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In-Press Preview

Breast cancer bone metastases often cause a debilitating non-curable condition with osteolytic lesions, muscle weakness and a high mortality. Current treatment comprises chemotherapy, irradiation, surgery and anti-resorptive drugs that restrict but do not revert bone destruction. In metastatic breast cancer cells, we determined the expression of sclerostin, a soluble Wnt inhibitor that represses osteoblast differentiation and bone formation. In mice with breast cancer bone metastases, pharmacological inhibition of sclerostin using an anti-sclerostin antibody (Scl-Ab) reduced metastases without tumor cell dissemination to other distant sites. Sclerostin inhibition prevented the cancer-induced bone destruction by augmenting osteoblast-mediated bone formation and reducing osteoclast-dependent bone resorption. During advanced disease, NF-κB and p38 signaling was increased in muscles in a TGF-β1-dependent manner, causing muscle fiber atrophy, muscle weakness and tissue regeneration with an increase in Pax7-positive satellite cells. Scl-Ab treatment restored NF-κB and p38 signaling, the abundance of Pax7-positive cells and ultimately muscle function. These effects improved the overall health condition and expanded the life span of cancer-bearing mice. Together, these results demonstrate that pharmacological inhibition of sclerostin reduces bone metastatic burden and muscle weakness with a prolongation of the survival time. This might provide novel options for treating musculoskeletal complications in breast cancer patients.

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Sclerostin inhibition alleviates breast cancer-induced bone metastases and muscle weakness

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**Abstract**

Breast cancer bone metastases often cause a debilitating non-curable condition with osteolytic lesions, muscle weakness and a high mortality. Current treatment comprises chemotherapy, irradiation, surgery and anti-resorptive drugs that restrict but do not revert bone destruction. In hormone receptor-negative breast cancer cell lines and human breast cancer tissue, we determined the expression of sclerostin, a soluble Wnt inhibitor that represses osteoblast differentiation and bone formation. In mice with breast cancer bone metastases, pharmacological inhibition of sclerostin using an anti-sclerostin antibody (Scl-Ab) reduced the metastatic burden. Furthermore, sclerostin inhibition prevented the cancer-induced bone destruction by augmenting osteoblast-mediated bone formation and by reducing osteoclast-dependent bone resorption. During advanced disease, NF-κB and p38 signaling was increased in muscles in a TGF-β1-dependent manner, causing muscle fiber atrophy, muscle weakness and tissue regeneration with an increase in Pax7-positive satellite cells. Scl-Ab treatment restored NF-κB and p38 signaling, the abundance of Pax7-positive cells and muscle function. These effects improved the health and expanded the life span of cancer-bearing mice. Together, these results demonstrate that pharmacological inhibition of sclerostin reduces bone metastatic burden and muscle weakness with a prolongation of the survival time. This might provide novel options for treating musculoskeletal complications in breast cancer patients.
Introduction

Breast cancer is the most common cancer in women worldwide with a prediction to further increase in the future, representing a tremendous medical and socio-economic burden (1). After treatment of the primary tumor, metastases often occur years or even decades later (1). Bone is a premier site for breast cancer metastases and about 70% of breast cancer patients with advanced disease suffer from osteolytic bone metastases, a stage at which the disease is incurable (2). Osteolytic metastases often cause skeletal-related events (SREs), including pathological fractures and pain that require palliative interventions (3, 4).

Destruction of the bone tissue in breast cancer-induced osteolytic disease occurs due to an increased number and activity of bone-resorbing osteoclasts (5). Osteoclasts are activated in a direct or indirect manner by cancer- and micro-environment-derived cytokines including parathyroid hormone-related peptide (PTHrP), receptor activator of NF-κB ligand (RANKL) and interleukins. Upon activation of bone resorption, matrix-derived growth factors and in particular transforming growth factor beta (TGF-β) further enhance tumor growth and osteoclast activity, constituting a ‘vicious cycle’ of bone metastasis (5). While it is firmly established that breast cancer cell- and matrix-derived growth factors stimulate osteoclast activity, evidence exists that breast cancer cells also provide molecular cues that suppress osteoblast function (6). Thus, it can be hypothesized that restoring osteoblast function might ameliorate metastatic bone disease. Nevertheless, the exact role of bone-forming osteoblasts in the context of breast cancer metastases-mediated bone destruction and their potential use for pharmacological treatment is not yet elucidated.

In addition to chemotherapy and radiation therapy, patients with osteolytic metastases are treated with anti-resorptive drugs such as bisphosphonates or an antibody against RANKL (Denosumab; XGEVA®, Amgen) that efficiently restrict osteoclast activity and therefore the
progression of bone destruction (7). Due to advances in surgery, adjuvant chemotherapy, hormone receptor blocking drugs and anti-resorptive therapies, the survival time of patients has been greatly prolonged (8). However, the overall morbidity and mortality are still very high, particularly once SREs occur (8). Furthermore, the disease remains irreversible and osteolytic lesions persist (8).

Since anti-resorptive drugs cannot revert osteolytic lesions, augmenting osteoblast function has been proposed as a potential approach to restore bone integrity in the context of metastases-induced osteolytic lesions (9). To date, only two bone anabolic drugs are available in the clinics for the treatment of osteoporosis, a debilitating disease that leads to loss of bone mass and ultimately fragility fractures. An intermittent administration of a recombinant fragment of Parathyroid Hormone (PTH 1-34) (Teriparatide; Forteo®/Forsteo®, Eli Lilly and Company) or of a PTH-related peptide analogue (PTHrP 1-34) (Abaloparatide; Tymlos®, Radius Pharma) increase osteoblast-mediated bone formation and bone mass but are not approved for use in cancer patients (6). Thus, there is currently no osteoblast-targeting drug available in the US or in Europe that could be used for the treatment of cancer-related bone disease.

Osteoblast differentiation and function is regulated by a network of transcription factors and signaling pathways (10). Canonical Wnt signaling is among the most prominent pathways promoting osteoblast differentiation, function and bone formation (11). Activation of the canonical Wnt pathway occurs upon binding of Wnt ligands to low-density lipoprotein receptor-related protein 5 and 6 (Lrp5/6) that form a complex with frizzled-related proteins. Sclerostin is a soluble Wnt antagonist that prevents binding of Wnt ligands to Lrp5/6 and therefore the activation of the pathway and ultimately osteoblast-mediated bone formation (11). Due to its strong inhibitory effect of Wnt signaling and pharmacological accessibility, sclerostin has emerged as a promising drug target and anti-sclerostin antibodies (Scl-Ab) have been developed to increase bone formation and bone mineral density (BMD) in the context of postmenopausal osteoporosis (12, 13). In clinical
trials, treatment of women with postmenopausal osteoporosis, Scl-Ab (Romosozumab; Evenity®, Amgen/UCB) increased bone formation while bone resorption was decreased, leading to an increase in BMD and a reduction of the fracture rate at several sites including the hip and spine (13, 14). Recently, Romosozumab has been approved in Japan for the treatment of severe postmenopausal osteoporosis and is expected to become available in the US and in Europe later this year. Thus far, activation of the canonical Wnt signaling pathway in the skeleton, neither in preclinical animal models nor by genetic or pharmacological means in humans, has caused any malignant side effect, demonstrating the overall safety of this approach in the musculoskeletal system. The strong effect of Wnt activation on increasing BMD and decreasing the fracture rate is also of interest in the context of bone fragility syndromes other than osteoporosis. For instance, osteogenesis imperfecta (OI) is often caused by mutations in the gene encoding collagen type 1, leading to brittle bones and fractures (15). In this context, another Scl-Ab (BPS-804, Setrusumab, Mereo BioPharma) is currently been investigated in clinical trials for the treatment of patients with OI (16).

