MRTF activity in breast cancer cells promotes osteoclastogenesis through a paracrine action of CTGF

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ABSTRACT

Bone is a frequent site for breast cancer metastasis. Conditioning of local tumor microenvironment through crosstalk between tumor cells and bone resident cells in the metastatic niche is a major driving force for bone colonization of cancer cells. This study demonstrates that Myocardin-related transcription factor (MRTF - a major cofactor for the transcription factor serum-response factor, SRF) activity in breast cancer cells is required for their ability to promote osteoclast differentiation of bone marrow-derived monocytes and colonize in bone. MRTF depletion in breast cancer cells affects a wide range of cell-secreted osteoclast-regulatory factors including connective tissue growth factor (CTGF), a prominent bone metastasis-associated gene that exhibit strong positive association in expression with MRTF activity in human breast cancer. Rescue experiments demonstrate that CTGF is an important paracrine mediator of pro-osteoclastogenic action of MRTF in breast cancer cells. Both SRF-dependent and -independent (SAP-domain directed) functions of MRTF are required for its ability to regulate CTGF expression and osteoclast differentiation. In conclusion, this study uncovers a novel MRTF-directed tumor-extrinsic mechanism of bone colonization of cancer cells and suggest that MRTF inhibition could be a novel strategy to suppress osteoclast activity and skeletal involvement in metastatic breast cancer.

Keywords: MRTF, SRF, bone, metastasis, osteoclast, CTGF

INTRODUCTION

Breast cancer is one of the leading causes of cancer-related death in women. The vast majority of breast cancer-related deaths are due to the metastatic spread of tumor cells to distant organs. Bone is a frequent site of breast cancer metastasis with the median survival of patients after tumor cells have metastasized to the bone being 2-3 years (1-4). Bone metastasis falls into three different categories, namely osteolytic (involves bone resorption), osteoblastic (involves deposition of new bone), and mixed (involves both formation and resorption of bone) with the vast majority of breast cancer-associated metastasis being osteolytic in nature (>70%) (5). Metastasis to the bone is complex process that involves chemotactic attraction and attachment of circulating tumor cells to the bone surface and interactions between cancer cells and various stromal cells in the bone microenvironment that lead to a vicious cycle of bone remodeling. Under normal conditions, bone-forming osteoblasts produce macrophage colony-stimulating factor (M-CSF) and receptor activator for nuclear factor κB (NFκB) ligand (RANKL) which stimulate pre-osteoclast cells (OCLp) to differentiate into osteoclasts (OCL), a type of cells that is responsible for degradation of mineralized bone. However, osteoblasts also produce osteoprotegerin (OPG) which binds to and sequesters RANKL thereby limiting OCL activity and regulating the levels of bone resorption (6-9). The balancing act of osteoblasts and OCL is dysregulated in the presence of tumor cells where osteoclastogenesis and OCL survival are greatly enhanced by increased secreted levels of RANKL and other soluble factors (e.g. IL6, IL11, IL1, TNF, PTHrP) contributed by tumor cells, osteocytes, osteoblasts and immune cells (10-12). Increased bone resorption promotes the release and activation of ECM-bound growth factors and cytokines, further amplifying this loop and stimulating tumor cell proliferation and metastatic colonization of tumor cells (13).

Myocardin-related transcription factors (MRTFs) are key coactivators of serum-response factor (SRF) that link actin dynamics to SRF-mediated transcription of a wide variety of genes including SRF itself and many

others which play important roles cytoskeleton remodeling, cell adhesion and ECM regulation (14-16). MRTF is also capable of transcriptionally regulating genes in an SRF-independent manner utilizing its chromatin-binding SAP domain and/or interactions with other transcription factors (16-18). There are two major isoforms of MRTF, namely MRTF-A (also known as MKL1) and MRTF-B (also known as MKL2). While these isoforms share structural and functional similarities and compensate for each other in many loss-of-function settings, phenotypic differences between mice deficient in MRTF-A vs-B suggest that these isoforms are not completely functionally redundant (19,20). Several studies have demonstrated MRTF's role in metastatic colonization of cancer cells. For example, MRTF depletion dramatically impairs lung colonization ability of breast cancer and melanoma cells in tail-vein injection model of experimental metastasis (21). Consistent with these findings, we recently demonstrated that MRTF-SRF interaction is critical for tumor-initiating ability and post-extravasation metastatic outgrowth of breast cancer cells (22). Interestingly, MRTF-induced actin polymerization and cell stiffening also increases susceptibility of tumor cells to T cell attack thereby limiting lung colonization of tumor cells through immunosurveillance when MRTF is overexpressed (23). Collectively, these findings suggest that a balanced MRTF activity is required for efficient metastatic colonization of cancer cells.

We previously showed that *in vivo* administration of CCG-1423, a small molecule that is widely used to inhibit MRTF's function (24-26), dramatically reduces development of skeletal metastases following systemic inoculation of breast cancer cells (22). However, CCG-1423 or its derivative compounds have poly-pharmacology and are known to target other proteins (example: MICAL and pirin)(27,28). In fact, CCG compounds bind to pirin at a much higher affinity than to MRTF. This is further underscored by recent findings of genome-wide transcriptional repression in cells by CCG-1423 that extends beyond MRTF's action (BioRxiv: https://doi.org/10.1101/2023.09.08.556810). Furthermore, in an *in vivo* administration setting, CCG compound's action is targeted globally in a non-cell type-specific manner. Therefore, to what extent MRTF-activity in tumor cells contributes to bone colonization remained unclear. In the present study, we provide genetic proof-of-concept for MRTF's essential role in bone colonization of breast cancer cells, and further demonstrate a novel role of tumor-extrinsic MRTF activity in osteoclastogenesis partly through a paracrine action of connective tissue growth factor (CTGF).

RESULTS

MRTF depletion impairs experimental bone colonization of breast cancer cells

To query MRTF's role in bone colonization of breast cancer cells, we genetically engineered our previously generated GFP/luciferase (luc) expressing subline of MDA-MB-231 (denoted as MDA-231 hereon), a widely used human triple-negative (lacks estrogen-, progesterone- and HER2-receptors) metastatic breast cancer cell line, to stably silence both MRTF-A and MRTF-B isoforms using a single shRNA. Non-targeting control shRNA-transfectants served as control. Immunoblots shown in **Fig 1A** confirm efficient depletion of both MRTF isoforms in MRTF-A/B-shRNA (referred to as MRTF-knockdown hereon) expressing subline of MDA-231 cells. We next performed intracardiac injection-based experimental metastasis assay (allows for wide-spread metastasis including in the bone) with control vs MRTF knockdown MDA-231 cells in immuno-deficient NOD-SCID-gamma (NSG) mice and monitored metastatic burden by bioluminescence imaging (BLI). We chose an experimental metastasis assay over orthotopic spontaneous metastasis assay to focus specifically on metastatic growth and decouple the effect of MRTF knockdown-induced changes in tumor cell dissemination. Use of NSG mice also enabled us to decouple the secondary effect of MRTF on immunosurveillance in metastatic colonization process.

Endpoint BLI imaging showed dramatic reduction in metastatic burden when mice were inoculated with MRTF knockdown cells vs control cells (**Figs 1B-C**). While 6 out 8 animals (75%) inoculated with control MDA-231 cells developed skeletal mice as judged by the presence of BLI signal (also confirmed by bone histology – **Fig 1D**) in the long bones (marked by asterisks in **Fig 1B**), inoculation of MRTF knockdown MDA-231 cells resulted in skeletal metastasis in only 1 out 8 (12.5%) animals. These *in vivo* data establish that MRTF activity is critical for bone colonization of breast cancer cells.

Substrate/ECM stiffness plays an important role in dictating the rate of proliferation tumor cells (29). Even isogenic pairs of dormant and outgrowth-proficient breast cancer cells (such as D2.0R vs D2.A1) proliferate at a similar rate on rigid 2D substrate but very differently when seeded on soft 3D ECM substrates (30-32). Although suppressing MRTF expression dramatically reduces proliferation of normal mammary epithelial and breast cancer cells when seeded within or top of soft ECM substrate (mimics stiffness of compliant organs such as lungs)(22,33), whether MRTF activity is equally critical for tumor cell outgrowth on relatively rigid bone microenvironment is not known. Therefore, we assessed the relative growth of control vs MRTF knockdown breast cancer cells (MDA-231) seeded on devitalized bone chips prepared from mouse calvaria. These experiments revealed that MRTF knockdown dramatically inhibits outgrowth of MDA-231 cells on bone surface (**Fig 2**), indicating anindispensable role of MRTF in tumor cell outgrowth in the bone microenvironment.

