Stromal Gli signaling regulates the activity and differentiation of prostate stem and progenitor cells

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ABSTRACT

Interactions between cells in the stroma and epithelium facilitate prostate stem cell activity and tissue regeneration capacity. Numerous molecular signal transduction pathways, including the induction of sonic hedgehog (Shh) to activate the Gli transcription factors, are known to mediate the crosstalk of these two cellular compartments. However, the details of how these signaling pathways regulate prostate stem and progenitor cell activity remain elusive. Here we demonstrate that while cell autonomous epithelial Shh-Gli signaling is essential to determine the expression levels of basal cell markers and the renewal potential of epithelial stem and progenitor cells, stromal Gli signaling regulates prostate stem and progenitor cell activity by increasing the number and size of prostate spheroids in vitro. Blockade of stromal Gli signaling also inhibited prostate tissue regeneration in vivo. The inhibition of stromal Gli signaling suppressed the differentiation of basal and progenitor cells to luminal cells, and limited prostate tubule secretory capability. Additionally, stromal cells were able to compensate for the deficiency of epithelial Shh signaling in prostate tissue regeneration. Mechanistically, suppression of Gli signaling increased the signaling factor TGFβ in stromal cells. Elevation of exogenous TGFβ1 levels inhibited prostate spheroid formation, suggesting that a stromal Gli-TGFβ signaling axis regulates the activity of epithelial progenitor cells. Our study illustrates that Gli signaling regulates epithelial stem cell activity and renewal potential in both epithelial and stromal compartments.

Stromal-epithelial cell interactions are essential for prostate development and adult prostate tissue regeneration (1). The prostate gland is composed of numerous connected tubules and the surrounding stromal microenvironment. Each tubule consists of three types of prostate epithelial cells (PrECs) including secretory luminal cells, basal cells, and neuroendocrine cells (2, 3). Luminal cells typically expressing cytokeratin (CK) 8 or 18 locate at the apical region of the epithelium, which are sensitive to androgen stimulation and produce secretory proteins. Basal cells expressing CK5 or p63 reside underneath the luminal cells and attach to the basement lamina (4). Both luminal and basal cells in the epithelial compartment contain stem/progenitor cells, which are capable of self-sustentation in tissue regeneration (5).

PrECs are surrounded by the stromal microenvironment providing an important niche to nurse epithelial progenitor cells (6). The stromal compartment comprises a variety of cell types including smooth muscle cells, subepithelial cells, wrapping cells, interstitial fibroblasts, and others (3, 7). The stromal cells secrete important...
signaling factors to stimulate prostate development and adult prostate tissue regeneration (1, 8). These paracrine signaling factors include stromal androgen, FGF, TGFβ, BMP, Sonic hedgehog (Shh), and others to induce prostatic secretion in luminal cells and maintain the self-renewal capacity of prostate stem/progenitor cells (9-12). Shh-Gli signaling exists in both prostate basal cells and stromal cells (7). However, how this signaling pathway controls the renewal capacity of stem/progenitor cells through the stromal-epithelial interaction remains elusive.

Shh-Gli signaling is an important signal transduction pathway in regulating the normal development of multiple organs including growth of prostatic tissues and differentiation of prostate epithelia (13, 14). It has been reported that Shh-Gli signaling facilitates prostate branching morphogenesis through regulation of hepatocyte growth factor (15). In mammalian cells, the Gli family consists of three members (Gli1, 2, and 3). While both Gli2 and Gli3 have C-terminal transcripational activation and N-terminal transcripational repression domains, Gli1 contains only a C-terminal transcripational activation domain. Shh signaling is primarily mediated through Gli2 and 3 (16). Gli3T is a mutant missing the C-terminal transcriptional activation domain and has been used as a constitutive transcriptional repressor of Gli signaling (17). In the Shh-dependent canonical pathway, the binding of Shh to a twelve-trans-membrane receptor Patched (Ptc/Ptch/Ptch1) induces trans-localization of Gli2/3 to the nucleus and regulates expression of downstream target genes such as Gli1, Bcl2, Ptc1, and others (16, 18, 19).

We have previously shown that epithelial Gli signaling regulates the expression of p63, and plays an essential role in the maintenance of the homeostasis and renewal potential of prostate stem/progenitor cells (20). Dys-regulation of Gli signaling by oncogenic events such as the synergy of Kras and androgen receptor (AR) promote the pathological expansion of basal/progenitor cells (20, 21). In this study, we demonstrate that the Shh-Gli signaling axis mediates the interaction of stromal-epithelial cells in prostate tissue regeneration under physiological conditions. Particularly, Gli signaling in stromal cells regulates the activity of prostate epithelial stem/progenitor cells and promoted prostate spheroid formation in vitro and tissue regeneration in vivo. Mechanistically, stromal Gli signaling regulates TGFβ1/2 expression and exogenous TGFβ1 inhibits prostate spheroid formation. Our study emphasizes the importance of stromal Gli signaling in the regulation of prostate stem cell activity and tissue regeneration capacity.

RESULTS

Cell autonomous Shh-Gli signaling regulates the renewal potential of prostate progenitor cells.

We have previously demonstrated that epithelial Gli signaling is essential in regulating the renewal potential of prostate stem/progenitor cells (20). To further examine the role of the cell autonomous Shh-Gli signaling axis in controlling prostate stem cell activity, lentiviral vectors over-expressing pre-Shh(WT) or pre-Shh(C25S) mutant were constructed (Fig. S1A-B). Shh(WT) undergoes both cholesterol modification at the C-terminus and palmitoylation at the N-terminus (Fig. 1A). Mutation of the cysteine to serine blocked palmitoylation of Shh without affecting its maturation (Fig. 1A-B and Fig. S1C), indicating that both pre-Shh(WT) and pre-Shh(C25S) were processed to form mature Shh protein (19 kDa).

