Surgery 166 (2019) 515–523

Contents lists available at ScienceDirect

Surgery

journal homepage: www.elsevier.com/locate/surg

Myeloid zinc finger-1 regulates expression of cancer-associated fibroblast and cancer stemness profiles in breast cancer

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ARTICLE INFO

Article history:
Accepted 6 May 2019
Available online 10 July 2019

ABSTRACT

Background: Osteopontin acts thru myeloid zinc finger-1 and transforming growth factor-β to drive the adoption of a cancer-associated fibroblast phenotype by local mesenchymal stem cells. Cancer-associated fibroblasts increase cancer cell stemness.

Methods: Mesenchymal stem cells were exposed to osteopontin or were cocultured with MB231 human breast cancer cells (high osteopontin producer) in the presence or absence of aptamer (inactivates extracellular osteopontin). Myeloid zinc finger-1 phosphorylation sites were identified, and phosphomutants of T134 (SCAN domain) and S453 (zinc finger DNA binding domain) were constructed. Transforming growth factor-β F and cancer-associated fibroblast markers (smooth muscle actin, vimentin, and tenascin-c) were measured in mesenchymal stem cells. In MB231, stemness markers Sox2, Nanog, and Oct4 were measured.

Results: Mesenchymal stem cells plus osteopontin increased transforming growth factor-β and cancer-associated fibroblast markers ($P < .05$ vs mesenchymal stem cells alone); this was abolished by aptamer inactivation of osteopontin. In mesenchymal stem cells transfected with phosphoresistant myeloid zinc finger-1, osteopontin did not increase cancer-associated fibroblast markers or transforming growth factor-β. In contrast, phosphomimetic myeloid zinc finger-1 increased cancer-associated fibroblast markers and transforming growth factor-β ($P < .05$ vs mesenchymal stem cells alone). In mesenchymal stem cells plus MB231, MB231 stemness markers were increased ($P < .05$ vs MB231 alone). In MB231 plus mesenchymal stem cells expressing phosphoresistant myeloid zinc finger-1, MB231 stemness markers were not increased in comparison with MB231 plus mesenchymal stem cells.

Conclusion: Myeloid zinc finger-1 phosphorylation in mesenchymal stem cells drives the osteopontin-mediated cancer-associated fibroblast phenotype, which then increases the cancer cell stemness profile.

Introduction

Cancer cells interact with the tumor microenvironment to create a milieu that enhances local and distant tumor potentiation.[1,2] Tumor growth and metastasis is highly dependent on complex dynamic interactions between cancer cells and tumor stroma, mediated by direct cell–cell contact and secreted growth factors and cytokines. Cancer-associated fibroblasts (CAFs), a tumor stromal element, are involved in tumor growth potentiation, extracellular matrix degradation, tumor cell motility, inhibition of host antitumor response, promotion of angiogenesis, and metastasis.[2,3] CAF origin is multifactorial and is derived from local fibroblasts, bone marrow–mesenchymal stem cells (MSCs), pericytes, and tumor cells. It is generally accepted that α-smooth muscle actin (SMA), tenascin-C (TEN), and vimentin (VIM) describe the CAF phenotype.[4] In addition, transforming growth factor-β (TGF-β) is critical for CAF activation and elaboration of a protumorigenic microenvironment.[5–7] Signaling by TGF-β regulates tumor initiation, progression, and metastasis through tumor cell–autonomous and host–tumor interactions. Osteopontin (OPN), a phosphoprotein secreted by malignant cells and tumor stromal cells, is a key mediator of tumor cell migration and metastasis and a marker of breast cancer progression and metastasis.[2,4] Recent findings suggest that tumor-derived OPN instigates bone marrow–derived MSC trafficking to the tumor microenvironment, which is characterized by the outgrowth of a

Presented at the Central Surgical Association 2019 Annual Meeting, Palm Harbor, FL.

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https://doi.org/10.1016/j.surg.2019.05.042
0039-6060/

desmoplastic stroma rich in CAFs that promotes cancer growth and metastasis.[4,8] Within the context of OPN-mediated CAF activation and tumor-microenvironment crosstalk, cancer stem cell (CSC) marker expression (Oct4, Nanog, and Sox2) and metastatic behavior of human breast cancer are enhanced.

Myeloid zinc finger-1 (MZF1) is a member of the SCAN–zinc finger transcription factor family, which has been discovered to play a complex and heterogeneous role in models of different types of cancer.[2,9] The cBioPortal database indicates the *MZF1* gene to be significantly amplified in certain solid tumors, such as those found in breast, uterine, lung, gastric, and bladder cancers, among others.[2,10] MZF1 protein has also been found to be regulated by phosphorylation to stabilize and upregulate N-cadherin expression.[11] We have previously demonstrated that cancer-derived OPN acts thru MZF1 and TGF-β to drive the adoption of a cancer-associated fibroblast (CAF) phenotype by local mesenchymal stem cells (MSCs).[4] However, the underlying molecular signaling mechanism by which MZF1 induces CAF transformation is unknown. Posttranslational phosphorylation of serine and threonine residues on target transcription factors is an extremely common signal transduction modality in this regard, and MZF1 is known to be regulated in this manner.[12]

In this study, we demonstrate that OPN-associated phosphorylation of MZF1 is required for the adoption of the CAF phenotype in MSCs within the tumor microenvironment with a resultant increase in cancer stemness markers in a model of human breast cancer.

