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Inhibition of STAT3 prevents bone metastatic progression of prostate cancer in vivo

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Abstract

Background: Prostate cancer (PC) metastasizes to the skeleton forming predominantly sclerotic lesions, and there is currently no cure for bone metastatic disease. The transcription factor signal transducer and activator of transcription 3 (STAT3) is implicated as a metastatic driver, but its potential as therapeutic target in bone metastasis has not been investigated. In this study, we evaluated for the first time a STAT3 inhibitor, Napabucasin, as a therapeutic option for bone metastatic PC.

Methods: Effects of STAT3 inhibitors, Stattic and Napabucasin, on metastatic potential in PC cells were studied in vitro by assessment of migration capacity, self-renewal potential, and tumorsphere formation. For evaluation of the role of STAT3 in initial skeletal establishment of PC cells as well as in progressed castration-resistant PC (CRPC) in bone, human VCaP prostate cancer cells were inoculated in the tibia of mice which subsequently were treated with the STAT3 inhibitor Napabucasin. Bone specimens were analyzed using computed tomography (CT), immunohistochemistry, and quantitative polymerase chain reaction.

Results: The small molecule STAT3 inhibitors Stattic and Napabucasin both effectively impaired metastatic potential of PC cells in vitro. Furthermore, treatment with Napabucasin prevented metastatic establishment in tibial bones in vivo and thereby also the tumor-induced sclerotic bone response seen in vehicle-treated VCaP xenografts. In addition, treatment with Napabucasin of established bone CRPC significantly decreased both tumor burden and tumor-induced trabecular bone volume compared with effects seen in vehicle-treated animals. Anti-mitotic effects were confirmed by decreased Ki67 staining in Napabucasin-treated xenografts compared with vehicle-treated xenografts. Alterations of gene expression in the femoral bone marrow (BM) niche toward the maintenance of hematopoietic stem cells and the myeloid lineage were demonstrated by quantitative real-time polymerase chain reaction and were further reflected by a substantial increase in the number of erythrocytes in BM of Napabucasin-treated mice. Furthermore, a

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unique pattern of STAT3 phosphorylation in osteoblasts/stromal cells surrounding the areas of tumor cells was demonstrated immunohistochemically in bone xenograft models using several different PC cell lines.

Conclusion: Inhibition of STAT3 activity disrupts the bone metastatic niche and targets both the skeletal establishment of PC and advanced bone metastatic CRPC in mice, suggesting STAT3 as a candidate for molecular targeted therapies of skeletal metastatic disease.

KEYWORDS
bone metastasis, CRPC, Napabucasin, prostate cancer, STAT3

1 | INTRODUCTION

Prostate cancer (PC) is one of the most common cancer types, and the 5-year survival rate for PC patients decreases from almost 100% when detected as localized cancer, to about 30% with the development of metastases.[1] In advanced stages, PC metastasizes to the skeleton, resulting in sclerotic metastases with local formation of excessive bone mass. This, in turn, can result in severe pain, nerve compressions, anemia, and impaired immune defense due to affected bone marrow (BM) space.

The signal transducer and activator of transcription 3 (STAT3) pathway is hyperactivated and implicated to drive the metastatic progression of several types of cancer.[2–5] STAT3 has been proven important for both formation of premetastatic niches and metastatic establishment of disseminated tumor cells (DTCs), as well as stem cell properties in embryonic and cancer stem cells.[4,5] In PC, constitutively active STAT3 is associated with advanced stages and metastatic progression.[6,7] Recently, it was reported that phosphorylated STAT3 (pSTAT3) is detected in lymph nodes and bone metastases to a greater extent compared with visceral metastases in PC patients,[8,9] suggesting STAT3 as a potential therapeutic target for castration-resistant PC (CRPC) metastasizing to the skeleton.[8] Interestingly, STAT3 also plays an important role in the bone remodeling process as several cytokines and growth factors regulating bone turnover are mediating their effects through STAT3.[10,11]

In a metastatic situation, the presence of tumor cells in the bone microenvironment affects the normal bone remodeling cycle resulting in skeletal complications related to the establishment of metastatic disease. The aim of this study was to evaluate the importance of STAT3 activity, and the effects of STAT3 inhibitors, on bone metastatic establishment and growth of PC cells in bone tissue. In this study, we use a novel small molecule inhibitor of STAT3, Napabucasin (BBI608), currently running in clinical phase III trials for metastatic stages of several cancers, for example, colon and pancreatic cancers (ClinicalTrials. gov Identifier NCT02753127 and NCT03721744, respectively). Napabucasin was initially reported in a preclinical study to have potent anti-tumorigenic and antimetastatic effects in several different cancer types, especially by targeting stemness.[12] The use of Napabucasin has, to the best of our knowledge, not been assessed in clinical trials for bone metastatic cancers.

In conclusion, we here show for the first time that STAT3 activity in the metastatic site is crucial for the establishment of PC cells in the skeleton. Furthermore, inhibition of STAT3 by Napabucasin also decreased bone metastatic growth in established CRPC xenografts. Together our data further establish STAT3 as a candidate for molecular targeted therapies of skeletal metastatic disease.

2 | MATERIALS AND METHODS

2.1 | Cell lines

Several prostate cancer cell lines, representing the heterogeneous landscape of PC in patients, were used in the in vitro experiments. The VCaP cell line, originally derived from a vertebral metastasis of prostate cancer, was used in the in vivo experiments. VCaP cells are androgen sensitive and represent many of the characteristics associated with advanced PC in men such as the expression of AR, the AR splice variant AR-V7, PSA, p53, and the TMPRSS2-ERG fusion.[13,14] The human PC cell lines 22Rv1 and VCaP were purchased from ATCC, PC-3M/Luc2 cells (here denoted PC-3M) were obtained from Caliper Life Sciences/PerkinElmer, and LNCaP-19 cells (castration-resistant subline of LNCaP) have been characterized elsewhere.[15–17] The 22Rv1 and LNCaP-19 cells were grown in RPMI-1640 medium, PC-3M in eagle's minimum essential medium (E-MEM) and VCaP cells in dulbecco's modified eagle medium (D-MEM). The murine preosteoblastic cell line MC3T3-E1, clone 4 (ATCC) was cultured in α-MEM in the absence of ascorbic acid. All cell culture media were supplemented with 10% fetal bovine serum (FBS; or 10% Dextran-charcoal stripped FBS [DCC] for LNCaP-19 cells) and 1% penicillin/streptomycin or gentamicin (all from Thermofisher). For three-dimensional (3D) tumoursphere culture of VCaP cells, serum-free StemXVivo tumoursphere culture medium (R&D Systems) was used. Conditioned media from osteoblast cultures (OCMs) were derived according to previous work.[16,17] All cell lines were cultured at 37°C in humidified air containing 5% CO_2 and routinely tested for mycoplasma.

