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Role of green tea catechins in modulating stromal-epithelial interaction in prostate cells

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Recent years have witnessed a significant interest in phytonutrients as treatment module for a range of diseases, including cancers. Catechins, predominant in green tea are potent antioxidants belonging to the flavonoid family that have been linked epidemiologically to a lower prevalence of prostate pathologies in Asian nations. In the present work, we investigated ameliorative potential of green tea catechins (epigallocatechin, EGC, and epigallocatechin 3-gallate, EGCG) on the aberrant proliferation of normal prostate epithelial cells driven by paracrine action of stromal medium, conditioned with estradiol (E_2) and dihydrotestosterone (DHT) (20 pM and 10 nM respectively). It was observed that EGC (5 µM) significantly inhibited the aberrant epithelial proliferation induced by stromal conditioned medium (CM), with a decreased expression of ERα, ERβ, AR, CK05, CK18, and PSA. Further, the CM collected after pre-treatment of stromal cells with EGCG (0.75 µM), had an inhibitory effect on epithelial cell proliferation, with reduction in the expression of ERα, AR, and CK05 while the expression of ERβ, CK18, and PSA were increased. These findings suggest that EGC helps in downregulating the aberrant epithelial proliferation while EGCG is able to downregulate the proliferative potential of stromal cell secretome. These insights will help in deciphering preventive measures against prostate pathologies involving stromal-epithelial cross-talk.

Keywords: BPH, Catechin, Flavonoid, Prostate, Sex steroids, Stromal-epithelial interaction

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Introduction

Human prostate gland is under the influence of sex steroids, dihydrotestosterone (DHT) and estradiol (E_2) for its development, functioning and maintenance of homeostasis[1]. During embryonic development, a cascade of molecular events initiated by SRY gene on the Y chromosome leads to the secretion of testosterone from the fetal testes (Sertoli cells). This is converted to DHT by the enzyme 5α-reductase to stimulate the differentiation and growth of the urogenital sinus (UGS) tissue at about 9 weeks of gestation[2]. The UGS composed of an outer layer of urogenital sinus mesenchyme (UGM) develop and differentiate into fibroblasts and smooth muscle cells in response to UGM androgen/androgen receptor (AR) signals[3]. The UGM then mediates epithelial budding in the inner layer of urogenital sinus epithelium (UGE) through mesenchymal-epithelial interaction[4]. The androgen/AR cascade signalling in stromal cells modulate the release of various andromedins (stromal-derived growth factors) such as fibroblast growth factor (FGF), keratinocyte growth factor (KGF), transforming growth factor β (TGF-β) etc. which promote further growth of complex ductal epithelium during puberty[5,6]. Estrogens have a synergistic effect in maintaining homeostasis along with DHT. ERα signalling cascade influences proliferation of both fibromuscular tissue of stroma and epithelial cells and ERβ influences apoptosis of the same[6,7].

Human prostate gland is the seat of three major causes of morbidity: prostatitis, benign prostatic hyperplasia (BPH), and prostate cancer (PCa) which are common with increasing age[8]. Changes in the stromal androgen/AR signal with ageing may influence the initiation and progression of these pathological conditions. In the ageing prostate, there is a decrease in concentration of DHT due to decreasing activity of 5α-reductase. Estrogen levels

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however, remain unchanged or are upregulated by the increased activity of the enzyme aromatase (CYP19A1/ cytochrome P45019A1)[9]. This causes an increased E_2:DHT ratio in the ageing prostate, that can be linked to an overall increase in cell proliferation and decrease in cell apoptosis, disrupting the normal homeostasis[6,10].

Epidemiological studies have long associated increased green tea consumption with decreased occurrence of prostate diseases in the Asian population[11]. Tea is the second most common beverage in the world and its usage in traditional medicine has been well documented since ancient times[12]. Green tea, produced from the leaves of *Camellia sinensis*, is composed of polyphenols namely catechins (flavan-3-ol) belonging to the family of flavonoid, low-molecular weight plant-derived anti-oxidative compounds. Catechins form around 30-40% of the dry weight of green tea, and are of 4 types: epigallocatechin 3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) amongst which EGCG is the most abundant. Tea catechins contain phenolic hydroxy groups, which are crucial for its antioxidant and radical-scavenging activity. A plethora of *in vitro* studies have indicated anti-proliferative, apoptotic, anti-angiogenic, anti-inflammatory, and anti-mutagenic efficacy of green tea catechins on various cancer cell lines[13–15].

