

## Tetraspanin CD151 mediates communication between PC3 prostate cancer cells and osteoblasts

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**Invasion and migration of cancer cells are crucial for the formation of secondary lesions. These require activation of signalling cascades modulated by the number of regulatory molecules. One such molecule is CD151, a member of evolutionary conserved tetraspanin family. CD151 is involved in cell adhesion, motility and cancer progression due to formation of complexes with laminin-binding integrins and regulation of growth factor receptors function (e.g. HGFR, TGFβR, EGFR). Recent studies point to correlation between CD151 expression and high tumour grade in prostate cancer (PCa). Herein, we investigated a possible role of CD151 in communication between PC3 cancer cells and either cancer-associated fibroblasts (CAFs) or osteoblasts, an interplay which is significant for metastasis. The analysis showed that although CAFs strongly enhanced both migration and invasion of PC3 prostate cancer cells, the effect was not dependent on CD151. On the other hand, CD151 was found to promote 3D migration as well as invasive growth in response to osteoblasts-secreted growth factors. Obtained data revealed that knockdown of CD151 abolished activation of pro-migratory/pro-survival kinases (i.e. FAK, Src, HSP27) triggered by osteoblasts, along with expression of matrix metalloproteinase-13. This suggests that CD151 participates in communication between PC3 cells and bone microenvironment and the process can be considered as a significant step of PCa progression and metastasis.**

**Key words:** CD151, prostate cancer, osteoblasts

**Received:** 04 June, 2016; **revised:** 19 July, 2016; **accepted:** 26 August, 2016; **available on-line:** 14 March, 2017

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**Abbreviations:** α-SM-actin, α-smooth muscle-actin; CAFs, cancer-associated fibroblasts; ECM, extracellular matrix; FAK, focal adhesion kinase; FAP-1, fibroblast activation protein-1; hFOB, human fetal osteoblasts; HRP, horseradish peroxidase; HSP27, heat shock protein 27; MMP-13, matrix metalloproteinase 13; NHPF, normal human prostate fibroblasts; PCa, prostate cancer; shRNA, short hairpin RNA

### INTRODUCTION

Prostate cancer (PCa) affects 11% of male population mostly at its elderly age. Bone metastases in PCa contribute primarily to high mortality and morbidity of patients (Bubendorf *et al.*, 2000). It is accepted that the development of carcinoma is strongly influenced by its microenvironment. In primary PCa, stromal fibroblasts were shown to influence profoundly the transition from pre-invasive to invasive growth of tumor cells and are regarded as crucial and active participants of tumorigenesis and metastasis (Olumi *et al.*, 1999). After homing in

bone niche, tumour cells start to exert numerous paracrine interactions with hosting bone cells (osteoblasts, osteoclasts), bone marrow stem cells and hematopoietic progenitors. Such interplay leads to formation of osteoblastic (bone-forming) rather than osteoclastic (bone-resorbing) lesions in metastases, which indicates marked deregulation of bone formation (Roodman, 2004). Number of growth factors/regulatory peptides including bone morphogenetic proteins (BMPs), endothelins (ETs), fibroblast growth factors (FGFs) and others, were earlier correlated with increased osteoblastic activity (Logothetis & Lin, 2005). Despite detailed characteristics of PCa cells action towards bone microenvironment deregulation (Hall *et al.*, 2005), a reverse process, i.e. impact of bone microenvironment on PCa cells behaviour has not been fully understood.

Tetraspanins are characterized by the presence of four trans-membrane domains and are known to play crucial role in a number of physiological (proliferation, motility, cell adhesion etc.) and pathological conditions (tumour progression, metastasis) (reviewed in Sądej *et al.*, 2014). Tetraspanin CD151 was the first member of this family associated with cancer progression. CD151 was found to promote invasion and metastasis in various *in vitro* and *in vivo* models of cancer (Chien *et al.*, 2008; Kohno *et al.*, 2002; Sądej *et al.*, 2009; Sądej *et al.*, 2010; Testa *et al.*, 1999). High level of CD151 was proved to correlate with a poor prognosis for patients with several epithelial malignancies including lung (Tokuhara *et al.*, 2001), breast (Kwon *et al.*, 2012; Romanska *et al.*, 2015b; Sądej *et al.*, 2009; Yang *et al.*, 2008), liver (Huang *et al.*, 2010), colon (Hashida *et al.*, 2003), pancreas (Zhu *et al.*, 2011), and glioblastoma (Lee *et al.*, 2013).

