The composition of prostate core matrisome *in vivo* and *in vitro* unveiled by mass spectrometric analysis

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Abstract

Background: The composition and organization of extracellular matrix (ECM) are important regulators of cell behavior. In particular in the prostate, this central role of the ECM is further stressed by the fact that several potential markers of prostate stem cells are matrix receptors.

Methods: We established 12 fibroblastic cell lines from cancerous and non-cancerous areas of 6 prostates and allowed the cells to produce ECM under cell culture conditions. We also performed a proteome wide analysis of the ECM components by mass spectrometry. To study the *in vitro* activation of fibroblastic cells we compared the differences between the ECM produced in cell culture by 6 non-cancerous-tissue-derived fibroblasts and the *in vivo* matrisome from the corresponding non-cancerous tissue of prostate.

Results: Our results suggest that at tissue level the ECM is mainly produced by fibroblastic cells and that it contains standard collagen I fibrils and fibril-associated proteins. Beaded-filament forming collagen VI is also abundant and basement membranes potentially contain 5 laminin subtypes and collagens XV and XVIII. As the main finding, we also detected differences when *in vivo* and *in vitro* matrisomes were compared. Only 65 out of 206 proteins were found to be common for both *in vivo* and *in vitro* samples. Majority of the 55 proteins, which were solely detected in *in vivo* samples, were considered to be plasma derived. Eighty-six proteins were solely found from *in vitro* fibroblast-derived ECM, and most of them were related to matrix remodeling or growth factor action, proposing that the activation of fibroblasts in cell culture may remarkably modify their gene expression profile. Finally, in comparison to traditional 2D *in vitro* cell culture, the ECM composition of 3D spheroid culture was analyzed. The matrisome in spheroid culture did not resemble the *in vivo* ECM more closely than in monolayer culture.

Conclusions: Artificial activation of ECM remodeling seems to be a distinctive feature in *in vitro* models. In conclusion the constitution of ECM produced by prostate derived fibroblasts *in vitro* is similar, but not identical to the prostate ECM *in vivo* as shown here by mass spectrometric analysis.

Keywords: extracellular matrix, integrin, collagen, stem cells

INTRODUCTION

The normal prostate tissue is built up by epithelial cells − luminal and basal − that are separated from fibromuscular stroma by basal membrane. The stroma contains mostly fibroblasts, smooth muscle cells and, to a lesser extent, endothelial, inflammatory and nerve cells [1]. Stromal cells together with the extracellular matrix (ECM) create supportive basis for epithelial glands and provide a proper microenvironment for differentiation, homeostasis and regeneration [2,3]. In prostate cancer, when epithelial cells lose their normal function, the tumor is formed by malignant epithelial cells and activated stromal cells. In addition, the ECM is considered to be an important regulator of disease progression, since it provides a platform for the malignant cell anchorage and migration. Furthermore, various ECM proteins can also promote cancer cell survival, proliferation and drug resistance.

The composition of the prostate ECM is extremely intriguing, since prostate stem cells have been shown to express high levels of three ECM receptors, namely CD44 [4], a receptor for hyaluronan and fibronectin [5], $\alpha2\beta1$ integrin [4,6], a receptor for collagens and $\alpha6\beta1$ integrin, a laminin receptor [7,8]. In addition to collagens and laminins, numerous other ECM glycoproteins are putative ligands for $\alpha2\beta1$ and $\alpha6\beta1$ integrins [9]. Thus, cell adhesion may be a major regulator of the prostate stem cells. Furthermore, we have previously shown that the expression levels of another collagen receptor, $\alpha11\beta1$ integrin, increase during prostate cancer progression [10]. Experiments with $\alpha11$ integrin null mice have also confirmed the essential function of this receptor during the growth of prostate cancer xenografts [11].

All cell types that are present in the prostate may participate in the production and modulation of the surrounding ECM. However, in normal tissue fibroblast-like cells and in tumors activated myofibroblasts, also called as cancer associated fibroblasts (CAFs), have been suspected to be the major producer and modulator of the ECM [12,13]. In addition, fibroblasts may regulate other cells by releasing soluble proteins, such as growth and differentiation factors [14].

It is not clear how well the ECM produced *in vitro* mirrors the ECM in tissues. However, the assumed involvement of fibroblasts and the ECM in tumor progression has generated a number of studies in which CAFs have been compared to "normal" fibroblasts [15–18]. Such comparisons have unveiled potential differential regulation of fibulin-1, fibronectin, osteoglycin [16] and tenascin C [17] as potentially differentially regulated genes in prostate CAFs and collagen X in breast cancer [15].

