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## Curcumin Inhibition of TGF $\beta$ signaling in bone metastatic breast cancer cells and the possible role of oxidative metabolites

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### Abstract

TGF $\beta$  signaling promotes progression of bone-metastatic (BMET) breast cancer (BCa) cells by driving tumor-associated osteolysis, a hallmark of BCa BMETs, thus allowing for tumor expansion within bone. Turmeric-derived bioactive curcumin, enriched in bone via local enzymatic deconjugation of inactive circulating curcumin-glucuronides, inhibits osteolysis and BMET progression in human xenograft BCa BMET models by blocking tumoral TGF $\beta$  signaling pathways mediating osteolysis. This is a unique anti-osteolytic mechanism in contrast to current osteoclast-targeting therapeutics. Therefore, experiments were undertaken to elucidate the mechanism for curcumin inhibition of BCa TGF $\beta$  signaling and the application of this finding across multiple BCa cell lines forming TGF $\beta$ -dependent BMETs, including a possible role for bioactive curcumin metabolites in mediating these effects. Immunoblot analysis of TGF $\beta$  signaling proteins in bone tropic human (MDA-SA, MDA-1833, MDA-2287) and murine (4T1) BCa cells revealed uniform curcumin blockade of TGF $\beta$ -induced Smad activation due to downregulation of plasma membrane associated TGF $\beta$ R2 and cellular receptor Smad proteins that propagate Smad-mediated gene expression, resulting in downregulation of PTHrP expression, the osteolytic factor driving *in vivo* BMET progression. With the exception of early decreases in TGF $\beta$ R2,

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inhibitory effects appeared to be mediated by oxidative metabolites of curcumin and involved inhibition of gene expression. Interestingly, while not contributing to changes in Smad-mediated TGF $\beta$  signaling, curcumin caused early activation of MAPK signaling in all cell lines, including JNK, an effect possibly involving interactions with TGF $\beta$ R2 within lipid rafts. Treatment with curcumin or oxidizable analogs of curcumin may have clinical relevancy in the management of TGF $\beta$ -dependent BCa BMETs.

## Keywords

curcumin; breast cancer; TGF $\beta$ ; Smad; JNK; bone metastases

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## 1. INTRODUCTION

Osteolysis is a hallmark of breast cancer (BCa) bone metastases (BMET), a tumor-driven process that promotes metastatic progression within the bone microenvironment [1,2]. Most women with advanced BCa develop osteolytic BMETs, which are essentially incurable, and may arise from asymptomatic disseminated tumor cells already present in bone at the time of initial diagnosis [3–5]. Tumoral TGF $\beta$  signaling in bone-disseminated BCa cells, fueled by TGF $\beta$  released from osteolytically-resorbed bone matrix, induces additional tumor-associated osteolysis by promoting the formation of more bone-resorbing osteoclasts, thus facilitating tumor expansion within bone in a feed-forward loop [1,2,6–21].

The importance of tumoral TGF $\beta$  signaling in promoting osteolytic BMET progression is supported by a large body of evidence from human BCa BMET xenograft models where neutralization of TGF $\beta$ , tumoral TGF $\beta$  signaling (including canonical Smad signaling, which is active in 75–100% of clinical BMET [18,22]), and/or TGF $\beta$ -inducible tumor-derived parathyroid hormone related protein (PTHrP), a osteolytic factor expressed in most clinical BMET [23–25], prevents tumor-associated osteoclast formation and *in vivo* metastatic progression. This inhibitory effect is independent of direct effects on tumor growth [1,2,6–21].

Of relevance for the current studies, curcumin, a naturally occurring turmeric-derived polyphenol, has also been reported by our laboratory and by others to block osteolysis *in vivo* and thus BCa BMET progression in these same pre-clinical models [26,27]. Notably, our laboratory has further demonstrated that curcumin inhibition of osteolysis and BMET progression, which contrasted with the lack of a direct effect on orthotopic tumor growth (i.e., proliferation), was associated with curcumin inhibition of tumoral TGF $\beta$  pathways driving osteolysis, most notably, TGF $\beta$ -inducible Smad signaling and PTHrP secretion [26,28,29].

Curcumin's TGF $\beta$ -targeted anti-osteolytic effects are intriguing since drugs currently used to block osteolysis in BCa, which are beneficial but not curative, target osteoclasts but not the tumoral pathways driving their formation. Also of note, curcuminoids circulate *in vivo* as inactive glucuronides that do not inhibit tumoral TGF $\beta$  signaling [28,30,31]. However, adding to the potential promise of curcuminoids in targeting of TGF $\beta$ -driven tumor-associated osteolysis is recent evidence that bioactive curcumin can readily be

formed *in situ* within the bone/tumor microenvironment via enzymatic deconjugation of curcumin-glucuronides [28,30,31], allowing for local targeting of TGF $\beta$ -signaling in bone while sparing systemic TGF $\beta$  activity. However, the mechanism by which curcumin suppresses BCa cell TGF $\beta$  signaling, critical for *in vivo* osteolysis and BMET progression [6,7,14,15,18,20,21], is not known.

Given the potential promise of curcuminoid polyphenols in blocking tumoral TGF $\beta$  signaling cascades mediating osteolysis and BCa BMET progression, current experiments were initiated to investigate the mechanism(s) responsible for the inhibitory effect of curcumin on Smad signaling in BCa cells forming TGF $\beta$ -dependent osteolytic BMET *in vivo* [6–9,14,15,18,20,21], and the uniformity of mechanism(s) across cell lines. In particular, these studies focus on MDA-SA cells, where pathologic roles of TGF $\beta$ -induced canonical (Smad) as well as non-canonical MAPK (p38, JNK, or ERK) signaling are well described, including the *in vivo* dependence of osteolysis and BMET progression on TGF $\beta$ -induced PTHrP [8,9,14,15,20], and *in vivo* protective effects of curcumin are established [26]. In addition, while deconjugation of circulating curcumin-glucuronide is required for curcumin bioactivity within bone [28,30,31], also explored here was the question of whether further oxidative metabolism of curcumin to form reactive intermediates is also required for inhibition of TGF $\beta$  signaling in bone metastatic BCa cells, as has been posited for other cellular curcumin targets [32–34].

## 2. MATERIALS AND METHODS

### 2.1 Materials

The chemical content of turmeric-derived curcumin (#218580100, Fisher Scientific) was verified using standard methods (see below), with assayed content 96.5% curcuminoids by weight, comprised of 80.6% curcumin with lesser amounts of demethoxycurcumin [13.5%] and bisdemethoxycurcumin [2.4%]) [35,36], and stock solutions prepared in DMSO. A non-oxidizable curcumin analog, diacetyl curcumin (DAC), was synthesized as previously described [34,37]. Cells were stimulated, as indicated, with recombinant human TGF $\beta$ 1 (#240-B, R&D Systems). N-acetylcysteine (NAC, #A1540914) and nystatin (#J6048606) were purchased from Alfa Aesar. Cycloheximide (CHX, #C7698), (#SML1169), okadaic acid (OA, #459620), chloroquine (CQ, #C6628), and MG132 (#474791) were purchased from Millipore-Sigma. The MAPK inhibitors SP600125 (#S1077) and SB202190 (#S1460) were purchased from Selleckchem. Mini-PROTEAN TGX-PAGE gels (#4568046) were purchased from BioRad and PVDF membranes (#IPFL0010) from Millipore.

