TRE17/Ubiquitin-specific Protease 6 (USP6) Oncogene Translocated in Aneurysmal Bone Cyst Blocks Osteoblastic Maturation via an Autocrine Mechanism Involving Bone Morphogenetic Protein Dysregulation

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Aneurysmal bone cyst (ABC) is a pediatric osseous tumor characterized by extensive destruction of the surrounding bone. The molecular mechanisms underlying its pathogenesis are completely unknown. Recent work showed that translocation of the TRE17/USP6 locus occurs in over 60% of ABC cases resulting in TRE17 overexpression. Immature osteoblasts are presumed to be the cell type harboring translocation of TRE17 in at least a subset of ABCs. However, the effects of TRE17 overexpression on transformation and osteoblast function are unknown. TRE17 encodes a ubiquitin-specific protease (USP) and a TBC (TRE2-Bub2-Cdc16) domain that promotes activation of the Arf6 GTPase. Here we report that TRE17 potently inhibits the maturation of MC3T3 pre-osteoblasts in a USP-dependent and Arf6-independent manner. Notably, we find that TRE17 function is mediated through an autocrine mechanism. Transcriptome analysis of TRE17-expressing cells reveals dysregulation of several pathways with established roles in osteoblast maturation. In particular, signaling through the bone morphogenetic protein (BMP) pathway, a key regulator of osteogenesis, is profoundly altered. TRE17 simultaneously inhibits the expression of BMP-4 while augmenting the BMP antagonist, Gremlin-1. Osteoblastic maturation is restored in TRE17-expressing cells by the addition of exogenous BMP-4, thus establishing a functional role for BMP-4 during TRE17-induced transformation. Because bone homeostasis involves a precise balance between the activities of osteoblasts and osteoclasts, our studies raise the possibility that attenuated osteoblast maturation caused by TRE17 overexpression may contribute to the bone loss/destruction observed in ABC.
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TBC (TRE2-Bub2-Dcl6) domain. TBC domains generally encode GTPase-activating proteins (GAPs) for Rab family GTPases (7). However, we and others have shown that the TBC domain of TRE17 lacks catalytic activity and that it functions instead to mediate interaction with the small GTPase, Arf6 (8–10). We further showed that TRE17 promotes activation of Arf6 in vivo (10). The TRE17(long) isoform uniquely encodes a ubiquitin-specific protease (USP) at its C terminus (11, 12). TRE17(short) is truncated, lacking a significant portion of the USP domain, and is thus catalytically inactive (11, 12).

As mentioned, little is known about the mechanism of TRE17 action in the etiology of ABC. In particular, the effects of TRE17 on cell transformation are poorly characterized. In this context, it is important to highlight that ABCs are complex lesions consisting of multiple blood-filled cysts, separated by fibrous stromal areas containing spindle cells, inflammatory cells, and numerous capillaries. Notably, TRE17 translocation occurs exclusively in spindle cells within the fibrous stroma. Although the cell of origin has not been definitively identified, a clue about their lineage is provided by the fusion partners of TRE17, whose promoters drive TRE17 expression. All of the partners of TRE17 are highly active in mesenchymal lineages such as fibroblasts and osteoblasts (the cell lineage responsible for bone formation) (3–5). Indeed, one fusion partner, osteomodulin (OMD), is expressed exclusively in osteoblasts. Furthermore, osteoid (the organic component of bone matrix produced by osteoblasts) is commonly present in these lesions. Together, these observations suggest that the cells affected by TRE17 rearrangement in at least a subset of ABCs are immature osteoblasts. A recent study demonstrated that multiple molecular and histological features of ABC are indeed recapitulated when immature osteoblasts expressing TRE17 are xenografted into nude mice (13), validating this as a promising model system for dissecting its mechanism of action in ABC pathogenesis. This prompted us to further examine the effects of TRE17 on osteoblast transformation and function. In the current study, we report that TRE17 elicits key hallmarks of the transformed state, including altered cell morphology and a block in differentiation. We further identify dysregulation of autocrine bone morphogenetic protein (BMP) signaling as an important mechanism by which TRE17 arrests osteoblastic maturation.

