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Extracellular matrix secreted by reactive stroma is a main inducer of pro-tumorigenic features on LNCaP prostate cancer cells

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ABSTRACT

Tumor microenvironment modifications are related to the generation of reactive stroma and to critical events in cancer progression, such as proliferation, migration and apoptosis. In order to clarify these cellular interactions mediated by reactive stroma, we investigated the effects of cell–cell contacts, and the influence of soluble factors and extracellular matrix (ECM) secreted by Benign Prostate Hyperplasia (BPH) reactive stroma over LNCaP prostate tumor cells. Using *in vitro* functional assays, we demonstrated that ECM strongly stimulated LNCaP cell proliferation and migration, while inhibiting apoptosis, and inducing a deregulated expression pattern of several genes related to prostate cancer (PCa) progression. Conversely, reactive stromal cells *per se* and their secreted conditioned medium partially modulated these

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1. Introduction

Prostate adenocarcinoma is the sixth most common cancer worldwide with prevalence among men [1]. In recent years, it has been demonstrated the importance of tumor microenvironment in many cancer diseases [2,3], and the role of the so-called reactive stroma has been consolidated itself as a key element in the progression of prostate cancer (PCa) [4]. Several studies have shown that reactive stroma is significantly different from non-tumoral stromal compartment [5,6]. The classic works of Olumi et al. [7,8] provided relevant data about the importance of the stroma in PCa progression, demonstrating that formation of prostate tumors in xenograft models was only possible in the presence of stromal cells. Subsequently, it was demonstrated that changes in tissue homeostasis caused by PCa lead to an increase in proliferation and invasion of tumor cells, triggering a response from prostatic stroma similar to that observed in the healing process [9].

Several factors secreted during the healing process are also released during the formation of reactive stroma in response to tumor growth [10]. These factors include Fibroblast Growth Factor (FGF-2) [11], Connective Tissue Growth Factor (CTGF) [12], Vascular Endothelial Growth Factor (VEGF), Platelet Derivated Growth Factor (PDGF) [5] and Transforming Growth Factor (TGF-β) [13]. In particular, TGF-β seems to play a key role in the formation of reactive stroma, triggering phenotypic changes on cells that compose this compartment. Under the influence of these factors, prostatic normal stroma, which was originally composed of a large number of smooth muscle cells, initially characterized by the expression of cytoskeleton proteins, such as desmin and calponin, becomes fibrous with a significant increase in the number of myofibroblasts [14]. This modified phenotype also induce stromal remodeling of the extracellular matrix (ECM), which leads to the release of new growth factors and ECM molecules, including type I and III collagens [15], tenascin-C, versican, [16,17], and metalloproteinases 2 (MMP-2) and 9 (MMP-9) [18], culminating with the formation of a reactive stroma.

Prostate reactive stroma formation is not an exclusive feature of malignant neoplasic growth. Benign Prostatic Hyperplasia (BPH) is characterized by benign prostatic tumor growth and occurs concomitantly with cancer in more than 80% of cases [19,20]. Both

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diseases are marked by the presence of a strong inflammatory process [21], secretion of TGF-β and interleukin 8, further contributing to the formation of a typical reactive stroma [22]. It has also been reported that BPH reactive stroma contributes to LNCaP prostate tumor cell formation *in vivo*, in a similar way as observed in PCa [23]. In the present study, using a co-culture system and *in vitro* functional assays, we investigated the role of different components of BPH reactive stroma over LNCaP tumor cells. Tumor progression features, such as cell proliferation, migration, apoptosis and the expression of malignancy related genes were analyzed. Our data indicated that ECM secreted by BPH reactive stroma was the most prominent element to promote the major alterations and characteristics associated with tumor progression.

2. Materials and methods

2.1. Isolation and characterization of reactive stroma

Transurethral resection fragments of prostate tissues obtained from 3 BPH surgeries were used to obtain the BPH reactive stromal cells. This procedure was approved by the Ethics Committee of Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, and registered with the Protocol-CAAE 0029.0.197.000-05. Fragments of 1–3 mm^3 were grown in 24-well plates containing Dulbeccós Modified Eaglés Medium (DMEM) (Sigma) culture medium supplemented with 10% of Fetal Bovine Serum (FBS) (Hyclone), 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma). The medium was changed every 2 days. After the cell attachment to the bottom of the culture plate, cells were trypsinized and then transferred to 25 mm^2 culture dishes. After six passages, a homogeneous stromal cell population was established. To identify if these stromal cells presented a reactive phenotype, these cells were immunocytochemically characterized for some cytoskeleton markers. Cells were washed twice with PBS and fixed with 4% paraformaldehyde-PBS (Sigma) for 10 min. After fixation, they were washed for additional three times with PBS and then incubated in a solution of 50 nM NH_4Cl (Vetec) for 30 min. The antibody nonspecific binding was blocked with PBS/BSA 5% and then primary antibodies were added over cell monolayers incubated overnight. We used antibodies against vimentin (Sigma), α-smooth muscle actin (Sigma), cytokeratin peptide 18 (Sigma) and desmin (Dako). To identify ECM components secreted by these stromal cells, antibodies against fibronectin (Sigma), laminin (Sigma), type I and III collagens (Novotec), type IV collagen (Sigma) and chondroitin sulfate (Sigma) were used. After incubation with primary antibodies, cells were washed with PBS and incubated for 2 additional hours with either goat anti-mouse Alexa 488 or goat anti-rabbit Alexa 488/546 secondary antibodies (Invitrogen). Cell nuclei were counterstained with DAPI (Santa Cruz Biotechnology). Finally, cells were washed in distilled water and mounted on histological slides with N-propylgallate (Sigma). Images were captured using a confocal microscopy (Olympus IX81) and a Hamamatsu orca^{R2} digital camera.

2.2. LNCaP cell culture

LNCaP cell line was obtained from the American Type Tissue Collection (Manassa, VA) and maintained routinely in RPMI 1640 (Gibco) culture medium supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin, except in control or experimental conditions, in which FBS supplementation was the minimum necessary for the maintenance of cell viability (0.5%), without interfering with the analysis.