Our present knowledge of the ECM composition in prostate is based on gene expression profiles, immunohistochemistry and *in vitro* models all of which have their shortcomings. Worryingly, the specificity of many of the frequently used antibodies has recently been challenged [19], which significantly questions the credibility of the existing information about the presence of various ECM components in prostate. Here we have analyzed the ECM content of 6 prostate tissue samples by mass spectrometry and also analyzed the ECM produced *in vitro* by cultured fibroblast-like cells from the same 6 samples using both monolayer culture and spheroid models. Our results unveil the composition of the prostate matrisome and, most importantly, reveal that there are significant differences in, the accumulation of proteins related to ECM remodeling and growth factors even though the basic components of matrisome *in vivo* and *in vitro* are quite similar.

MATERIALS AND METHODS

Tissue samples and primary human prostate fibroblasts.

Prostate cancerous and normal tissues were obtained in collaboration with Auria Biobank and Pathology Department of Turku University Hospital from prostate cancer patients (N = 6) who

underwent robotic assisted laparoscopic radical prostatectomy at the Turku University Central Hospital in 2013. The prostate samples were collected by the Turku Prostate Cancer Consortium Study (TPCCS) (Corresponding scientist: Dr. Kari Syvänen M.D., Ph.D., F.E.B.U., Turku University Hospital, Department of Surgery, Division of Urology) and the study was approved by the ethics committee of the Hospital District of Southwest Finland (ETMK: 3/180/2013). A signed consent from all patients was received. The fresh tissue specimens were processed within 30 min post-operatively. In brief, tissue cores from the peripheral zone of both lobes were removed in apical-basal axis using a MD5000 Tissue Coring Press (Alabama Research & Development, Munford, USA) and a coring tool with 5 mm diameter. A histological validation sample was cut from the middle of each core, fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin for histopathological analysis. The fresh tissue material next to validation samples was used for primary cell cultures and another sample was snap-frozen in liquid nitrogen and stored in -80°C for MS analyses. In the histological analysis of validation samples, the percentage of stroma, normal epithelium, prostatic intraepithelial lesion (PIN) and carcinoma, as well as Gleason grade and presence of inflammation (score 0-3) were determined for each tissue sample (see Table 1). The most representative cases containing similar percentage of carcinoma in one lobe and morphologically benign tissue in another lobe were selected for further analysis.

Cell culture

Primary prostate normal fibroblast and cancer associated fibroblast cultures were established by using a published method [20] with the exceptions that digestion of tissue samples was enhanced by the addition of 5.2 U/ml hyaluronidase and increasing the incubation from 2.5 h to overnight. Fibroblastic cells used in experiments represented passages up to six. For monolayer culture, 1.5 $x10^6$ fibroblasts were seeded on 100 mm cell culture plate in serum free Fibroblast basal media (FBM, Lonza, Basel, Switzerland). Cell plates reached to confluence were treated for 7 days with 50 µg/ml L-ascorbic acid in FBM in order to induce ECM production. The cells were removed from

the ECM by hypotonic lysis according to a previously described method [21]. The ECM proteins on plate were collected into lysis buffer (1% v/v Triton X-100, 0.5 M EDTA, Tris HCl (pH 7.4)) by mechanical scraping, sample treatment continued as described in section Mass spectrometry.

The 3D culture was performed as monoculture of fibroblasts or as co-culture with DU145 cells (ratio 3:1). For spheroid formation, cells were seeded into micro-molds (MicroTissues 3D Petri Dish micro-mold spheroids, Z764051, Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, 2.8×10^5 cells were seeded in 75 µl of serum-free RPMI 1640 culture medium supplemented 2 mM UltraGlutamine (Lonza) and 100 U/ml penicilin-streptomysin (PenStrep; Lonza) on each mold. Next day, the surrounding culture medium was replaced with 1 ml of new culture medium supplemented with L-ascorbic acid (50 µg/ml). This medium was changed daily with freshly made L-ascorbic acid. After 9 days, the tumor spheroids were centrifuged out of the agarose molds (1300 g, one min) to 24-well plate, each well containing 500 µl of PBS (Lonza) at the bottom. The spheroids were transferred to Eppendorf tubes and centrifuged $1000 \times g$ / 1 min. PBS was removed and the spheroid pellets were incubated with hypotonic lysis buffer (10 mM Tris, 1 mM EDTA, pH 7.4, 10 µg/ml deoxyribonuclease I (Sigma-Aldrich) on an orbital shaker at 4°C overnight. The pellets were washed twice with the hypotonic lysis buffer and frozen for mass spectrometry analysis.

