled anti-mouse IgG secondary antibody (cat. 7076, Cell Signaling) or peroxidase-coupled anti-rabbit IgG secondary antibody (cat. 7074, Cell Signaling) for 1 hour, and protein expression was detected with SuperSignal West Dura Chemiluminescent Substrate (cat. Prod 34075, Thermo Scientific, Rockford, IL). Membranes were reprobed with monoclonal anti-β-actin antibody (cat. 4970, Cell Signaling) to control for equal loading.

2.6 Immunohistochemistry and immunofluorescences

Cells and graft sections were used for immunofluorescence in cells and tissue sections. Cells were fixed and permeabilized with Perm/Wash Buffer (cat. 554723, BD Biosciences). Formalin fixed, paraffin embedded graft sections were blocked with Image-iT FX signal enhancer for 30 min and incubated for 2 hours at room temperature with primary antibodies combined with reagents of Zenon Alexa Fluor 488 (green) (cat. Z25002, Invitrogen) or 555 (red) (cat. Z25305, Invitrogen) labeling kit. For the CXCL12γ antibody, a rabbit polyclonal antiserum (anti-CXCL12γ) was raised against the peptide KVGKKEKIGKKKRQ, mapping to the specific C-terminal region of CXCL12γ. The N-terminal cysteine enables direct conjugation of the peptide to the protein carrier and is not present in the native sequence. Peptide synthesis, coupling, immunization, ELISA titration and affinity purification were done by BioGenes GmbH (Berlin, Germany).[43] PSA (cat. ab403, Abcam), androgen receptor (AR, cat. ab202432, Abcam), mucin 1 (MUC1, cat. ab70745, Abcam), estrogen receptor alpha (ERα, cat. ab108398, Abcam), CK18 (cat. ab7797, Abcam), CD24 (cat. MS-1279-P, Thermo Fisher Scientific, Fremont, CA), CK5 (cat. ab52635, Abcam), CD44 (cat. ab6124, Abcam), Collagen IV (cat. ab6311, Abcam), SYN (cat. ab23754, Abcam), and human leukocyte antigen (HLA, cat. ab70328, Abcam) antibodies were used as the primary antibody. After washing with PBS, cells and graft sections were mounted with ProLong Gold antifade reagent with DAPI (cat. P36931, Invitrogen). Images were taken with Olympus FV-500 confocal microscope. In some cases, H&E stain was used for the gross morphology of grafts. Images were taken with Olympus 51A microscope.

2.7 FACS analyses

For analyses of epithelial cell phenotypes, luminal ($EPCAM^{Hi}$/$CD49f^{Lo}$) or non-luminal ($EPCAM^{Lo}CD49f^{Hi}$) and an epithelial stem cell phenotype ($EPCAM^{Lo}$/$CD49f^{Hi}$/$CD24^{Lo}$/$CD44^{Hi}$)[8, 48, 49], control or CXCL12γ overexpressing epithelial cells (PNT2, MCF10A) (1 x 10^5) were seeded onto 12-well culture plates and were cultured for 3 days. The cells were incubated with FITC anti-human CD326 (EPCAM) antibody (cat. 324204, Biolegend, San Diego, CA), PE/Cy7 anti-human CD49f antibody (cat. 313622, Biolegend), APC/Cy7 anti-human CD24 antibody (cat. 311132, Biolegend), APC anti-CD44 antibody (cat. 559942, BD

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Biosciences, San Jose, CA) for 30 min at 4°C. DAPI was used for determining cell viability. The cell phenotypes were evaluated by FACS analyses (FACSAria IIu flow-cytometer, Becton Dickinson, Mountain View, CA). Assays were performed in triplicate and the results are representative of three independent experiments.

2.8 Sphere formation assays

Control or CXCL12γ overexpressing cells (PNT2, MCF10A) were dissociated to single cells by standard trypsinization and washed three times with PBS. The cells were plated in stem cell culture medium (DMEM:F12 plus 10 ng/ml bFGF, 20 ng/ml EGF, 5 mg/ml insulin, and 0.4% BSA) supplemented with 1% KO serum replacement (Invitrogen/Gibco, p/n 10828-028) at a density of 1,000 cells/ml in low attachment 6 well culture plates.[50] Seven day old spheres are enumerated as size >50 cells[51]

2.9 Xenotransplantation of normal human epithelial cells

To determine the ability of CXCL12γ to regulate the reprograming of normal epithelial cells, the control or CXCL12γ overexpressing cells (PNT2, MCF10A) (1×10^6) with irradiated human bone marrow stromal cells (BMSC) (2×10^5) (5:1 ratio) in growth factor-reduced Collagen Type I gel (cat. 354236, BD Bioscience, San Jose, CA) were transplanted into the fat pads of 5-7 week-old male or female SCID mice. The mice were sacrificed 4 weeks after grafting. Grafts were removed, fixed in formalin, embedded in paraffin, and sectioned.

2.10 Statistical analyses

Results are presented as mean ± standard deviation (s.d.) of mean. Significance of the difference between two groups was determined by unpaired Student's *t*-test. Analyses were conducted in with GraphPad Prism version 7 software. Values of $p < 0.05$ were considered significant.