### 2.2 Cell Culture

Bone-metastatic (“bone tropic”) MDA-MD-231 BCa (MDA-SA) cells that secrete TGF $\beta$ -inducible PTHrP that is curcumin-inhibitable [8,14,15,20,26,28,29], and a MDA-SA-derived cell line stably transfected to overexpress TGF $\beta$ R2 lacking the cytoplasmic domain required for TGF $\beta$ -mediated signal transduction (“TGF $\beta$ R2 cyt”) [20,38,39], were kindly provided by Dr. Theresa Guise, Indiana University [14,15]. The parent MDA-SA cells have been propagated from MDA-MB-231 cells initially obtained from Kent Osborne [40] (not ATCC) by Yoneda and Mundy at the University of Texas Health Sciences Center in San Antonio

(SA) [41], who were the first to describe the cell's bone tropic behavior and formation of osteolytic BMET following intracardiac injection. It should be noted that TGF $\beta$  induction of PTHrP in MDA-SA cells transfected with empty vector was indistinguishable from wild type cells used here [14]. Two bone-tropic cell lines derived from ATCC MDA-MB-231 cells by *in vivo* selection for an aggressive bone metastatic phenotype attributable to the overexpression of TGF $\beta$ -regulated genes (MDA-1833 and MDA-2287) were a kind gift from Dr. Joan Massagué, Sloan-Kettering [18,21]. Bone-tropic murine 4T1 cells, which form TGF $\beta$ -dependent BMET with evidence of active Smad signaling in immunocompetent mice [6,7,42–45] and secrete TGF $\beta$ -inducible PTHrP that is curcumin-inhibitable [28], were obtained directly from American Type Culture Collection (#CRL-2539, ATCC) and used within 10 passages. Human cell lines were authenticated using short tandem repeat profiling by the University of Arizona Genetics Core [46] and all cells were cultured in DMEM (#10–013-CV, Corning) supplemented with 10% FBS (#S11150, Atlanta Biologicals) and 1% penicillin/streptomycin (#15140, ThermoFisher). For analysis of curcumin effects on TGF $\beta$  signaling pathway proteins, unless otherwise stated, cells were pretreated with vehicle (DMSO) or curcumin followed by TGF $\beta$ 1 (5 ng/ml) (or media alone) for specified times prior to isolation of whole cell lysates with RIPA buffer (#R0278, Millipore-Sigma) for Western analyses. Membrane-associated proteins were isolated from cells using the ProteoExtract Native Membrane Protein extraction kit (#444810, Millipore-Sigma), and plasma membrane proteins were isolated using the Pierce Cell Surface Isolation Kit (#89881, ThermoFisher), according to the manufacturers' protocol. For analysis of effects on TGF $\beta$ -induced PTHrP, secreted PTHrP was assayed by commercial radioimmunoassay (RIA) as previously described [28]. Briefly conditioned media (treated with protease inhibitors prior to storage at  $-80^{\circ}\text{C}$ ) was obtained from cells pre-treated with curcumin, DAC, or vehicle for 4 hours prior to concurrent addition of TGF $\beta$ 1 (5 ng/ml) for 24 hours. PTHrP was then quantified in the conditioned media using a PTHrP RIA (Beckman Coulter, #DSL-8100). Results are expressed either as absolute concentrations or relative to basal control cells or to TGF $\beta$ -stimulated cells in experiments where control cells were also included to verify TGF $\beta$  induction.

### 2.3 Protein Turnover Studies

To determine curcumin effects on protein degradation, cells were concurrently treated with cycloheximide (50  $\mu\text{g}/\text{mL}$ ) and curcumin (30  $\mu\text{M}$ ) (vs media alone) for up to 48 hours. Lysosomal or proteosomal protein degradation pathways were also blocked by pre-treatment with chloroquine (40  $\mu\text{M}$ ) or MG132 (10  $\mu\text{M}$ ), respectively, for 2–3 hours followed by treatment with curcumin (30  $\mu\text{M}$ ) vs media alone for 4h (for TGF $\beta$ 2) or 16h (for Smad2). Curcumin effects on protein synthesis were determined by pre-treating cells with cycloheximide (50  $\mu\text{g}/\text{mL}$ ) for 24 hours to inhibit *de novo* protein synthesis and deplete endogenous protein levels, followed by treatment with curcumin (30  $\mu\text{M}$ ) vs media alone for up to 24 hours.

### 2.4 Western Blot Analyses

Proteins, isolated from whole cell lysates and quantitated by Bradford assay for normalized loading (#5000002, Bio-Rad), were separated on Mini-PROTEAN TGX-PAGE gels (BioRad), and transferred to Immobilon-FL PVDF membranes (Millipore-Sigma), with

even protein loading confirmed by stain-free imaging of UV-activated binding of gel trihalo compound binding to protein tryptophan residues (BioRad) and by detection of the housekeeping gene,  $\beta$ -actin (#4967, Cell Signaling Technology [CST]). Blots were probed with primary antibodies (obtained from CST, unless otherwise stated) directed against Smad2 (#5339), pSmad2 (S465/467, #3108), pSmad2-L (S245/250/255, #3104), Smad3 (#9523), pSmad3 (S423/425, #9520), Smad2/3 (#8685), SARA (#13285), Smad4 (#38454), Smad7 (#42–0400, ThermoFisher), JNK (#9252), p-JNK (T183/Y185, #4668), p38 (#8690), p-p38 (T180/Y182, #4511), ERK1/2 (#4695), p-ERK1/2 (T202/Y204, #4370), TGF $\beta$ R2 (#79424 [CST] or #ab184948 [Abcam], as indicated), TGF $\beta$ R1 (#ab31013, Abcam), or ETS-1 (#sc-350, Santa Cruz) followed by HRP-conjugated secondary antibody (#7074, CST). All anti-Smad and anti-MAPK antibodies recognize both human and murine proteins, as does the CST TGF $\beta$ R2 antibody. While the Abcam antibody used to verify curcumin effects on TGF $\beta$ R2 in human cells is only documented to recognize human. Effects of deglycosylation on the size of TGF $\beta$ R2 proteins detected by Western were determined by treatment of lysates with PNGase F (#P0704, New England Biolabs) according to the manufacturer's protocol. Blots were visualized with SuperSignal West Femto ECL substrate (#34095, ThermoFisher). Quantitative densitometry was determined using ImageJ (v2.0.0, NIH).

## 2.5 Real-time quantitative PCR

RNA was isolated from whole cell lysates using TRI-reagent (#T9424, Millipore-Sigma) with cDNA synthesized using BioRad iScript kit (#170–8890), as per manufacturers protocols. Real-time quantitative PCR (qPCR) was performed on a Roche LightCycler 96 system using BioRad iQ Supermix (#170–8860) and TaqMan primers against Smad2 (#Hs00183425\_m1), TGF $\beta$ R2 (#Hs00234253\_m1), and 18S (#Hs9999901\_s1, Thermo Fisher). Cycle thresholds ( $C_t$ ) values were normalized to an endogenous housekeeping control (18S RNA), with mRNA levels in curcumin-treated cells expressed relative to vehicle controls using the  $2^{-C_t}$  method, as previously described [47]. Statistical analysis (t-test) was performed using  $C_t$  values and confidence intervals (CI) were calculated, as described elsewhere [48].

## 2.6 Statistical Analysis

Statistical analyses were performed using Prism v6.0h software (GraphPad, San Diego, CA), with data expressed as mean  $\pm$  SEM, except where noted. Half-maximal inhibitory concentrations ( $IC_{50}$ ) were determined by using a four-parameter logistic equation. Significant differences were determined by t-test or by one-way or two-way ANOVA with post-hoc testing, as appropriate.

## 3. Results

### 3.1 Curcumin inhibition of TGF $\beta$ -induced Smad signaling was associated with decreased TGF $\beta$ R2 and Smad proteins and activation of MAPKs

Curcumin reduced levels of C-terminal phosphorylated Smad2 and Smad3, the receptor Smads (R-Smads), in response to TGF $\beta$  stimulation in a concentration-dependent fashion (Fig. 1A). This required >4 hours of curcumin exposure prior to TGF $\beta$  activation (Fig.

1B) in MDA-SA cells. Curcumin inhibition of receptor-mediated Smad2/3 phosphorylation could not be attributed to decreases in SARA, which stabilizes phosphorylated Smad2/3 [39], nor increases in Smad7, which blocks Smad2/3 phosphorylation (Fig. 1A). Dephosphorylation of the R-Smads also did not appear to be enhanced by curcumin, as pre-treatment with okadaic acid (OA), an inhibitor of the protein phosphatase (PP1/2A) targeting phosphorylated Smad2/3 [49], did not alter curcumin's inhibitory effect (Fig. S1). Smad4 and constitutively active ETS-1, two factors required for Smad-dependent TGF $\beta$  induction of PTHrP gene expression in MDA-SA cells [26,28,29,50], were also unchanged by curcumin (Fig. 1A).

