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Betaglycan drives the mesenchymal stromal cell osteogenic program and prostate cancer-induced osteogenesis

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Abstract

Bone metastatic prostate cancer provokes extensive osteogenesis by driving the recruitment and osteoblastic differentiation of mesenchymal stromal cells (MSCs). The resulting lesions greatly contribute to patient morbidity and mortality, underscoring the need for defining how prostate metastases subvert the MSC–osteoblast differentiation program. To gain insights into this process we profiled the effects of co-culture of primary MSCs with validated bone metastatic prostate cancer cell line models. These analyses revealed a cast of shared differentially induced genes in MSC, including betaglycan, a co-receptor for TGF β . Betaglycan has not been studied in the context of bone metastatic disease previously. Here we report that loss of betaglycan in MSC is sufficient to augment TGF β signaling, proliferation and migration, and completely blocks the MSC–osteoblast differentiation program. Further, betaglycan was revealed as necessary for prostate cancer-induced osteogenesis *in vivo*. Mechanistically, gene expression analysis revealed betaglycan controls the expression of a large repertoire of genes in MSCs, and that betaglycan loss provokes >60-fold increase in the expression of Wnt5a that plays important roles in stemness. In accord with the increased Wnt5a levels, there was a marked induction of canonical Wnt signaling in betaglycan ablated MSCs, and the addition of recombinant Wnt5a to MSCs was sufficient to impair osteogenic differentiation. Finally, the addition of Wnt5a neutralizing antibody was sufficient to induce the expression of osteogenic genes in betaglycan-ablated MSCs. Collectively, these findings suggest a betaglycan–Wnt5a circuit represents an attractive vulnerability to ameliorate prostate cancer-induced osteogenesis.

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Introduction

Prostate cancer metastasizes to bone more frequently than any other tissue [1]. In the bone, prostate cancer cells induce a vicious cycle of excessive osteolysis and osteogenesis, and indeed the latter is a hallmark of metastatic disease [1, 2]. Further, pathological bone formation induced by prostate cancer is of poor quality, causes severe pain and increased risk of fracture, and significantly compromises patient quality of life. Current therapies to treat this debilitating condition are mainly palliative, and there remains a need for understanding the mechanisms of how prostate cancer progresses in bone to inform new and effective treatments.

In the tumor–bone microenvironment, bone metastatic prostate cancer cells hijack and subvert the homeostatic processes of bone turnover, and this aberrant remodeling worsens during tumor progression [3, 4]. In particular, prostate cancer cells can promote osteoclast maturation that leads to bone resorption and the release of bone matrix-sequestered growth factors, including transforming growth factor beta (TGF β), that support prostate cancer cell survival and growth [5, 6]. Further, we and others have shown that increased levels of TGF β in bone metastatic prostate cancer promotes the recruitment of bone marrow-derived mesenchymal stromal cells (MSCs), which then undergo differentiation into bone-forming osteoblasts and contribute to the aberrant osteogenic features of the disease [7-9].

TGF β and other members of the TGF β superfamily, such as bone morphogenetic proteins (BMPs) play key roles in the development and turnover of the skeletal system, where they control the balance between bone resorption and bone formation [10, 11]. For example, loss of function studies have shown that ablation of TGF β ligands or receptors compromises proper skeletal development [12, 13], and pharmacological agents that block TGF β can promote increased bone formation under both normal and pathological conditions [14, 15]. TGF β mediates its effects via binding to TGF β receptor II (T β RII) and dimerization with TGF β receptor I (T β RI), a serine/threonine receptor tyrosine kinase that leads to phosphorylation and nuclear translocation of SMAD transcription factors and to the transcription of TGF β target genes. A third receptor, betaglycan (T β RIII), contains two separate TGF β ligand-binding sites and a short cytoplasmic domain that has no kinase function. As such, betaglycan has been designated a TGF β co-receptor [16]. Interestingly, betaglycan has been shown to both positively and negatively regulate TGF β activity, where its opposing effects have been attributed to glycosaminoglycan modifications and/or proteolytic shedding [17].

Despite several studies regarding the roles of TGF β in bone metastatic disease [5, 6, 18-22], the role of betaglycan in metastatic prostate cancer-associated bone disease has not been explored. Here, we report that prostate cancer cells promote the differential regulation of several TGF β -related genes in MSCs, including a marked upregulation of betaglycan. Further, MSCs in prostate cancer patient bone biopsies express high levels of betaglycan, whereas there is little to no expression of betaglycan in adjacent prostate cancer cells. To assess the role of prostate cancer-induced betaglycan expression on MSC function and recruitment, we utilized CRISPR-mediated gene editing to ablate betaglycan expression in

bone marrow-derived MSCs. These studies revealed key roles for betaglycan in controlling the MSC osteogenic program and mechanistically linked these effects to the control of Wnt5a expression. Collectively, the data presented herein support targeting the betaglycan-Wnt5a circuit to ameliorate aberrant osteogenesis in bone metastatic prostate cancer.

Results

Prostate cancer cells induce betaglycan expression in MSCs

To identify factors through which prostate cancer cells regulate MSC behavior, we incubated primary human MSCs with conditioned media derived from human prostate cancer cell lines having bone metastatic capabilities, C42B and PC3-2M. After 6 h of culture, MSC mRNA was isolated from biological replicates ($n = 3/\text{group}$) and used to perform gene microarray analysis. Of the 227 genes regulated by conditioned medium from both C42B and PC3-2M cells (Fig. 1a), gene ontology analyses (PANTHER [23]) revealed significant enrichment of MSC biological processes associated with development, cell receptor signaling, growth and differentiation (Fig. 1b). Several members of the TGF β superfamily, including betaglycan, were up or down regulated compared to controls (Fig. 1c).