Extraction of ECM proteins

Prostate tissue pieces were immediately snap-frozen in liquid nitrogen and subsequently stored at -150°C until later use. Prostate tissue pieces were partially thawed and weighted, diced into smaller pieces and immediately placed into ice-cold PBS, supplemented with protease and phosphatase (PP) inhibitor mixture (Thermo Fisher Scientific, Waltham, MA, USA) and 25 mM EDTA, and washed subsequently with PBS mixture for 5 times, 30 ml total per sample. The matrix proteins were extracted according to a published protocol [22]. Briefly, the tissue samples were treated with 0.5 M

NaCl solution to extract loosely bound ECM proteins, cellular material was removed by 0.08% SDS from tissue and the remaining ECM proteins were extracted by 4 M guanidine buffer.

Mass spectrometry

The ECM protein extracts from tissue and monolayer cultures were precipitated with six volumes of acetone at -20°C overnight. The precipitated proteins were recovered by centrifugation at 20 000 x g and +2°C for 15 min and dissolved in solution containing 8 M urea, 2 M thiourea and 50 mM ammonium bicarbonate. The ECM proteins from monolayer culture were purified with Pierce Detergent Removal Spin Column (Thermo Fischer Scientific). The cysteines were reduced in 10 mM dithiotreitol at 37°C for 1 h and alkylated in 40 mM iodoacetamide at room temperature for 1 h. The solution was diluted to 1:10 with 50 mM ammonium bicarbonate, and the proteins were digested with trypsin (Promega, Madison, WI, USA) at 37°C overnight. The proteins from 3D cultures were not acetone-precipitated and they were digested with LysC/Trypsin mixture (Promega), first for 4 h in 8 M / 100 mM ammonium bicarbonate and then for 16 h after dilution to 0.8 M urea with 100 mM ammonium bicarbonate and subsequently filtrated by a Microcon ultrafiltration device with 10 kDa cutoff (Merck-Millipore, Billerica, MA, USA). The peptides were desalted by StageTips [23] and loaded on a nanoflow HPLC system (Easy-nLCII, Thermo Fisher Scientific) coupled to the Orbitrap Velos Pro Hybrid Ion Trap-Orbitrap or QExactive (for 3D culture samples) mass spectrometer (Thermo Fisher Scientific) equipped with a nano-electrospray ionization source. Two or three repeated runs per sample were performed.

Data analysis

Tandem mass spectra were searched by Proteome Discoverer software (version 1.4, Thermo Scientific) and Mascot (version 2.4, Matrix Science, London, UK) for searching human SwissProt entries in UniprotKB database (release 2015_08) with a parent ion tolerance of 5.0 ppm and a fragment ion mass tolerance of 0.50 Da. Carbamidomethyl (C) as a fixed modification and

oxidation (M, P, K) and acetylation of the protein N-terminus as dynamic modifications were included. Maximum of one missed cleavage was allowed. Decoy database search using reversed human SwissProt sequences was used to assess false discovery rate. The result files were loaded into Scaffold software (version 3, Proteome Software Inc., Portland, OR, USA) to align the MS runs by the detected proteins. The false discovery rate threshold for the protein identification was set to 0.01. The protein was determined as detected in the sample if, at least in one replicate run of the sample, its identification had been derived from at least two unique peptide identifications. The tandem mass spectra obtained from 3D culture samples were analyzed by MaxQuant software [24] using the same database and amino acid modifications as with the Mascot search. Label-free quantitation with the "match between runs" option selected was performed. The protein was determined as detected if the LFQ intensity was higher than 10 000 with at least two identified peptides. Only the protein identifications present in the most recent list (updated August 2014) of human "core matrisome" or "matrisome-associated" proteins in the MatrisomeDB (http://www.matrisomedb.org/) [25] were selected for further analysis. The mass spectrometry data have been deposited to the ProteomeXchange Consortium [26] via the PRIDE partner repository with the dataset identifiers PXD006562 (username: reviewer31133@ebi.ac.uk, password: GFH9oFv5) and PXD006563 (username: reviewer60397@ebi.ac.uk, password: fvVHW8o0).