3 RESULTS

3.1 CXCL12γ regulates the phenotypic alteration of luminal to non-luminal phenotype in normal epithelial cells

Under normal culture conditions, little or no mRNA expression of CXCL12γ was detected in normal prostate epithelial cells (PNT2) (Figure 1A). To determine the role that CXCL12γ plays in prostate epithelial cell phenotype, *CXCL12γ* was overexpressed in PNT2 cells and overexpression of CXCL12γ was validated at the RNA and protein levels (Figure 1A,C). During passage of the CXCL12γ overexpressing PNT2 cells, their morphology changed from a predominantly cobblestone appearance tocells which were less tightly packed and had projections (Figure 1C; bright field images). In conjunction with the change in phenotype, significant alterations in gene expression were noted in CXCL12γ overexpressing PNT2 cells compared to control cells. We observed a reduction of the luminal cell markers (*EPCAM, PSA, AR, CD24, KRT8*, and *KRT18*) and a significant enhancement of the basal cell markers (*KRT5, CD44,* and *TP63*) and the neuroendocrine marker (*SYN*) (Figure 1E). At the protein level, reduction of the luminal cell markers (CK18, EPCAM, and CD24), enhancement of the non-luminal cell markers (CD44 and

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CK5), and the neuroendocrine cell marker (SYN) were detected in CXCL12γ expressing PNT2 cells compared to control PNT2 cells by immunoblot analyses (Figure 1G). Immunofluorescence confirmed that CXCL12γ expressing PNT2 cells had a less round morphology and less expression of luminal markers compared to their control cells (Figure 1I).

For the normal mammary epithelial cells (MCF10A), again little or no mRNA expression of CXCL12γ was detected in MCF10A cells in under normal culture conditions. We then overexpressed CXCL12γ and CXCL12γ overexpression was validated at both the RNA and protein levels (Figure 1B and D). CXCL12γ overexpressing MCF10A cells also changed their morphology from a predominantly luminal (cobblestoned) to non-luminal (spindle-like) appearance (Figure 1D; bright field images). We also found analogous patterns of lineage marker gene expression after CXCL12 overexpression in MCF10A cell, with the reduction of the luminal cell marker mRNAs (*EPCAM, MUC1, α-ER1, CD24, KRT8,* and *KRT18*) and enhancement of the basal cell markers (*COL4A1, KRT5,*and *CD44*) compared to control MCF10A cells (Figure 1F). A reduction of the luminal cell markers (α-ER1, CK18, EPCAM, and CD24) and enhancement of the non-luminal cell markers (CD44 and CK5) were detected in CXCL12γ expressing MCF10A cells compared to control MCF10A cells by immunoblot analyses and immunofluorescence staining at the protein level (Figure 1H, J). Together, these data show that CXCL12γ alters lineage marker expression in cultured epithelial cells.

3.2 CXCL12γ-mediated non-luminal epithelial cells have stem-like features

Epithelial stem cells are specified during development and are controlled by epithelial-mesenchymal interactions.[17] Growing evidence suggests that basal region is the source of prostate epithelial stem cells, which can give rise to all three types of prostate epithelial cells.[52] Likewise, a rare subset of basal cells are thought to give rise to both the luminal and basal type of the mammary epithelial cells and have high efficacy to generate ductal-lobular outgrowths *in vivo* mammary fat pads.[3, 19, 53, 54]

To further explore the role that CXCL12γ plays in regulating lineage phenotypes, we analyzed surface protein expression in PNT2 cells by FACS analysis (Figure 2A). Under basal conditions, high levels of the luminal cell markers ($EPCAM^{Hi}/CD49f^{Lo}$) were detected in control PNT2 cells (luminal: 98.3±0.15%; non-luminal: 0.83±0.06%) and high levels of the non-luminal cell markers ($EPCAM^{Lo}/CD49f^{Hi}$) were detected CXCL12γ expressing PNT2 cells (luminal: 0.7±0.01%; non-luminal: 94.1±0.52%) (Figure 2A,C). Next, we specifically examined a stem-like phenotype using additional cell surface markers by FACS analysis. We found that control PNT2 expressing luminal markers ($EPCAM^{Hi}/CD49f^{Lo}$) highly expressed CD24. In contrast, cells expressing a non-luminal phenotype ($EPCAM^{Lo}/CD49f^{Hi}$) highly expressed CD44 in CXCL12γ expressing PNT2 cells (Figure 2A). Importantly, the stem-like phenotype including the subpopulation of $EPCAM^{Lo}/CD49f^{Hi}/CD24^{Lo}/CD44^{Hi}$ cells was dramatically enhanced in CXCL12γ expressing PNT2 cells (78±0.96%) relative to control PNT2 cells (5.2±1.34%) (Figure 2D).