EXPERIMENTAL PROCEDURES

Cell Culture—MC3T3-E1 (Subclone 14) murine pre-osteoblasts (hereafter referred to as MC3T3) were obtained from the ATCC. Stable cell lines expressing TRE17 constructs were grown in ascorbic acid-free α-minimal essential medium supplemented with 10% tetracycline-free fetal bovine serum (FBS), penicillin and streptomycin GlutaMax (Invitrogen), and 400 μg/ml G418. Differentiation was induced by the addition of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate to the growth medium. Doxycycline (dox) was added at 2 μg/ml to induce expression of TRE17. HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, penicillin and streptomycin, and GlutaMax. Cultures were maintained at 37 °C in 5% CO₂.

Stable MC3T3-derived pooled cell lines expressing TRE17 alleles in a dox-inducible manner were generated as recently described (13). As a negative control, pBII-L encoding luciferase alone was ligated to pN1βactin-rTAT2S-M2-IRE5-GFP to generate pSMV (super module vector). pSMV- and TRE17-expressing constructs were transfected into MC3T3 with Lipofectamine 2000 (Invitrogen). Cells were selected in α-minimal essential medium containing tetracycline-free FBS and G418 (400 μg/ml). GFP-positive cells were isolated from the G418-resistant pool by flow cytometry.

Plasmids, Antibodies, and Reagents—Arf6 constructs were provided by Dr. Julie Donaldson (National Institutes of Health). TRE17 constructs subcloned into HA-pcDNA3 or pEBG have been previously described (10, 12, 14). The TRE17 point mutant deficient in Arf6 binding (TRE17/A6–) was generated using QuikChange site-directed mutagenesis (Stratagene) and contains the substitutions Leu-289 → Phe, Asp-329 → Ala and Trp-331 → Ser.

Anti-HA antibody was purchased from Santa Cruz Biotechnology or Roche Diagnostics. Anti-BMP-4 antibody (3H2.3) was from Santa Cruz Biotechnologies. Recombinant BMP-4 was from R&D Systems. Luciferase assays were performed using the Dual-Luciferase reporter assay kit (Promega).

TRE17 Binding and Arf6 Activation Assays—Assays to monitor TRE17 binding to Arf6 or calmodulin or Arf6 activation were performed as described previously (10, 12).

Differentiation of MC3T3 Cells and Assays to Monitor Osteoblast Maturation—MC3T3-derived cell lines were seeded at 2 × 10⁵ cells/35-mm well. The following day, doxycycline and ascorbic acid/β-glycerophosphate were added to induce TRE17 expression and osteoblast differentiation, respectively. Cells were grown for an additional 5–7 days, with medium and supplements being replaced every 2 days. For the conditioned medium (CM) transfer experiments, donor samples were grown in differentiation medium in the absence or presence of dox as indicated; the CM was collected and transferred to naive control MC3T3 cells, replacing it with fresh CM every other day.

Alkaline phosphatase (ALP) activity was monitored either by using a spectrophotometric assay or by in situ staining of cells. For the former, cells were solubilized in 100 μl of 0.01% SDS/phosphate-buffered saline (PBS) supplemented with protease inhibitors on ice for 10 min and then pelleted at 16,000 × g for 10 min at 4 °C; 40 μl of the supernatant was added to 300 μl of p-nitrophenyl phosphate liquid substrate (Sigma) and incubated for 1 h at 37 °C before the addition of 600 μl of 0.5 N NaOH to halt the reaction. The optical density at 405 nm was measured. To measure ALP activity in situ, cells were washed twice in PBS and fixed in 100% methanol for 10 min. BCIP/NBT liquid substrate (Sigma) was added to the cells and incubated at room temperature. Samples were washed and stored in distilled water.

