

RESEARCH PAPER

Prostate cancer cells specifically reorganize epithelial cell-fibroblast communication through proteoglycan and junction pathways

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ABSTRACT

Microenvironment and stromal fibroblasts are able to inhibit tumor cell proliferation both through secreted signaling molecules and direct cell-cell interactions but molecular mechanisms of these effects remain unclear. In this study, we investigated a role of cell-cell contact-related molecules (protein ECM components, proteoglycans (PGs) and junction-related molecules) in intercellular communications between the human TERT immortalized fibroblasts (BjTERT fibroblasts) and normal (PNT2) or cancer (LNCaP, PC3, DU145) prostate epithelial cells. It was shown that BjTERT-PNT2 cell coculture resulted in significant decrease of both BjTERT and PNT2 proliferation rates and reorganization of transcriptional activity of cell-cell contact-related genes in both cell types. Immunocytochemical staining revealed redistribution of DCN and LUM in PNT2 cells and significant increase of SDC1 at the intercellular contact zones between BjTERT and PNT2 cells, suggesting active involvement of the PGs in cell-cell contacts and contact inhibition of cell proliferation. Unlike to PNT2 cells, PC3 cells did not respond to BjTERT in terms of PGs expression, moderately increased transcriptional activity of junctions-related genes (especially tight junction) and failed to establish PC3-BjTERT contacts. At the same time, PC3 cells significantly down-regulated junctions-related genes (especially focal adhesions and adherens junctions) in BjTERT fibroblasts resulting in visible preference for homotypic PC3-PC3 over heterotypic PC3-BjTERT contacts and autonomous growth of PC3 clones. Taken together, the results demonstrate that an instructing role of fibroblasts to normal prostate epithelial cells is revoked by cancer cells through deregulation of proteoglycans and junction molecules expression and overall disorganization of fibroblast-cancer cell communication.

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Introduction

Tumor microenvironment (TME) has a crucial role in tumor initiation and progression.¹⁻⁴ Complex relationship between epithelial cancer cells and the organ-specific microenvironment is well documented for prostate cancer⁵⁻⁸ and targeting the stroma has been suggested as a promising and attractive therapeutic option for the cancer treatment.⁹⁻¹¹


The major cellular components of TME are composed of cancer-associated fibroblasts (CAFs) and cooperative interaction between heterotypic fibroblasts and tumor cells contribute to cancer progression.¹²⁻¹⁴ Molecular mechanisms of transition of normal fibroblasts to CAF during prostate carcinogenesis include changes in transcriptional activity of numerous genes. Comparative analysis of fetal human prostate, normal prostate human fibroblasts (NPFs) and CAFs identified 671 transcripts that were enriched in CAFs and 356

transcripts whose levels were decreased relative to normal fibroblasts.¹⁵ Twelve new proteins differentially expressed in cancer-associated fibroblasts versus normal fibroblasts were identified in our previous study and 4 of them were related to the Rho kinase signaling pathway suggesting a potential mechanism of fibroblast-CAF transition.¹⁶ Although the molecular changes in CAF phenotype are evidently associated with tumor growth, invasion, and metastasis,¹⁷ exact molecular mechanisms how CAF or normal fibroblast affect prostate cancer cells not clear. It is shown that normal human fibroblasts inhibit proliferation and motility of prostate cancer cells through both soluble factors and direct cell-cell interactions^{18,19} but involved genes are not identified yet as well as molecular mechanisms by which these changes actively induce or repress gene expression in normal and malignant cells.²⁰

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Among the numerous cell surface and extracellular matrix (ECM) molecules potentially involved in fibroblast-cancer cell interaction, proteoglycans and junction molecules as well as protein ECM components (collagens, fibronectin, elastin) look most perspective candidates responsible for normal or pathological cell-cell interactions.

Proteoglycans (PGs) are complex molecules consisting of protein core and covalently attached polysaccharide chains of glycosaminoglycans (GAG) which are present in every mammalian tissue.²¹ PGs are abundant at cell surface and extracellular matrix in any tissue and both core protein and polysaccharide chains contribute to functional activity of the molecules.^{22,23} Progressive changes in proteoglycans occur both in prostate cancer cells and tumor microenvironment, but neither the source nor consequences of those changes are well understood.²⁴ As to individual PGs in prostate carcinogenesis, an important functional role is shown for syndecan-1,²⁵⁻³⁰ syndecan-2,²⁹⁻³¹ perlecan,³²⁻³⁴ decorin,^{35,36} lumican,³⁷ versican,³⁸⁻⁴⁰ CD44,⁴¹ glypican-1.⁴²

Other key players responsible for intercellular contacts and communication are junctions pathways. Among them, adherens junction and tight junction are the most studied.

Adherens junctions are protein complexes in which cadherin adhesion receptors connect the actin cytoskeletons of neighboring animal cells mediating cell-cell adhesion and providing the tissue with mechanical continuity and barrier function.⁴³ Perturbations with their main components (cadherins, NOTCH1-4, PVRL1-3) affects expression of numerous downstream genes and is associated with aggressive phenotype of prostate cancer cells.⁴⁴⁻⁴⁶

Tight junctions represent the closely associated areas of 2 cells whose membranes join together via special transmembrane proteins (claudins, occludins). They help to maintain the polarity of cells by preventing the lateral diffusion of integral membrane proteins between the apical and lateral/basal surfaces, prevent the passage of molecules and ions through the space between plasma membranes of adjacent cells and precisely control the transcellular transport. Tight junctions has a vital role in maintaining cell to cell integrity and the loss of cohesion of the structure can lead to invasion and metastasis of cancer cells.^{47,48} Structure and function of tight junctions are controlled by the Rho GTPase family⁴⁹ and CLDN3 (claudin 3) was identified as potential diagnostic and prognostic biomarker for prostate cancer.⁵⁰

Overall, the extracellular molecules and cell surface junction molecules are considered as promising therapeutic targets in the treatment of prostate tumors.^{21,51,52}

A main aim of the work was to perform a complex analysis of the main extracellular and cell surface molecules involved in direct cell-cell and cell-matrix

interactions (collagens, fibronectin, proteoglycans and cell adhesion molecules) into crosstalk between fibroblasts and normal and cancer prostate epithelial cells.

Results

Fibroblasts inhibit proliferation of normal and cancer prostate epithelial cells in coculture system *in vitro*

Coculture model was used to study crosstalk between human fibroblasts and normal or cancer prostate epithelial cells *in vitro*. In the model, human recombinant telomerase-transfected immortalized human fibroblasts (BJhTERT) were cocultured with normal prostate epithelial cells (PNT2) and prostate cancer cell lines with different tumorigenic properties (hormone-dependent non-metastatic LNCaP cells and hormone-independent metastatic PC3 and DU145 cells) (Fig. 1). Upon coculture, different modes of interaction with fibroblasts were observed for normal (PNT2) and cancer (LNCaP, PC3, DU145) cells (Fig. 1A, B). No confrontation was observed between fibroblasts and normal cells, which formed tight intercellular contacts. However, adhesion of epithelial cancer cells to the surface was evidently impaired especially at the points of epithelial cell-fibroblast contacts.

