

The type III transforming growth factor- β receptor inhibits proliferation, migration, and adhesion in human myeloma cells

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ABSTRACT Transforming growth factor- β (TGF- β) plays an important role in regulating hematopoiesis, inhibiting proliferation while stimulating differentiation when appropriate. We previously demonstrated that the type III TGF- β receptor (T β RIII, or betaglycan) serves as a novel suppressor of cancer progression in epithelial tumors; however, its role in hematologic malignancies is unknown. Here we demonstrate that T β RIII protein expression is decreased or lost in the majority of human multiple myeloma specimens. Functionally, restoring T β RIII expression in myeloma cells significantly inhibited cell growth, proliferation, and motility, largely independent of its ligand presentation role. In a reciprocal fashion, shRNA-mediated silencing of endogenous T β RIII expression enhanced cell growth, proliferation, and motility. Although apoptosis was not affected, T β RIII inhibited proliferation through induction of the cyclin-dependent kinase inhibitors p21 and p27. T β RIII further regulated myeloma cell adhesion, increasing homotypic myeloma cell adhesion while decreasing myeloma heterotropic adhesion to bone marrow stromal cells. Mechanistically, live cell imaging of myeloma and stroma cell cocultures revealed that T β RIII-mediated inhibition of heterotropic adhesion was associated with decreased duration of myeloma/bone marrow stromal cell interaction. These results suggest that loss of T β RIII expression during multiple myeloma progression contributes to disease progression through its functional effects on increased cell growth, proliferation, motility, and adhesion.

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INTRODUCTION

Multiple myeloma is the second most common hematologic malignancy in the United States, accounting for 1–2% of all cancers and 10–15% of hematologic tumors (Jemal *et al.*, 2007). Despite ad-

vances in conventional and high-dose therapy, multiple myeloma remains incurable with a 5-yr survival of 31% (Brenner, 2002). Lack of insight into the mechanisms of chemotherapeutic resistance remains a significant barrier to treatment of this disease.

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Abbreviations used: 7-AAD, 7-aminoactinomycin D; APC, allophycocyanin conjugate; BMP, bone morphogenic protein; BMSC, bone marrow stromal cell; BSA, bovine serum albumin; CCD, charge-coupled device; cdk, cyclin-dependent kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; Ig, immunoglobulin; IHC, immunohistochemistry; IL-6, interleukin-6; KRH, Krebs-Ringer-HEPES; MGUS, monoclonal gammopathy of undetermined significance; NTC, nontargeting vector control; PBS, phosphate-buffered saline; SFM, serum-free RPMI medium; T β RIII, type III TGF- β receptor; TGF- β , transforming growth factor- β ; VEGF, vascular endothelial growth factor.

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Myeloma cells localize in the bone marrow, where their survival is dependent on the normal stromal cells that secrete cytokines and interact with malignant cells through adhesion molecules (De Vos and Klein, 2004). Several growth factors—including interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and transforming growth factor- β (TGF)- β —play an important role in multiple myeloma pathogenesis and mediate tumor cell proliferation, drug resistance, and migration in the bone marrow milieu (Yasui *et al.*, 2006). A better understanding of how these signaling pathways are functionally linked is required to design therapeutic strategies that target the myeloma cell in the bone marrow microenvironment.

The TGF- β signaling pathway plays an important role in regulating normal hematopoiesis, inhibiting proliferation while stimulating differentiation when appropriate (Dong and Blobe, 2006). TGF- β