RT-PCR—RNA was extracted with TRIzol (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed using the Easy-A one tube RT-PCR system (Stratagene) per the manufacturer’s protocol. Alternatively, for RT-qPCR, reverse transcription reactions were performed on 1–2 μg of total RNA using a SuperScript first-strand synthesis system kit (Invitrogen). Real time PCR was performed using TaqMan probes on an ABI Prism 7900 system. Relative expression levels were determined.
From a standard curve of serial dilutions of cDNA samples and normalized to GAPDH levels. Amplification reactions were performed using the following primers: ALP, forward, 5'-GGCC-TCTCCAAGACATATA-3'; reverse, 5'-CCATGACCTGCAG-GATAATCC-3'; bone sialoprotein (BSP), forward, 5'-ACGGCCA-CGCTACTTTCTTAT-3'; reverse, 5'-TCCAGCGCGGCTTT-CCTTACATT-3'; Col1A1, forward, 5'-GACGCCATCAAGG-GACGCCATCAAGG-3'; reverse, 5'-GACGGACACATTGGGGGTAG-3'; CTGATGACCGGTAG-3'; Grem1, forward, 5'-GCAAACGCGCCTATATC-3'; reverse, 5'-CCCAGTCGAGTGATATG-3'. Additional primer sequences are available upon request.

**Transplantation Assays**—To assess mitogen-independent growth, MC3T3 cells were seeded at 1 × 10^5/35-mm well in α-minimal essential medium containing 10% tetracycline-free FBS, and dox was added to induce TRE17 expression. The following day, cells were switched to medium containing 0.5% tetracycline-free FBS and dox. Cells were trypsinized and counted at the indicated time points. To document alterations in cell morphology, MC3T3 cells treated with doxycycline and then photographed using a Nikon Eclipse TE2000 inverted microscope using a 40× objective. For collagen invasion assays, Transwell filters (8.0-μm pore size, Corning) were coated with acid-denatured collagen. For migration assays, filters were left uncoated. In both cases, growth medium containing 10% FBS was placed in the distal chamber. Cells were placed in the upper chamber and allowed to migrate for 24 h, seeding triplicate wells for each sample. Total cells and cells migrated to the distal side of the filter were determined by fixing in 3.7% formaldehyde/PBS and staining with crystal violet. The stain was extracted from the filter with 1% deoxycholic acid, and migration or invasion was determined by measuring the optical density at 590 nm.

**Microarray Analysis**—MC3T3 cell lines were differentiated for 7 days. Total RNA was isolated using TRIzol (Invitrogen) and then subjected to an additional round of purification using the RNaseasy mini kit (Qiagen). Gene expression profiles were analyzed using the Affymetrix Mouse 1.0 ST microarray platform at the University of Pennsylvania Microarray Core facility. This array contains 28,853 genes, each of which is represented by ~27 probes spread across the full length of the gene, providing a comprehensive reflection of gene expression. The DAVID (Database for Annotation, Visualization and Integrated Discovery) bioinformatics database and the Ingenuity Pathway Analysis tool were used to classify the gene list into functionally related gene groups.

**Detection of BMP-4 in Conditioned Medium**—Cells were grown in differentiation medium for 5 days. The conditioned medium was subjected to low speed centrifugation to remove cell debris and then precipitated with 10% trichloroacetic acid on ice for 20 min. Pellets were washed twice in cold acetone. Samples were immunoblotted with anti-BMP-4 antibody (3H2.3) from Santa Cruz Biotechnology.

**RESULTS**

**TRE17 Mutants That Inactivate Its USP and TBC Domains**—To understand how TRE17 functions in the etiology of ABC, we initiated analysis of its oncogenic properties in immature osteoblasts, the cell type believed to harbor translocation and overexpression of TRE17 in this tumor. As part of these studies, we sought to dissect the roles of its USP and TBC domains in transformation. To assess the role of the USP domain, we used a mutant harboring a substitution in the key catalytic residue (Cys-541 → Ser; termed TRE17(long)/USP−), which we previously demonstrated to be inactive (12) (Fig. 1A). To assess the role of the TBC domain, more extensive mutagenesis was required because deletion of the entire domain altered the global conformation of the protein.3 Because we previously showed that this domain functions to mediate binding to the Arf6 GTPase, we sought to identify point mutations that abol-