The effect could be accounted for the decreased proliferation activity of PC3 cells reported earlier,²² so proliferation rates of the normal and different cancer epithelial cells before and after coculture were determined using CyQuant proliferation assay (Fig. 1B). Fibroblast presence decreased the proliferation rates of normal PNT2 epithelial cells and 2 of 3 cancer cell lines under the study (LNCaP and PC3). Surprisingly, the most aggressive PC3 and DU145 cancer cell lines differentially responded to coculture with fibroblasts. The fibroblasts inhibited the proliferation of PC3 cells but were not able to decrease the proliferation rate of DU145 cells *in vitro*.

Prostate cancer cells lose the ability to respond to fibroblasts in terms of proteoglycan and ECM proteins expression

As shown previously for BJhTERT-PC3 cells confrontation,²² both soluble factors secreted by PC3-confronted fibroblasts and cell surface/ECM molecules (responsible for contact-dependent inhibition of tumor cell proliferation) contribute to inhibition of tumor cell proliferation upon confrontation. To identify important genes that might be involved in the control of prostate epithelial cells proliferation by BJhTERT fibroblasts, the expression of key cell surface and cell/tumor microenvironment

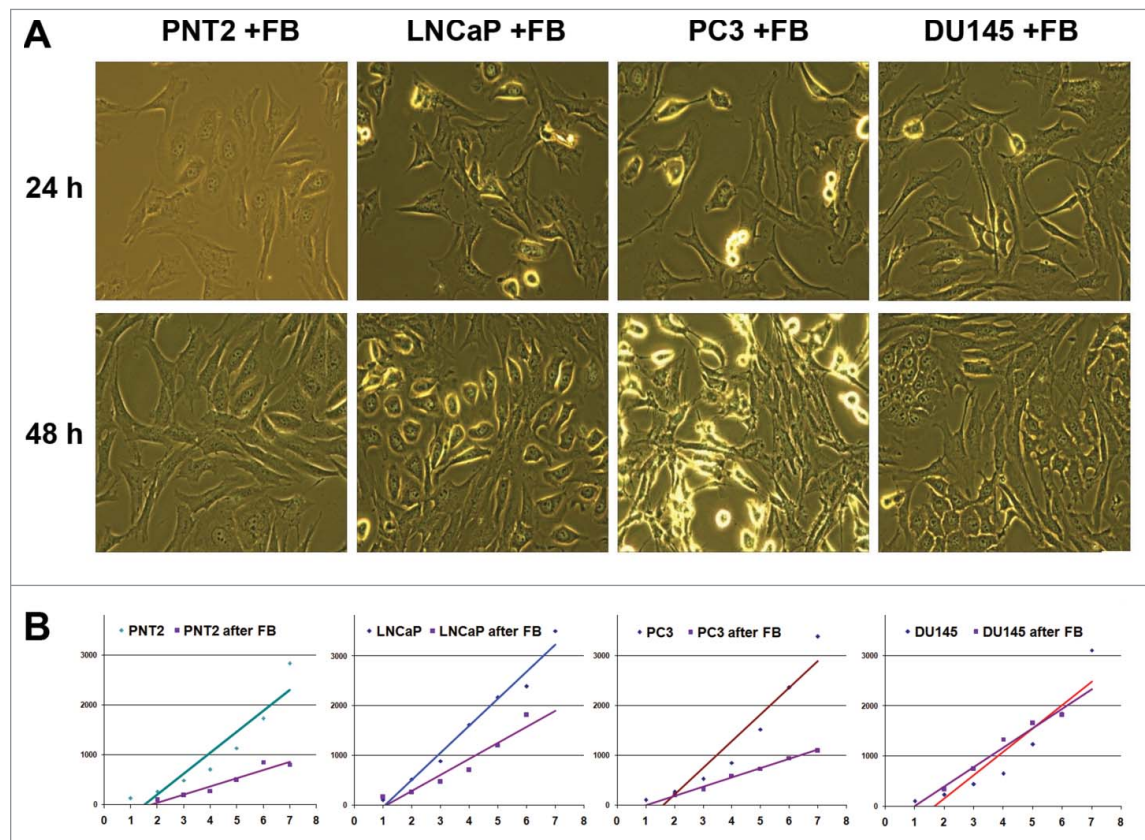


Figure 1. Effects of BJhTERT fibroblasts on proliferation of normal and cancer prostate epithelial cells *in vitro*. (A) Morphology of cocultures at day 1 and day 2 (light microscopy), magnification $\times 200$. (B) Proliferation rates of normal (PNT2) and cancer epithelial prostate cells before and after coculture with fibroblasts (CyQUANT NF Cell proliferation assay).

macromolecules (collagen, fibronectin, proteoglycans) was investigated in the prostate cell lines before and after coculture with BJhTERT by real-time RT-PCR analysis (Fig. 2). To analyze an overall ability of the studied cells to express a pool of different PG core proteins, the obtained results were presented as a conventional parameter which compose of a sum of expression levels for all the proteoglycans under the study.

For PNT2 cells, coculture with fibroblasts resulted in re-organization of the transcriptional activity of main microenvironment-coding genes - decrease of the overall transcriptional activity of PGs (mainly, due to NG2) and increase of collagen 1A1 and fibronectin expression (Fig. 2A and B). Similar effects were observed after culturing of PNT2 in fibroblast conditioned medium (Fig. 2A), although patterns of the expressed proteoglycans were slightly different supporting both direct cell-cell contacts and soluble factors as necessary contributors to the microenvironmental regulation of PG expression in epithelial cells.

As to prostate cancer epithelial cell lines, they demonstrated 2–3-fold decreased transcriptional activity for PG genes compared to normal PNT2 cells and did not respond significantly to coculture with BJhTERT

(Fig. 2A). Expression levels for protein ECM components (collagen, fibronectin, elastin) in the prostate cancer epithelial cells were similar to normal PNT2 cells when cultivated as monoculture but differentially affected by BJhTERT fibroblasts (Fig. 2B). Hormone-dependent non-metastatic LNCaP cells looked more similar to the normal PNT2 cells, responding to the fibroblast coculture by the significant increasing of ECM components expression although PGs expression was unaffected. In contrast, hormone-independent non-metastatic PC3 and DU145 cells were almost irresponsive to BJhTERT fibroblasts in terms of microenvironment-related genes expression in spite of different basic transcriptional activities of the PG- and ECM-coding genes. Possibly, retained expression of PGs and collagen 1A1 in PC3 cells could be related to their ability still to respond to BJhTERT fibroblasts by the decreasing of their cell proliferation rate, whereas DU145 cells almost did not express PGs and ECM components and did not respond to fibroblasts in terms of cell proliferation rate (Fig. 1B). The results support an important instructive role of fibroblasts in modulation of transcriptional activities of microenvironmental genes in surrounding epithelial cells.



Figure 2. Fibroblast coculture effect on normal and cancer prostate epithelial cells. Changes in expression of proteoglycans (A) and ECM components (B) in normal and cancer prostate cells upon coculture with fibroblasts. Intensity of the amplified DNA fragments normalized to that of *GAPDH*.

Fibroblasts reveal heterogeneous changes in proteoglycan expression coordinated with proliferation rate inhibition upon coculture with different cancer cells lines

The next intriguing question was whether prostate epithelial cells (normal or cancer) affect fibroblasts in terms of their proliferation rate and ability to express key cell surface molecules and microenvironmental components (Fig. 3).