As betaglycan can positively or negatively regulate TGF β signaling [17, 24-27] and has not been evaluated in the context of prostate cancer, we explored its potential functions in MSCs. Initially, we validated betaglycan induction in MSCs by prostate cancer-conditioned media via qRT-PCR, which demonstrated an 8–10-fold increase in betaglycan expression in response to C42B-conditioned and PC3-2M-conditioned media, and a four-fold increase in expression in response to conditioned media derived from the bone metastatic rodent prostate cancer cell line, PAIII (Fig. 1d). Notably, immunofluorescence analyses established the clinical relevance of these observations, where there were high levels of betaglycan expression in CD90+ MSCs surrounding the prostate cancer cells in the majority (70%) of bone metastatic prostate cancer samples analyzed ($n = 10$, Fig. 1e).

Ablation of betaglycan in MSCs enhances TGF β signaling

To assess the role of betaglycan in MSCs, we ablated gene expression using CRISPR-Cas9 editing (SI Appendix, Table S1). Control (CTRL) and betaglycan knockdown (BGKD) clones were isolated, and two of five BGKD clones (4 and 5) exhibited complete loss of the full-length glycosylated protein (Fig. 2a). qRT-PCR analyses revealed that loss of betaglycan in MSCs was associated with a significant increase in TGF β 1 expression but with a significant reduction in TGF β 2 expression (Fig. 2b), the TGF β isoform that has the highest affinity for betaglycan. Similarly, betaglycan loss had no effect on T β RI expression but did lead to increased levels of T β RII expression in MSCs (Fig. 2b).

Canonical TGF β signaling directs the phosphorylation and nuclear translocation of SMAD transcription factors [28]. Although the addition of exogenous TGF β 1 significantly elevated pSMAD2 and total SMAD2 in both CTRL and BGKD MSCs (Fig. 2c), we noted slightly elevated levels pSMAD2 in the BGKD MSCs under baseline serum-free conditions (Fig. 2c). Normalizing to actin, we also consistently observed higher baseline levels of total SMAD2 in BGKD MSCs (>5 fold) compared to CTRL. Plasminogen activator inhibitor 1

(PAI1) is a well-known TGF β transcriptional target [29], and we confirmed the increased TGF β signaling capacity in BGKD MSCs using a PAI1 promoter-reporter assay (Fig. 2d). We also validated these effects using an shRNA approach, where stable knockdown of betaglycan expression also augmented TGF β signaling MSCs (SI Appendix, Fig. S1). Thus, betaglycan suppresses TGF β signaling in MSCs.

Betaglycan promotes MSC differentiation

We and others have shown that TGF β promotes MSC migration and proliferation [3, 8, 9, 30-33]. To test the effects of betaglycan on MSC motility, equal numbers of CTRL and BGKD MSCs were seeded into the upper wells of a modified Boyden chamber and allowed to freely migrate under serum-free conditions for 5 h. Analyses revealed that BGKD MSCs were three-fold more migratory than the CTRLs (Fig. 3a). Further, in long-term growth assays over 7 days, BGKD MSCs grew at faster rates compared to the CTRL MSCs (Fig. 3b). TGF β is also a key mediator of cell stemness [34, 35], and given the inhibitory role for betaglycan in TGF β signaling, we assessed the differentiation capacity of BGKD MSCs [27, 34]. Osteogenic assays using alizarin red as a readout for matrix mineralization demonstrated that BGKD MSCs were totally defective in MSC–osteoblast differentiation compared to CTRL MSCs (Fig. 3c). Again, similar effects of betaglycan on MSC migration, proliferation, and differentiation were independently validated using an shRNA approach (SI Appendix, Fig. S1). Finally, as measured by Oil Red O staining, BGKD MSCs were also defective in adipogenic differentiation compared to CTRL MSCs (Fig. 3d). Therefore, betaglycan is necessary for MSC differentiation into osteoblasts and adipocytes.

Betaglycan drives prostate cancer-induced osteogenesis

A hallmark of bone metastatic prostate cancer is aberrant osteogenesis, and this significantly contributes to patient morbidity. Given our in vitro data, we tested if MSC-derived betaglycan would impact prostate cancer-induced bone formation using the C4-2B model of osteogenic bone metastatic prostate cancer [30, 36]. Immunocompromised mice were intratibially co-injected with a 1:1 ratio of C4-2B:CTRL MSCs ($n = 6$) or C4-2B:BGKD MSCs ($n = 7$ per group). Mice were euthanized after 7 weeks of tumor progression, a time point at which the C4-2B cells have not yet breached the cortical bone [30, 37]. Tumor-bearing tibias were isolated and high-resolution μ CT scan analysis revealed prostate cancer-induced osteogenesis was greatly reduced in the BGKD MSC group compared to the CTRL MSC-inoculated mice (Fig. 4a). We observed significantly reduced bone volume:total volume (BV/TV) ratios and decreased trabecular number and thickness in the C4-2B-BGKD MSC group vs. the C4-2B–CTRL MSC cohort (Fig. 4a). No effect on trabecular spacing was noted. Histomorphometry analysis for collagen using trichrome stain validated these μ CT BV/TV results, demonstrating significantly lower BV/TV ratios in the C4-2B–BGKD MSC group compared to C4-2B–CTRL MSCs (Fig. 4b). Furthermore, tartrate-resistant acid phosphatase (TRAcP) staining of osteoclasts revealed increases in the numbers of multinucleated osteoclasts lining the tumor–bone interface in the C4-2B–BGKD MSC group compared to the C4-2B–CTRL MSC cohort, but this difference was not statistically significant (Fig. 4c). Finally, to validate these observations, these in vivo studies were repeated using the PAIII model of bone metastatic osteogenic prostate cancer. Although significant decreases in bone volume were observed via histomorphometry in the PAIII-

BGKD MSC group compared to controls, the effects of betaglycan ablation were less robust than those observed with the C4-2B model (SI Appendix, Fig. S2). This may be due to the rapid nature of the PAIII model (2 weeks to endpoint) compared to that of C4-2B (7 weeks to endpoint). Overall, these data are consistent with the notion that MSC-derived betaglycan promotes prostate cancer-induced osteogenesis.