Immunofluorescence and confocal microscopy

Fibroblasts and fibroblast-derived cell-free matrices were fixed with 4% paraformaldehyde (Thermo Fischer Scientific) for 10 min RT, cells were permeabilized with 0,2% Triton X-100 in PBS for 5 min. Blocking and hybridization were performed in 3% bovine serum albumin in PBS. Antibodies against α-SMA (ab32575, Abcam, Cambridge, UK), pan-cytokeratin (C2562, Sigma-Aldrich), collagen I (ab6308, Abcam) and fibronectin (ab6328, Abcam) with dilution 1:100 were used. Nuclei were visualized with DAPI (Sigma-Aldrich). The imaging with Zeiss LSM780

confocal microscope (Zeiss, Jena, Germany) was performed at the Cell Imaging Core, Turku Centre for Biotechnology.

Real-time quantitative PCR

Total RNA was extracted using Trisure (Bioline, London, UK). cDNA was synthesized from 1 µg of total RNA using the First Strand cDNA Synthesis Kit (Thermo Fischer Scientific). TaqMan probe-based qRT-PCR was analyzed using ABI Prism 7900 HT (Applied Biosystems, Foster City, CA, USA). Primers and probes were designed using Universal Probe Library (Roche, Basel, Switzerland) and are listed in Supplementary Table 1. The target gene expression was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control and qPCR experiment was analyzed using ΔΔCT method.

RESULTS

Cell culture conditions activate all fibroblasts, but some cancer-related features may still remain *in vitro*

Cancerous and non-cancerous tissue samples (Fig. 1A) from peripheral zone of 6 radical prostatectomy specimens were used to initiate 12 cell lines. The details of histological analysis and clinical information related to the patients are presented in Table 1. Morphologically the cells represented fibroblasts and they were positive for α-smooth muscle actin (α-SMA). Immunofluorescence analysis revealed that in some primary cultures a small percentage (<10%) of cells also expressed keratin, an epithelial cell marker (Fig. 1B), indicating a minor contamination by tumor or non-transformed epithelial cells. The cell lines were further characterized by measurements of keratin 8 and 18 mRNA levels (Fig. 1C). The results confirmed the presence of small proportion of keratin expressing cells in some cell lines that represented both tumor-derived and control fibroblasts. However, the contamination was considered to be a minor one and not to compromise the further experiments as the number of epithelial cells decreased during extended

culture. The markers of fibroblast activation, α-SMA and fibroblast-activation protein (FAP), were also analyzed at mRNA level (Fig. 1D). As expected, α-SMA and FAP mRNA levels positively correlated to each other (Fig. 1E). Based on these results similar signs of activation were detected in cell lines derived from non-cancerous tissue samples and in CAFs.

All 12 fibroblastic cell lines were allowed to produce ECM for 7 days. Ascorbic acid was present to ensure the proper function of prolyl hydroxylases and the synthesis of stable collagens. Immunofluorescence with collagen I and fibronectin antibodies (Fig. 2A) and scanning electron microscopy (Fig. 2B) were used to visualize the fibrillar structure of the *in vitro* matrix. To test the functional properties of the ECM, fibroblastic cells were lysed and the remaining matrix was used in cell proliferation assays. Prostate cancer DU145 cells attached and spread on this matrix, indicating that the ECM proteins were in functional conformation. However, DU145 cells proliferated significantly slower on a fibroblast derived matrix compared to collagen I or FN coated 2D plastic surfaces (Fig. 2C).

Mass spectrometric analysis of matrix proteins produced in monolayers revealed the presence of 164 ECM proteins that have been defined in MatrisomeDB [25] as "core matrisome" or "matrisome-associated" (Supplementary Table 2). In accordance with the observation that both non-cancer-derived and tumor-associated fibroblasts are activated in cell culture, 132 out of the 164 detected matrisome proteins were produced by at least one cell line in both groups. Thirteen proteins were found in individual tumor-associated fibroblast lines, only, and 19 proteins were solely observed in individual non-cancer derived fibroblast matrices. However, none of these qualitative differences were statistically significant when the tumor and the non-tumor derived fibroblasts were compared as groups to each other. The semiquantitative mass spectrometric analysis did not allow detailed quantitative comparison of ECM component between tumor and non-cancerous tissue fibroblasts. To study whether the expression of three matrix proteins, namely collagen α1(I), collagen α1(III) and periostin, correlates with fibroblast activation we analyzed their mRNA levels (Fig. 3A-C). In accordance with previous papers we detected a large variation