In the present study, altered stromal-epithelial interaction corresponding to the ageing prostate *in vivo* were mimicked *in vitro* by culturing normal prostate stromal (WPMY-1) cells under 2:1 ratio of E_2 and DHT. The conditioned medium (CM) containing growth factors secreted by stromal cells was then transferred to normal prostate epithelial (RWPE-1) cells, to see if it induces aberrant proliferation. Further, catechins (EGCG and EGC), that have been reported to show anti-proliferative activity in various cancer cell lines were used to study their effects on this stromal cell mediated proliferation of epithelial cells.

Materials and Methods

Reagents

Dulbecco's modified Eagle medium (DMEM, high glucose), defined keratinocyte serum-free medium (K-SFM) and fetal bovine serum (FBS) were procured from Gibco, Thermo Fisher Scientific (Waltham, Massachusetts, USA). β-Estradiol (E_2), 5α-androstan-17β-ol-3-one (dihydrotestosterone, DHT), thiazolyl blue tetrazolium bromide (MTT), antibiotic-antimycotic solution (100X), DMSO (culture and molecular grade), EGCG, EGC and finasteride were from Sigma Aldrich (St. Louis, Missouri, USA). Cell culture flasks (T-25, T-75), multi-well plates (96 and 6) were obtained from Corning (New York, USA). Conical centrifuge tubes, microcentrifuge tubes, etc. were bought as per need.

Cell culture

Non-neoplastic prostate stromal cell line (WPMY-1) and non-neoplastic epithelial cell line (RWPE-1) were procured from American Type Culture Collection (ATCC, Manassas, Virginia, USA). RWPE-1 cells were maintained in keratinocyte-serum free medium (K-SFM) supplemented with 5 ng/mL epidermal growth factor (EGF) and 0.05 mg/mL bovine pituitary extract (BPE) at 37°C and 5% CO_2 in a humidified incubator. WPMY-1 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS) at 37°C and 5% CO_2 in a humidified incubator. Phase-contrast microscope (Olympus) was used to periodically monitor the morphology and growth of the cells and media was replaced every 2 days.

Preparation of stromal conditioned medium (CM)

Conditioned medium was prepared from WPMY-1 cells seeded in 75 cm^2 tissue-culture flasks with 15 mL complete DMEM. In 80% confluent flask, medium was replaced with 15 mL DMEM with 5% charcoal stripped FBS along with hormonal treatment of E_2 20 pM and DHT 10 nM for 72 h in 5% CO_2, 37°C incubator. After 72 h, the medium was collected and centrifuged (2000 g, 10 min, 4°C) to remove cellular debris, aliquoted, and stored at -80°C till further use.

Treatment of cells

Green tea catechins, EGC, and EGCG were diluted in DMSO. RWPE-1 cells were treated with various concentrations of catechins along with CM for 72 h to determine the non-toxic as well as efficient concentrations in 96-well plate via cell viability assay. Selected concentrations were further used for treatment of epithelial cells in 6-well plate for RNA and protein isolation. Finasteride (anti-BPH drug) was used as a positive control.

WPMY-1 cells were treated with different doses of green tea catechins for 24 h prior to CM preparation to determine their inhibitory effect on cell secretome. The pre-treated CM was then used to culture

epithelial cells for 72 h in 96-well plates($5x10^3$ cells/well)for cell viability assay and in 6-well plates($1.75x10^5$cells/well)for RNA and protein isolation.

Cell viability assay

MTT assay was performed to assess cell viability. Briefly, 96-well plates seeded with $5x10^3$ cells/well were incubated for 72 h (RWPE-1) and 24 h (WPMY-1) in 5% CO_2 at 37°C. After incubation with desired treatment, cells were exposed to 0.5 mg/ mLMTT (Sigma Aldrich, St. Louis, Missouri, USA) for 4 h and then formed formazone crystals were dissolved in DMSO for 2 h. The absorbance of coloured product was measured at 570 nm using a microplate reader (Tecan).

RNA isolation and real time PCR

Total RNA was isolated from cells using Trizol according to the manufacturer's instructions (Invitrogen). The RNA obtained was reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit, Thermo Fischer. qPCR for target genes' expression (ERα, ERβ, and AR) was performed using KAPA SYBR® FAST qPCR Master Mix (2X) Kit. The primer sequences used are mentioned in Table 1. C_t values obtained were used to calculate the relative mRNA expression of target genes with β-actin as endogenous control. Fold change was calculated using ΔΔct method and statistical significance was analyzed using Student t-test (unpaired, one tailed).

