Induction of integrin α₂ in a highly bone metastatic human prostate cancer cell line: roles of RANKL and AR under three-dimensional suspension culture

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Abstract

Background: Prostate cancer (PCa) bone metastasis can be markedly enhanced by increased receptor activator of NF kappa-B ligand (RANKL) expression in PCa cells. Molecular mechanisms that account for the increased predilection of PCa for bone include increased bone turnover, promotion of PCa cell growth and survival in the bone environment, and recruitment of bystander dormant cells to participate in bone metastasis. The current study tests the hypothesis that PCa cells acquire high adhesion to bone matrix proteins, which controls PCa bone colonization, under the RANKL/RANK and AR axes.

Methods: We used a highly bone metastatic RANKL-overexpressing LNCaP PCa cell line, LNCaP[RANKL], as a model to pursue the molecular mechanisms underlying the increased adhesion of PCa cells to collagens. A three-dimensional (3-D) suspension PCa organoid model was developed. The functions of integrin α₂ in cell adhesion and survival were evaluated by flow cytometry and western blot. AR expression and functionality were compared in 2-D monolayer versus 3-D suspension cultures using AR promoter- and PSA promoter-luciferase activity. AR role in cell adhesion was assessed using an adhesion assay.

Results: LNCaP[RANKL] cells were shown to adhere tightly to Coll matrix through increased α₂ integrin expression. This increased adhesion, concomitant with activation of the FAK and Akt pathways, was further enhanced by culturing LNCaP[RANKL] cells in 3-D suspension. Under the influence of 3-D suspension culture, AR was restored in LNCaP[RANKL] cells via downregulation of AP-4 transcription factor, and supported increased α₂ integrin expression and adhesion to Coll.

Conclusion: 3-D suspension culture and in vivo PCa tumor growth restore AR through downregulation of AP-4, enhancing integrin α₂ expression and adhesion to Coll which is rich in bone matrices. The interactions of PCa with Coll, mediated by integrin α₂ and AR expression, could be a key molecular event accounting for PCa bone metastasis.

Keywords: 3-D culture, Androgen Receptor, AP-4, Cell Adhesion, Collagen Type I, Integrin α₂, Prostate cancer
**Introduction**

Prostate cancer (PCa) has the highest incidence and is the second most common cause of cancer death among men in western countries [1]. The main clinical complication causing morbidity [2,3] and mortality in PCa patients is bone metastasis, which presents in over 80% of all men who die of PCa [4,5]. Despite the high occurrence of skeletal metastasis, the underlying molecular mechanisms determining the predilection of PCa cells for homing to bone are not well-understood. Previously, we hypothesized that the osteomimetic properties of PCa cells account for the predilection of PCa to metastasize and grow in the bone microenvironment [6]. We found that β-2 microglobulin (β-2 M), a major histocompatibility co-receptor, mediates the expression of non-collagenous bone matrix proteins such as osteocalcin and bone sialoprotein in metastatic human prostate cancer cell lines [7]. We found that upon the induction of β-2 M, prostate cancer cells overexpress RANKL, a protein intimately related physiologically to bone turnover [8]. RANKL drives PCa cells to undergo epithelial-to-mesenchymal transition (EMT) [9,10], and when expressed by human cancer cell lines like LNCaP RANKL, produces explosive skeletal and soft tissue metastases upon intracardiac administration in mice [11].

Overexpression of RANKL plays a role in the breast cancer osteolytic phenotype by binding to its RANK receptor on precursor osteoclasts [12]. Recent studies have shown that RANKL positively correlates with higher Gleason score in PCa [13] and predicts the survival of PCa patients [14]. Denosumab, an anti-RANKL antibody approved by the FDA for the management of osteoporosis and breast and prostate cancer bone metastasis, has been shown to improve or delay skeletal metastasis in breast and prostate cancer by 35% [15] and 18% [16], respectively. However, overall patient survival is not affected, indicating the critical roles of other potential factors affected by the RANK-mediated downstream signaling network in PCa bone metastasis.

Another important factor in the development and progression of PCa is androgen receptor (AR) [17]. AR has regulatory roles promoting PCa cell adhesion and survival in bone. PCa cells are initially androgen-sensitive (AS) and respond to androgen deprivation therapy (ADT) [17]. Overtime, while PCa cells remain AR positive, they progress to become androgen-insensitive (AI) and acquire increased invasiveness and metastatic potential [18,19]. AI tumors in hosts subjected to ADT become hypersensitive to residual intracrine androgen due in part to AR gene amplification, AR gene mutation, and/or higher AR regulating transcription factors (TFs) [20-22]. Recently, AR was found to induce cancer cell adhesion and survival through integrin expression [23-25]. Since AR plays a significant role in PCa metastasis, understanding how AR affects PCa adhesion to collagen matrix in bone could provide potential therapeutic approaches to block PCa bone homing and increase patient survival.

Multivariable tumor and microenvironmental factors are known to engage in tumor development and progression. Current 2-D monolayer culture lacks the relevant cell–cell and cell–matrix interactions that occur physiologically in the *in vivo* environment. This limitation makes it extremely difficult or potentially impossible to define the key cell signaling networks supporting essential cellular functions *in vitro* [26,27]. Extracellular matrix (ECM) mediates biological and physical cues external to the cell that result in altered cell proliferation, migration, invasion, and adhesion. Cell-ECM communication is initiated through the interaction of α- and β-integrin subunits to specific extracellular matrices [28,29] activating cell signaling pathways such as cell focal adhesion kinase (FAK) [30,31]. 3-D *in vitro* models have an invaluable ability to recapitulate some of the *in vivo* cell-cell and cell-ECM interactions governing tumor cell behavior [32,33].