It was shown that coculture of fibroblasts with normal PNT2 epithelial cells resulted in significant inhibition of their proliferation and re-organization of transcriptional activity of the studied genes (collagen, fibronectin and proteoglycans) toward a more active production of protein ECM components. Significant activation of fibronectin and collagen 1A1 expression and 2-fold downregulation of proteoglycans (especially CSPG4/NG2) resulted in completely different expression pattern of the microenvironmental genes in PNT2-exposed BJhTERT fibroblasts compared with BjTERT in monoculture (Fig. 3A and B). The observed changes correspond the functional role of fibroblasts in normal tissue stroma as key producers of protein and glycan ECM components to create appropriate microenvironment for other cell types. The ability of fibroblasts to activate collagen and fibronectin expression and fine-tune proteoglycans expression in response to epithelial cells presence seems

to provide a precise self-regulating system for creation of the optimal microenvironment for the cells and proper organization of normal tissue. Do inability of fibroblasts to properly respond to surrounding cells in terms of ECM production could be a key point of transformation of normal microenvironment into TME.

All studied prostate cancer cell lines affected the fibroblast proliferation rate and ability to express collagen 1A1 and fibronectin in the similar manner as normal cells, whereas their effects to the proteoglycan expression were various. The common trend was the decrease of the variety of the proteoglycans expressed by fibroblast - inactivation of glypican-1 expression upon coculture with LNCaP cells, and glypican-1 and versican after PC3 cells. Another hormone-independent metastatic DU145 cell line demonstrated additionally 2–4-fold downregulation of syndecan-1 and NG2 (Fig. 3A).

To test the effect at the protein level, immunostaining of the co-cultured cells for main PGs (members of the small leucine-rich proteoglycan (SLRP) family decorin and lumican, and cell surface heparansulfate proteoglycans syndecan-1 and glypican-1) was performed (Fig. 4). The presented results support transcriptional data on reorganization of PGs expression both in normal prostate epithelial PNT2 cells and BJhTERT fibroblasts upon their coculture. The changes were more pronounced in PNT2 cells where significant down-regulation of decorin and up-regulation of syndecan-1 and lumican occur

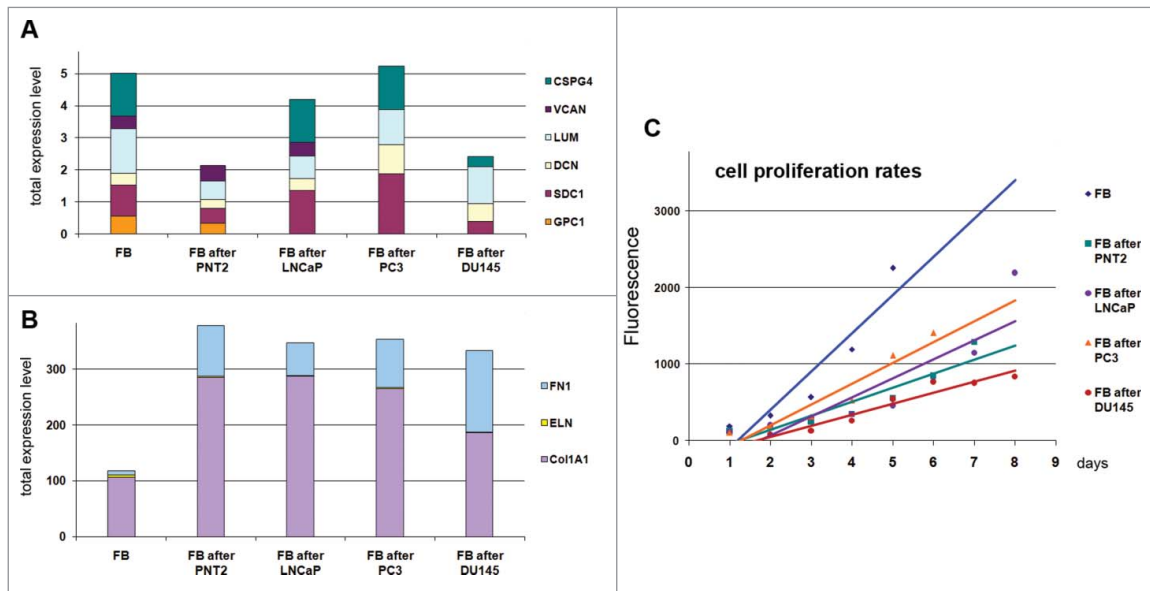


Figure 3. Effects of normal and cancer prostate epithelial cells to TERT-immortalized fibroblasts. (A) Proteoglycan expression. (B) ECM components expression. (C) Changes in fibroblast proliferation rate upon coculture with prostate epithelial cells.

(Fig. 4A and B). In their turn, BJhTERT fibroblasts were characterized by moderate redistribution of decorin and increase of syndecan-1 over their cell surface.

In contrast to PNT2 cells, neither LNCaP and PC3 prostate cancer cells nor BJhTERT did not demonstrated

evident changes in decorin, lumican, syndecan-1 and glypican-1 expression upon coculture. Surprisingly, only aggressive prostate cancer DU145 cells responded to the BJhTERT coculture by activation of syndecan-1 expression both in themselves and surrounding fibroblasts