Western blotting

For determination of protein expression [cytokeratin 05 (CK05), cytokeratin 18 (CK18) and prostate specific antigen (PSA)], epithelial cells were cultured and treated in 6-well plates as mentioned above. After 72 h of treatment, existing medium was discarded and cells were washed with 500 µL of ice-cold phosphate buffered saline (PBS) followed by 500 µL of 0.25% trypsin-EDTA. The trypsin was deactivated with cold FBS-PBS mixture and cells were scrapped using a cell scraper transferring the cell suspension to a 1.5 mL microcentrifuge tube. The tube was centrifuged at 125 g for 10 min, supernatant discarded and cell pellet was washed with ice-cold PBS, prior to resuspension in lysis buffer. 100 µL of RIPA buffer supplemented with 0.1 µL of 100X protease inhibitor and phosphatase inhibitor was added. The tube was incubated at 4°C for 30 min with constant shaking and then centrifuged at 12000 g for 20 min, 4°C. The supernatant was transferred to a fresh tube, quantified by Bradford Assay and stored at -80°C until further use.

SDS-PAGE was performed using 12% resolving gel and 4% stacking gel and proteins were transferred to PVDF membrane. Membranes were incubated with primary antibodies Keratin 5 rabbit mAb (1:1000), Keratin 18 mouse mAb (1:2000), KLK3 rabbit mAb (1:1000), and β-actin rabbit mAb (1:1000) overnight at 4°C. They were further incubated with HRP conjugated secondary antibodies anti-mouse IgG (1:3000) and anti-rabbit IgG (1:3000) for 2 h. A chemiluminescent substrate was used to develop blot by chemi documentation system (Azure).

Statistical analysis

The data was analysed using MS Excel and GraphPad Prism 8.01 with one way analysis of variance (ANOVA) or Student t-test (unpaired, one tailed). The level of statistical significance was set at $P < 0.05$.

Results

Effect of stromal derived cell secretome on the aberrant proliferation of prostate epithelium

The paracrine effect of stromal derived CM was investigated on the proliferation of epithelial cells using MTT. The epithelial cells cultured with stromal

Table 1 — List of forward and reverse primer sequence used in qPCR

S. No.	Gene			Primer sequence	
	Name	Official name and symbol (NCBI)	Expected amplicon size (bp)	Primer	5′ → 3′
1	Beta actin	Actin beta (ACTB)	191	Forward	ACAGAGCCTCGCCTTTGC
				Reverse	CCACCATCACGCCCTGG
2	Androgen receptor	Androgen Receptor (AR)	164	Forward	GACATGCGTTTGGAGACTGC
				Reverse	TTCCCTTCAGCGGCTCTTTT
3	Estrogen receptor alpha (ERα)	Estrogen receptor 1 (ESR1)	174	Forward	CTTGGACAGGAACCAGGGAAAA
				Reverse	TTCAGGGTGCTGGACAGAAAT
4	Estrogen receptor beta (ERβ)	Estrogen receptor 2 (ESR2)	217	Forward	GCATGCGAGATGGGGAGAAA
				Reverse	GGGCCTTTAAGCTGGTTTGC

derived CM showed significant upregulation in their proliferation (Fig. 1a). The effect of CM was further evaluated on the relative mRNA expression of steroid receptors where ERα and AR were found upregulated with statistical significance (Fig. 1b). Further, the protein levels of basal epithelial marker CK05 and luminal epithelial marker CK18 were increased as compared to the control (Fig.1c).

Effect of EGC and EGCG on the aberrant proliferation of prostate epithelium

The effect of green tea catechins in combination with stromal CM was analysed using different concentrations. EGC at 5 µM was effective in decreasing the proliferation caused by CM (Fig. 2b) and was significant in downregulating the relative mRNA expression of the steroid receptors ERα and AR (Fig. 2c). It was also effective in decreasing the protein levels of CK05 (basal marker) with not much effect seen in CK18 and PSA (luminal marker and secretory protein) (Fig. 2d). EGCG did not show significant anti-proliferative activity and hence was not investigated further (Fig. 2a).