In the present investigation, we used 3-D models to test the possibility that increased PCa adhesion to bone-derived ECM could promote PCa homing to bone. The objectives of this study were: 1) To investigate if RANKL overexpression promotes overexpression of integrins that support the adhesion of PCa cells to bone matrix proteins; 2) To determine if the levels of integrin expression are affected by growing PCa cells in 3-D suspension culture; 3) To determine if AR can be restored in RANKL-overexpressing LNCaP cells, and whether this restored AR modulates integrin expression/function to increase the growth, adhesion and survival of PCa cells in bone. To the best of our knowledge, we illustrated for the first time that overexpression of RANKL in human PCa cells induced dramatic upregulation of integrin α2 expression which facilitated the adhesion of PCa cells, specifically to collagen type I (ColI). We assessed and compared the adhesion of PCa cells to Coll in 2-D vs. 3-D culture, and determined the roles of FAK and Akt activation in PCa adhesion and survival. We further assessed the overall effects of AP-4, a newly identified regulator of AR, on cell adhesion to Coll via increased α2 integrin expression.

**Results**

Comparison of LNCaPNeo and LNCaPRANKL cell adhesion, integrated motility, and migration

Previous studies established that RANKL-overexpressing LNCaP or ARCaP cells metastasized to bone and soft tissues when inoculated intracardially [11,34]. We used the RANKL-transfected LNCaP cell line, LNCaP RANKL, to test the possibility that increased PCa cell homing to mouse skeleton could be due to increased cell adhesion and migration through a rise in integrin expression. We
determined differential adhesion, integrated motility, and migration between LNCaP<sub>Neo</sub> and LNCaP<sub>RANKL</sub> cells under 2-D versus 3-D growth conditions. Prior to the use of 3-D conditions, we extensively compared the pros and cons of culturing PCa cells under 2-D versus 3-D using different substrata consisting of Matrigel, Hydrogel, polymeric PLGA mesh, and suspension culture in the presence or absence of Coll. The morphologic features of PCa cells under 2-D and 3-D growth conditions and their pros and cons are presented in Additional file 1: Figure S1 and Additional file 2: Table S1. Based on these comparative studies, we concluded that 3-D suspension culture has the definitive advantages of simplicity, ease of expanding into large scale culture, low cost, and production of spheroid structures that can be easily handled for histopathologic and immunohistochemical analyses of the cultured cells. After these comparative studies, we compared the adhesion and migration of LNCaP<sub>Neo</sub> and LNCaP<sub>RANKL</sub> cells cultured in a 2-D monolayer rather than 3-D suspension. Figure 1A shows that LNCaP<sub>RANKL</sub> cells attached to the Coll and collagen IV (CollIV) extracellular matrices, better than LNCaP<sub>Neo</sub> cells. Results indicate that the higher adhesion of LNCaP<sub>RANKL</sub> cells to Coll-coated plates was further enhanced when they were pre-grown in 3-D suspension culture (Figure 1A; left panel). As expected, the increased adhesion of LNCaP<sub>RANKL</sub> cells to Coll can be antagonized by an anti-α<sub>2</sub>β<sub>1</sub> antibody, where a 55% reduction of cell adhesion to Coll was observed within 30 min

![Figure 1](http://www.molecular-cancer.com/content/13/1/208)
ColI-coated plates, when compared to LNCaPNeo cells, motility in 3-D mOBM containing ColI [35] than on 2-D that integrin file 4: Figure S3A). Using microarray data, we also found expression between LNCaPNeo and LNCaPRANKL cells revealed increased among the known receptors for ColI, 

Increased integrin α2 mediates activated phosphorylation of FAK and Akt in LNCaP RANKL cells under 3-D suspension growth

Among the known receptors for Coll, α2β1 subunits are shown to be specific to Coll and play a critical role in PCa [36,37]. Our preliminary comparative microarray analysis of 2-D monolayer grown LNCaPNeo and LNCaP RANKL cells revealed increased α2 integrin expression (Additional file 4: Figure S3A). Using microarray data, we also found that integrin α2 expression in LNCaP RANKL cells was further enhanced by subjecting LNCaP RANKL cells to 3-D suspension culture (Additional file 4: Figure S3A). mRNA and protein expression of integrin α2 was performed in each condition and confirmed the microarray data (Figure 2A, 2B). qRT-PCR analysis of cell embedded in suspension containing 0.1 mg/ml Coll suggests that higher expression of integrin α2 in cells could be further triggered in the presence of Coll (Figure 2A). These results were confirmed by FACS analysis comparing integrin α2 expression between LNCaPNeo and LNCaP RANKL cells cultured under 2-D monolayer and 3-D suspension conditions (Figure 2C). Quantitative analysis of FACS data revealed that integrin α2 expression of LNCaP RANKL/LNCaPNeo cells increased by 2.4 fold when cells were cultured in 3-D suspension as opposed to 2-D monolayer culture. FACS analysis did not show any significant changes in α1 and β3 integrin expression. Increase in integrin α2 expression appeared to be controlled by the RANKL/RANK axis, as the protein expression of RANKL correlates with integrin α2 expression (Figure 2B). This was further confirmed by using LNCaP RANKL cells with RANK knocked down. Disrupting the RANKL/RANK pathway resulted in reduced mRNA and protein expression of integrin α2 (Additional file 4: Figure S3B). Interestingly, the protein expression of RANKL of LNCaP RANKL cells grown in the 3-D suspension culture illustrates expression of the smaller band besides the total RANKL. This band could represent a soluble RANKL. In a parallel study using Elisa assay we have shown that soluble RANKL only increases by 7% in LNCaPNeo cells when compared 3-D suspension with 2-D monolayer. However, this difference increases to 30% in LNCaP RANKL cells. Higher soluble RANKL in 3-D suspension could be explained by potentially higher MMP7 expression in this condition, which is known to be responsible for the cleavage of RANKL [38]. Corresponding with the increased integrin α2, we also observed that LNCaP RANKL cells expressed higher levels of phosphorylated focal adhesion kinase (FAK) and phosphorylated Akt, when compared to 2-D monolayer (Figure 2D). Interestingly, under 3-D suspension conditions, LNCaPNeo parental cells showed slightly lower p-FAK expression, while Akt phosphorylation was significantly higher. These data in aggregate suggest that RANKL-expressing PCa cells grown in 3-D suspension have elevated cell adhesion and survival capability and this is likely mediated by activated α2β1 integrin.