Methods

Cell culture

Human MSCs (CD105+/CD73+/CD44+ and CD34−/CD45−/CD14−/HLA−DR−) were maintained in minimum essential medium α with 20% fetal bovine serum. The human MDA-MB231 breast cancer cell line is a high-OPN–producing cell line and was purchased from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's minimum essential medium with 10% fetal bovine serum. A 1:1 ratio of cancer cells to MSCs were plated in Boyden chamber (Corning, Inc, Corning, NY) wells with 0.4-μm pores that allow cytokine and growth factor passage but prevent cell movement. Experimental groups received 180 ng/mL OPN, 100 nmol/L OPN-R3 aptamer (APT), or mutant APT daily. The APT specifically binds and inactivates extracellular OPN. Cells were cocultured for a total of 72 hours, after which cells were removed and RNA was acquired using TRIzol (Thermo Fisher Scientific, Tampa, FL). The pharmacologic properties and sequences of OPN-R3 APT and mutant APT have been previously published.[13,14]

Characterization of MZF1 phosphorylation sites

MZF1 was isolated from cell lysates and separated by polyacrylamide gel electrophoresis. The resolving gel was stained, and the MZF1 band was excised and stored at −70 °C until use. The gel slices were subjected to in-gel tryptic digestion after reduction and carboxyamidomethylation. The resultant digests were pooled just before liquid chromatography–tandem mass spectrometry injection. Phosphorylated peptide sequences were identified and localized by modification of the liquid chromatography–tandem mass spectrometry protein identification procedure. Masses of posttranslational modifications are included as variable modifications in Mascot searches and incorporated into Scaffold files. Kinase-specific prediction was performed by submission of the MZF1 sequence to NetPhos.[15]

Phosphomimetic and phosphoresistant MZF1 mutations

Two phosphoresistant (T134A and S453A) and three phosphomimetic (T134E, S453D, and T134E+S453D) mutations of MZF1 were constructed by site mutagenesis and confirmed by sequencing. Transient transfection of pcDNA3.1 (Invitrogen, Carlsbad, CA) expression vectors containing mutant MZF1 was performed using lipofectamine.

Quantitative real-time polymerase chain reaction

Following the manufacturer's manual, total RNA was isolated using TRIzol (Thermo Fisher Scientific). cDNA was synthesized from 1.0 μg of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), according to the manufacturer's protocol. Reaction parameters for cDNA synthesis were as follows: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Quantitative real-time polymerase chain reaction (RT-PCR) was performed with iQ SYBR Green Supermix by using the iCycler iQ RT-PCR detection system (Bio-Rad). Parameters were as follows: 95°C for 3 minutes, 95°C for 30 seconds, 55°C for 35 seconds (40 cycles), 95°C for 1 minute, and 55°C for 10 minutes. ΔΔcycle threshold values were calculated using human β-actin as an endogenous control. Human-specific primers were used; primer sequences are as follows:

- VIM: 5′-AGGAAATGGCTCGTCACCTTCGTGAATA-3′; 5′-GGAGTGTCGGTTGTTAAGAACTAGAGCT-3′
- SMA: 5′-TAGCACCCAGCACCATGAAGAT-3′; 5′-GAAGCATTTGCGGTGGACAATG-3′
- TEN: 5′-AGCATCACCCTGGAATGGAGGA-3′; 5′-TGTGGCTTGTTGGCTCTTTGGA-3′
- TGF-β: 5′-TGGCGATACCTCAGCAACC-3′; 5′-CTCGTGGATCCACTTCCAG-3′
- Sox2: 5′-GCCTGGGCGCCGAGTGGA-3′; 5′-GGGCGAGCCGTTCATGTAGGTCTG-3′
- Oct4: 5′-GCTCGAGAAGGATGTGGTCC-3′; 5′-CGTTGTGCATAGTCGCTGCT-3′
- Nanog: 5′-TCTGGACACTGGCTGAATCCT-3′; 5′-CGCTGATTAGGCTCCAACCAT-3′
- β-actin: 5′-AGCGGGAAATCGTGCGTGAC-3′; 5′-CAATGGTGATGACCTGGCCGT-3′

Statistical analysis

Data are means ± SEM.

Results

MZF1, cancer stemness, and CAF markers in a mouse xenotransplant model

In a model using the low-OPN–expressing MZF1 human breast cancer cell line, we have previously performed a series of orthotopic xenotransplant experiments in severe combined immunodeficiency mice using dual-labeled MCF7 or MCF7 cells that constitutively express OPN (RFP-luc-MCF7-lvOPN) coimplanted with green fluorescent protein-MSCs.[4] Another set of green fluorescent protein-MSCs had MZF1 constitutively knocked down (MSC(dMZF1)). The various MZF1 cells and MSCs were coimplanted in the R3 position, and animals were treated with saline, APT that specifically binds and inactivates extracellular OPN, or mutant inactive APT. After 8 weeks, the animals were killed, their cells sorted, and their cDNA collected. We used these samples to determine the roles of OPN and MZF1 in the crosstalk between cancer