Effect of EGC and EGCG pre-treated stromal CMon the epithelial proliferation

The effect of green tea catechins on stromal cells were then analysed by pre-treating the cells with non-toxic doses of catechins(0.5 and 0.75 µM of EGCG and 0.15 and 0.25 µM of EGC) (Fig. 3a and b). After pre-treatment, the culture medium was discarded and CM was prepared in fresh DMEM to ensure that no residual catechin remained in the culture supernatant. The CM prepared thereafter was used to treat epithelial cells and were analysed for proliferative effect. CM pre-treated with 0.75 µM EGCG significantly inhibited the proliferation of RWPE-1 cells as compared to the control (Fig. 3c). Further analyses showed it was effective in downregulating the mRNA levels of Erα and AR as well as protein levels of CK05. However, ERβ, CK18, and PSA were upregulated post-treatment (Fig. 3e and f). EGC did not show any significant inhibition of epithelial cells' proliferation (Fig. 3d), therefore was not utilized for further analyses.

Discussion

Prostate is an endocrine-regulated gland dependent on sex hormones, testosterone and estrogen for development and functionality. The prostate micro-environment also plays a key role in influencing the heterogeneity and growth dynamics and is composed of stromal and epithelial compartments[16]. According to experimental findings, both these hormones and the cross-talk between the compartments are crucial for development of the prostate during pre-natal stage, with their absence resulting in abnormal/retarded growth[2]. In a normal adult prostate, cellular homeostasis is maintained mostly by DHT (active form of testosterone), with minor synergistic effect of estradiol (E_2). Testosterone and DHT interact with androgen receptors in the prostate epithelial and stromal cells to stimulate the release of andromedins (growth factors) essential for cell proliferation, differentiation, and maintenance[17]. Similar interaction is observed for estrogen, via ERα (proliferative effect) and ERβ (apoptotic effect) to maintain the prostate milieu[6,7]. The hormonal equilibrium of the prostate changes significantly as men age, as a consequence of

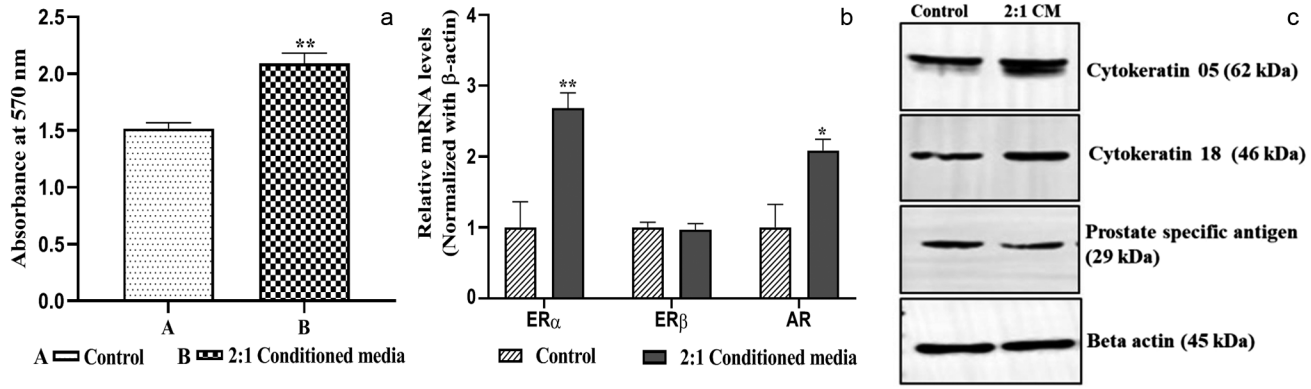

Fig. 1 — Effect of stromal derived conditioned media on the proliferation of prostate epithelium. a) Effect of 2:1 stromal CM on the growth of RWPE-1 cells, data are shown as mean±SD (n = 3); ** indicates P <0.01;b) Relative gene expression of ERα, ERβ and AR in RWPE-1 cells under the effect of 2:1 stromal CM; data are shown as mean±SEM (n=3); ** indicates P <0.01, * indicates P <0.05 compared to control group; and c) Western Blots of CK05, CK18 & PSA from RWPE-1 cells under the effect of 2:1 stromal CM (β-actin was used as loading control).