In breast cancer patients, SREs are often associated with muscle weakness, a debilitating condition that decreases mobility, well-being and life quality of breast cancer patients (17, 18). The disease mechanisms underlying cancer-associated muscle weakness are largely unknown and no therapy is available to date (17, 18). Thus, novel therapeutic approaches targeting breast cancer bone metastases and muscle weakness are urgently needed.

Here we report that metastatic breast cancer cells secrete sclerostin, thereby inhibiting canonical Wnt signaling in osteoblasts and osteoblast differentiation, indicating that breast cancer cells might impair bone formation while promoting metastases and bone resorption. Treatment of bone metastases-bearing female mice with Scl-Ab (Setrusumab) alleviated tumor growth in bone and bone destruction without increasing metastases at other sites. Surprisingly, Scl-Ab treatment
protected from cancer-induced muscle fiber atrophy and loss of muscle function in tumor-bearing mice and prolonged the life span of these animals. These findings reveal that therapeutic inhibition of sclerostin reduces breast cancer-induced bone metastases and muscle weakness in mice, indicating its potential as novel therapeutic option for breast cancer patients.
Results

Breast cancer cell-derived sclerostin inhibits Wnt signaling in osteoblasts

To address the hypothesis that metastatic breast cancer cells impair osteoblast differentiation, we collected conditioned medium from MDA-MB-231 metastatic breast cancer cells and differentiated primary calvarial cells into osteoblasts in the presence or absence of medium that had been conditioned by breast cancer cells. In support of our hypothesis, conditioned medium inhibited osteoblast differentiation and matrix mineralization, demonstrated by a reduced expression of the osteoblast marker genes Runx2 and Ocn and a weaker Alizarin red staining (Figure 1, A and B). Furthermore, using a TopFlash reporter gene assay we determined that cancer cell-conditioned medium suppressed the activity of the canonical Wnt signaling pathway in osteoblasts in a dose-dependent manner (Figure 1C). These findings indicate that metastatic breast cancer cells may secrete factors that inhibit canonical Wnt signaling in osteoblasts in a paracrine fashion.

Breast cancer cells have been shown to express Dickkopf 1 (Dkk1), a soluble antagonist of canonical Wnt signaling. However, antagonizing cancer cell-derived Dkk1 did not fully restore the activity of the Wnt pathway (19), suggesting that additional mechanisms might exist. Indeed, expression analysis revealed a significantly higher expression of the Wnt inhibitor sclerostin in metastatic MDA-MB-231 breast cancer cells compared to non-metastatic MCF-7 breast cancer cells (Figure 1D). To determine whether sclerostin expression is a general feature of breast cancer cells, we performed an in silico analysis using the EMBL-EBI Expression Atlas (20). In addition to cells of the MDA-MB-231 cell line, expression of sclerostin was found in cells of the SUM159PT, CAL51, HCC 1187, HCC 1197, HCC 1395, HCC 1806 and KPL-4 breast cancer cell lines. Interestingly, six of these cell lines (SUM159PT, CAL51, HCC 1187, HCC 1197, HCC 1395 and HCC 1806) neither express the estrogen receptor (ER) nor the progesterone receptor (PR) and
do not bear an amplification of HER-2/Neu gene (referred to as triple-negative breast cancer cells) (21)(22). Furthermore, although KPL-4 cells have a HER-2/Neu amplification, they do not express the ER or the PR (23), suggesting that sclerostin expression is a common feature of hormone receptor-negative breast cancer cells. To address the question whether sclerostin is expressed in primary breast cancer tissue from patients, we quantified the sclerostin expression in tissue biopsies obtained from 48 breast cancer patients and from four healthy individuals. While sclerostin expression was not detected in healthy breast tissue, 21% of primary breast cancers expressed sclerostin (Fig 1E). Interestingly, 56% of triple-negative breast cancer tissues and 43% of ER-negative and PR-negative breast cancer tissues expressed sclerostin (Fig 1E). Furthermore, 2 out of 3 (66 %) metastatic breast cancers with unknown receptor status were positive for sclerostin expression. Tumors expressing either the ER or the PR or both receptors did not express sclerostin (data not shown). To determine if sclerostin expression is a specific feature of breast cancer or if it is also expressed by other types of cancer, we analyzed sclerostin expression in human colon (n=9), kidney (n=9), liver (n=9), lung (n=9), prostate (n=9), ovary (n=9) and thyroid (n=9) cancer biopsies and the respective healthy tissue. Sclerostin expression was detected in two colon (22%), one ovary (11%) and two lung (22%) cancer tissues, suggesting that although sclerostin expression is not specific to breast cancer, it is not a general oncogenic feature of all types of cancer (Table 1).

To investigate whether breast cancer cell-derived sclerostin reduces Wnt signaling activity in osteoblasts, we restricted sclerostin expression in MDA-MB-231 cells using three different siRNAs designed to target SOST mRNA (Supplemental Figure 1A and B). Next, conditioned medium was obtained from MDA-MB-231 cells transfected with scrambled control siRNA or siRNA targeting SOST mRNA. Indeed, antagonizing sclerostin expression in metastatic breast cancer cells partially but significantly restored the impaired Wnt signaling activity in calvarial osteoblasts (Figure 1F).
Sclerostin inhibits the activation of the canonical Wnt signaling pathway in osteoblasts by binding to the first \(\beta\)-propeller domain of the extracellular region of the LRP5 receptor (24). Heterozygous missense mutations (G171V and A214V) within this domain of LRP5 cause a high bone mass phenotype in patients and in mice due to a reduced binding of sclerostin (25, 26). To determine whether cancer cell-derived sclerostin inhibits Wnt activity in osteoblasts through Lrp5, we obtained osteoblasts from genetically modified mice heterozygous for the \(Lrp5\) mutation G171V. In support of our hypothesis, osteoblasts bearing a mutant \(Lrp5\) with a disabled sclerostin binding site were resistant to breast cancer-mediated repression of Wnt signaling (Figure 1G). These data suggest that breast cancer cells impair osteoblast differentiation, at least in part, by a sclerostin/Lrp5-mediated inhibition of the canonical Wnt signaling pathway.