Notably, while changes in R-Smad phosphorylation/dephosphorylation did not appear to account for curcumin-induced decreases in TGF $\beta$ -stimulated R-Smad activation, constitutive Smad2/3 protein levels were reduced. This effect was concentration-dependent and required >4h of curcumin exposure (Fig. 1A,B). In addition, reduced pSmad2/Smad2 levels were preceded by curcumin-induced changes in TGF $\beta$  receptor 2 (TGF $\beta$ R2) and MAPK signaling (Fig. 1A–C). Curcumin caused an early (4h), profound, and persistent (16h) decrease in TGF $\beta$ R2 (but not TGF $\beta$ R1), independent of TGF $\beta$  treatment (Fig. 1B,C). These findings were confirmed using antibodies directed against two different TGF $\beta$ R2 epitopes with collapse of Western bands into an anticipated ~60 kDa protein [51] following deglycosylation (Fig. S2). The curcumin-mediated decrease in constitutive TGF $\beta$ R2 protein levels, which continued for 12 hours after initiation of TGF $\beta$  Smad signaling (Fig. 1C), was associated with persistent activation of MAPK signaling (Fig. 1B,C). ERK signaling was constitutively active and unaltered by curcumin and/or TGF $\beta$  in MDA-SA cells (Fig. S3). However, p38 MAPK signaling (phosphorylation of p38), which is also induced by TGF $\beta$  in MDA-SA cells and contributes to TGF $\beta$ -regulated expression of PTHrP and other osteolytic factors [8,20,26], was independently activated by curcumin. JNK signaling, a pathway unaltered by TGF $\beta$  (Fig. 1B,C), was also independently activated by curcumin.

Essentially identical curcumin-induced changes in TGF $\beta$  signaling pathways were confirmed in all BCa cell lines forming TGF $\beta$ -dependent osteolytic BMET *in vivo* (Fig. 2). An associated decrease in constitutive Smad2/3 and TGF $\beta$ -induced pSmad2/3 levels was documented in human MDA-1833, human MDA-2287, and murine 4T1 cells (Fig. 2A), such that constitutive Smad2 levels in all four BCa cell lines (including MDA-SA) were reduced by 36.5% ( $p < 0.05$ ,  $n = 3-4$ ) and pSmad2 levels by > 54.5% ( $p < 0.05$ ,  $n = 3-4$ ) after 16 hours of curcumin (30  $\mu$ M) treatment. Early and persistent curcumin-mediated decreases in TGF $\beta$ R2 (Fig. 2B) confirmed using both antibodies, and activation of JNK and p38 MAPK signaling (Fig. 2B), were similarly documented in the entire panel of bone-tropic BCa cell lines forming TGF $\beta$ -dependent BMET *in vivo*. JNK signaling in human MDA-2287 cells was an exception, being constitutively active and unaltered by curcumin treatment.

### 3.2 Possible cross-talk between curcumin-activated MAPKs (p38 and JNK) and Smad-dependent TGF $\beta$ signaling

Experiments were next conducted to determine whether curcumin activation of MAPKs contributed to changes in TGF $\beta$ R2 and/or R-Smads. A known MAPK-mediated means of altering the fate of pSmad2/3 [52–54], phosphorylation of serine residues in the linker

region of Smad2/3 [Smad2/3-L]), was first examined as one possible mechanism. Curcumin stimulated Smad2-L phosphorylation in TGF $\beta$ -naïve cells, an effect evident within 4 hours of treatment (Fig. S4) that persisted for 16 hours (Fig. 3A). At both 4 and 16 hours, this effect was additive with TGF $\beta$ 's previously reported stimulatory effects on Smad linker phosphorylation [53] (Fig. 3A and S4). Curcumin-induced Smad2-L phosphorylation was mediated by JNK activation, as inhibition of JNK, but not p38 activity, reversed this effect (Fig. 3B). However, neither induction of p38 or JNK signaling by curcumin, including JNK-stimulated Smad linker phosphorylation, mediated curcumin reductions in TGF $\beta$ R2, nor decreases in Smad2 or TGF $\beta$ -stimulated pSmad2, as pretreatment with JNK or p38 inhibitors, whose efficacy was otherwise confirmed (data not shown), did not prevent these curcumin inhibitory effects (Fig. 3B). Additionally, while Smad linker region phosphorylation can alter nuclear trafficking/effects of activated R-Smads [54], JNK-mediated phosphorylation of Smad2-L did not appear responsible for curcumin inhibition of TGF $\beta$ -stimulated PTHrP expression. Inhibition of JNK suppressed constitutive (but not TGF $\beta$ -inducible) PTHrP secretion in MDA-SA cells, but did not prevent curcumin inhibition of TGF $\beta$ -inducible PTHrP (Fig. 3C), a driver of *in vivo* metastatic progression in the MDA-SA BMET model [15]. Additionally, p38 inhibition did not alter curcumin blockade of TGF $\beta$ -inducible PTHrP in MDA-SA cells (Fig 3C).

### 3.3 Effect of curcumin on TGF $\beta$ R2 trafficking

TGF $\beta$ R2 protein was primarily membrane-associated (e.g. plasma membrane or endocytic vesicles), in agreement with prior reports [55], and was reduced by curcumin in MDA-SA cells (Fig. 4A) independent of TGF $\beta$  stimulation in both the membrane and soluble fractions. Soluble localization of Smad2/3 and MAPK proteins confirming successful fractionation (Fig. 4A). TGF $\beta$ R2 within the plasma membrane, critical for initiation of TGF $\beta$  signaling, was also specifically reduced by curcumin (Fig. 4B). Because TGF $\beta$ R2/R1 complexes in lipid rafts vs. clathrin-coated pits are reported to mediate MAPK vs. Smad signaling, respectively [39,55–58], MDA-SA cells were treated with inhibitors of each endocytic process to assess effects on curcumin-induced changes. Nystatin treatment to disrupt lipid rafts [39] did not prevent early (Fig. 4C and S5) or late (Fig. 4D) curcumin reductions in TGF $\beta$ R2. However, nystatin did block curcumin-induced JNK activation (Fig. 4C and S5) and subsequent Smad2-L phosphorylation (Fig. S5), while having no effect on curcumin-induced p38 activation (Fig. 4C and S5). Treatment with an inhibitor of clathrin-coated pit endocytosis (Pitstop) did not alter curcumin effects on TGF $\beta$ R2, TGF $\beta$ -mediated Smad2 phosphorylation, or MAPK signaling (Fig. 4C,D).

TGF $\beta$ -stimulated Smad2/3 phosphorylation was considerably reduced in MDA-SA cells overexpressing TGF $\beta$ R2 with a truncated cytoplasmic region (“TGF $\beta$ R2 cyt”) vs wild type cells) but remained curcumin inhibitable (Fig. 4E). This truncation prevents TGF $\beta$ R2-mediated signal transduction [20,38] but likely does not alter the predominant lipid raft location of plasma membrane TGF $\beta$ R2 receptors [39,59]. Notably, JNK activation by curcumin was also prevented by overexpression of TGF $\beta$ R2 cyt, suggesting a possible role for ligand-independent TGF $\beta$ R2 signaling in mediating curcumin induction of JNK (Fig. 4E). In contrast, activation of p38, which was increased at baseline relative to wild type cells, was no longer induced by curcumin or TGF $\beta$  in TGF $\beta$ R2 cyt-transfected cells

(Fig. 4E). TGF $\beta$ -stimulated secretion of PTHrP in TGF $\beta$ R2 cyt-transfected cells was also completely abrogated and unaffected by addition of curcumin (Fig. 4F).