Other ECM receptors have been shown to play a role in PCa invasion and migration including CollIV receptor, α1β1 [39], laminin receptors, α2β1 [40] and α6β1 [25], and fibronectin receptor, α2β3 [41]. We compared the microarray expression of α1, α3, α4, α5, β1, and β3 between LNCaPNeo and LNCaP RANKL cells. Other than increased integrin α2 expression, only integrin α6β3 showed significantly increased expression in 3-D suspension versus 2-D monolayer culture (Additional file 4: Figure S3A). However, we could not confirm the differential expression of this integrin by FACS analysis (data not shown). Additionally, we analyzed the cell surface expression of integrin α2 in the androgen-refractory PCa cancer cell line, ARCaP. Upon malignant progression, ARCaP M cells are known to express high endogenous RANKL [9] and fail to express functional AR [42]. ARCaP M cells were found to express lower levels of integrin α2 than indolent ARCaP E cells (Additional file 4: Figure S3C).

Restoration of AR expression in LNCaP RANKL cells in vivo and in 3-D suspension culture enhances cell adhesion to Coll

Since AR is diminished in metastatic LNCaP RANKL cells compared to their parental LNCaPNeo cells, we tested the possibility of restoring AR activity by growing LNCaP RANKL cells in vivo as tumor xenografts or 3-D suspension
cultures. As shown in Figure 3A, AR protein expression in LNCaP RANKL cells is undetectable when grown as a 2-D monolayer. IHC staining of AR revealed positive staining of LNCaP RANKL cells grown subcutaneously (Figure 3A). Further, AR IHC staining was also observed in 3-D suspension cultures of LNCaP RANKL cells and this was confirmed by Western blot (Figure 3A, 3B). The restored AR in LNCaP RANKL cells was shown to be biologically functional as revealed by increased PSA promoter luciferase activity in LNCaP RANKL cells (Figure 3C). PSA promoter luciferase activity was significantly elevated in LNCaP RANKL cells grown in 3-D suspension culture as opposed to 2-D monolayer culture. Because AR was shown to drive integrin α2 expression in PCa cells [23,43], we asked if restoration of AR expression in LNCaP RANKL cells, grown in 3-D suspension, enhanced cell adhesion to...
ColI LNCaP cells grown on 2-D monolayer or in 3-D suspension were treated with 10 nM R1881, an androgen agonist, or 10 nM R1881 plus an AR antagonist, Casodex (Bicalutamide, 20 nM). Cell adhesion to ColI was examined relative to plastic as a control. Under R1881 treatment, in either 2-D monolayer or 3-D suspension culture, LNCaP cells compared to plastic control was significantly higher by 4- and 9-fold, respectively. Casodex was found to block the adhesion of LNCaP cells to ColI by 1.3-fold in 3-D suspension culture. As expected, AR antagonist did not affect the ColI binding of LNCaP cells when grown in a 2-D monolayer because of the absence of detectable AR expression under this culture condition (Figure 3D). These results suggest that activated AR, in the presence of R1881 treatment, induces integrin α2 expression and is responsible for the increased adhesion of LNCaP cells to a ColI substratum.

Restoration of AR expression by downregulating transcription factor AP-4

Our previous publication using site-directed mutagenesis and transcription factor deletion/interference assays identified the suppressive action of AP-4 on AR expression [11]. We hypothesized that decreased AP-4 expression could contribute to increased AR expression in LNCaP cells grown in 3-D suspension. The qRT-PCR study of AP-4 and AR expression, in LNCaP cells grown in 3-D suspension or in LNCaP cells after transient transfection with AP-4 siRNA in 2-D monolayer, revealed an inverse relationship between AP-4 and AR expression. Unlike LNCaPNeo cells, LNCaP cells grown as 2-D
monolayer expressed higher AP-4 with corresponding lower AR. Upon AP-4 knockdown or in cells grown in 3-D suspension, AP-4 expression is reduced and this corresponds with increased AR expression (Figure 4A, B). AR restoration was confirmed by both Western blot and increased AR promoter luciferase activity by 1.6 fold (Figure 4E). In resemblance to AP-4 which induced EMT in colorectal cancer [44], AP-4 siRNA transfected LNCaP<sub>RANKL</sub> cells exhibited mesenchymal-to-epithelial transition (MET), a reversal of EMT biomarker expression and decreased cell invasion (Additional file 5: Figure S4A, B). Taken together, AP-4 could be the molecular basis of AR restoration in LNCaP<sub>RANKL</sub> cells cultured in 3-D suspension. Interestingly, however, enhanced AR expression by gene transfer into LNCaP<sub>RANKL</sub> cells did not affect AP-4 expression (Additional file 5: Figure S4C), suggesting that there is no established feedback loop between AR and AP-4.

**Discussion**

The bone environment is enriched with cytokines, growth factors, progenitor cells, and hematopoietic cells, providing a suitable metastatic microenvironment to promote PCa tumor cell adhesion, proliferation, migration, and survival. Despite this supportive microenvironment, cancer bone metastasis is a highly inefficient process and occurs infrequently in cancer patients [45]. However, 80% of all PCa metastatic lesions exist in the bone [4,5]. To understand the interactions between the tumor and its microenvironment, we engineered an indolent human PCa cell line, LNCaP<sub>RANKL</sub>. We examined LNCaP<sub>RANKL</sub> and ARCaP<sub>M</sub> cells, which endogenously expressed a high level of RANKL, for their metastatic potential to bone and soft tissues. The results consistently showed that RANKL drives these cells to undergo EMT and assume many characteristics considered as metastatic cancer cell phenotypes, including the expression of mesenchymal and stem cell biomarkers, neuroendocrine and osteomimetic properties [46], gaining the propensity for metastasis to bone and
soft tissues in mice [11]. Further, Chu et al. [11] showed that RANKL protein administered by the intra-peritoneal route can induce prostate cancer bone colonization in mice, confirming the importance of the pathophysiological role of RANKL as both autocrine and paracrine factor. In this study, we specifically examined the effects of the RANKL-RANK mediated signal network that drives PCa cells to express selected integrin isotypes favoring their adhesion to collagens, known to be rich in the bone microenvironment. Our work reveals the importance of the 3-D culture environment that determines integrin expression via functional AR and ultimately affects the pathophysiology of PCa metastases. The pathophysiologic significance of our findings is depicted in Figure 5. 1) RANKL/RANK signaling augments integrin α2 expression in RANKL-transfected LNCaP cells but not in ARCaP cells overexpressing RANKL intrinsically (Figure 2, Additional file 4: Figure S3). This observation could possibly be due to the nearly undetectable levels of AR expression in the ARCaP cell line [42].

