The roles of RUNX2 and osteoclasts in regulating expression of steroidogenic enzymes in castration-resistant prostate cancer cells

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ABSTRACT

Intratumoral steroidogenesis is involved in development of castration-resistant prostate cancer (CRPC) as bone metastases. The osteoblast transcription factor RUNX2 influences steroidogenesis and is induced in CRPC cells by osteoblasts. This study investigates osteoclastic influence on RUNX2 in intratumoral steroidogenesis.

Steroidogenic enzymes and steroid receptors were detected with immunohistochemistry in xenograft intratumoral tumors from CRPC cells. In vitro, expression of RUNX2 was increased by osteoclasts in osteoblastic LNCaP-19 cells, but not in osteolytic PC-3. Silencing of RUNX2 downregulates expression of CYP11A1, CYP17A1 and HSD3B1 in LNCaP-19 cells co-cultured with osteoclasts, leading to inhibition of KLK3 expression. Osteoclasts promoted CYP11A1 and RUNX2 promoted AKR1C3, HSD17B3 and CYP19A1, but suppressed ESR2 in PC-3 cells.

This study shows that osteoclasts promote RUNX2 regulated induction of key steroidogenic enzymes, influencing activation of androgen receptor in CRPC cells. The potential of RUNX2 as a target to inhibit progression of skeletal metastases of CRPC needs further investigation.

1. Introduction

The skeleton is the major site for metastatic prostate cancer (PC), which is the main cause for morbidity and mortality from PC. While local PC can be readily treated with surgery or radiation, there is no curative therapy for metastatic PC. Androgen ablation in the form of medical or surgical castration limits the availability of testosterone, resulting in inhibition of metastatic growth. However, a castration-resistant form (CRPC) inevitably develops, leading to the high mortality of metastatic PC (Mohler et al., 2004; Nishiyama et al., 2004). Despite the recurrent growth under castrated conditions, a large proportion of CRPC still depends on activation of and signaling by the androgen receptor (AR). This is demonstrated by the increased levels of the AR driven prostate-specific antigen (PSA) in the circulation of relapsing patients, as well as the clinical response to further targeting of the AR pathway (Fizazi et al., 2012).

One resistance mechanism is the capacity of PC cells to synthesize testosterone in the circulation after androgen deprivation therapy (ADT). Increased intratumoral levels of testosterone and dihydrotestosterone (DHT) have been demonstrated in several studies (Montgomery et al., 2008; Titus et al., 2005) and the increased levels of steroidogenic enzymes converting precursors into androgens have been the proposed mechanism (Hoiland et al., 2010; Holzbeierlein et al., 2004; Jernberg et al., 2013; Stanbrough et al., 2006).

The relapse from ADT mainly happens at the metastatic site, and thus the microenvironment in the bone is likely to play a supporting role. PC metastasis in bone generally results in osteoblastic lesions with increased bone formation detectable with bone scintigraphy (Roudier et al., 2003a), in contrast to several other malignancies, such as breast and lung cancer, where bone degradation and osteolytic metastases dominates (Roodman, 2004). However, even if bone formation is the dominating phenotype of PC metastases, dysregulated bone resorption is prevalent, and lesions most often are heterogenic comprising both osteoblastic and osteolytic activity (Keller and Brown, 2004; Roudier et al., 2003b).

Abbreviations: ADT, androgen deprivation therapy; AR, androgen receptor; CRPC, castration-resistant prostate cancer; ER, estrogen receptor; PC, prostate cancer; PSA, prostate specific antigen; RUNX2, runt-related transcription factor 2.

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The phenotype of the bone metastases depends on the response of the bone cells to signals from the tumor cells, altering the balance between bone formation and resorption. Interaction with bone cells has also been demonstrated to promote PC growth (Fitzazi et al., 2003; Lee et al., 2003; Wang et al., 2009). A vicious cycle has been described in which osteoclasts degrade bone matrix, resulting in release of growth factors promoting osteolytic tumor growth, which in turn stimulate osteoclast development by secretion of receptor activator of nuclear factor kappa-B ligand RANKL (Clines and Guise, 2005; Vessella and Corey, 2006).