In prostate cancer, the expression of CD151 was found to be significantly higher in poorly differentiated PCa specimens and had a better prognostic value for predicting clinical outcome of PCa patients than the traditional histological grading (Gleason grading) (Ang *et al.*, 2004). CD151 was shown to promote migration and invasion of PCa cells *in vitro* (Ang *et al.*, 2010) and development of metastasis in experimental animal models of prostate cancer (Copeland *et al.*, 2013; Detchokul *et al.*, 2014). Whilst contribution of CD151 to cancer progression has been well established, it was also reported that in certain cell types the presence of CD151 is associated with suppression of both cell motility (Chien *et al.*, 2008), tumour growth (Baldwin *et al.*, 2014) and patients survival (Romanska *et al.*, 2015a).

Functional interactions of CD151 with multiple proteins indispensable for cancer progression e.g. laminin-binding integrins (Kazarov *et al.*, 2002; Sterk *et al.*,

2002; Yauch *et al.*, 1998) or matrix metalloproteinases (Sugiura & Berditchevski, 1999; Yanez-Mo *et al.*, 2008) indicate mainly a modulatory role of this tetraspanin. Another feature of CD151 is associated with regulation of growth factors function, e.g. TGF- $\beta$ 1/R (Sadej *et al.*, 2010), soluble growth factors secreted by endothelial cells (Sadej *et al.*, 2009), HGF/R (Klosek *et al.*, 2009) and EGF/R (Baldwin *et al.*, 2008; Yang *et al.*, 2008). This allows the estimation of CD151 contribution in modulation of activity of receptors for growth factors secreted by tumor microenvironment cells. Although few papers reported CD151 role in PCa progression (Ang *et al.*, 2010; Copeland *et al.*, 2013; Detchokul *et al.*, 2014), the aspects of CD151 function in mediating signal from growth factors present in a niche of primary tumour and/or metastatic lesion and its consequences for PCa cells behaviour were never studied.

Herein, we focused on CD151 role in progression of PCa. Two distinct setups were assessed to determine CD151 role in communication between prostate cancer cells and primary tumour microenvironment - represented by cancer-associated fibroblasts - CAFs, or bone metastasis niche - represented by osteoblasts. PC3 cells derived from metastasis to the bone, therefore 'primed' to invade and target skeleton, were used as a model in the study. CD151-regulated growth, migration and invasion were studied. Although there was no CD151-dependent effect, we found a very strong promotion of prostate cancer cell migration following exposure to CAFs-conditioned medium. On the other hand, CD151 was involved in migration and invasive growth of PC3 cells in the presence of growth factors secreted by osteoblasts. This seems to be related to CD151-dependent activation of FAK, Src and HSP27 as well as expression of MMP-13. Therefore, CD151 function in regulation of prostate cancer cells metastasis to bones requires further studies.

## MATERIALS AND METHODS

**Cell lines, antibodies and reagents.** PC3 - human prostate carcinoma cell line derived from bone metastasis and hFOB - conditionally immortalized human osteoblastic cell line were purchased from ATCC. PC3 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100  $\mu$ g/ml, respectively). hFOB cells were cultured in 1:1 mixture of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium, with 2.5 mM L-glutamine (without phenol red), penicillin/streptomycin and 10% of fetal bovine serum. hFOB cells were grown either at 34°C (allowing rapid osteoblasts cell division) or at restrictive 39°C (resulting in cells differentiation towards more mature osteoblast phenotype). All media and their supplements were from Sigma-Aldrich. The following antibodies were obtained from Santa Cruz Biotechnology: anti-phospho-FAK (Tyr397) and anti-Src. Anti- $\beta$ -actin, anti- $\alpha$ -smooth muscle-actin, anti-CD151 (polyclonal), anti-FAP1 and anti-vimentin were from Sigma-Aldrich. Anti-phospho-Hsp27 (Ser82), anti-phospho-Src (Tyr416, produced by immunization of animals with a synthetic phosphopeptide corresponding to residues surrounding Tyr419 of human Src) and anti-MMP-13 were purchased from Cell Signaling Technology. Anti-CD151 antibody (5C11) used for cell sorting and flow cytometry was kindly donated by Dr. Fedor Berditchevski (University of Birmingham, UK). Secondary antibodies conjugated with AffiniPure DyLight 549 and AffiniPure DyLight 488 were from Jackson ImmunoResearch.