Figure 5 Schematic summary of the role of RANKL-overexpression in promoting cancer cell adhesion. In LNCaP RANKL cells, RANKL overexpression induced the expression of α2 integrin and AP-4 transcription factor. The later may account for the suppressed AR expression. In 3-D suspension culture, α2β1 integrin was activated through RANKL expressed by PCa cells or soluble RANKL expressed by stromal cells in the bone microenvironment. This activation would elicit FAK and Akt phosphorylation, resulting in enhanced cell motility, adhesion and survival. α2β1 integrin activation was further enhanced through AP-4 downregulation, resulting in AR accumulation that could play a role in LNCaP RANKL cell adhesion to Coll. We propose a possible positive feedback loop (dotted line) between AR and integrin α2 regulation that is further enhanced under 3-D conditions to support cell anchoring and survival in the bone microenvironment.
other cell surface receptors, such as discoldin domain receptors [47,48], glycoprotein V1 receptor [49], leukocyte-associated Ig-like receptor [50] or mannose receptor [51,52], could be downstream targets of the RANK-mediated signal network that controls ARCaP	extsubscript{M} cell progression and metastasis by interacting with collagen matrices. 2) Consistent with the high bone metastatic behavior of LNCaP	extsuperscript{RANKL} cells, we have shown for the first time that integrin α2 expression is significantly enhanced in a 3-D suspension model in a RANKL-dependent manner (Figure 2). Exacerbated integrin α2 expression increases the binding of these cells specifically to Coll, the most abundant bone matrix protein (Figure 1). Their profound cell binding to Coll and migration can clearly discriminate indolent LNCaP	extsuperscript{Neo} and metastatic LNCaP	extsuperscript{RANKL} cell lines when cultured in 3-D suspension. Concurrently, we observed that anti-αβ1 antibody effectively antagonized LNCaP	extsuperscript{RANKL} cell binding to Coll matrix (Figure 1). Enhanced integrin α2 expression was shown to facilitate the adhesion and survival of PCa cells through activated FAK and Akt phosphorylation (Figure 2). High expression of integrin α2 in metastatic PCa and its important role in cell survival and adhesion in the bone microenvironment is supported by recent experimental and clinical publications [53,54]. 3) Concomitant with enhanced integrin α2 expression, we also observed that LNCaP	extsuperscript{RANKL} cells grown in 3-D suspension exhibited elevated functional AR expression, a result not seen in 2-D monolayer culture (Figure 3). It worth mentioning, while functional assay of AR on LNCaP	extsuperscript{RANKL} cells showed 4.3 fold increases in 3-D suspension/ 2-D monolayer, the fold difference in AR protein level seemed to be higher. However, we would not expect a linear relationship between AR and its responsive promoter-reporter activity due largely to the efficiency of AR and its accessory transcriptional factors binding to the promoters and also the efficiency of the translational machinery of proteins in cells that ultimately determine the promoter reporter activity. Moreover, inhibition of AR nuclear translocation by Casodetx treatment reduced LNCaP	extsuperscript{RANKL} cell adhesion to Coll (Figure 3), suggesting that LNCaP	extsuperscript{RANKL} cell adhesion through integrin α2 is potentially AR-dependent. Our data are in agreement with those of Nagakawa et al. [23] who showed that integrin α2 expression and Coll adhesion could be elevated by AR expression in an AR-transfected PCa cell line, DU145. In support of experimental studies, we used the publicly available human prostate cancer genome data listed in TCGA [55], to confirm a direct correlation between mRNA expression of AR and integrin α2 (Spearman’s correlation = 0.60, N = 302). Therefore, the adhesion of PCa cells in the bone microenvironment could be enhanced by modulating AR expression and function. While our study and others suggest that AR could regulate integrin α2, we were unable to find evidence that integrin α2 directly increases AR activity. Our studies of AR promoter did not reveal any binding sites for integrin α2. However, further studies are required to finally conclude whether integrin α2 could directly or indirectly regulate AR expression. 4) We further illustrated that AR restoration in LNCaP	extsuperscript{RANKL} cells under 3-D suspension condition is at the transcriptional level via downregulation of a key TF repressor, AP-4 (Figure 4). AP-4 overexpression converts the upregulation of c-Myc/Max in RANKL-overexpressing PCa cells [11] and drives EMT in colorectal cancer [44,56]. Similarly, downregulation of AP-4 with a concomitant increased expression of AR and integrin α2 in PCa cells results in the reversal of EMT and reduced PC invasion (Additional file 5: Figure S4). Because enhanced AR activity was frequently observed in clinically advanced PCa specimens [57,58], we hypothesize that enhanced AR expression in LNCaP	extsuperscript{RANKL} tumors could enhance the adhesion and survival of LNCaP	extsuperscript{RANKL} cells in mice. In agreement with clinical observations and the role of AP-4 downregulation, our preliminary data showed that LNCaP	extsuperscript{RANKL} cells overexpressing AR did have enhanced growth when inoculated as subcutaneous tumor xenografts in mice (data not shown). Further in vivo studies are warranted to determine if AR expression in LNCaP	extsuperscript{RANKL} cells confer increased α2 integrin expression and bone colonization through adhesion of PCa cells to collagen matrix in the skeleton.