Pharmacological inhibition of sclerostin reduces the bone metastatic burden and prolongs the survival time

Given the negative effect of cancer-derived sclerostin on osteoblasts, we hypothesized that inhibition of sclerostin and subsequent activation of osteoblast function might protect from the progression of metastatic bone disease. To investigate the therapeutic potential of Scl-Ab treatment in breast cancer-induced bone destruction, an in vivo metastasis model was used in which MDA-MB-231 breast cancer cells stably expressing the luciferase gene (MDA-MB-231-\(luc\)) were inoculated by cardiac injection into 8-weeks old female SCID mice. To recapitulate the clinical situation of breast cancer patients, bone metastases were allowed to form in mice prior to treatment. Two weeks after breast cancer cell injection, micro-metastases were detected in the long bones by bioluminescence imaging and mice were randomized into two treatment arms. One group was treated by Scl-Ab (100 mg/kg) while the other group received vehicle control (50 \(\mu\)l/10g) i.v. once a week for four weeks. Non cancer-bearing mice without treatment, with vehicle or Scl-Ab
treatment served as healthy controls. Weekly bioluminescence imaging of cancer-bearing mice revealed a progressive growth of bone metastases in vehicle-treated mice (Figure 2, A and B). Although metastases developed also in mice that received Scl-Ab, tumor growth in bones was significantly reduced compared to vehicle-treated controls (Figure 2, A and B). The reduced tumor growth was confirmed by histological analyses of the metastases area in the tibiae (Figure 2, C and D) and in the femur (Supplemental Figure 2A) of cancer-bearing mice. To evaluate the potential effects of Scl-Ab treatment on cancer cell relocation and metastases formation at other sites, several organs including lung, liver and brain were imaged by ex vivo bioluminescence after sacrifice (data not shown). Furthermore, the expression of the human leukocyte antigen (HLA) was determined by qRT-PCR and immunohistochemistry in the brain and in the lung, which are the most common sites of breast cancer metastases after bone. Importantly, inhibition of sclerostin did not change the abundance of breast cancer cells at extra-skeletal sites such as the lung and the brain (Figure 2, E and F, Supplemental Figure 2B), indicating that Scl-Ab treatment of mice with bone metastases does not cause breast cancer cell dissemination into other organs. Due to the strong reduction of bone metastatic burden, we hypothesized that Scl-Ab treatment might be beneficial for the overall life span. Indeed, Scl-Ab treatment significantly prolonged the survival time of breast cancer-bearing mice (Figure 2G).

*Sclerostin antibody treatment protects from breast cancer-induced bone destruction*

To test the hypothesis that inhibition of sclerostin might be a therapeutic approach to prevent bone destruction or to restore bone integrity in the context of breast cancer-mediated bone destruction, bone mass of mice with and without bone metastases was quantified by micro-computed tomography (µCT). Consistent with previous reports (27), Scl-Ab treatment of non-tumor-bearing mice increased the trabecular bone mass of the distal femur (Supplemental Figure 3, A and B) and
of the proximal tibia (Supplemental Figure 3, C and D), as well as the cortical thickness of the femur midshaft (Supplemental Figure 3, E and F). In mice with bone metastases, Scl-Ab treatment protected from breast cancer-induced osteolytic lesions and subsequent loss of bone mass of the distal femur (Figure 3A) and of the proximal tibia (Figure 3B). Furthermore, histological analyses of the proximal tibia revealed a significantly reduced bone formation rate and bone mass in cancer-bearing vehicle-treated mice compared to healthy vehicle-treated control animals (Figure 3, C to E). Intriguingly, Scl-Ab treatment of mice with bone metastases not only restored bone mass comparable to the bone mass of healthy vehicle-treated control animals, but also increased both bone mass and bone formation to the level of healthy Scl-Ab-treated mice (Figure 3, D and E). Detailed histomorphometric analysis of the bone surfaces nearby metastases revealed that the presence of breast cancer cells blunted bone formation in vehicle-treated mice, which was significantly restored by Scl-Ab treatment (Figure 3, F and G). These results suggest that Scl-Ab prevents bone loss in the context of bone metastases at least in part by restoring breast cancer-induced inhibition of bone formation at the bone-tumor interface.

To further analyze the mode of action of Scl-Ab in mice with breast cancer bone metastases, amino pro-peptide of type 1 collagen (P1NP) and Tartrate-resistant acid phosphatase (TRAP) 5b were measured in the serum of cancer-bearing mice as biomarkers for bone formation and bone resorption, respectively. While the P1NP serum concentration was higher in Scl-Ab-treated mice, the serum concentration of TRAP5b was decreased compared to vehicle-treated animals, suggesting that Scl-Ab treatment activates bone formation and reduces bone resorption (Supplemental Figure 4A and B). This dual mode of action has been consistently reported in the context of the treatment of postmenopausal osteoporosis (13, 14). To further investigate this finding at a cellular level, parameters of osteoblasts and osteoclasts were quantified in cancer-bearing mice. As expected, the number and size of bone-forming osteoblasts was significantly increased in Scl-
Ab-treated cancer-bearing mice compared to vehicle-treated control animals (Figure 3, H and I). Furthermore, the number and size of bone-resorbing osteoclasts was strongly reduced in response to Scl-Ab treatment (Figure 3, J and K), suggesting that the increase in bone mass was due to a concomitant increase in bone formation and a reduction of bone resorption. Together, these data strongly indicate that Scl-Ab treatment does not only increase bone mass through its anabolic action by restoring the tumor-induced impairment of osteoblast function, but also reduces the breast cancer-mediated increase in osteoclast activity, thereby reverting osteolytic disease.

**Inhibition of sclerostin prevents breast cancer-induced loss of muscle function**

Patients with bone metastases often suffer from muscle weakness (17, 18). Similarly, mice with breast cancer-induced metastatic bone disease have a reduced ex vivo muscle contractility compared to healthy animals (Figure 4A) (17). Given the beneficial effect of Scl-Ab in reducing tumor burden and bone destruction, we postulated that inhibition of sclerostin might also affect muscle function. To test this hypothesis, we analyzed the specific muscle force and endurance of the extensor digitorum longus (EDL) muscles of healthy mice and of mice with bone metastases treated with Scl-Ab or vehicle control. Although Scl-Ab treatment increased bone mass in healthy mice (Figure 3C and D, Supplemental Figure 3), muscle function was not altered in these animals (Figure 4A). In contrast, Scl-Ab treatment of mice bearing bone metastases protected from cancer-induced muscle weakness, determined by quantification of specific muscle force (Figure 4A) and endurance (Figure 4B). To better understand the disease mechanism of the altered muscle function, the tibialis anterior (TA) muscles were stained for succinate dehydrogenase and the cross-sectional area (CSA) of oxidative and non-oxidative fibers was quantified. Consistent with an unchanged muscle function, Scl-Ab treatment of healthy mice did not affect the CSA of neither oxidative nor non-oxidative muscle fibers (Figure 4, C and D). Interestingly, bone metastases caused a significant
reduction of the CSA, which was fully restored by Scl-Ab treatment (Figure 4, C and D). Since the oxidative muscle fibers were affected in cancer-bearing mice (Figure 4D), it appears to be likely that metastatic bone disease may cause an oxidative stress and muscle fiber atrophy in skeletal muscles, which is prevented by treatment with Scl-Ab.