For mammary epithelial MCF10A cells, the luminal lineage phenotype, as assessed by surface markers, was found in control MCF10A cells (luminal: 96.75±0.35%; non-luminal:

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1.55±0.35%), whereas a predominantly non-luminal lineage phenotype was found in CXCL12γ expressing MCF10A cells (luminal: 0.9±0.07%; non-luminal: 90.4±0.42%) (Figure 2B,C). We also found that control MCF10A cells expressing luminal markers ($EPCAM^{Hi}/CD49f^{Lo}$) highly expressed CD24. In contrast, cells expressing a non-luminal phenotype ($EPCAM^{Lo}/CD49f^{Hi}$) highly expressed CD44 in CXCL12γ expressing MCF10A cells (Figure 2B). Furthermore, for the stem-like phenotype, the mammary epithelial cells also showed a similar pattern as the prostate epithilal cells; CXCL12γ expressing MCF10A cells (69.7±0.95%) compared to control MCF10A cells (3.1±1.97%) (Figure 2D).

To functionally validate that CXCL12γ-expressing non-luminal subpopulations demonstrate stem-like properties, we examined the ability of the cells to form spheroids in culture. The number and size of spheres were significantly enhanced in CXCL12γ expressing PNT2 cells as compared to their control cells (Figure 3A,B). Similar results of the sphere formation were obtained in MCF10A cells (Figure 3A,B).These findings suggest that the intracellular CXCL12γ-mediated non-luminal epithelial cells are associated with the acquisition of the stem-like properties, which may contribute to glandular development.

3.3 CXCL12γ induces epithelial gland development *in vivo*

To determine if CXCL12γ has the capacity to induce normal epithelial cells towards a stem-like phenotype with gland forming properties, control or CXCL12γ overexpressing PNT2 cells were transplanted with irradiated human bone marrow stromal cells (hBMSC) into the fat pads of male SCID mice (Figure 4A). After 4 weeks, the mice were sacrificed and tissues were recovered for histologic analysis. A significantly greater range of prostate gland-like structures and significantly higher numbers of glands or acini were observed following the transplantation of CXCL12γ overexpressing PNT2cells compared with their control cells (Figure 4B,C). To verify that the structures were of human origin, the tissues were stained for human leukocyte antigen (HLA), confirming that the contributing cells from glands or acini were derived from the transplanted cells (Figure 4D). We further verified that the non-luminal type of CXCL12γ overexpressing PNT2 cells gave rise to the luminal, basal, and neuroendocrine cell types in the glands or acini from transplanted grafts, whereas the luminal type of control PNT2 cells gave rise to only the luminal cell type (Figure 5A). Specifically, the luminal markers (PSA, AR, CK18, and CD24) were detected in the gland or acini formed from CXCL12γ overexpressing or control cells. However, basal markers (CK5 and CD44) and a neuroendocrine marker (SYN) were only detected in glands or acini formed from grafts of CXCL12γ overexpressing PNT2 cells (Figure 5A).

Furthermore, control or CXCL12γ overexpressing MCF10A cells were transplanted with irradiated hBMSC into the fat pads of female SCID mice (Figure 4A). After 4 weeks, the mice were sacrificed and tissues were recovered for histologic analysis. The size of gland-like structures were dramatically increased and significantly higher numbers of glands or acini were observed following the transplantation of CXCL12γ overexpressing MCF10A cells compared with their control cells (Figure 4B,C). HLA staining was again used to confirm that the cells constitution the gland or acini were derived from the transplanted mammary epithelial cells (Figure 4D). CXCL12γ overexpressing MCF10A cells gave rise to the luminal and basal/myoepithelial cell types in the glands or acini from implanted grafts,

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whereas the control MCF10A cells gave rise to only luminal cell type in the glands or acini from implanted grafts (Figure 5B). Importantly, higher levels of the luminal markers (MUC1, α-ER1, CK18, and CD24) and the basal/myoepithelial markers (Collagen IV, CK5, and CD44) were detected in grafts of CXCL12γ overexpressing MCF10A cells compared to grafts of control cells (Figure 5B).

Together these data suggest that CXCL12γ functions to support the development of epithelial tissue stem cells, which facilitates glandular development.

4 DISCUSSION

Here, we show for the first time that an intracellular chemokine CXCL12γ reprogrammed luminal cells into the non-luminal cells, which have a multipotent stem-like activity, resulting in the development of robust gland-like structures in both prostate and mammary epithelial cells in an *in vivo* xenotransplantation animal model.