2.3. Experimental conditions

The experimental conditions used in all the assays were the following: (a) LNCaP cells cultivated over the established reactive stromal cells, (b) LNCaP cells cultivated in the presence of conditioned medium secreted by reactive stromal cells, (c) LNCaP cells cultivated over the ECM secreted by reactive stromal cells and (d) LNCaP cells cultivated in 0.5% FBS (control condition). For experimental condition (a), reactive stromal cells were cultured in six well plates in complete medium for 72 h. Then, culture medium was removed, cells were washed with balanced saline solution (BSS) and incubated with 5 μM of the vital green fluorescent dye CMFDA (Molecular Probes) for 45 min. After this step, cells were washed and incubated for 1 h in DMEM supplemented with 10% FBS before, being washed again with BSS. After fluorescent dye incorporation by the reactive stromal cells, 25 × 104 LNCaP cells were seeded over them and the new established co-culture was maintained in DMEM with 0.5% FBS for a period of 72 h [7]. After this period, LNCaP cells were separated from stromal cells by flow cytometry (MoFlo, Dako Cytomation), using an argon laser with a wavelength of 488 nm.

In order to obtain the conditioned medium and the ECM secreted by reactive stromal cells mentioned on experimental conditions (b) and (c), respectively, we employed protocols previously established [24]. Briefly, 5 × 104 reactive stromal cells were grown in 24-well plates with complete culture medium for 96 h. Then, medium was removed, cells were washed with BSS and maintained for more 24 h in DMEM with 0.5% FBS. After this period, the supernatant (conditioned medium) was collected, centrifuged (800 g) and finally added to 105 LNCaP cells that were being cultured apart, and cultivated for 72 h. The ECM secreted by reactive stromal cells was obtained culturing 5 × 104 stromal cells in 24-well plate with complete medium for 96 h. After that, the medium was removed, cells were washed with PBS supplemented with calcium chloride (PBS-Ca) and then 300 μL of lysis buffer (PBS-Ca, 20 μM Leupeptin (Sigma), 1 mM PMSF (Sigma), 0.1% Triton X-100 (Sigma) and 0.1 nM Ammonium Hydroxide (Vetec)) was added for 15 min and then washed again tree times with PBS-Ca. This method removes the cells without disrupting the cell matrix. Finally, 105 LNCaP cells were seeded and cultured over this secreted matrix for 72 h. For experimental control condition (d), LNCaP cells were seeded and cultured for 72 h in DMEM supplemented with 0.5% FBS.

2.4. Cell proliferation assays

In order to evaluate cell proliferation, crystal violet assay was employed. Cells were fixed in ethanol for 10 min, then stained with crystal violet 0.05% (Vetec) for 10 min and finally solubilized with methanol. The supernatant was collected and its absorbance was measured in an ELISA reader (BIO-RAD iMARKE) at 595 nm. Cell proliferation was also accessed by immunostaining LNCaP cells with anti-histone H3p (Milipore) using the same protocol described above to characterize the reactive stromal cells. Cell cycle analysis was performed using propidium iodide assay. Briefly, cells were resuspended in 500 μL of propidium iodide solution (PBS, 0.1% Triton X-100, 0.1% RNAse and 50 μg/mL propidium iodide – Sigma) and incubated for 5 min on ice. Cell cycle analysis was assessed by flow cytometry (FACScalibur BD Bioscience) after 20,000 events acquisition and the data were analyzed in Cell Quest software.

2.5. LNCaP apoptosis assays

LNCaP cells were resuspended in 400 μL of binding buffer containing 5 μL of annexin V FITC and 5 μL propidium iodide (Apoptosis Detection Kit II BD Biosciences) for 15 min at room temperature. Annexin V binding was evaluated by flow cytometry (FACScalibur BD Biosciences), after 20,000 events acquisition and the data were analyzed in Cell Quest software.

2.6. LNCaP migration assays

LNCaP cell motility was characterized for each one of the experimental conditions used so far. To do that, 1×10^5 LNCaP cells were plated and samples were moved to a culture chamber, with controlled conditions of temperature and CO_2 (37 °C and 5%, respectively), adapted to an inverted microscope Nikon Eclipse TE 300 (Nikon). During 12 h, phase contrast images were captured every minute using a Hamamatsu C2400 CCD camera (Hamamatsu, Japan). After the movie assembly, 10 different cells were marked with black dots 10 times smaller in diameter than the cell at intervals of 10 min (72 images by movie), using the Image J software (National Institutes of Health, USA). The labeled cell trajectories were obtained by imaging analysis performed with the softwares Image J and Kaleidagraph (Synergy Software, USA). From the trajectories, the tangencial velocity, (V_T), and the spreading velocity, (V_S), were defined (see Supplemental materials). The tangencial velocity is proportional to the total cell displacement in the period of 12 h and the spreading velocity is proportional to the maximum cell displacement relating to its initial position for the same time period.

2.7. Gene expression analysis

Total RNA from LNCaP cells was extracted using RNeasy Mini Kit (Qiagen) according to the manufacture's instructions. RNA yield and quality were determined by a spectrophotometer NanoDrop ND-1000 V3.2 (Nanodrop Technologies, Inc., USA). Equal amounts (1 μg) of RNA from cells were reverse transcribed with cDNA Syntesis kit "Superscript II First-Strand Synthesis System for RT-PCR" (Invitrogen) and Oligo (dT) primer (Invitrogen). The cDNA was used as a template for subsequent real-time polymerase chain reaction (PCR). Quantitative real-time PCR was done on a CFX96 Real Time System (BIORAD) C1000 Thermal Cycler using SYBR Green (Applied Biosystems) following the manufacturer's instructions. The primers used for real-time PCR are listed in Supplementary Table 1. The expression level of MMP2, MMP9, E-cadherin, N-cadherin, Vimentin, CdK1, CdK2, CK8, CK18, Bax and Bcl-2 mRNA were all normalized with β-actin expression level. To evaluate the quality of the real-time PCR products, melt curve analyses were performed after each assay. Relative expression was determined using the ΔΔCT method with β-actin rRNA as the reference genes.

2.8. Statistical analysis

Statistical analysis was performed using the Graph Pad Prism 5.0 following by Student *t* test, except in migration assay, where the nonparametric Kruskal–Wallis, with Gaussian approximation in determining the assays values was used. Values considered statistically significant were those in which $p < 0.05$.