### 3.4 Effect of curcumin on TGF $\beta$ R2 and Smad2 protein stability and synthesis

TGF $\beta$ R2 protein had a short 2.2h half-life in cycloheximide chase experiments, in agreement with previous estimates [61], and receptor stability was unchanged by curcumin treatment (Fig. 5A, top). Similarly, Smad2 protein levels (14.5h half-life), while trending slightly lower in response to curcumin treatment, were not statistically different from controls (Fig. 5A, bottom). Consistent with a lack of effect on protein degradation, blockade of proteasomal or lysosomal degradation pathways with MG-132 or chloroquine, respectively, did not prevent curcumin-mediated reductions in TGF $\beta$ R2 or Smad2/3 proteins (Fig S6). In experiments examining curcumin effects on protein synthesis following 24h of cycloheximide pre-treatment to deplete proteins, increases in TGF $\beta$ R2 significantly lagged in curcumin-treated (vs. control) cells and remained 2-fold lower at 24h (Fig. 5B, top). Similarly, Smad2 protein levels were significantly lower in curcumin-treated cells (Fig. 5B, bottom), remaining statistically unchanged, while levels in control cells increased significantly within 24h ( $p < 0.05$ ). In parallel with decreased protein synthesis, TGF $\beta$ R2 and Smad2 mRNA levels were significantly lower in curcumin-treated (vs. control) MDA-SA cells (Fig. 5C).

### 3.5 Possible role for oxidative metabolites of curcumin in blocking TGF $\beta$ signaling

After 4 hours of 10  $\mu$ M curcumin treatment, bicyclopentadione (BCP, 0.9  $\mu$ M), a stable end product of multistep curcumin oxidation [32], was detected in MDA-SA cell conditioned media as curcumin levels decreased (Fig. S7). Pre-treatment of MDA-SA cells with N-acetylcysteine (NAC) – a precursor of glutathione that scavenges and neutralizes unstable curcumin-derived oxidative intermediates [32,62] – reversed all changes induced by curcumin at 16 hours, normalizing TGF $\beta$ R2 and Smad2 levels, restoring TGF $\beta$ -induced pSmad levels, and preventing curcumin activation of MAPK signaling (Fig. 6A). All early curcumin effects were also reversed by NAC, with the notable exception of early decreases in TGF $\beta$ R2 (Fig. 6B). To further assess a possible role of oxidative metabolites, curcumin effects were compared to those of diacetyl curcumin (DAC) [34], a curcumin analog (Fig. 6C) that did not undergo oxidative metabolism during 24 hours of incubation with MDA-SA cells (data not shown). In contrast to curcumin-induced JNK and p38 phosphorylation and decreasing TGF $\beta$ R2 levels, non-oxidizable DAC was without effect (Fig. 6B). Similarly, while curcumin inhibited TGF $\beta$ -stimulated PTHrP secretion, DAC was without effect (Fig. 6D).

## 4. Discussion

Effects of TGF $\beta$  in cancer are complex, with possible beneficial effects as a tumor suppressor during early oncogenesis vs. detrimental effects at later stages, including a well-defined role in promoting tumor-associated osteolysis in BCa metastatic progression within bone [1,63]. In metastatic breast cancer, where osteolytic bone metastases occur in most cases, TGF $\beta$  signaling in bone-disseminated cells is a well-documented driver of osteolysis and metastatic progression, independent of effects on tumor cell proliferation



[6–8,14,15,18–21]. Because of recent evidence of *in situ* metabolism of inactive circulating curcumin-glucuronides to form the active aglycone within bone [30,31], as well as inhibitory effects of curcumin on breast cancer cell osteolytic TGF $\beta$  signaling pathways and TGF $\beta$ -dependent osteolysis and BMET progression *in vivo* [26,28,29], the current experiments were undertaken to examine the mechanism(s) by which curcumin inhibits TGFs signaling in breast cancer cells known to form TGF $\beta$  dependent BMET *in vivo*, all of which represent the triple negative (TNBC) BCa subtype, for which targeted therapeutics (e.g. antiestrogens) are not available. Curcumin uniformly decreased TGF $\beta$ -stimulated Smad signaling in TNBC bone-tropic cell lines dependent on this signaling pathway for osteolytic *in vivo* BMET progression [6,7,14,15,18,20,21]. Decreased TGF $\beta$ -stimulated, receptor-mediated (C-terminal) Smad phosphorylation in curcumin-treated BCa cells were not attributable to altered accessory proteins (e.g., SARA or Smad7) or phosphorylation stability (e.g., phosphatases). Instead, curcumin specifically targeted TGF $\beta$ R2 and R-Smads (Fig. 7), two key signaling proteins required for continuous propagation of TGF $\beta$ -induced Smad signaling [39,55,64,65], which persisted for up to 12 hours in bone metastatic cells. Early and persistent curcumin-mediated decreases in total and plasma-membrane associated TGF $\beta$ R2 protein, the latter being the initiation site for TGF $\beta$  binding and signal transduction [55,65], were documented in all tested BCa cell lines. In contrast, TGF $\beta$ R1 levels forming an active complex with TGF $\beta$ -bound TGF $\beta$ R2, were unchanged. Decreased TGF $\beta$ R2 did not appear mediated by curcumin-induced changes in receptor trafficking or degradation, but rather occurred secondary to decreased synthesis of this short lived ( $t_{1/2} = 2\text{h}$ ) receptor (Fig. 7). A targeted decrease in R-Smad protein levels (Smad2 and Smad3) was also documented in all curcumin-treated bone-tropic BCa cell lines, which was most apparent at later times following curcumin treatment likely due to their longer half-life [65], and was also attributable to decreased synthesis (Fig. 7).

While regulated changes in TGF $\beta$ R2 and R-Smad attributable to protein trafficking and degradation are well described following TGF $\beta$  stimulation [39,55], much less is known about regulation of their constitutive expression [66,67]. While future studies are needed to elucidate the specific cellular target(s) of curcumin responsible for reducing constitutive levels of TGF $\beta$ R2 and R-Smad, results here clearly demonstrated that inhibitory effects of curcumin on the synthesis of these proteins were: 1) specific, targeting only TGF $\beta$ R2 and R-Smads; 2) uniform across all BCa cell lines forming Smad-mediated TGF $\beta$ -dependent BMETs; and 3) due, at least in part, to curcumin inhibition of TGF $\beta$ R2 and Smad2 gene expression.

A role for reactive curcumin oxidative metabolites as mediators of curcumin effects on TGFs signaling pathways in bone tropic BCa cells was elucidated using MDA-SA cells, whose TGF $\beta$ -dependent signaling pathways driving *in vivo* BMET progression are particularly well described and emblematic of the other BCa BMET models [6,7,9,14,15,18,20,21]. NAC has previously been used to scavenge and block the effects of reactive curcumin oxidative intermediates on protein function in other curcumin-treated cell types [32,62]. Thus, this evidence that NAC prevents reductions in TGF $\beta$ R2 and pSmad/Smad levels in response to curcumin treatment in MDA-SA cells (with the exception of early decreases in TGF $\beta$ R2) suggests that reactive intermediates of curcumin oxidative degradation may play an important role in blocking the Smad signaling driving MDA-SA BMET progression *in*

*in vivo*. Alternatively, it is possible that NAC is simply achieving this effect by decreasing oxidative stress. In the absence of a specific target(s) of reactive curcumin metabolites mediating effects on TGF $\beta$  signaling, it is difficult to elucidate which NAC mechanism is prevailing. However, the detection of BCP, a final stable end-product of curcumin oxidation [32] and biomarker for reactive “upstream” intermediates (e.g., quinone methide and spiroepoxide) [34], in curcumin-treated BCa cell conditioned media and the lack of activity of a non-oxidizable curcumin analog, provide additional support for a possible role of reactive curcumin metabolites in blocking Smad-signaling that drives BMET progression in these, and potentially other, TGF $\beta$ -dependent bone metastatic BCa cells. Since reactive curcumin intermediates are capable of forming stable adducts with proteins that alter protein function, as has been reported for DNA topoisomerase and IKK $\beta$  [33,34], future studies will be required to query the possible role of specific protein/curcumin adduct(s) in mediating curcumin reductions in TGF $\beta$ R2 and Smad protein gene expression and synthesis (Fig. 7).