*Antagonizing sclerostin restores breast cancer-induced increase of NF-κB signaling*

To further elucidate the molecular mechanisms underlying the effect of Scl-Ab on skeletal muscles of cancer-bearing mice, we investigated various signaling pathways involved in oxidative stress. Western blot analysis revealed an increased phosphorylation of p38, ERK1/2 and STAT3 in the gastrocnemius (GAS) muscle of cancer-bearing mice compared to healthy control animals (Figure 5A, Supplemental Figure 5A). Interestingly, the cancer-induced phosphorylation of p38 was restored in the muscles of Scl-Ab-treated animals (Figure 5A). Since activation of the NF-κB pathway has been shown to be a critical component of skeletal muscle atrophy (28), we investigated whether NF-κB signaling is also implicated in breast cancer-induced bone metastases. Under steady-state conditions, NF-κB is sequestered in the cytoplasm through an interaction with members of IκB family of inhibitor protein termed IκBs (Inhibitor of κB). Upon activation, the IKK complex, which contains the two IκB kinases IKKα and IKKβ, phosphorylates the IκB proteins thereby targeting them to ubiquitination and proteasomal degradation (28). Interestingly, IKKα and IKKβ were strongly phosphorylated in the muscles of cancer-bearing mice treated with vehicle compared to healthy animals. Furthermore, phosphorylation of the NF-κB subunit p65 was strongly increased in cancer-bearing mice, indicating an activated NF-κB signaling. Phosphorylation of IKKα, IKKβ and p65 was greatly reduced by Scl-Ab treatment (Figure 5A), suggesting that the presence of breast cancer bone metastases activates the p38-NF-κB signaling cascade, which is attenuated by the inhibition of sclerostin.
To determine whether sclerostin directly activates the p38-NF-κB signaling pathway in myoblasts, we stimulated C2C12 myoblasts with recombinant sclerostin. Sclerostin treatment did not induce phosphorylation of p38 or the components of the NF-κB pathway (Supplemental Figure 5B, data not shown), suggesting that the pathway is activated indirectly by cytokines present in the metastatic micro-environment. TGF-β1 is an abundant growth factor released from the bone matrix during breast cancer-induced bone resorption. Interestingly, stimulation of undifferentiated C2C12 myoblasts with TGF-β1 activated the p38-NF-κB pathway, thus recapitulating the effect observed in the muscles of cancer-bearing mice (Figure 5B). Inhibition of the NF-κB pathway abrogated the TGF-β1-induced phosphorylation of p38 (Figure 5C), suggesting that the effect of TGF-β1 is at least in part mediated via NF-κB. Consistently, TGF-β1 stimulation inhibited the differentiation of C2C12 myoblasts into myocytes as determined by a reduced expression of Myogenin and MyoD (Figure 5D). These data suggest that TGF-β1 released from the bone matrix during osteolytic bone resorption reduces muscle function, which is prevented by Scl-Ab treatment. Indeed, the expression of the TGF-β1 target gene Pai1 was significantly increased in muscles of bone metastases-bearing mice compared to healthy animals (Figure 5E). However, Pai1 expression was not increased in the muscles of Scl-Ab-treated tumor-bearing mice (Figure 5E), indicating that Scl-Ab treatment restores the breast cancer-induced activation of the TGF-β1 and p38-NF-κB pathway.

In colon cancer, NF-κB accumulation prevents the downregulation of Pax7, leading to a compromised muscle regeneration and impaired skeletal muscle function (29). To address the question whether this also occurs in mice with breast cancer bone metastases, we analyzed the number of Pax7-positive satellite cells in the tibialis anterior muscles. Interestingly, the number of Pax7-positive cells was significantly increased in cancer-bearing mice compared to healthy animals (Figure 5, F and G). This cancer-induced increase was partially but significantly restored by Scl-Ab treatment (Figure 5G). Together, these data suggest that pharmacological inhibition of
sclerostin protects from breast cancer-induced loss of muscle function by preventing the cancer-mediated activation of NF-κB signaling and the subsequent increase of Pax7-positive satellite cells.

**Discussion**

In the present study we report that MDA-MB-231 metastatic breast cancer cells secrete sclerostin, a soluble glycoprotein that binds to Lrp5/6 on osteoblasts and prevents the activation of the Lrp5/6-frizzled receptor complex by soluble Wnt ligands and subsequent activation of the canonical Wnt signaling pathway (11). In vitro assays revealed that metastatic breast cancer cell-derived sclerostin restricts osteoblast differentiation. In vivo inhibition of sclerostin using a monoclonal anti-sclerostin antibody (Scl-Ab) reduced bone metastatic burden and protected from breast cancer-induced bone destruction. At the tissue level, inhibition of sclerostin increased the bone formation rate and bone mass in both healthy and cancer-bearing mice. In mice with bone metastases, sclerostin inhibition increased the osteoblast number and the bone formation rate, while the number and activity of osteoclasts was decreased. Together these effects restricted the tumor burden and osteolytic bone destruction, thereby increasing the survival time of cancer-bearing mice.

Muscle weakness is known to accompany metastatic breast cancer disease (17, 30), which was confirmed in our model system. Interestingly, while Scl-Ab treatment did not affect the function of healthy muscle, the decreased strength and endurance of muscles obtained from mice with breast cancer bone metastases were reconstituted to the performance of muscles obtained from healthy mice. At the tissue level, sclerostin inhibition restored the relative smaller size of the oxidative and non-oxidative muscle fibers in cancer-bearing mice. Molecularly, similar to the results found by stimulation with TGF-β1, metastatic bone disease activated p38/NF-κB signaling and increased the number of Pax7-positive satellite cells in the muscle tissue, which was largely abrogated by Scl-Ab treatment.
Breast cancer is a devastating disease of high prevalence with a great potential to metastasize to bone. Bone metastases cause osteolytic lesions that lead to pain, pathologic fractures and muscle weakness (17, 18). These conditions are accompanied with a high morbidity and mortality and an overall reduced survival rate (8). Current treatments are effective in reducing the progression of osteolytic lesions but do not reverse bone destruction or improve muscle weakness, demonstrating the need for additional therapeutic options. In breast cancer-induced bone disease it is firmly established that breast cancer cells create a hostile micro-environment by secreting soluble factors, which increase the number and activity of bone-resorbing osteoclasts that eventually cause bone destruction (5). While some secreted factors including interleukins directly activate osteoclasts, other factors like PTHrP stimulate osteoblasts to secrete RANKL, which in turn augments osteoclast activity and bone resorption (5). The activated bone resorption releases matrix-derived growth factors like TGF-β1 that enhance cancer cell proliferation and further activate osteoclast activity and bone resorption, thereby establishing a feed-forward loop (5). In this context, osteoblasts mainly assume a passive role as recipients and sources of growth factors. However, recent evidence and data reported in this study also suggest an active role of osteoblasts during the establishment and expansion of multiple myeloma and breast cancer (27, 31). For instance, myeloma cells have been reported to inhibit bone formation, which is relieved by Scl-Ab treatment (27, 31). In contrast to breast cancer cells, myeloma cells do not express sclerostin and the inhibition of bone formation is likely mediated by Dkk1 and other factors (27, 31). Thus, augmentation of bone formation by Scl-Ab treatment is likely caused by antagonizing osteocyte-derived sclerostin.