Tumor-cell intrinsic activity of MRTF promotes osteoclast activation

Since bone metastasis associated with breast cancer is predominantly osteolytic in nature (5), and OCLs are the key players in bone resorption, we asked whether tumor-intrinsic MRTF activity has any role in modulating the osteoclastogenesis process. To address this question, we performed breast cancer-OCL indirect co-culture assays. Specifically, we assessed RANKL-induced OCL differentiation of OCL progenitor (OCLp) cells prepared from primary bone marrow derived monocytes (BMDMs) with or without exposure to the conditioned media (CM) harvested from 2D cultures of control vs MRTF knockdown MDA-231 cells (schematized in **Fig 3A**). From these experiments, we quantified two metrics including the total cell number /well and OCL count as a % of total number of cells, the results of which are summarized in **Fig 3B-C**. OCLs were identified by tartrate-resistant alkaline phosphate (TRAP)-positive multi-nucleated (3 or more nuclei) cells. Although we did not see any significant impact of RANKL addition on the total cell count in the culture, addition of CM harvested from tumor cells regardless of the status of MRTF expression suppressed cell proliferation (**Fig 3B**). As expected, RANKL addition dramatically promoted OCL differentiation, and this was further enhanced upon exposure to the CM of control shRNA cells (**Fig 3C**). However, MRTF-depletion severely dampened OCL differentiation even to a level below that seen in the group representing RANKL addition alone suggesting that MRTF-depletion in breast cancer cells has a suppressive effect on osteoclastogenesis (**Fig 3C;** representative images of TRAP staining of RANKL-stimulated BMDMs exposed to CM of control vs MRTF knockdown MDA-231 cells are shown in **supplementary Fig S1**). DC-Stamp (a transmembrane protein) and Cathepsin-K (a protease) are major regulators of osteoclastogenesis, and deficiency of either is linked to decreased OCL differentiation and/or activity (34,35). By quantitative RT-PCR analyses of parallel cultures, we further demonstrated that expressions of both Cathepsin-K and DC-Stamp in RANKL-stimulated OCLp are dampened when exposed to the CM of MRTF knockdown MDA-231 cells (**Figs 3D-E**). To establish generality of MRTF's role in modulating osteoclastogenesis, we repeated similar experiments with CM harvested from the cultures of D2A1 (a murine breast cancer cell line) cells that were also genetically engineered for shRNA-mediated

stable suppression of MRTF isoforms (knockdown of MRTF isoforms in D2A1 cells shown later in **Fig 5**). Similar to our findings in MDA-231 cells, we also a saw a general trend of reduced total cell count in D2A1 CM-exposed cultures but the difference was statistically significant only between RANKL-deprived culture and RANKL stimulated culture exposed to the CM of knockdown cells (**supplementary Fig S2A).** However, RANKL-induced OCL differentiation was further enhanced by CM derived from control shRNA-bearing D2A1 cells, and dramatically inhibited when MRTF-expression was silenced, recapitulating our findings in MDA-231-BMDM co-culture studies (**Fig S2B**). These results demonstrate that MRTF-activity of breast cancer cells promotes OCL differentiation in a paracrine signaling manner.

MRTF activity promotes OCL differentiation partly through CTGF regulation requiring both SRF-dependent and –independent functions

To gain insight into how MRTF activity in breast cancer cells in OCL differentiation/bone colonization, we followed an analysis pipeline as schematized in **Fig 4A**. First, we performed RNAseq analyses to transcriptionally profile 2D and 3D cultures of our previously described GFP/Luc expressing subline of MDA-231 cells with or without doxycycline-inducible overexpression of various forms of MRTF-A: wild-type (WT) MRTF-A, ΔSRF-MRTF-A (impaired in SRF's interaction) or ΔSAP-MRTF-A (deficient in SAP-domain function) (22)(details of the RNAseq results are part of another manuscript under preparation). From the 2D RNAseq data, we identified differentially expressed genes (DEGs - based on false discovery rate (FDR) and absolute log_2fold-change set to <0.001 and >1.5, respectively) upon MRTF-A overexpression. We then overlapped those DEGs with previously published DEGs of 2D cultures of MDA-231 cells with or without stable co-silencing of MRTF isoforms restricted to identical FDR and fold-change criteria (21). From these analyses, we identified a subset of genes that were downregulated in MRTF-A/B knockdown and upregulated in WT-MRTF-A overexpression settings to generate a high-stringency MRTF gene signature comprising of 33 genes. Second, we compiled a signature of 21 genes based on two previously published findings (12,36) that are associated with bone metastasis of breast cancer cells. This list (referred to as breast cancer bone metastasis gene signature or BCBMgs in short) included 10 upregulated (*CXCR4, MMP1, ADAMTS1, FGF5, CTGF, IL11, FST, NFAT1, Ph4, and BBS1*) and 11 downregulated (*APOBEc3B, NUP155, MFAP3L, NIP7, C6orf17, KCNS1, STEAP3, C16orf61, PALM2, ATL2, and SFT2D2*) genes. Third, we mapped high-stringency MRTF gene signature against the BCBMgs to identify candidate MRTF-regulated bone-metastasis associated genes that are directionally consistent with impaired skeletal metastasis phenotype in MRTF knockdown setting as seen in our in vivo studies. As per these analyses, we identified three bone metastasis-associated genes that are positively upregulated by MRTF activity including CTGF, IL11, and FGF5, and further validated these findings from RNAseq data of 3D cultures of MDA-231 cells. As per transcript per million (TPM) analyses, overexpression of WT-MRTF-A induced all three genes reproducibly in both 2D and 3D cultures of MDA-231 cells (**Fig 4B**), although the effect of functional disruption of MRTF's ability to induce these genes was found to be gene-specific. MRTF-A's ability to induce IL11 expression was primarily related to its SRF-related function while CTGF induction required both SAP-domain and SRF-related functions of MRTF. Fold-increase in FGF5 expression resulted from overexpression of MRTF-A was only partly diminished when its SAP-domain function was disrupted. In general, there was good concordance in the patterns of expression of these three genes between the 2D and the 3D culture conditions.

Interestingly, all three genes (CTGF, IL11 and FGF5) encode for cell-secreted factors that are known to promote OCL differentiation and/or activity (37-39). However, when we analyzed three large human breast

cancer transcriptome databases (METABRIC [n=1981] (40), TCGA [n=1208] (41), and SCANB [n=3207] (42); n represents the number of clinical samples), only CTGF expression showed a strong positive correlation (spearman's correlation coefficient >0.6) with MRTF gene signature score (GSS ssGSEA score (43)of the aforementioned MRTF signature of 33 genes) in primary tumors consistently in all three transcriptome databases (**Fig 4C**). Furthermore, between the two isoforms, expression of only MRTF-A (not MRTF-B – data not shown) has a significant positive association with CTGF expression in human breast cancer (**Fig 4D**). Based on these clinical correlation data, we further investigated CTGF as a potential mediator of MRTF-dependent regulation of OCL differentiation. First, by immunoblot analyses we confirmed that MRTF depletion in either MDA-231 or D2A1 cells results in dramatic downregulation of CTGF expression suggesting that MRTF's critical requirement for CTGF expression (**Fig 5A**). Next, we performed CTGF rescue experiments where we compared RANKL-induced OCL differentiation of BMDMs exposed to the CM of control vs MRTF knockdown MDA-231 cells with or without exogenous addition of 50 ng/mL or 100 ng/mL CTGF in the culture media. We chose these two concentrations based on previously published studies in which CTGF supplementation at these concentrations at the time of RANKL addition enhanced OCL activation (37,44). As expected, application of tumor cell CM of control MDA-231 cells led to reduced proliferation but enhanced OCL differentiation of RANKL-treated BMDMs, and silencing MRTF expression in MDA-231 cells dramatically inhibited OCL differentiation. Although addition of recombinant CTGF at either concentration did not affect BMDM proliferation (as judged by the total cell count), it was able to partly reverse the suppressive effect of MRTF depletion on OCL differentiation (**Fig 5B-D)**. Note that increasing CTGF concentration from 50 to 100 ng/ml did not result in any further increase in OCL differentiation in the culture. These experimental data demonstrate that CTGF is an important paracrine mediator of pro-osteoclastogenic action of MRTF in breast cancer cells.