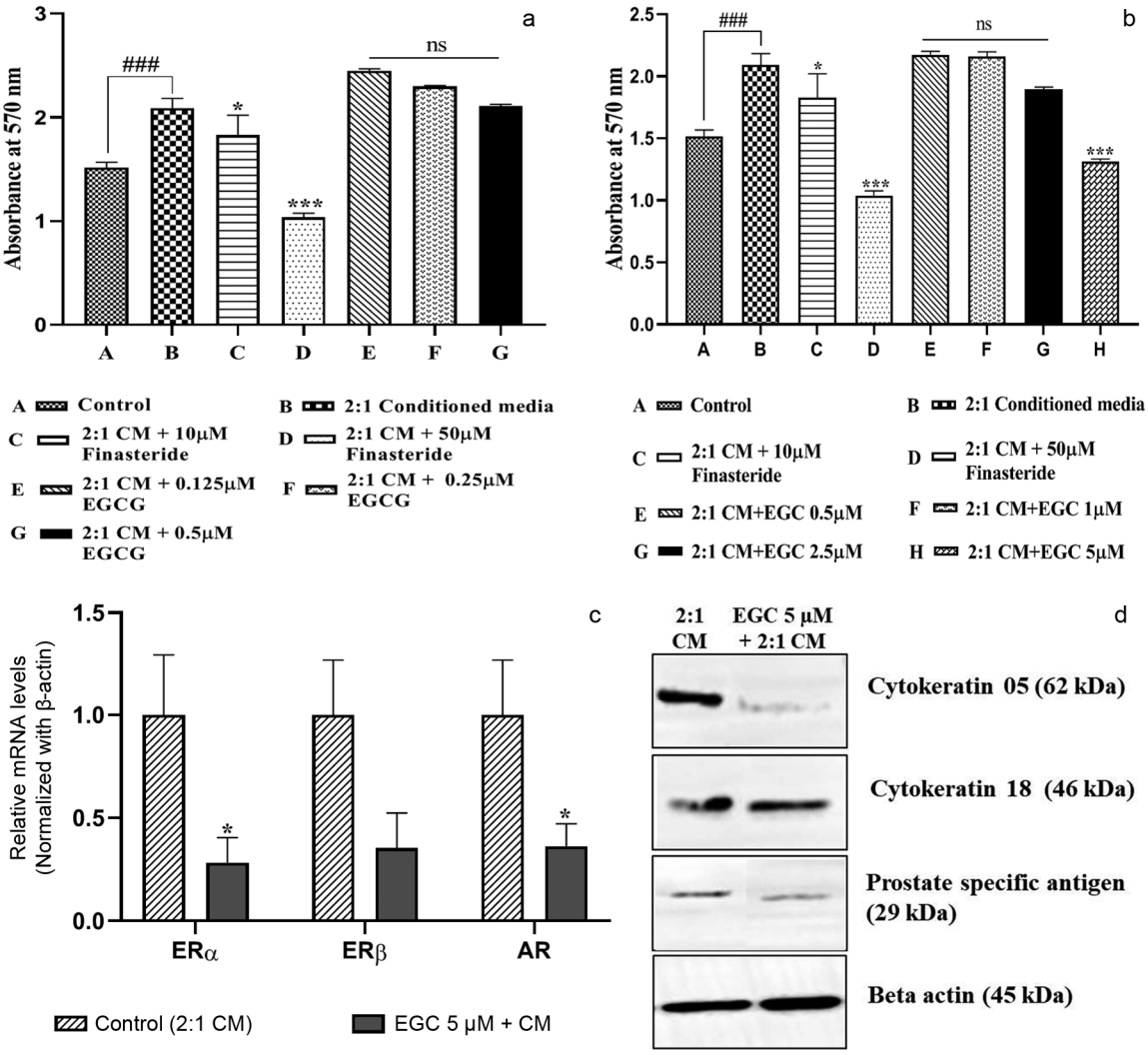

Fig. 2 — Effect of catechins on the proliferation of epithelial cells cultured with stromal derived conditioned media. a) Effect of EGCG on the growth of RWPE-1 cells cultured with stromal derived medium; data are shown as mean±SD (n = 3); ### indicates significant change (P <0.001) in growth of cells in group B as compared to A; *** indicates significant change (P <0.001) in growth of cells in group D as compared to B; *indicates significant change (P <0.05) in growth of cells in group C as compared to B; ns indicates non-significant change in growth of cells in group E, F and G as compared to B; b) Effect of EGC on the growth of RWPE-1 cells cultured with stromal derived medium; data are shown as mean±SD (n = 3); ### indicates significant change (P <0.001) in growth of cells in group B as compared to A; *** indicates significant change (P <0.001) in growth of cells in group D and H as compared to B; * indicates significant change (P <0.05) in growth of cells in group C as compared to B; ns indicates non-significant change in group E, F and G as compared to B; c) Relative gene expression of ERα, ERβ and AR in RWPE-1 cells under the effect of EGC; data are shown as mean±SEM (n = 3); * indicates P <0.05 compared to control group; and d) Western Blots of CK05, CK18 & PSA from RWPE-1 cells under the effect of 2:1 stromal CM and EGC (β-actin was used as loading control).

decrease in DHT level (reduced 5α-reductase activity) and increase in the estrogen level (increased aromatase activity)[5,9]. This imbalance has been implicated in the emergence and maintenance of prostate pathologies, namely benign prostate hyperplasia and prostate cancer. Both of these diseases disrupt the stromal epithelial cross-talk, which leads to an unrestrained cellular proliferation.