Our work reveals the importance of the 3-D culture environment that determines integrin expression via functional AR and ultimately affects the pathophysiology of PCa metastases. Our significant findings are as follows: 1) The ability of PCa cells to adhere, survive and metastasize to bone could be masked by culturing PCa cells as a 2-D monolayer. We observed that RANKL-overexpressing PCa cells have barely detectable AR when cultured on plastic. When these cells were grown as 3-D suspensions or in mice, AR was found to be restored and to activate PSA promoter-luciferase activity. Additionally, we observed higher adhesion of LNCaP	extsuperscript{RANKL} cells to Coll in an AR-dependent manner, most likely through increased expression of α2 integrin. These results are consistent with the high levels of AR expression in clinical PCa bone metastasis specimens. 2) The TF repressor, AP-4, was found to be a negative regulator of AR at the transcriptional level and is modulated in a cell context-dependent manner. We speculate that AP-4 downregulation, epigenetically via promoter methylation or genetically via AP-4 regulators such as c-Myc, could play a decisive role in upregulating the levels of AR. This could profoundly control the responses of prostate tumors to androgen deprivation therapy. 3) Upregulation of integrin α2 may be a common path for human PCa to develop castration resistance and bone metastasis. Balasubramaniam et al. recently studied BAF57, a component of the switching-defective and
sucrose nonfermenting (SWI/SNF) chromatin-remodeling complex conglomerate [59]. They found that BAF57 deregulation circumvented androgen-mediated signaling, upregulated α2 integrin expression, altered other SWI/SNF complex components at the α2 integrin locus and conferred a prometastatic migratory advantage on PCa cells that could contribute to castration resistance and bone metastasis in patients [60]. Hall et al. [36] demonstrated experimentally that LNCaP cells selected for Coll binding exhibit higher integrin α2 expression, become more adhesive and migratory in *in vitro* and acquire the capacity to grow within the bone compared to non-collagen binding parental cells. These results are in agreement with work of Colombel et al. [54] who showed that higher α3β1 protein expression in primary PCa tissues correlates with bone metastasis. Additionally, Sotnik et al. [53] demonstrated elevation of α3β1 protein level in PCA skeleton metastases when compared to primary site or soft tissue metastases. These data along with our observations (Figure 2) suggest that Coll can re-program cell fates by forcing the expression of α2. Ultimately, through a RANKL- and α2-mediated downstream signal network Coll can transdifferentiate or reprogram non-metastatic PCa cells to gain increased adhesion, growth and survival potential in the bone microenvironment. More work is needed to build clinically relevant alternative cell signaling network models that could improve cancer diagnosis and prognosis and offer targets for therapeutic intervention.

**Conclusion**

The survival of patients with skeletal metastasis is very poor and more efficient prevention therapies are urgently needed. Our understanding of the role of the RANKL/RANK and AR axes in cancer cell adhesion is evolving. Our study supports direct regulation of integrin α2 and adhesion to Coll through RANKL/RANK signaling. Previous experimental and clinical studies in agreement with our data show the direct role of AR in Coll adhesion, possibly through integrin α2 expression. Our findings suggest that increased integrin activity enhances bone adhesion in a RANKL/RANK and AR dependent manner. Since there are studies supporting the regulation of AR through cell-ECM interaction, it is plausible that a positive feedback loop between AR and integrin α2 is induced under 3-D and *in vivo* conditions to support the growth and survival of PCa cells through activated p-FAK and p-Akt. We anticipate expanding the described 3-D suspension culture into co-culture models where relevant cancer cells and normal cells of different lineages can be constructed, studied and fully characterized. We believe that 3-D suspension culture and the co-culture of cancer cells with relevant cells in the tumor microenvironment could provide important additional insights into cancer plasticity and progression.

**Materials and methods**

**Cell culture**

**Cell lines and 2D culture conditions**

LNCaP human prostate cancer progression models were established by our laboratory as previously described [61]. The LNCaPNeo, LNCaP*RANKL* and LNCaP*RANKL* cells with RANK knockdown (LNCaP*RANKL*RANK KD*) cell lines were established by Chu et al. [11]. LNCaP was maintained in RPMI 1640 supplemented with 5% FBS. LNCaPNeo, LNCaP*RANKL*, and LNCaP*RANKL*RANK KD* cells were also maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 5% FBS with 200 ng/ml of Geneticin selector. ARCaP̂ and ARCaP̂ M cells established by our laboratory [34,42] were maintained in T-medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. MC3T3 cells (kindly provided by Dr. Neale Weitzmann, Emory University, Atlanta, GA) were maintained in DMEM supplemented by 10% FBS. All cells were incubated in 5% CO₂ atmosphere at 37°C.

**3D culture conditions**

**Hydrogel**

Hydrogel was prepared using the Hy Stem Hydrogel kit (Glycosan BioSystem Inc. CA) according to the manufacturer’s instructions. In brief, Hy Stem and Extralink solutions were prepared by dissolving the lyophilized solids in DG water under aseptic conditions (1% w/v). Extralink was added to the Hy Stem in 1:4 ratio. The final solution was then incubated for 10 min before encapsulating the cell pellet (10,000 cells/ml). 200 μl of the final solution was then plated in ultra-low attachment 24-well plates (Sigma) within 20 min of encapsulation for full polymerization in the 37°C incubator for 30 min before adding 1 ml complete medium per well. Collagen type I (BD Biosciences, 100 mg/ml rat tail) was added to the cell pellet at the 0.1 or 0.3 mg/ml final concentration (PH 7.0) before encapsulation, when specified. Medium was changed every 3 days by removing 500 μl/well of the medium and replacing it with 500 μl of fresh complete medium.

**Suspension**

LNCaPNeo and LNCaP*RANKL* cells, cultured on plastic, were trypsinized at the log phase, washed and resuspended to a final concentration of 20,000 cells/ml. One ml of the final solution was then plated in ultra-low attachment 24-well plates (Sigma) within 20 min of encapsulation for full polymerization in the 37°C incubator for 30 min before adding 1 ml complete medium per well. Collagen type I (BD Biosciences, 100 mg/ml rat tail) was added to the cell pellet at the 0.1 or 0.3 mg/ml final concentration (PH 7.0) before encapsulation, when specified. Medium was changed every 3 days by removing 500 μl/well of the medium and replacing it with 500 μl of fresh complete medium.
Mesh
Poly (D,L-lactide-co-glycolide) (PLGA) fiber sheets (Mesh) were kindly provided by Dr. Jurgen Groll, University Hospital, Würzburg, Germany [62]. Sheets 120 μm thick were trimmed to 8 mm circles using a disposable biopsy punch (Kia Medical, Inc) and placed in 48-well plates. Wells were washed with 70% EtOH and twice with 1X PBS. Plates were left under UV light for 30 min before use. LNCaPNeo and LNCaP RANKL cells were trypsinized at the log phase, washed and resuspended to a final concentration of 10,000 cells/ml. 500 μl of the final suspended cells were plated in each well and grown for 7 days before they were fixed or collected for further analysis. To study the Coll interaction with cells, mesh fibers were coated with 50 ng/μl of rat tail Coll (BD Biosciences).