**Isolation of fibroblasts from clinical material.** Cancer-associated fibroblasts (CAFs) and normal human prostate fibroblasts (NHFPs) were isolated from adult patients (with advanced stage of PCa) who underwent radical prostatectomy at Clinic of Urology, Medical University of Gdansk (approved by the Ethics Committee of the Medical University of Gdansk). CAFs were derived from neoplastic tissues whereas NHFPs from adjacent non-transformed tissue (confirmed by pathologist). Specimens were sliced into about 1×1×1 mm fragments and enzymatically digested with collagenase type I (225 U/ml) and hyaluronidase (125 U/ml) (Sigma-Aldrich) in complete medium with overnight rotation at 37°C. Purified fibroblasts were used for further studies up to 6–8 passages. Conditioned medium was collected from fibroblast culture grown for minimum 2 days at high confluence.

**Knockdown of CD151 in PC3 cells.** CD151 silencing was performed with lentiviral transfer of shRNA targeting CD151 expression as previously described (Baldwin *et al.*, 2008; Sadej *et al.*, 2009) followed by cell sorting of CD151-negative population. Efficiency of knockdown was verified by Western blotting and flow cytometry analyses. As a control, PC3 cells were infected with virus carrying siRNA with a nonsense sequence.

**Immunostaining.** CAFs and NHFPs cells were fixed with 2% paraformaldehyde for 10 minutes and permeabilized with 0.1% Triton X-100 for 1 minute. After blocking with 3% BSA solution, cells were washed with PBS and incubated with specific primary antibodies. Antibodies against FAP1 (1:1000), vimentin (1:5000) and  $\alpha$ -smooth muscle-actin (1:500) followed by appropriate secondary antibodies (coupled with AffiniPure DyLight 549 or DyLight 488, Jackson ImmunoResearch) were used. Images were acquired with ZEISS AxioVision fluorescent microscope.

**Western blotting.** Cells grown up to 80–90% confluence were lysed with Laemmli buffer (2x concentrated) containing 2 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 5 mM EGTA, 2 mM Na<sub>4</sub>PO<sub>7</sub>, 5 mM NaF and 5 mM Na<sub>3</sub>VO<sub>4</sub>. Samples containing equal amounts of proteins per lane were loaded, resolved in SDS-PAGE and then transferred onto nitrocellulose membrane. The membranes were blocked in 5% skimmed milk and probed with specific antibodies overnight at 4°C followed by incubation with secondary antibodies conjugated with HRP (Sigma-Aldrich).  $\beta$ -actin was used as a protein load control. For signalling experiments cells were serum starved overnight and then stimulated with conditioned medium for indicated time points.

**3-Dimensional cell cultures.**  $1.5 \times 10^3$  cells were resuspended in 1:1 growth factors-reduced matrigel (BD Biosciences) and standard culture medium. 40  $\mu$ l drops were placed in 12-well plates, incubated for 30–40 minutes in 37°C until matrigel solidified and then covered with 800  $\mu$ l of medium. For feeding experiments, conditioned medium (CM) from CAFs or osteoblasts was mixed 1:1 with DMEM. Cells were cultured in conditioned media up to 7–8 days. For morphological analyses of colonies 3D pictures were taken using a Zeiss Primovert microscope coupled with a camera (AxioCam ERc 5s, Zeiss). At least 50 representative colonies were included in morphometric analyses. Experiments were performed in triplicates. Comparative data were analysed with the unpaired Student's *t*-test using the Statistica 7.1 software.

**Boyden Chamber Assay - analysis of cell transmigration or invasion.** To analyse cell invasion, the lower compartment of inserts (8- $\mu$ m pore size; BD Falcon)

was coated with 30  $\mu$ l of matrigel (diluted in DMEM to final concentration of 1 mg/ml). In case of cell transmigration studies, the porous membrane of insert remained non-coated. Cells were detached with enzyme-free dissociation solution.  $2 \times 10^5$  cells were dissolved in 400  $\mu$ l of serum-free DMEM and placed in upper chamber. Each well was filled with 1200  $\mu$ l complete DMEM or DMEM/conditioned-medium (1:1). Nuclei of cells which migrated or invaded through matrigel-coated membrane after approximately 24 h were stained with Hoechst 33342 (Sigma Aldrich). Cells were counted under the ZEISS AxioVision fluorescent microscope. Comparative data were analysed with the unpaired Student's *t*-test using Statistica 7.1 software. Differences for which  $p < 0.01$  were considered as statistically significant.