The bone microenvironment, like PC cells, is highly dependent on steroids for its function. Estrogen inhibits bone resorption by osteoclasts by shifting the balance of osteoprotegerin (OPG)/receptor activator of RANKL secretion from osteoclasts as well as limiting the number of tumor necrosis factor-producing T-cells (Eghbali-Fatourechi et al., 2003; Hofbauer and Khosla, 1999; Hofbauer and Schoppet, 2004; Munder et al., 2011; Reggia et al., 2001). Even if testosterone mainly serves as a substrate for the aromatization into estrogen catalyzed by CYP19A1, AR signaling in osteoclasts has been reported to play a role in the maintenance of the trabecular bone (Chiang et al., 2009; Notini et al., 2007).

We have previously demonstrated that osteoclasts increase the expression of steroidogenic enzymes in osteoblastic CRPC cells (HagbergThulin et al., 2016). Yang et al. recently showed that the osteoblastic transcription factor runt-related transcription factor 2 (RUNX2) mediates induction of the steroidogenic enzymes CYP11A1 and CYP17A1 in phosphatase and tensin homolog (PTEN)-deficient PC cells (Yang et al., 2018). Ectopic expression of RUNX2 has been shown to be a negative prognostic factor for tumors and is associated with the bone metastatic progression of breast cancer and PC (Barnes et al., 2004; Pratap et al., 2006). In PC cells, RUNX2 activates expression of bone matrix and adhesion proteins, matrix metalloproteinases and angiogenic factors that is strongly associated with PC metastasis (Banival et al., 2010).

The role of RUNX2 in experimental osteolytic bone metastases of PC is to promote osteolytic tumor growth together with metastasis-related genes such as matrix metalloproteinase 9 (MMP-9), vascular endothelial growth factor (VEGF), MMP-13, and secreted bone-resorbing factors such as interleukin-8 (IL-8) and parathyroid hormone-related protein (PTHrP) (Akech et al., 2010). However, the role of RUNX2 in bone-cell induced expression of steroidogenic enzymes in mCRPC cells is not well defined.

The capacity of tumor cells in bone metastases to synthesize steroids play an important role in CRPC development and drug resistance and may also influence the bone microenvironment. In the present study, we investigate whether osteoclasts influence steroidogenesis, and whether the function of RUNX2 in this aspect is affected, in both osteoblastic and osteocytic CRPC cells.

2. Materials and methods

2.1. Cell culture

LNCaP-19 is an in house osteogenic CRPC cell line developed from LNCaP that has been characterized previously (Gustavsson et al., 2005; HagbergThulin et al., 2014; Jemmabekken et al., 2006). Osteolytic CRPC PC-3 cells were obtained from the European Collection of Cell Cultures ECCC (Wiltshire, UK). LNCaP-19 and PC-3 cells were maintained in RPMI 1640 medium supplemented with glucose, sodium pyruvate medium and 10% charcoal-dextran stripped serum (CCS), Invitrogen) or 10% fetal bovine serum (FBS), Invitrogen). Osteoclast precursors, RAW 264.7 cells, were obtained from ATCC (ATCC TIB-71) (Rockville MD) and maintained in DMEM (ATCC® 30–2002™) with 10% FBS. All cultures were supplemented with 1% penicillin/streptomycin and confirmed mycoplasma free.

2.2. Formation of osteoclasts

Osteoclasts were transformed from murine RAW 264.7 macrophages by the stimulation of soluble RANKL (EMD Millipore GF091). The RAW 264.7 cells were seeded with 5 x 10^5 cells/cm^2 concentration in DMEM (ATCC 30–2002) with 10% FBS in 6 well plates, 10 ng/ml of sRANKL was added when seeding. Medium, as specified above, was changed with the same concentration of sRANKL every 72 h for 6 days. At day 7, cells were washed with PBS three times and fixed with 4% paraformaldehyde for 15 min. Mature osteoclasts were defined by tartrate-resistant acid phosphatase (TRAP) positivity. The staining was performed using the Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich 387A-1 KT), and positivity was verified with microscope (Fig. S1).