In metastatic breast cancer it was shown that sclerostin is derived by the metastatic tissue in addition to osteocytes (32). This is consistent with our findings showing that metastatic MDA-MB-231 breast cancer cells and several other triple-negative breast cancer cells express sclerostin while
non-metastatic or hormone receptor-positive breast cancer cells do not express sclerostin. Estrogen receptor signaling has been shown to reduce sclerostin expression (33). Whether low sclerostin expression in ER-positive breast cancer cells is due to estrogen signaling remains to be elucidated. However, it is possible that the lack of the estrogen receptor is permissive for sclerostin expression in aggressive triple-negative cells. In the present study, sclerostin expression was analyzed in a restricted number of primary breast cancer tissue biopsies. Thus, investigating the expression of sclerostin in a larger patient cohort and in bone metastases would be useful to determine whether sclerostin expression correlates with the hormone receptor status and bone metastatic burden. Nevertheless, our results suggest that sclerostin expression might be an important functional feature of malignancy in triple-negative breast cancer that is likely to contribute to the deregulation of the local metastatic micro-environment and potentially to distant effects of bone metastases. Triple-negative breast cancer represents approximately 10-15% of all breast cancers and patients have high risk of relapse and poor survival compared to breast cancers expressing hormone receptors or HER-2/neu (34). The good prognosis of hormone receptor-positive and HER-2/neu amplified tumors is largely due to advances made in targeted therapies i.e. agents that interfere with hormone production/action or inhibit HER-2/neu, respectively (35). Unfortunately, there are no targeted therapies recommended for triple-negative breast cancer and the prevailing treatment remains to be standard chemotherapy. Thus, identification of sclerostin expression in a subset of triple-negative breast cancers might be of translational relevance for the development of improved future treatment options.

Our experiments reveal that MDA-MB-231 metastatic breast cancer cell-derived sclerostin binds to Lrp5 on osteoblasts, leading to an inhibition of canonical Wnt signaling and osteoblast differentiation. By this mechanism, metastatic breast cancer cells are likely to suppress osteoblast activity and bone formation in vivo, a component that might contribute to metastatic bone
destruction by favoring osteoclast-dependent bone resorption over osteoblast-mediated bone formation. In addition to augmenting osteoblast function and bone formation, sclerostin inhibition using Scl-Ab strongly reduced the osteoclast number and activity. These findings are consistent with Phase 2 and Phase 3 clinical trials in which postmenopausal women with osteoporosis were treated with an anti-sclerostin antibody to increase BMD (36, 37). The decline in bone resorption might be due to an increased expression of the Wnt target gene osteoprotegerin (OPG), which acts as a decoy receptor to RANKL and decreases the RANKL/OPG ratio and bone resorption (38). The dual osteoanabolic and anti-resorptive effect most likely contributes to the increase in bone mass and to the attenuation of bone destruction in the context of the osteolytic disease. Indeed, Scl-Ab treatment of mice bearing bone metastases greatly reduced the metastatic burden compared to vehicle-treated mice. Reduction of bone metastases did not increase metastases formation at other sites, leading to a decrease of the overall metastatic burden. Importantly, Scl-Ab treatment of metastases-bearing mice prolonged the overall survival rate.

Patients with advanced metastatic breast cancer often suffer from a wasting condition in which skeletal muscle undergoes atrophy, leading to a pronounced weight loss and ultimately cachexia (39, 40). In the muscle micro-environment, Pax7-positive satellite cells are the main resident stem cells that can give rise to myocytes (41). In addition, other interstitial and perivascular cell populations can also enter the myogenic lineage and contribute to muscle repair in response to tissue damage (42). Muscle damage can be a consequence of cachexia that is associated with an expansion and activation of Pax7-positive interstitial satellite cells and type 2 fiber atrophy (43). Satellite cell activation is indicated by an increased abundance of desmin and phospho-p38α (44). Under normal conditions, satellite cells are positioned between the myofiber sarcolemma and the basal lamina, but exit from quiescence upon muscle damage along with other myogenic progenitor populations and subsequently expand as myoblasts to repair damaged fibers (45). Perturbation of
the sarcolemma and the basal lamina as well as satellite cell activation in cachectic muscle is not due to the spreading of tumor cells but rather caused by circulating factors including TGF-β1 (17). Serum-derived cachectic factors activate NF-κB signaling in myogenic progenitors upstream of Pax7, which becomes upregulated and suppresses the expression of MyoD and myogenin. This mechanism blocks myogenic differentiation, myoblast fusion and promotes muscle wasting (29).

Furthermore, it has been demonstrated that the increase of circulating TGF-β1 in the context of osteolytic disease upregulated the NADPH oxidase 4 in skeletal muscle, causing an oxidation of the ryanodine receptor and calcium (Ca^{2+}) release channel (RyR1) (17). Oxidation of RyR1 leads to Ca^{2+} leakage and an impairment of muscle contraction (17). These findings strongly indicate that several molecular mechanisms exist that contribute to a cancer and bone metastases-mediated decline of muscle function and ultimately cachexia.

In the present study, we report that mice with breast cancer bone metastases have a reduced muscle strength and endurance and demonstrate that the muscle weakness is fully abrogated by a treatment with Scl-Ab. Our data are consistent with the findings reported by others and demonstrate that advanced stages of malignancy and bone metastases cause muscle weakness (17). However, the therapeutic concept of sclerostin inhibition on muscle function has not been reported thus far and is of great clinical interest. At the tissue level, advanced malignancy with bone metastases caused a reduction of the size of oxidative and non-oxidative muscle fibers. Antagonizing sclerostin activity restored the muscle fiber atrophy, thereby normalizing the tissue micro-architecture. Molecularly, cancer-induced muscle fiber atrophy was associated with an increased NF-κB and p38 signaling that was also induced by TGF-β1 and normalized by Scl-Ab treatment. Stimulation with TGF-β1 also reduced the myogenin expression and myoblast differentiation. These findings suggest that the enhanced osteoclast activity in the bone-cancer micro-environment increases the serum concentration of bone matrix-derived TGF-β1, impairs myogenin expression and muscle
regeneration downstream of NF-κB and p38 signaling. Indeed, the TGF-β1 pathway activity was increased in the muscle tissue evidenced by an increased Pai-1 expression, which was at least in part reconstituted by sclerostin inhibition. Furthermore, the number of Pax7-positive satellite cells was strikingly increased in tumor-bearing mice and almost normalized by the Scl-Ab treatment. We therefore propose that the bone-forming effect and the anti-resorptive properties of the anti-sclerostin treatment both contribute to the reduction of osteolytic bone destruction and the extension of the survival time, while the anti-catabolic effect mainly contributes to the restoration of the muscle function by preventing an increase in TGF-β1 concentration.