Recent reports demonstrate that the signaling pathway of CXCL12 and its receptor CXCR4 play pivotal roles in prostate or mammary epithelial cell fate and tissue regeneration. CXCL12 promotes epithelial branching and/or canalization during the prostate epithelial morphogenesis.[55] CXCR4 signaling permits lumen formation from human epithelial cell RWPE1 and blocking CXCR4 signaling with AMD3100 impairs the initial stages of acinar morphogenesis (spheroid formation) and reduces the average number of spheroids in a 3D Matrigel *ex vivo* model.[55] CXCL12 signaling plays a critical role in stem cell activities, which is enabled by progesterone-dependent upregulation of CXCR4, resulting in the generation of mammary epithelial progenitors in the adult mammary gland.[56] Further, progesterone-stimulated expansion of epithelial subsets is blocked by CXCR4 inhibition, leading to decreased mammary progenitor numbers in the adult gland.[56] Recently, we have demonstrated that the role of CXCL12γ in induction of CSCs and small cell neuroendocrine phenotypes through CXCR4-mediated PKCalpha/NFkappaB signaling, which promotes prostate or breast tumor outgrowth, metastasis and chemoresistance.[47] Our findings show the similar functional capabilities of non-luminal stem-like cells by CXCL12γ expression in epithelial gland development and CSCs by CXCL12γ expression in cancer progression[47]. It will be interesting to elucidate the role of CXCL12γ on the transformation of ProSCs or MaSCs to CSCs and subsequent cancer cell progression, which might identify an important therapeutic target.

Substantial knowledge has been gained in many aspects of prostate or mammary tissue development. Prostate-induced pluripotent stem cells (Pro-iPSC) or mammary-induced pluripotent stem cells (M-iPSC) have been induced to develop glands using a combination of defined transcription factors.[57-59] Adult multipotent stem cells (aMSC) or induced multipotent stem cells (iMSC) from differentiated prostate or mammary epithelial cells have also been advanced for the reprograming into a multipotent state, which could generate multiple epithelial cell lineages.[7, 9, 16, 18] Here, we have demonstrated that an intracellular chemokine, CXCL12γ, reprograms non-luminal stem-like cells, resulting in the development of robust gland-like structures from both prostate and mammary epithelial cells.

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The identification of ESCs remains controversial. Recent work suggests that basal multipotent stem cells differentiate into their lineages and maintain tissue development and homeostasis.[16] Human prostate stem cells (ProSCs) can be distinguished by expression of CD133, α2β1 integrin, p63, Trop2, CD49f, and delta homolog 1 (DLK1).[9, 48, 60-63] Most of these studies have used one or two markers, for example, a subset of basal cells expressing α2β1-integrin was selected by rapid adhesion to collagen type I, which were able to generate prostate-like acini *in vivo* [9]. Similarly, α2β1^{hi}/CD133+ basal cell populations have a high *in vitro* proliferative rate, which can reconstitute prostate-like acini.[60] Other work showed that CD133+ cells isolated from prostates are identified as putative stem/progenitor cell populations, which are able to self-renew and to regenerate different lineage cells.[61, 62] Further, Goldstein et al. proposed that the prostate stem cells are a basal cell expressing Trop2 and CD49f, which produce a multipotent progenitor that generates all three types of epithelial cells.[48] Another group proposed that delta homolog 1 (DLK1)$^{+VE}$CD49^{hi}CD26^{-VE} cells are basal stem cells, which generate multi-lineage spheroids and fully differentiated prostate gland architecture.[63] In prostate tissues, stem cells/progenitors give rise to differentiated luminal cells expressing CK8, CK18, CD57, AR, PSA, and prostatic acid phosphatase (PAP). They also give rise to basal cells expressing the markers CK5, CK14, p63, CK19, CD44, and Bcl-2.[64, 65]

Human mammary stem cells (MaSCs) have been defined by using a number of surface markers including aldehyde dehydrogenase (ALDH), CD24, CD29, CD44, CD49f, and EPCAM.[66, 67] For example, ALDH expressing stem cells in normal breast epithelium have the potential to differentiate to multiple lineages and exhibit significant growth capacity following transplantation in animal models.[67] The study shows the role of BRCA1 *in vitro* systems and a humanized NOD/SCID mouse model. BRCA1 regulates the maintaining of ALDH1+/ER– stem cells/progenitors and the loss of BRCA1 blocks differentiation of ALDH1+/ER– stem cells/progenitors into ER+ luminal epithelial cells.[68] Mammary stem cells have also been identified using expression of EPCAM$^{neg\text{-}low}$CD49f^{hi} in the basal cells, which develop into all mammary gland lineages and aid in the development of mammary glands in animal models.[8, 18, 69] In mammary tissues, stem cells/progenitors give rise to luminal cells expressing CK8 and CK18 and the basal/myoepithelial cells expressing CK5 and CK14. [7]

Here, we defined ProSCs and MaSCs based on expression of several cell surface markers, EPCAM^{Lo}/CD49f^{Hi}/CD24^{Lo}/CD44^{Hi} and evaluated their potential for gland development. Interestingly, a recent report demonstrated that CD49f (integrin α6) is a conserved biomarker of more than 30 different stem cells including ProSCs and MaSCs involved in their self-renewal.[28, 70] Although the molecular mechanism of the role of the common expression of CD49f in stem cells remains unclear, in our study we found that CXCL12γ-mediated non-luminal type of epithelial cells dramatically enhance the CD49f expression, suggesting that CXCL12γ may be a key regulator of stem cell function.