The self-renewal potential of prostate stem/progenitor cells can be examined by prostate sphere formation (Fig. 1C) (22). We examined if the cell autonomous Shh-Gli signaling axis regulates prostate stem cell activity by the sphere formation assay. Over-expression of Gli3T or knockdown of Gli1 and Gli2 were confirmed by the down-regulation of Ptc1 and Bcl2, or Gli1/2 expression, respectively (Fig. S2). As reported previously (20), over-expression of Gli3T, a dominant-negative repressor of Gli signaling, in PrECs significantly inhibited primary prostate sphere formation (Fig. 1D). While over-expression of Shh(C25S) inhibited the number of primary prostate spheres, over-expression of Shh(WT), shRNA-Gli1, or shRNA-Gli2 resulted in no change in the sphere number (Fig. 1D). However, the number of secondary spheres was significantly inhibited by over-expression of Gli3T, Shh(C25S) or shRNA-Gli2, but not shRNA-Gli1 (Fig. 1E). The data indicate that suppression of Shh-Gli2/3 signaling by loss of Shh palmitoylation, knockdown of Gli2, or over-expression of the repressor Gli3T inhibits the renewal activity of prostate stem cells.
P63, CK5, and CK14 are the well-established markers of prostate basal/progenitor cells, and CK8 and CK18 are known luminal markers. We have previously shown that over-expression of Gli3T and shRNA-Gli2 inhibited p63 expression (20). Therefore, we analyzed if Shh(WT) or Shh(C25S) regulates the levels of these proteins in PEB cells, a cell line isolated from mouse prostate basal cells (23). While overexpression of Shh(WT) in PEB cells did not further increase expression levels of basal or luminal markers, overexpression of Gli3T down-regulated expression levels of p63, CK5, CK14, and AR, but not CK8 or CK18. Similarly, Shh(C25S) decreased levels of p63, CK5, and CK14, but not CK8, CK18, or AR (Fig. 1F). Collectively, the data suggest that the cell autonomous Shh-Gli signaling axis regulates the renewal potential of prostate spheroids by potential regulation of basal or stem/progenitor cells.

**Mesenchymal stromal cells enhance prostate spheroid formation and elevate epithelial Gli and p63 expression.**

To evaluate the contribution of stromal cells in the regulation of prostate epithelial stem/progenitor cell activity, an in vitro stromal cell-sphere co-culture assay was developed in which the formation of prostate spheres is under the stimulation of mesenchymal stromal cells (Fig. 3A). The number and size of prostate spheroids were significantly increased when PrECs were co-cultured with UGSM cells (Fig. 3B-C). Additionally, stromal cells significantly increased the number of prostate spheroid aggregates. The aggregates contained 2-6 spheroids, with some aggregates having more than 6 spheroids (Fig. 3B).

Further analysis showed that expression levels of progenitor and basal markers (p63, CK5, or CK14), AR, and luminal markers (CK8 or CK18) were increased in the PrECs+UGSM group in comparison with PrECs alone group (Fig. 3D). Expression levels of Gli1, 2, and 3 in prostate spheres grown with or without the stimulation of UGSM were also compared. While Gli1 was barely detected in spheres (not shown), levels of Gli2 and Gli3 were significantly elevated in the PrECs+UGSM group in comparison with PrECs alone group (Fig. 3D). Additionally, the number of single spheres and spheroid aggregates derived from the PrECs+UGSM group was significantly higher than that from PrECs alone in the secondary spheroid passage, suggesting UGSM cells enhance sphere renewal potential (Fig. 3E). Collectively, the data suggest that mesenchymal stromal cells enhance the activity of prostate stem/progenitor cells, which is associated with epithelial Gli signaling.

**Stromal Gli signaling promotes prostate stem/progenitor activity.**

We studied the contribution of stromal Gli signaling in supporting epithelial stem/progenitor cell activity. To exclude the endogenous stromal cells from the PrECs preparation, prostate basal cells were isolated based on Lin-CD49f+Sca1+ markers (4) (Fig. 4A-C). Additionally, UGSM cells were transduced with Gli3T, shRNA-Gli1, or shRNA-Gli2 by lentiviral infection (Fig. 4A). The number of spheres increased more than 2-fold in the basal cells+UGSM or UGSM-vector groups in

**The presence of mesenchymal stromal cells compensates for the deficiency of epithelial Shh signaling in prostate tissue regeneration.**

The interactions between stromal and epithelial cells is essential in prostate tissue regeneration (1). Epithelial stem/progenitor cell activity was impaired by over-expression of Shh(C25S) or Gli3T. We examined if wild type mesenchymal cells could compensate for the deficiency of cell autonomous Shh signaling in epithelial cells. In contrast to the inhibition of primary prostate sphere formation by Shh(C25S) or Gli3T (Fig. 1D), epithelial cells over-expressing Shh(WT) or Shh(C25S) showed no difference in the size of regenerated prostate tissue in the presence of wild type urogenital sinus mesenchyme (UGSM) cells (Fig. 2A). RFP-expressing tubules indicated the tubules were infected with control vector, Shh(WT), or Shh(C25S). Histologically, regenerated prostate tubules showed no difference in expression levels of CK5/CK8 or p63 (Fig. 2B). Similarly, the regenerated prostate tissues derived from over-expression of Gli3T in epithelial cells showed similar tubule structure as the control vector (Fig. S3). The data suggest that the impaired epithelial Shh signaling could be rescued by the presence of normal mesenchymal cells.
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Comparison with the basal cells alone or the basal cells+3T3 cells (Fig. 4D). The size of spheres increased in the presence of UGSM, UGSM+Gli3T/shRNA-Gli2/shRNA-Gli1, or 3T3 in comparison with basal cells alone (Fig. 4D). The UGSM-induced sphere number was significantly inhibited by over-expression of Gli3T or shRNA-Gli2, but not by shRNA-Gli1 (Fig. 4D). Additionally, the number of spheres and spheroid aggregates significantly decreased in the PrECs+UGSM-Gli3T group in comparison with the PrECs+UGSM-control group (Fig. 4E), suggesting that over-expression of Gli3T inhibited spheroid renewal potential. The data suggest that the expression of stromal Gli2/3 promotes epithelial stem cell activity.