Consistent with other reports, we demonstrated that Scl-Ab treatment has a bone-sparing effect and reduces bone destruction in the context of a malignant bone disease that disturbs the balance of the bone micro-environment. Muscle fiber atrophy and muscle weakness, which often accompany metastatic breast cancer, was also prevented by pharmacological inhibition of sclerostin. These features are clinically relevant and may improve the current treatment options of patients with breast cancer bone metastases. In all pre-clinical and clinical studies that are available thus far, Scl-Ab treatment has been proven safe with regard to malignant side effects. Its clinical use might therefore be possible even with a history of a malignant disease or an actual cancer diagnose, which is a great advantage over the currently available bone-anabolic drugs PTH 1-34 (Teriparatide; Forteo®/Forsteo®, Eli Lilly and Company) and PTHrP (Abaloparatide; Tymlos®, Radius BioPharma) (6). It would therefore be of great interest to investigate in a clinical context, if sclerostin inhibition is also beneficial for patients with breast cancer bone metastases. This would expand the repertoire of oncological treatment options and improve the bone health of breast cancer patients.
Methods

Animal experiments. MDA-MB-231 breast cancer cells stably expressing the luciferase gene were described elsewhere (46) and injected into the left ventricle of 8-weeks old female immune-compromised CB-17/lcr-Prkdc\textsuperscript{scid}/Rj mice (Janvier Laboratories). Bioluminescence imaging (BLI) was performed two weeks after breast cancer cell injection using an IVIS 200 imaging system (Perkin Elmer) and mice were randomized into two treatment arms. One group (n=8) received vehicle (50 µl/10g mouse) and the other group (n=8) anti-sclerostin antibody (Scl-Ab) (100 mg/kg, BPS-804, Setrusumab, Mereo BioPharma) intravenously (i.v) once a week for four weeks. Tumor burden was measured weekly by BLI. Mice were sacrificed one week after the last injection. In the survival study, mice were treated once a week with vehicle (n=6) or Scl-Ab (n=6), monitored daily and sacrificed once well-defined criteria such as 20% weight loss were reached. Healthy CB-17/lcr-Prkdc\textsuperscript{scid}/Rj mice without treatment (n=10), treated with vehicle (n=10) or with Scl-Ab (n=10) served as control. Investigators were blinded to the group allocation.

Micro-computed tomography. Micro-computed tomography (μCT) was used for three dimensional analyses of long bones. Long bones of mice were analyzed using high-resolution μCT with a fixed isotropic voxel size of 10 µm (70 peak kV at X µA 400 ms integration time; Viva80 micro-CT; Scanco Medical AG). All analyses were performed on the digitally extracted bone tissue using 3D distance techniques (Scanco Medical AG) as reported previously (47). Region of interest (ROI) was defined manually by drawing contours in slices. Due to cancer-induced bone destruction and absence of intact bone surfaces in cancer-bearing mice, the ROI contained both the cortical and the trabecular bone. In non-cancer-bearing bones only trabecular bone was analyzed using a standard method (47).
**Ex vivo analysis of muscle function.** *Ex vivo* contractility of the *Extensor digitorum longus* muscle (EDL) was analyzed using a device dedicated to the measurement of mouse muscle properties in situ, *ex vivo* and in vivo (Aurora Scientific). For this purpose, EDL was dissected from the hind limb, loops were tied to the tendons of the muscles and mounted to a force transducer. Muscles were stimulated to contract using a supramaximal stimulus applied by two electrodes. For fatigue studies, EDL was stimulated with 70 Hz for 50 repeats and the maximum tetanic force was detected. Data were collected and analyzed using Dynamic Muscle Control/Data acquisition (DMC) and Dynamic Muscle Control Data Analysis (DMA) programs (Aurora Scientific). Specific muscle force was calculated as described previously (48). Investigators were blinded during data analysis.

**Histological and immunohistochemical analyses.** For bone analyses, mice were injected seven and two days prior to sacrifice with calcein (20 mg/kg) and demeclocycline (20 mg/kg; both Sigma-Aldrich), respectively. Tibiae were collected and fixed in 4% paraformaldehyde (PFA) for 48 hours. For histomorphometric analysis, tibiae were embedded in methylmethacrylate. Toluidine blue, von Kossa/van Gieson and Tartrate-resistant acid phosphatase (TRAP) staining were performed using 5 µm sagittal sections. Quantitative bone histomorphometric measurements were performed according to standard protocols using an OsteoMeasure system (Osteometrics) (49). Femora were cleaned from soft tissue, fixed in 4% PFA for 24 hours at +4°C, decalcified with 4% EDTA for 4 days and 20% EDTA for 24 hours and embedded in paraffin. Sections were cut and immuno-histochemical staining was performed using an anti-Osterix antibody (Rabbit polyclonal; Santa Cruz). Brain and lung tissues were fixed in 4% PFA for 24 hours at +4°C. Tissue samples were embedded in paraffin, cut and immuno-histochemical staining was performed with an antibody against HLA Class 1 ABC (Mouse monoclonal, Abcam).
For histological analyses of the muscle tissues, the *tibialis anterior* (TA) muscle was dissected from the hind limb, embedded in 10% Gum Tragacanth and snap-frozen in cooled 2-Methylbutane. Cryo sections were performed using a cryotome and sections were stained for succinate dehydrogenase (SDH). Pax7 staining was performed using anti-Pax7 antibody (mouse monoclonal; Developmental Studies Hybridoma Bank (DSHB)). The muscle fiber area and the number of Pax7-positive cells were quantified using the Osteomeasure system (Osteometrics). The investigators were blinded regarding the treatment.