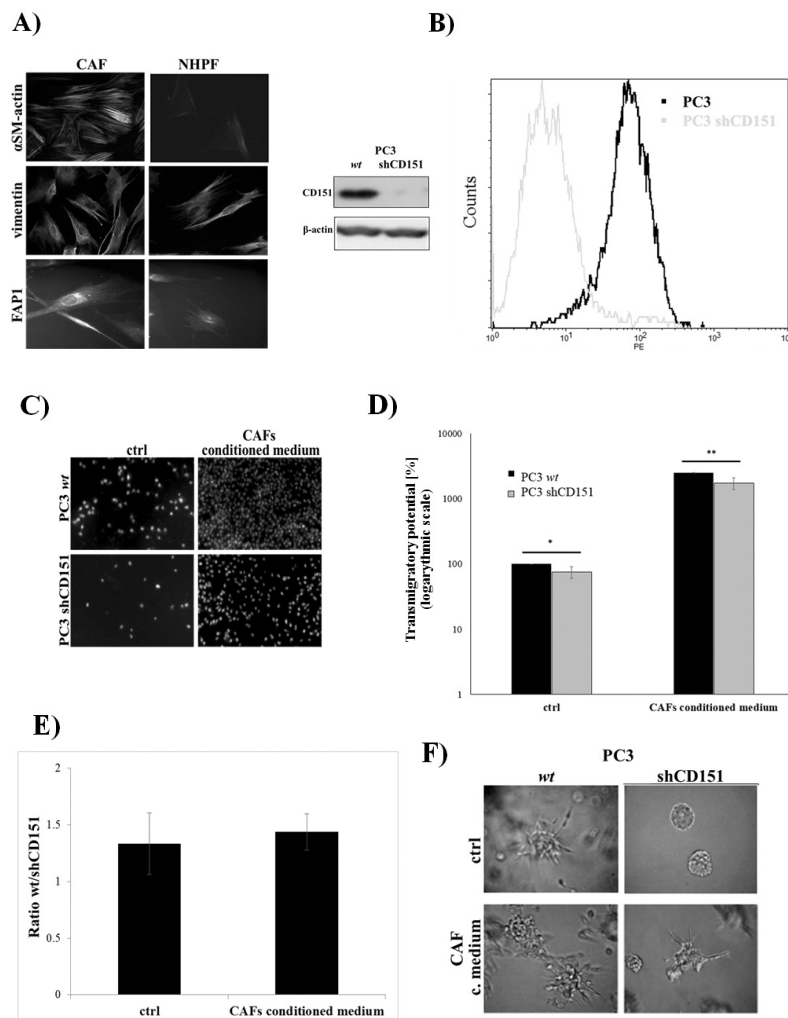
**Analysis of MMPs activity – zymography assay.** Conditioned medium was collected from cells grown for 48 h, centrifuged and supernatants were mixed with loading buffer (non-boiled, non-reduced samples). For a negative control, samples were dissolved in  $2 \times$  Laemmli buffer with  $\beta$ -mercaptoethanol and boiled at 90°C for 10 minutes. All samples were applied on 8% resolving gel supplemented with gelatin type B (0.1% w/v) and

separated in SDS-PAGE. Then gels were incubated in a renaturing buffer (containing 2.5% Triton X-100) for 90 minutes to restore native structure of matrix metalloproteinases and left overnight in developing buffer (50 mM Tris-HCl, pH 7.8, 0.2 M NaCl, 5 mM CaCl<sub>2</sub> and 0.02% Brij 35) which allows digestion of proteases substrates (37°C). To visualise activity of MMPs (i.e. cleaved gelatine) gels were stained in Coomassie Brilliant Blue solution (0.5%) followed by standard destaining procedure.