Our CTGF TPM data from RNAseq analyses as shown in **Fig 4B** demonstrated that disruption of either SAP-domain or SRF-related function blunts MRTF's ability to induce CTGF expression. To validate these TPM data, we performed qRT-PCR and immunoblot analyses for CTGF expression in MDA-231 sublines with various perturbations of MRTF-A. Consistent with the TPM data, CTGF expression was robustly increased at both mRNA and protein levels upon overexpression of WT-MRTF-A relative to control, and this was reversed when either SAP-domain or SRF's interaction of MRTF-A was disrupted, more robustly by the latter (**Figs 6A-B**). Given these results, we next assessed the relative importance of SRF- vs SAP-domain-related activities of MRTF in OCL differentiation of BMDMs. Although MRTF-A overexpression in MDA-231 cells did not further augment OCL differentiation vs control, disruption of either SRF's interaction or SAP-domain function of MRTF-A significantly reduced OCL differentiation but without impacting BMDM proliferation (**Figs 6C-D;** representative images of TRAP-stained cells are shown in **supplementary Fig S3).** Collectively, these data suggest that both SRF-dependent and -independent (SAP-domain directed) functions of MRTF are required for its ability to regulate CTGF expression and osteoclast differentiation.

Loss of MRTF expression in breast cancer cells has a broad impact on osteoclast regulatory factors

Since exogenous addition of CTGF only partially rescued the defect in osteoclastogenesis induced by MRTF deficiency, we speculated that MRTF depletion may also impact other osteoclast-regulatory cell-secreted factors. Since many immunomodulatory cytokines and chemokines play prominent role in both positively and negatively regulating proliferation, survival and differentiation of OCL (45), we next performed luminex analyses of CM harvested from control vs MRTF knockdown cultures of MDA-231

cells to profile a panel of 48 cytokines and chemokines. We detected a total of 13 differentially abundant factors between the two groups with 7 factors upregulated (IL1RA, IP10/CXCL10, IL1β, TGFα, fractalkine/CX3CL1, IL27, RANTES/CCL5) and 6 factors downregulated (IL8, G-CSF, FGF2, IL3, IL17E/IL25, FLT3L) in the CM of MDA-231 cells upon MRTF knockdown within statistical significance (**Fig 7A**). The fold-changes and the p-values for each of these differentially abundant factors are listed in a tabular form in **Fig 7b**. While the direction of changes of some of these factors is not consistent with reduced OCL differentiation in MRTF knockdown setting, upregulation of anti-osteoclastogenic factors (IL1RA and IL27) and conversely, downregulation of pro-osteoclastogenic factors (IL8, G-CSF, and FLT3L) are consistent with repression of OCL differentiation in MRTF knockdown setting. Based on these findings, we propose a schematic model representing how MRTF activity in tumor cells could potentially regulate OCL differentiation in a paracrine manner (**Fig 8**).

DISCUSSION

In the present study, we demonstrate several new findings. First, we show that MRTF's presence is absolutely essential for outgrowth and metastatic colonization of breast cancer cells in bone microenvironment. Second, we uncover a novel tumor-extrinsic function of MRTF in promoting osteoclastogenesis in a paracrine manner. Third, we causally link regulation of CTGF expression to MRTF-dependent control of osteoclast differentiation process. Fourth, we identify a host of additional cell secreted OCL-regulatory factors that are impacted upon loss of MRTF expression in breast cancer cells, providing additional mechanistic insights into how MRTF-deficiency in breast cancer cells interferes with osteoclastogenesis.

OCL differentiation and activation is a multistep process that is dependent on a multitude of growth factors liberated from the bone matrix and secreted by tumor cells in the metastatic niche. This involves proliferation and differentiation of hematopoietic stem cells to RANK-/TRAP- OCLp cells, differentiation of OCLp cells to RANK+/TRAP+ mononuclear OCLs (a process that is dependent on the activities of NF-κb and NFATc transcription factors), and finally fusion of mononuclear OCLs to multi-nucleated and bone-resorption competent mature OCLs that are characterized by the expressions various markers (DC-STAMP [dendritic cell-specific transcription protein], Atp6v0d2 [v-ATPase subunit d2], cathepsin K, and OSTM1[osteoclastogenesis-associated transmembrane protein] (46). DC-STAMP, a transmembrane protein originally identified in dendritic cells, is essential for cell-cell fusion in OCLs (35,47). CTGF plays a key role in both induction of and interaction with DC-STAMP resulting in multi-nuclear OCL formation (44). This study demonstrates that MRTF deficiency dramatically diminishes CTGF expression in breast cancer cells (D2A1 and MDA-231) suggesting that MRTF plays an indispensable role in driving CTGF expression. This finding is consistent with MRTF-A overexpression-induced robust increase in CTGF expression in breast cancer cells and a strong positive correlation between MRTF activity and CTGF expression in human tumors in our study as well as a similar in vivo overexpression-related finding in mouse model of angiogenesis (48). Our studies also find that RANKL-induced DC-STAMP expression is prominently enhanced (by ~3 fold) when exposed to breast cancer cell CM, an effect that is completely reversed when MRTF expression is silenced in breast cancer cells. We cannot definitely rule out MRTF's role in early aspects of OCL differentiation process. Since exposure to tumor cell CM (regardless of the MRTF expression status) reduces the total count of OCLp cells (by ~50%) (a differential that cannot be explained by 5% increase in multi-nucleated OCL formation in RANKL-stimulated cells when exposed to the CM of MRTF-proficient breast cancer cells), we speculate that cell-cell fusion of mononuclear OCLs

is enhanced by secreted factors from tumor cells, in part by MRTF/CTGF signaling-driven augmentation of DC-STAMP expression in a paracrine manner. However, we also found that addition of recombinant CTGF only partially recoups the OCL differentiation differential between the control and MRTF knockdown groups, and that % of OCL differentiation in RANKL-treated culture is in fact diminished significantly when treated with CM of MRTF-deficient breast cancer cells. Therefore, MRTF-depletion in breast cancer cells clearly has a suppressive effect on OCL differentiation process through a paracrine action that extends beyond the effect of CTGF. To this end, RNA-seq analysis identified MRTF-dependent upregulation of pro-osteoclastogenic factors IL11 and FGF5 (these factors were not represented in Luminex assay). Our Luminex studies additionally discovered that MRTF-depletion in breast cancer cells leads to reduced secreted levels of several pro-osteoclastogenic cytokines (IL8, G-CSF, IL25, FLT3L) with concomitant upregulation of anti-osteoclastogenic cytokines (IL1RA, IL27 and CCL5) (note that these genes were not altered transcriptionally). Among the pro-osteoclastogenic factors identified by Luminex analyses, IL8 stimulates OCL differentiation through inducing RANK-induced NFATc1 activation in an autocrine-signaling manner (49). G-CSF accelerates osteoblast turnover and suppresses the expression OPG (a negative regulator of RANK/RANKL signaling) (50) while eliciting increased OCL activity and bone resorption in both cancer (51)and periodontitis models(52). Although FL3T3L can substitute for M-CSF in supporting OCL induction(53), since our studies were performed in cultures supplemented with M-CSF, we presume that reduced FL3TL secretion, if at all, has minimal role, in suppression of OCL differentiation in MRTF knockdown setting. Among the anti-ostoeclastogenic factors, IL1RA (IL1 receptor antagonist) inhibits the signaling elicited byIL1,an inducer of RANKL expression as well as an activator of various hallmark gene expression programs [NFκβ, p38, ERK] required for OCL differentiation (54)). Il27 is known to inhibit RANKL-induced signaling pathway (55). Therefore, we will need to look into potential contributions of these select candidate differentially abundant cytokines and changes in associated signaling pathways as additional future directions of mechanistic investigations underlying MRTF-regulated paracrine control. It will be interesting to prioritize on anti-osteoclastogenic factors (IL1RA and IL27) given that MRTF-deficiency robustly suppresses RANKL-induced OCL differentiation.