Investigators have made significant advances in the use of transplantation models to quantify ESCs. However, because conditions can always be further optimized, these assays might be better used to measure relative rather than absolute numbers of ESCs. Therefore, in the above data, we do not propose to quantify the total number of cells with ESC potential in the

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population. Common methodologies for transplantation of human epithelial cells or ESCs uses Collagen type I or Matrigel containing the epithelial cells in combination with supportive fibroblasts under the kidney capsule or humanized fat pad of highly immune-deficient, hormone (e.g.,estrogen, testosterone)-supplemented mice [8, 18, 49]. We also evaluated formation of glands/acini from xenograft of SCID animal models. Overall, we found 2-20 glands/acini per low power field with 4-5 fold increases from CXCL12γ overexpressed PNT2 or MCF10A cell implanted grafts compared to their control cell implanted grafts. An analogous work to ours studying a different molecular mechanism had similar findings (2 to 5 fold changes between groups)[49]. To date, there is limited studies of graft efficacy of prostate or mammary tissue regeneration. While in this study we mainly focused how CXCL12γ function to glands/acini formation in animal models, we speculate that many experimental conditions and/or factors such as cell types with specific subpopulations, target molecules (ECM)-mimicking microenvironment, duration of experiments, hormone supplements etc. will affect the ability of primed epithelial cells to form glands/acini appropriately. Clearly, more advanced cell transplantation strategies desire to improve cell retention, survival, and engraftment in the transplantation, result in the effective epithelial gland regeneration.

In this study, our findings demonstrate that CXCL12γ promotes a cellular reprogramming of terminally differentiated epithelial cells to non-luminal cell-derived stem-like states, which facilitate robust new tissue development.

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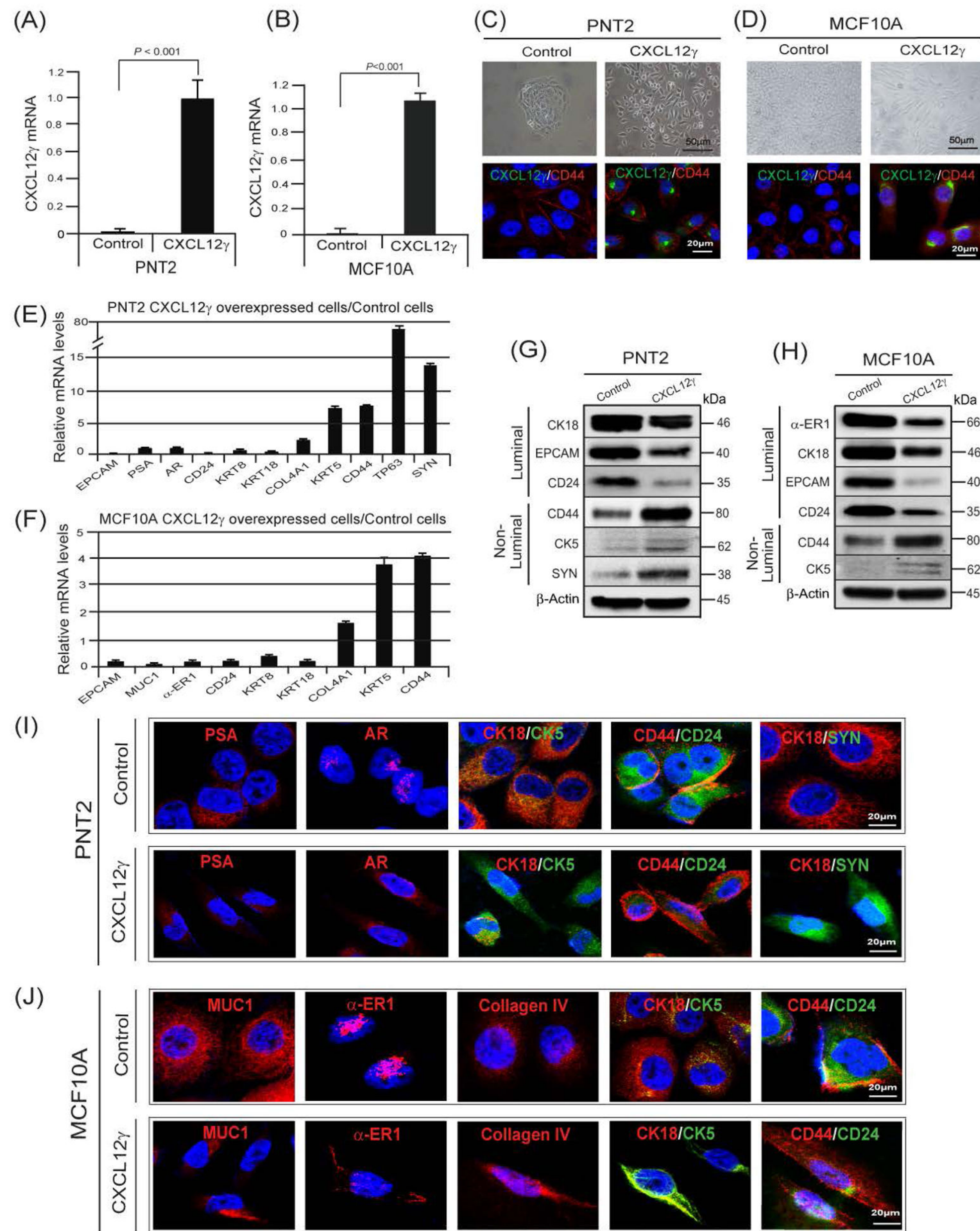

FIGURE 1. CXCL12γ regulates the transition of a luminal to non-luminal phenotype.