In this study, in presence of CM derived from stromal myofibroblasts which were cultured under 2:1 (E_2: DHT) treatment, the normal prostate epithelial cells showed aberrant proliferation (Fig. 1a) with a significant upregulation of ERα and AR at mRNA level (Fig. 1b). Previous studies reported that growth factors increase endogeneous AR expression and transcriptional activity in epithelial cells via activation

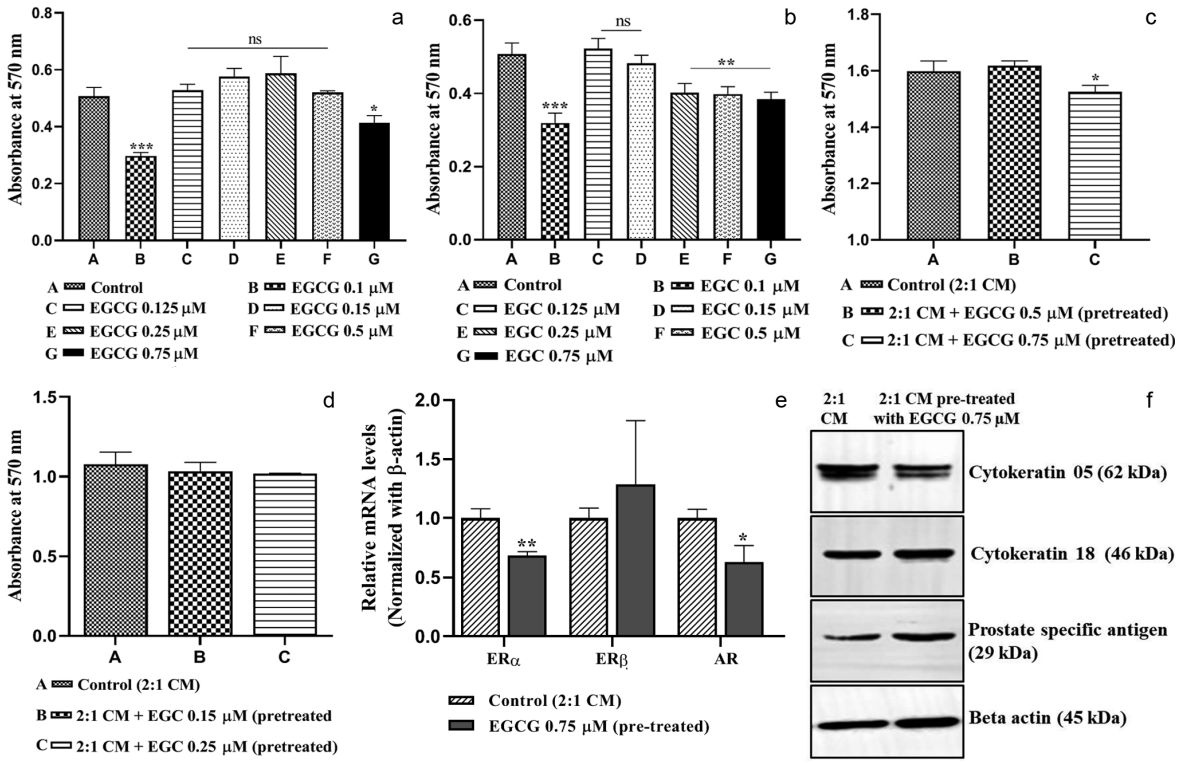

Fig. 3 — Effect of stromal derived conditioned media pre-treated with catechins on epithelial proliferation. a) Growth of WPMY-1 cells under different concentrations of EGCG; data are shown as mean±SD (n = 3); *** indicates significant change (P <0.001) in growth of cells in group B as compared to A; * indicates significant change (P <0.05) in growth of cells in group G as compared to A; ns indicates non-significant change in group C, D, E and F as compared to A; b) Growth of WPMY-1 cells under different concentrations of EGC; data are shown as mean±SD (n = 3); *** indicates significant change (P <0.001) in growth of cells in group B as compared to A; ** indicates significant change (P <0.01) in growth of cells in group E, F and G as compared to A; ns indicates non-significant change in group C, and D as compared to A; c) Effect of stromal CM pre-treated with EGCG on RWPE-1 cells; data are shown as mean±SD (n = 3); * indicates significant change (P <0.05) in growth of cells in group C as compared to A; d) Effect of stromal CM pre-treated with EGC on RWPE-1 cells; data are shown as mean±SD (n = 3); e) Relative gene expression of ERα, ERβ and AR in RWPE-1 cells under the effect of CM pre-treated with EGCG; data are shown as mean±SEM (n=3); ** indicates P<0.01, * indicates P<0.05 compared to control group; and f) Western Blots of CK05, CK18 & PSA from RWPE-1 cells under the effect of 2:1 stromal CM pre-treated with EGCG (β-actin was used as loading control).

of multi-signalling cascades[18–22]. Upregulation of Erα expression in epithelial cell has also been suggested to be involved in prostate pathologies[23] and henceforth, the growth factors in 2:1 CM could have been implicated in inducing aberrant epithelial proliferation mediated by the increased expression of ERα at mRNA level. However, there was no significant change in the expression of ERβ.

Further, there have been contradictory studies focussing on the origin of cell lineage underlying PCa, so we were interested in identifying the same in aberrant epithelial proliferation caused by stroma-mediated cross-talk. Studies in renal grafting models have found transformed human basal cells giving rise to prostate cancer[24–26], whilst another study found population of luminal stem cells in regressed mice prostate to serve as an *in vivo* cell of origin[27]. However, few studies have also reported both basal and luminal cells to have the ability to serve as cells of origin, indicated by lineage tracing in mice with the *Pten* tumour suppressor gene specifically removed in one of the two cell types[28–30]. In this study, both cell specific protein markers for basal epithelia (CK05) and luminal epithelia (CK18) were upregulated (Fig. 1c), with CK05 increasing substantially suggesting that the basal cells might serve as the progenitor for aberrant proliferation brought about by the age dependent alterations in prostate. Prostate specific antigen (PSA), a secretory protein released from the luminal cells is usually upregulated in prostate pathologies and acts as a bio-marker for the same. In this study, PSA was downregulated at the protein level under the effect of 2:1 CM which has been well supported by a study which highlighted that