One additional and unanticipated effect of curcumin was its early and persistent induction of MAPK signaling in all BCa cell lines studied (Fig. 7), also attributable to oxidative intermediates. While crosstalk between Smad and MAPK signaling pathways is well documented [52,54,58], in MDA-SA cells neither JNK nor p38 activation by curcumin was responsible for curcumin’s inhibitory effects on TGF $\beta$ R2 or Smad signaling, or for curcumin inhibition of TGF $\beta$ -inducible PTHrP, the primary TGF $\beta$ -regulated gene responsibility for BMET progression in the MDA-SA *in vivo* model [15]. This, of course, does not rule out a possible role for curcumin activation of MAPK signaling in altering the expression of other TGF $\beta$ -regulated genes in this or other bone tropic cell lines. In this regard, it is of interest that curcumin induction of JNK signaling led to phosphorylation of the linker region of the R-Smads, a modification that can alter the nuclear trafficking of receptor-activated Smad complexes and thus, Smad-mediated gene expression [52,54]. Evidence that curcumin activation of JNK signaling was dependent on intact lipid rafts and abrogated by overexpression of TGF $\beta$ R2 lacking the cytoplasmic domain was also intriguing. Prior studies suggest that loss of the TGF $\beta$ R2 cytoplasmic domain does not alter the receptor’s preferential location in lipid rafts [39,59], a compartment mediating TGF $\beta$ R2/R1 MAPK signaling (contrasting with clathrin-coated pit TGF $\beta$ R2/R1 complexes mediating Smad signaling) [57,58], but does abrogate TGF $\beta$ R2/R1 signal transduction (ligand-dependent or -independent) [20,38,68]. Therefore, the lipid raft/TGF $\beta$ R2 cytoplasmic-domain dependence of JNK activation by curcumin in the absence of TGF $\beta$  suggests the possibility that ligand-independent activation of TGF $\beta$ R2/TGF $\beta$ R1 complexes by curcumin, or more specifically oxidized intermediates of curcumin degradation, within lipid rafts could be mediating JNK activation in bone-tropic BCa cells (Fig. 7). Consistent with this postulate, structural changes to plasma membrane lipid rafts or proteins within these domains in response to curcumin treatment have been reported in other cell types [69–71].

While *in vivo* bioactivity of curcumin, including bone protective effects, has been reported in humans and in animal models [26,35,72,73], the relationship between *in vivo* curcumin metabolism and bioactivity appears complex. Following curcumin ingestion, the major circulating metabolite, curcumin-glucuronide is inactive, but can be enzymatically deconjugated within bone to form bioactive aglycone curcumin [30,31]. We have posited

that this enzymatic enrichment of the active aglycone within bone could promote bone bioactivity, including TGF $\beta$  inhibitory effects of curcumin in BCa cells disseminated to this site, which also is notably enriched for the ligand driving tumor-induced osteolysis (TGF $\beta$ ) due to its release from resorbed bone matrix [30]. These current studies suggest that curcumin formed *in situ* in bone must also undergo subsequent, irreversible metabolism to form short-lived reactive oxidative intermediates to block Smad-mediated TGF $\beta$  signaling in tumor cells metastatic to this site, further increasing the bone-specificity of curcumin's anti-TGF $\beta$  effects. To date, *in vivo* evidence of curcumin oxidation is lacking, potentially due to the insensitivity of current methods, which rely on detection of an end product (BCP) that is only formed if upstream intermediates do not covalently attach to biomolecules (e.g., protein) [32–34]. However, given the potential clinical utility of a bone-specific TGF $\beta$  inhibitor in BCa [9,10] and curcumin's uniform inhibitory effects on TGF $\beta$ -induced Smad signaling in all TGF $\beta$ -dependent bone-metastatic BCa cell lines studied, the results reported here suggest potential promise for curcumin or oxidizable curcumin analogs in specifically blocking BMET progression in BCa. The necessity of oxidative metabolism in the bioactivity of curcumin has been previously reported for other curcumin effects [34], including inhibition of NF- $\kappa$ B signaling [74] or poisoning of topoisomerase [33]. However, to our knowledge this is the first report of oxidative metabolites of curcumin being required for the inhibition of TGF $\beta$  signaling.

Given the ready availability of curcumin for over the counter use [75], it is important to note that osteolytic effects of tumoral TGF $\beta$  signaling have primarily been documented in pre-clinical “triple-negative” BCa (TNBC) BMET models lacking expression of estrogen receptor (ER-), progesterone receptor (PR-), and HER2, including the cell lines studied here [1,2,6–10,14–21,26,28,76]. However, most breast cancer bone metastases are associated with tumors expressing ER, PR, or HER2 [77], which provides a clinical caveat for the applicability of the current findings. As research examining TGF $\beta$  signaling in non-TNBC osteolytic BMET progression progresses [78,79], additional research will be warranted to determine the utility of TGF $\beta$  inhibition and, thus, use of curcumin or similar oxidizable analogs, for the management of BMET risk across a range of clinical BCa subtypes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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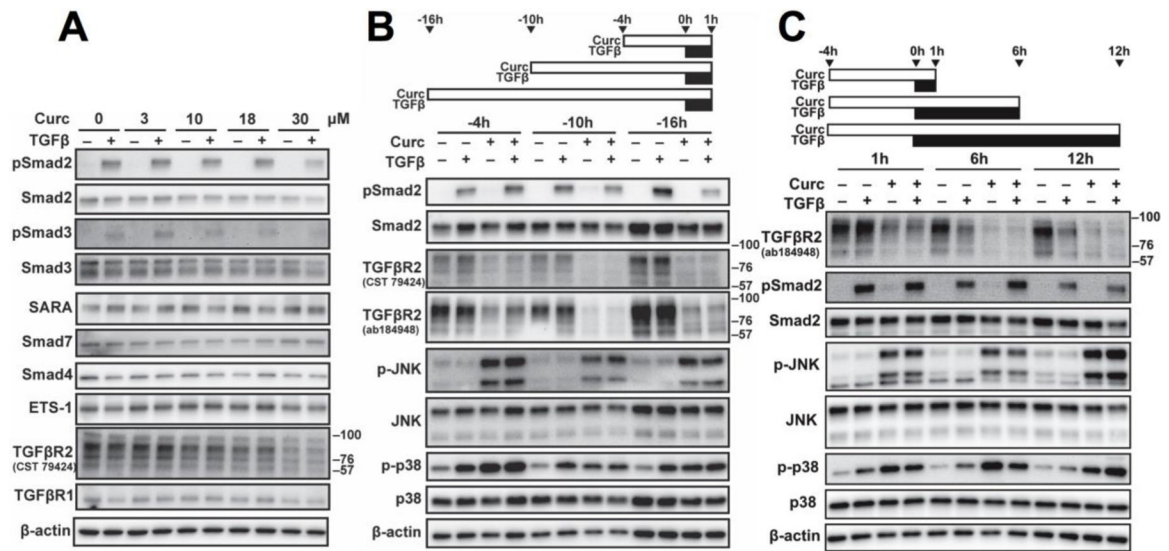
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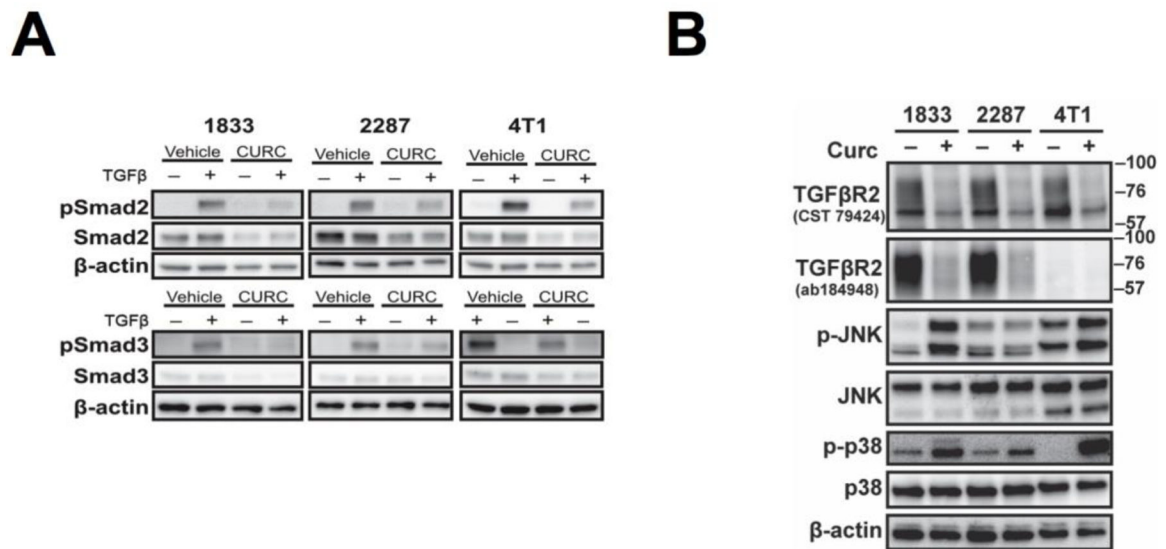
**Highlights**