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ish this interaction. A number of sites within the TBC domain were mutated, either rationally (based on the published high resolution structures of other TBC domains) or randomly. Through this analysis, we identified a triple point mutant involving residues Leu-289, Asp-329, and Trp-331 that significantly reduced binding to Arf6 (Fig. 1A). Binding studies were performed using HeLa cells because their high transfection efficiency allowed robust expression. As we previously reported (10), an N-terminal fragment of TRE17 comprising the TBC domain was able to co-precipitate with a GTP-locked Arf6 mutant (Arf6TN), but not a GTP-locked mutant (Arf6QL) (Fig. 1B). In contrast, binding of Arf6TN to the corresponding N-terminal fragment of TRE17 containing the three point mutations in the TBC domain (denoted TRE17(A6−)) was significantly reduced (Fig. 1B). The TRE17(A6) mutant tended to be expressed at somewhat lower levels than wild type (WT) TRE17 in these transient transfections. Nevertheless, longer exposures always revealed a true qualitative difference in Arf6 binding (not shown). To ensure that loss of binding was not due to gross misfolding of the mutant protein, we examined whether TRE17(A6−) could associate with calmodulin, which we previously identified as a direct binding partner of TRE17 (12). As seen in Fig. 1C, calmodulin (CaM) binding was not significantly or reproducibly altered in the TRE17(A6−) mutant, confirming that loss of Arf6 binding was specific and not due to global conformational perturbations.

We next sought to determine whether the TRE17(A6−) mutant was also defective in inducing activation of Arf6 in vivo. Extracts were prepared from HeLa cells expressing either WT TRE17 or TRE17(A6−), and active Arf6 was affinity-purified using GST-GGA3, which binds to Arf6 in a GTP-dependent manner (10, 15). Consistent with our previous work, WT TRE17 activated Arf6 when compared with cells transfected with control vector (Fig. 1D). However, TRE17(A6−) was defective in activation of Arf6. Thus, TRE17(A6−) is defective in both binding to Arf6 and inducing its activation in vivo and stands as a crucial reagent in dissecting the requirement for Arf6 and the TBC domain in TRE17-mediated responses.

Generation of Pre-osteoblastic Cell Lines Reveals USP-dependent Morphological Transformation by TRE17—Transformation involves multiple genomic alterations that interfere with normal cellular differentiation, proliferation, and/or survival. To determine whether any of these processes were dysregulated by TRE17, we generated stable cell lines expressing WT TRE17 or the mutant alleles above, using MC3T3-E1 cells (referred to hereafter as MC3T3). MC3T3 are an extensively characterized murine pre-osteoblastic cell line that can be induced to differentiate in response to physiological stimuli, such as ascorbic acid and BMP family ligands (16–19). Pooled MC3T3 cell lines stably expressing HA-tagged TRE17 alleles were treated with dox for 24 h; TRE17 peptides were detected using anti-TRE17 (left) or anti-HA (right); extracts were blotted for p70S6k (left) or actin (right) as loading controls. Arrowheads mark migration of TRE17 peptides; asterisks denote nonspecific bands detected by the HA antibody. vect, vector; USP−, TRE17(long)/USP−; A6−, Arf6 binding-deficient mutant. B, MC3T3 cell lines expressing HA-tagged TRE17(long) or TRE17(A6−) were treated with dox to induce TRE17 expression. Lysates were subjected to anti-HA immunoprecipitation followed by anti-Arf6 immunoblotting (top panel). Alternatively, extracts were subjected to GST-GGA3 pulldowns to monitor levels of active Arf6 (second panel). Asterisk denotes a nonspecific band that derives from the GST-GGA3 affinity reagent. Whole cell lysates were immunoblotted for total levels of Arf6 and TRE17. C, MC3T3 cells stably expressing the indicated TRE17 mutants were treated with dox for 24 h and then photographed using a Nikon Eclipse TE2000 inverted microscope; 40× magnification.