2.2 | Compounds

The small molecule STAT3 inhibitors Stattic and Napabucasin (BBI608) were purchased from Selleck Chemicals and used at non-toxic doses below the reported IC50 (<10 µM). The inhibitors were dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock solutions and stored in aliquots at −20°C until use.

2.3 | Cell viability assay

VCaP cells were plated at a density of 5×10^3 cells/well in 96-well plates in either 100 µl of control media or control media containing 30% OCM. After attachment overnight, cells were treated with vehicle (DMSO) or graded concentrations of Napabucasin. Cell viability was determined after 48 and 72 h by MTT assays according to the manufacturer's instructions (Promega).

2.4 | Transwell migration assay

Tumor cell migration was evaluated using Transwell migration assay. Cells were seeded at a density of 5×10^3 cells in serum-free cell culture medium in Transwell inserts (pore size 8.0 µm; Corning) in 24-well companion plates containing complete medium including 10% FBS. After 24 h, migrated cells on the bottom side of the membrane were fixed with ice-cold methanol for 10 min and stained with 0.1% crystal violet. Migrated cells were counted using a standard microscope, where two microscopic fields were evaluated for every Transwell insert.

2.5 | Clonogenic assay

For studies on self-renewal potential, PC cells were seeded for clonogenic assays at a density of 3×10^2 cells per well, in 6-well plates. Cell culture media were changed every 3 days until colonies formed after 10–14 days. When visible clones were formed, cells were fixed and stained as for Transwell migration described above. Photographs were taken using a standard digital camera and clones were counted macroscopically by eye.

2.6 | Tumoursphere formation in 3D culture

For evaluation of anchorage-independent growth and formation of tumourspheres, VCaP cells were cultured in semi-solid culture medium in ultralow binding plates. Cells were seeded in StemXVivo serum-free tumoursphere medium at a density of 1.5×10^4 cells/ml in 6-well plates and cultured for 10 days in the absence or presence of test substances before microphotographs were taken using an EVOS FL Cell Imaging System (Invitrogen). Analysis of spheroid size was performed using the IMARIS software (Bitplane).

2.7 | Animals and intratibial implantations

Eight-weeks-old male athymic BALB/c nude mice were purchased from Charles River Laboratories International, Inc. Human VCaP cells (6×10^5 cells) were resuspended in 7 µl Matrigel (BD) and injected into the left tibial BM of mice according to previous work.[16,17] Mice were divided into two groups ($n = 7$ per group) to receive vehicle or Napabucasin treatment. Napabucasin was used in vivo at a dose of 5 mg/kg according to manufacturer's instructions, and administered in 2% DMSO in corn oil every second day by intraperitoneal (ip) injections.

For studies on the effect of Napabucasin on tumor establishment of PC in bone, treatment with Napabucasin was initiated in conjunction with tumor cell implantation and continued for 3 weeks in noncastrated mice. To evaluate the effect of Napabucasin on established CRPC tumors, treatment was initiated on established tumors 6 weeks after surgical castration and tumor cell implantation. The experiments were terminated after 9 weeks in total. After sacrifice, all tumor-bearing and contra-lateral tibiae were excised, fixed in 4% neutral buffered formalin for 48 h followed by fixation in 70% ethanol. Subsequently, bones were decalcified in EDTA and embedded in paraffin. Tumor establishment was assessed in 4 µm-thick hematoxylin and eosin (H&E) stained sections of the central part of the BM. For evaluation of effects in the adjacent BM niche, femoral BM from the tumor-bearing leg was washed, separated by a brief centrifugation and stored in RNAlater solution (Thermo Fisher) at −80°C for subsequent mRNA extraction.

The use of animals was approved by the local Committee on the Ethics of Animal Experiments of Gothenburg (approval no. 172-2014) and all efforts were made to minimize animal suffering.

2.8 | Peripheral quantitative computed tomography (pQCT) and microCT (µCT)

Tumor-induced bone response and effect of treatment with Napabucasin were quantified by measurements of bone mineral density (BMD) obtained from CT scans of tibiae by pQCT using an XCT RESEARCH M instrument (version 4.5B: Norland Medical Systems). Measurements were performed on tumor-bearing tibiae and contralateral tibiae to obtain total BMD and trabecular BMD, at a distance of 1.5 mm distal to the proximal growth plate. The sclerotic response induced by VCaP cells was validated on vehicle-treated animals by the measurement of BMD on tumor-bearing tibia versus nontumor-bearing tibia.

In the study on established intraosseous CRPC tumors, tumor-bearing tibiae and contralateral control tibiae from both Napabucasin-treated and vehicle-treated animals were subjected to specimen µCT analysis using a Skyscan 1072 instrument (Bruker), to assess treatment effects on tissue and trabecular bone mass. Tumor tissue volume was measured with CTAn analysis software v. 1.18.4.0 (Skyscan, Bruker), by utilizing the region of interest (ROI) Shrink Wrap command to exclude non-radiocontrasting tissue surrounding the mineralized tumor, from

the top of the growth plate spanning 50 layers (approximately 0.57 mm) toward the distal end of the tibiae. The trabecular analysis was done from a region starting 0.16 mm below the bottom of unmineralized proximal growth plate and extending 75 layers (approximately 0.85 mm) distal to the proximal growth plate.