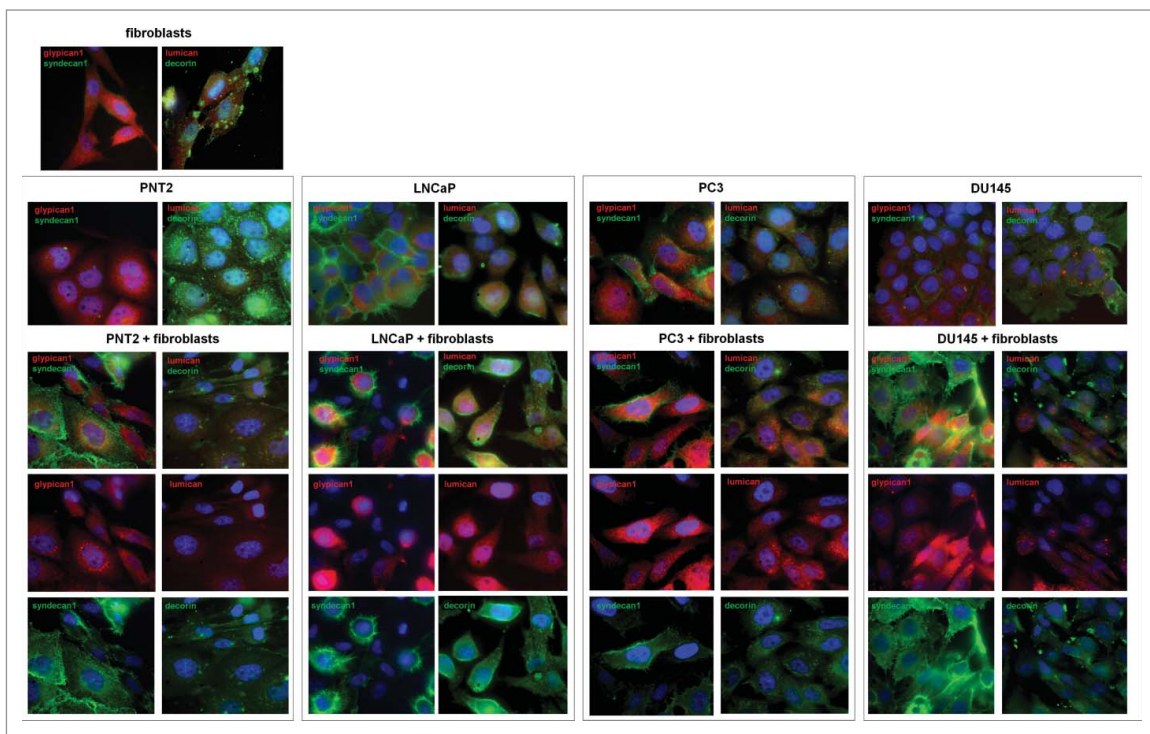


Figure 4. Immunocytochemical analysis of proteoglycans expression in fibroblasts (FB) and normal (PNT2) and cancer prostate cells (LNCaP, PC3, DU145) before and after coculture with fibroblasts. Decorin (green), lumican (red), syndecan-1 (green), glypican-1 (red). Nuclear counterstain - DAPI (blue).

(Fig. 4B). The results have something in common with similar morphology of the cocultures (Fig. 1A) and effects of PNT2 and DU145 to proteoglycan expression in BJhTERT fibroblasts (Fig. 3A) and BJhTERT proliferation rate (Fig. 3C). Possibly, retaining a specific proteoglycan expression pattern at their surface, DU145 cells escape a microenvironmental control (through some unknown molecular mechanisms related to cell-cell and cell-matrix interaction and recognition) and propagate and metastasize *in vivo* in spite of the not so high proliferation activity *in vitro*.

Cancer cells demonstrate increased expression of junction-related molecules and inhibit that in surrounding fibroblasts

Another interesting observation is related to the weak intercellular contacts between prostate cancer cells and BJhTERT fibroblasts upon their confrontation arguing for the disruption some important pathways for their communication. To study the matter further, we investigated a panel of genes involved in different types of intercellular contacts (PCR Array Human Cell Junction Pathway Finder array) in normal and cancer prostate epithelial cells and BJhTERT fibroblasts before and after coculture (Fig. 5, Table 1, Table S2).

Human Cell Junction Pathway Finder array let to determine the expression levels of 84 genes involved in different types of cell-cell and cell-matrix contacts (focal adhesions, tight junctions, gap junctions, adherens junctions, desmosomes, hemidesmosomes). Normal prostate epithelial PNT2 cells did not show significant changes in the transcriptional activity of the genes upon coculture with BJhTERT fibroblasts being consistent with the observation about no confrontation between the normal prostate epithelial cells and BJhTERT (Fig. 1A).

However, coculture of prostate cancer PC3 cells with BJhTERT resulted in upregulation of different junction-related genes in PC3 cells (especially Focal Adhesions, Tight Junctions and Adherens Junctions) (Fig. 5, Table 1).

In parallel, similar experiments were performed to study expression of junctions-related genes in BJhTERT fibroblasts before and after coculture with normal or cancer prostate cells (Fig. 6, Table S1). The obtained results showed that BJhTERT cells responded to the coculture with normal (PNT2) or cancer (PC3) prostate cells in a different manner. PNT2-exposed BJhTERT cells demonstrated increased transcriptional activity of the genes involved in different types of cell-cell contacts (Fig. 6A), among which focal adhesions (CAV1, ITGA3, ITGA5, ITGB1, ITGB5), adherens junctions (CDH1, CDH2, Notch2, PVRL2, PVRL3) and tight junctions

(Cldn1, Cldn4, Cldn12, F11R, ICAM1, JAM3, OCLN, TJP1, TJP2) were the main up-regulated pathways (Fig. 6B). On the contrary, PC3-confronted BJhTERT were characterized by selective inhibition of focal adhesion genes and re-organization of transcriptional patterns of adherens junctions and tight junctions-related genes with a common tendency to their downregulation (Fig. 6A, B). As the result, PC3-confronted fibroblasts demonstrate significantly decreased expression levels of the genes involved in different types of cell-cell contacts (especially focal adhesions and adherens junctions) compared with fibroblasts cocultured with normal PNT2 cells (Fig. 6A, right panel). Transcriptional inhibition of the junction pathways along with the down-regulation of some proteoglycans (glypican-1, versican) (Fig. 2) suppose that the ability of PC3-confronted fibroblasts to establish proper intercellular contacts with surrounding cancer cells and regulate their proliferation rate could be compromised. Taken together with significant up-regulation of the known CAF marker α -smooth muscle actin (SMA) in PC3-confronted fibroblasts (Fig. 6C), the obtained data suggest transcriptional deregulation of junction proteins and proteoglycans as a potential molecular mechanism contributing to reprogramming of normal fibroblast to cancer-associated fibroblast during malignant transformation.

Discussion

From the obtained results, we suggested a possible scheme for interference of human BjTERT fibroblasts and normal or cancer prostate epithelial cells (Fig. 7).

Normal epithelial PNT2 cells and BjTERT fibroblasts establish tight heterotypic intercellular contacts (with no signs of confrontation) upon coculture in the experimental model *in vitro*. Complex changes in the expression of key contact-related ECM and cell surface molecules (COL1A1, FN1, PGs, junctions molecules) occur both in PNT2 cells and fibroblasts, and could be interpreted as a necessary adaptation of the cells to coexistence. Unfortunately, there are almost no literature data on normal prostate epithelial cell-fibroblast interactions to compare with. Anyway, this is a very important point to start with because it allows to outline the described expression changes as a normal physiological reaction to coculture and perform subsequent comparative analysis with prostate cancer cell-fibroblast interactions.

Prostate cancer cell lines (LNCaP, PC3, DU145) demonstrate completely different behavior in their interactions with BjTERT fibroblasts compared with normal PNT2 cells. Cancer cells seem to be independent from the regulating signals from fibroblasts in terms of proteoglycan and protein ECM components expression (Fig. 2)