between different fibroblastic cell lines [16]. Still we made the surprising observation, that collagen α1(III) mRNA levels were in 5 out of 6 cases lower in cancer derived fibroblasts when compared to the fibroblasts cultured from the non-affected area in the same prostate (p=0.026). Finally the mRNA levels of the three ECM proteins where compared to the activation marker mRNA levels. Collagen α1(I) or α1(III) mRNA levels did not correlate with α-SMA mRNA levels (Fig. 3D-E), whereas the correlation of periostin to α-SMA was statistically significant (p=0.005) (Fig. 3F). Thus, some properties of the fibroblastic cells may also reflect their origin rather than the level of activation.

Mass spectrometric analysis reveals the core *in vivo* matrisome of prostate

We applied a three-step extraction protocol [22], to extract both loosely and tightly bound ECM proteins from tissue samples obtained from the non-cancerous areas of the surgically removed prostates. In accordance with the variation in the histological picture (Fig. 1A), the composition of ECM varied between tissues samples. Altogether, 120 ECM proteins were reliably recognized (Fig. 4A, Supplementary Table 3). Based on the data it was possible to reconstitute the basic structure of ECM in prostate. Typical components of loose connective tissue collagen fibrils were present in all samples: Collagens I, III and V, fibril-binding proteoglycans decorin and biglycan as well as fibril-associated collagens XII and XIV. Similarly, typical basement membrane components were recognized: Collagen IV (α1 and α2 chains), laminins (α2, α4, α5, β1, β2 and γ1 chains, suggesting the presence of laminin-211, -411, -421, -511 and -521), nidogens (1 and 2), heparan sulphate proteoglycan 2 (perlecan), fibulin 1 and basement membrane-associated multiplexins (collagens XV and XVIII). Abundantly expressed ECM proteins also included beaded-filaments forming collagen VI (α1, α2, α3 chains), fibulins (1 and 2), fibrillin-1, dermatopontin, fibronectin, tenascins (C, XB) and proteoglycans lumican and osteoglycin (Supplementary Table 3).

Comparison of the *in vivo* matrisome to the ECM protein pattern produced *in vitro* by fibroblastic cells unveils major differences

Fifty-five ECM proteins were detected solely in *in vivo* samples. These polypeptides were considered to represent ECM proteins produced by cell types other than fibroblasts or genes suppressed by 2D monolayer culture conditions. This group contained proteins, such as fibril-associated collagen XII, anchoring fibril-forming collagen VII, dermatopontin and proteoglycans lumican and osteoglycin (Fig. 4C, Supplementary Table 3). However, most of the differentially present proteins were derived from plasma, namely fibrinogens (α, β, γ), plasminogen, β2-macroglobulin, coagulation factors, histidine rich glycoprotein etc. Thus, fibroblasts may still be the main source of *in vivo* ECM. Indeed, 65 ECM proteins that were produced by fibroblasts were also detected in *in vivo* samples (Fig. 4C, Supplementary Table 3). This number also included all the major basement membrane components that were recognized in at least some of the fibroblast cultures. The observation suggests that also the fibroblasts participate in the production of the basal laminas.

Eighty-six ECM proteins were recognized solely in *in vitro* samples, indicating that fibroblasts may undergo major changes in their gene expression pattern when cell cultures are established. These proteins included e.g. hexagonal lattices forming collagen α1(VIII) (recognized in all *in vitro* samples and 0/6 *in vivo* samples), elastin, COMP, thrombospondins (1 and 2) and fibrillin-2 (Supplementary Table 3). However, when the ECM proteins were divided into different categories, it was obvious that the major difference between *in vivo* and *in vitro* matrix was in the presence of ECM regulators (Fig. 4C). This group contains proteins, such as proteinases and their inhibitors, which participate in matrix remodeling and also growth factors and regulators of growth factor action (Supplementary Table 3). The proteins that were abundant in *in vitro* fibroblast matrix, but not recognized *in vivo,* included e.g. connective tissue growth factor, collagen triple helix repeat containing protein 1, ADAMTS (1, 2, 5 and 14), HtrA serine peptidase 1, tissue type plasminogen activator, fibroblast growth factor 2, insulin like growth factor 2, serpin peptidase inhibitor (clade

E) etc. Notably, tissue inhibitor of metalloproteinase 1 (TIMP1) was abundantly found *in vivo*, but not *in vitro*, whereas TIMP3 was found *in vitro* only. These observations are in agreement with the hypothesis that in *in vitro* cultures the fibroblastic cells are activated, they produce growth factors and intensively remodel ECM around them.