2.3. Co-culture of osteoclasts and prostate cancer cells

LNCaP-19 and PC-3 cells were co-cultured with osteoclasts using the Transwell permeable support (24 mm insert, 0.4 μm pore size, polyester membrane) in 6 well plates (Corning 3450, Kennebunk, ME, USA). Transwell inserts were pre-incubated with the culture medium for LNCaP or PC-3 cells, as specified above, for 24 h before seeding 3 x 10^5 cells/insert of PC-3 or LNCaP-19 cells on the membrane. After culturing for 36 h, the inserts with PC cells were moved into plates with mature osteoclast (after the formation procedure described above) in the lower compartments. RAW 264.7 cells was used in control wells. Both osteoclasts and RAW 264-7 cells were washed with PBS, and LNCaP-19 or PC-3 culture medium was added prior to the co-culture. Co-culturing was performed for 48 h after which cells were harvested for later analysis. All culture experiments were repeated at least three times.

2.4. siRNA transfection

Small interfering RNA (siRNA) targeting RUNX2 or non-silencing control siRNA (Silencer select, Applied Biosystems) were used to transfect LNCaP-19 and PC-3. Knockdown of endogenous RUNX2 and its effect on steroidogenic targets were examined after 48 h. Cells (1 x 10^5) were transfected using the Amaxa®Nucleofector® system using transfection reagents from LONZA (Cologne, Germany), according to manufacturer’s instructions. After transfection, cells were suspended in 500 μl fresh media and seeded in 6-well plates. For osteoclast stimulation, prostate cancer cells were suspended in 250 μl fresh media after the transfection and seeded in the Transwell permeable support wells with mature osteoclasts in the lower compartment and co-cultured for 48 h. In control wells, RAW 264.7 cells were used.

2.5. RNA isolation and reverse transcription

Total RNA was extracted from tumor cells after 48 h of bone cell stimulation using the RNeasy Mini Plus kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA concentration was measured on a NanoDrop (Thermo Fisher Scientific Inc, Wilmington, DE). A total of 1 μg RNA per reaction was reverse transcribed into cDNA using the Vilo Superscript cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions.

2.6. Screening for osteoclast and RUNX2 influence on androgen-related gene expression

To screen LNCaP-19 and PC-3 cells for changes in steroidogenesis related gene expression induced by osteoclasts and silencing of RUNX2, a human androgen gene signature array (Applied Biosystems; TaqMan® Array Gene Signature plate # 4418728) comprising 92 androgen-related genes and 4 endogenous control genes was used. The present study focuses only on the steroidogenic part of the array. Tumor cells were transfected with either siScramble (Ctrl) or siRUNX2 and stimulated by osteoclasts (OC) and compared to their control situation (C). In the
screen, one sample from each setting (Ctrl C, siRUNX2, Ctrl OC, and siRUNX2 OC) was analyzed. Five ng cDNA was used for the PCR reactions with the parameters set according to the manufacturer’s instructions. ΔΔCt values were calculated using GUSB and GAPDH as endogenous controls. If the fold change in relative expression between controls and osteoclast treated in each comparison was >2 the expression of the gene was considered changed.

2.7. Verification of expression changes with quantitative RT-PCR

Selected genes affected by RUNX2 silencing in the screen were verified with single TaqMan assays. The specific TaqMan gene expression assays (Applied Biosystems) were used in Supplementary Table 1 (S1). Gene expression analysis was performed on an ABI Prism 7500 Fast Sequence Detector (Applied Biosystems, Foster City, CA). The expression levels of each sample were normalized against GAPDH. PCR parameters were according to the manufacturer’s protocol, and the ΔΔCt method was used for relative mRNA quantification. PCR reactions were performed in duplicates for all samples, originating in at least three independent biological replicates.

2.8. In vivo intratibial tumors

The protocol was approved by the local Committee on the Ethics of Animal Experiments of Gothenburg (Permit Number: 11672/2019). Intratibial injections were performed as previously described (Back et al., 2020; Jennbacken et al., 2011). Briefly, Balb/c nude mice (6–8 weeks old; Charles River Laboratories International, Inc., Wilmington, MA) were anesthetized with isoflurane. The right leg was flexed and a 29-gauge needle was inserted via the knee joint, into the proximal end of the tibia. 1 × 10⁵ LNCaP-19 cells or 5 × 10⁵ PC-3 cells in 7 μl Matrigel (BD Bioscience, Bedford, MA) were injected into the bone marrow cavity. The mice were castrated directly before cell implantation. The experiment was ended after 8 weeks. For histological evaluation, the tibiae were dissected and fixed in formalin, decalcified in EDTA and embedded in paraffin.