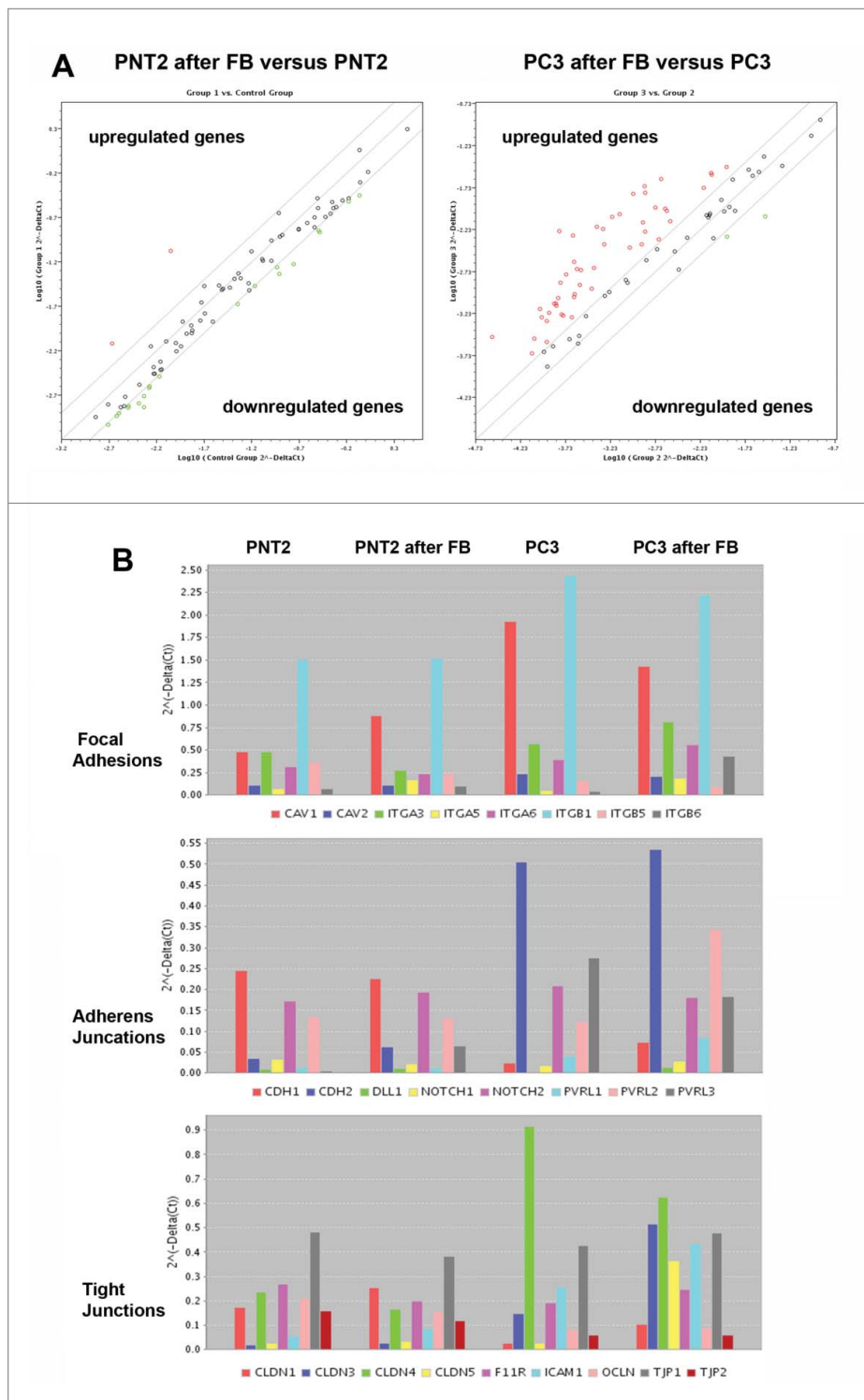


Figure 5. RT² ProfilerTM PCR Array Human Cell Junction Pathway Finder analysis of normal and cancer prostate epithelial cells before and after coculture with BJhTERT fibroblast. (A) The relative expression levels for each gene in PNT2 cells (Group 1) or PC3 cells (Group 3) after co-culture with fibroblasts are plotted against the same gene from the control PNT2 cells or PC3 cells (Control Group or Group 2, respectively). The middle line shows the similar expression in both groups with 2-fold change boundaries. Genes up-regulated >2-fold lie above the middle line and the down-regulated genes lie below the line. (B) Gene expression levels for PNT2 cells (Control group), BJhTERT-exposed PNT2 cells (Group 1), PC3 cells (Group 2) and BJhTERT-exposed PC3 cells (Group 3) (RT² ProfilerTM PCR Array Data Analysis Program version 3.5).

Table 1. Changes in the expression of genes relevant to the Cell Junction pathway. Listed up- and down-regulated genes in the Test Sample (after coculture) divided by the normalized gene expression in the Control Sample (before coculture). Two-fold change is taken as significant.

Cells	Genes Over-Expressed	Genes Under-Expressed
FB after PNT2	CAV1,CAV2,CAV3,CDH1,CDH2,CLDN1 CLDN11,CLDN12,CLDN4,CLDN7,CLDN9 DLL1,DSC2,DSC3,DSG2,DSP,F11R,GJA1 GJA5,GJB1,GJB3,GJB4,GJB5,GJC2,ICAM1 ICAM2,ITGA1,ITGA4,ITGA5,ITGAV,ITGB1 ITGB3,ITGB4,ITGB5,ITGB6,JAM2,JUP,NOTCH1 NOTCH2,OCLN,PVRL2,PVRL3,TJP1,TJP3	
PNT2 after FB FB after PC3	GJA1,ITGA5,JAM3,PVRL3 CDH1,CDH2,CLDN1,CLDN14,CLDN3,CLDN4 DSC2,DSC3,DSG2,ESAM,F11R,GJA1,GJB3 ICAM1,ITGB2,ITGB4,JUP,OCLN,PVRL1,TJP1	GJC3 CAV1,CLDN10,CLDN11,CLDN16,CLDN17 CLDN18,CLDN2,CLDN6,DLL1,DSC1,DSG1 DSG3,DSG4,DST,GJA4,GJA5,GJA8,GJB6 GJD2,GJC3,ITGA2,ITGA4,ITGA7,ITGA8 ITGA9,JAM2,JAM3,PVRL3,TJP2,TJP3
PC3 after FB	CAV3,CDH1,CLDN1,CLDN10,CLDN11,CLDN17 CLDN19,CLDN3,CLDN5,CLDN6,CLDN7,CLDN8 CLDN9,DLL1,DSC1,DSG1,DSG3,DSG4,DSP GJA1,GJA3,GJA4,GJA5,GJA8,GJB1,GJB2,GJB3 GJB4,GJD2,ICAM2,ITGA1,ITGA2,ITGA4,ITGA5 ITGA7,ITGA8,ITGA9,ITGAL,ITGAM,ITGB2 ITGB3,ITGB6,JAM2,JAM3,JUP,NOTCH3 PVRL1,PVRL2,TJP3,HGDC	DSG2,ITGB4

and fail to establish direct heterotypic intercellular contacts (LNCaP, PC3) (Fig. 1A). At the same time, cancer cells possess an ability to affect surrounding fibroblasts, different cancer cells lines induce heterogeneous changes in proteoglycan expression in cancer cell-exposed fibroblasts which correlate with the fibroblasts proliferation rate (Fig. 3A and C). Expression of junction molecules is simultaneously downregulated in BjTERT and upregulated in prostate cancer PC3 cells suggesting higher propensity of the cancer cells to aggregation and clonal growth with no or minimal involvement of BjTERT. The observation has something general with recently proposed 3D model for analysis of cancer cells aggregation which suggest that tumorigenesis *in vitro* is a developmental process involving coalescence of cancer cells in 3D facilitated by specialized cells (named “facilitators” and “probes”) that culminates in large hollow spheres with complex architecture.⁶⁶

All the described effects result in completely different structure of fibroblast interactions with normal or cancer epithelial cells, where do failure to respond to stromal fibroblasts by physiological reorganization of expression of cell-cell contact-related molecules and establishment of heterotypic contacts could be a key point.

