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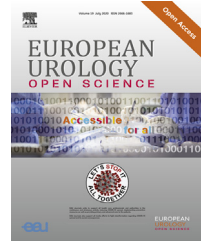
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## Brief Correspondence

# Overexpression of Placental Growth Factor in Stromal Cells from Benign Prostatic Hyperplasia: Another Piece in the Puzzle?

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Lower urinary tract symptoms associated with benign prostatic hyperplasia (BPH) have a European prevalence of 24% and are a significant economic burden for the society (>£200 million annually in the UK) [1].

BPH is a disease of ageing, the incidence and prevalence of which increase as men get older. However, the exact cause(s) remains disputed despite much research. Aetiological studies have focussed on inflammatory processes within the prostate, which lead to growth factor (GF) production, stem cell activation, and cellular proliferation [2].

The 5 $\alpha$  reductase inhibitors, which target the androgen signalling axis in prostate cells, decrease the impact of androgen receptor on cell proliferation in BPH [3]. Whilst symptomatic improvement and disease regression are observed, a significant treatment time is required and a proportion of patients fail treatment and require surgery.

These long response times imply that the true driver(s) of the disease may not be targeted by this treatment; that is, what is the overall dependence of BPH on androgens? Thus, targeting of the wrong “driver” cell population and indirect activation of AR via intermediate intracellular pathways through inflammatory signalling could explain equally the variable success of 5 $\alpha$ RI.

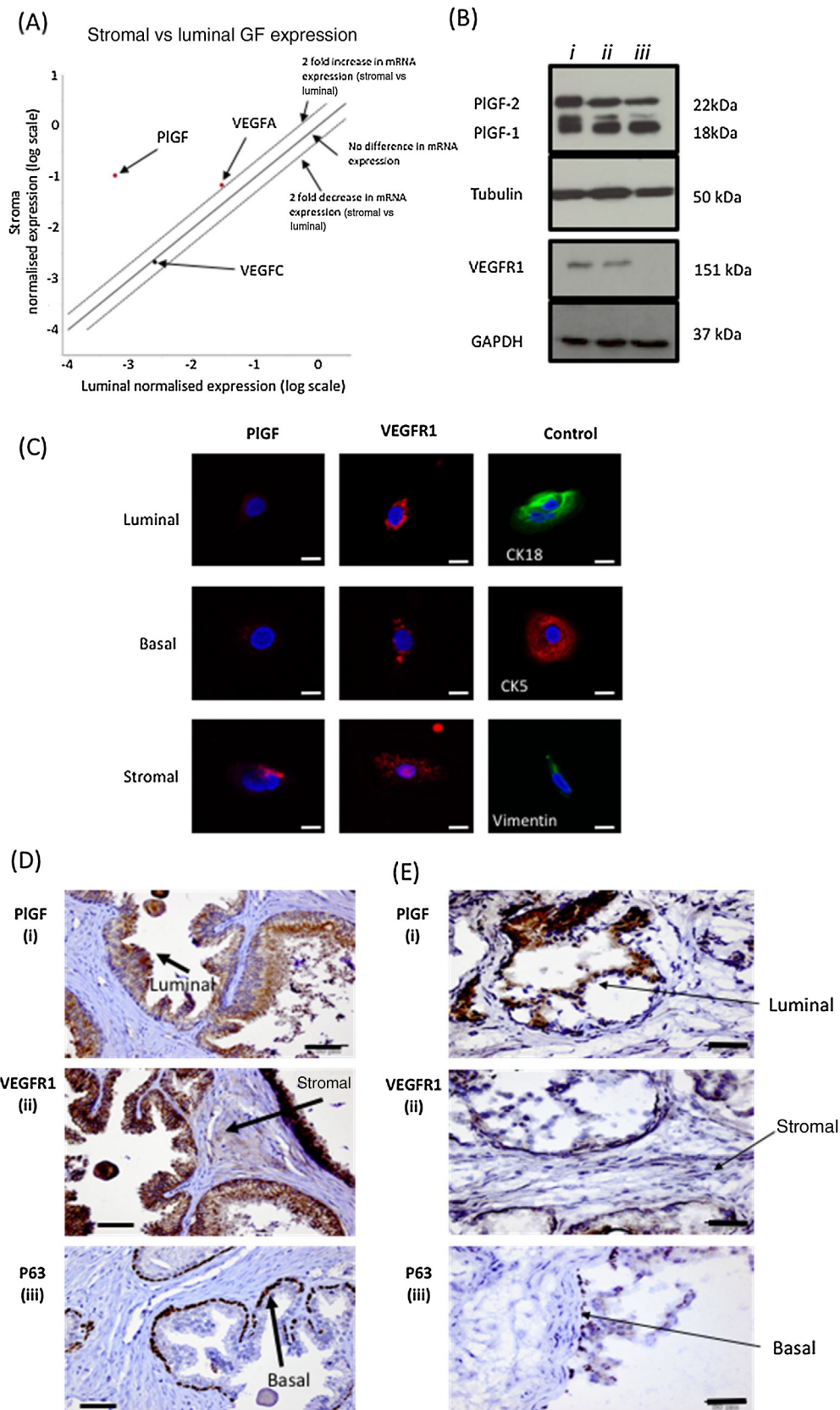
Since the characteristic cellular overgrowths, of both epithelial and stromal components, in BPH are dependent on either autocrine or paracrine stimulation by GF, we sought to survey a fuller range of human GF genes for their potential influence on BPH. GF mRNA array (Qiagen, Hilden, Germany) analyses were performed on RNA from *uncultured* prostate cell subpopulations, luminal cells (LCs; EpCAM<sup>+</sup>/CD49<sup>+</sup>), committed basal cells (CBs; EpCAM<sup>+</sup>/CD24<sup>+</sup>), and stromal cells (StCs; EpCAM<sup>−</sup>/Vimentin<sup>+</sup>) freshly purified from BPH tissue. The direct approach, more closely applicable to *in vivo* conditions, was prompted by earlier studies [4], which revealed a number of cell culture artefacts stimulated by GF in culture media. Separation is important,