2.9. Immunohistochemistry

Immunohistochemistry was performed using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA). Tissue sections (4 μm) were pre-heated at 60 °C for 10 min, deparaffinized and rehydrated in ethanol. For antigen retrieval and endogenous peroxidase blockage, sections were heated in unmasking solution (Vector Laboratories, Inc.) and incubated in 0.3% hydrogen peroxidase in methanol. Sections were blocked with normal serum (1.5% in PBS) and then incubated with primary antibodies at 4 °C overnight. The antibodies used are specified in Supplementary Table 2 (S2). The sections were incubated with biotinylated secondary antibodies (1/200 dilution in blocking solution) followed by ABC reagent. The peroxidase reaction coupled to the secondary antibody was visualized with diaminobenzidine. Finally, sections were counterstained with Mayer’s hematoxylin, dehydrated and mounted in Pertex. EDTA decalcified tibia was used as a control to formic acid-treated tibia. As a negative control, immunohistochemistry was performed in the absence of primary antibodies.

Evaluation of RUNX2 staining was performed using a semi-quantitative scoring system multiplying staining intensity and area of positive tumor cells. Intensity and area was scored as 0 to 5 and multiplied to give a value for five different microscopic fields per section. Five tumors per group were evaluated by two investigators independently, and a mean of those evaluations are presented (mean ± SEM).

2.10. Statistics

Statistical calculations were performed using the SPSSv20 software package (SPSS, Chicago, IL). Statistical differences between individual treatments were measured with Student’s t-test. All data are presented as mean ± SEM. A P-value of <0.05 was considered significant.

3. Results

3.1. Bone microenvironment alters RUNX2 levels

The expression of RUNX2 were investigated in LNCaP-19 and PC-3 cells. In vitro the mRNA levels of RUNX2 were lower in LNCaP-19 than in PC-3 (Fig. 1A). Osteoclast stimulation induced RUNX2 expression in LNCaP-19, while the RUNX2 expression in PC-3 was not significantly affected by osteoclast stimulation (Fig. 1B). Growing as intratibial tumors in vivo, LNCaP-19 forms osteoblastic lesions with extensive formation of trabecular bone, while PC-3 cells fill the bone marrow and have the capacity to degrade the cortical bone (Fig. 1C). In the intratibial model, RUNX2 was expressed in both LNCaP-19 and PC-3 xenografts (Fig. 1D), with the highest expression in PC-3 although the difference was not as large as in vitro, indicating that the bone microenvironment, including osteoclasts, increase RUNX2 expression in LNCaP-19 also in vivo. Nuclear staining of RUNX2 was most prominent in PC-3 (Fig. 1E).

In vivo, the steroidogenic enzymes CYP11A1 and AKR1C3 were strongly expressed in both LNCaP-19 and PC-3 intratibial xenograft tumors in castrated male nude mice. Aromatase (CYP19A1) was detected in both types of tumors, with a marked staining in osteoblasts in LNCaP-19 (Fig. 2A). AR was strongly expressed in LNCaP-19, but in PC-3, levels were negligible (Fig. 2B), as expected since PC-3 has previously been reported to lack AR expression and to be an androgen independent cell line (Buchanan et al., 2004). LNCaP-19 cells show negligible staining for estrogen receptor alpha (ERα (ESR1)), while a proportion of cells were positive in PC-3 tumors. In contrast, ERβ (ESR2) was strongly expressed in both tumor types (Fig. 2B). In addition, ERα was expressed in osteoblasts in LNCaP-19 tumors.

3.2. Osteoclasts induce differential expression patterns of steroidogenic enzymes in osteogenic and osteolytic CRPC cells

To compare the effects of osteoclasts on the androgen related gene expression in PC cells with different bone metastatic phenotypes, we performed a screening using an androgen signature array plate on osteoblastic LNCaP-19 cells and osteolytic PC-3 cells stimulated by osteoclasts.

Osteoclasts affected the majority of the steroidogenesis related genes in LNCaP-19, but up- or down regulation of the genes were evenly distributed, and no general effect of osteoclasts on expression levels could be observed (Fig. 3A). In contrast, steroidogenesis related genes were largely affected by osteoclasts in the osteolytic PC-3 cells, which responded to co-culture with osteoclasts almost exclusively with increased steroidogenesis related gene expression (Fig. 3B). These results contrast to previous data on osteoblast stimulation, where effects on PC3 were negligible and LNCaP-19 generally responded with increased expression of steroidogenesis related genes (HagbergThulin et al., 2016).