3. Results

3.1. Reactive stroma characterization

A homogeneous reactive stromal cell population derived from BPH surgery resections was established. The primary cell culture was characterized at each passage and it was observed a gradual decrease in the number of rounded cells with epithelial appearance and an increase in the number of elongated cells with fibroblastic aspects, as demonstrated by the phase contrast micrographs (Fig. 1A and B). In previous studies it has been demonstrated that prostate reactive stroma phenotype is characterized by an increase in expression of cytoskeleton proteins, such as vimentin and α-smooth muscle actin, followed by a decrease or absence of muscle markers such as desmin [10,25]. We observed after six passages in our culture system, a cell population positively staining for vimentin (Fig. 1C and D) and α-smooth muscle actin (Fig. 1E and F), but negative for cytokeratin 18 (Fig. 1G) and desmin (Fig. 1H). These results indicated that a homogenous BPH stromal cell population presenting a reactive phenotype was isolated. Moreover, ECM secreted by these reactive stromal cells presented immunostaining for type I, III and IV collagens, laminin, fibronectin and chondroitin sulfate (Supplementary Fig. 2).

3.2. Influence of reactive stroma on LNCaP cell morphology

Cell morphology analysis is an important tool that could provide relevant information about cell features or its microenvironment [26]. LNCaP cells seeded in optimum culture condition (10% of FBS supplementation) presented an elongated morphology, with many cytoplasmic extensions (Supplementary Fig. 1A). However, when LNCaP cells were cultivated in minimum medium (0.5% FBS; control, Fig. 2A and B) or over reactive stromal cells (Fig. 2C and D), they presented a more rounded shape, forming small clusters. This morphological organization pattern was also similar to that observed when LNCaP cells were cultured with the conditioned medium secreted by reactive stromal cells (Fig. 2E and F). Alternatively, when LNCaP cells were cultured over the ECM produced by reactive stromal cells, they presented major cytoplasmic expansions, with a consequent increase in the number of cell interconnections (Fig. 2G and H). These results are similar to that observed when LNCaP cells were seeded in complete medium culture conditions indicating that ECM supports typical LNCaP cell morphology, even in minimum serum supplementation.

3.3. Soluble factors and ECM secreted by reactive stroma induce LNCaP cell proliferation

Among numerous cellular events related to tumor progression, one of them is the increase in cell proliferation. To address this, we first labeled reactive stromal cells with green CMFDA. Then, LNCaP cells were layered over these labeled reactive stromal cells (Supplementary Fig. 1). After 72 h, cell sorting efficiency of this model was evaluated by flow cytometry, thus allowing the segregation of both cell populations with a high efficiency of 99.30% (Supplementary Fig. 1B). Next, sorted LNCaP cells were evaluated for cell proliferation by crystal violet assays 72 h after being co-cultured over the reactive stromal cells (Fig. 3A). Our data demonstrated that when co-cultured over reactive stromal cells, LNCaP cell proliferation was not activated. However, when LNCaP cells were cultured in the presence of conditioned medium secreted by reactive stromal cells, there was a significant increase in LNCaP cell proliferation, as compared to control condition ($p < 0.01$ Fig. 3B). Notably, the most pronounced effect over cell proliferation was observed when LNCaP cells were cultured over ECM secreted by stromal cells, which induced an increase on growth rate ($p < 0.001$ Fig. 3C). These data are all in agreement with the histone H3p staining (Fig 3D) and the cell cycle analysis, in which the percentage of LNCaP cells at S and G2/M phases were increased when these cells were cultured over the ECM secreted by the reactive stroma, indicating that ECM activate LNCaP cell proliferation by stimulating cell cycle entry (Fig. 3E and F).

3.4. Cell–cell and cell–ECM interactions modulate the survival of LNCaP cells

The balance between cell proliferation and apoptotic cell death is a well-established cancer imprint [27]. In order to understand the influence of reactive stromal cells on LNCaP apoptotic cell death, the proportion of positive annexin V^+ LNCaP cells were evaluated under each experimental condition. LNCaP cells co-cultured with reactive stromal cells presented a lower percent of apoptotic cell death when compared to control situation (medium with minimal FBS) (Fig. 4A). The LNCaP cell apoptotic index was also lower when these cells were seeded over the ECM secreted by reactive stroma when compared with the control situation (Fig. 4C and D). However, when LNCaP cells were cultured in the presence of conditioned medium secreted by reactive stromal cells, the same values of apoptotic cell death as that for the control was observed (Fig 4B). Hence, these data indicate that reactive stromal cells and its secreted ECM are both positively modulating the survival of LNCaP cells.

3.5. LNCaP cell motility is modulated by the reactive stroma

The videomicroscopy assays employed to characterize the LNCaP motility revealed that the interactions between the stroma and its derived components with LNCaP cells produced different responses. When compared to the control situation (Fig. 5A), the spreading velocity was significantly enhanced ($p < 0.05$) for each tested situation. The highest increase in spreading velocity was observed when LNCaP cells were plated over the ECM ($p < 0.001$ Fig 5A). The tangential velocity was also significantly enhanced when LNCaP cells were cultured over the ECM and stromal cells ($p < 0.001$ Fig. 5B). No significant differences were observed for tangential velocity values for LNCap cells cultured in the presence of conditioned medium when compared to the control situation (Fig. 5B).

3.6. ECM secreted by reactive stroma induce a pro-tumorigenic gene expression profile

In order to delineate putative molecular mechanisms by which secreted ECM is evoking LNCaP cell tumor progression features, we then investigated gene expression profiling of several genes related to PCa progression. LNCaP cells cultured over the ECM secreted by reactive stroma promoted a significant upregulation of MMP-2, MMP-9, E-cadherin N-cadherin, vimentin, CDK1 and CDK2 genes, while the expression pattern of CK8, CK18, Bcl-2 and BAX genes was unaltered (Fig. 6). These data indicated that the effects of secreted ECM on inducing LNCaP pro-tumorigenic features are partially mediated by the net result of an upregulated expression of several genes typically involved on PCa progression, such as those that activate cell migration, invasion and epithelial-mesenchymal transition.

4. Discussion

The fundamental role of tissue stroma on tumorigenesis and tumor progression has been extensively investigated [2,3,27,28].