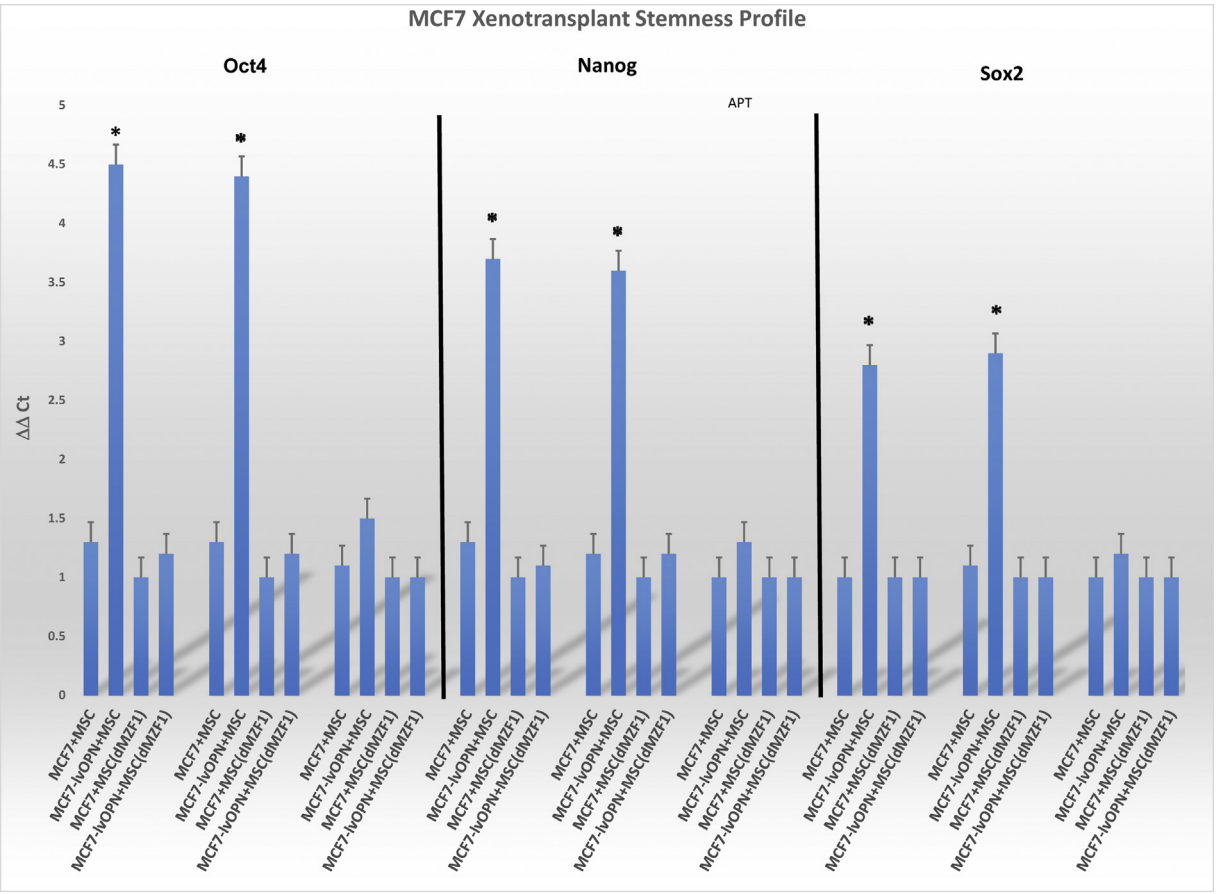

Fig 1. Ex vivo stemness profile of MCF7 cells from a xenotransplant model. Orthotopic xenotransplant experiments in severe combined immunodeficiency mice using dual-labeled MCF7 or MCF7 cells that constitutively express OPN (RFP-luc-MCF7-lvOPN) coimplanted with GFP-MSCs. Another set of GFP-MSCs had MZF1 constitutively knocked down (MSC(dMZF1)). The various MZF1 cells and MSCs were coimplanted in the R3 position, and animals were treated with saline, APT that specifically binds and inactivates extracellular OPN, or mutant inactive APT. After 8 weeks, the animals were killed, their cells sorted, and their cDNA collected. Oct4, Nanog, and Sox2 mRNA were measured with RT-PCR. *$P < 0.05$ vs MCF7+MSC and MCF7-lvOPN+MSC(dMZF1). *RT-PCR*, real-time polymerase chain reaction; *MSC*, mesenchymal stem cells; *APT*, aptamer; *OPN*, osteopontin; *GFP*, green fluorescent protein.

cells and MSCs in the local microenvironment to induce the CAF phenotype and increase cancer stemness (Fig 1).

In setting of a low-OPN–producing breast cancer cell line, MCF7, coimplanted with wild-type MSCs, the tumor cells expressed a low level of the stemness markers, Oct 4, Nanog, and Sox2. This was not altered among the saline, APT, and APT treatment groups. In contrast, when MCF7 cells expressing high OPN levels (MCF7-lvOPN) were coimplanted with MSCs, cancer stemness markers were significantly increased in the tumor cells from animals treated with saline or the APT mutant. This was ablated when extracellular OPN was inactivated by APT treatment. Further, when the high-OPN–expressing MCF7 cells were coimplanted with MSCs with constitutive knockdown of MZF1, the increase in the level of stemness markers was ablated in the saline-treated animals.

In our previous work in this model, we demonstrated that OPN acts on MSCs via the transcription factor MZF1 to express the CAF phenotype with increased expression of SMA, VIM, TEN, and TGF.[4] These current ex vivo data suggest CAF feedback to the local tumor cells to increase expression of stemness markers.

Characterization of MZF1 phosphorylation sites

The underlying molecular signaling mechanism by which MZF1 induces CAF transformation is unknown. Posttranslational phosphorylation of serine and threonine residues on target transcription factors is a common signal transduction modality, and MZF1 is known to be regulated in this manner. We then sought to investigate the role of phosphorylation in the present setting. MSC lysates from MSCs cocultured with high-OPN–expressing MB231 human breast cancer cells in the presence and absence of APT underwent polyacrylamide gel electrophoresis, and the MZF1 protein band was submitted to determine the pattern of differential amino acid phosphorylation. Five sites (T134, T173, S448, S453, and T585) in MZF1 were found to be phosphorylated in the MSC+MB231 cells, indicating induction in the presence of extracellular OPN. Two sites were selected: T134, which corresponded to the SCAN domain, and S453, which resided within the zinc finger DNA binding domain. Phosphoresistant (T134A, S453A, and T134A+S453A) and phosphomimetic (T134E, S453D, and T134E+S453D) MZF1 mutants were constructed for MSC transient transfection.