TRE17(A6−) mutant was defective in both binding and activation of endogenous Arf6 in the MC3T3 stable cell line (Fig. 2B). A classic feature of transformation is an alteration in cell morphology. Control vector-expressing MC3T3 cells exhibited a well spread appearance that was identical to the parental cell line and that was unaffected by dox. TRE17(long) cells appeared grossly similar to control cells under non-induced conditions but showed dramatic changes in morphology upon the addition of dox, appearing much more spindle-shaped and refractile (Fig. 2C). This effect was dependent on the USP activity of TRE17 because MC3T3 lines expressing TRE17(long)/USP− were indistinguishable from control MC3T3 cells. TRE17(A6−)/MC3T3 exhibited an intermediate morphology, suggesting that the ability of TRE17 to activate Arf6 contributes to generation of the spindle-shaped, refractile phenotype, although not as prominently as its USP activity.
TRE17 Blocks Osteoblastic Maturation in a USP-dependent Manner—Upon treatment with ascorbic acid, MC3T3 cells undergo a differentiation program in which they express most genes associated with osteoblastic maturation in vivo, including ALP, BSP, and various collagen isoforms (16–19). To test whether TRE17 affects maturation, control or TRE17-expressing MC3T3 cells were grown in osteogenic medium containing ascorbic acid and dox, and ALP activity was monitored by staining of cultures with the coupled BCIP/NBT chromogenic substrate. As shown in Fig. 3A, control cells grown in differentiation medium exhibited strong ALP activity. Staining was significantly reduced in MC3T3 cells expressing TRE17(long) and TRE17(A6−), Arf6 binding-deficient mutant. ALP activity was measured using a spectrophotometric-based assay with p-nitrophenyl phosphate substrate as described under “Experimental Procedures.” Data represent the mean ± S.D. of eight experiments; *, p < 0.05; **, p < 0.01. vect, vector.

C, RNA was isolated from MC3T3 cells expressing the indicated TRE17 mutants, grown under differentiation-inducing conditions for 5 days. RT-PCR was performed using primers against ALP, BSP, and Col1A1. D, real time PCR was performed to quantify changes in gene expression, using the ABI Prism 7900 system. For each gene, two independent primer pairs were used (denoted ALP-1 and ALP-2, etc.), and expression was normalized against GAPDH levels. E, MC3T3 cells co-expressing TRE17(long) and TRE17(short) were blotted with anti-TRE17, and ALP activity was monitored by BCIP/NBT staining.
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determine whether these variations arose from changes in ALP gene expression, RT-PCR was performed. As seen in Fig. 3, C and D, the alterations in ALP activity largely reflected the effects of TRE17 on ALP expression. Together, the above data strongly suggest that TRE17(long) blocks osteoblastic maturation. To strengthen this conclusion, we examined the expression of two additional genes induced upon maturation, BSP and collagen 1A1 (COL1A1). Both of these genes were strongly suppressed in MC3T3 cells expressing TRE17(long) and TRE17(A6−) (Fig. 3, C and D). In contrast, levels of these differentiation markers were not inhibited by TRE17(short). Together with the transcriptome analysis shown below, these results confirm that TRE17 blocks osteoblastic maturation in a manner dependent on its USP activity, but not its ability to activate Arf6.

TRE17(long) Functions Dominantly over TRE17(short) in Inhibiting Osteoblast Maturation—We recently determined that primary ABC tumors express both TRE17(long) and TRE17(short),4 raising the critical question as to which isoform is transforming. Moreover, the observation that TRE17(short) not only failed to block differentiation but actually enhanced it raised the question of which isoform functions dominantly. Therefore, we generated MC3T3 cell lines co-expressing TRE17(long) and TRE17(short) at comparable levels (Fig. 3E). Strikingly, osteoblastic maturation was strongly suppressed in these cells, as determined by ALP staining (Fig. 3E). This result indicates that TRE17(long) functions dominantly over TRE17(short) to inhibit osteoblast differentiation. Moreover, this suggests that a maturation block would likely be exerted in ABC tumor cells in which the two isoforms are co-expressed.

Effects of TRE17 on Mitogen-independent Growth and Invasion—In addition to the effects of TRE17 on osteoblast cell morphology and differentiation, we asked whether TRE17(long) might induce other in vitro hallmarks of the transformed phenotype. For example, morphological alterations and enhanced refractility are often associated with anchorage-independent growth and increased cell motility. However, neither TRE17(long) nor any of the other alleles induced colony formation in soft agar (data not shown) or enhanced serum-induced motility or invasion through collagen gels (supplemental Fig. 1A). Furthermore, none of the TRE17 alleles conferred a significant proliferative advantage when compared with MC3T3 cells expressing control vector, under conditions of limiting serum (supplemental Fig. 1B) or full serum (data not shown). In fact, growth rates were modestly reduced by TRE17(long) and TRE17(A6−) (supplemental Fig. 1B). Cell death was not observed under any conditions (data not shown). Thus, among the in vitro parameters typically associated with transformation, TRE17 selectively affected cellular morphology and differentiation, in a manner dependent on its USP activity.