Mouse osteoblast matrix (mOBM)
MC3T3-E14, mouse osteoblast precursor cells were grown on 12-well plates (VWR) for 10 days to beyond confluence. Cells were then treated with osteogenic medium (100 mmol/L dexamethasone, 10 mmol/L beta-glycerophosphate, and 0.05 mmol/L L-ascorbic acid-2-phosphate) for an extra 3 weeks with medium changes every 4 days, and then decellularized using 20mM of sterilized ammonium hydroxide (NH₄OH) for 30 min, and washed extensively prior to seeding the cells [35,63].

In vivo experiments
All animal procedures were performed according to an approved protocol from the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center. LN RANKL, LN RANKL-AR cells (2×10⁶ cells/100 μl PBS) were inoculated subcutaneously in 4-week-old male nude mice (Taconic, Oxnard, CA). All mice were followed for total of 45 days. Tumor volume was measured every 3 days.

Microarray data analyses for AR and integrin α₂ gene signature
To identify potential correlations between AR and integrin α₂ in human samples, we used a dataset that primarily included adenocarcinoma prostate cancer samples, the Cancer Genome Atlas (TCGA) dataset (n = 336). Expression data for the TCGA dataset was downloaded from the TCGA data portal (http://www.cbioportal.org/public-portal/index.do).

Cell morphology
Samples were fixed with 3.7% formaldehyde permeabilized with 0.2% Triton X-100 (Sigma) for 30 min and blocked with 5% Bovine Serum Albumin (BSA) (Sigma) for 1 hour. Samples were then washed with phosphate-buffered saline (PBS), pH 7.4 and incubated with 4′-6-diamidino-2-phenylindole (DAPI) (100 ng/ml, Invitrogen) and Alexa Fluor® 488 and 568 Phalloidin (4 μl/ml, Life Technologies) for 1 hr in the dark for nucleus and f-actin cytoskeleton staining, respectively. Phase-contrast images were then captured using Nikon Eclipse Ti (NIKON instruments Inc.). Organoids were placed on coverslip-bottom chambers (Lab-Tek) and fluorescent confocal images were captured using Leica TCS-SP5 Xconfocal microscopy (Leica Microsystems).

Microscopic live cell imaging and analyses
2×10⁴ cells/ml were seeded on 12-well plates coated with 50 ng/μl or decellularized mOBM wells. A Nikon Eclipse Ti inverted microscope equipped with an automotive x-y-z stage was used for multiposition and perfect focus system time-lapse microscopy. An environmental chamber was used to maintain humidity, 5% CO₂, and 37°C temperature. FITC and TRITC filters with a shutter control (Lambda SC, Smart Sutter Controller) and a CCD Head camera (Andor Technology) were used for fluorescent imaging. All imaging was performed using a 10× phase contrast (Nikon Plan Fluor Ph1) objective. All the images were also converted to TIF files for analysis of shape and integrated traveled using CellProfiler 2.1.0 (Broad Institute, Boston, MA).

RNA isolation and quantitative real-time PCR (qRT-PCR)
Total RNA from cells was isolated using a RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA concentration was quantified using the Nanodrop-2000 (ThermoScientific). Samples with a 260/280 ratio higher than 1.8 were used for subsequent procedures. Complementary DNA (cDNA) was generated from 1 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI), as instructed. 20 ng of cDNA was subjected to PCR analyses using an AB 7500 Fast detection system (Applied Biosystems, Foster City, CA) at 95°C for 10 min and 40 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, followed by a dissociation curve. The sequences of all primers used are listed in Table 1.

Western blot analysis
LNCaPNeo and LNCaP RANKL cells were cultured in 6-well plates under 2-D monolayer conditions to 70% confluence or in 3-D suspension conditions for 7 days. The cells were then pelletized and washed with PBS before being lysed in RIPA buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA and 25 mM NaF) containing 1× protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Samples were then centrifuged and the supernatants collected and quantified using the Bradford Protein Assay (Thermo Fisher Scientific, Waltham, MA). 20 μg of cell lysate were resolved on 4-15% Bis-Tris gradient SDS-PAGE (BioRad, Hercules, CA), followed by transblotting onto nitrocellulose membrane (BioRad, Hercules, CA).
The membranes were blocked in 5% non-fat milk in TBST for one hour at room temperature (RT) and incubated with diluted primary antibodies in blocking buffer at 4°C overnight. The primary antibodies used were AR (1:500), integrin α2 (1:500), β-actin (1:2000), AP-4 (1:500, Santa Cruz), FAK (1:1000, Abcam), p-FAK (1:1000), Akt (1:1000), p-Akt (1:2000), c-Met (1:1000), and p-c-Met (1:1000, Cell Signaling). The membranes were washed with TBST three times before incubating with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10000, Santa Cruz). The membranes were visualized using Kodak Image Station 4000MMPro instrument (AFAB Lab resources, Frederick, MD) and Carestream MI SE Network software. Images were cropped to improve the clarity of the figures. Each image is representative of two separate studies.

**Fluorescence Activated Cell Sorter (FACS)**

LNCaPNeo and LNCaP\textsuperscript{RANKL} cells were detached from 2-D monolayer using accutase (Millipore) to preserve membrane receptors. Organoids from 3-D suspension culture were made into single cells using a final concentration of 1 mg/10 ml collagenase in accutase and 20 min incubation at 37°C. Cells were then washed and resuspended into single cell suspension in 1 × PBS containing 1% FBS (FACS buffer). After two washes with cold FACS buffer, cells were incubated for 30 min on ice with FITC-tagged anti-human CD49a, CD49b, CD51/61 and PE anti-human CD29 (BioLegend) or isotype control FITC mouse IgG1, k (eBioscience). Antibodies were washed twice with FACS buffer. Cell fluorescence signals were determined immediately after staining using a BD Accuri C6 flow cytometer (BD Biosciences) equipped with an argon laser emission of 488 nm. FITC and PE were identified using a 530 ± 15 nm and 585 ± 20 nm band pass filter, respectively. The analysis was performed using FlowJo software (TreeStar Inc.). A primary gate was set excluding dead cells or debris based on physical parameters (forward and side light scatter, FSC and SSC, respectively).