MZF1 phosphomutants and the OPN-dependent CAF phenotype in MSCs

MSCs were transfected with wild-type or mutant phosphoresistant (T134A and S453A) MZF1 and were exposed to OPN, and RT-PCR was performed for TGF and the CAF markers SMA, VIM, and TEN (Fig 2, *A* and *B*). MSCs exposed to OPN exhibited a 10- to 16-fold increase in CAF marker and TGF expression. Wild-type MZF1 alone did not alter the expression profile of the MSCs; however, OPN added with wild-type MZF1 resulted in increased CAF marker and TGF expression in a manner similar to that of OPN

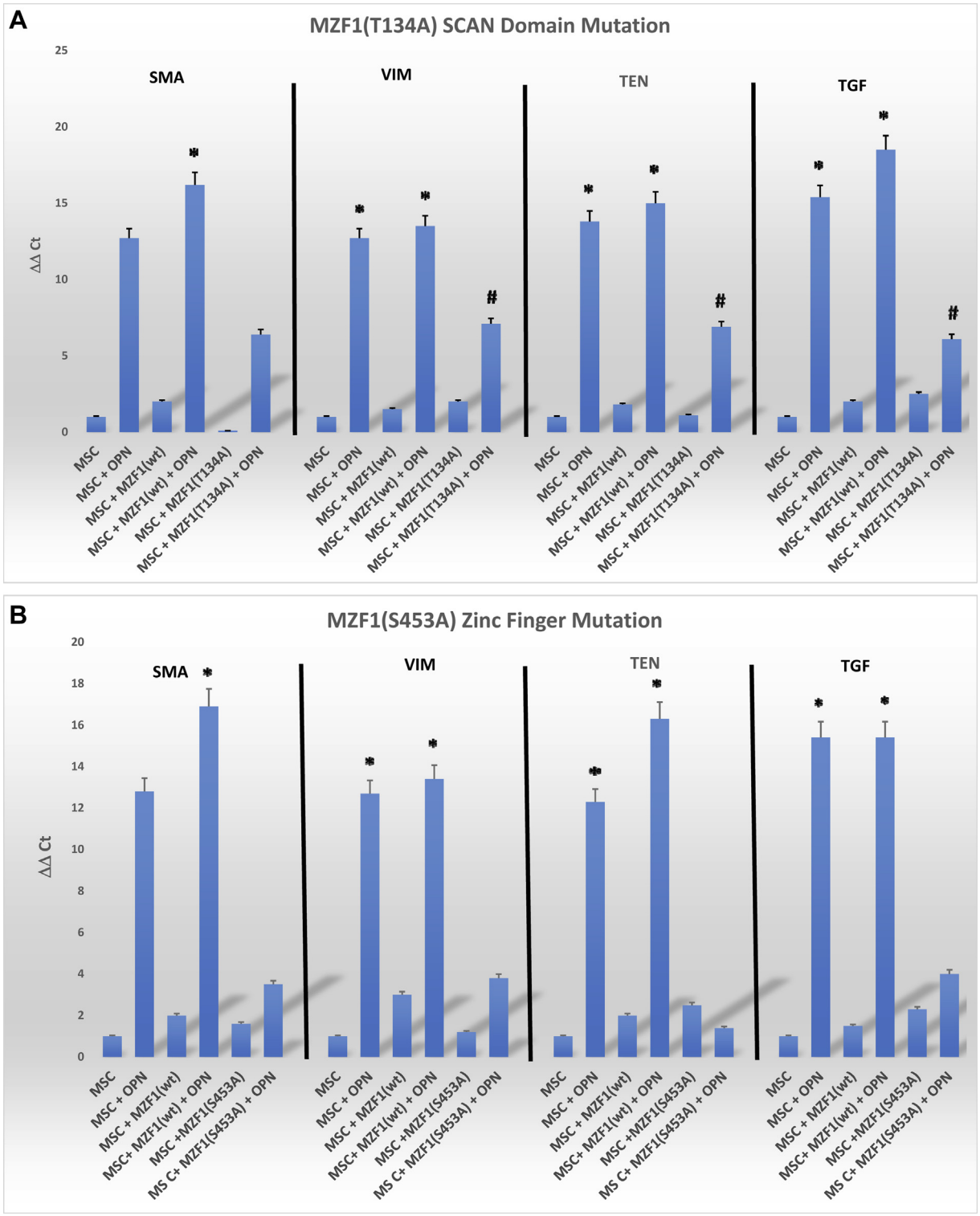

Fig 2. Effect of phosphoresistant MZF1 in MSCs exposed to OPN on expression of CAF markers (SMA, VIM, and TEN) and TGF. T134A and S453A phosphoresistant mutants were transfected into MSCs to determine the role of MZF1 phosphorylation on MZF1 function. *(A)* T134A SCAN domain mutation of MZF1. *$P < 0.05$ vs MSC alone, MSC+MZF1(wt), MSC+MZF1(T134A), and MSC+MZF1(T134A)+OPN; #$P < 0.05$ vs MSC alone, MSC+OPN, MSC+MZF1(wt), MSC+MZF1(wt)+OPN, and MSC+MZF1(T134A). *(B)* S453A zinc finger mutation of MZF1. *$P < 0.05$ vs MSC alone, MSC+MZF1(wt), MSC+MZF1(S453A), and MSC+MZF1(S453A)+OPN. *SMA*, α-smooth muscle actin; *VIM*, vimentin; *TEN*, tenascin-C; *MSC*, mesenchymal stem cells; *OPN*, osteopontin; *TGF*, transforming growth factor.

alone. In contrast, when either T134A or S453A was transfected and OPN added, the resultant CAF marker and TGF expression profile was decreased by 2–3 fold but not to the baseline levels seen in MSCs alone.