In literature, there are scattered data on expression changes for individual proteoglycans, protein ECM components or junctions molecules in prostate cancer cell-fibroblast model systems *in vitro*. It was shown that prostate tumor cells (LNCaP, PC3, DU145) induce fibroblasts to secrete increased versican levels via a paracrine

mechanism mediated by transforming growth factor beta1;⁵³ metastatic cancer cells PC3 and DU145 create a metastatic niche by altering the phenotype of local fibroblasts, leading to changes in the ECM (significant down-regulation of collagens I, II, III, IV, decorin, biglycan, lumican, fibromodulin, TGF- β and upregulation of vimentin, $\alpha 5\beta 1$ integrin, MT1-MMP).⁵⁴ Cocultivation with PC3 cells make the cancerous and hyperplastic fibroblasts more alike each other (only 26 differentially expressed genes vs. 383 differentially expressed genes between fibroblasts from cancerous and hyperplasia areas before cocultivation with PC-3 cells);⁵⁵ PC3 conditioned media increase α -smooth muscle actin (α -SMA) and vimentin expression in human prostate fibroblasts, and their differentiation into CAF-like phenotype through the TGF $\beta 2$ -dependent mechanism.⁵⁶ Transformation of normal fibroblasts into CAFs seems occur in a heterogeneous manner and might be strongly dependent on biochemical characteristics of adjacent cancer cells.⁵⁷ All the data support our results on the ability of prostate cancer cells to affect stromal fibroblasts and transform them into a myofibroblastic phenotype similar to that found in CAFs.

Together with the induced morphological changes of fibroblasts, all the prostate cancer cell lines (LNCaP, PC3, DU145) decrease fibroblast proliferation rates in our coculture system *in vitro*, and the published data to compare with are controversial. It was shown that conditioned media from the cancer cell lines stimulate fibroblast proliferation⁵⁸ possibly via down-regulation of cell adhesion, cell-cell contact, and cell cycle regulation

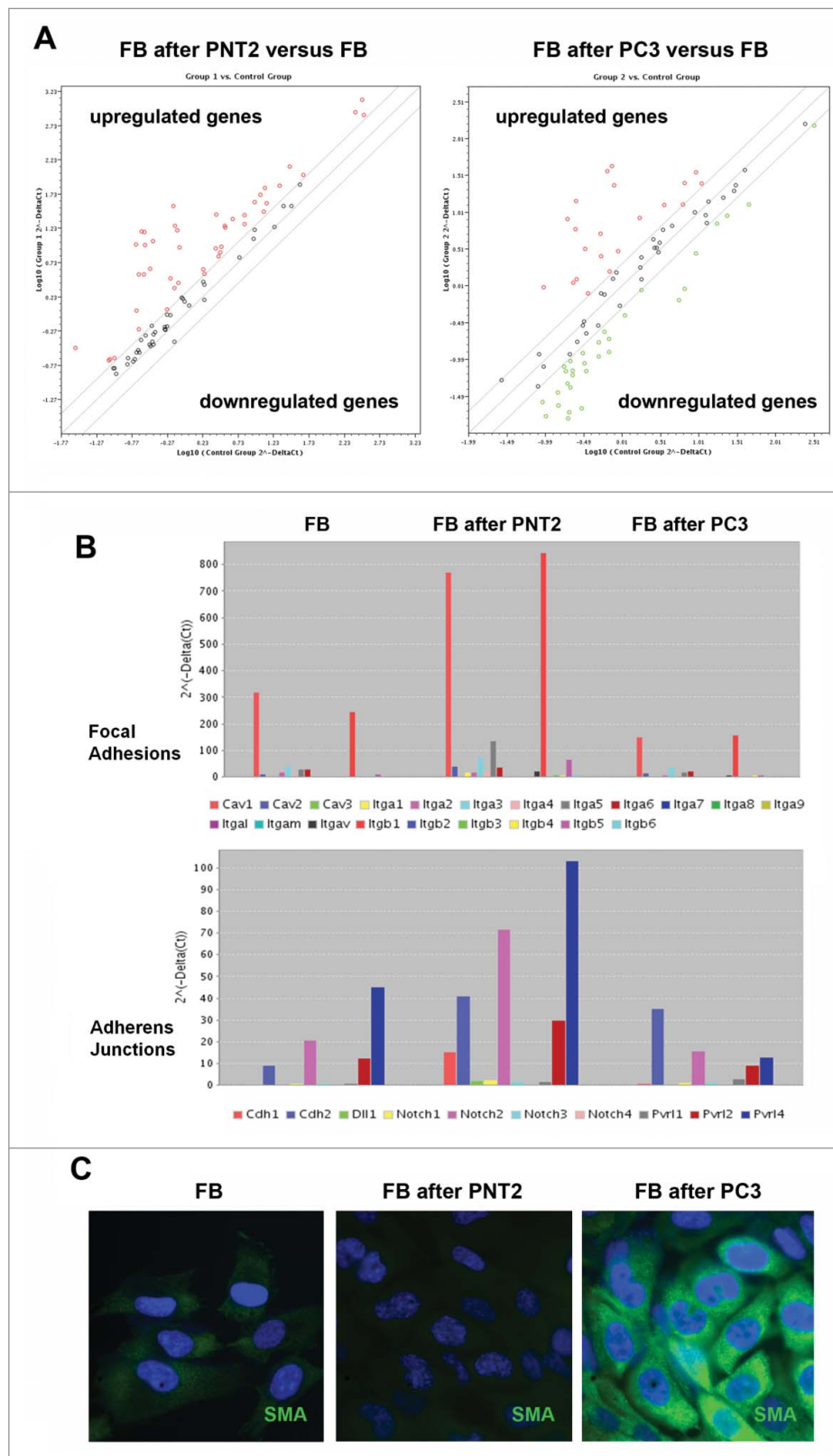


Figure 6. Expression profiling of cell junction-related genes of BJhTERT fibroblasts before and after coculture with normal and cancer prostate epithelial cells. (A) RT² ProfilerTM PCR Array Human Cell Junction Pathway Finder analysis. The relative expression levels for each gene in fibroblasts after co-culture with PNT2 cells (Group 1) or PC3 cells (Group 2) are plotted against the same gene from the control fibroblasts (Control Group). The middle line shows the similar expression in both groups with 2-fold change boundaries. Genes upregulated >2-fold lie above the middle line and the downregulated genes lie below the line. (B) Gene expression levels for fibroblasts, PNT2- and PC3-confronted fibroblasts for junctions pathways with highest expression levels. (C) Immunostaining for α -smooth muscle actin (SMA) (green). Nuclear counterstain - DAPI (blue).