**Adhesion assay**

The adhesion assay was a modification of a previously published protocol [64]. For each condition, 1×10^5 cells/ml were placed in 15-ml conical tubes and 10 μg/ml α5β1 blocking antibody (VLA-2 Millipore) or IgG1 isotype control (BioLegend) was added and incubated for 20 minutes at room temperature. Binding assays were performed by seeding 5000 cells in 100 μl of complete medium on plastic or fibronectin-α, collagen-IV-β, or collagen 1-precoated 96-well plates (BD Biosciences). At 30 min, 1-, 3-, 6-, 12- and 24-hr time points, wells were washed twice with PBS, 100 μl of complete medium was replaced, and 10 μl of alamarBlue (Invitrogen) was added, according to the manufacturer’s instructions. After 12 hrs incubation at 37°C in the dark, the plates were read using the Spectra max M2 microplate reader (Molecular Devices, Sunnyvale, CA) at 590 nm with Softmax Pro software. All the readings were normalized to the reading of the well with no cells as a background measurement. The initial activity of the cells was measured by adding 10 μl of alamarBlue directly to the well without washing the cells.

For cells treated with R1881 and/or Casodex, serum-starved medium with 5% dextran-coated charcoal was used instead of complete medium. Plates were washed at 30 min and 1 hr time points and read after 12 hrs of alamarBlue assay. Each condition was performed in triplicate and two independent experiments were carried out per condition.

**Immunohistochemical (IHC) analysis**

**Sample preparation**

IHC staining was applied to cells grown as in vitro 2-D monolayers. Cells were grown directly on 8-chamber slides to 80% confluence. In some cases, cells were grown as in vitro 3-D organoids. Organoids harvested from the 3-D suspension culture were carefully collected into 1.5 ml eppendorf tubes and spun down to a pellet. 1% LMP agarose solution was prepared and added to the pellet. After solidification, using a micro spatula, agarose-cell pellets were wrapped in tissue paper, placed in a plastic tissue cassette, and tissue processing was performed overnight using an automated tissue processor. For in vivo tissues, subcutaneous tumors were collected and fixed in 4% formaldehyde immediately for 24 hrs. The next day, tumors were processed for paraffin embedment as described above.

**Histology analysis**

IHC was followed according a previously published protocol [10]. All reagents from the DAKO system (Carpinteria, CA) were used for immunoperoxidase staining of the
sectioned slides. Paraffin-embedded slides were rehydrated and antigenic epitopes were retrieved in citrate buffer using a pressure cooker. After antigen retrieval, slides were blocked with dual endogenous enzyme block (DEEB) at RT for 10 min and incubated with primary antibodies against AR (Santa Cruz) at 4°C overnight. The slides were placed at RT for 1 h, rinsed in Tris-buffered saline with 0.05% Tween (TBST) and incubated with Envision + Labeled Polymer-HRP at RT for 30 min. The slides were incubated with peroxidase substrate buffer with a chromogen, diaminobenzidine (DAB), to detect the staining signal, followed by hematoxylin counterstaining of nuclei. After dehydration and cover-slipping, the slides were examined by light microscopy. For monolayer samples, slides were blocked without the peroxidase step.

**Transient transfection and luciferase reporter assays**

AR [65] promoter-luciferase plasmid DNA and control CMV-TK plasmid DNA (for transfection efficiency control) were transiently transfected into prostate cancer cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 48 hrs. The cells were then harvested and protein lysate extracted using 1× passive lysis buffer (Promega, Madison, WI). The lysate was centrifuged at 13,200 rpm at 4°C for 10 min, and the supernatant was collected for luciferase assay. Promoter and TK activity was measured using Dual-Glo luciferase assay, as instructed (Promega, Madison, WI). In short, 20 μl of protein lysate was mixed with 100 μl of substrate (luciferin) and luciferase activity was measured using a BD Monolight 3010 luminometer (BD Pharmingen, San Diego, CA). TK activity was measured by immediately adding 100 μl of Stop&Glo buffer with 50× Stop&Glo substrate to the mix and re-measuring the samples. The relative luciferase activity of each sample was calculated by normalizing to the TK activity. To assess PSA [66] promoter-luciferase activity, we followed the same procedure as above. In addition, cells were serum-starved for 24 hrs before treatment with either 10 nM ethanol or R1881 for another 48 hrs before harvest. Each condition was done in quartet and two independent experiments were carried out per assay.

**Migration and invasion assays**

Cell migration and invasion analysis were performed in a 24-well plates using Transwell™ chambers (BD Biosciences). As described previously, transwells were coated with collagen type I or growth factor reduced Matrigel (BD Biosciences) for migration or invasion assay, respectively. LNCaPNeo, LNCaP-RANKL-control and LNCaP-RANKL-AP-4 KD were serum-starved in RPMI 1640 overnight. The next day, transwells were placed on 24-well plates with 400 μl of complete medium. 100 μl of serum-free RPMI 1640 containing 5x10⁴ cells were seeded inside the chambers for 24 hr (migration) or 48 hr (invasion) at 37°C. At each time point, cells remaining on the transwell were fixed with 10% formaldehyde and stained with 0.5% crystal violet. Cells inside the chamber were cleared and remaining cells where quantified [67].

**In vitro healing assay and 3D migration assay**

24-well plates were coated with 50 ng/μl rat tail Coll and stored at 4°C overnight. Wells were washed twice with PBS before use. For the 2-D wound healing assay, cells were seeded in 24-well plates and cultured to 90% confluence. A straight scratch was made using a 1,000 μl pipette tip to simulate a wound. Wells were washed with 1X PBS to remove unattached cells. Wells were imaged at time zero and after 24 hrs using the 4x objective. Images were analyzed using ImageJ and the distanced traveled was measured. Three images were taken of each triplicate well for two independent experiments. For the 3-D migration assay, organoids were taken from 7-day suspension culture and placed on 24-well Coll-coated plates. Images were captured right after seeding and at 24, 48, and 72 hrs. The area covered by the cells was measured using ImageJ and compared between the two cell lines at each time point. The study was done in triplicate for two independent experiments.