3.3. RUNX2 regulates expression of steroidogenic enzymes in osteoblastic LNCaP-19

Next, we wanted to elucidate the role of RUNX2 in regulation of gene expression related to steroidogenesis and steroid signaling. We transiently knocked down the expression of RUNX2 with siRNA in LNCaP-19 and PC-3 cells (Fig. 4A and B) and analyzed the transcriptome related to steroid metabolism using the same PCR-based array as a screen for induced changes. PC-3 and LNCaP-19 cells responded largely opposite to RUNX2 silencing, with an overall stimulatory role of RUNX2 in LNCaP-19 and a suppressive in PC-3 (Fig. 4C, E). In osteoclast-stimulated LNCaP-19, the pattern dominated by RUNX2 promoting gene expression was even more pronounced, with the exception of for example AR
and ESR2 (Fig. 4D). In osteoclast-stimulated PC-3 cells, the suppressive role of RUNX2 was not as distinct as in the control setting, with almost the same number of genes upregulated as downregulated by silencing of RUNX2 (Fig. 4F).

A subset of these genes was chosen for further analysis based on their expression pattern in the screening experiment and their function in steroid synthesis and signaling.

3.4. RUNX2 promotes expression of steroidogenic genes and AR activation in LNCaP-19 cells co-cultured with osteoclasts

Using single Taqman assays a selection of genes where assessed in replicate cultures to verify and quantify the differences indicated in the screening analysis focusing on the androgen signaling pathway. As indicated by the relatively weak effect seen in the screen, osteoclasts did not significantly promote expression of any of the steroidogenic genes in LNCaP-19, despite the osteoclast-induced increased in RUNX2 expression. However, the role of RUNX2 for steroidogenic gene expression was obvious, since levels of CYP11A1, AKR1C3, HSD3B1, and CYP17A1 all were decreased by silencing of RUNX2. For CYP11A1 (P < 0.01) and HSD3B1 (P < 0.001), this effect was specific for the osteoclast-stimulated situation in this full scale experiment, although not fully in line with the screening experiment, while AKR1C3 (P < 0.05) was regulated by RUNX2 in the control setting. RUNX2 affected CYP17A1 independently of osteoclasts (P < 0.05 and < 0.01) (Fig. 5A). AR was downregulated by RUNX2 silencing in the control setting (P < 0.01), while osteoclast stimulation overruled this effect. KLK3, the target gene for AR, encoding PSA, was promoted by osteoclasts (P < 0.01). Silencing of RUNX2 increased KLK3 expression in the control setting (P < 0.05), while AR activation was inhibited by siRUNX2 in co-culture with osteoclasts (P < 0.01) (Fig. 5B).

3.5. Osteoclasts and RUNX2 influence steroidogenesis in osteolytic PC-3 cells

In contrast to the osteoclast induced increase of RUNX2 in
osteoblastic LNCaP-19 cells, the existing high RUNX2 expression in osteolytic PC-3 cells was not further increased by osteoclasts (Fig. 1B). Nevertheless, expression of CYP11A1 was increased by osteoclasts (p < 0.05) in PC-3. This indicates a RUNX2-independent mechanism for this induction, supported by the unchanged expression levels after RUNX2-silencing. The expression of the enzymes more downstream in the steroid synthesis pathway, AKR1C3, HSD17B3, and CYP19A1 were all downregulated by silencing of RUNX2 in the presence of osteoclasts (all p < 0.05). HSD17B3 was downregulated by RUNX2-silencing also in the control setting (p < 0.01), while, in contrast, CYP19A1 expression was increased by RUNX2-silencing in co-culture with the control cells (Fig. 6A).

Since PC-3 cells do not express AR, an altered steroidogenesis may instead influence estrogen signaling in these cells. Neither RUNX2 nor osteoclasts affected expression of ESR1, encoding ERα. ESR2, coding for the estrogen receptor beta (ERβ), was more highly expressed after silencing of RUNX2 (p < 0.05) independent of osteoclast stimulation, indicating a general repressing role of RUNX2 on its expression (Fig. 6B). There was no significant difference in the ESR2 levels in the siRUNX2 treated cells in the control setting compared to the osteoclast stimulated situation. Neither the expression of estrogen receptors nor CYP19A1 were significantly affected by osteoclasts or RUNX2 in LNCaP-19 (data not shown).