## RESULTS

### Tetraspanin CD151 mediates PC3 cells migration and invasion but is not involved in CAF-driven effects

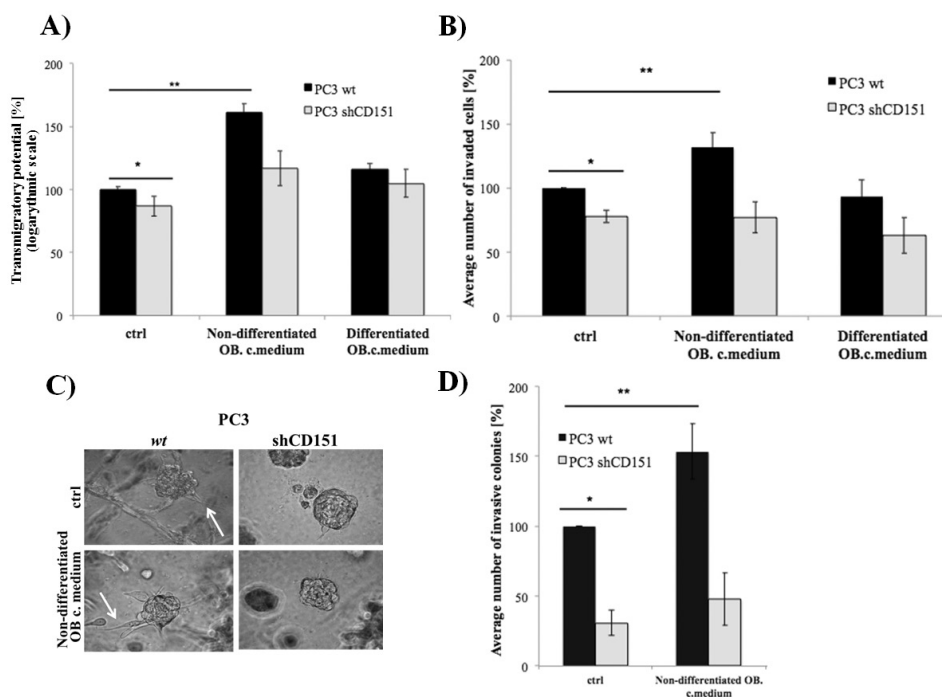
Majority of cancer-associated fibroblasts originate from 'normal' residing fibroblasts but also from bone marrow-derived circulating cells, myeloid precursors as well as myofibroblasts activated during the process of tumorigenesis (reviewed in Bhowmick *et al.*, 2004; Karagiannis *et al.*, 2012; Tommelein *et al.*, 2015). Cancer-asso-



**Figure 1.** Analysis of CD151 involvement in CAFs impact on behaviour of PC3 cells.

(A) CAFs and NHPF were isolated from PCa patients. Cells were immunostained for  $\alpha$ -SM-actin, vimentin and FAP-1 to verify their phenotype. (B) CD151 knockdown in PC3 cells was confirmed by Western blotting and flow cytometry. (C–E) Investigation of PC3 cells (wt vs. shCD151) transwell migration towards CAFs-conditioned media in Boyden chamber assay. Number of cells which migrated in control conditions was considered as 100%. Data are presented as the mean  $\pm$  S.D., \* $p < 0.01$ , \*\* $p < 0.005$ , (F) CD151-dependent PC3 cell growth in 3D matrigel  $\pm$  CAFs-conditioned medium.





**Figure 2. Analysis of CD151 involvement in osteoblasts impact on behaviour of PC3 cells.**

Boyden chamber assay was performed in order to analyse PC3 cells (wt vs. shCD151) transwell migration (A) ( $*p < 0.01$ ,  $**p < 0.005$ ) and invasion (B) ( $*p < 0.006$ ,  $**p < 0.001$ ) towards osteoblasts-conditioned media (non-differentiated and differentiated). Number of cells which migrated/invaded through the membrane in control conditions was considered as 100%. Data from three independent experiments are presented as the mean  $\pm$  S.D. (C) Cells were seeded in three-dimensional matrigel and cultured with/without hFOB (non-differentiated or differentiated)-conditioned media for 7–8 days. Representative pictures were taken under Zeiss Primovert Microscope (magnification 200x). (D) Quantification of invasive colonies number. Data from three independent experiments are presented as the mean  $\pm$  S.D.,  $*p < 0.001$ ,  $**p < 0.003$ .