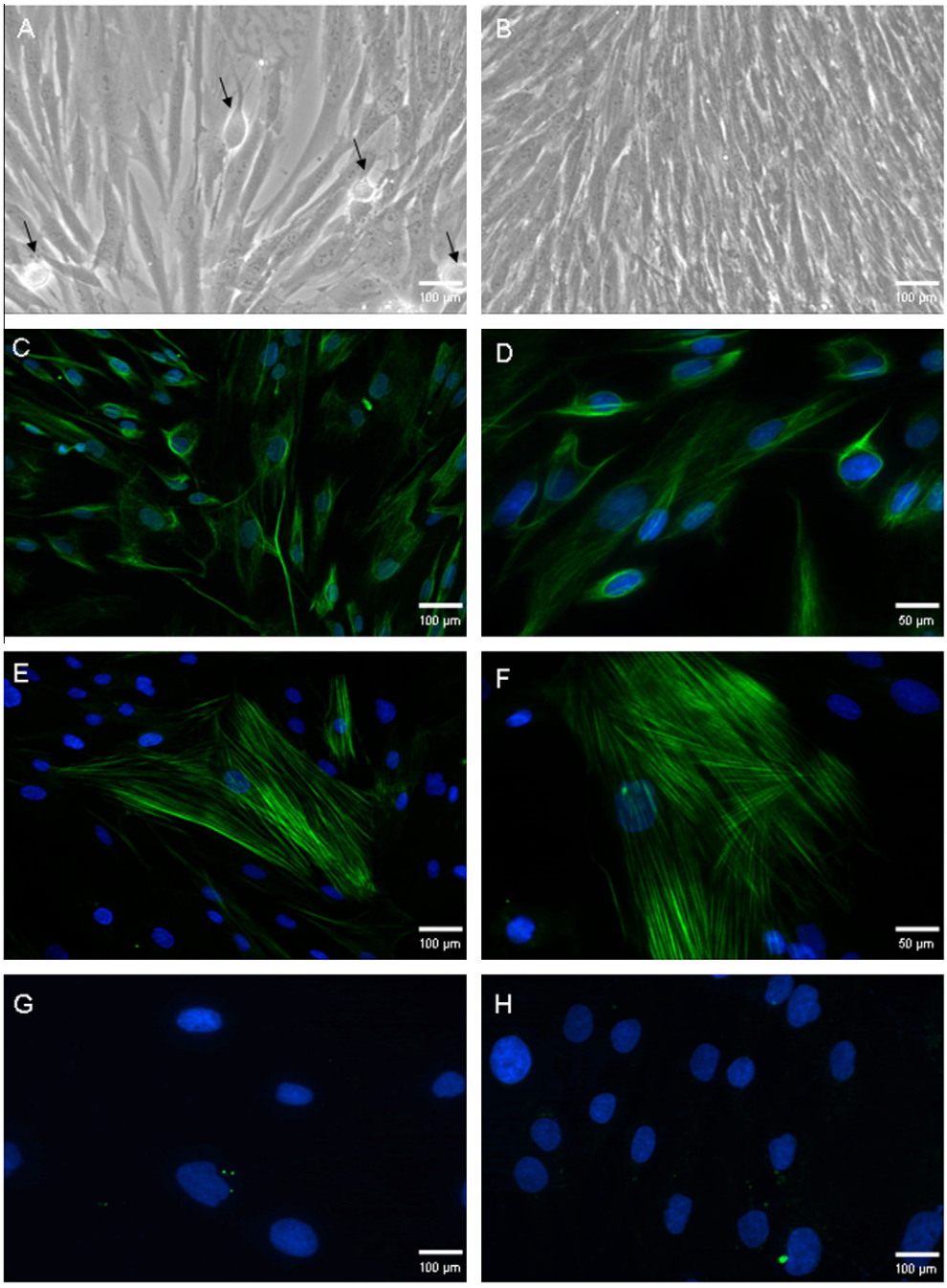

Fig. 1. Characterization of the stromal cell lineage. Phase-contrast micrographs of primary prostatic cells (A and B) and immunostaining for vimentin (C and D), α-smooth muscle actin (E and F), cytokeratin 18 (G) and Desmin (H). Note the presence of cells with rounded conformation typically showed by epithelial-like cells in the first passage (A – arrow) and at the sixth passage a predominance of cells with typical mesenchymal appearance (B) and with a reactive phenotype (C–H) as showed by cytoskeleton elements immunostaining.

Additionally, the development of an altered stromal microenvironment in response to tumor development is a common feature of neoplastic tissues [26]. Under the nomenclature of reactive stroma, the relevance of this compartment in PCa was reported by several groups, demonstrating its role on modulating key events, such as cell survival [9], proliferation [8] and angiogenesis [23]. A common feature of most of these studies was the use of *in vivo* xenograft models [7–9,12,23]. Apart from advantages of *in vivo* experimental models, a notable disadvantage of these approaches corresponds to difficulties to isolate the interacting variables. Therefore, we used an *in vitro* system to investigate the contribution of the distinct microenvironment stromal components on PCa progression, analyzing the role of cell–cell, cell–ECM and cell–soluble factors interactions. In order to better understand the interactions mediated by prostate reactive stroma, we established and characterized herein a homogeneous population of stromal cells derived from BPH presenting reactive phenotype. Our *in vitro* assays demonstrated that secreted ECM significantly induced LNCaP cell proliferation and migration, preserving its typical morphology, while inhibiting apoptosis. On the other hand, reactive stromal cells *per se* or their conditioned medium only partially modulated these events. Furthermore, the use of BPH derived tissue in order to obtain the reactive stroma *in vitro*, represents a reliable model to assess how the interactions mediated by this compartment