ligands regulate cellular processes by binding to three high-affinity cell surface receptors: the type I TGF- β receptor (T β RI), the type II TGF- β receptor (T β RII), and the type III TGF- β receptor (T β RIII). T β RIII, the most abundant TGF- β receptor, has traditionally been thought to function as a coreceptor, enhancing ligand binding to associated receptors (Kirkbride et al., 2005, 2008). Substantial evidence, however, supports an essential role for T β RIII, including the embryonic lethal phenotype of T β RIII knockout mice, in part due to ineffective erythropoiesis (Stenvers et al., 2003), and the essential and nonredundant role for T β RIII in the endothelial-to-mesenchymal transition of chick heart development (Brown et al., 1999). T β RIII has been demonstrated to serve as a suppressor of cancer progression in cancers of the breast, kidney, lung, ovary, pancreas, and prostate (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008; Gordon et al., 2008; Margulis et al., 2008). Like other coreceptors, T β RIII undergoes ectoderm shedding, releasing a soluble form of T β RIII (sT β RIII), which has the potential to serve as an antagonist of T β RIII signaling (Lopez-Casillas et al., 1994). Moreover, work in our laboratory has established a role for sT β RIII in decreasing cancer cell migration and invasion (Dong et al., 2007). The mechanisms by which T β RIII and sT β RIII receptors regulate TGF- β superfamily signaling remain poorly understood.

TGF- β normally functions to suppress the proliferation and immunoglobulin (Ig) production of B cells (Kehrl et al., 1986). In multiple myeloma, resistance to the homeostatic functions of TGF- β signaling develops, perhaps through defective trafficking of T β RI and T β RII to the cell surface (Amoroso et al., 1998; Fernandez et al., 2002). In response, both human myeloma and bone marrow stromal cells (BMSCs) from myeloma patients secrete higher levels of TGF- β compared with normal plasma cells (Urashima et al., 1996; Hayashi et al., 2004), contributing to the immune dysfunction present in multiple myeloma patients (Hayashi et al., 2004). Importantly, TGF- β -neutralizing antibodies or a T β RI inhibitor can block IL-6 and VEGF secretion and decrease myeloma cell growth and cell adhesion to the BMSCs (Urashima et al., 1996; Hayashi et al., 2004). These results establish resistance of myeloma cells to the homeostatic effects of TGF- β and its effect on the bone marrow stroma as important factors in the pathogenesis of multiple myeloma. Here we investigate the role of T β RIII in multiple myeloma.

RESULTS

T β RIII expression is decreased in human multiple myeloma

T β RIII expression is decreased or lost in several human epithelial tumors, including cancers of the breast, lung, ovary, and prostate (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008), with loss of expression correlating to disease progression and predicting a poorer prognosis for patients. Functionally, loss of T β RIII results in increased motility and invasion in vitro and increased tumorigenicity, angiogenesis, and invasiveness in vivo (Dong et al., 2007; Hempel et al., 2007; Turley et al., 2007; Finger et al., 2008). To investigate T β RIII expression during multiple myeloma progression, we initially examined genomic data available through the publicly available Oncomine Cancer Profiling Database. T β RIII mRNA expression was decreased during multiple myeloma progression, with decreased expression in bone marrow specimens from patients with monoclonal gammopathy of undetermined significance (MGUS) relative to normal patients, and was decreased in bone marrow from multiple myeloma patients relative to bone marrow from MGUS patients (Mattioli et al., 2005; Zhan et al., 2006) (Supplemental Figure S1, summarized in Supplemental Table S1). In contrast, there was little to no change in the expression of the other major TGF- β

receptors examined, including T β RII, T β RI (ALK-5), endoglin, and ALK-1 (unpublished data).

We previously demonstrated that T β RIII protein expression is largely regulated at the message level and that altering levels of T β RIII protein expression was sufficient to regulate TGF- β signaling (Blobe et al., 2001; Chen et al., 2003). To examine T β RIII protein expression in multiple myeloma, we performed immunohistochemistry (IHC) analysis on human bone marrow biopsy specimens from multiple myeloma patients. Using a tissue microarray (US Biomax, Rockville, MD) containing 10 cases of myeloma, 11 normal tissue controls, and an anti-T β RIII specific antibody as previously reported (Dong et al., 2007), we established that T β RIII expression was decreased in multiple myeloma specimens compared with normal controls (Figure 1, A–C). T β RIII expression decreased by 60% when comparing normal controls to multiple myeloma specimens ($p < 0.0001$, Figure 1B). When examining T β RIII expression based on level of expression (low IHC score = 0–1, medium IHC score = 2–3, high IHC score = 4–5), the percentage of samples with high T β RIII expression decreased from 81% of normal controls to 15% of multiple myeloma specimens ($p < 0.0001$), whereas the percentage of samples with low T β RIII expression increased from 0% of normal controls to 40% of multiple myeloma specimens ($p < 0.001$, Figure 1C). In addition, three of four myeloma cell lines demonstrated decreased/lost T β RIII expression at the mRNA (unpublished data) and protein level (Figure 1D).