Matrisome in spheroid-type culture differs from the matrisome in *in vitro* monolayer and prostate tissue

To further study the effect of cell culture conditions on ECM production and composition we selected one fibroblast cell-line (patient 2 in Table 1, cancer location) and used, in addition to the usual monolayer culture, 3D spheroid cultures with and without DU145 prostate cancer cells (Fig. 5). ECMs produced in these conditions were compared (Supplementary Table 4, Fig. 6A). ECM produced by fibroblasts in monolayer shared 62 proteins with the matrix produced by the same cells in 3D spheroid culture, whereas more than 86 proteins were specific for the culture condition (Fig. 6A). The most different groups were ECM regulators, secreted factors and glycoproteins. Laminin isoforms α3 and γ2 and collagen VII were only recognized in spheroids. Interestingly, collagen VII anchors basement membranes to connective tissue. Thus, some of the prominent differences between 2D and 3D cells culture models seem to be relate to formation of basal laminas. Additionally the formation of collagen fibrils may be different, since lysyl oxidase (LOX) was found to be present in 3D cultures only.

Co-culture with DU145 prostate cancer cells only slightly changed the ECM produced by fibroblasts, since 98 of the identified proteins were common (Fig. 6B). Most notable changes were seen in ECM regulators, for example in the presence of metalloproteinases ADAM10 and ADAM17 and absence of TIMP-3 (Supplementary Table 4). Laminin β3 isoform was present in co-cultures only. Spheroid co-cultures of fibroblasts and prostate cancer cells have been used to mimic tissue-like conditions.

The results show that the basic matrisome produced by monolayer cultures of fibroblasts resembles ECM in prostate tissue, but is not identical. Fibroblasts activated by *in vitro* conditions express major changes in activity of ECM remodeling. Spheroid-type 3D cultures of fibroblasts resemble *in vivo* conditions, when the presence of the basement membrane associated collagens is studied, but in general, matrisome in spheroid culture did not resemble the *in vivo* ECM more closely than in monolayer culture. Co-culture of DU145 cells with fibroblasts only slightly changes the composition of ECM in spheroid culture.

DISCUSSION

Three hallmark proteins of the presumed prostate stem cells [4–8] are receptors for ECM components, such as hyaluronan, fibronectin, collagens and laminins. Since the ECM likely plays an essential role in the regulation of this crucial cell type, it is important to recognize their ligands at tissue level. The critical uncertainties related to specificity of antibodies [19] especially complicates the analyses focused on the expression of protein with complex molecular composition, such as collagen and laminin subtypes. Mass spectrometry and proteomics have been used to reveal the composition of ECM in many tissues, including lung [27], articular cartilage [28], colon [29], aorta [22] and breast [30]. Here, a similar analysis was performed to unveil the most abundant components in prostate ECM. The basic matrisome in prostate tissue suggested the presence standard collagen I fibrils, basement membranes and beaded-filaments (collagen VI). The presence of collagens XV and XVIII is also of interest, since endostatin, a proteolytic fragment of these collagens, may act as an endogenic inhibitor of androgen receptors [31]. We could not recognize collagen XIX or XXIII related peptides despite the fact that their presence has been suggested based on immunohistochemistry [32,33].

Glycoprotein dermatopontin and proteoglycans lumican and osteoglycin were abundantly in prostate ECM, but were not recognized in ECM produced *in vitro* by fibroblasts cultured in monolayers. Dermatopontin is a 22 kDa Tyrosine Rich Acidic Matrix Protein (TRAMP) known to interact with transforming growth factor beta [34] and also regulate the architecture of ECM