A, Confirmation of *CXCL12γ* mRNA expression following *CXCL12γ* overexpression in normal prostate epithelial cells (PNT2 cells) quantified by real-time PCR. B, Confirmation of *CXCL12γ* mRNA expression following *CXCL12γ* overexpression in normal mammary epithelial cells (MCF10A cells) quantified by real-time PCR. C, Bright-field microscopy of control or CXCL12γ overexpressing PNT2 cells (top, Bar=50 μm) and verification of CXCL12γ protein expression in CXCL12γ overexpressing PNT2 cells as detected by immunofluorescence (bottom). CXCL12γ (green), CD44 (red), and DAPI nuclear stain (blue). Bar=20μm. D, Bright-field microscopy of control or CXCL12γ overexpressing MCF10A cells (top, Bar=50 μm) and verification of CXCL12γ protein expression in CXCL12γ overexpressing MCF10A cells as detected by immunofluorescence (bottom).

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CXCL12γ (green), CD44 (red), and DAPI nuclear stain (blue). Bar=20 μm. E, Relative mRNA levels expressed by PNT2 cells following overexpression of CXCL12γ normalized to control cells; the luminal markers (*EPCAM, PSA, AR, CD24, KRT8*, and *KRT18*), the basal markers (Collagen IV (*COL4A1*), *KRT5, CD44*, and *TP63*), and the neuroendocrine marker (*SYN*). F, Relative mRNA expression in CXCL12γ overexpressing MCF10A cells normalized to control cells; the luminal cell markers (*EPCAM, MUC1,* α-estrogen receptor 1 *(α-ER1*), *CD24, KRT8*, and *KRT18*) and the basal cell markers (*COL4A1, KRT5*, and *CD44*). G, Immunoblot analysis for luminal cell markers (CK18, EPCAM, and CD24), basal cell markers (CD44 and CK5), and neuroendocrine cell marker (SYN) in control or CXCL12γ overexpressing PNT2 cells. H, Immunoblot analysis for luminal cell markers (α-ER1, CK18, EPCAM, and CD24) and the basal cell markers (CD44 and CK5) in control or CXCL12γ overexpressing MCF10A cells. I, Immunofluorescence of control or CXCL12γ overexpressing PNT2 cells for luminal cell markers (PSA, AR, and CK18), basal cell markers (CK5 and CD44), and neuroendocrine cell marker (SYN). Bar=20 μm. J, Immunofluorescence of control or CXCL12γ overexpressing MCF10A cells with the luminal cell markers (MUC1, α-ER1, CK18, and CD24) and basal cell markers (Collagen IV, CK5, and CD44). Bar=20 μm. Quantitative data are presented as mean ± SD, with *p* values calculated by Student's *t*-test.

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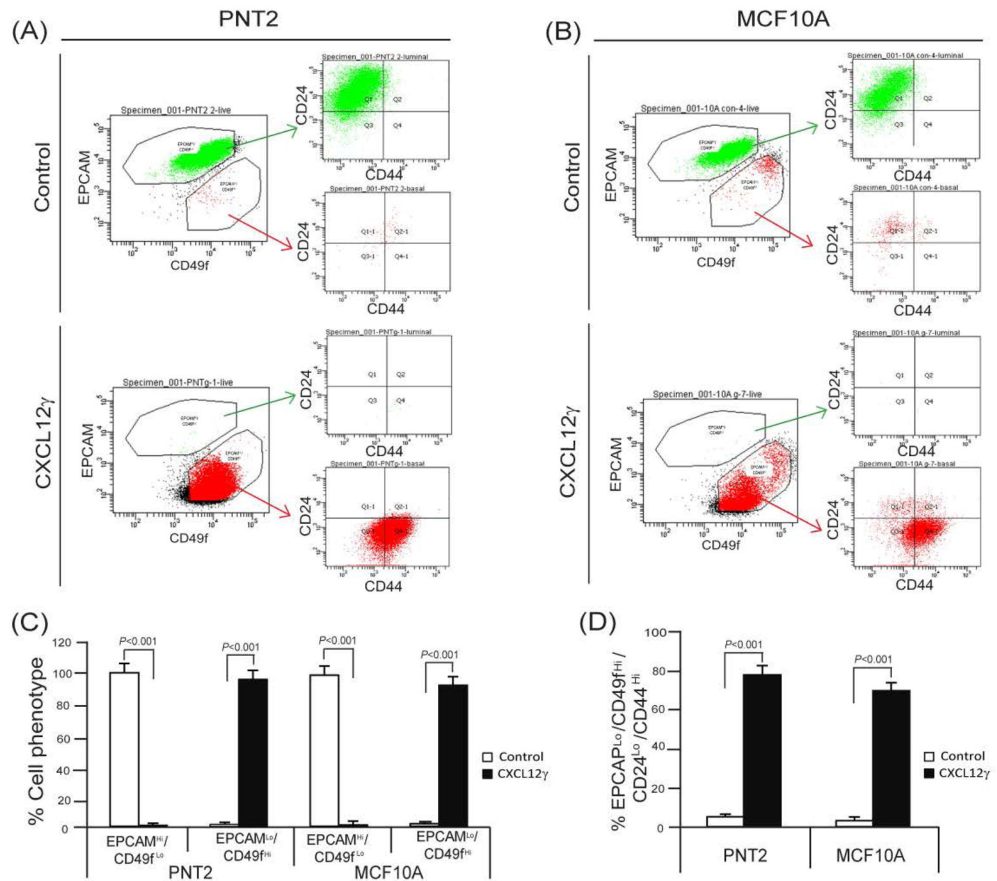