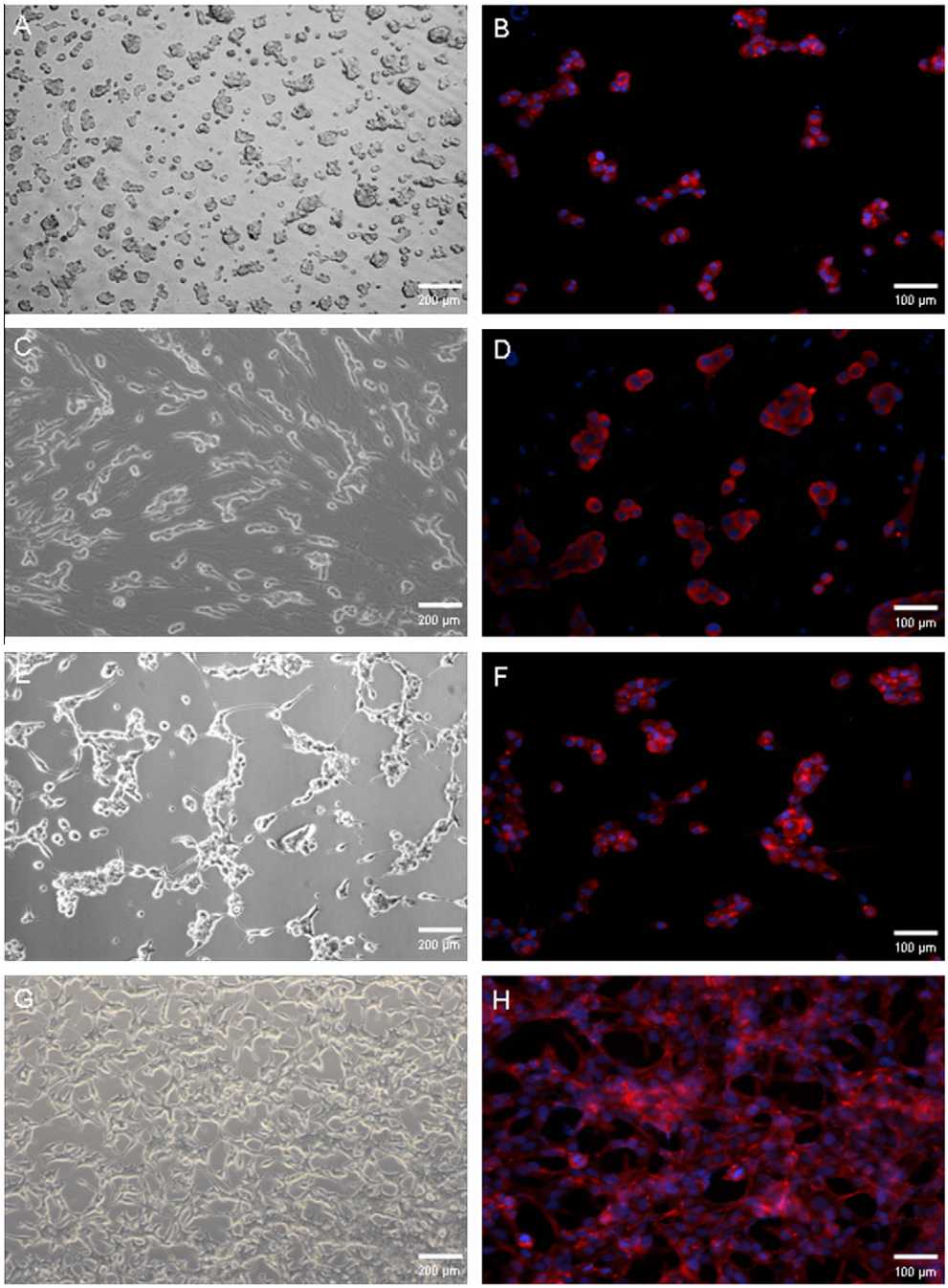

Fig. 2. The LNCaP cell morphology is only supported by ECM. Phase-contrast micrographs (A, C, E, G), phalloidin staining (B, F, H) and immunostaining for cytokeratin 18 (D). The morphology of LNCaP cells was evaluated 72 h after following culture conditions: Control (0.5% FBS) (B), upon the stromal cells (C and D), in the presence of stromal cells conditioned medium (E and F) and over the ECM (G and H). The morphological aspects of LNCaP cells were observed only after cells were seeded over the ECM.

contribute to prostate tumor growth, once it presents a well established reactive phenotype [22], besides being also validated in previous studies [14,23]. Among the interactions evaluated in our study, those related to cell–ECM interactions represented the most pronounced effects on positively modulating tumor events such as proliferation, survival and migration.

Trying to identify molecules able to promote those effects of ECM on LNCaP tumorigenic features we also investigated herein which proteins are being expressed in the secreted ECM and also the gene expression profile this stromal component could induce on LNCaP cells, enabling a pro-tumorigenic phenotype. By immunocytochemistry analysis, we identified some ECM components produced by reactive stromal cells (Supplementary Fig. 2A–F), such as type I, III and IV collagens, laminin, fibronectin and chondroitin sulfate. Recent data has shown that increased cross-links between collagen molecules activated growth and invasion of mammary tumor cells [29]. Type I collagen is regarded as a natural barrier against the invasion of tumor cells into the adjacent stroma, although the increase in its synthesis is detected during the process of metastasis [30]. It has been previously observed that in PCa metastasis at bone tissues, the resident stromal cells decreased type I, II, III and IV collagen synthesis [31]. However, the opposite response was observed at early stages of tumor growth, like those involving the reactive stroma generation. In this context, tumor cells apparently induce collagen synthesis by prostate stromal cells [10]. A prominent cancer ECM molecule intensely studied has been