The phosphomimetic mutants (T134E, S453D, or T134E+S453D) were then transfected in a similar fashion, and CAF marker and TGF expression were measured (Fig 3). Again, exposure to OPN increased the expression of CAF markers and TGF in MSCs by 15- to

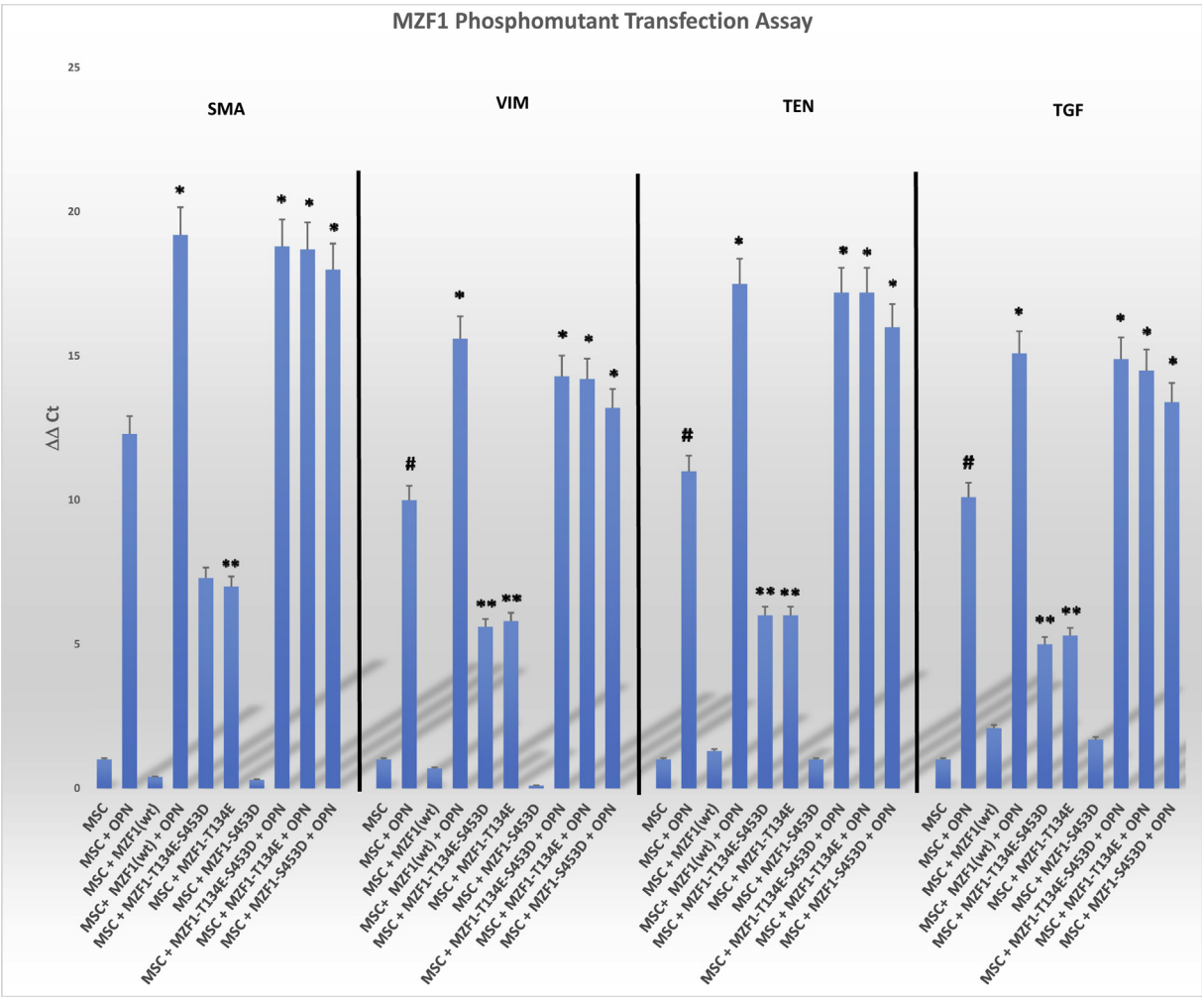

Fig 3. Effect of phosphomimetic MZF1 in MSCs exposed to OPN on expression of CAF markers (SMA, VIM, and TEN) and TGF. T134E and S453D phosphomimetic mutants were transfected into MSCs to determine the role of MZF1 phosphorylation on MZF1 function. #$P < 0.05$ vs MSC, MSC+MZF1(wt), MSC+MZF1(wt)+OPN, MSC+MZF1-T134E-S453D, MSC+MZF1-T134E, MSC+MZF1-S453D, MSC+MZF1-T134E-S453D+OPN, MSC+MZF1-T134E+OPN, and MSC+MZF1-S453D+OPN; *$P < 0.05$ vs MSC, MSC+OPN, MSC+MZF1(wt), MSC+MZF1-T134E-S453D, MSC+MZF1-T134E, and MSC+MZF1-S453D; **$P < 0.05$ vs MSC, MSC+OPN, MSC+MZF1(wt), MSC+MZF1(wt)+OPN, MSC+MZF1-S453D, MSC+MZF1-T134E-S453D+OPN, MSC+MZF1-T134E+OPN, and MSC+MZF1-S453D+OPN. *CAF*, cancer-associated fibroblasts; *SMA*, α-smooth muscle actin; *VIM*, vimentin; *TEN*, tenascin-C; *MSC*, mesenchymal stem cells; *OPN*, osteopontin; *TGF*, transforming growth factor.

20-fold. Transfection of T134E or T134E+S453D in MSCs enhanced CAF marker and TGF expression by 5- to 10-fold versus MSCs alone and MSCs with wild-type MZF1. However, the level of increase was less than that noted in the presence of OPN, both in MSCs and MSC+MZF1. In contrast, transfection of S453D in MSCs did not alter the CAF marker and TGF profile. It was only when OPN was added to MSC+MZF1(S453D) was there an significant increase.