FIGURE 2. CXCL12γ induces stem-like features.

A and B, FACS plots for epithelial cell type and epithelial stem-like phenotype in control or CXCL12γ overexpressing PNT2 cells (A) or MCF10A cells (B). C, Analyses of % luminal cell populations (EPCAM^{Hi}/CD49f^{Lo}) and % non-luminal cell populations (EPCAM^{Lo}/CD49f^{Hi}) in control or CXCL12γ overexpressing PNT2 cells or MCF10A cells. D, Analyses of % epithelial stem-like phenotype (EPCAM^{Lo}/CD49f^{Hi}/CD24^{Lo}/CD44^{Hi}) in control or CXCL12γ overexpressing PNT2 cells or MCF10A cells. Quantitative data are presented as mean ± SD, with *p* values calculated by Student's *t*-test.

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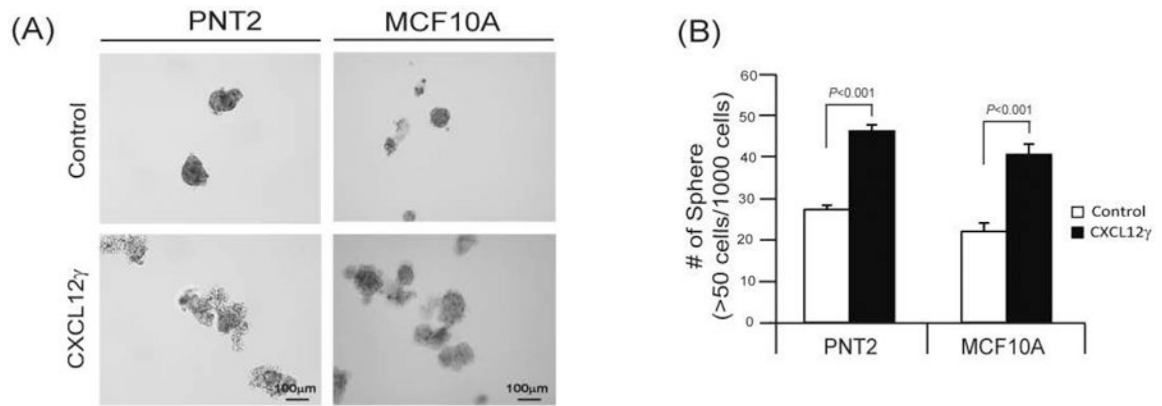

FIGURE 3. CXCL12γ enhances sphere formation.

A, Sphere formation for control or CXCL12γ overexpressing PNT2 or MCF10A cells (bright-field microscopy). Bar=100μm. B, Quantification of Figure 3A. Assays were performed in triplicate and the results are representative of three independent experiments. Quantitative data are presented as mean ± SD, with *p* values calculated by Student's *t*-test.

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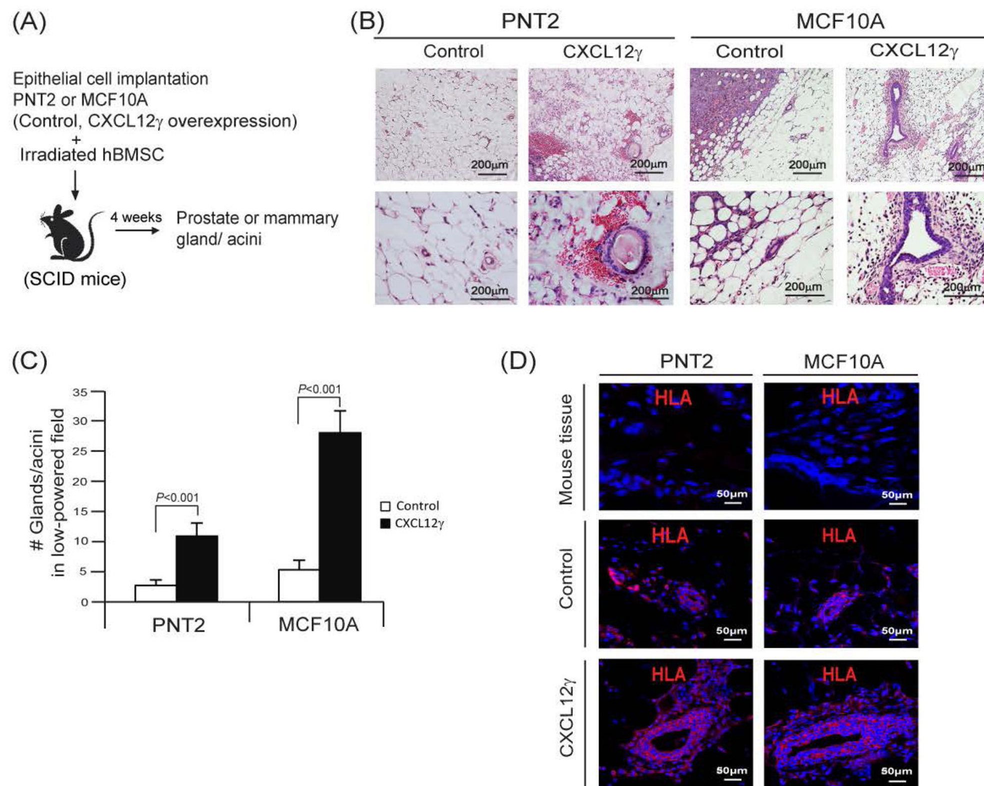