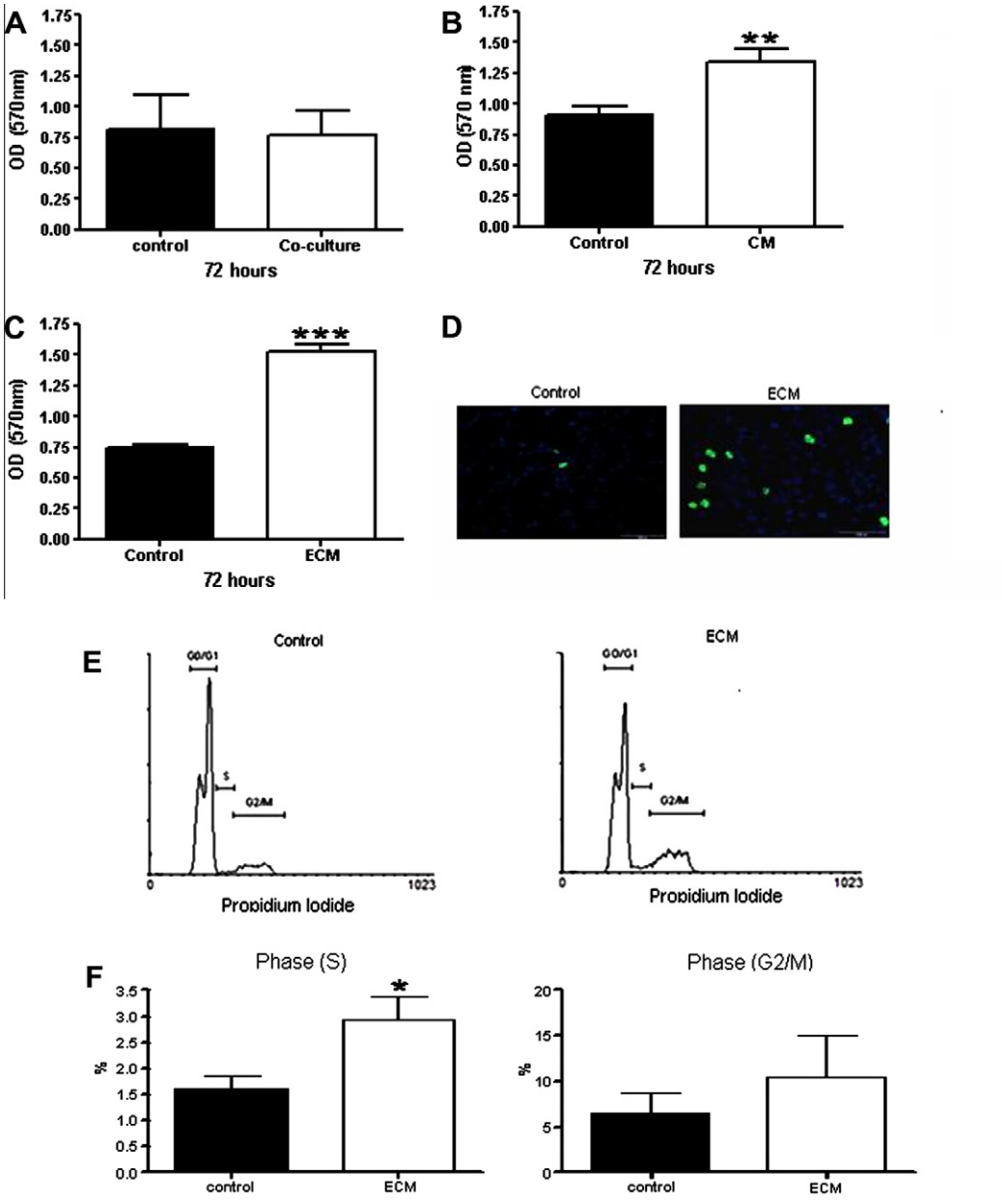

Fig. 3. LNCaP cells proliferation analysis. LNCaP cell proliferation was measured by colorimetric analysis of the incorporation of crystal violet 72 h after cultivation of LNCaP cells upon the stromal cells (A), in the presence of conditioned medium (B) and upon the ECM (C). The conditioned medium and ECM induced the proliferation of LNCaP cells, but the ECM did so more strongly and moreover the cells seeded over ECM showed a typical profile of cells than are actively dividing with a pronounced percentage of cells at S and G2/M phases as revealed by cell cycle (E and F). The dividing potential of LNCaP cells seeded upon ECM was confirmed too by immunostaining for histone H3p (D). D O = Optical Density; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

the protein laminin family. It has been reported that the expression of laminin-5 becomes absent during progression of intraepithelial neoplasia to PCa [32]. The downregulation of laminin-5 seems to be related to the formation of a more passive microenvironment to tumor growth, leading to changes in the expression of genes related to signaling, adhesion and cell cycle [33]. The cleavage of β-3 chain of laminin-5 matrix metalloproteinase by MT-1 generates a 80-kDa peptide responsible for the increased motility of prostate tumor cell lines [34]. On the other hand, the study of DeOcampo et al. [35], demonstrated that the interaction between laminin-1 and integrin α6β1 promotes phosphorylation of FAK (focal adhesion kinase) and subsequent activation of MAP kinase, both cell proliferation signaling pathways. In fact, these reports about the relevance of laminin in the progression of PCa could explain some of our results, in which we observed that when cultured over ECM secreted by stromal cells, LNCaP cells were able to present a higher proliferation rate, increased survival and migration capacities, followed by positive modulation of genes involved with these events.

The gene expression pattern observed on LNCaP cells as a result of secreted ECM interaction are in accordance to a model in which stromal and PCa epithelial cells present close relationships, favoring tumor progression. Stroma–epithelium interactions in the prostate has been well documented and prostate stromal compartment has demonstrated a pivotal role in PCa invasion and metastasis [5], conferring activation of cell growth and metastatic lesions of PCa cells in an *in vivo* transplantation experiment [36]. It is also well known that some stromal factors further induce epithelial cells to produce several gene products related to tumor progression [37,38]. Some of these molecules are importantly involved on ECM remodeling [36] and epithelial-mesenchymal plasticity [39]. We observed herein