Our studies also show that both SRF- related and SAP-domain activities of MRTF contribute to driving CTGF expression, a finding that parallels with reduced OCL differentiation in the settings of CM of mutant vs wild-type MRTF-A overexpressing breast cancer cells. Interestingly, overexpressing MRTF-A did not further augment OCL differentiation relative to control condition despite increasing CTGF expression at the mRNA level by ~20-fold, a scenario that can be potentially explained by MRTF-dependent changes in both pro- and anti-osteoclastogenic secreted factors as revealed by our Luminex analyses. Although both SAP-directed and SRF-related activities of MRTF-A in breast cancer cells appear to be important in its ability to regulate OCL differentiation in a paracrine manner, we previously demonstrated severe impairment in metastatic colonization of breast cancer cells caused by disruption of SRF's interaction but not the SAP-domain function of MRTF (22). This is most likely due to fact the general tumor-initiating ability of breast cancer cells, a critical aspect of metastatic colonization of tumor cells in any organ, is critically dependent on SRF's interaction rather than SAP-domain function of MRTF(22).

In conclusion, this study uncovers a novel MRTF-directed tumor-extrinsic mechanism of bone colonization of cancer cells involving OCL differentiation partly through regulation of CTGF expression. It would be interesting to investigate in the future whether CTGF administration could partly restore defects of bone colonization of MRTF-deficient tumor cells *in vivo*.

EXPERIMENTAL PROCEDURES

Animal Studies

All animal experiments were conducted in compliance with an approved IACUC protocol, according to University of Pittsburgh Division of Laboratory Animal Resources guidelines. Briefly, 10^5 MDA-231 cells, suspended in 100 µL PBS, were injected into the left cardiac ventricle of fully anesthetized NSG mice. Bioluminescence imaging (BLI) of anesthetized mice was performed with IVIS Spectrum 15 minutes after intraperitoneal injection of D-luciferin.

Cell culture and OCL differentiation assay

Generation and culture of variants of MDA-231, stably co-expressing GFP and luciferase, and various MRTF-A constructs have been previously described (22). D2A1 cells stably expressing luciferase (a gift from Dr. William Schiemann, Case Western Reserve University) were cultured in DMEM media (Lonza, catalog# BW12-604F) supplemented with 10% (v/v) FBS and 1% antibiotics (100 U/ml penicillin and 100 µg/mL streptomycin). For stable silencing of MRTF isoforms, MDA-231 and D2A1 cells were transduced with lentivirus encoding either MRTF-A/B shRNA (Vectorbuilder vector ID - VB200825-1246dwj) or non-targeting control shRNA (Vectorbuilder vector ID - VB151023-10034), and selected with puromycin. Primary BMDMs were isolated from the long bones of female FVB mice and cultured in αMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS; Corning, catalog# MT35011CV) and 1% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin; Thermo Fisher, catalog# 15070063) as described previously (56). Primary BMDMs were seeded in the wells of a 96 wells plate in triplicate and expanded in αMEM supplemented with M-CSF (20 ng/mL) for 3 days (d-3 to d0) to generate OCLp cells. OCL differentiation was induced by addition of RANKL (10 ng/mL) and M-CSF (10 ng/mL) on d0 and subsequent culture for 4 days, with or without the addition of breast cancer cell CM at a 10% v/v ratio. To prepare CM, breast cancer cells were seeded in a 10 cm plate and cultured in serum containing media for 4 days prior to collection. For the CTGF rescue experiments, CTGF (PeproTech, 120-19) was added to the culture at either 50 ng/ml or 100 ng/ml concentration on d0. To assess OCL differentiation, cells were fixed with 37% formaldehyde for 15 minutes, washed with PBS and stained using leukocyte acid phosphatase kit (Sigma-Aldrich, 387A-1KT) following the manufacturer's instructions. OCLs (marked by TRAP+ multinucleated cells with three or more nuclei) were counted using the "OC_Finder" program which uses an automated cell segmentation approach before applying deep learning to classify the cells as either OCLs or non-OCLs (57). Total number of cells was counted using ImageJ.

RNA extraction, quantitative RT-PCR and RNA-sequencing

Total RNA was extracted from either 2D or matrigel-on-top (MoT) 3D (protocol for MoT 3D culture is detailed in (22)) cultures using RNeasy mini kit (Qiagen, catalog #74104) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 1 ug of RNA using the QuantiTect reverse transcription kit (Qiagen, catalog #205311) following the manufacturer's instructions. Each PCR was prepared with 50 ng of cDNA, 12.5 µL of SYBR Select Master Mix (ThermoFisher, catalog #4472903), 1 µM forward and reverse primers, and water for a total volume of 25 µL. Thermal cycling and data analysis were performed using the StepOne Plus Real-Time PCR System and StepOne Software (Applied Biosystems) to detect quantitative mRNA expression of Cathepsin-K, DC-STAMP and CTGF. The primer sequences for Cathepsin-K were 5'-GAAGAAGACTCACCAGAAGCAG-3' (sense) and 5'-

TCCAGGTTATGGGCAGAGATT-3' (antisense). The primer sequences for DC-STAMP were 5'-TTGCCGCTGTGGACTATCTG-3' (sense) and 5'-GAATGCAGCTCGGTTCAAAC-3' (antisense). The primer sequences for CTGF were 5′-GCAGGCTAGAGAAGCAGAGC-3′ (sense) and 5′-GGTGCAGCCAGAAAGCTC-3′ (antisense). RNA sequencing was performed at the University of Pittsburgh HSCRF Genomics Research Core. Fastq files from SRP097696 archive were downloaded using SRA Toolkit version 2.9.2. Transcripts from the Ensembl v.93 transcriptome (hg38) were quantified with Salmon v0.14.0. Gene-level expression was calculated using tximport v.1.12.0 and differential gene expression was calculated with DESeq2 v.1.24.0. Quality control principle component and heatmap plots demonstrated one potential outlier sample, which was removed prior to final differential gene expression analysis. All scripts are available upon request.

Protein extraction and Western blotting

Cell lysates were prepared by a modified RIPA buffer (25 mM Tris–HCL: pH 7.5, 150 mM NaCl, 1% (v/v) NP-40, 5% (v/v) glycerol), 1 mM EDTA, 50 mM NaF, 1 mM sodium pervanadate, and protease inhibitors (Sigma, catalog P8340) supplemented with 6× sample buffer diluted to 1× with the final SDS concentration in the lysis buffer equivalent to 2%. Conditions for the various antibodies were: polyclonal MRTF-A (Cell Signaling, catalog #14760S; 1:1000), MRTF-B (Cell Signaling, catalog #14613S; 1:1000), polyclonal GAPDH (Sigma, catalog #G9545, 1:2000), polyclonal CTGF (Abcam, catalog #ab6992), monoclonal Tubulin (Sigma, T9026, 1:1000). monoclonal anti-rabbit HRP (Jackson Immunoresearch, catalog #211–032-171, 1:1000), and polyclonal anti-mouse HRP (BD Biosciences, catalog #554002, 1:1000).

Calvaria Preparation/tumor cell outgrowth assay

Female C57Bl/6 post-natal day 21 mice were injected intraperitoneally with 20 mg/kg Alizarin complexone dihydrate (CAS 455303-00-1, 2.0 mg/ml in bicarbonate buffer) and sacrificed 24 hours post injection. Calvaria were immediately excised and placed in phosphate buffered saline. The periosteum was removed by scraping with a scalpel. Calvaria were then digested in 0.5 mg/mL collagenase (Sigma Aldrich, catalog #C9697-50), 0.25% trypsin-EDTA for 10 minutes at a time at 37ºC and stored in PBS at -20°C until used. Two thousand cells were seeded in duplicates in a 96-well plate on approximately 2 mm x 2 mm sized squares of calvaria. After 72 hours of culture, cells were imaged at 4x magnification and outgrowth was quantified by the total calvarial area covered by GFP+ cells using ImageJ.