expression of PSA was negatively regulated by growth factor (EGF) in cancer epithelial cells[31].

Green tea catechins in combination with prepared stromal CM were then administered to epithelial cells where EGC exhibited significant inhibitory effect on the proliferation (Fig. 2b), with no significant inhibitory action by EGCG (Fig. 2a). It was observed that EGC treatment significantly downregulated the expression of sex steroid receptors (ERα, ERβ, and AR) at mRNA level (Fig. 2c). Basal cell marker, CK05 was seen to be profoundly decreased in cells treated with EGC while luminal cell marker, CK18 did not exhibit any such change (Fig. 2d). These observations appear to be novel, as to the best of our knowledge there is no report on the anti-proliferative effect of EGC (in isolation) with either normal or cancerous cell lines of prostate or other origins. Androgen-dependent secretory protein PSA found downregulated in this study is consistent with a previous randomized clinical trial wherein usage of brewed green tea found significant reduction in PSA levels[32]. Another phase II clinical trial with crude green tea catechins also found decreased expression of PSA levels[33]. Altogether, the results are suggestive towards the potential role of EGC in preventing aberrant epithelial proliferation (with respect to basal epithelial cells) of the prostate gland.

Various experimental evidences have also suggested that EGCG downregulates expression of AR in prostate cancer cells and represses androgen signalling by suppressing the translocation of AR into the nucleus[34,35]. A previous study has also found EGCG to be anti-estrogenic for ERα and stimulatory for ERβ-mediated estrogenic activities in HeLa cells transiently transfected with an estrogen response element (ERE)-regulated luciferase reporter and ERs[36]. Another study also suggested EGCG to downregulate ERα pathway in the breast carcinoma cells[37]. The effect of EGCG on PSA was also studied in a placebo-controlled phase II randomized trial which showed that EGCG did not decrease PSA levels[38]. These research findings have been well corroborated with our experimental results wherein it was observed that the inhibitory effect of 2:1 CM pre-treated with EGCG significantly downregulated the expression of sex steroid receptors ERα and AR while expression of ERβ was upregulated at mRNA level (Fig. 3e). Further, basal cell marker, CK05 was seen to be profoundly decreased in cells treated with EGCG as compared to the control cells (treated with 2:1 CM), while luminal cell marker, CK18, and PSA were upregulated (Fig. 3f). These findings suggest the potential role of EGCG in regulating epithelial proliferation in the prostate by modulating paracrine signalling (via stromal secretome) (Fig. 4).