through acceleration of collagen and fibronectin fibrillogenesis [35,36]. Furthermore, dermatopontin may enhance cell adhesion mediated by α3β1 integrin [37–39]. In a transgenic mouse model, dermatopontin promotes the formation of intra-epithelial neoplasia in prostate [40]. Lumican is a small leucine-rich proteoglycan (SLRP), recently reported to play a restrictive role on primary prostate cancer progression [41]. Another SLRP, osteoglycin, is also a regulator of collagen fibrillogenesis and its expression is often decreased in malignant tumors including prostate cancer [42]. The fact that dermatopontin, lumican and osteoglycin were not recognized in ECM produced by neither spheroid nor monolayer cultured prostate derived fibroblasts leaved open the question of the *in vivo* origin of these ECM components. The three proteins may have significant influence on the collagen fibrillogenesis in prostate tissue and also influence on the α2β1 integrin mediated cell adhesion. Prostate stem cells are highly integrin α2β1 positive [4,6], and this receptor may also be essential for prostate cancer bone metastasis [43–45]. In addition to collagens, other prostate ECM proteins that may act as ligands for α2β1 include various laminins, decorin and endorepellin, the COOH-terminal domain of perlecan [46].

Despite the fact that the most basic components of ECM were also found in the matrix produced by prostate derived fibroblasts in cell culture, there were several differences when tissue matrisome was compared to that in *in vitro* conditions. The main differences were detected in proteins associated to matrix remodeling and growth factor action, which is in accordance with the hypothesis that in cell culture the fibroblasts are constantly activated. Indeed, we could see the presence of the activation markers also in cultures derived from the non-cancerous tissue areas. Furthermore, our data indicate that the *in vitro* production of periostin correlates with fibroblast activation. Periostin is suggested to be a marker of aggressive prostate cancer and also to regulate epithelial–mesenchymal transition [47–49].

In general our results suggest that the composition of ECM produced *in vitro* by prostate fibroblasts may be affected by many secondary factors and be functionally very different when compared to *in vivo* ECM. In the end, this is not surprising as fibroblasts are exposed to constant

cellular stress, migration and proliferation *in vitro,* compared to relatively "steady state" *in vivo*. We also unveiled the matrisome in 3D spheroid cultures containing prostate fibroblasts alone or in combination DU145 prostate cancer cells. ECM in spheroid cultures had differences in e.g. the expression of some basement membrane related proteins, but it was not possibly to claim that spheroid ECM would be significantly more "tissue-like" when compared to monolayers. When the presence of putative ligands for prostate stem cell adhesion receptors were compared, we noticed remarkable changes in specific laminin isoforms and collagen subtypes, again indicating that *in vitro* models have significant differences when compared to prostate tissues.

In addition to the analysis of the basic matrisome in prostate, we also compared fibroblastic cells derived from the non-cancerous areas to the potential CAFs. Such comparison may be affected by the fact that in prostate cancer the stromal activation seems to take place also outside the tumor area (50). Furthermore, it is not obvious what cells are actually selected by the cell culture conditions. There is some evidence, that fibroblast like cells derived from both non-cancerous area and tumors have mesenchymal stem cell and mesenchymal progenitor cell like properties (51). Still, we made an interesting observation that collagen III mRNA expression was significantly lower in tumor site derived cells. Decreased immunostaining for collagen III in prostate tumor when compared to benign areas has been reported [52]. Furthermore, data in Proteinatlas/Pathology [53] suggest very low expression levels of collagen III in prostate cancer. Thus our result may explain these previous *in vivo* observations. A recent paper has shown the restrictive role of collagen III in a mouse model of breast cancer [54], and indicated that collagen III may play an important role in the tumor microenvironment by suppressing metastasis-promoting characteristics, such as adhesion, invasion, and migration. Decreased amounts of collagen III in tumor stroma may also increase cell proliferation and decrease apoptosis [54]. However, it is too early to speculate, whether the same mechanism is relevant in prostate cancer.

CONCLUSIONS

To conclude, our results have unveiled the basic matrisome of prostate tissue. Furthermore, we report many similarities in the composition of ECM produced by prostate derived fibroblasts in cell culture, but also many important differences which should be taken into account studying the crosstalk between prostate cancer cells and ECM *in vitro*. The most fundamental differences were found to be related to the molecular mechanisms that remodel and organize ECM. Consequently *in vivo* and *in vitro* matrixes may have fundamental functional differences.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Characterization of primary prostate fibroblastic cell-lines established from patient-derived prostate tissue. (**A**) Examples of hematoxylin and eosin stained prostate tissue samples, obtained for establishment of fibroblastic cultures. Representative pictures show some variation in the ratios of epithelial and stromal compartments. High magnification images indicate the cancerous areas. Scale bars 1 mm and 100 µm for low and high magnification, respectively. P -patient, Ca - cancer, NC - non-cancerous. (**B**) Immunofluorescent staining of fibroblast cultures. All cells are highly positive for a-SMA (green) while a single cell is positive with Pan-Cytokeratin antibody (red). The nuclei were stained with DAPI (blue). Scale bar 20 µm. (**C**) Gene expression analysis of keratin 8 and 18 by q-PCR in primary human prostate fibroblast cell lines. (**D**) Gene expression analysis of fibroblast activation markers α-SMA and fibroblast activation protein (FAP) by qRT-PCR in human prostate