Cytokine Analyses

CM harvested from 10 cm culture plates and concentrated to 100 µL was probed for cytokine/chemokine expression levels by HD48A (48-plex human discovery assay) Luminex panel using a service provided by Eve Technologies (Calgary, AB, Canada). Values were normalized to the average value of the control group per analyte basis.

Statistics

All statistical tests were performed using GraphPad Prism 9 software. ANOVA (for more than 2 groups) and Student's t-test (for 2 groups) were performed for comparing the means between various experimental groups. For ANOVA, we performed Tukey's post hoc test for individual group comparisons. A p-value less than 0.05 was considered to be statistically significant.

DATA AVAILABILITY

RNA-sequencing data are in the process of GEO submission. All other data are included in the manuscript and supporting information.

SUPPORTING INFORMATION

The article contains supporting information.

ACKNOWLDEGMENTS

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AUTHOR CONTRIBUTION

PC and DG performed experiments, analyzed data, and wrote manuscript; DB, NW and FC performed data analyses, JT contributed to generation of experimental tools; AVL oversaw some aspects of data analyses, DLG conceived study design and supervised some aspects of the project, PR conceived the overall study design, supervised the project, oversaw experiments, edited/wrote manuscript and acquired funding.

CONFLICT OF INTEREST: The authors declare no conflict of interest.

Figure Legends

Figure 1. MRTF depletion impairs experimental bone colonization of breast cancer cells *in vivo*. A) Immunoblot-based confirmation of knockdown of MRTF isoforms in MDA-231 cells (C – control shRNA, M – MRTF A/B shRNA) **B)** Representative end-point BLI images of NSG mice 2 weeks after intracardiac injection of control vs MRTF knockdown MDA-231 cells. **C)** Quantification of distant metastasis using end-point BLI imaging readout for the two groups (n=8 animals/group, *** p<0.001). **D)** Hematoxylin and Eosin staining of BLI signal positive bones confirm presence of metastases (indicated by the arrow) harvested from mice injected with control shRNA-bearing cells. Scale bar represents 200 µm.

Fig 2. Loss of MRTF expression impairs outgrowth of breast cancer cells on bone surface *in vitro*. A) Representative fluorescence images of GFP+ control and MRTF-A/B shRNA expressing MDA-231 cells 72 hrs after seeding on alizarin red+ mouse calvarial bone. **B)** Quantification of area of bone covered with GFP+ cells 72 hours post-seeding (data summarized from 3 independent experiments, **** p<0.0001; scale bar – 50 µm).

Fig 3. Tumor cell-derived factors promotes osteoclastogenesis requiring MRTF's action A) Schematic depicting workflow of breast cancer-OCL indirect co-culture experiments (*created with BioRender*). **B-C)** Quantifications of total cell count and % OCL in the settings of BMDMs treated with or without RANKL along with CM harvested from control vs MRTF knockdown MDA-231 cell culture (OCLs were defined as those which were TRAP+ and had >=3 nuclei; data summarized from 3 independent experiments). **D-E)** Quantitative RT-qPCR analyses show the fold-changes in DC-Stamp (*panel D*) and Cathepsin-K (*panel E*) expressions in parallel culture settings (data summarized from 3 independent experiments; **** p<0.0001, ** p<0.01, * p<0.05).

Fig 4. Identification of candidate bone metastasis-associated genes inducible by MRTF. A) Schematic depicting the workflow to identify breast cancer-associated bone metastasis genes inducible by MRTF. **B)** TPM (transcripts per million – normalized to housekeeping genes) of candidate bone metastasis associated genes (IL11, CTGF, FGF5) based on RNA-sequencing data of 2D and 3D cultures of MDA-231 cells subjected to acute doxycycline-induced overexpression of wild-type (WT) vs various mutant forms of MRTF-A. **C)** Correlation between select genes and MRTF gene signature score (GSS) in TCGA, MRTABRIC and SCANB databases (Spearman's co-efficient (S) and p-values are included for each of these analyses). **D)** Correlation between CTGF and MRTF-A mRNA expression in human breast cancer samples (based on TCGA data analyses).

Fig 5. **MRTF-deficiency in breast cancer cells inhibits osteoclastogenesis partly through suppressing CTGF expression**. **A)** CTGF immunoblot of total cell lysates of MDA-231 and D2A1 cells with or without stable knockdown of MRTF expression (GAPDH and tubulin blots serve as loading controls; D2A1 lysates were probed with anti-MRTF-A and anti-MRTF-B antibodies to confirm knockdown of the two MRTF isoforms). **B-D)** Quantification of total cells per well *(panel B*) and % OCL (*panel C*) in RANKL-supplemented cultured of BMDMs treated with the indicated MDA-231 CM (c – control; M – MRTF-A/B knockdown) and recombinant CTGF. Panel D shows representative TRAP-stained cells in different groups (data summarized from 3 independent experiments; *** p<0.001; **** p<0.0001; scale bar – 200 □m)

Fig 6. Both SAP-domain and SRF-related functions of MRTF contribute to CTGF regulatory and osteoclastogenesis-promoting activities of MRTF. A) Quantitative RT-PCR analyses showing the mean ± SD values of the fold-changes in CTGF mRNA levels in 3D cultures of MDA-231 cells following overexpression of the various forms of MRTF-A (data summarized from three independent experiments, $p < 0.05$). **B)** CTGF immunoblot of total lysates of MDA-231 cells subjected to overexpression (OX) of various forms of MRTF-A relative to control (the numbers indicate fold-change in CTGF expression; tubulin blot – loading control). **C-D)** Quantification of total cells per well *(panel C*) and % OCL (*panel D*) in RANKL-supplemented cultures of BMDMs treated with CM derived from the various MRTF-A overexpressing MDA-231 cultures relative to control (data summarized from 3 independent experiments, **** $p<0.0001$).

Fig 7. MRTF-dependent changes in secreted immunomodulatory factors. A) Heat plot of differentially abundant cytokines/chemokines in the CM of control vs MRTF knockdown (KD) MDA-231 culture. **B)** Fold changes and p-values for differentially abundant cytokines/chemokines with pro- vs anti-osteoclastogenic activities.

Fig 8. A proposed schematic model of how MRTF activity in breast cancer cells promotes osteoclastogenesis through a paracrine action.

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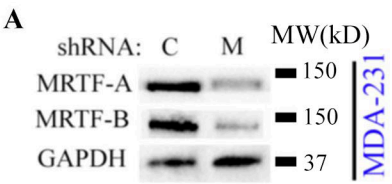
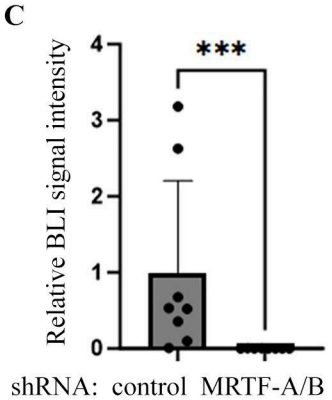
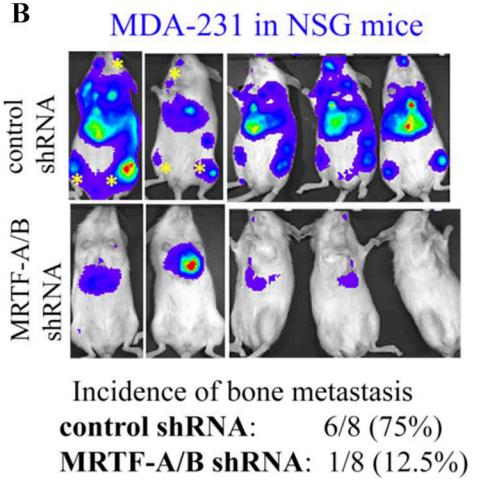
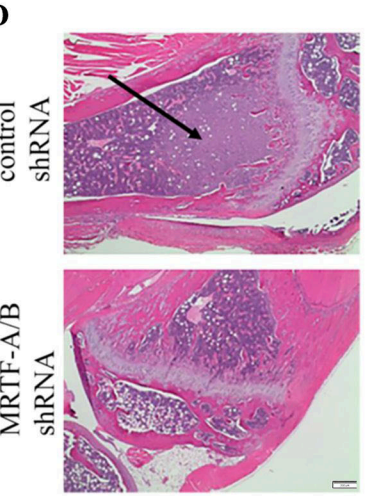
Chawla et al. Figure 1