T β RIII inhibits cell growth and proliferation in multiple myeloma cells

We previously demonstrated that T β RIII enhanced TGF- β 1-mediated growth inhibition in L6 myoblasts and that the cytoplasmic domain of T β RIII contributed to this response (Blobe et al., 2001; Chen et al., 2003; You et al., 2007), whereas T β RIII had little effect on the proliferation of breast, lung, or prostate cancer cells. To investigate the role of T β RIII in non-epithelium-derived cancer cells, we transiently expressed T β RIII in the RPMI-8226 myeloma cell line, which lacks endogenous T β RIII expression (Figure 1D), using T β RIII-expressing adenovirus tagged with green fluorescent protein (GFP) or GFP-expressing adenovirus alone as a control (Figure 2A). Here and throughout, infected RPMI-8226 cells were sorted and gated on GFP, and the adenovirally infected cells were used. Restoring expression of T β RIII in RPMI-8226 cells resulted in a 50–60% reduction in cell growth (Figure 2B; $p < 0.05$) and a 40–50% reduction in proliferation (Figure 2C; $p < 0.001$) relative to control cells. We next investigated the effect of shRNA-mediated silencing of endogenous T β RIII expression in the U266 myeloma cell line (Figure 1D), with a nontargeting vector control (NTC) shRNA as control (Figure 2A). Here and throughout, U266 cells were sorted and gated on dsRED, and the adenovirally infected cells were used. Reducing T β RIII expression in U266 cells increased cell growth by 60% (Figure 2D; $p < 0.05$) and increased proliferation by 75% (Figure 2E; $p < 0.001$) relative to control. These data indicate that T β RIII inhibits multiple myeloma cell growth and proliferation.

T β RIII serves as a coreceptor for multiple TGF- β superfamily ligands, including TGF- β and bone morphogenic proteins (BMPs) (Kirkbride et al., 2008). To determine whether T β RIII-mediated growth inhibition of myeloma cells was ligand-dependent, we examined the effect of several TGF- β superfamily ligands (BMP-2, BMP-4, TGF- β 1) on cell growth and inhibition in the presence or absence of T β RIII. Whereas BMP-2, BMP-4, and TGF- β each modestly inhibited myeloma cell proliferation, in each case T β RIII inhibited proliferation to an equivalent extent in the presence and absence of ligand (Supplemental Figure S2). We further examined the