Next, we further examined if sphere formation could also be regulated by adult prostate stromal cells. The primary adult stromal cells were isolated based on Lin-CD49f+Sca1+ markers (Fig. 4B-C). The isolated adult stromal cells grew similar to UGSM cells. Stromal cells were transduced with control or Gli3T by lentiviral infection (Fig. 4F). Similar to the UGSM-sphere assay (Fig. 4D), adult stromal cells promoted the number and size of prostate spheroids. The number, but not the size, of prostate spheroids was inhibited by the suppression of Gli signaling through over-expression of Gli3T (Fig. 4G). Collectively, the data indicate that adult stromal Gli signaling regulates epithelial stem cells as well.

**Blockade of stromal Gli signaling inhibits prostate tissue regeneration in vivo.**

We examined the contribution of stromal Gli signaling in prostate tissue regeneration in vivo. To exclude the potential of endogenous stromal contribution in tissue regeneration, basal cells were isolated based on Lin-CD49f+Sca1+ markers (Fig. 5A-C). The isolated cells were mixed with/without UGSM expressing control vector or Gli3T (Fig. 5A) and subjected to the prostate tissue regeneration assay. The number of regenerated tubules was significantly elevated in the regenerated tissue derived from the basal cells+UGSM group in comparison with the basal cells alone group (Fig. 5D-E and Fig. S4). However, it was significantly inhibited in the basal cells+UGSM-Gli3T group in comparison with the basal cells+UGSM group (Fig. 5D-E). Additionally, the secretion (stained as pinkish color by H&E staining) was largely not visible in the lumen of tubules in the basal cells+UGSM-Gli3T group in comparison with those in the basal cells+UGSM group (Fig. 5D, b-c).

We characterized cell types in the regenerated tubules from the different groups. The number of p63+ and/or CK5+ cells in the tubules derived from the basal cells alone or basal cells+UGSM-Gli3T group were significantly elevated in comparison with the basal cells+UGSM group (Fig. 5D, F, H, and S4). In contrast, the number of CK8+ cells in the tubules derived from the basal cells alone or basal cells+UGSM-Gli3T groups were significantly lower (Fig. 5D, G, and S4). Collectively, the data indicate that blockade of Gli signaling in UGSM inhibited the tissue regeneration potential and the differentiation of prostate basal/progenitor cells into luminal cells.

Similarly, by using PrECs (un-sorted cells, endogenous stromal cells not excluded) for prostate tissue regeneration, the number of tubules and branching morphogenesis of the regenerated tubules were also inhibited in the PrECs+UGSM-Gli3T group in comparison with the PrECs+UGSM-control group (Fig. S5).

**Mesenchymal stromal Gli signaling regulates TGFβ expression levels to affect stem cell activity.**

We investigated how blockade of stromal Shh-Gli signaling would regulate other signaling pathways, which are related to stem cell activity in stromal cells. Over-expression of Gli3T or knockdown of Gli3 was confirmed by decreased mRNA levels of Gli downstream genes such as Gli1 and Bcl2 (Fig. 6A and C). While over-expression of Gli3T resulted in a significant increase of TGFβ2 levels in UGSM cells (Fig. 6B), shRNA-Gli3 increased expression levels of TGFβ1 and TGFβR1/2/3 (Fig. 6D). The difference in regulation of TGFβ1 or 2 expression levels might be due to a broader effect of Gli3T, which could block both Gli1/2 or Gli3 signaling, considering they have different downstream targets (24, 25). Interference of Gli signaling in UGSM cells also affected AR, Notch, and BMP expression (Fig. S6).

We examined how exogenous TGFβ1 affected the activity of prostate progenitor cells using the sphere formation assay. An increase of TGFβ1 in the medium inhibited the number (Fig. 6E) and size (Fig. 6F) of prostate spheres and sphericoid
aggregates (Fig. 6G) stimulated by UGSM cells. A high concentration of TGFβ1 (10 ng/mL) completely inhibited the UGSM-stimulated prostate spheroid formation (Fig. 6E). The results were consistent with the inhibitory effect of sphere formation in vitro (Fig. 4D and 4G) and tubule formation in vivo (Fig. 5D). The data indicate that stromal Shh-Gli signaling modulates the TGFβ signaling pathway in stromal cells, thereby regulating epithelial stem cell activity.

DISCUSSION

Our study demonstrates that cell autonomous Shh-Gli signaling in the epithelial compartment dictates the renewal potential of prostate stem/progenitor cells. Prostate basal cells are a major pool of stem/progenitor cells in prostate tissue (4). The cells maintain high expression levels of Shh and Gli3 (7). Blockade of Shh signaling by over-expression of the Shh(C25S) mutant inhibits expression of p63 or CK5, a marker of progenitor/basal cells in prostate tissue (26, 27), and prostate spheroid formation. This result is consistent with our previous experimental evidence that over-expression of Gli3T, a dominant negative suppressor of Gli signaling, inhibits the renewal capacity of prostate progenitor cells (20). Our studies illustrate that the Shh-Gli2/3-p63 signal transduction pathway is essential in maintaining the homeostasis of prostate stem cells in the epithelial compartment (Fig. 7).

Our study also illustrates that stromal Gli signaling regulates epithelial stem cell activity (Fig. 7). Stromal-epithelial cell interactions are important for prostate development and adult prostate regeneration (1). We show that mesenchymal stromal cells elevate expression of Gli2/3 and p63/CK5 in prostate spheroids from the co-culture assay. The epithelial CK8/CK18 levels are also elevated through stimulation of stromal cells, suggesting that stromal cells promote the differentiation process as well. Blockade of stromal Gli signaling inhibits the number of spheres in vitro and regenerated prostate tubules in vivo. Our data support Shh-Gli signaling as an important pathway that facilitates the crosstalk of stromal cells with epithelial basal/progenitor cells in prostate development and tumor initiation (7). In particular, stromal Gli2/3-TGFβ1/2 signaling promotes the activity of prostate stem/progenitor cells. In contrast to previous reports (28), the experimental design in this study allows us to clearly dissect the contribution of Shh signaling in either the epithelial or stromal compartment towards the regulation of prostate tissue regeneration. Similar to other epithelial stem cells, the microenvironment is essential in promoting the renewal activity of prostate progenitor cells (6, 29).