ciated fibroblasts express all markers of myofibroblasts (including alpha SM-actin) (Bhowmick *et al.*, 2004; Karagiannis *et al.*, 2012). In the vast majority of papers the term 'CAF' involves all fractions of fibroblasts present in tumor's reactive stroma and is actually interchangeable with the term 'activated myofibroblasts' (De Wever *et al.*, 2008; Karagiannis *et al.*, 2012; Tommelein *et al.*, 2015). A series of excellent papers clearly suggests involvement of activated myofibroblasts/CAFs in PCa progression (Comito *et al.*, 2014; Liao *et al.*, 2010; Olumi *et al.*, 1999; Taddei *et al.*, 2014). To analyse impact of cancer-associated fibroblasts on motility and invasion of PC3 prostate cancer cell, a set of few primary fibroblast cell lines was established from cancerous and adjacent tissue samples taken from radical prostatectomies of PCa patients. CAFs were confirmed to express specific, previously reported markers (Garin-Chesa *et al.*, 1990) i.e. elevated expression of  $\alpha$ -SM-actin ( $\alpha$ -smooth muscle-actin) and FAP-1 (Fibroblasts Activation Protein-1) which distinguishes them from normal fibroblasts (Fig. 1A). The study itself was focused on elucidating the CD151 involvement in mediating communication between prostate cancer cells and cancer-associated fibroblasts – crucial contributors of the progression of the disease. To this end CD151-negative variant of PC3 cells was generated by transfer of specific shRNA. High efficiency of CD151 knockdown (over 95%) was assessed by Western blot and flow cytometry (Fig. 1B). Analysis of cell migration in control conditions showed that depletion of CD151 in PC3 cells impaired their transwell migratory potential by over 25% ( $p < 0.01$ ) (Fig. 1C–D). Importantly, CAF-conditioned medium (containing all secreted growth factors) had a profound impact on PC3 cells migration, giving over 35-fold increase in a number of migrated cells. Due

to tremendous promotion of cell migration in response to CAFs-conditioned medium, the results of experiments are presented in logarithmic scale (Fig. 1D). Noteworthy, calculation of PC3 (CD151-positive)/PC3 shCD151 cell number ratio revealed that depletion of CD151 reduced cell migration down to similar extent in both experimental setups (control *vs.* CAF-conditioned medium) (Fig. 1E). Further analysis demonstrated that PC3 cells formed invasive 3D matrigel-penetrating colonies and such phenotype was abolished by knockdown of CD151 (Fig. 1F). CAFs conditioned medium enhanced invasive properties of PC3 cells which was also impaired following CD151-silencing. Despite promotion of invasiveness, there was no significant impact on those colonies size or cells proliferation (in both 2D and 3D conditions, data not shown). The results clearly show that although CD151 participates in PC3 cells migration and invasion, it does not take part in those activities driven by cancer-associated fibroblasts.

#### CD151 facilitates osteoblast-driven PC3 migratory and invasive phenotype

Osteoblasts contribution to bone metastasis of prostate cancer was previously suggested (Roodman, 2004). PC3 cells transwell migration and invasion were analysed towards both non-differentiated and differentiated human fetal osteoblasts (hFOB)-conditioned media to determine CD151 function in putative osteoblasts impact on these activities. Firstly, we found that knock-down of CD151 affected not only cell migration but also cell invasion (Fig. 2A–B). Secondly, non-differentiated osteoblasts were shown to stimulate both transwell migration ( $\sim 1.61x$ ) and invasion ( $\sim 1.32x$ ) of PC3 cells (Fig. 2A

and 2B) in CD151-dependent manner. Surprisingly there was no influence of growth factors/cytokines secreted by differentiated osteoblasts on the behaviour of PC3 cells. Further analyses, focused on pattern of PC3 cells 3D matrigel growth, confirmed promotion of invasive phenotype by non-differentiated hFOB (Fig. 2C). Although colonies formed by PC3 cells displayed invasive protrusions, their length was greater in response to signal from osteoblasts (due to lack of differentiated hFOBs impact in transwell migration/invasion assays here we tested only non-differentiated hFOBs). In addition, the number of invasive colonies was larger following exposure to osteoblasts-conditioned medium (Fig. 2D). As expected CD151-silencing strongly affected impact of growth factors secreted by osteoblasts on PC3 cells type of growth (Fig. 2C and 2D).

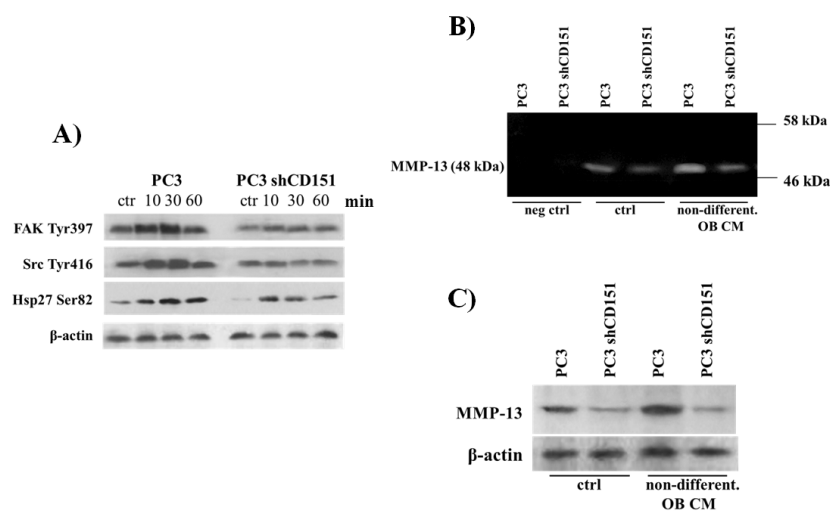
### CD151 is involved in regulation of promigratory and proinvasive signalling pathways in response to osteoblasts-secreted growth factors