Betaglycan controls expression of *Wnt5a* and Wnt signaling effectors in MSCs

To identify candidates through which betaglycan controls MSC differentiation, microarray analysis of CTRL and BGKD MSC cell lines cultured under normal serum-containing conditions was performed. These analyses revealed that ablation of betaglycan caused significant up-regulation and down regulation of genes associated with select biological processes, most prominently development and differentiation, as determined by heat maps and gene ontology analyses (Fig. 5a and SI Appendix, Fig. S3). Using a cutoff of greater than two-fold differential expression and positivity for at least two independent probe sets, we identified genes that were significantly up-regulated and down regulated in the BGKD MSCs compared to CTRL MSCs (Fig. 5b). Notably, *Wnt5a* expression was, on average, elevated to 63-fold in BGKD MSCs.

Given the interplay between TGF β and the Wnt pathway in controlling stemness [35, 38-40], the relevance of the significant up-regulation of *Wnt5a* in BGKD MSCs was explored. A marked, 60-fold increase in *Wnt5a* levels in BGKD MSCs was verified by qRT-PCR (Fig. 5c). Further, analysis of conditioned media confirmed the presence of high levels of *Wnt5a* in the culture of MSC BGKD whereas no *Wnt5a* was detected in the MSC CTRLs (Fig. 5d). Immunoblot analyses revealed both the short and long isoforms of *Wnt5a* were selectively expressed at high levels in MSC BGKD cells [41].

To test if the increased *Wnt5a* expression in MSC BGKD cells was due to increases in TGF β signaling, we incubated the MSC CTRL and MSC BGKD cells with a TGF β inhibitor, 1D11, which blocks the binding of all TGF β isoforms to T β RII [14, 15]. qRT-PCR analysis demonstrated that 1D11 treatment provoked a two-fold reduction in *Wnt5a* expression in MSC BGKD compared to MSC CTRL cells (Fig. 5e). Furthermore, consistent with increased *Wnt5a* signaling, *β -catenin* expression was also elevated in MSC BGKD cells vs. MSC CTRL cells (Fig. 5f). Interestingly, although there were no significant changes in the expression of Wnt receptors *Ror2*, *Lrp5*, or *Lrp6*, the expression of several Wnt signaling effectors was significantly upregulated in the MSC BGKD cells, including *casein kinase 1 epsilon (CSK1e)*, *adenomatous polyposis coli (APC)*, and *Axin* (Fig. 5g, h and (SI Appendix, Table S2)). Thus, betaglycan-dependent differentiation of MSCs is associated with its control of genes that regulate stemness, such as *Wnt5a* and Wnt signaling effectors.

A betaglycan-*Wnt5a* circuit controls MSC osteoblast differentiation

Wnt signaling limits osteoblast differentiation by suppressing genes required for osteoblast maturation and the deposition of extracellular matrix [42]. qRT-PCR analysis revealed significantly reduced levels of runt-related transcription factor 2 (*RUNX2*), type-1-alpha collagen (*COL1A1*), and osterix (*OSX*) in MSC BGKD cells (Fig. 6a-c). Notably, the addition of a *Wnt5a* neutralizing antibody restored *RUNX2* and *COL1A1* levels in MSC

BGKD to levels found in MSC CTRL cells (Fig. 6c). Furthermore, treatment of MSC CTRL cells with recombinant Wnt5a totally blocked their ability to mineralize the extracellular matrix cultured in osteogenic media, and in MSC BGKD cells (Fig. 6d), which are defective in osteogenesis (Fig. 3c, d). Thus a betaglycan-to-Wnt5a circuit controls the osteoblast differentiation program of MSC.

Discussion

A hallmark of bone metastatic prostate cancer is excessive bone formation, a phenomenon that is poorly understood relative to other tumor–bone microenvironmental processes such as osteolysis. Here we identified a mechanism of pathological bone formation by focusing on how prostate cancer cells regulate MSC function. We and others have previously found that the pleiotropic cytokine TGF β recruits MSCs to sites of active bone remodeling [8, 9, 30]. Here we observed that bone metastatic prostate cancer cells induce the expression of the Type III TGF β receptor, betaglycan, in MSC, and that betaglycan in turn promotes MSC osteoblast differentiation and the development of osteogenic lesions. Accordingly, CRISPR editing or shRNA knockdown studies revealed that betaglycan signaling is essential for osteogenic differentiation of MSCs. Finally, our expression analyses and functional studies revealed that a key mediator of betaglycan signaling in controlling MSC osteogenesis is Wnt5a, where betaglycan signaling represses the expression of Wnt5a and Wnt signaling effectors, and where blocking Wnt5a is sufficient to induce osteogenic gene expression in betaglycan-deficient MSCs, and treatment with Wnt5a blocks osteogenesis in control MSCs. Thus, a betaglycan–Wnt5a circuit is necessary for MSC osteogenesis.

Although not resolved here, it is plausible that TGF β itself produced by prostate cancer cells is responsible for inducing the expression of betaglycan in MSC, particularly since binding sites for SMAD3, SMAD4, and SP1 are present in the *betaglycan* promoter region [43]. Regardless, betaglycan has been shown to play both activating and inhibitory roles in TGF β signaling. This may in part be explained by post-translational modifications to this TGF β receptor, where glycosaminoglycan chain modifications alter the ability of betaglycan to sequester TGF β thereby regulating ligand interactions with T β RII. Germane to our work, recent studies have also shown that differences in the proteoglycan state of betaglycan can inhibit Wnt canonical signaling via the binding of Wnt ligands to heparin sulfate chains of the receptor. Therefore, ablating betaglycan in MSCs can lead to enhanced TGF β and Wnt signaling and, as a consequence, promote cell stemness [24].

Here, our data show that betaglycan is a regulator of MSC stem-like properties where genetic ablation augments TGF β signaling, cell motility, and proliferation, and totally blocks the ability of MSC to differentiate into osteoblasts. These findings establish roles for betaglycan in the biology of osteoblasts, where this receptor is highly expressed [44, 45]. In accord with our findings, betaglycan loss in mice provokes a lethal, diminutive embryonic phenotype that is associated with marked defects in skeletal ossification [46]. Interestingly, it is also notable that our studies revealed that betaglycan loss in MSCs leads to a significant reduction of TGF β 2. TGF β 2–null mice exhibit significant skeletal deformation as opposed to other TGF β isoforms, supporting a role for the TGF β 2–betaglycan signaling axis in skeletal development [47]. Furthermore, TGF β 2 has the highest binding affinity for

betaglycan and is known to induce betaglycan expression. This suggests that an interplay between TGF β 2 and betaglycan is potentially important in mediating bone development and the osteoblast differentiation program in MSCs [43]. In accord with this, a recent study demonstrated that betaglycan ablation prevented MSC commitment to osteoblast lineage in a mouse model of palatogenesis [48].