- Curcumin blocks breast cancer TGF $\beta$ -mediated Smad signaling driving osteolysis in bone
- Curcumin mediates this effect by downregulating TGF $\beta$ R2 and Smad2/3 gene expression
- Oxidative curcumin metabolites appear responsible for the majority of these effects.
- Curcumin also activated JNK signaling in a lipid-raft dependent manner



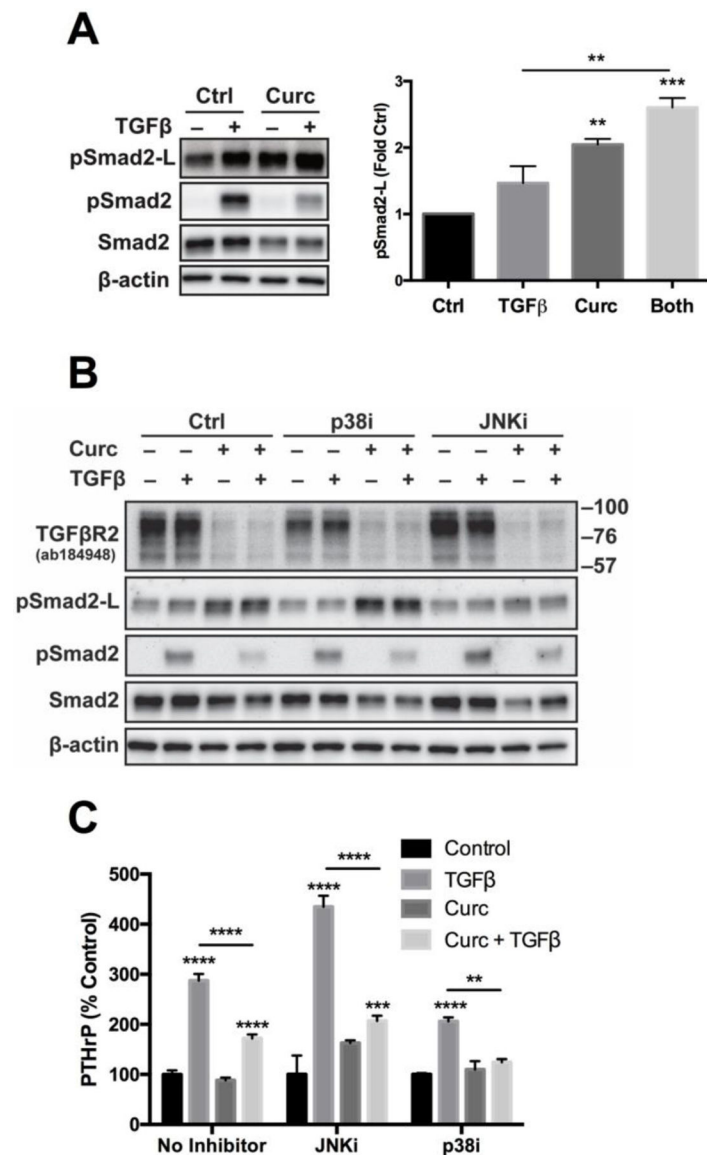
**Figure 1. Curcumin effects on Smad and MAPK signaling proteins in control and TGFβ-stimulated MDA-SA cells.**

**A)** Concentration-dependent effects of curcumin (16h-pretreatment followed by 1h of concurrent TGFβ). **B)** Time-dependent effects of curcumin (30 μM) pre-treatment (indicated by the white bar), followed by 1h of concurrent TGFβ (black bar). **C)** Time dependent effects of TGFβ following 4h of curcumin (30 μM) pre-treatment. Results are representative of two or more experiments.

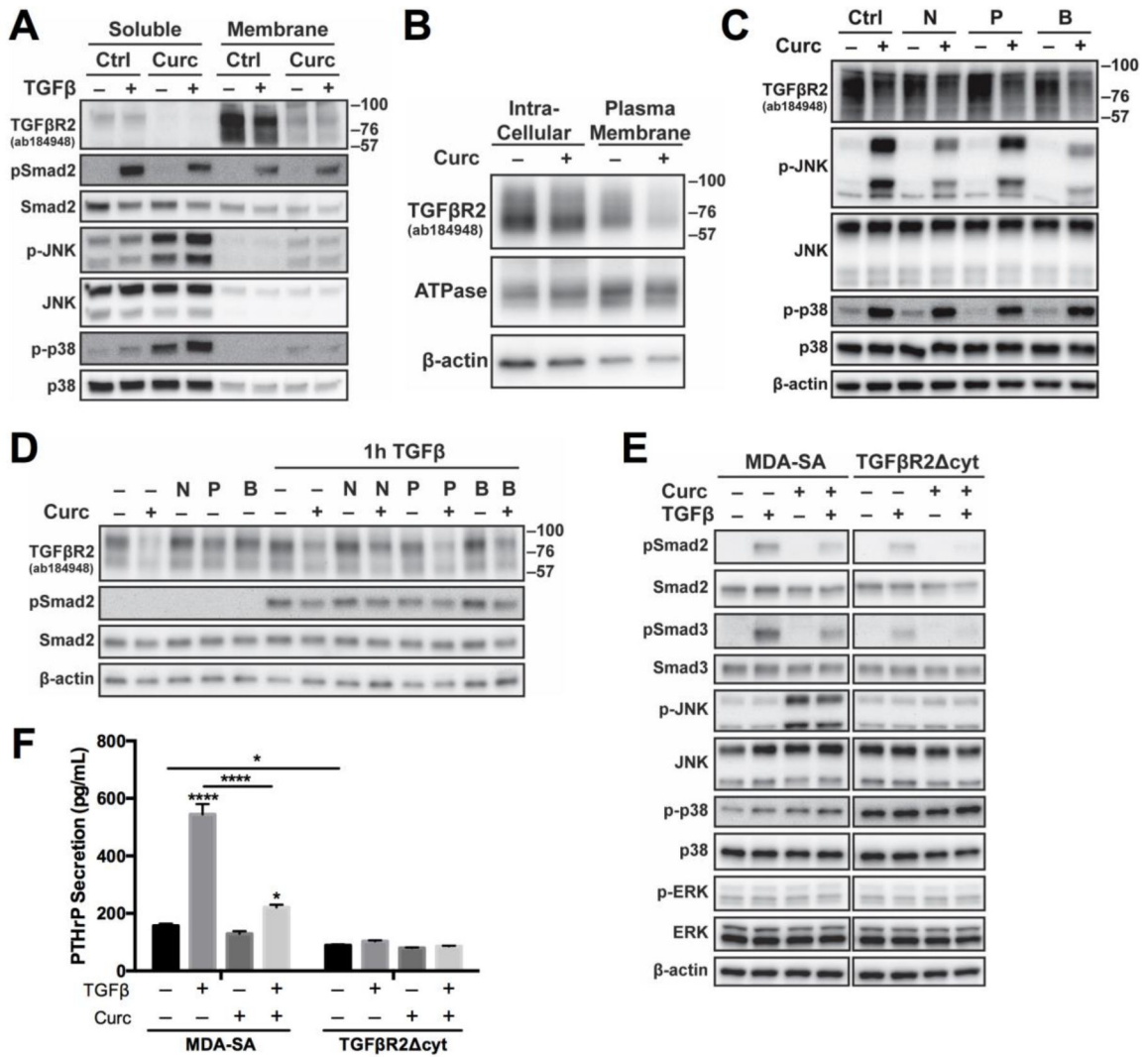


**Figure 2. Curcumin effects on TGFβ signaling proteins in other breast cancer cell lines forming TGFβ-dependent bone metastases**

**A)** Smad signaling in human MDA-1833, MDA-2287, and murine 4T1 cells pre-treated with curcumin (30 μM) for 16h followed by 1h of concurrent TGFβ. **B)** TGFβ receptor and MAPK signaling following curcumin (30 μM) treatment for 16h. Note: As in MDA-SA cells, ERK signaling was constitutively active and unaltered by curcumin and/or TGFβ in these cell lines (data not shown).