To investigate the possible role of RUNX2 for the signaling by ERβ in PC-3 cells, possible effector genes downstream of ERβ was assessed. Generally, ERβ is viewed upon as a tumor suppressor in breast and prostate cancer (Cheng et al., 2004), which is in line with the observation of reduced Ki-67 expression together with silenced RUNX2 and increased expression of ESR2 (Fig. 6B).

4. Discussion

Increased intratumoral steroidogenesis is a resistance mechanism for PC metastases to evade tumor growth inhibition by therapies targeting AR activation and signaling. This paper points out the role of RUNX2 in regulation of expression of genes coding for steroidogenic enzymes in both osteoblastic and osteolytic CRPC cells. Specifically, our data indicates that RUNX2 plays an important role for modulating AR activation by promoting intratumoral expression of steroidogenic enzymes in osteoblastic CRPC cells stimulated by osteoclasts.

Both PC cells and bone cells are largely regulated by steroids. Besides the AR activating role of testosterone, its major role in the bone is as precursor for estradiol, the main anabolic hormone for the bone microenvironment (Chiang et al., 2009; Manolagas et al., 2013). Increased expression of steroidogenic enzymes is a common feature for CRPC (Montgomery et al., 2008; Stanbrough et al., 2006). Although dedifferentiated cases of PC may lose their AR, intratumorally
synthesized steroids may activate other steroid receptors, or be secreted to influence the bone microenvironment. The strong dependence of steroids for both bone and PC makes the interactions taking place in bone metastases of specific interest. ADT affects the bone microenvironment to promote a hospitable niche for PC to thrive, since the castration driven osteolysis results in release of growth factors supporting tumor proliferation (Michaelson et al., 2004). However, the contribution from the microenvironment in terms of influencing the intratumoral steroid synthesis has not so far drawn that much attention.

In this study, we observed that osteoclasts affect the gene expression of steroidogenic enzymes. The osteolytic CRPC cell line PC-3 was more responsive to osteoclastic signaling, compared to the osteoblastic cell...

Fig. 4. Screening of steroid related gene expression changes in LNCaP-19 and PC-3 in response to osteoclast stimulation and RUNX2 silencing. A, B) Relative expression levels of RUNX2 in cells treated with control siRNA (Ctrl) or siRNA for RUNX2 (siRUNX2) in co-culture with control cells (C) or osteoclasts (OC). C, D) Illustration of changes induced in LNCaP-19 by silencing of RUNX2 in the control setting (C), and with osteoclast stimulation (D). E, F) Illustration of changes induced in PC-3 by silencing of RUNX2 in the control setting (E) and with osteoclast stimulation (F). The horizontal lines divide the upregulated, downregulated, and not changed (NC) genes compared to the control setting in question, with a fold change in relative expression >2 defined as a change in either direction.

Fig. 5. Illustration of relative gene expression changes in LNCaP-19 by RUNX2 silencing. A) Expression changes of genes related to steroidogenesis in LNCaP-19 after silencing of RUNX2 in the control setting and with stimulation by osteoclasts. B) Expression changes of genes related to AR signaling in LNCaP-19 after silencing of RUNX2 in the control setting and with stimulation by osteoclasts. Data shown are mean ± SEM of samples analyzed in triplicates. To enable easier comparisons, expression levels of RUNX2 are duplicated from Fig. 4 without markers for statistical significance.*: P < 0.05, **: P < 0.01.
line LNCaP-19. Specifically, CYP11A1, involved in de novo steroidogenesis was induced in PC-3 by osteoclasts, indicating that intratumoral steroidogenesis may serve other functions than activating AR to overcome castration therapy since AR is absent in PC-3. The difference in response to osteoclasts between the two cell lines in this study is complementary to our previous observation that osteoblasts primarily affected the osteoblastic LNCaP-19 and not osteolytic PC-3 (HagbergThulin et al., 2016). However, even if direct effects of osteoclasts on LNCaP-19 steroidogenesis was not prominent, osteoclasts increased the expression of RUNX2, which in turned displayed a regulative function of steroidogenesis also in these cells.