primary fibroblast cell lines. E, FAP mRNA levels correlated significantly with α-SMA mRNA levels within samples. r$_s$, Spearman's correlation coefficient; p, statistical significance.

Fig. 2. Fibroblasts produce functionally proper ECM *in vitro*. (**A**) Immunofluorescence analysis of structural ECM proteins in fibroblast cultures. Intact fibroblast culture as control and according lysed plates were immunostained with antibodies against collagen I and fibronectin. Scale bar 20 μm. (**B**) Representative SEM images of intact fibroblast culture and of cell-free ECM. Scale bar 10 μm. (**C**) Proliferation analysis of DU145 cells on collagen I (ColI), on fibronectin (FN) and on fibroblast derived matrix (FDM), n = 3, * p < 0.05 determined by Student's t-test.

Fig. 3. Analysis of mRNA expression levels of matrix proteins in primary prostate fibroblast culture. Relative mRNA expression levels of collagen I (**A**), collagen III (**B**) and periostin (**C**) measured by q-PCR. Correlation analysis of matrix protein mRNA expression levels with expression of activation marker α-SMA showed positive correlation between periostin and α-SMA mRNA levels (**F**); there was no correlation between collagen I and collagen III and α-SMA mRNA levels (**D - E**). r$_s$, Spearman's correlation coefficient; p, statistical significance, n.s., non-significant.

Fig. 4. *In vivo* matrisome of prostate differs from the ECM protein pattern produced *in vitro* by fibroblastic cells. (**A**) Characterization of the ECM protein categories of human prostate tissue analyzed by LC-MS/MS proteomics. Pie chart shows distribution of the ECM proteins in different ECM categories from non-affected areas of 6 prostate cancer patients. (**B**) Characterization of prostate derived fibroblast produced ECM composition by LC-MS/MS proteomics. The pie diagram shows the number of proteins in each subgroup Number of identified proteins from six non-cancerous tissue-derived fibroblast culture are shown. The proteins categorized as basement membrane (BM) proteins are not included in any other category. (**C**) Venn diagrams show the numbers of ECM proteins overlapping among the tissue ECM-enriched fraction and fibroblast

produced ECM of the six non-cancerous tissue-derived samples. The lower panel of pies shows group division of ECM protein recognitions found *in vivo* only, common ECM protein recognitions between prostate tissue and fibroblast produced matrix in monolayer and proteins recognized solely in prostate fibroblast culture produced ECM as monolayer *in vitro*.

Fig. 5. Representative images of DU145 cells grown alone or mixed with prostate fibroblasts in scaffold-free 3D spheroid culture system. (**A**) DU145 cells form rounded spheroids alone and mixed with prostate fibroblasts. Eight thousand cells/ spheroid were seeded into agarose molds to provide scaffold-free 3D environment and followed for 2, 24, 72 hours. In co-culture the ratio DU145 cells to fibroblasts was 1:2. Scale bar 100 µm. (**B**) Confocal images of fluorescently labelled DU145 cells (red) and patient-derived fibroblasts (green) in co-culture spheroids. Cells were stained using cell tracker dyes, seeded into spheroids at the 1:2 ratio, and the arrangement of cells was followed over 6 days. The middle z-slice images are shown. Scale bar 50 µm.

Fig. 6. Matrisome in spheroid-type culture. (**A**) The number of common and different proteins in fibroblast produced matrix in monolayer and 3D spheroid culture. Pie charts show the ECM proteins recognized by the groups. (**B**) The number of common and different proteins in matrix produced by fibroblast monoculture or by fibroblasts in co-culture with DU145 prostate cancer cells in 3D spheroids. The group division of proteins recognized in ECM is shown in pie charts. Fibroblasts from patient #2 used for these analyses.