2.9 | Immunohistochemistry (IHC)

Before staining, tissue sections were preheated at 60°C for 1 h, deparaffinized, and rehydrated in graded ethanol. For antigen retrieval and endogenous peroxidase blockage, sections were heated in unmasking solution (Vector Laboratories) and incubated in 0.3% hydrogen peroxidase in methanol. Sections were blocked with normal serum (1.5% in tris-buffered saline with tween (TBST)) and then incubated with either anti-human pSTAT3 (Ab76315 diluted 1:100, Abcam) or anti-Ki67 (MA5-14520 diluted 1:200, Thermo Fisher) primary antibody at optimized dilution at 4°C overnight. The sections were then incubated with biotinylated secondary antibody diluted in blocking solution, followed by ABC reagent (Vector Laboratories). The peroxidase reaction was visualized with DAB (DAKO). Sections were counterstained with Mayer's hematoxylin. As negative controls, IHC was performed in the absence of primary antibody or with IgG-matched isotype control. The number of erythrocyte clusters in tibial BM was calculated from three microscopic fields per total BM area. Clusters of erythrocytes are defined as numbers of erythrocytes > 25% per microscopic field (original magnification ×200).

2.10 | Western blot

Proteins were extracted from whole cells on ice with CelLytic™ M cell lysis reagent (Sigma-Aldrich) with the addition of phosphatase inhibitors (PhosSTOP; Roche) and protease inhibitors (cOmplete Mini; Roche) followed by centrifugation and collection of cell lysates. For Western blot analysis, samples containing 20 μg of protein were separated on 4%–12% Bis-Tris gradient gels with MOPS running buffer and transferred onto polyvinylidene fluoride (PVDF) membranes using the iBlot gel transfer system (Invitrogen). The membranes were blocked in 2% blocking solution (Amersham/GE Healthcare) in TBST for 1 h at room temperature and incubated with primary antibodies, anti-pSTAT3 (Ab76315 diluted 1:100, Abcam) and anti-Actin (A5441 diluted 1:10,000, Sigma-Aldrich) overnight at 4°C. Membranes were washed and incubated with a HRP-labeled secondary antibody for 1 h at room temperature. The immunoreactions were detected using the ECL Advance Western blot analysis detection system (Amersham/GE Healthcare).

2.11 | Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from in vitro cell cultures and femoral BM from tumor-bearing mice using the Allprep kit (Qiagen) and converted into complementary DNA (cDNA) using a Vilo superscript kit (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using an ABI 7700 sequence detector instrument and TaqMan Universal PCR Master Mix according to the directions of the manufacturer (Applied Biosystems). C_t values and relative expression levels were calculated using the $\Delta\Delta C_t$ method. TaqMan MGB probes (Applied Biosystems) are listed in Tables S1 (human genes) and S2 (mouse genes).

2.12 | Statistical analysis

For in vitro cell experiments, statistical analysis was performed using Levene's homogeneity test and Mann–Whitney *U* test. Pearson χ^2 test was used to determine relationships between drug treatment and observed effects in bone. Two-tailed Student´s *t* test was used to compare means of BMD and for means in mRNA expression. Results are expressed as means ± standard error of the mean (*SEM*), and are representative data of three independent experiments if not otherwise stated. Values for $p < .05$ were considered statistically significant.

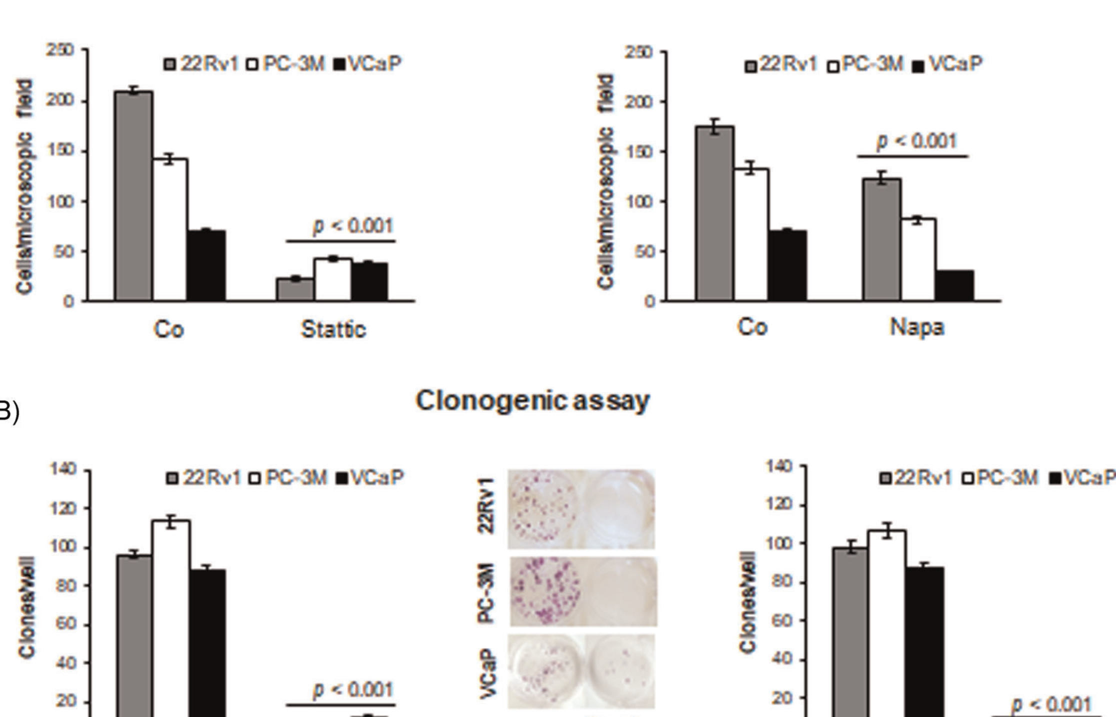
3 | RESULTS

3.1 | Inhibition of STAT3 signaling impairs metastatic potential in prostate cancer cellsin vitro

The importance of STAT3 activity in early stages of the metastatic process was evaluated in vitro. Indeed, transwell migration assay showed that migration in the presence of STAT3 inhibitors Stattic or Napabucasin (both at a nontoxic concentration of 0.5 μM) resulted in a statistically significant ($p < .001$) impairment of migration potential in all three PC cell lines studied (Figure 1A). Furthermore, self-renewal potential was also efficiently reduced by both Stattic and Napabucasin as assessed by clonogenic assay (Figure 1B). Incubation with either Stattic or Napabucasin totally abolished the ability of both the 22Rv1 and PC-3M cells to form colonies, while VCaP cells retained some clonogenic capacity in treated cells although with still statistically significant ($p < .001$) differences between the groups (Figure 1B). Interestingly, the culture of VCaP cells in semi-solid medium revealed that both Stattic and Napabucasin significantly ($p < .001$) decreased the capacity of the VCaP cells to perform anchorage-independent 3D growth (Figure 1C).