FIGURE 4. CXCL12γ induces gland development.

A, Xenograft animal model for prostate or mammary epithelial cell *in vivo* regeneration. Control or CXCL12γ overexpressing cells (PNT2, MCF10A) ($1x10^6$) with irradiated human bone marrow stromal cells (BMSC) (2×10^5) (5:1 ratio) were implanted into the fat pads of 5-7 week-old male or female SCID mice. After 4 weeks, the mice were sacrificed. B, H&E stain of the grafts. Bar=200 μm. C, Quantification of number of glands/acini from Figure 4B. Data are presented as mean ± SD (n=7), with *p* values calculated by Student's *t*-test. D, Immunofluorescence to determine the species of origin using an antibody against human leukocyte antigen (HLA). Bar=50 μm.

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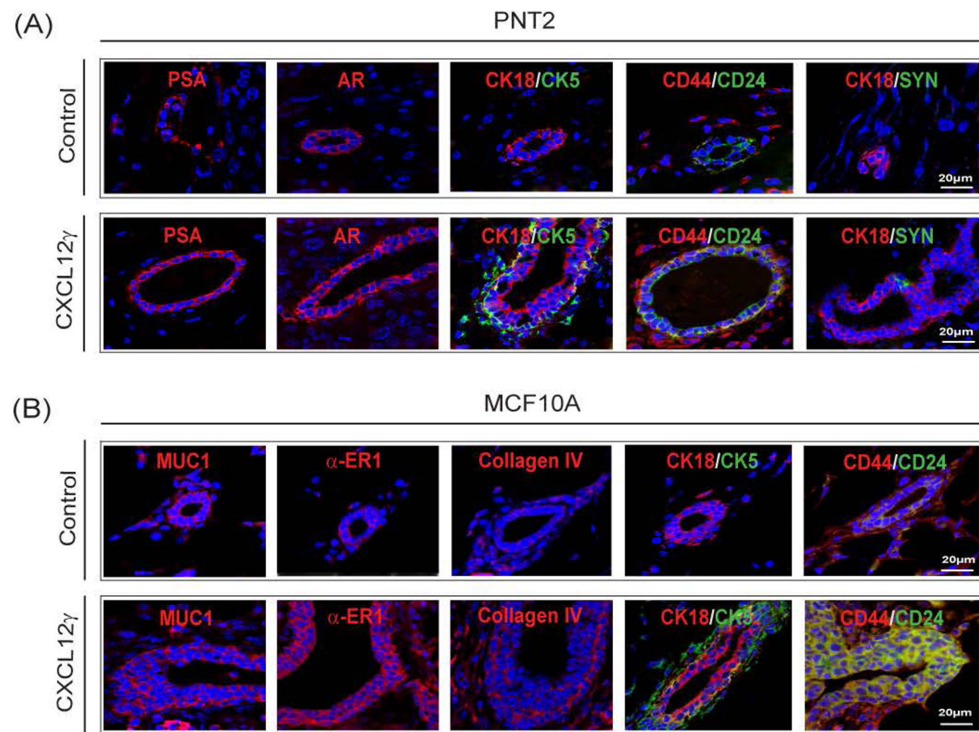

FIGURE 5. Characterization of the epithelial lineage phenotype in the glands or acini from implanted grafts.

A, Immunofluorescence for luminal cell markers (PSA, AR, CK18, and CD24), basal cell markers (CK5, and CD44), and neuroendocrine cell marker (SYN) of the resulting tissues derived from transplantation of control or CXCL12γ overexpressing PNT2 cells. Bar=20 μm. B, Immunofluorescence for luminal cell markers (MUC1, α-ER1, CK18, and CD24), basal cell markers (Collagen IV, CK5, and CD44) of the glands or acini produced by control or CXCL12γ overexpressing MCF10A cells. Bar=20 μm.