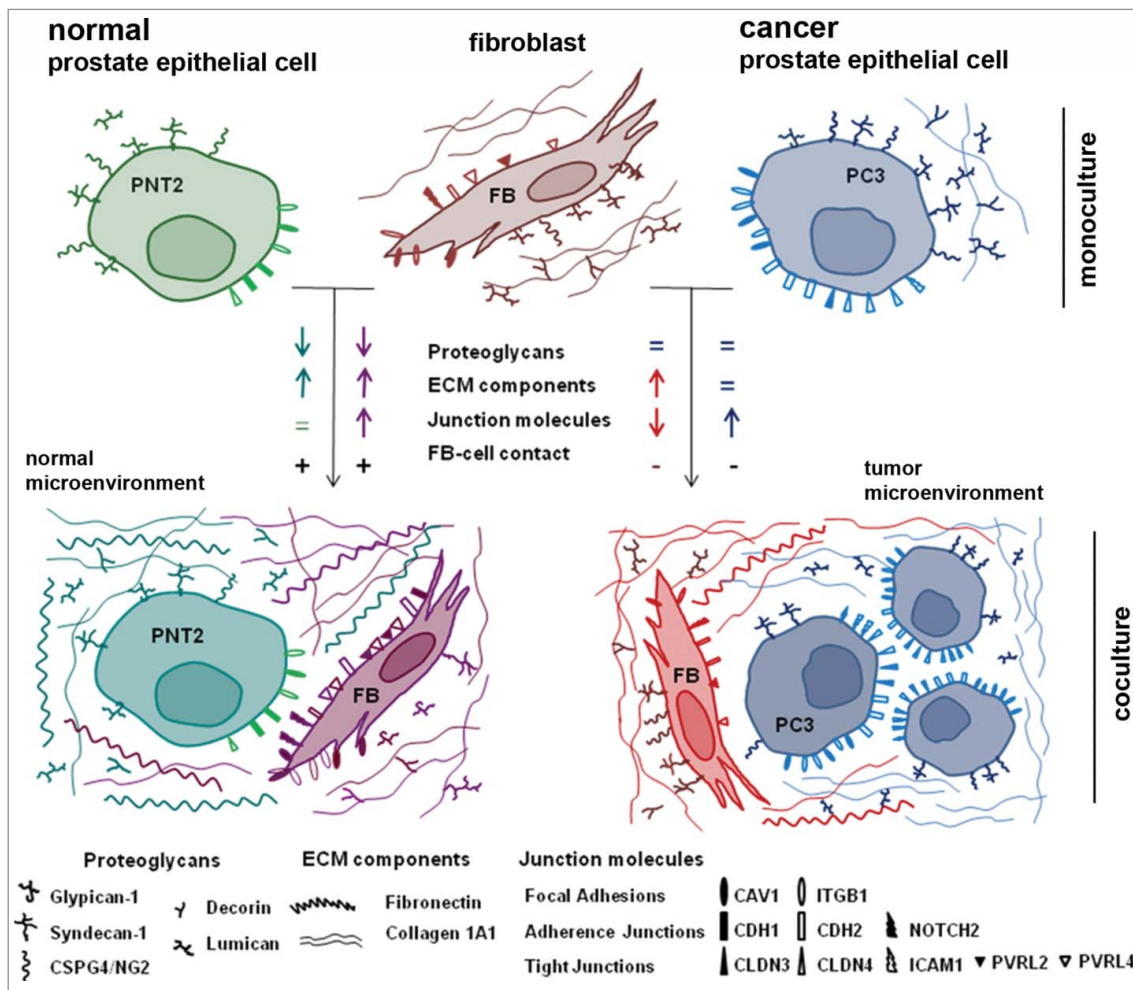


Figure 7. Schematic representation of a running hypothesis for fibroblast – prostate epithelial cells interactions.

proteins⁵⁹ or inhibit it⁶⁰ and the matter needs further investigation.

As to an ability of stromal fibroblasts to affect prostate cancer cells, there are much less data concerning cell-cell and cell-ECM contact molecules. It is shown that gingival fibroblasts do not express E-cadherin, while prostate cancer cells LNCaP, PC3, DU145 demonstrate differential E-cadherin staining and coculture of PC3 cells with fibroblasts results in a reduction in the nuclear E-cadherin reactivity in fibroblast-exposed PC3 cells.⁶⁰ From the other side, P-cadherin but not E-cadherin was identified as important component for maintaining adherens junctions in DU145 cells, and depletion of any of the cadherin-associated proteins (p120ctn, β -catenin or α -catenin) was sufficient to disrupt adherens junctions in DU145 cells and increase migration and cancer cell invasion.⁶¹

Presented results on the ability of BjTERT fibroblasts to differentially affect proliferation rate of different prostate cancer cell lines upon coculture need to be analyzed in context of already published data. According

Kaminski et al., fibroblast conditioned medium enhance proliferation and anchorage-independent growth of DU145 cells and increase migration of PC3 cells,⁵⁸ fibroblasts of different origin and their conditioned media stimulate proliferation of normal or malignant prostate epithelial cell, while fixed fibroblast monolayers and extracellular matrix prepared from fibroblast cultures failed to stimulate prostatic epithelial growth.⁶² In our previous work, we showed that primary human fibroblasts inhibit proliferation of 6 human cancer cell lines¹⁸ and ECM/other surface proteins of the fibroblasts seems to be involved in contact-dependent inhibition of tumor cell proliferation.¹⁹ Possibly, the apparent contradictions depend on the experimental conditions such as an initial ratio of fibroblasts/cancer cells or analysis time-points. For example, it was demonstrated that conditioned medium from gingival fibroblasts inhibit proliferation of LNCaP cells, after 3 days by 33% and after 6 days by up to 82%, but has no effect on the PC3 and DU145 cell growth.⁶⁰ Taking into account long duration of such coculture experiments (up to 6 days), one should keep in

mind a process of potential transformation of the fibroblasts to CAFs during the experiment, which could significantly influence functional interactions between the cells. According Paland et al., whereas normal human fibroblasts inhibit the growth of immortalized prostate epithelial cells but promote the growth of metastatic PC3 cells, CAFs promote the growth of prostate epithelial cells but not of PC3. The results suggest that normal fibroblast cells have a protective function at very early stages of carcinogenesis by preventing immortalized epithelial cells from proliferating whereas CAFs aid immortalized epithelial cells to further develop.⁶³ Finally, they co-evolute with cancer cells and affect every prostate cancer cell line in different ways, which may be because of their different origin.⁵⁹

Our data stay in line with this hypothesis and support heterogeneous mechanisms for interaction of different prostate cancer cell subtypes (LNCaP, PC3, DU145) with BjTERT- fibroblasts.

In co-culture, androgen-sensitive non-metastatic LNCaP cells grow in a network on the top of the monolayer formed by fibroblasts and both cell types undergo morphological changes, while colonies of androgen-insensitive metastatic PC3 and DU145 cells were surrounded by fibroblasts.⁶⁰ They respond to prostate fibroblasts by alterations in their cytogenetic and biologic profiles in xenograft model *in vivo*.⁶⁴

Androgen-independent metastatic cell lines PC3 and DU145 demonstrate relative independence from the regulating signals from fibroblasts and molecular mechanisms of the effect seem to be different. Among them, DU145 cell line is the most intriguing, and paradoxical combination of different physiological and molecular parameters of DU145 cells takes a special attention to the cell line. DU145 cells look as a least sensitive to regulating effect by fibroblasts (Figs. 1 and 2) but are the most potent in their effects to surrounding fibroblasts among the studied prostate cancer cell lines (Fig. 3A and C) underlying their aggressive metastatic nature. Coculture with fibroblasts do not change DU145 proliferation rate (Fig. 1) and fibroblast conditioned medium results in an enhanced proliferation and anchorage-independent growth of this cell line in soft agar.⁵⁹ At the same time, the cells demonstrate a lowest proliferation rate similar to that of PNT2 and retain the ability to respond to BjTERT by activation of syndecan-1 at their surface (Fig. 4). These observations suggest that do combination of evident oncogenic properties with unchanged cell surface architecture could be a favorable feature for successful dissemination of such cancer cells. Interestingly, a similar paradox was described for breast cancer cell subtype with high hyaluronan (HA) binding capacity. The HA(high) subpopulation exhibit lower

proliferation activity along with significantly higher local invasion and lung micrometastases than either unsorted parental cells or the HA(-/low) subpopulation. The results revealed a previously undetected form of heterogeneity that predicts invasive/metastatic behavior.⁶⁵ Possibly, DU145 could be an appropriate model to study molecular mechanisms of prostate cancer metastasis further.