Prostate epithelium and reactive stromal cells co-evolve to regulate prostate development (3). TGFβ and hedgehog signaling are two well-characterized pathways in embryonic development (30). Numerous studies have reported that TGFβ regulates hedgehog signaling and Gli expression. Reciprocally, Gli signaling positively regulates TGFβ levels (24, 31). Our data reveal a transcriptional regulation of TGFβ by Gli signaling in stromal cells. Blockade of Gli signaling by over-expression of Gli3T inhibits stromal-stimulated basal cell differentiation in prostate tissue regeneration. Particularly, stromal Gli signaling regulates numerous signaling routes including TGFβ expression levels, which inhibits the differentiation of basal/progenitor cells (p63+ or CK5+) to CK8+ luminal cells. As a result, it further controls the fate of epithelial stem cells.

Blockade of Gli signaling by Gli3T (Fig. 6A-B) or shRNA-Gli3 (Fig. 6C-D) results in an increase of TGFβ2 and TGFβ1 levels in UGSM, respectively. The differential effect of Gli3T and shRNA-Gli3 on regulation of TGFβ isoforms might be due to the different genetic approaches for suppression of Gli signaling. Gli3T is a constitutive repressor form of Gli3 that antagonizes in a dominant fashion the transcription of Gli factors including Gli1 and Gli2. The over-expression of Gli3T leading to elevation of TGFβ2 expression is also reported in pancreatic cancer cells (32) or with activation of Smoothened (24). On the other hand, microarray analysis suggests that Gli3 alone regulates TGFβ1 expression levels in thymocytes at the embryonic stage E18.5 (33). Gli3 is bi-functional, meaning that it acts as a transcriptional activator as Gli3A, or as a repressor as Gli3R. Gli3 is mostly expressed in the fetal stage (34), and is predominantly the Gli3R isoform in stromal cells (35). In contrast to Gli3T, shRNA-Gli3 potentially suppresses Gli3R levels, thus
leading to up-regulation of TGFβ1 in UGSM cells (Fig. 6C-D).

Targeting palmitoylation of Shh is a therapeutic approach for inhibition of Shh-Gli signaling. Our studies demonstrate that loss of Shh palmitoylation inhibits the renewal potential of prostate epithelial stem/progenitor cells. Although palmitoylation is not required for the auto-cleavage of pre-Shh protein to mature Shh (36), it is essential for the formation of the multimeric form of Shh to perform its physiological activity such as the regulation of embryonic development (37, 38). Shh-Gli signaling is associated with numerous types of cancer including prostate cancer (39). The up-regulation of Shh in prostate cancer cells modulates the tumor microenvironment and facilitates osteoblastic cells in metastatic prostate cancer (40). The majority of inhibitors targeting Shh-Gli signaling are based on blockade of Shh downstream signaling including Smo, Ptc, or Gli transcriptional activators (41, 42). Therefore, targeting palmitoylation of Shh provides a direct therapeutic approach to inhibit the ligand activity. Palmitoylation of Shh is catalyzed by Hhat, which transfers palmitoyl-CoA to the N-terminal cysteine of Shh (36). It has been shown that mutation of Hhat, which disrupts the palmitoylation of Shh, interferes with testicular organogenesis (43). Recently identified inhibitors such as RU-SKI 39/41/43/50 show promising inhibitory effects on Shh signaling (44, 45). Our results provide biological evidence that although inhibition of Shh palmitoylation suppresses cell autonomous Shh-Gli signaling, the normal stromal microenvironment is sufficient to overcome the deficit of epithelial Shh-Gli signaling. Therefore, the efficacy of the inhibitors in targeting stromal Gli signaling should also be investigated.

EXPERIMENTAL PROCEDURES

Plasmid and lentiviral production - The open reading frame (ORF) of mouse Shh was PCR amplified from the parental vector, pBS-Shh(WT) (Addgene, Plasmid #13999) (primers listed in Supplemental Table 1) and inserted into the XbaI site of FUCRW vector. The C25S mutation was introduced by sequential PCR site-directed mutagenesis technique. In the first round of the PCR reaction, Pre-mShh-5’(XbaI)/Pre-mShh-C25S-R and Pre-mShh-C25S-F/Pre-mShh-3’ (XbaI) fragments were amplified individually with pBS-mShh as the template. In the second PCR, full-length Shh-C25S was amplified by the two flanking primers Pre-mShh-5’(XbaI) and Pre-mShh-3’(XbaI) with the two fragments generated in the first PCR as the template. PCR was performed in a 20 μL reaction mixture containing 100 ng DNA template and 20 ng of each primer. The thermocycler steps were 2 min at 95 °C, 30 cycles of 30 seconds (s) at 95 °C, 30 s at 58 °C and 1 min extension at 72 °C, followed by a final extension at 72 °C for 5 min. The PCR products were gel-purified, digested by XbaI, and inserted into the XbaI site of FUCRW vector. Since all the lentiviral vectors were derived from the FUCRW parental vector, they carry an RFP marker under the CMV promoter. Lentivirus production and infection were performed as described previously (21). All procedures followed the safety guidelines and regulations of the University of Georgia.

Isolation of primary prostate epithelial cells and prostate basal cells - Whole prostate tissues were isolated from five C57BL/6J mice (2 months old) and minced. The minced tissues were further digested by collagenase in DMEM medium for at least 1 hour at 37 °C. The collagenase-digested cell suspension was further digested with 0.05% trypsin for 5 min to dissociate cell clusters into single cells (22). After washing with DMEM to remove trypsin, the dissociated primary prostate epithelial cells (PrECs) were re-suspended in 1 mL PrEGM medium.