3.2 | Targeting STAT3 prevents establishment of PC in the bone microenvironment

To further determine the role of STAT3 in PC bone metastatic disease, mice with intratibial xenografts of human VCaP cells were treated with the small molecule STAT3 inhibitor Napabucasin. As shown in Figure 2, Napabucasin-treated mice had intact tibial BM with no detectable tumor establishment or sclerotic response, in contrast to vehicle-treated animals where the BM cavity comprised large areas of tumor cells



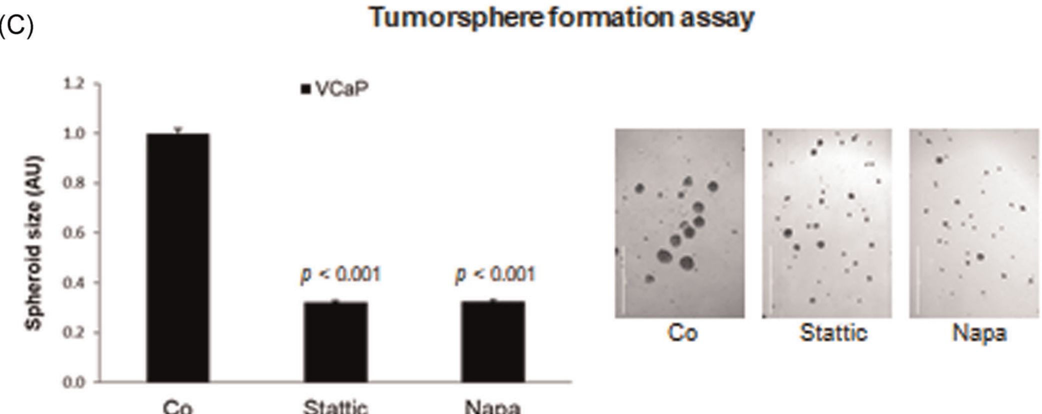


FIGURE 1 Inhibition of STAT3 activity impairs metastatic potential in prostate cancer cell lines. (A) Transwell migration was performed by seeding of 5×10^3 cells per migration insert in the presence of vehicle (DMSO; Co), Stattic or Napabucasin (both at 0.5 μM) and left to migrate for 24 h before staining with crystal violet and counting of migrated cells in eight microscopic fields per condition. (B) Cells were seeded for clonogenic assay at very low density (300 cells per well in 6 well plates), and cultured in the presence of vehicle (DMSO; Co), Stattic or Napabucasin (both at 0.5 μM) for 6–10 days before evaluation. Clones were stained with crystal violet, photographed, and counted manually ($n = 4$ wells per condition). (C) For evaluation of anchorage-independent 3D growth, VCaP cells were seeded at a density of 1.5×10^4 cells/ml in semisolid StemXVivo tumoursphere culture medium in 6 well ultralow binding plates. After 10 days, microphotographs were taken and analysis of spheroid size was performed. Data are represented as mean ± *SEM*, $n = 100$ spheroids per condition. Statistical analyses were performed comparing Stattic- or Napabucasin-treated cells to vehicle-treated control cells. DMSO, dimethyl sulfoxide; STAT3, signal transducer and activator of transcription 3 [Color figure can be viewed at wileyonlinelibrary.com]