Notably the ablation of betaglycan in MSCs had profound effects on gene expression and leads to marked increases in the expression of Wnt5a, which is known to be induced by TGF β signaling [39]. Consistent with these findings, we observed that inhibiting TGF β signaling in the BGKD MSCs reduces Wnt5a expression [49, 50]. However, novel findings reported here are that inhibiting Wnt5a activity restores osteogenic gene expression and osteoblastic differentiation of BGKD MSCs, whereas treatment with recombinant Wnt5a blocks MSC osteogenesis. These findings are in accord with those showing that Wnt5a inhibits periodontal osteoblast differentiation and regulates the expression of bone-related genes [51]. Of note, however, Wnt5a has also been reported to promote osteoblast differentiation [52]. We suspect these differential effects may be due to the specific Wnt pathway node utilized and/or on the differentiation state of the MSC–osteoblast precursor at the time of exposure to Wnt5a. Further, the long and short isoforms of Wnt5a may also have differential effects on MSCs, and here it is notable that BGKD MSCs express both isoforms.

While our studies focused on Wnt5a given the interplay between TGF β and Wnt pathways in development, it should be noted that betaglycan loss led to differential regulation of several other genes having roles in the MSC osteogenic program. For example, betaglycan loss was associated with a marked down-regulation (500-fold on average) in the levels of the ABI gene family member 3 (NESH)-binding protein (*ABI3BP*), a key mediator of MSC differentiation [53], as well as a 150-fold repression on average in pleiotrophin, also known as osteoblast-stimulating factor-1 (*OSF-1*). Thus, betaglycan signaling is necessary for a cast of genes that control MSC osteogenesis.

Importantly, our studies establish betaglycan as a key regulator of prostate cancer-induced osteogenesis, where betaglycan loss in MSCs significantly reduces aberrant osteogenesis in the intratibial C42B and PAIII models of bone metastatic prostate cancer. Our findings demonstrate that bone metastatic prostate cancer cells express little to no betaglycan but induce its expression in bone marrow MSCs which in turn leads to increased bone formation. Interestingly, other reports have shown that decreased betaglycan expression in cancer cells drives stemness, progression, and metastasis indicating differential roles for betaglycan in the cancer and host microenvironment [25, 54, 55]. Based our studies, we hypothesize that the induction of betaglycan in host MSCs by prostate cancer-derived factors also significantly suppresses the expression of Wnt5a. Clinically, high Wnt5a levels in prostate cancer correlate with favorable prognosis and out-comes [56], and causal roles for Wnt5a in limiting prostate cancer cell growth in bone have been described. For example, forced Wnt5a expression in PC3 cells significantly reduces cancer growth in bone [56, 57], and the induction of Wnt5a expression in osteoblasts has been reported to inhibit metastatic prostate cancer to growth [56-59]. Thus, the induction of betaglycan expression in MSCs may not only promote an osteogenic response but also suppress factors, such as Wnt5a that inhibit prostate cancer cell growth. Collectively, these data suggest that agents that mimic

Wnt5a may be of therapeutic benefit administered alone or in combination with other targeted therapies. In this regard, the Wnt5a mimetic Foxy-05 reduces the invasion and metastasis of DU-145 prostate cancer cells in vivo [60] and is the subject of ongoing clinical trials (NCT02020291, NCT02655952). On the other hand, some studies have indicated Wnt5a contributes to prostate cancer disease progression, and Wnt5a expression in circulating tumor cells and in the bone environment correlates with development of castration-resistant disease [61, 62]. We speculate that the divergent roles for Wnt5a in prostate cancer reflects selective effects of Wnt5a isoforms. In particular, the short isoform (Wnt5a-S) augment cancer cell proliferation and is highly expressed in several cancers, whereas the long isoform (Wnt5a-L) suppresses cancer cell growth and is frequently suppressed in tumors [41].

Heretofore, how prostate cancers induce osteogenic responses is poorly understood. The findings presented herein explain this phenomenon, where we show that prostate cancer cells induce betaglycan signaling in MSCs and that a betaglycan-to-Wnt5a circuit is both essential for MSC osteoblastic differentiation and promotes prostate cancer-induced osteogenesis. Importantly, these findings support targeting this circuit for the treatment of bone metastatic osteogenic prostate cancer.

Materials and methods

Cell culture and patient specimens

Human MSCs were purchased from commercial vendors (Lonza, Cat# PT-2501) and kept at low passage. Mouse MSCs were previously isolated from bone marrow flushes of female FVB mice, as described [63] and characterized by flow (R&D Systems, #FMC003). MSCs stably transfected with CRISPR plasmids were cultured in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, platelet-derived growth factor (PDGF-BB; 10 ng/ml), and puromycin (5 µg/ml). For differentiation, MSCs were cultured with lineage-specific supplements for 3–21 days, according to protocol (osteogenic, adipogenic, chondrogenic assays; R&D MSC Differentiation kits #CCM009, CCM011, CCM006). For analysis of effects of Wnt5a on differentiation, osteogenesis assays were supplemented with recombinant Wnt5a (0.5 µg/ml; 645-WN, R&D Biosystems) or anti-Wnt5a (1 µg/ml; #AF645, R&D Biosystems). Luciferase-expressing PAIII and C42B prostate cancer cell lines were cultured in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and puromycin. All cell lines were periodically tested for mycoplasma (R&D, #CUL001B) and short tandem repeat (STR) verified at the Moffitt Molecular Genomics Core. De-identified tissue sections of bone metastatic prostate cancer were obtained from the University of Washington Rapid Autopsy Program (kind gift of Dr. Colm Morissey).