Taken as a whole, the phosphomimetic and phosphoresistant MZF1 mutant data indicate that MZF1 phosphorylation of T134 is necessary and sufficient to induce the CAF phenotype, whereas S453 phosphorylation is additive but not necessary or sufficient. In addition, there are OPN-dependent pathways for expression of the CAF phenotype that are parallel to and independent of MZF1.

MZF1 and MSC-MB231 coculture

The previous studies were then repeated in a coculture setting using the double mutants (phosphomimetic T134E+S453D or phosphoresistant T134A+S453A). MSCs were cocultured with the high-OPN–producing MB231 breast cancer cell line to determine the role of MZF1 in MSC adoption of a CAF phenotype and the resultant effects on the stemness profile of MB231 cells (Fig 4). In MB231+MSC, MSCs expressed significantly increased levels of SMA, TEN, and VIM; however, in the presence of APT, which specifically binds and inactivates extracellular OPN, the CAF markers were no different from those of MSCs alone. Finally, when MSC(T134A+S453A) was cocultured with MB231, the expression of the CAF markers was not different from MSCs alone. In a setting of MB231+MSC(T134E+S453D), the CAF profile was not different from that of MB231+MSC.

The MB231 cells were examined for cancer stemness markers in this context (Fig 4, *B*). MB231 cells were first cultured in the presence of APT to determine whether OPN feeds back to induce enhanced cancer stemness. The resulting levels of Oct4, Nanog, and Sox2 were not different from MB231 cells alone. In MB231+MSC, there was a 4- to 5-fold increase in expression of all 3 stemness markers. (The subsequent translated proteins were also confirmed to increase in a parallel fashion; data not shown.) The stemness markers all decreased when APT was added to the MB231+MSC coculture. Finally, in MB231+MZF1(T134A+S453A), the stemness markers decreased to levels that were 2- to 3-fold less than those of MB231+MSC. In setting of MB231+MSC(T134E+S453D), the stemness profile was not different from that of MB231+MSC.