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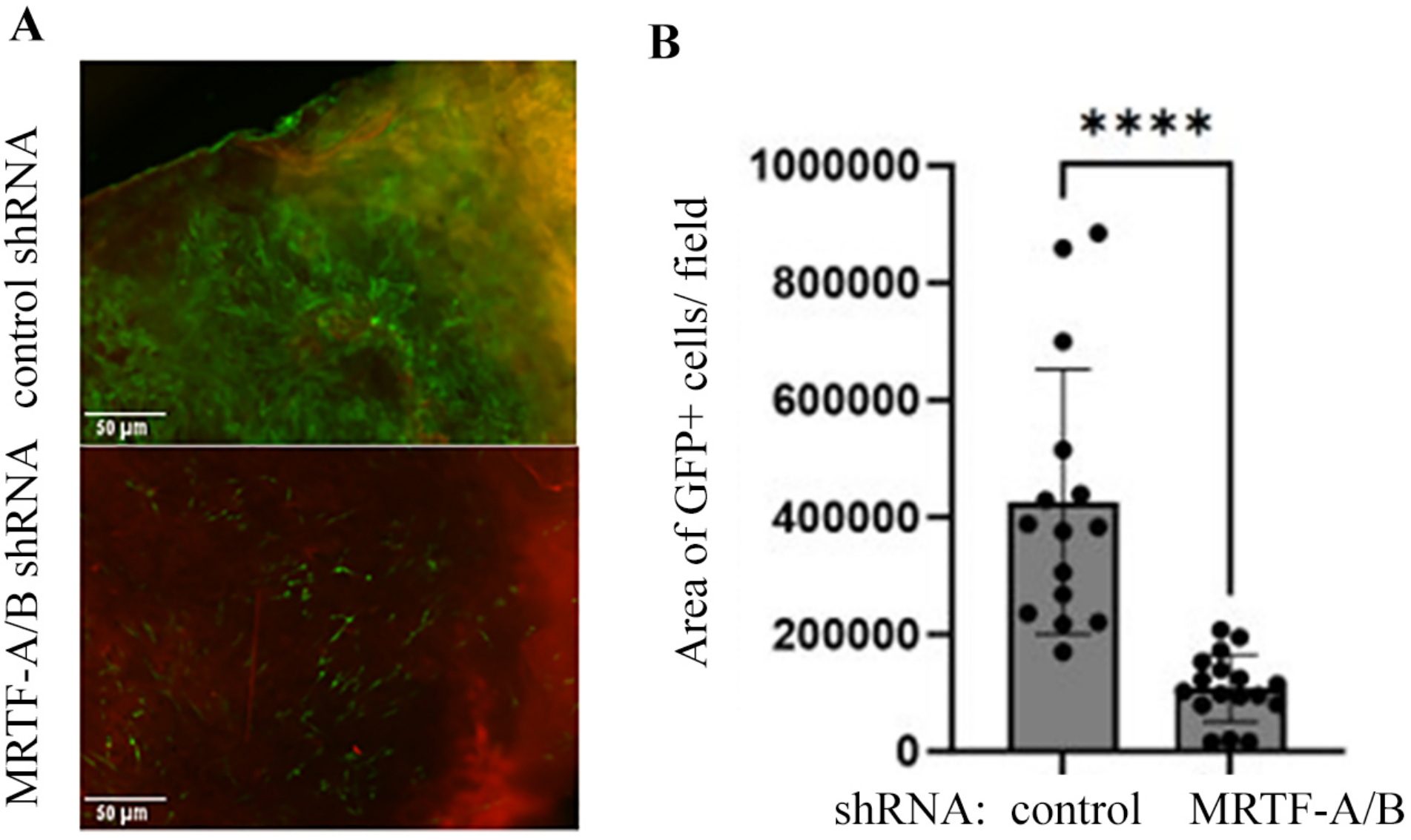

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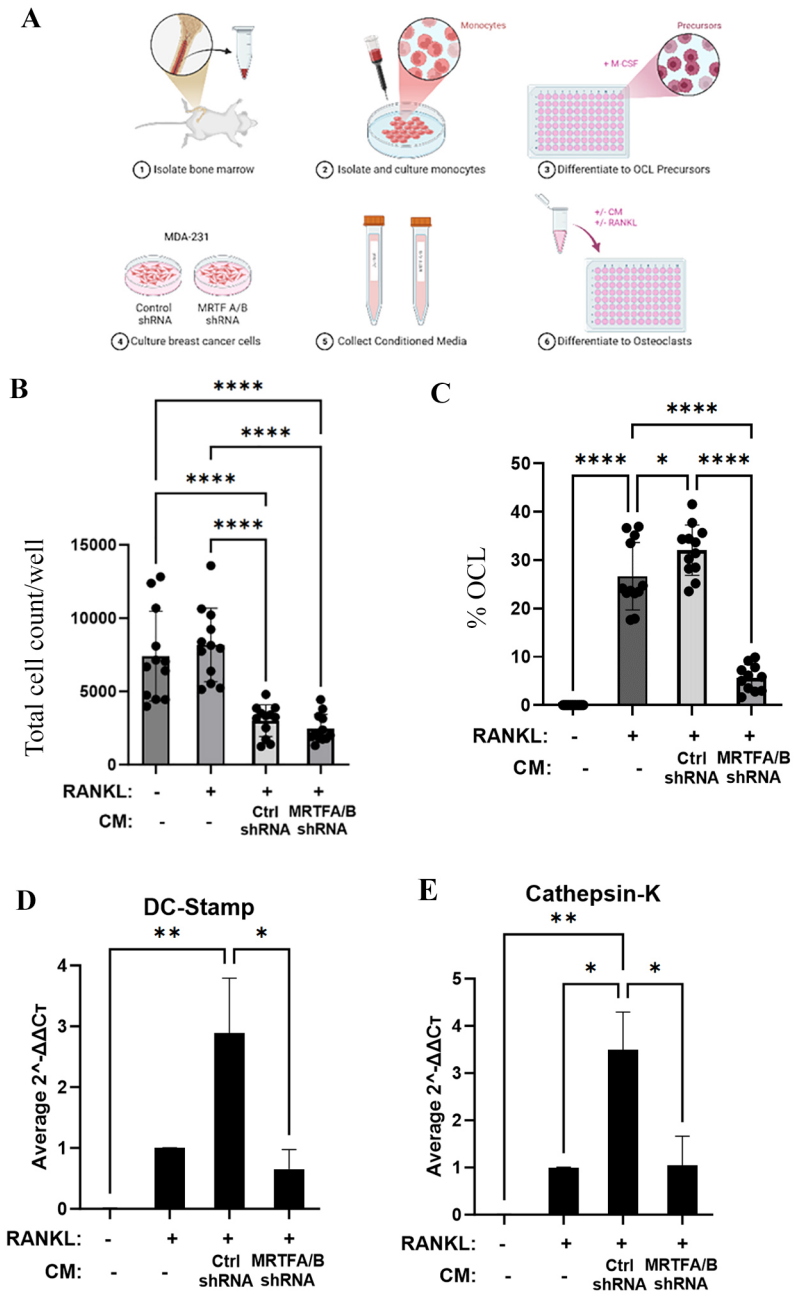