Inhibition of Maturation by TRE17(long) Is Mediated through an Autocrine Mechanism—We next explored the mechanism by which TRE17(long) inhibits osteoblastic maturation. One means by which it might function is through the production of autocrine factors. For example, TRE17(long) might suppress the expression of secreted factors that promote osteogenesis or induce expression of factors that inhibit it. Involvement of an autocrine mechanism was explored by treating control MC3T3 cells with CM collected from cells expressing the various TRE17 alleles. Vector control or TRE17-expressing MC3T3 cell lines were grown in differentiation-inducing medium containing dox for 2 days to allow the accumulation of putative autocrine factors. Naïve MC3T3 were treated with the CM from these cells, replacing it with fresh CM every 1–2 days for a period of 5 days, after which cells were stained for ALP activity. As seen in Fig. 4A, CM from TRE17(long)- and TRE17(A6−)-expressing cells significantly suppressed ALP production, whereas CM from MC3T3 expressing TRE17(long)/USP or TRE17(short) did not. These data indicate that the ability of TRE17(long) to block osteoblastic maturation arises, at least in part, through an autocrine mechanism.

Microarray Analysis Reveals Perturbations in Multiple Pathways That Regulate Osteoblast Differentiation in TRE17(long)/MC3T3 Cells—To identify candidate autocrine factors that mediate suppression of osteoblast maturation by TRE17(long), RNA was isolated from the various MC3T3 cell lines grown under differentiation conditions. Gene expression profiles were analyzed using the Affymetrix Mouse 1.0 ST microarray chip. This analysis confirmed that in addition to the specific maturation-associated genes we examined in Fig. 3, C and D, above, other markers of osteoblastic differentiation, such as OMD and multiple collagen isoforms, were down-regulated in cells expressing TRE17(long) but not TRE17(long)/USP− (Fig. 4B). Real time PCR analysis confirmed that expression of Col2A1, Col8A1, and OMD was significantly inhibited by TRE17(long), in a USP-dependent manner (Fig. 4C). Notably, their degree of inhibition was even greater than initially indicated by the microarray analysis.

Using the DAVID bioinformatics database and Ingenuity Pathway Analysis program, it was further found that multiple pathways with established roles in modulating osteoblastic maturation were deregulated in TRE17(long)/MC3T3 cells, including the insulin-like growth factor (IGF), Wnt, and BMP pathways. Expression of multiple components in each of these signaling pathways was altered by TRE17(long) when compared with control MC3T3 cells, in a dox- and USP-dependent manner (Fig. 4B). Within the IGF pathway, the ligands IGF-1 and IGF-2 have been documented as positive regulators of osteoblast differentiation (20). Messenger RNA levels of both of these growth factors were decreased in TRE17(long)-expressing cells (Fig. 4B). Components of the Wnt pathway also exhibited changes in expression in a manner consistent with blocking maturation; Daam2, a transducer of Wnt signaling and a positive regulator of osteoblast differentiation, and Tcf3, a transcription factor important in alkaline phosphatase expression (21), were both decreased (Fig. 4B). Similarly, multiple proteins in the BMP pathway were dysregulated (see next section). Together, these data indicate that TRE17(long) has broad effects on the expression of genes regulating osteoblastic differentiation.

BMP Pathway Dysregulation Plays a Key Role in the Maturation Block Induced by TRE17(long)—Among the pathways identified above, BMP has a particularly important role in