Microarray and gene expression analysis

MSC RNA was isolated from cells in serum-containing growth media using Trizol reagent, according to the manufacturer's protocol, and genomic DNA removed using the RNeasy MinElute CleanUp kit (Qiagen). 100 ng of total RNA was amplified and labeled with biotin

using the Ambion Message Amp Premier RNA Amplification Kit (ThermoFisher) [64]. Hybridization with the biotin-labeled RNA, staining, and scanning of the chips has been previously described [65]. The oligonucleotide probe arrays used were Affymetrix Human U133A 2.0 and Mouse 430 2.0 Arrays, which contain over >45,000 probe sets representing more than >39,000 transcripts. The array output files were visually inspected for hybridization artifacts and then analyzed using Affymetrix Expression Console v 1.4 using the MAS 5.0 algorithm, scaling probe sets to an average intensity of 500. Using the Affymetrix Transcriptome Analysis Console v3.0, a one-way unpaired ANOVA analysis was performed and differentially expressed probe sets with fold changes >2.0 and FDR adjusted *p*-values < 0.05 were retained. Gene ontology analyses were performed using PANTHER [23].

Intratibial prostate cancer studies

All animal experiments were performed with University of South Florida IACUC approval (R1762; CCL) and were conducted in accordance with the guidelines set forth in the *Guidelines for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Six-week-old male C57BL/6 Rag-2 or SCID Beige mice were injected intratibially with luciferase-expressing PAIII or C4-2B cell lines (5×10^4 or 5×10^5 , respectively, in 20 μ l of saline) in a 1:1 ratio with either scrambled control (CTRL; *n*=6) and betaglycan CRISPR ablated (BGKD; *n*=7) clonal MSC populations. Contralateral limbs were injected with saline to serve as an injury control. Bioluminescence was measured (relative light units: RLU) longitudinally as a correlate of tumor growth (IVIS™ Perkin Elmer). Three days subsequent to inoculation, mice were randomized into groups based on obtained RLU values. Sample size was estimated based on our previous results with the C4-2B model [30]. The study endpoint was predetermined as 1×10^6 RLU. Mice were excluded in the event of histological evidence demonstrating tumor breach of cortical bone. At the study endpoint, mice were euthanized and tumor and saline-injected limbs were harvested and fixed in 10% neutral-buffered formalin for 24 h.

Statistical analysis

To determine statistical significance among groups, analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison test. Variance was further measured using the Brown–Forsythe test. Sample size was chosen based on a confidence level of 95% with a 5% margin of error. *p*-value<0.05 was considered as statistically significant. Error bars represent standard error from the mean (SEM). All statistical analyses were performed with Graph Pad Prism 6.0 (Graphpad Inc., LaJolla, CA, USA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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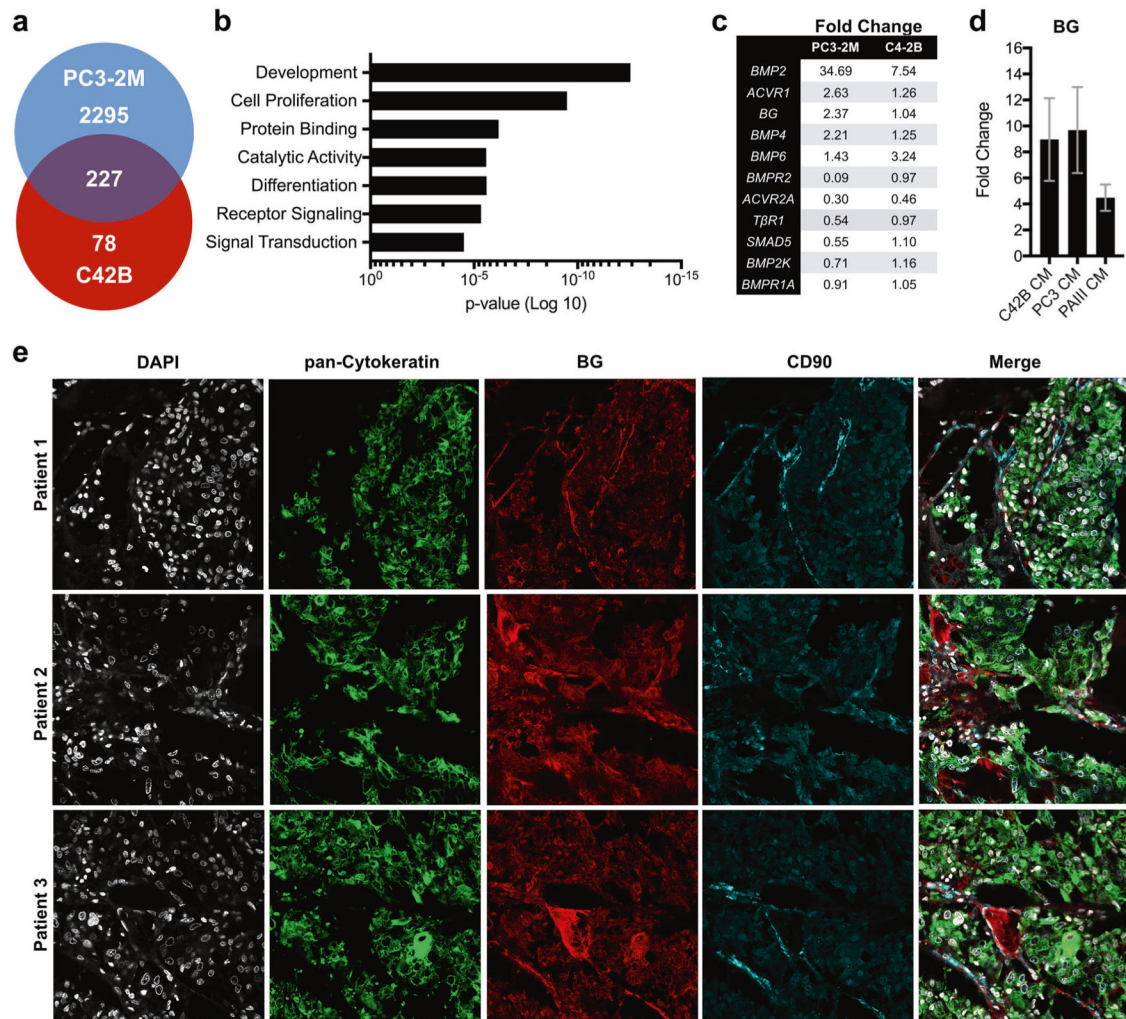


Fig. 1.