since whole tissue homogenates can mask the origin and impact of a GF or cytokine, and dilute the magnitudes of differential RNA expression [5].

GF mRNA expression was significantly higher within the StC population than in the epithelial layer (LCs and CBs), for example, fibroblast growth factors 2 and 7 and insulin growth factor 1, as previously demonstrated for BPH. However, the GF with the highest expression disparity was placental growth factor (PlGF; Fig. 1A, and Supplementary Fig. 3 and 4), a member of the vascular endothelial growth factor (VEGF) family that binds exclusively to VEGF receptor 1 (VEGFR1) [6], which had previously shown negligible associations with BPH pathology.

Other VEGF (A and C) family GFs did not demonstrate significant expression upregulation. However, whole tissue homogenates consistently expressed both active isoforms of PlGF protein (Fig. 1B), together with VEGFR1 in 66% of samples (Fig. 1B-iii), implying that some heterogeneity is nevertheless apparent within different BPH disease processes.

Immunocytochemical analysis of uncultured, fractionated, paraformaldehyde (PFA)-fixed individual cell populations was next carried out for PlGF and VEGFR1 (Fig. 1C and Supplementary Fig. 5). In agreement with the GF array data, higher PlGF protein expression is seen in fixed StCs than in LCs and CBs. These high levels of expression suggest that stromal PlGF is released into the extracellular matrix to propagate its effect on other cell types. In PFA-fixed cells, VEGFR1 expression was strong in all prostate cell subtypes. When we immunostained formalin-fixed, paraffin-embedded BPH tissue (tissue microarrays—24 patients, from the Orchid Research tissue bank, Barts Hospital, London, UK), PlGF expression was observed in LCs and StCs, with the highest expression in the luminal epithelia (Fig. 1D and Supplementary Fig. 6), unlike the freshly PFA-fixed cells. We validated this observation by staining frozen fixed BPH sections that also demonstrated high PlGF expression in LCs



**Fig. 1 – Placental growth factor (PIGF) expression in uncultured benign prostatic hyperplasia (BPH).** (A) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of mRNA fold expression for PIGF and vascular endothelial growth factor (VEGF) A and C in fractionated and uncultured stromal versus luminal cells from fresh BPH tissue ( $n=6$ ). A scatter plot of expressed mRNA was generated from Qiagen (Hilden, Germany) web-based PCR-array data analysis software. The X axis represents the log expression of mRNA GFs in the combined ( $n=6$ ) stromal cell samples, whilst the Y axis represents the log expression of mRNA GFs in the combined ( $n=6$ ) luminal cell samples. Three points on the graph are shown, which represent PIGF,