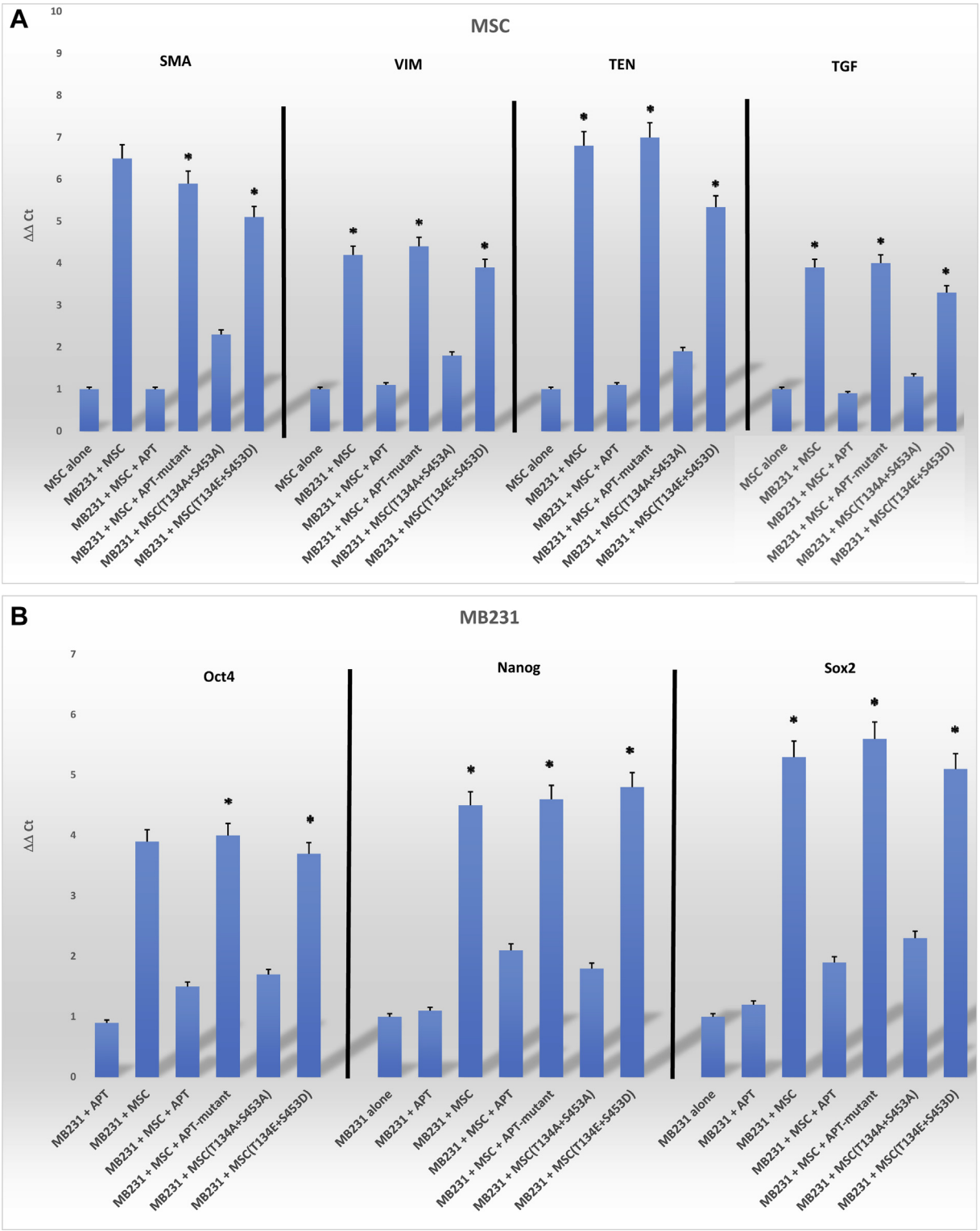

Fig 4. Coculture of MSCs with MB231 to determine the role of MZF1 phosphorylation mutants on MSC expression of CAF markers (SMA, VIM, TEN, and TGF) and MB231 cancer cell expression of stemness markers (OCT4, NANOG, and SOX2). (*A*) CAF marker expression in MSCs. *$P < 0.05$ vs MSC alone, MB231+MSC+APT, and MB231+MSC(T134A+S453A). (*B*) Stemness markers in MB231. *$P < 0.05$ vs MSC alone, MB231+MSC+APT, and MB231+MSC(T134A+S453A). *APT*, aptamer; *CAF*, cancer-associated fibroblasts; *MSC*, mesenchymal stem cells.

These results indicate that MB231-derived OPN acts on MSCs in coculture via phosphorylation of MZF1 at T134 and S453 to induce MSCs to express a CAF phenotype with a concomitant feedback to MB231 to increase their stemness profile.

Discussion

In this study, we demonstrated that the OPN-mediated CAF phenotype in MSCs is dependent upon T134 and S453

phosphorylation of the transcription factor MZF1. Blockade of T134 phosphorylation in MSCs inhibits expression of the CAF phenotype and the stemness profile of cocultured MB231 human breast cancer cells. We conclude that increased cancer stemness in OPN-producing human breast cancer cells occurs as a result of cross-talk with MSCs in the tumor microenvironment.

MZF1 is a member of the SCAN–zinc finger transcription factor family.[4,9,10,16] The human genome contains approximately 70 SCAN–zinc finger factors. It has recently been implicated in a number of cancers, including human breast, uterine, lung, bladder, and colorectal cancer. A repeated theme among these studies is the interplay between myc and MZF1 because myc may bind to the MZF1 promoter. The intracellular mechanisms of MZF1 regulation in these cancers are not well characterized. However, in contrast to the existing studies that associate MZF1 with tumorigenesis, this is one of the few studies that has demonstrated a role for MZF1 in the development of the CAF phenotype within the microenvironment MSCs and the subsequent effects in enhancement of cancer cell stemness.[17] Five phosphorylation sites were identified. We selected T134 and S453 because they corresponded to functional regions of MZF1. T134 corresponded to the SCAN domain and S453 resided within the zinc finger DNA binding domain. Regarding the remaining phosphorylation sites, they certainly could play a role, but the data show that when the T134A+S453A double phosphoresistant mutant is transfected, MSC expression of the CAF and TGF markers in the presence of MB231 are not different from that seen in wild-type MSCs alone. In addition, MB231 cells correspondingly do not express increased levels of stemness markers. So, as a result, we surmise that the remaining 3 phosphorylation sites do not significantly contribute to the mechanism underlying the CAF expression profile.

The CSC model has received increased attention during the past 2 decades.[18] According to this model, primary tumors are heterogeneous and comprise multiple distinct cell subpopulations that arise from a small number of pluripotent cancer cells named CSCs or tumor-initiating cells. CSCs are characterized as the small proportion of cancer cells that have an indefinite ability for self-renewal and pluripotency. As a result, CSCs are responsible for initiating and sustaining cancer growth. This, in turn, enhances tumor initiation, propagation, metastasis, recurrence, and chemotherapy resistance. However, the validity of this model remains a topic of some controversy.[19] For example, the precise definition of CSCs is not clear. According to a consensus definition, CSCs are defined experimentally by the ability to recapitulate the generation of a continuously growing tumor.[20] Nevertheless, investigators use a variety of markers, including CD44+ CD24−/low, CD44v, CD133, and ALDH, in addition to the markers in this study, Sox2, Nanog, and Oct4.[21]

Two models are proposed to explain the presence of CSCs within a tumor.[22] In the stochastic model, each cancer cell has the capability to dedifferentiate into a CSC; specific cell signaling dysregulation leads to increased stemness of tumor cells during neoplastic transformation. In contrast, in the hierarchical model, CSCs are the progenitors of differentiated tumor cells. According to this hierarchical model, CSCs can self-renew to expand the CSC pool and differentiate into heterogeneous cancer cell types to form the bulk of the tumor. Neither model is mutually exclusive, and it is thought that both may exist within a tumor. In the current set of studies, we demonstrate that increased cancer stemness, as measured by increased expression of the pluripotency-associated transcription factors, Sox2, Nanog, and Oct4, in OPN-producing cancer cells occurs as the result of crosstalk with MSCs in the tumor microenvironment. It is unclear whether this reflects the stochastic model, the hierarchical model, or both. Regardless, the increased stemness is associated with enhanced tumor properties of initiation, propagation, metastasis, recurrence, and chemotherapy resistance.

Finally, this study points to the importance of the relationship between the cancer cell and the tumor microenvironment and its constituent cells.[2,23] The microenvironment is complex and is capable of both combatting the growth of malignant cells and abetting disease progression. Historically, much attention had focused on the cancer cell, itself. However, the local microenvironment, which consists of the interactions between malignant cells and the nonmalignant cells of the surrounding area, also regulates the growth and development of the tumor, in part through altering the relative stem cell profile of the tumor cells. In this study, cancer cells express OPN to activate local MSCs to become CAFs and subsequently feeds back by an as-yet-undefined signaling pathway to increase tumor stemness. This finding suggests that the totality of the cancer cell, its interactions with the microenvironment, and the overall host response dictates the progression of the disease. Thus, research efforts should consider this entire set of complex interactions.