For the isolation of prostate basal cells, the above PrECs were sorted based on Lin−Sca1+CD49f+ markers as described previously (4). In brief, 10 μL of cell suspension (around 5x10^4 cells) was aliquoted into 4 tubes each containing 0.5 mL PrEGM medium. One microliter of CD49f-PE (0.2 mg/mL), 1 μL Sca-1-APC (0.2 mg/mL), 1 μL Lin-FITC containing a cocktail mixture of CD45 (0.17 mg/mL), CD31 (0.17 mg/mL), and Ter119 (0.17 mg/mL), or none were added to each tube, respectively. The samples were used for a gating control in FACS sorting. Additionally, a mixture of 5 μL CD49f-PE (0.2 mg/mL), 4 μL Sca-1-APC (0.2 mg/mL), and 5 μL Lin-FITC containing a cocktail mixture of CD45 (0.17 mg/mL), CD31 (0.17 mg/mL), and Ter119 (0.17 mg/mL) were added to the parental tubes. All tubes were incubated on ice for 30 min. Stained cells were
subjected to cell sorting by the MoFlo XDP (Beckman Coulter). Basal cells (LinCD49f+Scal+) were collected and counted for the sphere formation assay.

Primary sphere formation from PrECs and the secondary sphere formation from the dissociated primary spheroid cells (without stromal cells) - Isolated primary PrECs from BL6 mice as described above were counted and seeded at 5x10^5 cells per well in a 12-well plate. Lentivirus carrying the control vector, over-expression of Shh(WT), Shh(C25S), Gli3T, shRNA-Gli1, or shRNA-Gli2 were added to dissociated primary prostate cells with MOI=10-20, respectively. After 2 hours spin-infection (at 1500 rpm), lentivirus was removed from each well. The transduced cells were re-suspended from the well and washed twice, and finally re-suspended in 50 μL PrEGM medium (Lonza, Cat# CC-3166). Fifty microliters of cell suspension were mixed with 50 μL of matrigel and plated around the rims of the wells in a 12-well plate. The rims of a well allowed matrigel to create a 3D-space for PrECs to grow as prostate spheres. After the cell-matrigel mixture solidified at 37 °C for 20 minutes, 1 mL of PrEGM was added. The sphere number and size were recorded after 10 days incubation.

For the experiments of sphere formation in the presence of TGFβ1 ligand, isolated primary PrECs from BL6 mice as described above were counted. 7.5x10^3 cells were re-suspended in 50 μL PrEGM medium and mixed with 50 μL of the matrigel and plated around the rims of the wells in a 12-well plate. Once solidified at 37 °C for 20 minutes, 1 mL of PrEGM was added. After over-night incubation, the medium was replaced with fresh PrEGM medium containing 0, 1, 5, or 10 ng/mL of TGFβ1 (a gift from Dr. Peter Sun’s lab), respectively. The number and size of spheres were recorded at day 10.

For the secondary spheres, dispase was added to digest the matrigel matrix. The primary spheres were collected and treated sequentially with collagenase and trypsin as described above. Digested cells were passed through a 22-gauge syringe 3 times and filtered by a 40 μm cell strainer. Cells were then re-suspended in 400 μL PrEGM medium and sorted for RFP positive cells by flow cytometry. Fifty microliters of 8x10^3 RFP+ cells in PrEGM were mixed with 50 μL of matrigel and re-seeded in a well of 12-well plate. The number of spheres was counted after 10 days incubation. Growth media and matrigel conditions were the same as the primary sphere assay (22).

Sphere-stromal cells co-culture assay - In this assay, spheres were formed under the induction of urogenital mesenchymal (UGSM) cells or adult stromal cells. Of note, UGSM or stromal cells alone did not form spheres in the matrigel in the co-culture assay.

For the sphere-UGSM cells co-culture, UGSM cells were isolated from 16.5 day of BL6 mouse embryos (46). 5x10^3 unsorted primary PrECs or LinCD49f+Scal+ basal cells as isolated above were aliquoted into a tube and mixed with PrEGM medium or PrEGM medium containing 5x10^3 UGSM, UGSM-vector, UGSM-Gli3T, UGSM-shRNA-Gli1, UGSM-shRNA-Gli2, or NIH3T3 cells (as a control) to a final volume of 50 μL. The basal cells+UGSM cell mixture were then mixed with 50 μL of matrigel and plated around the rims of the wells in a 12-well plate. After the cell-matrigel mixture solidified at 37 °C for 20 minutes, 1 mL PrEGM medium or PrEGM medium containing 5x10^3 of UGSM, UGSM-vector, UGSM-Gli3T, UGSM-shRNA-Gli1, UGSM-shRNA-Gli2, or NIH3T3 cells were added to the corresponding wells, respectively.

For the sphere-adult stromal cells co-culture, UGSM isolated as described above was replaced with adult stromal cells to perform similar experiments. Adult stromal cells were isolated based on LinCD49f+Scal+. The isolated cells were grown in the same medium as UGSM cells (DMEM, 5% Nu-serum, 5% FBS, 0.05% of insulin, 0.01 μM dihydrotestosterone, and with penicillin-streptomycin), and could be passaged in cell culture for 3-5 passages. Adult stromal cells were transduced with control vector, Gli3T, shRNA-Gli1, or shRNA-Gli2 by lentiviral infection depending on the experimental settings. The number of spheres was counted 10 days later.

Cell culture and antibodies for Western blot analysis - 293T and 3T3 cells were grown in DMEM medium with 10% FBS. Normal mouse prostate epithelial basal cells (PEB) were isolated and immortalized as a cell line (a gift from Dr. Wilson’s lab) (23). The cells were grown in PrEGM medium with supplement growth factors
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(Lonza) and 5% FBS. PEB cells transduced with control vector, Shh(WT), Shh(C25S), or Gli3T (Control) by lentiviral infection were grown in PrEGM with 5% FBS. To exclude uninfected cells, RFP-positive cells were isolated by cell sorting (BD Biosciences). Three days after cells were transduced, culture medium was removed and cells were washed with PBS. Cells were lysed by RIPA buffer containing protease inhibitors. Cell lysate was subjected to Western blot analysis for expression levels of Gli3/Gli3T (Abcam, Ab69838, 1:1000), ERK2 (Santa Cruz, sc-154, 1:5000), p63 (Santa Cruz, 8431, 1:250), CK5 (Biolegend, 905501, 1:500), and Shh (Cell Signaling, 2207, 1:1000), AR (Santa Cruz, sc-816, 1:1000), Gli1 (Cell Signaling, 3538, 1:1000), Gli2 (Abcam, ab26056, 1:1000), GAPDH (Cell Signaling, 2118, 1:5000), and γ-tubulin (Sigma, T6557, 1:5000).