Our next aim was to define molecular mechanism by which CD151 might promote migration or invasion following stimuli from osteoblasts. According to the literature, CD151 can influence growth factors-mediated communication with the tumour microenvironment only when it is in a complex with integrins (Deng *et al.*, 2012; Franco *et al.*, 2010; Sadej *et al.*, 2009; Sadej *et al.*, 2010). PC3 cells with various status of CD151 were exposed to hFOBs-conditioned media and analysed for activation of FAK, Src (main mediators of integrins-triggered signalling) and HSP27 (chosen due to profound contribution to PCa progression shown *in vitro*, *in vivo* (Voll *et al.*, 2014) and in clinical studies (Cornford *et al.*, 2000)). Under stimulation by non-differentiated osteoblasts-conditioned medium, a marked increase in phosphorylation of these promigratory kinases was observed (Fig. 3A). As expected, knockdown of CD151 strongly attenuated activation of FAK, Src and HSP27. FAK/Src are direct, fairly upstream mediators of integrin- and/or growth factor receptors-triggered signalling whereas HSP27 is a downstream effector of p38 kinase. Therefore it is not surprising that kinetics of FAK/

Src *vs.* HSP27 in CD151-dependent activation is different. CD151 involvement in regulation of these kinases following exposure to osteoblasts-conditioned media can be linked directly to the observed differences in cell migration (Fig. 2A). Invasive behaviour of cancer cells requires activity of matrix metalloproteinases, capable of degrading ECM proteins. These enzymes were found to be regulated by CD151 according to few studies (Hong *et al.*, 2006; Yue *et al.*, 2013). In order to examine contribution of CD151 to the control of matrix metalloproteinases activity upon osteoblasts stimulation, zymography assay was performed. We found that increased invasive behaviour of PC3 cells following their exposure to osteoblasts-conditioned medium correlated with the elevated activity of ECM-degrading matrix metalloproteinase (Fig. 3B). The obtained zymogram showed that although there was no bands associated with function of MMP-2 (72 kDa) or MMP-9 (92 kDa) (both shown to be regulated by CD151 (Yanez-Mo *et al.*, 2008; Zhang *et al.*, 2016)), a clear CD151-dependent digestive activity, located at molecular weight of approx. 48 kDa, typical for MMP-13 was observed (Fig. 3B). This was elevated following treatment with hFOBs-conditioned media, primarily in PC3 (CD151-positive) cells. Analysis of MMP-13 expression in PC3 *vs.* PC3 shCD151 cells revealed engagement of tetraspanin in regulation of MMP-13 level (Fig. 3C). Moreover, cells treatment with non-differentiated osteoblasts-conditioned media resulted in enhanced MMP-13 expression in CD151-dependent manner. Therefore we conclude that promotion of PC3 cells invasion in response to signal from osteoblasts depends on CD151-driven expression rather than activity of MMP-13.

### DISCUSSION

The occurrence of prostate cancer is estimated as the second most frequent disease in elderly male population. Despite enormous effort, which has been put in understanding the mechanism governing PCa development, the specificity of the routine prognostic approaches is still raising controversies (Collette *et al.*, 2006). Therefore, the primary aim of this study was to investigate



**Figure 3. Osteoblasts activate pro-migratory signalling pathway and expression of MMP-13 in CD151-dependent manner.**

(A) PC3 cells were starved overnight and stimulated with osteoblasts-conditioned media for indicated time points (10, 30, 60 minutes). Activation of specific proteins was analysed by Western blotting as described in Materials and Methods. (B) PC3 cells were seeded in equal numbers on 60 mm plates and incubated with osteoblast conditioned-medium for 48 h prior overnight starvation. Samples of media with secreted MMPs collected from cell culture media were analysed by zymography for proteolytic activity of MMPs. (C) Detection of MMP-13 in PC3 wt vs. PC3 shCD151 ± osteoblasts-conditioned media. All experiments were reproducible.

possible role of one candidate – tetraspanin CD151 in PCa progression and metastasis.