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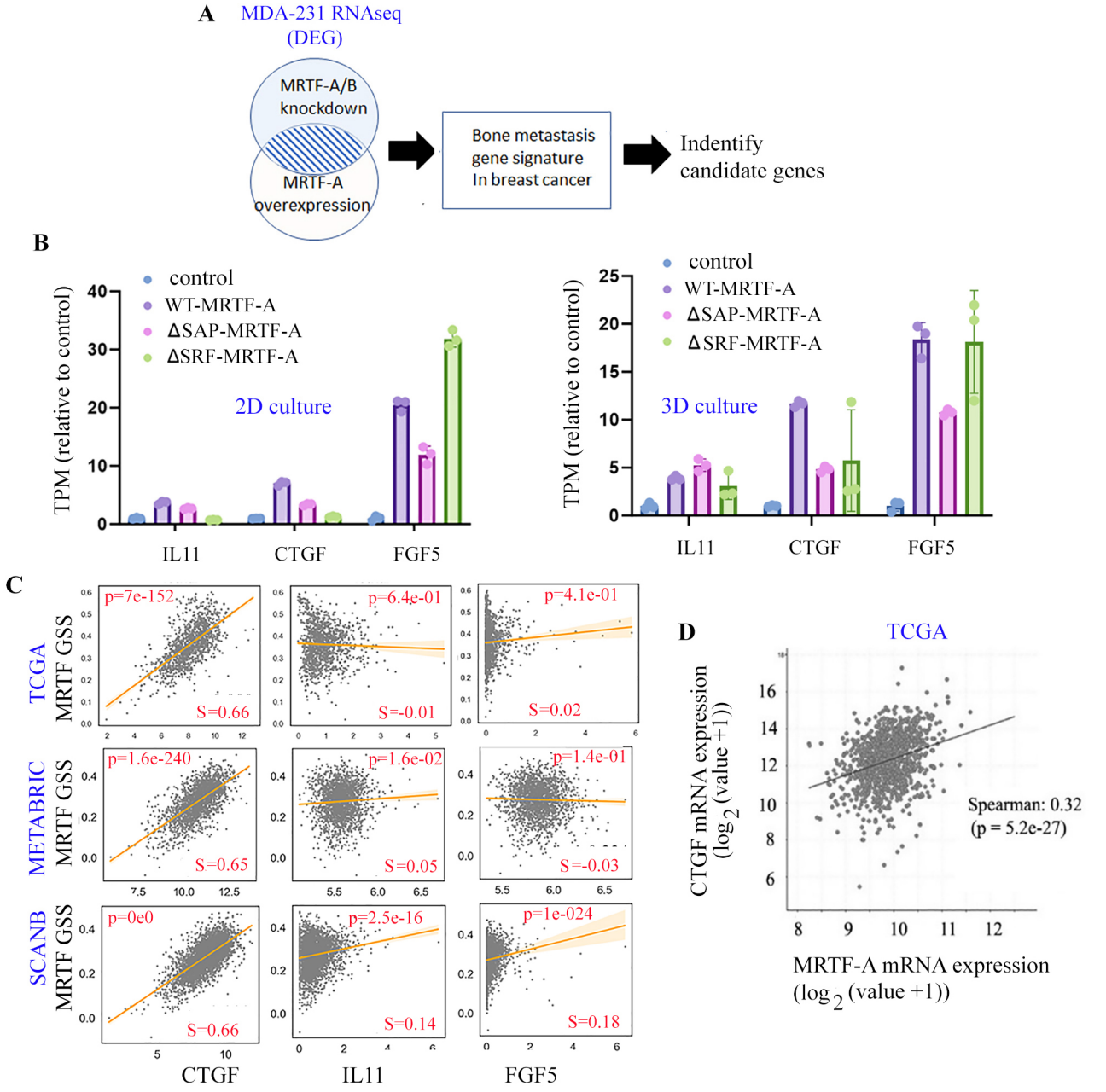

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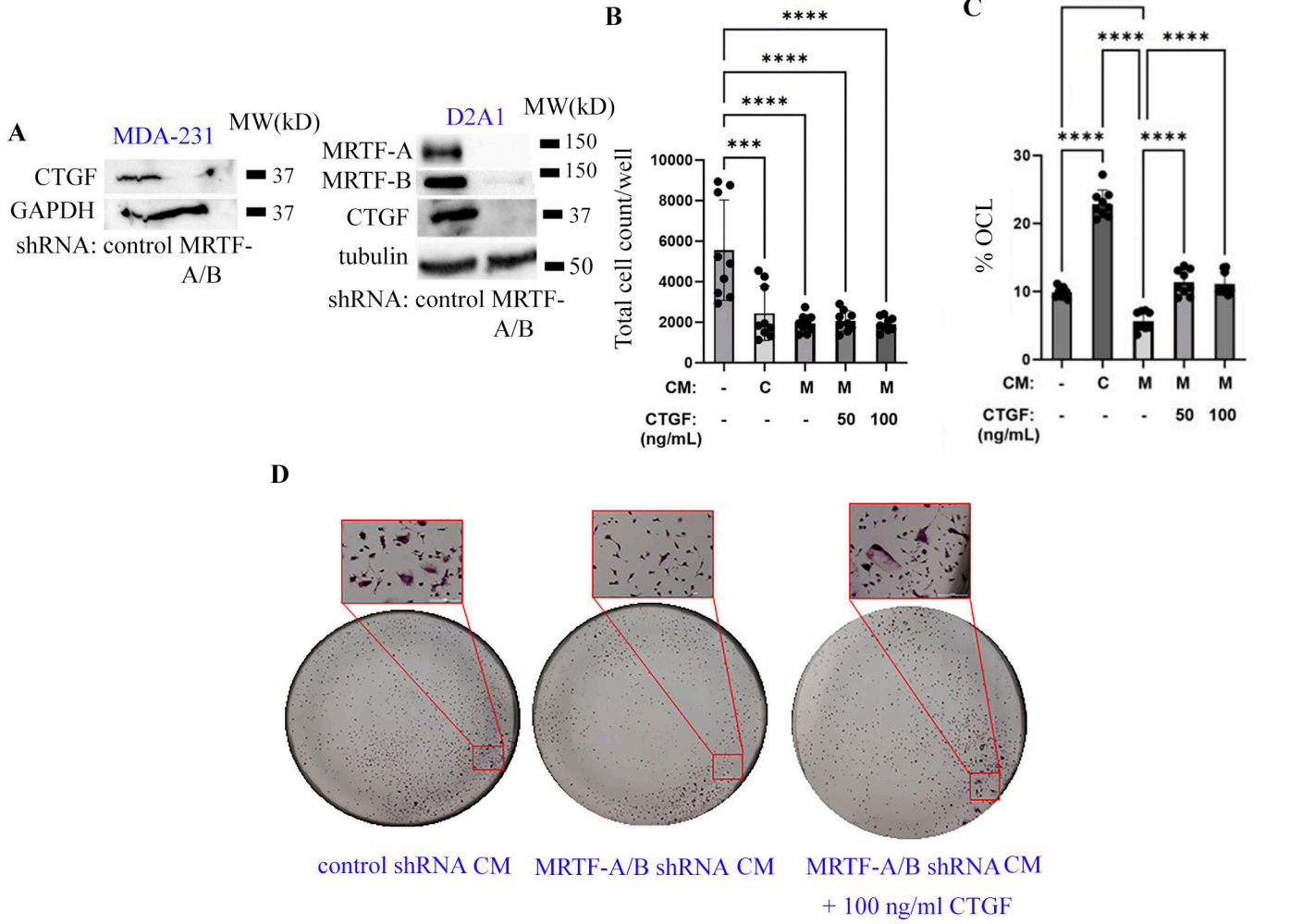