**Table 1 – Expression of PlGF and VEGFR1 in each cell population in formalin-fixed paraffin-embedded BPH tissue sections on tissue microarrays (Bart's Hospital, London).**

Sample No.	Prostate tissue layer					
	Luminal		Basal		Stroma	
	PlGF	VEGFR1	PlGF	VEGFR1	PlGF	VEGFR1
1	++	++	–	++	–	++
2	+++	+++	–	+++	–	++
3	+	+++	–	++	–	+
4*	–	–	–	–	–	+++
5	+	++	–	–	–	++
6	++	++	++	++	–	++
7	–	–	+++	+++	+	+
8	+++	+++	+++	+++	+	+
9*	–	–	–	–	–	++
10	+++	++	–	++	+	+++
11	+++	+++	–	+++	+	+
12*	–	–	–	–	–	+
13	+++	+++	–	+++	+	+
14	+++	+++	–	–	–	–
15	++	++	–	–	+	+
16	+	+++	–	+++	++	++
17	++	++	–	++	++	++
18	++	++	–	++	+	++
19*	–	–	–	–	–	++
20	++	++	–	++	–	+
21	–	–	++	++	–	++
22	+++	+++	–	+	–	++
23*	–	–	–	–	–	+
24	+	++	–	+	–	++

BPH = benign prostatic hyperplasia; PlGF = placental growth factor; VEGFR1 = vascular endothelial growth factor receptor 1.

The symbol +++ denotes strong staining, ++ moderate, + weak, and – absent of staining. The strongest and most abundant expression of PlGF in the array was in the luminal cell layer, with occasional expression in the stroma in nine samples. VEGFR1 expression was higher in all cell population layers than in PlGF expression, and all samples within the array demonstrated VEGFR1 expression within at least one cell population. Only one stromal sample [14] did not express the receptor. There was a strong correlation between PlGF expression and VEGFR1 expression within the luminal cell layer. Committed basal cell VEGFR1 expression did not correlate with PlGF expression levels.

\*Next to the sample number, indicated that no epithelial layer was present on the tissue section.

(Fig. 1E), so paraffin embedding of tissue had not affected the PlGF expression pattern. This contrast in PlGF location between isolated cells and fixed tissue arrays could be the result of epitope masking or destruction during processing, but could equally be due, in part, to hypoxia-induced expression of PlGF during the cellular isolation process.

Although a potential artefact, this demonstrates that hypoxic stress (known to occur in BPH) will drive the overexpression of PlGF, where it has not been identified before, or indeed a paracrine effect of the epithelium on the stroma. Expression of VEGFR1 in the tissue microarrays was widespread throughout all the cell subtypes, similar to the