RUNX2, or CBFA2, is a crucial transcription factor for osteoblast differentiation (Lian et al., 2004), but is often ectopically expressed in prostate cancer cells (Pratap et al., 2006). RUNX2 has previously been shown to regulate expression of enzymes involved in sterol- and steroidogenesis and was shown to bind to the promoter region of CYP11A1 indicating a direct transcription regulating function (Teplyuk et al., 2009). In PC cells, it has been shown that RUNX2 promotes the expression of both CYP11A1 and CYP17A1, emphasizing its role in intratumoral steroidogenesis (Yang et al., 2016). The induction of both RUNX2 and CYP11A1 in PC cells by osteoblasts has previously been demonstrated (HagbergThulin et al., 2016; Sieh et al., 2010), suggesting the important role for osteoblasts in the capacity of de novo steroidogenesis of CRPC growing as skeletal metastases. In the present study, RUNX2 was induced by osteoclasts in LNCaP-19 cells, without increasing the expression of CYP11A1 significantly. Together with the observation that osteoclasts induced CYP11A1 but not RUNX2 in PC-3, this points to other mechanisms influencing the association between RUNX2 and CYP11A1. However, osteoclasts still appear to influence the function of RUNX2 in this aspect, since silencing of RUNX2 resulted in a significant downregulation of CYP11A1 exclusively in co-culture with osteoclasts.

This specific role of RUNX2 for intratumoral steroidogenesis in co-culture with osteoclasts was also evident in LNCaP-19 cells, where expression of CYP17A1 and HSD3B1 were distinctly downregulated after silencing of RUNX2. The functional importance of this was supported by the efficient inhibition of the AR-regulated KLK3 expression by silencing of RUNX2, again only in the osteoclast co-culture setting, implying the inhibited steroidogenesis as the mediator. The mechanism behind the specific importance of RUNX2 for the expression of certain steroidogenic enzymes in tumor cells interacting with osteoclasts is not known. Intratumoral steroidogenesis has been identified as an important resistance mechanism to ADT, since the tumor cells will not be dependent on testosterone synthesized within the hypothalamic-pituitary-gonadal axis. The importance of intratumoral steroidogenesis is demonstrated by the good response to inhibition of CYP17A1 by abiraterone in approximately 60–70% of patients with CRPC (Ryan et al., 2013). Thus, targeting RUNX2 may be useful in further interfering with intratumoral steroidogenesis in treatment of metastatic PC and CRPC.

RUNX2 interacts with the AR signaling pathway on different levels which increases the complexity in interpretation of the results from the present study, as illustrated in Fig. 7. In the absence of altered expression of steroidogenic enzymes, the increased KLK3 expression observed in LNCaP-19 cells in co-culture with osteoclasts could possibly attributed to the increased levels of RUNX2 induced by osteoclasts, which may directly promote KLK3 expression by its promoter sequence (Fowler et al., 2006). In contrast, KLK3 was increased by RUNX2 silencing in the control setting, which possibly could be attributed to an inhibitory function of RUNX2 on AR activity. It is known that RUNX2 functionally interacts with AR affecting expression of its target genes, with both promoting and inhibitory effects reported (Baniwal et al., 2010; Kawate et al., 2007). More detailed studies demonstrated a locus-specific regulation of AR by RUNX2, indicating a repressive function of RUNX2 on AR regarding for example NKX3.1 and TMRPS2 (Little et al., 2014).

The comparatively different effects of osteoclasts and RUNX2 in the two cell lines in this study may reflect their different osteo-related phenotypes, but may also depend on the different AR-status of the cells. LNCaP-19, although being castration-resistant, expresses AR and is still responsive to androgens (Gustavsson et al., 2005), while PC-3 lacks AR expression and does not respond to androgens (Buchanan et al., 2004). In a similar manner as RUNX2 interacts with AR, AR has been shown to inhibit the activity of RUNX2 by direct binding and thereby preventing its localization to its target sequences in gene promoter regions (Baniwal et al., 2009). Thus, in LNCaP-19, the AR could moderate the effect of RUNX2, while in PC-3 RUNX2 acts without AR mediated limitation. Most reports suggest RUNX2 to promote metastasis in both PC and other cancer forms (Barnes et al., 2004; Pratap et al., 2006). Likewise, an experimental study inhibiting RUNX2 expression in PC-3 led to decreased invasiveness and survival in the bone microenvironment (Akech et al., 2010). However, recently loss of RUNX2 was detected in a large proportion of circulating tumor cells from patients with PC bone metastases, suggesting a selection pressure favoring low RUNX2 expression in metastatic cells (Gupta et al., 2020), possibly by the resulting more active AR.