Collectively, the obtained results suppose that

- decrease of the proliferation rate and increase of ECM components expression could be a common reaction of fibroblasts to the presence of normal or cancer epithelial cells;
- transcriptional patterns of junction-related molecules are associated with malignant transformation of cell and homotypic/heterotypic intercellular contacts;
- normal or cancer epithelial cells specifically re-program PG expression in fibroblasts to provide themselves with a suitable microenvironment.

We hypothesized that proteoglycan patterns seem to be peculiar “fingerprints” in cell-cell communication and specific “sensors” to dynamically react into their microenvironment. However, a functional contribution of their protein cores and polysaccharide chains into this process is still unknown what can be a future step in the investigation of their involvement in cell-cell and cell-matrix interactions in normal or cancer tissues.

In summary, the presented data suggest deregulation of proteoglycans and junction molecules in prostate cancer cells and nearby fibroblasts as a potential molecular mechanism for autonomous growth of cancer cells in their microenvironment. The ability of different prostate cancer cell sub-types differentially affect their microenvironment lies within the context of our knowledge of the heterogenic structure of prostate tumors and contribute to the understanding of the biology of prostate cancer.

Materials and methods

Materials and antibodies

Mouse monoclonal anti-human Syndecan-1, rabbit polyclonal anti-human Glypican-1, mouse monoclonal anti-human β -actin antibodies (Abcam, UK); rabbit polyclonal anti-human Lumican, mouse monoclonal anti-human Decorin, mouse monoclonal anti-human HS antibodies (Abnova, USA); mouse monoclonal anti-human CS antibody (Sigma, USA), FITC-conjugated antibody against mouse IgGs, TexasRed-conjugated antibody against rabbit IgGs (Vector Laboratories, USA). Prolong Gold antifade reagent with DAPI (ThermoFisher Scientific, USA).

Cell lines and coculture assay

The human TERT-immortalized fibroblasts (BjTERT) and prostate cancer cell lines LNCaP, PC3, DU145 were obtained from MTC (Karolinska Institute, Stockholm, Sweden). The normal human prostate epithelial cell line PNT2 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). All cell lines were maintained in RPMI medium supplemented with 2 mM L-glutamine, 100 units penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% (v/v) fetal bovine serum at 37°C in a humidified 5% CO₂ incubator. Cells were harvested for analysis using trypsin/EDTA. For coculture experiments, fibroblasts and prostate epithelial cells were seeded with 1:1 ratio with 25–30% confluency at the start point and separated after 72h of incubation.

Magnetic separation of different cell types upon coculture

After coculture, epithelial cells and BjTERT fibroblasts were separated on OctoMACS Separator using MS Columns and Anti-Fibroblast MicroBeads human (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, cell suspension was mixed with Anti-Fibroblast MicroBeads conjugated to monoclonal mouse anti-fibroblast human antibodies and incubated for 30 minutes at room temperature. Cells suspension was applied onto the MACS column and flow-through, containing unlabeled prostate epithelial cells was collected. Magnetically labeled fibroblasts were eluted from the MACS column using elution buffer.

Total RNA isolation and reverse transcription

Total RNA was extracted from the cells using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The integrity and quality of the isolated RNA were checked by agarose gel electrophoresis and total RNA concentration was measured with Qubit-iT RNA Assays Kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. cDNA was synthesized from 1–2 μg of total RNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, USA), and 1/10th of the product was subjected to PCR analysis.

Analysis of ECM molecules and proteoglycan genes expression by Real-Time PCR

Real-time PCR was performed using the CFX96 Real-Time PCR Detection System (BioRad, USA) under the following conditions: 95°C for 3 min, 95°C for 20 sec, 59°C for 15 sec and 72°C for 50 sec. The total reaction volume was 25 μl . *GAPDH* was used as the housekeeping

gene. The PCR primers and conditions used are listed in Table S1.

In vitro cell proliferation assay

Cell proliferation rate was determined using the CyQUANT NF Cell Proliferation Assay (ThermoFisher Scientific, USA) according to the manufacturer's protocol. Briefly, cells were plated in a 96-well microplate at densities of 100–500 per well (8–12 identical wells in total) and the DNA content of the wells was measured every 24 h. This was achieved by removing the medium and adding 50 μl of fluorescent dye followed by incubation for 30 min at 37°C. The fluorescence intensity of each sample was measured at 485/530 nm using fluorescence microplate reader (SPECTRA max, Molecular Devices, Sunnyvale, CA, USA).

Human Cell Junctions PathwayFinder RT² Profiler PCR array

The Cancer PathFinder RT² Profiler PCR array (SABioscience, USA) was used to determine changes in the expression of 84 Junctions pathway-focused genes upon TSA treatment in fibroblasts and prostate epithelial cells after their coculture. Briefly, total RNA was isolated using a RNeasy Plus Mini Kit (Qiagen). The RNA concentration was determined using a Quant-iT Assay Kit for RNA quantification (ThermoFisher Scientific, USA) and was verified by electrophoresis. cDNA was synthesized from 1–2 μg of total RNA using a Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoFisher Scientific, USA). Real-Time PCR was performed using an RT² Profiler PCR Array Human Cell Junctions PathwayFinder System (PAHS-213Z) with SYBR Green Fluor q-PCR Master Mix (Qiagen) and an CFX96 Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions. All data were analyzed using Excel-based RT² PCR Array Data Analysis Software (SABioscience, USA). This integrated web-based software package automatically calculates ddCt-based fold changes in genes expression from the uploaded raw threshold cycle data. Each replicate cycle threshold (Ct) was normalized to the average Ct of 5 endogenous controls (B2M, HPRT1, RPL13A, GAPDH and ACTB) on a per plate basis.

Immunocytochemistry

For immunofluorescence analysis, cells were grown on glass coverslips and then fixed with phosphate-buffered 4% formaldehyde. Mouse monoclonal anti-syndecan-1 (Abcam; 1:150), rabbit polyclonal anti-glypican-1 (Abcam;

1:150), mouse monoclonal anti-decorin (Abnova; 1:150), rabbit polyclonal anti-lumican (Abnova; 1:150) were used for immunostaining. Staining patterns were visualized with Alexa 488-conjugated goat anti-mouse IgG (ThermoFisher Scientific; 1:1000) and Alexa 568-conjugated goat anti-rabbit IgG (ThermoFisher Scientific; 1:2000) antibodies. The cells were mounted and counterstained with DAPI using Prolong Gold SlowFade Gold with DAPI mounting medium (ThermoFisher Scientific, USA) and observed by fluorescent microscopy (LEICA DMRE).

Statistical analysis

Statistical analyses were performed using a computer program ORIGIN Pro 8.0; a value of $p < 0.05$ was considered to indicate a statistically significant difference. Data are expressed as the means \pm SEM.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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