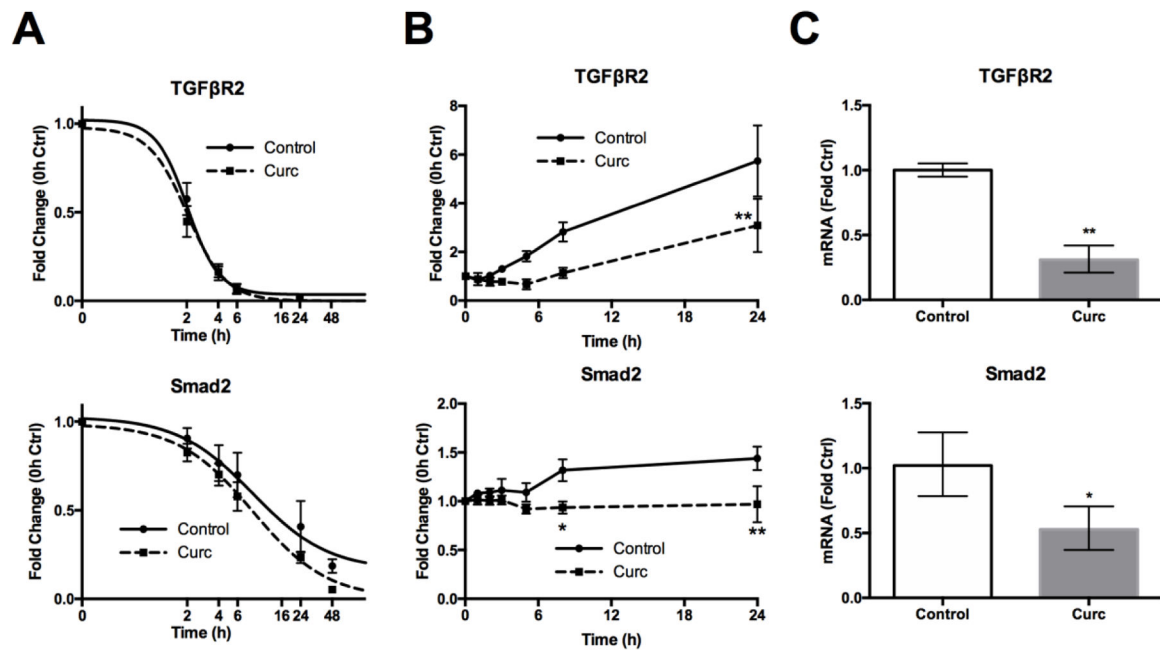
**Figure 3. Effect of MAPK inhibitors on Smad2 phosphorylation (linker or C-terminus), TGFβR2, and PTHrP secretion in MDA-SA cells treated with curcumin and/or TGFβ.**  
**A)** Effect of curcumin (30 μM, 16h pre-treatment) and/or TGFβ (1h) on both Smad2 linker-region phosphorylation (pSmad2-L) and receptor-mediated C-terminal phosphorylation (pSmad2) (densitometric quantitation [n=3] of pSmad2-L, right panel). **B)** Effect of MAPK inhibitors (3 h pre-treatment prior to curcumin) of p38 (SB202190, 10 μM) or JNK (SP600125, 25 μM) on Smad2 linker or C-terminal phosphorylation or TGFβR2 protein in curcumin and/or TGFβ-treated cells (16h curcumin followed by 1h TGFβ). Representative of two experiments. **C)** Effect of JNK or p38 inhibitor (1h pre-treatment, 25 μM SP600125 or 10 μM SB202190, respectively) on PTHrP secretion in cells treated with curcumin and/or TGFβ (30 μM curcumin, 4 hr prior to addition TGFβ; n=3–8/group). Because inhibitors decreased constitutive PTHrP levels, data are expressed as % change relative to constitutive (control) levels. \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001 vs control or as specified.



**Figure 4. Effects of curcumin on TGFβR2 localization and signaling in cells expressing wildtype TGFβR2 (MDA-SA) vs. a truncated TGFβR2 lacking the cytoplasmic domain (MDA-SA-TGFβR2 cyt).**

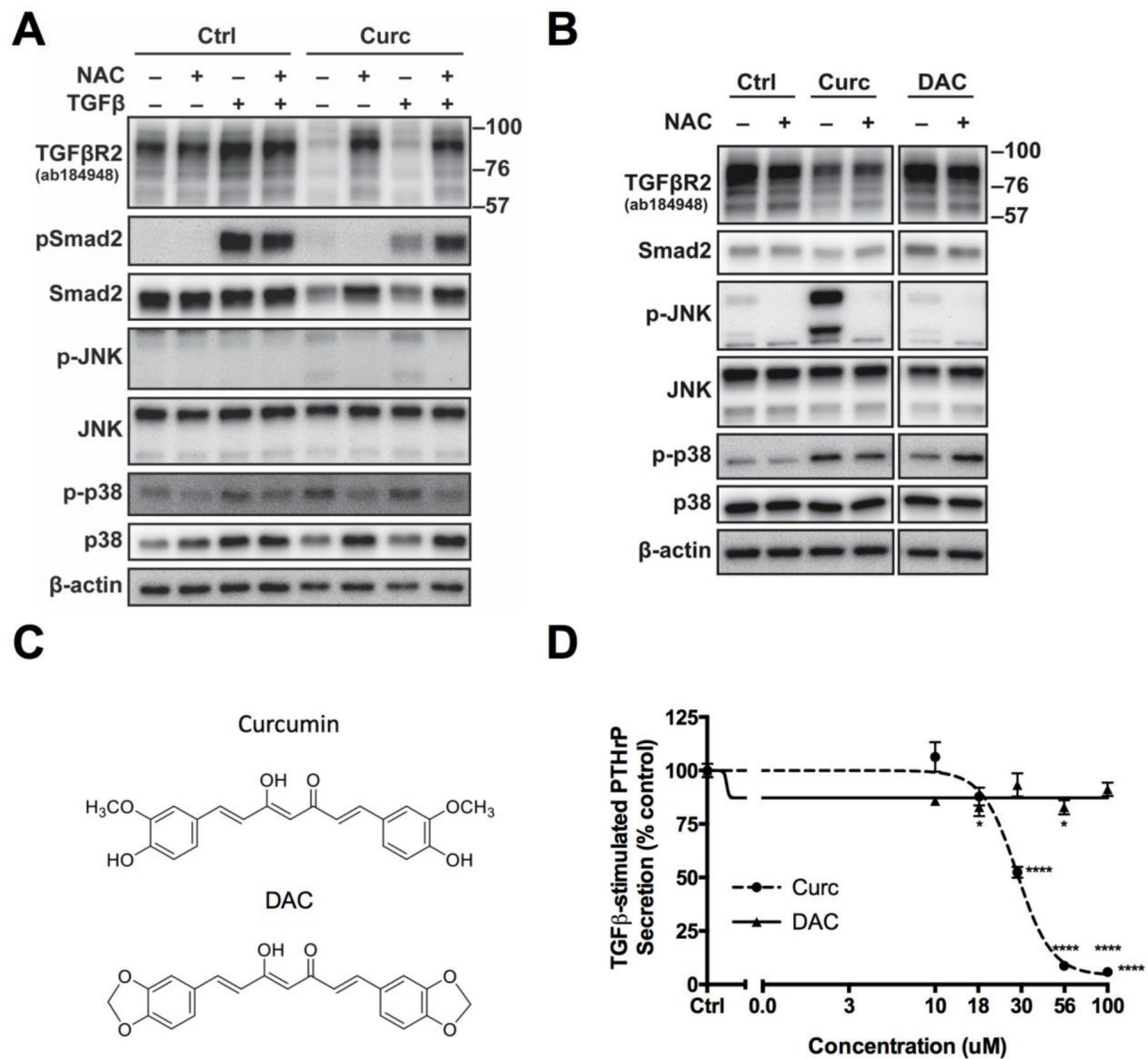
**A)** Membrane-associated (vs soluble) TGFβR2, Smad2, and MAPK proteins in MDA-SA cells treated with curcumin (30 μM, 4h pre-treatment) and/or TGFβ (1h). **B)** TGFβR2 in sodium/potassium ATPase-enriched plasma membrane (vs. intracellular) actin-enriched fractions from curcumin-treated (30 μM, 4h) MDA-SA cells. **C)** Early (4h) or **D)** late (16h) effects of curcumin (30 uM) on TGFβR2, MAPK and/or Smad signaling in cells pre-treated for 1h with a lipid raft disrupter (nystatin, 50 μg/mL [39]) and/or inhibitor of clathrin-dependent endocytosis (Pitstop 2, 20 μM [60]). Note: A single blot is shown in D with intervening unrelated data omitted. **E)** Effect of absent TGFβR2 cytoplasmic domain on Smad and MAPK signaling proteins or **F)** PTHrP secretion (n=4/group) in MDA-SA vs. MDA-TGFβR2 cyt cells pre-treated with curcumin (30 μM) prior to TGFβ stimulation. \* p < 0.05, \*\*\*\* p < 0.0001 vs control or as specified. N = nystatin, P = Pitstop 2, B = nystatin and Pitstop 2