The lack of AR-expression and the osteolytic phenotype of PC-3 present a different role of steroidogenesis and the interaction with bone cells compared to LNCaP-19. In contrast to in LNCaP-19, osteoclasts did not induce RUNX2 expression in PC-3, but RUNX2 still was highly expressed both in vitro and in vivo. Despite the general suppressive effect of RUNX2 on steroidogenesis-related gene expression in PC-3, it was shown to promote expression of specific steroidogenic enzymes. In addition to increased expression of AKR1C3 and HSD17B3, converting the adrenal androgen dehydroepiandrosterone (DHEA) into testosterone, RUNX2 also promoted expression of aromatase (CYP19A1) converting testosterone into estrogen in co-culture with osteoclasts. Since PC-3 is androgen-independent and lacks AR expression, the
importance of any steroids resulting from these steroidogenic enzymes is not as obvious as for LNCaP-19, but they may play a role in supporting the bone microenvironment or intratumoral signaling by other steroid receptors.

Although RUNX2 increase expression of several steroidogenic enzymes in PC-3, it suppresses the expression of ESR2 encoding ERβ. This specific suppression of ESR2, but not ESR1, by RUNX2 observed in our study, was in line with a study in breast cancer (Das et al., 2009). ERβ has been described as a tumor suppressor in breast cancer and PC (Cheng et al., 2004), and its expression decreases with PC progression, but is still frequently expressed in PC metastases (Lai et al., 2004; Leav et al., 2001). ERβ has been demonstrated to inhibit proliferation and induce apoptosis of PC cells both in vitro and in vivo (Chaurasiya et al., 2020; Tao et al., 2017; Zhou et al., 2018) as well as to inhibit epithelial to mesenchymal transition (Silva et al., 2018). In the present study, decreased expression of the proliferation marker Ki-67 was observed in PC-3 cells together with increased ESR2 levels after silencing of RUNX2. This supports a proliferative role of RUNX2 in PC-3, possibly mediated by suppressed ERβ, especially since decreased Ki-67 was not observed in the same pattern in LNCaP-19 cells, where ESR2 levels were not significantly affected by RUNX2 silencing.

Therapeutic targeting of PC metastasis is important to limit morbidity and mortality of the disease. Today, treatment with the alpha emitter radium-223 increases overall survival, and targets the bone building metastases by incorporation of the isotope in the bone tissue based on its similarities with calcium (Parker et al., 2013). Targeting of osteoclastogenesis by a monoclonal antibody towards RANKL has been shown to prolong the time to skeletal progression in PC patients (Smith et al., 2012). The present study indicates that interfering with osteoclasts may influence steroidogenesis, and possibly also the response to drugs targeting the AR signaling pathway. Further, the results supports the role of RUNX2 as a target to limit intratumoral steroidogenesis, in addition to the invasive and metastatic properties previously associated with RUNX2 (Pratap et al., 2006).

In conclusion, this study reports RUNX2 as an important mediator of steroidogenesis in CRPC cells with potential to influence castration resistant AR signaling. Its transcriptional function is affected by the interactions with osteoclastic stimuli and possibly by the presence of AR. Its promoting role in the expression of certain steroidogenic enzymes involved in steroidogenesis and androgen signaling makes it a putative therapeutic target for inhibition of intratumoral steroidogenesis. These findings need to be further explored.

CRediT authorship contribution statement

Junchi Huang: Investigation, Methodology, Writing – original draft, Writing – review & editing. Malin Hagberg Thulin: Conceptualization, Writing – review & editing. Jan-Erik Damber: Conceptualization, Supervision, Writing – review & editing. Karin Welén: Conceptualization, Formal analysis, Visualization, Resources, Supervision, Funding acquisition, Writing – review & editing.

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