VEGFA, and VEGFC. Other GF data points have been removed for clarity; however Supplementary Figures 3 and 4 demonstrate the additional GF investigated in the array. Data were normalised to *RPLPO* and *Actin beta* gene expression. The scatter plot of fold expression differences demonstrated that PlGF expression was 190× greater in stromal BPH cells than in luminal BPH cells, which was statistically significant ( $p < 0.05$ ). VEGFA and VEGFC did not demonstrate significant expression fold changes ( $p > 0.05$ ). Data analysis of each array cell sample was performed using the SABiosciences PCR array data analysis centre (Qiagen, Hilden, Germany). Fold expression differences of  $<2$  were considered as not significantly different in expression, which is shown in the graph by the parallel expression lines, that is, values above the dashed black lines are  $>2$  times that of the control (luminal cells) and are statistically significant. The solid line represents fold expression that is equal between the control (luminal) and the comparative (stromal) group. (B) Western blot analysis of PlGF expression (isoforms 1 and 2) and its receptor VEGF receptor 1 (VEGFR1) in unfractionated homogenised fresh BPH tissue ( $n = 3$ ). Both isoforms of PlGF were expressed in all patient samples; interestingly, VEGFR1 expression was not seen in patient (iii), which demonstrated the heterogeneity in BPH pathology. Tubulin and GAPDH were used as loading controls for PlGF and VEGFR1, respectively. (C) Immunofluorescence analysis of paraformaldehyde-fixed fractionated uncultured cell subpopulations from fresh BPH tissue showing highest expression of PlGF within stromal cells (red stain, first column) and widespread VEGFR1 expression (red stain, second column) in all cell populations, particularly luminal cells, suggesting a paracrine influence of stroma on the proliferating epithelial layer. Cytokeratin 18 and vimentin were controls for luminal and stromal cells, respectively (green stain, third column). Cytokeratin 5 was a control for committed basal cells (red stain, third column). Nuclei are stained blue (DAPI). These cells were a representation of 100 cells in each well of a chamber slide. Images were captured on a Zeiss upright 710 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The scale bar is 20  $\mu\text{m}$ . (D) Immunohistochemical analysis of formalin-fixed paraffin-embedded (FFPE) BPH tissue sections from tissue microarrays (Barts Hospital, London, UK) showing the highest expression of PlGF was within the luminal cell layer (i), which was in contrast to that seen by immunofluorescence. VEGFR1 was expressed within luminal, basal, and stromal cells (ii). P63 was used as a committed basal cell marker (iii). Sections were stained using ImmPRESS Excel Amplified HRP Polymer Staining Kit (anti-rabbit IgG kit: MP7601 and anti-mouse kit: MP7602; Vector Laboratories, Peterborough, UK). The scale bar is 100  $\mu\text{m}$ . (E) Immunohistochemical analysis of fresh frozen BPH tissue section showing the FFPE process did not contribute to the findings of highest expression of PlGF within the luminal cell layer (i), as this matched with the findings in Figures 1D-i and 1D-ii. P63 was again used as a committed basal cell marker (iii), and sections were stained using the ImmPRESS Excel Amplified HRP Polymer Staining Kit (Vector Laboratories, Peterborough, UK). The scale bar is 50  $\mu\text{m}$ . GF = growth factor.

fixed isolated cells. In the tissue arrays, VEGFR1 protein was most abundant within the StC population. No patient sample within any of the three cell subpopulations lacked VEGFR1 expression (Table 1).

The PlGF/VEGFR1 complex can both directly and indirectly activate pathways involved in angiogenesis, which follow a number of current disease pathways hypothesised for BPH. The recruitment of angiogenesis-competent myeloid bone marrow progenitors to growing vessel sprouts and collateral blood vessels [7] could be viewed as the use of stem cells to drive this processes forward, for example, the role of bone marrow-derived mesenchymal stem cells described recently by Brennen and Isaacs [8] in relation to the embryonic reawakening theory of BPH.

As a response to stress, PlGF recruits monocytes, which then differentiate into macrophages and foam cells. These cells then release proinflammatory cytokines such as interleukin (IL) 6 and IL1 $\beta$ , leading to further recruitment of angiogenic/inflammatory cells and further secretion of PlGF [9]. Such a “vicious cycle” of inflammation was proposed previously by Ficarra et al [2] for BPH. Interestingly, StCs that are activated by PlGF release both FGF2 and IL8 [9], two factors previously highlighted in the inflammation cycle linked to the formation of BPH [2]. We therefore propose a hypoxia/stress cycle induced by PlGF cross stimulation in BPH promoting prostate growth and also neovascularisation, important in the expansion of new tissue. A similar mechanism has been detected (and treated experimentally with anti-PlGF therapy) between chronic myeloid leukaemia and bone marrow StCs [10].

This research forms a basis for the investigation into another piece of the complex BPH puzzle. Further studies on the impact of PlGF-induced growth on in vitro cultured primary cells may potentially open a new avenue for the medical treatment of this disease, since PlGF expression is highly restricted in normal human adult tissues.

**Author contributions:** Norman J. Maitland had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

*Study concept and design:* Devlin, Collins, Frame, Maitland.

*Acquisition of data:* Devlin, Archer, Walker.

*Analysis and interpretation of data:* Devlin, Archer, Frame, Collins, Maitland.

*Drafting of the manuscript:* Devlin, Maitland.

*Critical revision of the manuscript for important intellectual content:* Maitland, Devlin, Collins, Frame.

*Statistical analysis:* Devlin, Archer, Frame, Maitland.

*Obtaining funding:* Maitland.

*Administrative, technical, or material support:* Mann, Simms, Berney, Frame, Collins.

*Supervision:* Maitland.

*Other:* None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.euro.2020.08.003>.

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