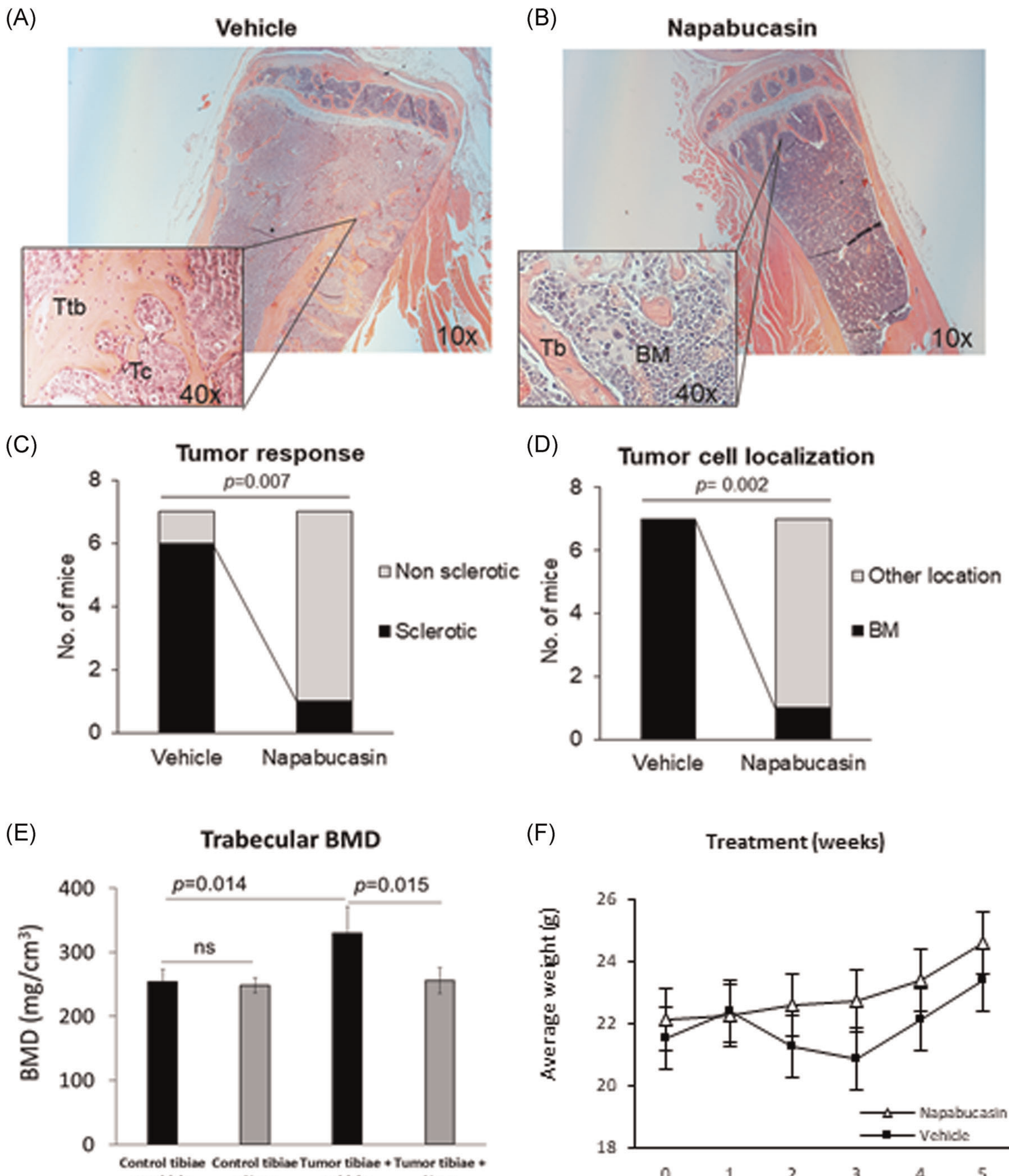


FIGURE 2 Targeting STAT3 prevents skeletal establishment of prostate cancer cells and attenuates the osteoblastic tumor response. VCaP cells (6×10^5 cells in Matrigel) were injected intratibially into the bone marrow of BALB/c nude mice. Treatment with the STAT3 inhibitor Napabucasin was initiated in conjunction with tumor cell implantation and mice were matched into two treatment groups; Napabucasin (5 mg/kg) or vehicle control (2% DMSO in corn oil). Treatment was administered by ip injection every second day for 3 weeks. The experiment was terminated after 9 weeks in total. (A–D) Immunohistochemical analysis of tibial VCaP xenografts stained with H&E for evaluation of tumor take, tumor-induced bone response and tumor cell localization. (E) Trabecular bone mineral density (BMD) was evaluated in excised tibial bones ex vivo using peripheral quantitative CT (pQCT). (F) Animal weight was analyzed throughout the treatment period. Data represents mean ± *SEM*, *n* = 7 per group. BM, bone marrow; computed tomography; H&E, hematoxylin and eosin; ip, intraperitoneal; STAT3, signal transducer and activator of transcription 3; Tb, trabecular bone; Tc, tumor cells; Ttb, tumor-induced trabecular bone [Color figure can be viewed at wileyonlinelibrary.com]

incorporated between newly formed trabecular bone, as demonstrated with H&E staining (Figure 2A,B). The histological evaluation demonstrated that only one out of seven of the mice in the Napabucasin-treated group displayed a sclerotic tumor response, compared with six out of seven mice in the vehicle-treated group (Figure 2C). All mice in the vehicle group displayed tumor cells in the BM, whereas the Napabucasin-treated animals had very few tumor cells in total and those were distributed in other locations outside the BM compartment in six out of seven mice (Figure 2D). As expected, VCaP cells induced a sclerotic bone response demonstrated by a significant increase in tumor-induced trabecular bone mineral density (tBMD; $p = .014$) as measured ex vivo by pQCT (Figure 2E). Intriguingly, treatment for 6 weeks with Napabucasin prevented the tumor-induced increase in tBMD ($p = .015$) seen in tumor tibiae from vehicle-treated animals (Figure 2E). In contrast, treatment with Napabucasin did not have any general effect on either total BMD including cortical bone tissue (data not shown) or tBMD in control tibiae (Figure 2E). In line with previous studies on xenografts treated with Napabucasin,[12] no signs of toxicity were observed from treatment as evidenced by body weight measurements (Figure 2F). Taken together, these results indicate that STAT3 activity is important for the engraftment of sclerotic PC cells in bone.

3.3 | Stromal expression of STAT3 in PC bone metastases

To further examine STAT3 activity in PC bone metastases, the phosphorylation (Y705) of STAT3 was evaluated in the intratibial VCaP xenografts and in previously collected tissue from intratibial xenografts using LNCaP and LNCaP-19 cells, cell lines that all elicit an overall sclerotic response in bone.[16,17] Indeed, IHC analysis confirmed a specific pattern of pSTAT3 staining in osteoblasts/stromal cells surrounding islands of tumor cells and in areas of tumor-induced de novo formation of trabecular bone in the BM cavity, as well as a consistent absence of staining of grafted tumor cells (Figure 3A–C). Although Napabucasin-treated animals displayed very few detectable VCaP cells in the tumor-injected tibiae, there was a clear decrease in pSTAT3 staining of Napabucasin-treated tumor tibiae compared with vehicle-treated tibiae (Figure 3D). In support of our in vivo findings, Western blot demonstrated that phosphorylation of STAT3 in "bone-naïve" LNCaP-19 cells in vitro was reversed when the tumor cells were cultured in the presence of osteoblast-derived factors in conditioned media from MC3T3-E1 osteoblast cultures (OCM; Figure 3E). As demonstrated in Figure 3F, gene expression analysis of VCaP cells cultured in OCM demonstrated that Napabucasin did not regulate any of the stemness markers *Aldha1*, *Cd44*, *Nanog*, *Oct4*, or *Sox2*, in contrast to previous studies on STAT3 inhibitors and solid tumors in nonskeletal metastatic situations.[12,18] Interestingly, Sonic hedgehog (*Shh*) was significantly upregulated in response to Napabucasin, which may indicate the activation of an alternative signaling pathway in response to decreased pSTAT3. Altogether, these results propose a unique role for STAT3 in bone metastases, with stromal STAT3 activation as metastatic mediator and suggested treatment target within the bone microenvironment.

3.4 | STAT3 inhibition alters the bone microenvironment by affecting BM stem cells and increasing erythropoiesis

As STAT3 is known to be important for the formation of premetastatic niches, we next investigated the impact of STAT3 inhibition on the bone microenvironment and the BM niche in vivo. Gene expression analysis was performed on femoral BM from the hindleg with tibial tumor implantation, based on a panel of 20 selected genes known to be involved in the regulation and establishment of the metastatic niche (Figure 4A and Table S2). Gene expression analysis strengthened STAT3 as regulator of the BM niche composition as treatment with Napabucasin increased the expression of genes important for the maintenance of hematopoietic stem cells (HSCs) and the myeloid lineage. The chemokine C-X-C motif chemokine ligand 12 (*cxcl12; sdf1)* which is essential for HSC homing and mobility,[19] lysyl oxidase (*lox*), cadherin-11 (*cdh11*), cadherin-2 (*cdh2*), interleukin-6 (*il6*), and receptor activator of NF-κB ligand (*tnfsf11)* were all significantly upregulated (Figure 4A). In contrast, transforming growth factor beta *(tgfβ)* and inducible nitric oxide synthase (*nos2*), both involved in the regulation of mesenchymal stem cell (MSC) recruitment and properties,[20–22] were significantly downregulated (Figure 4A). In support of the observed effect of Napabucasin on transcriptional activity in the BM niche and the suggested increase in HSC activity, a statistically significant ($p < .001$) increase in erythrocyte number was demonstrated in BM of Napabucasin-treated tumor tibiae (Figure 4B,C). Together, these data strongly indicate that STAT3 is involved in the regulation of the stem cell composition and activity in the BM as part of the role played by STAT3 in the regulation of the bone metastatic niche.