Real-time PCR - UGSM cells were infected with lentivirus depending on the experimental setting and cultured for five days. Cells were washed with PBS for RNA or protein extraction. Total RNA was isolated by a RNaseasy Kit (QIAGEN) following the protocol of the manufacturer. Complementary DNA was reverse-transcribed from 1.5 µg of total RNA in a 20 µL reaction with a high-capacity cDNA reverse transcription kit (Life Technologies). The RT products were diluted 30 times with distilled H2O, and 2 µL was used as template for each real-time PCR reaction. The reactions were performed using the PerfeCTa SYBR Green FastMix (Quanta Biosciences). The thermal cycling conditions were composed of an initial denaturation step at 95 °C for 1 min, 40 cycles at 95 °C for 10 s, and 60 °C for 50 s. The experiments were carried out in triplicate. The relative quantification in fold changes of gene expression was obtained by 2−ΔΔCt method with GAPDH as the internal reference gene. The sequences of primers used for RT-PCR are listed in the Supplemental Table 1.

Click Chemistry for the detection of Shh palmitoylation - PEB or 293T cells infected with virus carrying control vector, Shh(WT) or Shh(C25S) were grown in 6-cm dishes to 80-90% confluence. To metabolically label palmitoylated proteins, cells were further cultured in medium containing 2% BSA (fatty acid free) and 50 µM palmitic acid azide (15-Azidopentadecanoic Acid) (Thermo Fisher, C10265) for 293T cells or 50 µM 17-Octadecynoic Acid (17-ODYA) (Cayman, 90270) for PEB cells for 24 h. Cells were washed twice with PBS, and proteins were extracted with lysis buffer (100 mM sodium phosphate, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) containing protease and protein thioesterase inhibitors (0.2 mM hexadecylsulfonoyl fluoride and 10 µM palmostatin B). To perform click chemistry reactions, 50 µg cell lysate were incubated with reaction buffer (0.2 mM TAMRA-azide, 5 mM sodium L-ascorbate, 1 mM BTTP, 2 mM CuSO4) at a 1:1 ratio (v/v) for 1 h at room temperature in the dark. 4×SDS gel loading buffer containing 150 mM β-mercaptoethanol was added and samples were heated for 10 min at 70 °C and separated by SDS-PAGE. After gel electrophoresis, the gel was soaked in a fixation solution (40% methanol, 10% acetic acid, 50% water) for 1 h, followed by rinsing with deionized water (3x’s, 5 min) at room temperature. The fluorescence signal was detected using a Typhoon TRIO+ variable mode imager (GE Healthcare). The image was analyzed with ImageQuant (GE Healthcare). The expression of Shh was also confirmed by Western blot, and γ-tubulin was used as the loading control.

Prostate regeneration assay and immunohistochemistry - For the prostate regeneration assay, primary prostate cells were isolated from 8-12 week-old BL6 male mice. The isolated primary prostate cells were transduced with control vector, Shh(WT), or Shh(C25S) by lentiviral infection. Basal cells were also isolated based on LinCD49f+ Sca1+ from primary prostate cells as described above. The transduced cells (2x105 cells/graft) including control vector or Gli3T, isolated basal cells, or unsorted cells were combined with UGSM (2-3x105 cells/graft) followed by 25 µL of collagen type I (adjusted to pH 7.0). For Shh-induced transformation, UGSM cells were transduced with control, Shh(WT), or Shh(C25S) by lentiviral infection. The transduced and UGSM cells were combined with PrECs. After overnight incubation, grafts were implanted under the kidney capsule in CB.17Scid/Scid (SCID) mice by survival surgery. After 8 weeks, regenerated prostate tissues were obtained for histological and immunohistochemistry analysis.
All animals were maintained and used according to the surgical and experimental procedures of the protocol A2013-03-008 approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia. Formalin-fixed/paraffin-embedded specimens were sectioned at 4 µm thickness and mounted on positively charged slides. Sections were stained with H&E and immunohistochemistry (IHC) analysis was performed as described previously (21, 46, 47).

Statistical Analysis - Prism software was used to carry out a statistical analysis. The data are presented as mean ± SEM and analyzed by Student’s t test. All t tests were performed at the two-sided 0.05 level for significance.

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LEGENDS

FIGURE 1. Cell autonomous Shh signaling inhibits the renewal potential of prostate epithelial stem/progenitor cells. (A) Schematic of the maturation of wild type Shh [Shh(WT)] and Shh(C25S) mutant, a mutation of cysteine to serine. Pre-Shh(WT) and pre-Shh(C25S) were cloned into a lentiviral vector. Pre-Shh(C25S) is processed and cholesterolized at the C-terminus as the pre-Shh(WT), but not palmitoylated at the N-terminus. (B) Loss of palmitoylation in the Shh(C25S) mutant. PEB cells, a mouse prostate basal cell line, were transduced with pre-Shh(WT) and pre-Shh(C25S) by lentiviral infection. The cells were grown with 17-ODYA (60 μM). The expression of pre-Shh protein (~50 kDa) or mature protein (19 kDa) was detected by immunoblotting (right panel), and lysates were subjected to Click chemistry and palmitoylated-proteins were detected by fluorescence (left panel). (C) Schematic of primary and secondary spheroid formation. Prostate tissues were isolated from BL6 mice and dissociated into single cells. Prostate epithelial cells (PrECs) were transduced with pre-Shh(WT), pre-Shh(C25S), Gli3T, or shRNA-Gli1/2. Only PrECs with stem cell activity (indicated as the orange color) could form primary spheroids after embedding within matrigel. Primary spheres were digested into spheroid cells and embedded in matrigel to form secondary spheres. (D) Primary PrECs transduced with control vector, pre-Shh(WT), pre-Shh(C25S), Gli3T, or shRNA-Gli1 were mixed with matrigel and plated in separate wells in a 12-well plate. Sphere number was counted after 10 days incubation. (E) The primary spheres were dissociated and re-plated in the same way as described in (D) to generate secondary spheres. (F) PEB cells were transduced with control vector, pre-Shh(WT), pre-Shh(C25S), or Gli3T (positive control). The protein lysates were analyzed for p63, CK5, CK14, AR, CK8, CK18, Gli3T, Shh (mature form), and tubulin expression by immunoblotting. *, P<0.05; ***, P<0.001; N.S., not significant.