**Cell culture.** For calvarial osteoblast cultures, calvariae were dissected from 1 to 3-day old mice and digested sequentially in α-MEM containing 0.1% collagenase and 0.2% dispase (both Roche). Cell fractions 2 to 4 were combined and expanded in α-MEM (Invitrogen) containing 10% Fetal Bovine Serum (FBS; Invitrogen) and 100 U/ml penicillin; 100 µg/ml streptomycin (Invitrogen). C2C12 cell were purchased from DSMZ and cultured in D-MEM (Invitrogen) containing 10% FBS and 1% Penicillin/streptomycin. Cells were stimulated with 10 ng/ml TGF-β1 (R&D) or 100 ng/ml recombinant sclerostin (R&D). To inhibit NF-κB signaling in C2C12 cells, cells were pre-treated for 1 h with NEMO-binding domain peptide (NPD, Enzo) or L-TAT control peptide (Enzo). Myocyte differentiation was induced using D-MEM supplemented with 2% Horse Serum (Invitrogen) and 1% Penicillin/streptomycin. MCF-7 and MDA-MB-231 breast cancer cells were purchased from ATCC. MCF-7 cells were cultured in D-MEM (Invitrogen) and MDA-MB-231 cells were grown in α-MEM, both supplemented with 10% FBS and 1% Penicillin/streptomycin. All cell lines were tested for mycoplasma contamination. MDA-MB-231 cells were transfected with scrambled control siRNA or siRNA against sclerostin (Origene) using Lipofectamine 3000 (ThermoFischer) according to manufacturer’s instructions. For collection of conditioned medium (CM), MDA-MB-231 cells were cultured in the presence of 1% FBS for 24 hours and CM was
collected and stored at -80°C. Osteoblast differentiation was induced by supplementing α-MEM with 0.2 mM L-ascorbic acid and 10 mM β-glycerophosphate (both Millipore). Osteoblast differentiation was determined by staining the cells with 40 mM Alizarin Red S (AR-S; Sigma-Aldrich) solution at pH 4.2 for 10 min at room temperature after fixing the cells in 4% neutrally buffered formaldehyde solution. To determine Wnt signaling activity, calvarial osteoblasts were transfected with TopFlash and Renilla plasmids using the Neon System (Invitrogen). Luciferase activity was measured using the Promega Dual Luciferase kit following manufacturer’s guidelines.

**RNA isolation and qRT-PCR.** Lung and brain tissues were snap frozen in liquid nitrogen and RNA was isolated using Trizol. RNA was isolated from cultured cells using the RNeasy Plus Mini-kit (Qiagen). cDNA was synthesized using the NEB ProtoScript II First Strand cDNA Synthesis kit according to manufacturer’s instructions. Expression of *Myogenin*, *MyoD*, *Pai1*, *SOST* and the human leukocyte antigen (*HLA*) gene was quantified by qRT-PCR. After normalization to *GAPDH* mRNA, relative expression level of each target gene was calculated using the comparative CT method.

**Expression analysis in human tissue.** *SOST* expression in 48 human breast cancer tissues was analyzed using the TissueScan Breast Cancer Array III (Origene) according to the manufacturer’s instructions. A TissueScan Cancer Survey Array 96 I (Origene) consisting of 96 samples from 72 tumor samples and 24 non-malignant tissue samples from 8 different primary organs was utilized to analyze the expression pattern of *SOST* in various malignant tissues. The expression of *SOST* was quantified using qRT-PCR. *BETA-ACTIN (ACTB)* was used an internal control.
**Immunoblotting.** Cells were lysed in low salt lysis buffer (pH 7.6) containing 50 mM Tris base, 150 mM NaCl, 0.5% Nonindet P-40 (NP-40), 0.25% Sodium deoxycholate and complete protease and phosphatase inhibitors (Roche). Muscle tissue was homogenized in lysis buffer (20 mM Tris, pH 7.8, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol and protease and phosphatase inhibitors) using a dounce homogenizer. Lysates were separated on 12% polyacrylamide gels and subjected to immunoblot analysis. Immunoblots were incubated over night at 4°C with primary antibodies listed in Table 2. Peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies (1:10 000, Santa Cruz, Cat. No: W401B, W402B) were used to visualize bands using the Clarity Western ECL Substrate (BioRad). Images of the immunoblots were acquired using the ChemiDoc imaging system and Image Lab software (BioRad).

**ELISA.** Enzyme-linked immunosorbent assay (ELISA) was used to determine P1NP and TRAP concentration in the mouse serum. All procedures were performed according to the manufacturer’s (immunodiagnostics systems) instructions.

**Statistics.** Parametric data were analyzed using a two-tailed Student’s t-test when two groups were compared. A one-way analysis of variance (ANOVA) was used when more than two groups were compared, followed by a Tukey’s post-hoc analysis to compare the groups. Probability values were considered statistically significant at p<0.05. Experiments were repeated at least three times as biological replicates with minimum two technical replicates. All quantitative data are represented as mean ± SEM.
Study approval. All animal experiments were conducted in compliance with ethical regulations and according to the protocols approved by the local authority for animal welfare.

Author contributions: E.H. designed research studies, analyzed data and wrote the manuscript, S.S. conducted experiments, acquired and analyzed data, D.B. conducted experiments, acquired and analyzed data, J.P. conducted experiments, acquired and analyzed data, H.S. conducted experiments and analyzed data, H.T. conceived the study, designed research studies, conducted experiments, acquired and analyzed data and wrote the manuscript.