3.5 | Inhibition of STAT3 reduces metastatic growth of established CRPC in bone

Finally, we investigated the effects of Napabucasin on established CRPC bone tumors, a condition mimicking late-stage disease in PC patients. Micro-CT analysis of tumor tibiae was used to quantify the effect of Napabucasin treatment on tumor burden and tumor-induced bone formation of VCaP xenografts. Indeed, the results showed that treatment with Napabucasin significantly decreased tumor volume and tumor surface by 36% ($p = .035$) and 24% ($p = .022$), respectively, compared with the vehicle-treated group (Figure 5A,B). In line with these findings, H&E staining demonstrated that tumor mass was decreased in tumor tibiae from Napabucasin-treated animals compared with vehicle-treated (Figure 5C). In addition, staining for the proliferative marker Ki67 revealed a clearly decreased expression of Ki67 in Napabucasin-treated tumors (Figure 5C). Furthermore, the sclerotic effect induced by VCaP cells

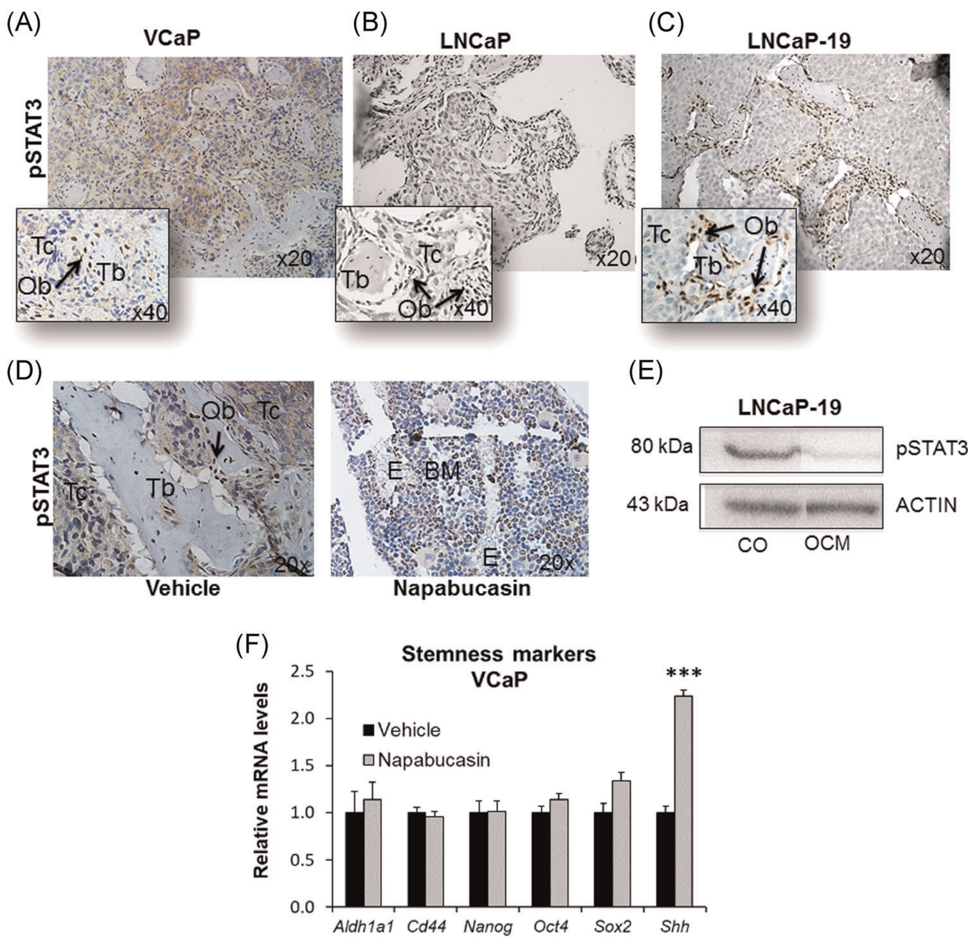


FIGURE 3 Stromal expression of phosphorylated STAT3 (pSTAT3) in sclerotic PC bone metastases. (A–D) Immunohistochemical (IHC) staining of pSTAT3 in intratibial PC xenografts of VCAP, LNCaP, and LNCaP-19 cells. (D) Treatment with STAT3 inhibitor Napabucasin (5 mg/kg) or vehicle control (2% DMSO in corn oil) was initiated in conjunction with tumor cell implantation and mice were treated by ip injection every second day for 3 weeks. The experiment was terminated after 9 weeks in total. (E) Western blot analysis of pSTAT3 in LNCaP-19 cells cultured in the absence (Co) or presence of osteoblast-conditioned culture media (OCM) for 48 h followed by protein isolation and subsequent analysis. (F) Quantitative real-time PCR analysis of mRNA expression of stem cell markers in VCaP cells cultured in OCM in the presence of vehicle control (DMSO) or Napabucasin (1 μM) for 48 h followed by RNA isolation, cDNA synthesis and qRT-PCR analysis. Data represent mean ± *SEM*, $n = 4$ per group, ***$p \leq .001$. BM, bone marrow; cDNA, complementary DNA; DMSO, dimethyl sulfoxide; E, erythrocyte; ip, intraperitoneal; Ob, osteoblast; OCM, osteoblast culture; PC, prostate cancer; PCR, polymerase chain reaction; Tb, trabecular bone; Tc, tumor cells [Color figure can be viewed at wileyonlinelibrary.com]

was reduced by treatment with Napabucasin also in established bone xenografts as demonstrated by a decrease in trabecular bone volume and bone surface, within the ROI of mineralized bone tissue, by 37% ($p = .041$) and 26% ($p = .040$), respectively, compared with vehicle-treated mice (Figure 5D,E). Notably, the weight of mice during treatment was unaffected, hence showing no signs of toxicity (Figure 5F). Together, these results show that STAT3 inhibition also exerts antitumoral effects on progressed CRPC in bone.