Previous reports suggested CD151 function in communication with tumour microenvironments at both primary tumour and distant metastases (Franco *et al.*, 2010; Klosek *et al.*, 2005; Sadej *et al.*, 2009; Sadej *et al.*, 2010). Herein we analysed CD151 involvement in the behaviour of prostate cancer cells exposed to growth factors secreted by CAFs (derived from primary tumour) or osteoblasts (representing bone niche). We found that silencing of CD151 impaired both migration and invasion of PC3 cells. On the other hand CAFs-conditioned medium vastly promoted cell motility and 3D invasion without obvious dependence on CD151 status (Fig. 1C–F). Therefore, we conclude that tumour fibroblasts undoubtedly promote prostate cancer cell migration and invasion (probably contributing to their escape from the primary site) however CD151 *per se* does not participate in these events. In the course of the study, the interplay between PC3 cells and osteoblasts (representing bone niche) was also analysed. There was no clear effect of differentiated osteoblasts on cancer cell migration and invasion which suggests lack of motility/invasion-promoting growth factors secreted by this pool of osteoblasts. On the other hand, non-differentiated, actively proliferating osteoblasts did enhance activity of both tested PC3 cells. Importantly, CD151 exerted clear growth and invasion promoting effect (at Boyden chamber and 3D culture systems) (Fig. 2A–D). CD151 was previously proved to regulate motility and invasion of PC3 cells (Ang *et al.*, 2010), however our finding suggests that these process can be additionally enhanced by osteoblasts-secreted growth factors in CD151-dependent manner. Certainly, it would be valuable to analyse CD151 function in response of androgen-sensitive PCa cell lines (e.g. LnCap). This approach could define a potential function of CD151 in early steps of PCa progression.

It was reported that promigratory function of CD151 in PC3 cells was based on regulation of ERK activity (Yang *et al.*, 2012). We additionally showed that presence of CD151 facilitates phosphorylation of FAK, Src and HSP27 (considered as directly involved in regulation of cell migration) in response to osteoblasts-conditioned medium. In addition, we analysed mechanism of CD151-promoted cell invasion following stimulation originating from osteoblasts. CD151-positive PC3 cells were characterised by higher activity of MMP-13 than CD151-negative cells and the difference was enhanced by osteoblasts action. We proved that it was based on CD151-regulated expression, rather than activity, of MMP-13. Limited information concerning osteoblasts influence on PC3 cells has been so far reported. This interaction was studied i.e. in mineralized human primary osteoblasts matrices which stimulated acquisition of invasive phenotype and proliferation of PC3 cells (Reichert *et al.*, 2010). Others showed that coculturing of both cell types in tissue-engineered bone construct elevated MMP-9 level (Sieh *et al.*, 2010). These data are in agreement with our studies showing osteoblasts-dependent promotion of prostate cancer cells migration and invasion and involvement of CD151 in regulation of these processes. The interdependence between CD151 and MMP-13 was previously shown in rat pancreatic carcinoma (Yue *et al.*, 2013). Our finding that CD151 participates in regulation of MMP-13 expression in response to bone niche stimuli, seems to be particularly important in the context of MMPs significance in extracellular matrix remodelling during formation of PCa metastases (Hornebeck *et al.*, 2002; Nemeth *et al.*, 1999).

Certainly, it would be tempting to identify growth factor(s) secreted by osteoblasts whose function is

regulated by CD151 in PCa cells. One of candidate is TGF- $\beta$ 1 proved to mediate chemotaxis of PC3 cells after treatment with osteoblasts-conditioned medium (Festuccia *et al.*, 1999). Although CD151 was shown to regulate TGF- $\beta$ 1-driven lung metastasis of breast cancer (Sadej *et al.*, 2010) we did not manage to identify CD151 involvement in TGF- $\beta$ 1 in osteoblasts-triggered PC3 cells migration or invasion (data not shown). This aspect i.e. a putative mediation of growth factors-based stimuli from bone niche by CD151 and subsequent consequences for metastasis requires further studies.

## Acknowledgements

The authors are grateful to Dr Fedor Berditchevski (University of Birmingham, UK) for antibody and plasmid sharing.

## Acknowledgements

This work was supported by research grants PRELUDIUM 3 (ID No. 2012/05/N/NZ3/00290) from National Science Center and HOMING PLUS (2010-2/12) from The Foundation for Polish Science.

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