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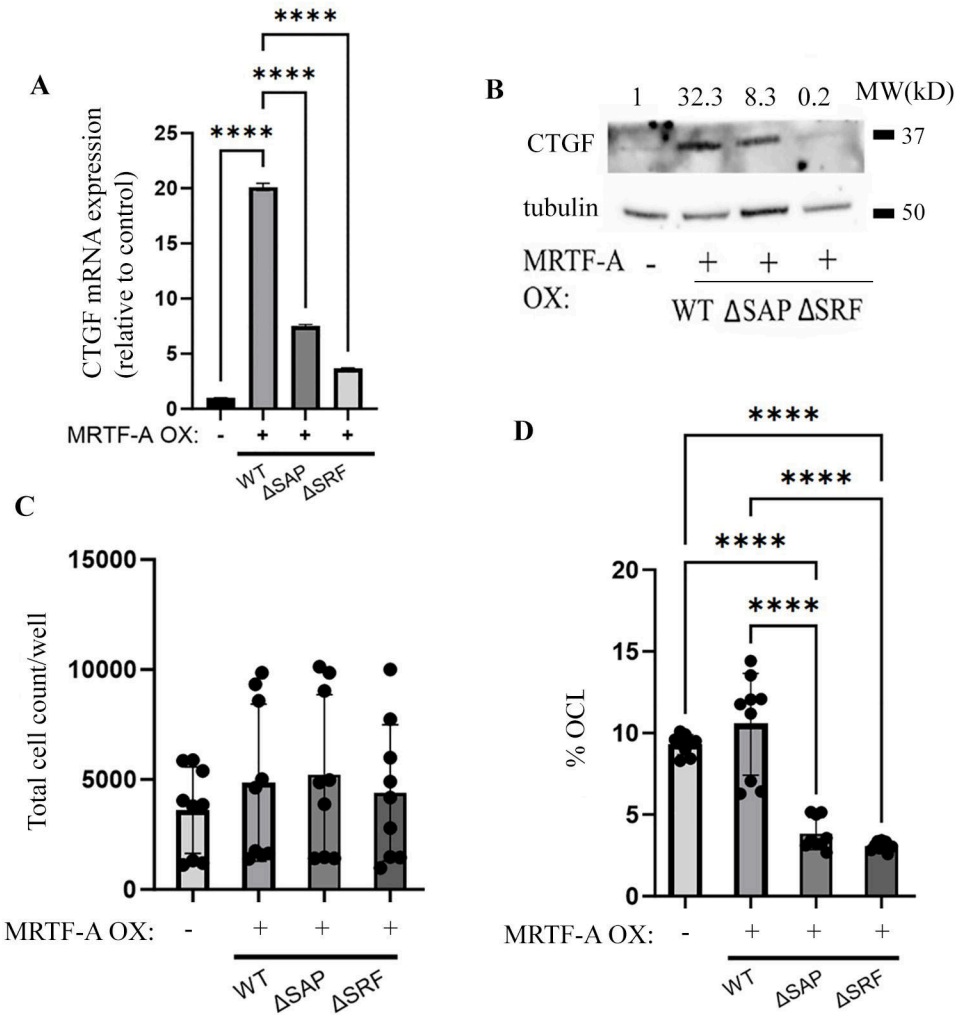
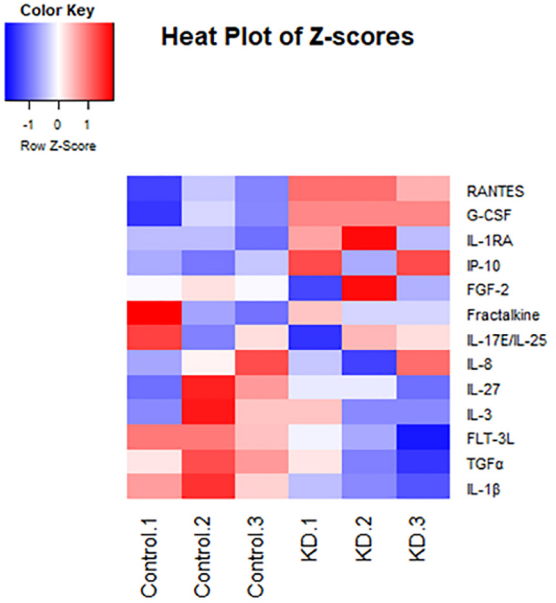


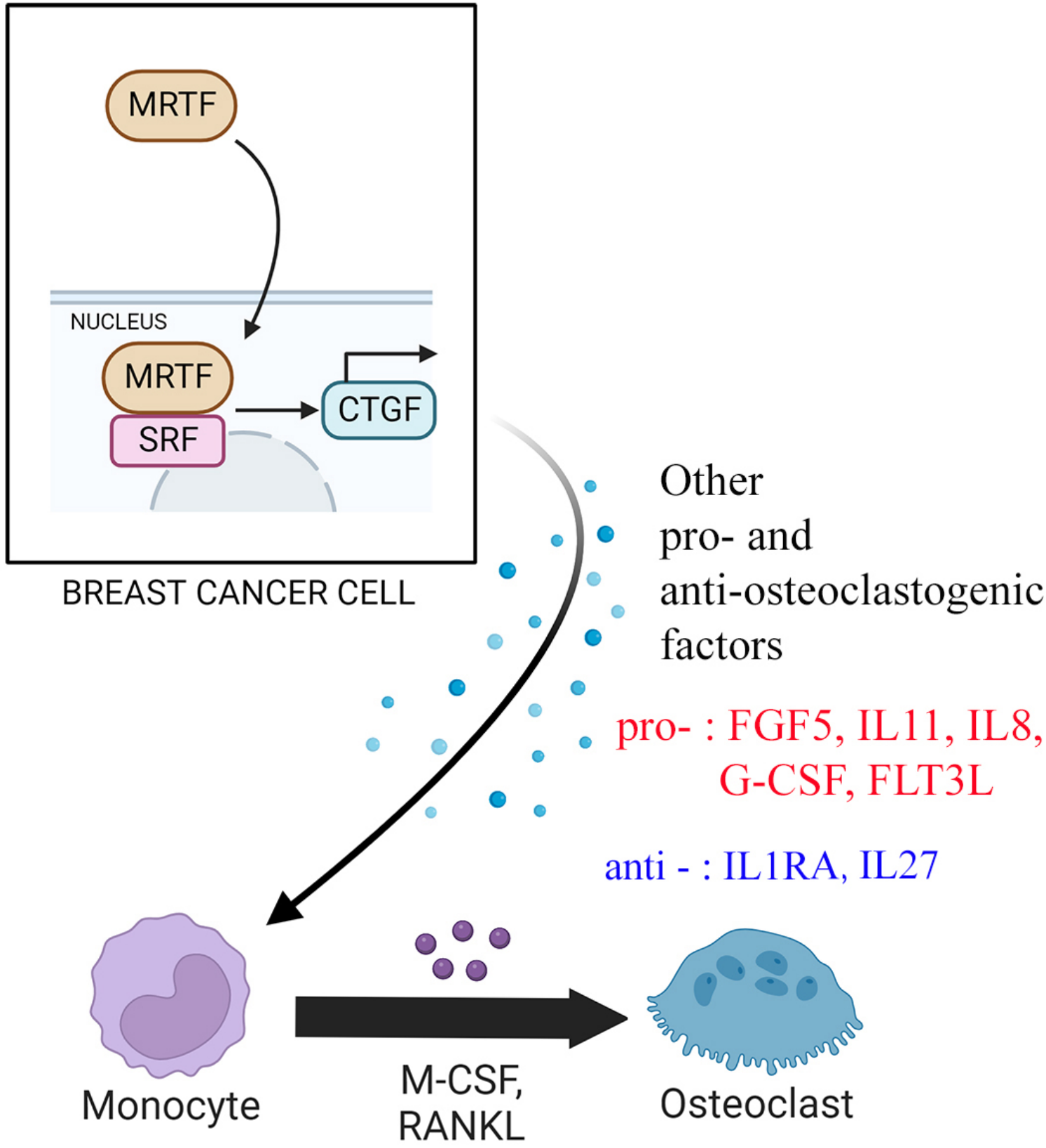
Chawla et al. Figure 7

A



B

Gene	Fold Change	p-value
Anti-Osteoclastogenic		
IL-1RA	3.01 ± 0.36	<0.0001
IL-27	6.64 ± 0.82	<0.0001
IL-3	0.44 ± 0.26	0.0475
Pro-Osteoclastogenic		
FGF-2	0.44 ± 0.47	0.0463
FLT-3L	0.42 ± 0.04	0.0059
Fractalkine	4.73 ± 5	<0.0001
G-CSF	0.21 ± 0.06	<0.0001
IL-17E/IL-25	0.36 ± 0.34	<0.0001
RANTES	2.15 ± 0.34	<0.0001
IL-1β	3.43 ± 0.31	<0.0001
IL-8	0.68 ± 0.28	0.0053
IP-10	7.8 ± 2.6	<0.0001
TGFα	3.84 ± 0.56	<0.0001

Note: Values are mean ± SD (n=3)

Chawla et al. Figure 8