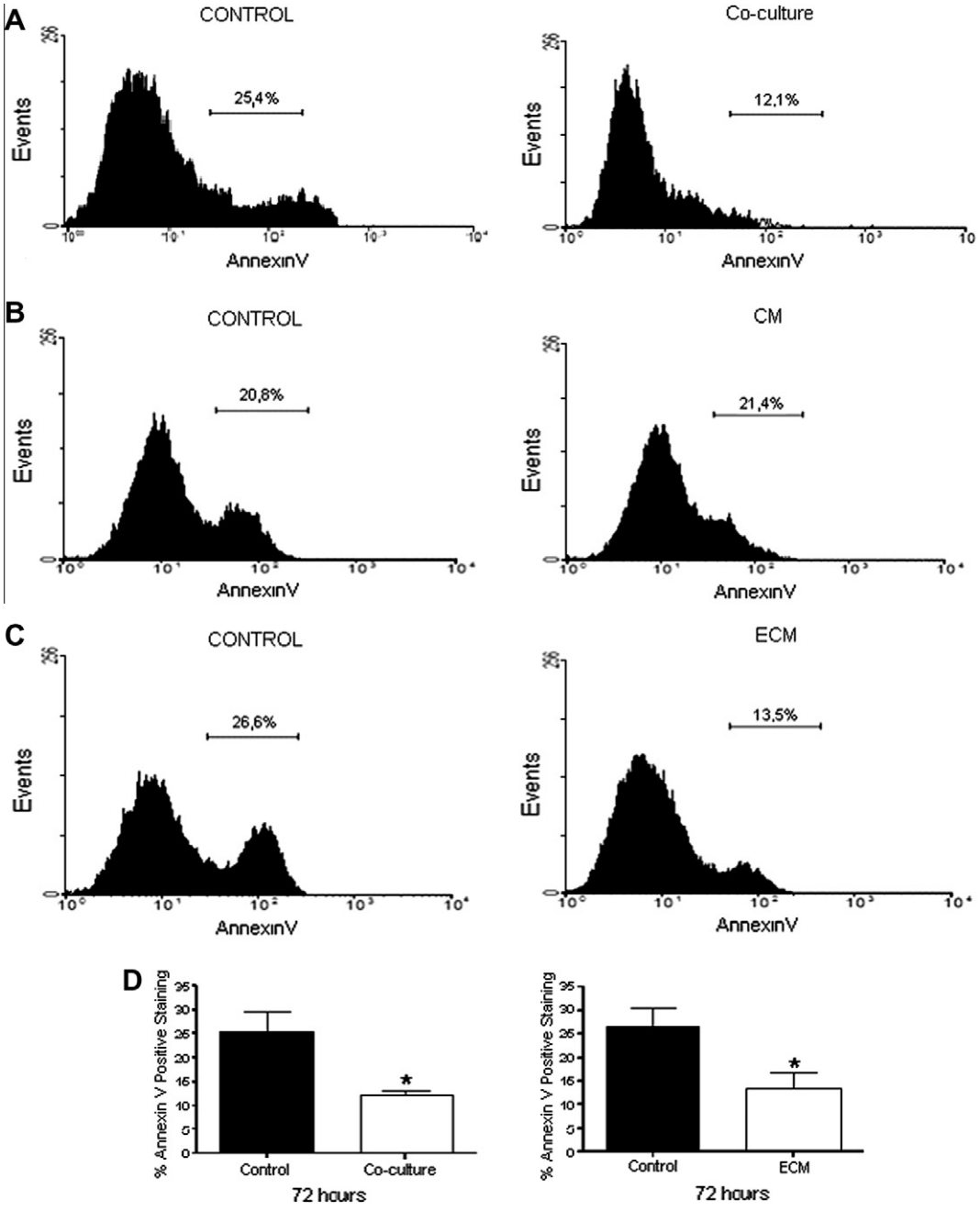

Fig. 4. LNCaP annexin V apoptosis assay. Representative Histograms relating the fluorescence emission for Annexin-V in LNCaP cells cultured for 72 h upon the stromal cells (A) in the presence of conditioned medium (B) and upon the ECM (C). Percentage of Annexin-V$^+$ cells(D).

that several genes related to these processes were significantly upregulated when LNCaP cells were cultured over ECM secreted by stromal cells, and possibly being involved on favoring LNCaP pro-tumorigenic features. The proteolytic degradation of ECM mediated by extracellular proteases, such as MMPs, is required for PCa cell migration and invasion. Among them, MMP-2 and MMP-9 present critical roles in PCa progression, by presenting proteolytic activity against a variety of ECM substrates, including collagens, proteoglycans, elastin, laminin, fibronectin, and casein [40]. We also found that secreted ECM positively modulates the expression of several epithelial-mesenchymal (EMT) and mesenchymal-epithelial (MET) transition markers, also more recently described as proteins conferring phenotypic plasticity. These phenotypic changes has been interpreted as the ability to easily transit between epithelial-like and mesenchymal-like states. These transitions have been discussed as being linked to stem cell–like properties and as more important determinants of aggressive metastatic behavior than the properties of the end states. It has been reported that metastatic PCa cells co-express epithelial and mesenchimal markers, such E-cadherin, N-cadherin, vimentin and cytokeratins, indicating phenotypic plasticity in these cells [41]. Our data presented similar results on cell cultured upon secreted ECM. Although it has been demonstrated that in human PCa occurs loss of E-cadherin expression and overexpression of N-cadherin, indicating EMT and metastasis activation [41], other authors have described that in PCa, attachment of metastatic cells to bone cells correlates with overexpression of E-cadherin [42] and that exit from EMT is marked by increased E-cadherin expression, which has been considered necessary for tumor growth at sites of metastasis [43]. CDK1 and CDK2, which function at different stages of the mammalian cell cycle,

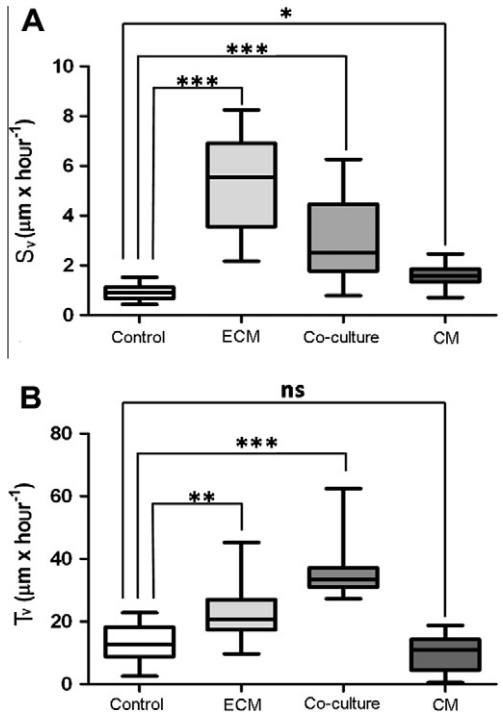

Fig. 5. Cell migration analysis by videomicroscopy. LNCaP cells spreading velocity (A) and tangential velocity (B). LNCaP spreading velocity rate was higher when these cells were seeded upon the ECM and tangential velocity was more pronounced over the stromal cells. NS = no significance.

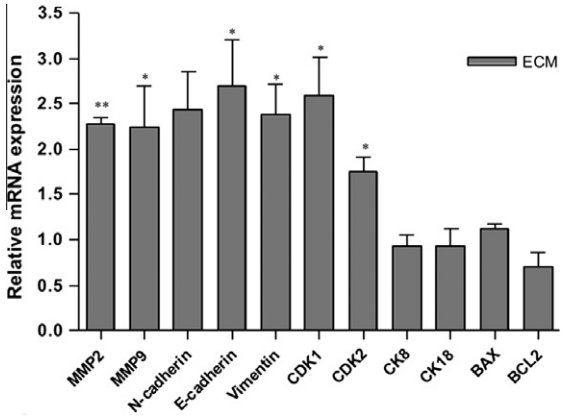

Fig. 6. Gene expression profile in EMC. Quantitative real-time PCR analysis of different genes studied in ECM, after 72 h of incubation. Gene expression is presented as fold expression compared with LNCaP cells cultivated in 0.5% FBS (control condition). MMP2, MMP9, E-cadherin, Vimentin, CdK1 and CdK2 gene expression were significantly increased in ECM compared with control cells. β-actin RNA transcript levels were used for the internal control. The values are expressed as means ± S.D. ($n = 4$), and the P-values were <0.05 (*) and <0.01 (**). MMP, matrix metalloproteinase; CdK, Cyclin dependent kinase; CK, cytokeratin; Bax, Bcl-2-associated X protein; BCl, B-cell lymphoma.

has also been reported as presenting important roles on PCa cell proliferation and survival [44] also were upregulated as a result of LNCaP cultured upon ECM. Although Bax and Bcl-2 transcripts did no presented upregulated expression on LNCaP cell cultured over secreted ECM, it is possible that other apoptosis regulatory targets are mainly controlling the pro-survival role of this stromal compartment.