FIGURE 2. Mesenchymal stromal cells compensate for the deficiency of epithelial Shh signaling in prostate tissue regeneration. (A) Primary PrECs were transduced with control vector, Shh(WT), or Shh(C25S), and mixed with UGSM and implanted under the renal capsule. After regenerated prostate tissues were harvested, phase and RFP fluorescence images were taken (scale bar, 2 mm). Grafts derived from Shh(WT) or Shh(C25S) had less RFP, likely due to lower transfection efficiency in comparison with the control vector. (B) H&E, RFP fluorescence (a-f: Scale bar, 100 μm), and IHC staining of CK5 (red)/CK8 (green)/DAPI(blue), p63, and AR of the generated tissue (g-o: Scale bar, 50 μm). Red arrows indicate p63+ cells. Of note, the RFP signal is usually bleached after antigen retrieval so that the staining for CK5 (red) will not be interfered with the RFP in tissues (also see Figure S7).

FIGURE 3. Mesenchymal stromal cells enhance prostate spheroid formation and increase expression levels of Gli transcription activators and basal and luminal markers of prostate spheres. (A) Schematic of the UGSM-sphere co-culture assay. Primary PrECs were isolated from prostate tissue. UGSM cells were isolated from 16.5 days mouse embryos. PrECs were mixed with 5000 UGSM cells and matrigel and plated in the rim of a 12-well plate, and another 5000 UGSM cells were inoculated in the center of a well. Of note, only PrECs with stem cell activity (indicated as the orange color) are able to form spheres. UGSM cells remained as cells and are not able to form spheres in the co-culture assay. (B-C) The number of single spheres and spheroid aggregates (2, 3, 4, 5, 6, and >6 spheroid aggregates) (B), and size of single spheres (C) were recorded at day 10. Representative images are shown. The presence of UGSM cells increased the size and number of prostate spheres and spheroid aggregates. (D) Prostate
spheres grown with/without UGSM cells were collected. After UGSM cells were removed from the mixture of spheres and UGSM cells by filtering through 40 μm mesh, spheres from both groups were collected and compared. Proteins were extracted from the isolated prostate spheroids (UGSM cells were excluded) for the analysis of Gli2/3 and GAPDH expression. Cell lysate derived from embryonic stem cells was used as a positive control for the expression of Gli 2 and 3. Expression levels AR, p63, CK8, CK18, CK5, CK14, and GAPDH in two sets of experiments are shown. (E) Prostate spheres from PrECs and PrECs+UGSM groups were dissociated into single cells. 6000 dissociated cells were mixed with matrigel and evaluated for secondary sphere formation. The number of single spheres (labeled as “1” on the X-axis) and spheroid aggregates (with 2, 3, or 4 spheres) were recorded. *, P<0.05; ***, P<0.001; ND, not detected.

FIGURE 4. Mesenchymal stromal Gli signaling promotes the activity of prostate stem/progenitor cells. (A) Schematic of the co-culture of prostate basal cells with UGSM. Prostate tissues were dissociated into PrECs. PrECs were co-stained with Lin-FITC, CD49f-PE, and Sca1-APC antibodies. Lin-CD49f+Sca1+ basal cells were further isolated by flow cytometry as shown in panels B-C. Additionally, UGSM cells were transduced with control vector (FUCRW), Gli3T, shRNA-Gli1, or shRNA-Gli2 by lentiviral infection. Basal cells and transduced UGSM cells were mixed in the co-culture assay. (B-C) Isolation of primary prostate basal cells. Primary PrECs were first gated for the negative staining of Lin-FITC (B), then the Lin- cell population was further gated for positive staining of Sca1-APC and CD49f-PE (C). The Lin-CD49f+Sca1+ cell population was further gated for positive staining of Sca1-APC and CD49f-PE (C). The Lin-CD49f+Sca1+ cells represent the basal cells. (D) Lin-CD49f+Sca1+ basal cells were mixed with the UGSM-vector/Gli3T/shRNA-Gli1/shRNA-Gli2 or 3T3 (control) for sphere formation. The sphere number (left panel) and size (the right panel) were recorded at day 10, and sphere images were taken (the bottom panel). (E) The prostate spheroids derived from UGSM-control or UGSM-Gli3T groups were dissociated into single cells. Cells were mixed with matrigel and subjected to the secondary sphere assay. The X-axis shows single spheres (labeled as “1”) or spheroid aggregates (spheroid aggregates with 2, 3, or 4 spheres). The Y-axis represents the ratio of sphere formation. 1000:1 means 1000 epithelial cells forming 1 prostate sphere. (F) Schematic of the co-culture of Lin-CD49f+Sca1+ basal cells with adult stromal cells. Adult stromal cells were isolated based on Lin-CD49f+Sca1+ population as shown in panel C. The stromal cells were grown in the same medium used for growing UGSM cells. The adult stromal cells were transduced with control vector (FUCRW) or Gli3T by lentiviral infection. Lin-CD49f+Sca1+ basal cells were mixed with adult stromal cells in the co-culture assay. (G) The sphere number and size were recorded at day 10, and sphere images were taken. **, P<0.01; ***, P<0.001; N.S., not significant; ND, not detected.