Prostate cancer cells induce betaglycan expression in bone marrow-derived MSCs. **a** Microarray analysis of differential gene expression induced in bone marrow MSCs in response to culture in C42B-conditioned and PC3-conditioned media (CM, 6h). **b** Gene ontology analysis of biological processes. **c** Expression of TGF β pathway members in MSCs that are regulated by culture in CM from PC3-2M and C4-2B cells. **d** qRT-PCR analysis of *betaglycan* (*BG*) mRNA levels in MSCs after 6 h incubation in CM derived from C4-2B and PC3-2M, and from CM from the independent rodent prostate cancer cell line, PAIII. Data shows fold increase over basal *BG* expression. **e** Betaglycan (*BG*; red) localization in human bone metastatic prostate cancer specimens ($n = 10$). Representative images from three individual patient specimens are illustrated. CD90 (blue) was used to localize MSCs while pan-cytokeratin (green) was used to localize the prostate cancer metastases. DAPI (gray) was used as a nuclear counterstain

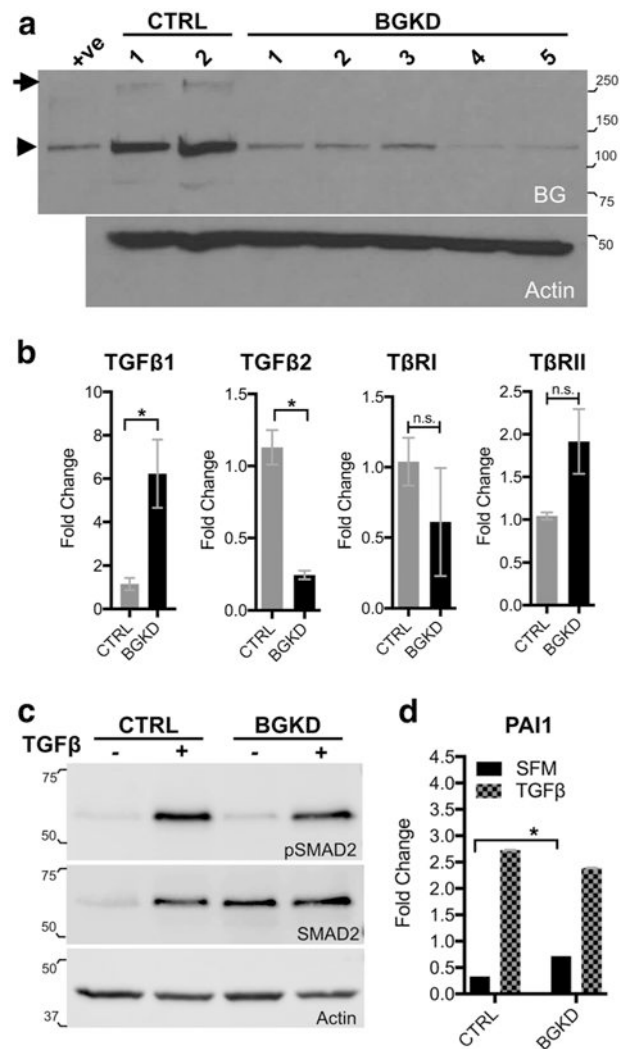


Fig. 2. Betaglycan knockdown enhances TGFβ signaling in MSCs. **a** Analysis of Betaglycan protein levels in scrambled control (CTRL) and betaglycan CRISPR ablated (BGKD) clonal MSC populations. Arrow indicates full-length betaglycan protein while arrowhead indicates the core protein. Recombinant BG was used as a positive (+ve) control while the actin immunoblot was used as a loading control. **b** qRT-PCR analysis of TGFβ ligand isoforms (*TGFβ1* and *TGFβ2*) and TGFβ receptor I and II (*TβRI*, *TβRII*) mRNA levels in betaglycan knockdown (BGKD) MSCs normalized to those in control (CTRL) MSCs. **c** Phosphorylated SMAD2 (pSMAD2) at basal levels (–) and in response to TGFβ treatment (+, 5 ng/ml for 30 min). Actin was used as a loading control. **d** *PAI1* promoter-reporter outputs at basal levels in serum-free media (SFM) and in response to TGFβ treatment (5 ng/ml for 24 h). Molecular weights are in kDa