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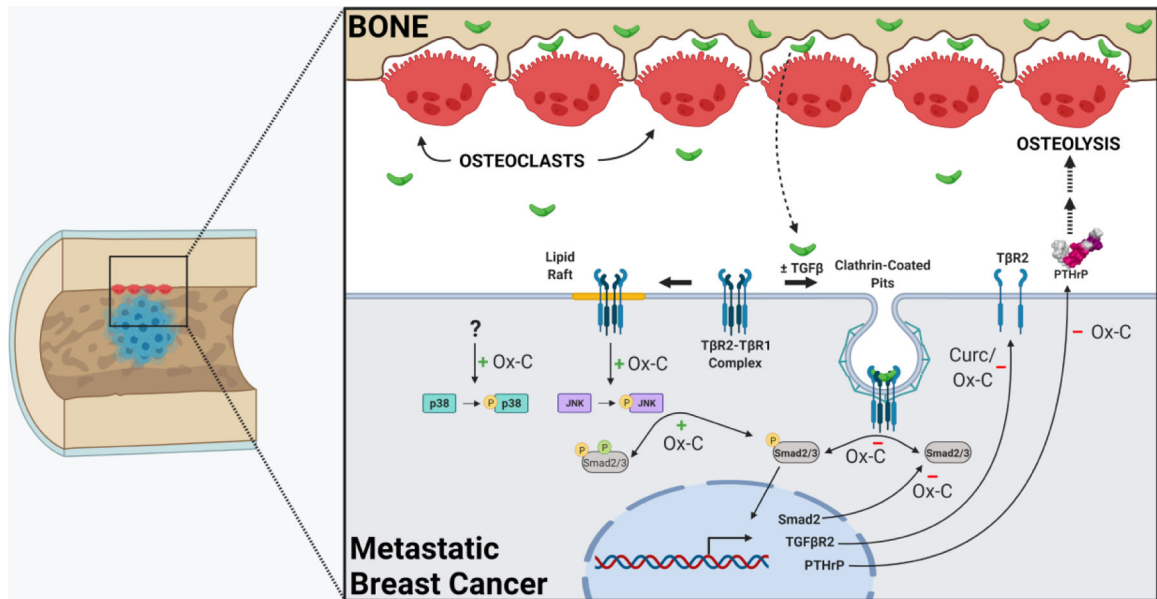
**Figure 5. Effect of curcumin on TGFβR2 and Smad2 stability, synthesis and expression.**

A) TGFβR2 and Smad protein stability (Western blot densitometry,  $n = 3/\text{time}$ ) in control vs curcumin-treated ( $30 \mu\text{M}$ ) MDA-SA cells concurrently treated with cycloheximide (CHX) to block protein synthesis. B) TGFβR2 and Smad protein synthesis (Western blot densitometry,  $n = 4/\text{time}$ ) in control vs curcumin-treated ( $30 \mu\text{M}$ ) MDA-SA cells pre-treated with cycloheximide for 24 h. C) TGFβR2 and Smad2 mRNA levels in curcumin-treated ( $30 \mu\text{M}$ , 16h) vs. control MDA-SA cells, reported as mean  $\pm$  CI \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. control.



**Figure 6. Possible role of oxidative metabolites in mediating curcumin effects.**

**A)** Effect of N-acetylcysteine (10 mM NAC, 1 h pretreatment) on late (16 h curcumin [30  $\mu$ M] prior to 1h TGF $\beta$  stimulation) curcumin-induced changes in TGF $\beta$ R2, Smad and MAPK signaling in control vs. TGF $\beta$ -stimulated cells **B)** Early (4h) effects of curcumin vs. diacetyl curcumin (DAC) (30  $\mu$ M) on TGF $\beta$ R2, Smad2, or MAPK signaling proteins in control vs NAC-pretreated MDA-SA cells. Note: This is a single blot with unrelated intervening data omitted. **C)** Structure of curcumin vs. DAC, a non-oxidizable analog of curcumin. **D)** Effect of curcumin vs. DAC (30  $\mu$ M, 4h pre-treatment) on TGF $\beta$ -stimulated PTHrP secretion in MDA-SA cells. \*  $p < 0.05$ , \*\*\*\*  $p < 0.0001$  vs. control.



**Figure 7. Overview of curcumin effects on TGF $\beta$  signaling pathways in bone-disseminated human breast cancer cells that drive tumor-associated osteolysis and promote BMET expansion.** Curcumin inhibited TGF $\beta$ -stimulated receptor-mediated Smad activation (e.g. phosphorylation, denoted by yellow P), which requires internalization of TGF $\beta$ -bound TGF $\beta$ R2/R1 complexes via clathrin-coated pits, directly (via decreased-constitutive Smad proteins) and indirectly (via decreased TGF $\beta$ R2 proteins). Curcumin-mediated reductions in TGF $\beta$ R2 and Smad2 proteins were likely attributable to decreased gene expression and were dependent on curcumin oxidation, with the exception of early reductions in TGF $\beta$ R2. Curcumin inhibition of TGF $\beta$ -inducible Smad signaling had the ultimate effect of blocking TGF $\beta$ -stimulated secretion of osteolytic PTHrP, without which osteolysis and BMET progression do not occur *in vivo*. Independent of curcumin effects on Smad-mediated TGF $\beta$ -inducible PTHrP, oxidative intermediates of curcumin induced (green positive sign) JNK activation (see yellow P), and subsequent Smad linker region phosphorylation (green P), in a manner possibly dependent on lipid raft localization of TGF $\beta$ R2 with functional cytoplasmic domains, albeit independent of ligand binding. Curcumin also induced phosphorylation of p38 through an unknown mechanism also dependent on curcumin oxidation. Created with [BioRender.com](https://www.biorender.com).