4 | DISCUSSION

Skeletal metastatic disease is a clinical challenge foremost because the mechanisms underlying metastatic disease are unclear and thereby limit the development of novel therapeutic approaches. In this study, we show unique findings on the transcription factor STAT3 as a target for bone metastatic PC. By using an intratibial implantation model, we here demonstrate for the first time that blocking STAT3 with the small molecule inhibitor Napabucasin efficiently impaired intraosseous establishment of PC cells. Indeed, treatment with Napabucasin totally prevented the tumor-induced increase in trabecular bone without affecting normal bone tissue. Recently, Zou et al., showed that Napabucasin has inhibitory effects on osteosarcoma and tumor cell-induced osteolysis,[23] a phenotype in contrast to the excessive bone formation seen in most PC bone metastases. Accordingly, this emphasizes the potential of Napabucasin and STAT3 inhibition in therapeutic applications for cancer-related bone disease irrespective of tumor phenotype. Furthermore, the fact that Napabucasin not only prevented tumor cell establishment but also the tumor cell-induced local effects on the bone tissue

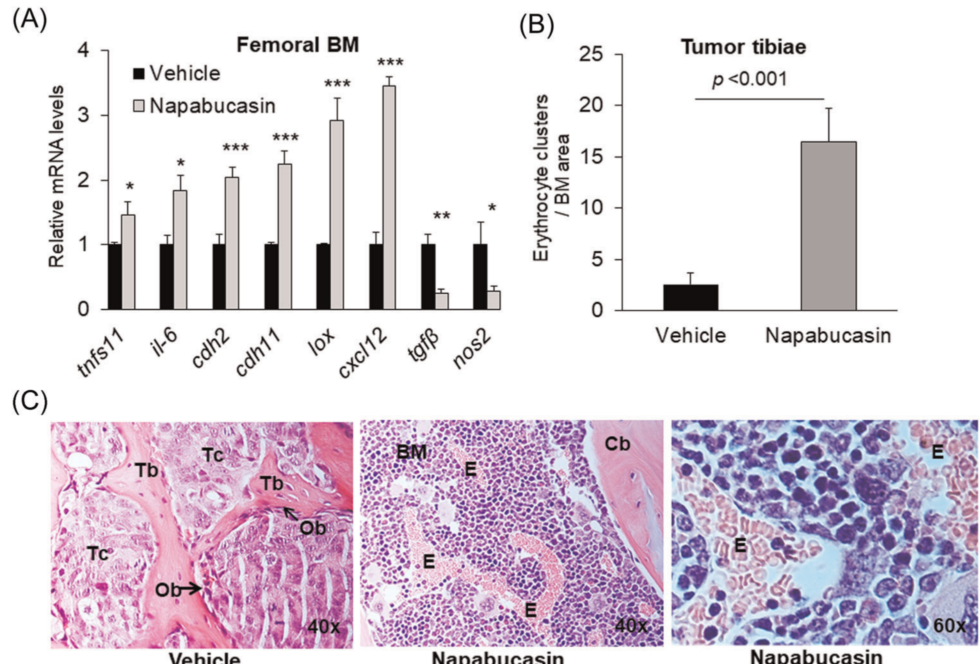


FIGURE 4 STAT3 inhibition alters the bone metastatic niche and affects stem cell properties promoting erythropoiesis. (A) A panel of >20 genes associated with the bone metastatic niche was evaluated in femoral bone marrow-derived from intratibial VCaP xenografts treated with Napabucasin (5 mg/kg) or vehicle control (2% DMSO in corn oil) by ip injection every second day for 3 weeks. Bone marrow was isolated from femur from the contralateral hindleg with intratibial implantation of VCaP cells at termination of the experiment and RNA was extracted for subsequent qRT-PCR analysis. Data represent mean ± *SEM*, $n = 6$ per group, $^{*}p \le .05$, $^{**}p \le .01$, $^{***}p \le .001$. (B,C) Immunohistochemical analysis of tibial VCaP xenografts stained with H&E for evaluation of erythrocyte numbers in the bone marrow compartment. Erythrocytes are quantified as erythrocyte clusters per bone marrow (BM) area. Data represent mean ± *SEM*, $n = 7$ per group. BM, bone marrow; Cb, cortical bone; E, erythrocyte; H&E, hematoxylin and eosin; ip, intraperitoneal; qRT-PCR, quantitative real-time polymerase chain reaction; STAT3, signal transducer and activator of transcription3; Ob, osteoblast; Tb, trabecular bone; Tc, tumor cells [Color figure can be viewed at wileyonlinelibrary.com]

in our xenograft model can be of great importance in a clinical situation to decrease the development of skeletal-related events (SREs) affecting survival in patients with skeletal metastatic disease. The dual positive effect by Napabucasin on both tumor growth and bone tissue parameters suggests a protective effect by STAT3 inhibition in metastatic sites. Treatment with Napabucasin reversed the tumor-induced local bone formation which, in a clinical situation, possibly also can improve the quality of life of PC patients by decreasing SREs such as metastasis-related bone pain and pathological fractures.

To the best of our knowledge, there are only three previous reports evaluating STAT3 activity in PC bone metastases. In two cases, nuclear staining of pSTAT3 in PC cells was detected in the metastatic site, and expression of pSTAT3 was increased in clinical bone metastases compared with metastases in other organs.[8,9] The majority of analyzed samples from bone metastases in those studies were, however, taken from PC patients who had undergone androgen-deprivation therapy and died from their disease, hence representing late-stage bone metastatic disease. In contrast, this study was performed under noncastrated conditions to mimic earlier stages of metastatic progression of PC and evaluate the importance of STAT3 signaling for the first contact of PC cells with the BM niche. Hence, the pSTAT3 staining pattern of the patient samples described above[8] was not in line with our current findings showing exclusive expression of pSTAT3 in osteoblasts/stromal cells and not in the tumor cells. However, a third study by Schulze and co-authors support our findings by demonstrating both induction by PC cells of STAT3 phosphorylation in osteoblasts in vitro, as well as pSTAT3 staining in osteoblasts in sample collections of bone metastases from both prostate and breast cancer patients.[24] Interestingly, we here demonstrate that LNCaP cells, originally derived from a lymph node, reverse their activation of STAT3 upon stimulation with osteoblast-derived factors in vitro, suggesting the role of the osteoblasts as regulators of STAT3 activity in invading tumor cells in bone. Communication and physical interactions with cells in the bone microenvironment is also crucial for the survival and growth of tumor cells invading the skeleton (reviewed in Croucher et al.[25]). Supporting the observed pattern of pSTAT3 in the bone stroma in sclerotic PC, pSTAT3 has also been reported to be present on the leading edge of tumors in association with stromal, immune, and endothelial cells, suggesting that STAT3 plays a critical role in the communication between cancer cells and the tumor microenvironment.[26] Hence, our work emphasizes STAT3 as a potential therapeutic target also for the early stages of PC bone metastasis.