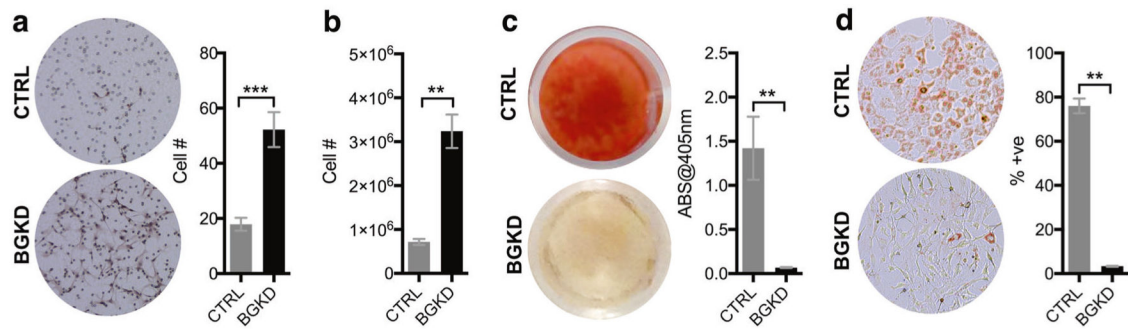
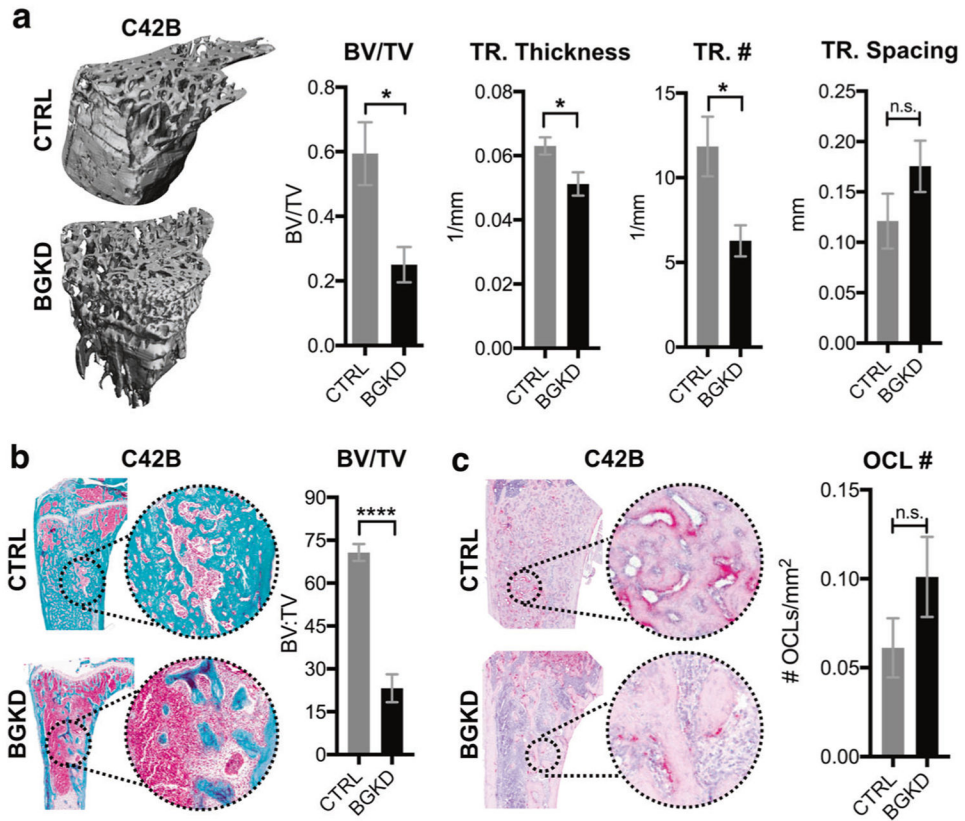


Fig. 3.

Betaglycan inhibits MSC migration and proliferation and is necessary for osteogenic and adipogenic MSC differentiation. **a** The number of migrating CTRL and BGKD MSCs in serum-free media was measured over a 5 h period using a Boyden chamber. Representative images from each group are shown. **b** CTRL and BGKD cell proliferation at 7 days was measured using cell counts. **c** Representative images of Alizarin Red staining for mineralization in CTRL and BGKD MSCs incubated with osteogenic media for 21 days. The amount of solubilized Alizarin Red was also measured in CTRL and BGKD MSCs. **d** The number of Oil Red O-positive cells as a function of total number of cells was measured in CTRL and BGKD MSCs incubated for 3 days in adipogenic media. Representative images from each group are shown. Asterisks denote statistical significance (** $p < 0.01$)

**Fig. 4.**

Betaglycan controls prostate cancer-induced osteogenesis. **a** Representative μ CT images of tibias 7 weeks following co-inoculation with C4-2B prostate cancer cells and CTRL or BGKD MSCs. Graphs illustrate μ CT values in each group for bone volume as function of total volume (BV/TV), trabecular thickness (TR. Thickness), trabecular number (TR. #), and trabecular spacing (TR. Spacing). **b, c** Representative images of trichrome **b** and TRAP **c** stained sections derived from CTRL/C4-2B and BGKD/C4-2B groups. BV/TV ratios **b** and osteoclast numbers/mm of tumor-bone interface (OCL#; red) were quantitated in non-sequential sections derived from each group. Asterisks denote statistical significance ($*p < 0.05$, $****p < 0.001$) while n.s. indicates non-significance