**Cell transfection and transduction protocol**

LNCaP-RANKL cells were grown in 6-well plates to 60% confluence, then transfected with 100 pmol final concentration of AP-4 siRNA or control siRNA (Santa Cruz Biotechnology, Inc.) for 48 hrs, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Samples were collected for qRT-PCR and western blot analysis or further transfected with AR or PSA promoter for the luciferase promoter assay as described above.

For cell transduction, LNCaP-RANKL cells were grown in 48-well plates to 50% confluence 24 hrs before transduction. Next day, the complete medium was replaced with complete medium containing Polybrene at a 5 μg/ml final concentration. Cells were infected with AP-4 or control sh-RNA lentiviral particles (Santa Cruz) for 24 hrs. Cells were cultured for an extra 24 hrs before being split to 1:3 ratio. Cell selection was started after an additional 24 hrs with 2 μg/ml of Puromycin.

**Microarray analysis**

RNA was isolated as above, hybridized to human U133plus2.0 array, and Affymetrix Gene Chip Expression Analysis was performed (UCLA Clinical Microarray Core). The microarray data was first pre-processed with quantile normalization. Genes were selected based on Students T- tests with P <0.05 and fold changes >2.
Statistical analysis
Differences between groups were analyzed using Student’s t-test. A p-value < 0.05 was considered statistically significant (denoted by an asterisk). At least three independent in vitro experiments were conducted in triplicate for all assays and analyses, unless otherwise specified.

Additional files

Additional file 1: Figure S1. Morphological features of prostate cancer cells in 2-D monolayer and 3-D suspension cultures. The growth of RANKL-overexpressing LNCaP cells was evaluated in 2-D monolayer or in 3-D embedded in hydrogel, on polymeric meshes, and in suspension cultures, in combination with the addition of Coll. The control LNCaPshRNA cells formed massive spheroids with hollow lumens (not shown) and exhibited clear invadopodia in the presence of Coll. In comparison, LNCaPRANKL cells formed only loosely-aggregated organoids in 3-D suspension culture, but were mostly in dispersed growth in other cultures. DAPI staining is shown in blue, and F-actin staining is green in 2-D monolayer but yellow in Mesh or 3-D suspension culture.

Additional file 2: Table S1. Assessments of 3-D culture conditions. 2-D monolayer culture on plastic was compared with models of 3-D cultures in matrigel, hydrogel, Mesh, and in suspension. 3-D suspension culture was found to be superior in terms of biological relevance, sample production, and further molecular analysis, time and cost efficiency, and ease of operation.

Additional file 3: Figure S2. Transient differences in the adhesion of LNCaPshRNA and LNCaPRANKL cells to ECM proteins. Cells grown on a 2-D monolayer or in 3-D suspension were harvested in single-cell preparation. For each group, 5,000 cells were seeded on 96-well plates coated with Coll, CollIV, or FN. Adhered cells at different times of incubation were determined by alamarBlue assay. Each value is the mean ± SD of 2 independent experiments done in triplicate.

Additional file 4: Figure 3. Integrin expression was regulated by RANKL and by the 3-D suspension culture condition. (A) The expression of integrin isoforms was profiled by microarray analysis. Values represented fold changes in LNCaPshRNA cells compared to the LNCaPRANKL control. As shown in red, α2, α6, and β3 integrins had more than 2 fold increases when grown in 3-D suspension. (B) The expression of α2 integrin appeared to be dependent on the RANKL/RANK pathway, as reduced expression was seen by qRT-PCR and western blot when the pathway was interfered with RANK-knockdown (RANK-KD). (C) Top panels, human prostate cancer ARCaP and ARCaPshRNA cells grown on a monolayer were stained for α2 and α3 integrins for FACS analysis. Bottom Panel, quantification of the FACS detection suggested that α2 integrin expression was lower in the more aggressive cell line ARCaPshRNA compared to ARCaP cells.

Additional file 5: Figure S4. Suppressing AP-4 led to a reversal of EMT and a decrease in cell invasion. (A) LNCaPshRNA cells treated with AP-4 siRNA were studied for EMT markers at the mRNA and protein level. Upon AP-4 KD, vimentin expression was reduced while E-cadherin was increased. (B) LNCaPshRNA cells treated with AP-4 siRNA showed significantly decreased invasion potential, while no changes in migration were observed. (C) AR expression vector was used to express AR in LNCaPshRNA cells (LNCaPshRNA.ARx). No changes in AP-4 expression were found by qRT-PCR analysis, compared to cells transfected with an empty vector (LNCaPshRNA.EV).

Abbreviations
DAP: 4'-diamidino-2-phenylindole; ADT: Androgen deprivation therapy; AR: Androgen receptor; AP: Androgen-insensitive; AS: Androgen-sensitive; BSA: Bovine Serum Albumin; Coll: Collagen type I; CollIV: Collagen type IV; EMT: Epithelial-to-mesenchymal transition; ECM: Extracellular matrix; FN: Fibronectin; FACS: Fluorescence Activated Cell Sorter; FAK: Focal adhesion kinase; IHC: Immunohistochemical; MET: Mesenchymal-to-epithelial transition; mOBI: Mouse osteoblast matrix; PBS: Phosphate-buffered saline; PLGA: Poly (DL-lactide-co-glycolide); Mesh: PLGA fiber sheets; PCa: Prostate cancer; qRT-PCR: Quantitative real-time PCR; RANKL: Receptor activator of NF kappa-B ligand; 3-D: Three-dimensional; TF: Transcription factor.

Competing interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors’ contributions
SZ participated in the design of the study, carried out all the experiments, drafted the manuscript, and performed statistical analysis. LWC contributed to the design of the study and editing of the manuscript. Both authors read and approved the final manuscript.

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