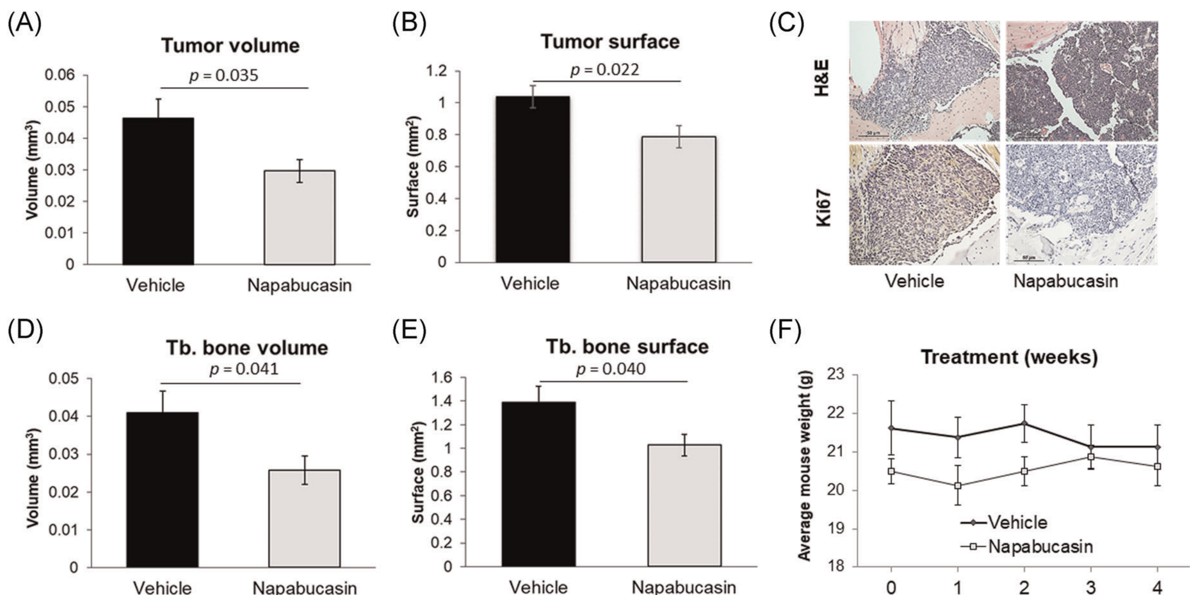


FIGURE 5 Inhibition of STAT3 reduces metastatic tumor burden of established CRPC, and reverses tumor-induced trabecular bone formation. VCaP cells (6×10^5 cells in Matrigel) were injected intratibially into the bone marrow of castrated BALB/c nude mice. For studies on established CRPC tumors in bone, treatment with the STAT3 inhibitor Napabucasin was initiated 6 weeks after surgical castration and tumor cell implantation on established tumors. Mice were matched into two treatment groups; Napabucasin (5 mg/kg) or vehicle control (2% DMSO in corn oil). Treatment was administered by ip injection every second day for 3 weeks. The experiment was terminated after 9 weeks in total. (A,B), (D–E) Volume and surface measurements of tibial tumor xenografts where trabecular bone was assessed in excised tibial bones ex vivo using micro-CT (μCT). (C) Immunohistochemical analysis of tibial VCaP xenografts stained with H&E and Ki67 antibody for evaluation of tumor take and tumor cell proliferation, respectively. (F) Animal weight was analyzed throughout the treatment period. Data represents mean ± *SEM*, *n* = 7 per group. CRPC, castration-resistant PC; H&E, hematoxylin and eosin; ip, intraperitoneal; STAT3, signal transducer and activator of transcription 3 [Color figure can be viewed at wileyonlinelibrary.com]

In addition to direct inhibitory effects on tumor cells, we also detected transcriptional regulation by Napabucasin in the femoral BM in xenografted hindlegs, suggesting a Napabucasin-induced shift in stem cell populations suppressing MSCs and promoting HSCs. One of the genes upregulated by Napabucasin was *cxcl12*, coding for the chemokine CXCL12 established as a key regulator of HSC homing to the BM.[19] Intriguingly, both PC cells and HSCs express the CXCL12 receptor, CXCR4, and migrate toward CXCL12-expressing organs, including the BM.[27,28] Moreover, Napabucasin-induced increase in erythrocyte numbers in the tibial BM suggests that STAT3 modulates the cell composition of the BM niche. In terms of clinical importance, our data indicate that STAT3 plays a regulatory role in the distribution and differentiation of stem cells in the BM. This is in line with a study by Chung et al. demonstrating that enforced STAT3 activity promotes HSC self-renewal and maintenance of immature hematopoietic cells.[29] This finding is especially important considering the prevalence of anemia associated with bone metastatic disease, suggesting a possible effect by Napabucasin treatment that can also counteract this symptom.

5 | CONCLUSION

This study provides novel insights into how STAT3 signaling mediates interactions between tumor cells and the bone microenvironment to drive skeletal metastatic disease. Our findings indicate that stromal STAT3 activation is more important than STAT3 signaling in the invading tumor cells in the initial stages of PC bone metastatic disease. We show for the first time that the STAT3 inhibitor Napabucasin both prevents tumor cell establishment in bone tissue and target late-stage CRPC in bone by decreasing tumor burden and tumor-induced increase in bone mass. Our work suggests Napabucasin as a promising candidate for molecular targeted therapies of skeletal metastatic disease.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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