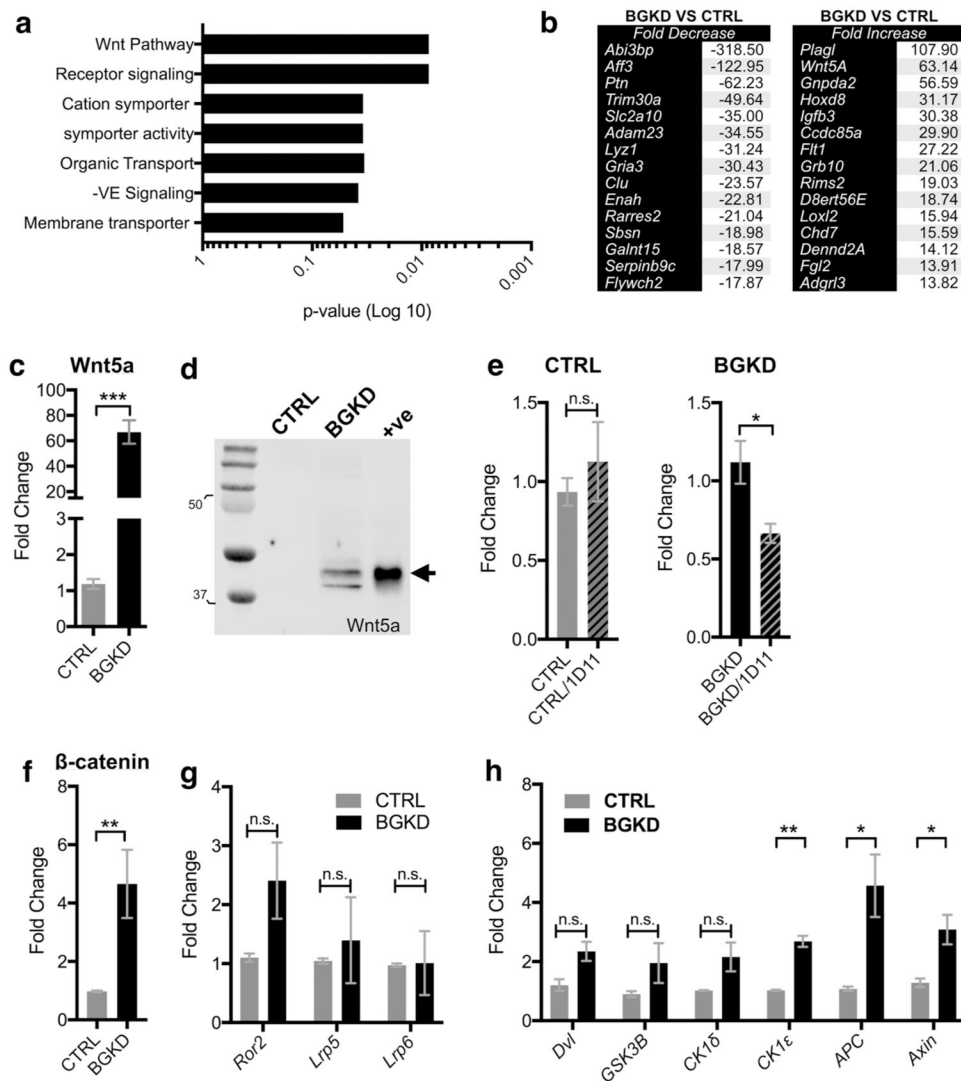


Fig. 5. Betaglycan controls *Wnt5a* expression in MSCs. **a** Gene ontology analysis of microarray data obtained from CTRL and BGKD MSC grown under normal serum-containing culture conditions. **b** Microarray analysis of average gene expression identified the top-15 up-regulated and down-regulated genes in the BGKD MSCs compared to CTRL. **c, d** qRT-PCR **c** and immunoblot **d** analysis of *Wnt5a* expression in CTRL and BGKD MSCs. Arrow indicates *Wnt5a*. Recombinant *Wnt5a* was used as a positive control (+ve). Molecular weights shown are in kDa. **e** qPCR of *Wnt5a* mRNA levels following incubation of CTRL and BGKD MSCs with the TGF β inhibitor 1D11 (10 μ g/ml for 6 h). 1D11 effects are normalized relative to respective non-treatment controls. **f–h** qPCR analysis of β -catenin **f**, Wnt receptors **g** and Wnt signaling effectors **h** at baseline in CTRL and BGKD MSCs. Asterisks denote statistical significance (* p < 0.05, ** p < 0.01, *** p < 0.005) while n.s. denotes non-significance

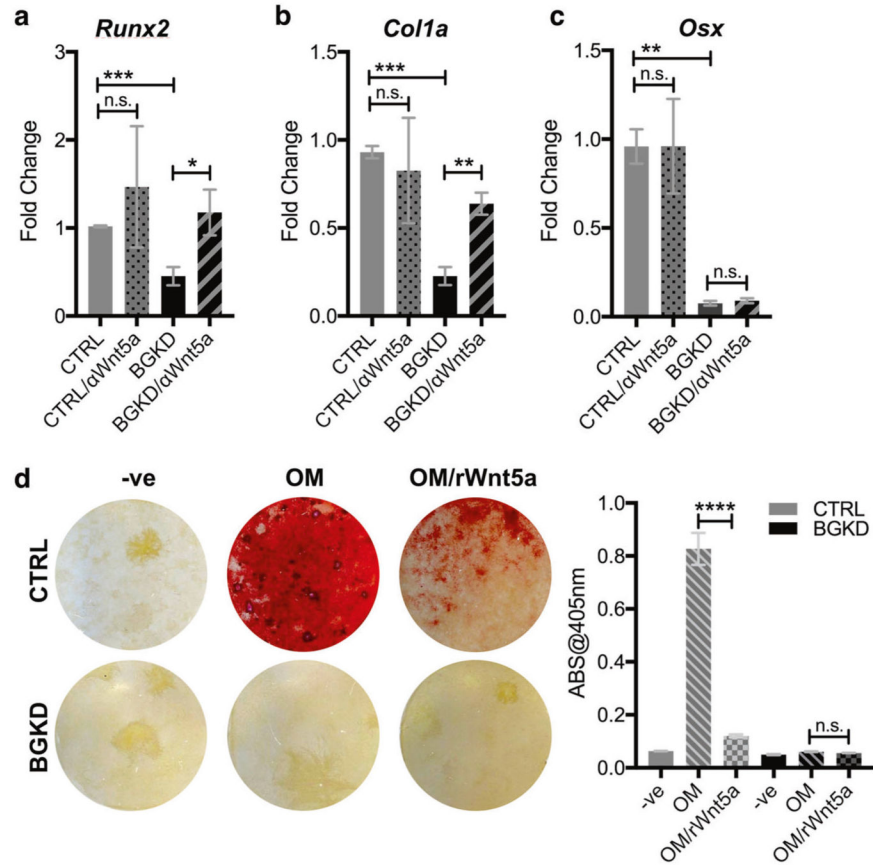


Fig. 6. Betaglycan regulation of Wnt5a contributes to the MSC-osteoblast differentiation program. **a–c** qPCR analysis of *RUNX2* (*RUNX2*; **a**), α -type I collagen (*COL1A1*; **b**), and osterix (*OSX*; **c**) in CTRL and BGKD MSCs treated in the presence or absence of a Wnt5a blocking antibody (α Wnt5a; 1 μ g/ml; 6 h). Fold changes are normalized to each respective non-treated control. **d** Representative images of Alizarin Red staining for mineralization in CTRL and BGKD MSCs incubated with osteogenic media (OM) for 21 days in the presence or absence of recombinant Wnt5a (rWnt5a; 0.5 μ g/ml). Cells were also treated with non-OM media as a negative control (–ve). The amount of solubilized Alizarin Red was measured in the treated CTRL and BGKD MSCs. Asterisks denote statistical significance (** $p < 0.01$, **** $p < 0.0001$)