Within the realm of cancer therapy, surgery falls under the umbrella of locoregional therapies. In combination with the work of others, our results indicate that both the tumor and its surrounding environment function in a synergistic fashion to enhance markers of local growth and metatstasis. In this context, simply relying on metrics of margin distance to achieve an R0 resection maybe insufficient. Questions that arise include "What is the extent of the microenvironment changes?," "Does the CAF phenotype extend to the edge of currently accepted measures of R0 resection?," and "Does the CAF phenotype resolve following resection of the tumor?" Certainly, preliminary data from our lab suggest that the initiation signal and the maintenance signal pathways are different.[8] What is the effect of the mature CAFs on seemingly normal cells? In addition, our previous work indicates that cancer can establish a premetastatic niche in bone marrow that is OPN and CAF dependent. As a result, surgeons must understand that macroscopic measures of extirpative success may be insufficient for the future. An overall strategy directed toward a cancer cure must account for the tumor and its surrounding microevironment.

Funding/Support

The authors have indicated that they have no funding regarding the content of this article.

Conflict of interest/Disclosure

None of the authors have any conflicts of interest to disclose.

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Discussion

Dr Stephen Grobmyer (Cleveland, OH): I would like to thank the Association for the privilege of the floor. I would also like to congratulate the authors on another in a series of important contributions to our understanding of the cancer biology and the complex tumor microenvironment as it relates to crosstalk between breast tumor cells and the parenchymal stem cells. I think this paper highlights nicely how complex this interaction is and the interdependence of these cells on each other in sustaining themselves. It certainly suggests some new opportunities to treat cancer that I will ask you about.

I have several questions. The first 3 questions relate to some of the basic work and the final 3 questions relate to some issues related to the potential clinical translation.

To begin with, your characterization of the myeloid zinc finger-1 phosphorylation sites in the manuscript identified 5 sites which were phosphorylated. You seem to have selected 2 for additional study, which you highlighted in this presentation. How and why were these particular phosphorylation sites selected, and is there a plan to investigate the other phosphorylation sites on the myeloid zinc finger protein, and might they be important?

Your work suggests that there is some zinc finger–independent expression of the cancer-associated fibroblast phenotype. Could you postulate what mechanism might be at play here in this scenario?

Finally, related to the basic mechanisms, your work suggested that there is a feedback from cancer-associated fibroblasts to increase the stemness profile of the cancer cells MB231. Do you have a hypothesis as to what this might be or how you might approach this important scientific question?

Related to the clinical issues, is osteopontin a good biomarker in human breast cancer? It's been shown to be a biomarker in some gastrointestinal cancers.

You also, in the manuscript, do some studies involving treatment of the mouse model with an aptamer APT, which blocks osteopontin and mediates an increase in cancer stemness. Is there any potential therapeutic role of this aptamer APT in reducing cancer stemness and aggressiveness that could be applied in a clinical situation?

Finally, based on your work and the work of others for a general audience, how close are we to developing specific cancer therapeutics that target cancer stem cells?

Dr Zhiyong Mi: Thank you, Dr. Grobmeyer, for your questions and comments. For the first question, we identified 5 phosphorylation sites based on the protein sequencing results. However, we focused on 2 of them. The reason is 1 of these 2 sites is located on the SCAN domain, and the other is on the zinc finger domain. Based on the kinase protein recognition sequence prediction analysis (NetPhosk 1.0), these 2 sites match the PKC/PKA kinase recognition sequence.

We do have a plan to investigate the other 3 sites' function. Currently, we're using RNA-seq transcriptome analysis to search for an increased protein kinase expression profile and will try to narrow down the potential protein kinases for these phosphorylation sites. Using the coculture system, we've harvested total RNA and sent for the RNA-seq sequencing analysis. That will help us to characterize more signal pathways related to OPN-induced MSC transformation into the CAFs, along with much more regulation information.

The next question: Are there any independent signal pathways? We think, yes. So, that's another reason why we're using the global transcriptome analysis to analyze the data to narrow down some interesting kinase signal pathways for MSC/CAF transformation regulation.

For the third question about the feedback-loop signal pathways to increase the cancer stemness, yes, I do think that's a very interesting question. Although we have not finished this study yet, our plan is to label the cancer cell with selected stemness gene promoter regulated fluorescent protein reporters so we can pull the cancer stemness cell out and do the single cell RNA-seq transcriptome sequencing and find the signal pathways for how the cancer stemness cell can be activated. I think that's the most important thing we are doing currently.

For the fourth question, is osteopontin a good biomarker in human breast cancer? More than 15 years ago, Dr. Ann Chambers' lab started the clinical trial to screen osteopontin concentration in breast cancer patients' plasma and reported it was correlated to breast cancer progression. Currently, I think there are more than 30 published papers that report that OPN expression is correlated to breast cancer patient prognosis, not only with breast cancer but with other cancers also. Based on these reports, the OPN expression is correlated to more than 20 different kinds of cancers. So I

think OPN has great potential to be a biomarker because OPN is located in the circulation system; it should be very easy to monitor and quantify. However, the current problem is the complexity of OPN. The human OPN have 5 different mRNA transcripts with many posttranslational modifications, such as phosphorylation, glycosylation, and transglutaminase cross-linking and cleavage fragments. The analysis mentioned previously correlates total OPN expression to cancer progression. So, currently, we are not exactly sure what kind of the modification of OPN or which of the isoforms or fragments are the real biomarkers and correlated to which cancer stages, and we do not really understand OPN's function yet. So I think we still need to do more basic research on OPN.

For the fourth question about the OPN aptamer, the 3D structure of OPN-aptamer binding complex was published last year and provides more clues about how to regulate binding and make extra modifications to facilitate its clinical applications.

For the fifth question, to decrease cancer stemness or clinical treatment to the cancer stem cell microenvironment, I think there is still a long way to go.