Our results showed a significant increase in survival, but not in the proliferative activity of LNCaP cells, when they were co-cultured over the reactive stromal cells. In fact, data from Olumi et al. [7] demonstrated that the formation of tumors in *nude* mice by LNCaP cells occurred primarily by a decrease in apoptosis of these tumor cells under the influence of stromal cells. Moreover, the morphology of LNCaP cells in contact with reactive stromal cells was quite different from the elongated morphology, as compared to optimum culture conditions (Supplementary Fig. 1A). This observation may partially explain the absence of LNCaP cells proliferation when in co-culture, since cells derived from solid tumors require a proper grounding in order to proliferate [26]. Therefore, whenever these cells do not appear to be firmly anchored, the signaling dependent on this interaction will not occur and the proliferation will be inhibited. One potential target for future studies may reside in the protein N-cadherin, which can be expressed by both tumor and stromal cells [24]. A possible down-regulation of this protein in reactive stromal cells might reduce the interaction of this compartment with tumor cells, thereby altering adhesion between both populations as observed in our data. On the other hand, the *in vitro* apparently lack of interactions between different cell populations could just reflect a well known behavior of tumor cells *in vivo*, which is the lack of adherence and the consequent increase in cell migration associated with metastasis [26]. The increased survival of LNCaP cells when co-cultured over reactive stromal cells may be related to other adhesion proteins such as cadherins and integrins that can interact to each other, forming heterotypic connections able to activate signaling pathways such as AKT, which are responsible for cell survival [45]. Analysis of cell migration by videomicroscopy revealed the absence of cytoplasmic expansions of LNCaP cells when cultured upon the stromal cells, which could be related with the lack of structures necessary for cell migration as lamellipodia, filopodia and invadopodia [46]. Moreover, when cultured over the reactive stromal ECM, LNCaP cells presented a directed movement (higher values of spreading velocity V_S, Fig. 5A). This kind of movement may be a consequence of an eventual protein trail that was left in the coverslip after reactive stromal cell lysis. LNCaP cells possibly adhered preferentially over proteins in this trail. By evaluating LNCaP cell tangential velocity V_T (Fig. 5B), we demonstrated that when co-cultured with reactive stromal cells or cultivated over reactive stromal ECM, the cell machinery is activated to move faster, so that, the cell motility is increased in those situations. The reactive stromal cells may be signaling to LNCaP cells, increasing their motility. The biggest values of V_T were found when LNCaP cells were co-cultured with the reactive stromal cells. In this situation, there is also a contribution of LNCaP tangential velocity due to reactive stromal cell movement. Aiming to verify the influence of the stromal cell displacement, images of stromal cell culture were captured following the same procedure described at the motility assays (see Section 2). Stromal cells were attached to uncoated polystyerene spheres of diameter 3 µm, mimicking LNCap cells. In this situation, it was not observed significant differences for the tangential velocity values when compared to control (data not shown). These results indicated that reactive stromal cell proteins or soluble factors released by these stromal cells increase LNCaP motility activating the proteins and signaling involved with this process. LNCaP cells cultured in the presence of conditioned medium produced by reactive stromal cells presented a significant increase on proliferation level. Regarding apoptosis index and migration behaviors, no difference has been observed as compared to control condition. Increased proliferation when LNCaP cells were cultured in conditioned medium secreted by reactive stromal cells may be a result of the influence of several growth factors. Among them, b-FGF was highlighted as the most potent mitogen among those mapped by Gleave in conditioned medium secreted by prostate fibroblasts, being also capable of inducing tumor formation in nude mice [47]. In PCa tumors, FGF-2 is upregulated in stroma compartment, as compared to epithelial ones [48] and is able to stimulate the proliferation and

secretion of proteases related to metastasis [10]. TGF-β seems to be used by tumor cells, since they decrease the expression of TGF-β receptors and increase the binder production which will operate in the adjacent stroma promoting the generation of reactive stroma that ultimately promotes tumor growth. Our results apparently demonstrated that classical anti-proliferative signaling pathway mediated by TGF-β is not happening, since there was no proliferation inhibition of LNCaP cells when cultured in the presence of the conditioned medium. A reasonable explanation could reside in the TGF-β ability to induce the expression of receptors for growth factor-derived stroma (SDF) CXCR4, which then activate the AKT pathway, thus contributing to tumor growth as observed by Mingfang et al. [25]. A large amount of IL-6, another important cancer-related cytokine, has been detected in conditioned medium produced by BPH stromal cells. It has also been demonstrated it was able to induce tumor cell apoptosis [49]. These reports could explain part of our results, since in our data it has not been observed a significant survival difference for LNCaP cells cultured in the presence of conditioned medium, as compared to control condition. Moreover, the altered morphology and low motility of LNCaP cells cultured in the presence of conditioned medium could be also related with IL-6 as previously showed [50].

5. Conclusions

This *in vitro* study contributes to a better understanding of the roles of reactive stromal compartment over PCa progression features. The interactions between this compartment and tumor cells can generate antagonistic responses mediated by direct contact with reactive stromal cells or their secreted soluble factors. In contrast, the signaling mediated by the extracellular matrix secreted by the reactive stroma activates the malignant phenotype of tumor cells used in our study. These data could reflect the existence of some heterogeneous biological responses in the stromal microenvironment that positively or negatively control some key stages of PCa progression. Finally, we propose that the ECM produced by reactive stroma contain key molecules mediating part of tumor microenvironment effects over prostate tumor progression, by activating the expression of target genes involved in these processes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2012.02.026.

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