4 M. M. Chou and A. M. Oliveira, unpublished observations.
FIGURE 4. Microarray analysis reveals broad dysregulation of pathways controlling maturation in TRE17(long)-expressing cells. A, control vector (vect)-expressing MC3T3 cells were incubated with CM from MC3T3 cells expressing the indicated TRE17 allele for 5 days under differentiation conditions, and ALP activity was measured. Data represent the mean ± S.D. of three experiments. Statistically significant differences relative to cells treated with control CM are indicated: *, p < 0.05; **, p < 0.01. USP, TRE17(long)/USP; A6, Arf6 binding-deficient mutant. B, heat map showing signaling pathways with altered expression of multiple components in MC3T3 expressing TRE17(long), but not TRE17(long)/USP. -Fold changes in expression for individual genes are indicated in green and red, respectively. -Fold changes in expression for individual genes are specified in the table. vec, vector; osteoblast diff., osteoblast differentiation; osteo, osteoblast. C, real time PCR was performed to confirm expression changes in select genes identified from the microarray. For each gene, two independent primer pairs were used (denoted -1 and -2 and), and expression was normalized against GAPDH levels.
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osteogenesis. Previous work has shown that maturation of MC3T3 cells is regulated by autocrine production of BMP-4. Our microarray analysis revealed that expression of gene products that play a negative role in BMP signaling (such as Smurf1 and Gremlin-1 (Grem1)) was increased, whereas products that play a positive role (such as BMP-4 and Runx2) was decreased (Fig. 4) (22). We focused on two of these proteins, BMP-4 and Grem1, for further analysis because they are secreted factors and thus candidates for mediating autocrine inhibition of osteoblastic maturation. BMP-4 is a key osteogenic factor that functions through TGFβ receptors I and II (TGFβRI/II). Grem1 is a BMP antagonist that heterodimerizes with BMP family ligands and prevents their association with TGFβRI/II (23, 24). Alterations in BMP-4 and Grem1 expression were confirmed by RT-PCR; Fig. 5A shows that TRE17(long) inhibits Bmp-4 and stimulates Grem1 expression in a USP-dependent manner. Again, RT-qPCR analysis revealed that the degree of their dysregulation was even greater than indicated by the microarray. Furthermore, immunoblotting revealed that BMP-4 levels in the CM of cells expressing TRE17(long) and TRE17(A6−), but not TRE17(short) or TRE17(long)/USP−, were reduced (Fig. 5B). Alterations in Grem1 protein levels could not be confirmed as antibodies that function for immunoblotting were not available. We next explored whether the dysregulation of BMP-4/Grem1 played a significant role in the inhibition of differentiation by TRE17(long). Toward this end, we examined whether maturation could be rescued by the addition of exogenous BMP-4. As shown in Fig. 5, C and D, the addition of recombinant BMP-4 to TRE17(long)/MC3T3 restored maturation, as measured by induction of ALP. Both ALP expression (Fig. 5C) and activity (Fig. 5D) were significantly rescued. Thus, among the regulatory factors identified in the microarray analysis, BMP-4 and Grem1 appear to play a pivotal role in mediating autocrine inhibition of maturation by TRE17.

DISCUSSION

Since the discovery of TRE17 translocation in ABC in 2004, virtually nothing has been elucidated about the mechanism by which its overexpression contributes to the formation of this complex tumor. The present study is the first to analyze the effects of TRE17 on osteoblastic differentiation and morphology. The presumed cell type affected in at least a subset of ABCs. Our key findings can be summarized as follows. (a) TRE17 inhibits osteoblast differentiation and alters osteoblast morphology without stimulating cell proliferation or invasiveness; (b) these functions require the USP activity of TRE17, but not its Arf6 stimulatory activity; (c) TRE17(long) functions dominantly over TRE17(short) in inhibiting osteoblast maturation; (d) inhibition of differentiation by TRE17 occurs at least in part through an autocrine mechanism; (e) transcriptome analysis points to multiple osteoblast regulatory pathways that are affected by TRE17; and (f) dysregulation of the BMP pathway plays a key role in the maturation block. We believe that in concert, these findings provide important new insights into the function of TRE17 in osteoblasts and provide a strong foundation for future studies on TRE17 during osteoblast differentiation and bone homeostasis in normal and pathological conditions.

Our data indicate that not only is maturation blocked in TRE17(long)/MC3T3, but further, that CM from these cells can dominantly inhibit differentiation of parental MC3T3 osteoblasts. Notably, the differentiation block can be overcome by the addition of exogenous BMP-4. There are two implications of these results that deserve comment. First, they raise the possibility that in vivo, TRE17-expressing cells might exert cell non-autonomous effects, inhibiting the function of neighboring osteoblasts via a paracrine mechanism to enhance bone loss even further. Second, the striking observation that BMP-4 can overcome the TRE17-induced differentiation block invokes the possibility of using localized BMP-4 or Grem1 inhibitors to treat ABC lesions in patients.