FIGURE 5. Blockade of Gli signaling in mesenchymal stromal cells inhibits prostate regeneration potential. (A) Schematic of the in vivo prostate regeneration assay. Lin-CD49f+Sca1+ basal cells isolated by flow cytometry shown in panels B-C were mixed with/without UGSM-vector or UGSM-Gli3T. The cell mixture was implanted under the renal capsule of SCID mice. (B-C) Isolation of primary prostate basal cells. Primary PrECs were first gated for the negative staining of Lin-FITC (B), then the Lin- cell population was further gated for positive staining of Sca1-APC and CD49f-PE (C). Lin-CD49f+Sca1+ basal cells were used for prostate tissue regeneration experiments. (D) The regenerated tissues were subjected to histological analysis. H&E (a-c: scale bar, 100 μm) and IHC staining (d-i: Scale bar, 50 μm) of CK5 (red)/CK8 (green)/DAPI (blue) and p63 were analyzed in the regenerated tissues. Yellow stars indicate the secretion in the lumen of regenerated prostate tubules by the H&E staining, and red arrows indicate p63+ cells. (E) The number of tubules in the regenerated grafts was counted. The tubule number in the basal cells+UGSM-vector was set as 100%. (F-H) The number of CK5+(F), and CK8+, (G) or p63+ (H) cells per tubule was counted, and the percentage calculated. *, P<0.05; ***, P<0.001.

FIGURE 6. Mesenchymal stromal Gli signaling regulates expression levels of TGFβ. (A-D) UGSM cells were transduced with vector control, Gli3T (A-B), or shRNA-Gli3 (C-D) by lentiviral infection. Total RNA in UGSM-control, UGSM-Gli3T or shRNA-Gli3 cells were extracted. Over-expression of
Gli3T (A) or down-regulation of Gli3 (C) was confirmed by decreased expression levels of Gli1, Gli3, Ptc1, and/or Bcl2 by RT-PCR. Expression levels of TGFβ1/2/3 and TGFβR1/2/3 were measured by RT-PCR (B and D). (E-F) Prostate tissues were dissociated into PrECs. PrECs alone or PrECs mixed with UGSM cells in the co-culture sphere assay were grown in the prostate sphere growth medium with control, 1, 5, or 10 ng/mL of TGFβ1. The number (E) and size (F) of spheres were counted at day 10. (G) The number of single spheres (labeled with “1” on the X-axis) and spheroid aggregates (aggregates with 2, 3, 4, 5, or 6 spheres) in PrECs cultured with UGSM with/without 1 ng/mL of TGFβ1 were recorded. *, P<0.05; **, P<0.01; ***, P<0.001; ND, not detected.

FIGURE 7. Shh/Gli signaling mediates the crosstalk of stromal-epithelial interaction to regulate the activity of prostate stem/progenitor cells. In cell autonomous Shh-Gli signaling, prostate basal cells secrete epithelial Shh and induce the dissociation of Ptch1 from Smo, which then activates the transcriptional activator Gli2/3 to regulate the expression of p63, Gli1, Bcl2, and Ptc1. Over-expression of Gli3T blocks Gli signaling and inhibits p63 expression and the renewal potential of prostate stem/progenitor cells (20). Additionally, epithelial stem cell activity is influenced by stromal Gli signaling. Blocking stromal Gli signaling (by over-expression of Gli3T or shRNA-Gli3) increases expression levels of TGFβ1 or 2, and other signaling. Elevation of TGFβ inhibits proliferation and/or differentiation of progenitor cells, subsequently inhibiting prostate tissue regeneration or spheroid formation. As a result, stromal Gli signaling through regulation of TGFβ levels contributes to the differentiation and activity of epithelial stem cells.
Figure 2

A

Vector Shh(WT) Shh(C25S)  
Phase  

Vector Shh(WT) Shh(C25S)  
RFP

B

Vector  Shh(WT)  Shh(C25S)

H&E

a b c

d e f

g h i

CK5/ 
CKB/ 
DAPI

j k l

p63

m n o

AR

Scale bars represent 100 μm.
Figure 3

A. Scheme of the experimental setup showing the isolation and culture of PrECs from the fetal urogenital sinus (UGSM), followed by Matrigel-based sphere formation.

B. Graph showing the sphere number (primary) with a significant difference between PrECs alone and PrECs + UGSM conditions. Prostate spheroids aggregation images are also provided.

C. Comparison of the diameter of spheres formed by PrECs alone and PrECs + UGSM, indicating a significant difference.

D. Western blot analysis of mESC spheroids aggregation showing the expression of Gli3, Gli2, and GAPDH proteins. Two sets of spheres are shown: 1) isolated spheres from PrECs alone group and 2) isolated spheres from PrECs + UGSM cells group.

E. Bar graph showing the sphere number (secondary) with a significant difference between PrECs alone and PrECs + UGSM conditions.
Figure 4

A. Cell sorting and culturing process. PrECs were sorted for Lin- Sca1+ CD49+ cells, followed by sorting for Basal cells in Matrigel. Virus of vector or Gl13T/shGl11/shGl2 was used.

B. Flow cytometry analysis showing SSC and Lin-FITC, Sca1-APC for identifying basal and stromal cells.

C. Cell population analysis showing CD49f/PE for basal and adult stromal cells.

D. Graph showing the number of spheres and sphere size. Legend: 1: Basal cell alone, 2: Basal+UGSM, 3: Basal+UGSM(vector), 4: Basal+UGSM(Gl13T), 5: Basal+UGSM(shGl1), 6: Basal+UGSM(shGl2), 7: Basal+Ni13T3.

E. Prostate spheroids aggregation. Legend: UGSM-Control, UGSM-Gl13T.

F. Cell culturing process for adult stromal cells. PrECs were sorted for Lin- Sca1+ CD49f+ cells, followed by culturing for Basal cells in Matrigel. Virus of vector or Gl13T was used.

G. Graph showing the number of spheres and diameter of spheres. Legend: 1: Basal only, 2: Basal+adult stromal cells, 3: Basal+adult stromal cells (vector), 4: Basal+adult stromal cells (Gl13T).
Stromal Gli signaling regulates the activity and differentiation of prostate stem and progenitor cells
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