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**Figure 1. Breast cancer-derived sclerostin inhibits Wnt signaling in osteoblasts.** (A and B) Calvarial cells were differentiated into osteoblasts in the presence of control medium or cancer-conditioned medium (CM) collected from MDA-MB-231 metastatic breast cancer cells. Osteoblast differentiation was determined by quantification of Runx2 and Osteocalcin (Ocn) gene expression (A) and by Alizarin Red staining (B) (n=4 independent experiments). (C) Calvarial osteoblasts were cultured in the presence of the indicated amount of MDA-MB-231-derived CM. Wnt signaling activity was determined by TopFlash reporter assay (n=4 independent experiments). (D) Sclerostin mRNA expression was quantified in non-metastatic MCF-7 and metastatic MDA-MB-231 breast cancer cells by qRT-PCR (n=6 independent experiments). (E) SOST mRNA expression was analyzed in breast cancer tissue from 48 patients. Proportion of Sclerostin-positive and Sclerostin-negative tissue samples is shown in all patients and in triple-negative (ER-, PR-, HER-) and in hormone receptor-negative (ER-, PR-) patients. All, n=48; ER-, PR-, HER-, n=9; ER-, HER-, n=7. (F) Wnt signaling activity in calvarial osteoblasts cultured with control medium or with CM from MDA-MB-231 cells transfected with scrambled control siRNA (si-ctrl CM) or siRNA against sclerostin (si-sclerostin CM) (n=6 independent experiments). (G) Wnt signaling activity in calvarial osteoblasts isolated from mice heterozygous for the LRP5 mutation G171V (LRP5-G171V+/+) and from control littermates (LRP5-G171V+/+) stimulated with control medium or cancer CM (n=4 independent experiments). Data are represented as mean ± SEM. Two-tailed Student’s t-test was used to compare two groups (A, D), and ANOVA followed by Tukey’s post-hoc analysis was used to compare three or more groups (C, E, F), *p<0.05, **p<0.01, ***p<0.001.
Figure 2. Pharmacological inhibition of sclerostin reduces bone metastatic burden in mice. MDA-MB-231 breast cancer cells stably expressing the luciferase gene were injected into the left ventricle of 8-week old female immune-compromised SCID mice. Micro-metastases were detected two weeks after breast cancer cell injection by bioluminescence imaging (BLI). Mice were randomized and received either vehicle (n=8) or anti-sclerostin antibody (Scl-Ab; n=8) once a week for four weeks. (A and B) Tumor growth in bone was visualized after 4 weeks of treatment (A) and quantified (B) by BLI. Values are represented in a log10 scale. (C and D) Quantification of the metastases area (C) in the tibia (D) of cancer-bearing mice treated with vehicle (n=16 tibiae) or Scl-Ab (n=16 tibiae) using histological sections. Scale bar indicates 1 mm. (E and F) Quantification of the human leukocyte antigen (HLA) mRNA expression in the lung (E) and in the brain (F) by qRT-PCR (n=8). (G) Kaplan-Meier survival curve of cancer-bearing mice treated with vehicle (n=6) or Scl-Ab (n=6). Data are represented as mean ± SEM. Two groups were compared using 2-tailed Student’s t-test, *p<0.05.
Figure 3. Anti-Sclerostin antibody treatment protects from breast cancer-induced bone destruction. (A and B) Micro-computed tomography (µCT) analysis of bone mass (BV/TV, bone volume/total volume) of the femur (A) and of the tibia (B) of cancer-bearing mice treated with vehicle (n=8) or anti-sclerostin antibody (Scl-Ab; n=8). (C) Von Kossa/van Gieson staining of the proximal tibiae and fluorescence double labeling (insets) from healthy mice and cancer-bearing mice treated with vehicle or Scl-Ab. Scale bars indicate 1 mm (black) and 50 µm (white). (D) Histomorphometric analysis of the bone mass (BV/TV, bone volume/tissue volume) of the proximal tibia (healthy non-treated n=5, vehicle treated n=10, Scl-Ab treated n=10; cancer-bearing vehicle treated n=8, cancer-bearing Scl-Ab treated n=8). (E) Analysis of the bone formation rate (BFR/BS, bone formation rate/bone surface) of the proximal tibia. (F) Von Kossa/van Gieson staining (two left panels) of the proximal tibia of mice with bone metastases and calcein double-labeling (two right panels) at the bone-cancer interface. Scale bars indicate 50 µm. (G) Quantification of the bone formation rate per bone surface (BFR/BS) at the bone-cancer interface (vehicle n=6, Scl-Ab n=3). (H) Immunohistochemical staining of Osterix in the distal femur of cancer-bearing mice treated with vehicle or Scl-Ab. Scale bar indicates 50 µm. (I) Histomorphometric analysis of the distal femur (N.Ob/B.Pm, number of osteoblasts/bone perimeter; Ob.S/BS, osteoblast surface/bone surface) (vehicle n=6, Scl-Ab n=3). (J) Tartrate-resistant acid phosphatase (TRAP) staining of the distal femur of cancer-bearing mice treated with vehicle or Scl-Ab. Scale bar indicates 50 µm. (K) Quantification of the number of osteoclasts per bone perimeter (N.Oc/B.Pm) and of the osteoclast surface per bone surface (Oc.S/BS) (vehicle n=8, Scl-Ab n=8). Data are represented as mean ± SEM. Two-tailed Student’s t-test was used to compare two groups (A, B, G, I, K), and ANOVA followed by Tukey’s post-hoc analysis was used to compare three or more groups (D, E), *p<0.05, **p<0.01, ***p<0.001.
Figure 4. Inhibition of sclerostin prevents breast cancer-induced loss of muscle function. (A) Specific force of the extensor digitorum longus (EDL) muscle from healthy mice without treatment (n=5), treated with vehicle (n=10) or anti-sclerostin antibody (Scl-Ab, n=10) and from cancer-bearing mice treated with vehicle (n=8) or Scl-Ab (n=8). (B) Endurance of the EDL muscle of mice with bone metastases treated with vehicle (n=8) or Scl-Ab (n=8). (C) Tibialis anterior muscle sections from healthy mice without treatment, vehicle or Scl-Ab treatment as well as from mice with bone metastases treated with vehicle or Scl-Ab stained for succinate dehydrogenase (SDH). Two representative muscles are shown/group. Scale bar indicates 50 µm. (D) Quantification of the cross-sectional area (CSA) of all muscle fibers, oxidative fibers (dark) and non-oxidative (light) fibers using SDH-stained muscle sections from healthy mice without treatment (n=5), vehicle (n=10) or Scl-Ab treatment (n=10) and from cancer-bearing mice treated with vehicle (n=8) or Scl-Ab (n=8). Data are represented as mean ± SEM. Three or more groups were compared using ANOVA followed by Tukey’s post-hoc analysis, *p<0.05, **p<0.01, ***p<0.001.
Figure 5. Treatment with an anti-sclerostin antibody restores breast cancer-induced activation of NF-κB signaling and increased number of Pax7-positive cells. (A) Immunoblot analysis of phosphorylated IKKα and IKKβ (p-IKKα and p-IKKβ), phosphorylated NF-κBp65 (p-NF-κBp65), phosphorylated p38 (p-p38) and total p38 in the gastrocnemius (GAS) muscle of healthy non-treated mice (n=5) and cancer-bearing mice treated with vehicle (n=8) or Scl-Ab (n=8). Actin was used as loading control. Representative samples are shown. (B) Immunoblot analysis of phosphorylated NF-κBp65 (p-NF-κBp65), phosphorylated p38 (p-p38) and total p38 in C2C12 myoblasts stimulated with vehicle (veh) or TGF-β1. Actin was used as loading control. Representative image of 6 independent experiments is shown. (C) Immunoblot analysis of phosphorylated NF-κBp65 (p-NF-κBp65), phosphorylated p38 (p-p38) and total p38 in C2C12 cells treated with a control peptide or an NF-κB blocking peptide (NPD) and stimulated with vehicle or TGF-β1. Actin was used as loading control. Representative image of 4 independent experiments is shown. (D) Myogenin and MyoD mRNA expression was quantified by qRT-PCR in C2C12 cells after 10 days of myogenic differentiation (n=3). (E) Pai1 mRNA expression was quantified in the GAS muscle from healthy non-treated mice (n=5) and from cancer-bearing mice treated with vehicle (n=8) or Scl-Ab (n=8). (F) Immuno-histochemical staining of Pax7 in the TA muscle from healthy non-treated mice and from mice with bone metastases treated with vehicle or Scl-Ab. Scale bar indicates 50 µm in the upper panel and 100 µm in the lower panel. (G) Quantification of Pax7-positive cells in the TA muscle from healthy non-treated mice (n=5) and from mice with bone metastases treated with vehicle (n=8) or Scl-Ab (n=8). Data are represented as mean ± SEM. Two-tailed Student’s t-test was used to compare two groups (C), and ANOVA followed by Tukey’s post-hoc analysis was used to compare three or more groups (D, F), *p<0.05, ***p<0.001.
Table 1. Sclerostin expression in different cancers

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<td>2 (22%)</td>
<td>7 (78%)</td>
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<td>9 (100%)</td>
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Table 2. Antibodies used in this study.

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IHC, Immuno-histochemistry; IB, Immunoblot