Our transcriptome and RT-qPCR analyses demonstrate that the USP activity of TRE17 is required for changes in gene expression, and studies are currently underway to identify its relevant substrate(s). It can be envisioned that TRE17 might deubiquitinate and rescue from proteasomal degradation signaling molecules or nuclear factors that inhibit the osteoblastic maturation program. Two key master regulators of osteogenesis are Runx2 and ATF-4, both of which can be regulated by ubiquitination (25, 26). However, these factors are pro-osteogenic and are thus unlikely targets of TRE17. Indeed, we confirmed that neither of these transcription factors was dysregulated in TRE17(long)/MC3T3 cells. Thus, further work will be required to determine the mechanisms of altered gene expression induced by TRE17(long). Other important questions that are under study involve the mechanism by which TRE17 regulates the expression of BMP-4 and Grem1, as well as other components of the BMP, Wnt, and IGF pathways. This includes the investigation of the relative roles that each of these pathways might play during osteoblast differentiation.

Despite the dramatic effects of TRE17 on osteoblast maturation and morphology, it was insufficient to induce other in vitro hallmarks of the transformed phenotype, such as enhanced proliferation. Although somewhat surprising, this is nevertheless consistent with the multihit model of tumorigenesis. These results are also consistent with our finding that in primary cultures derived from ABC tumors, cells harboring the TRE17 translocation/overexpression did not display a growth advantage over cells lacking TRE17 expression. TRE17 also failed to induce motility and invasiveness in MC3T3 cells. Although these in vitro assays need to be interpreted with caution, it is nevertheless worth noting that ABCs are benign lesions that do not metastasize (with one recent exception (27)), and therefore, TRE17 might not entail enhanced motility/invasiveness as is the case with metastatic neoplasms.

In addition to inhibiting osteoblastic maturation, we recently found that TRE17 induces the production of matrix metalloproteinases (13) and inflammatory cytokines5 when expressed in osteoblasts. Among the cytokines induced by TRE17 were granulocyte-macrophage colony-stimulating factor and interleukin-1α, which have varied functions in the inflammatory response, including the ability to promote the differentiation and/or activation of osteoclasts, the cells that mediate degrada-

5 L. M. Pringle and M. M. Chou, unpublished observations.
tion of bone (28–36). Thus, TRE17 appears to simultaneously inhibit osteoblast maturation and stimulate osteoclast activity. Because bone homeostasis requires a precise balance between the bone-forming activity of osteoblasts and the bone-resorptive activity of osteoclasts, our work raises the possibility that the bone loss/destruction observed in ABC may arise from per-

**FIGURE 5.** BMP pathway is dysregulated in TRE17(long)/MC3T3 and can be rescued by exogenous BMP-4. **A,** RNA was isolated from MC3T3 cells expressing the indicated TRE17 alleles under differentiation-inducing conditions, in the absence or presence of dox as indicated. RT-PCR was performed using primers against *Grem1* and *BMP-4* (left panel and right panel, respectively). Real time PCR was also performed to quantify changes in expression (graphs). For each gene, two independent primer pairs were used (denoted −1 and −2), and expression was normalized against GAPDH levels. **B,** MC3T3 cell lines were treated with dox under serum-free conditions. The conditioned medium were precipitated with trichloroacetic acid and then subjected to immunoblotting with anti-BMP-4. Purified recombinant BMP-4 (*reBMP4*), which has a slightly reduced electrophoretic mobility, was used as a positive control for blotting. **C and D,** TRE17(long) or vector control MC3T3 were grown in differentiation medium, in the presence or absence of dox and the indicated concentration of recombinant BMP-4 for 5 days. In C, real time PCR was performed to quantify ALP expression levels, using two independent primer pairs and normalizing against GAPDH. In D, ALP activity was measured by BCIP/NBT staining.
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turbations in both of these processes. In aggregate, our findings suggest that TRE17 functions predominantly through the modulation of secreted proteins, which might function in an autocrine and perhaps paracrine fashion to alter cell maturation, induce inflammation, and degrade the extracellular matrix.

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