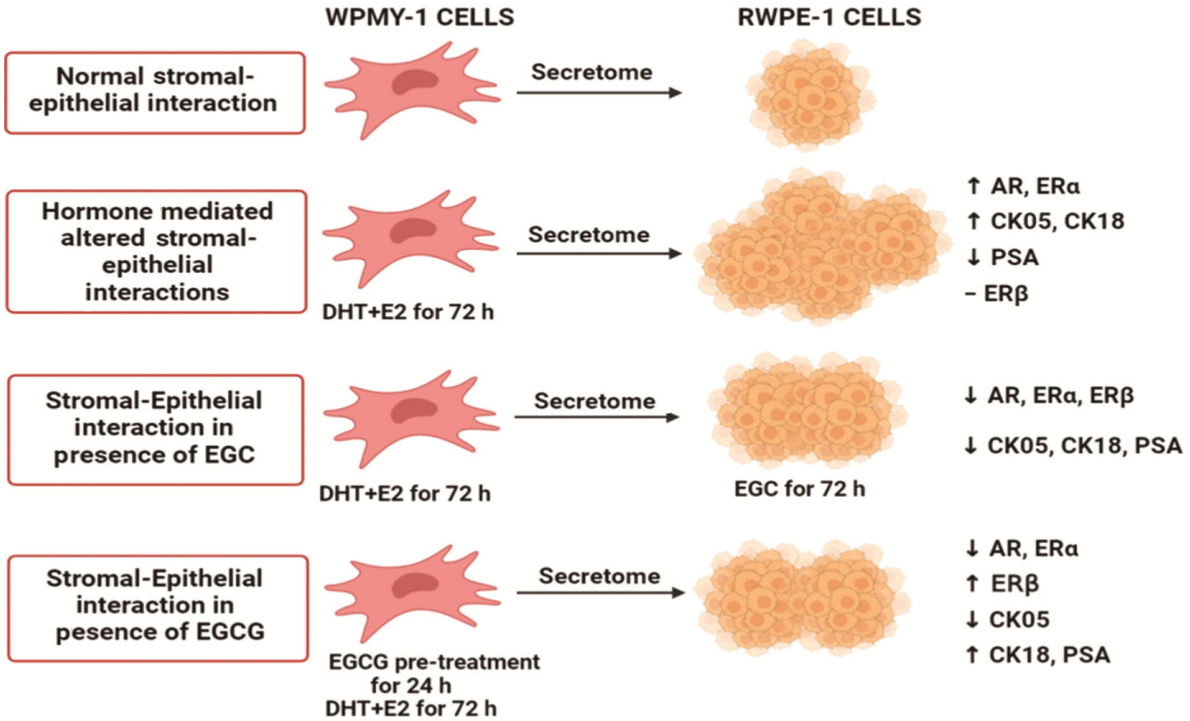

Fig. 4 — Illustration of effect of green tea catechins on modulating steroid mediated stromal-epithelial interaction.

Conclusion

This study aimed to test the hypothesis that alteration of the hormonal milieu during ageing induces prostate stromal cells to drive excessive growth of prostate epithelial cells; to comprehend the effects of catechins on the proliferation of prostate epithelia and the secretion of growth factors from stroma. Our findings suggest that EGC helps in downregulating the aberrant epithelial proliferation while EGCG is able to downregulate the cell secretome released from stromal cells suggesting anti-proliferative effects of EGC on epithelial cells and EGCG on stromal cells. The current experimental set-up can be further explored with combined effect of EGC and EGCG, by utilizing a co-culture model to study the stromal-epithelial cross-talk more effectively and the effect on the sub-population of epithelial cells can be analyzed with immunocytochemistry studies. Further, the signaling mechanisms underlying the anti-proliferative activity of these catechins need to be deciphered, to establish them for preventive phytotherapy.

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Conflict of interest

The authors declare that there is no conflict of interest.

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