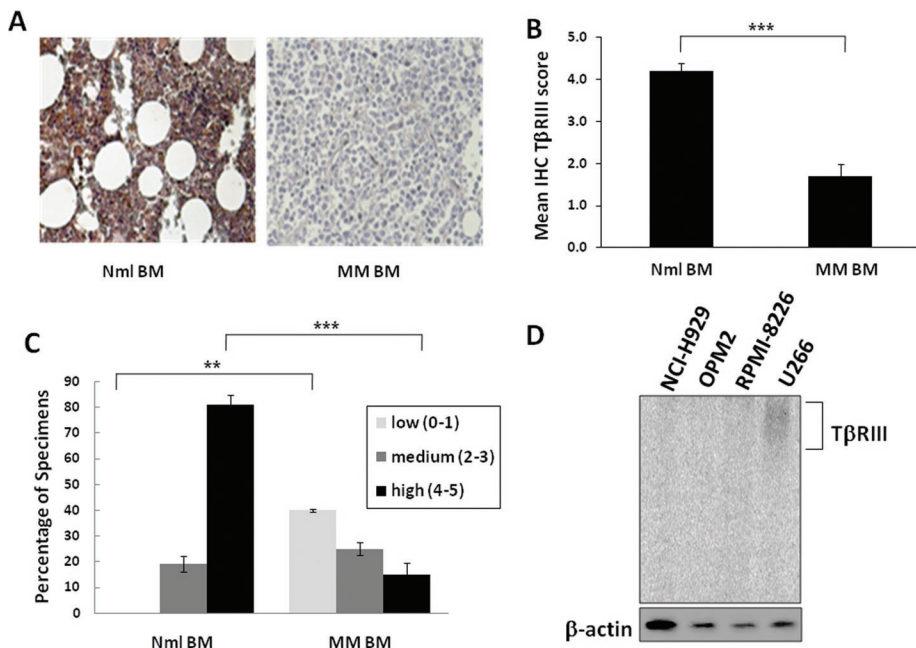


FIGURE 1: Decreased TβRIII expression in human multiple myeloma. TβRIII protein levels were detected by IHC using an α-TβRIII antibody on a Biomax tissue array. (A) Representative IHC analysis (image selected based on mean IHC score) of TβRIII expression (original magnification, 40×) in normal (Nml) bone marrow (BM) and in multiple myeloma (MM) bone marrow biopsy specimens. Immunoreactivity for TβRIII was scored as 0–5 and categorized as low (0–1), medium (2–3), or high (4–5). (B) Summary of IHC results from normal (11 specimens, duplicate cores per case) and multiple myeloma (10 specimens, duplicate cores per case) tissues, based on mean IHC score. (C) Summary of IHC, with percentages shown. The percentages of low vs. medium vs. high in each sample type are represented graphically. ** $p < 0.001$; *** $p < 0.0001$. Error bars represent SEM. (D) The indicated cells were exposed to 100 pM of ^{125}I -TGFβ1, cross-linked, immunoprecipitated with an α-TGF-βRIII antibody, separated by SDS-PAGE, and detected by phosphorimaging. β-Actin is shown as a loading control. Assay shown is representative of experiments performed at least three times.

effect of BMP-2 and TGF-β1 on Smad1/5 and Smad2 phosphorylation in control and TβRIII-expressing myeloma cells. Whereas BMP-2 induced Smad1/5 phosphorylation and TGF-β1 induced Smad2 phosphorylation, expression of TβRIII only modestly inhibited BMP-2-induced Smad1/5 phosphorylation (Supplemental Figure S3A), and had no effect on TGF-β1-induced Smad2 phosphorylation (Supplemental Figure S3B). These results suggest that although myeloma cells are BMP-2/TGF-β1-responsive (Supplemental Figure S3), the effects of TβRIII on multiple myeloma cell growth and proliferation are largely independent of its ligand presentation role.

TβRIII increases cyclin-dependent kinase inhibitor expression in multiple myeloma cells

Cell growth is a balance of proliferation and apoptosis. To investigate whether TβRIII regulates apoptosis, we stimulated apoptosis with the proteasome inhibitor bortezomib. Transiently increasing TβRIII expression in RPMI-8226 cells or shRNA-mediated silencing of TβRIII expression in U266 cells did not significantly alter bortezomib-induced apoptosis compared with control cells (unpublished data), suggesting that TβRIII regulates cell growth primarily through regulation of proliferation. To investigate the mechanism by which TβRIII regulates proliferation, we examined the effect of TβRIII on cell-cycle regulatory proteins known to be regulated by the TGF-β signaling pathway, including the cyclin-dependent kinase (cdk) inhibitors (Elbendary *et al.*, 1994; Hannon and Beach, 1994; Datto *et al.*, 1995; Li *et al.*, 1995; Warner *et al.*, 1999). TβRIII expression significantly increased the levels of cdk inhibitors, p21 and p27, indepen-

dent of exogenous ligands in RPMI-8226 cells (Figure 3). Although the known TβRIII ligands, BMP-2, BMP-4, and TGFβ-1, also increased p21 and p27 expression (Figure 3), the magnitude of increase was similar in the presence and absence of TβRIII, suggesting that, consistent with the proliferation data, the effects of TβRIII are largely independent of its ligand presentation role. Taken together, these data suggest that TβRIII-mediated inhibition of cell growth and proliferation in myeloma cells is mediated, in part, by an increase in expression of the cdk inhibitors p21 and p27.

TβRIII decreases adhesion of multiple myeloma cells to BMSCs

Binding of myeloma cells to BMSCs triggers expression of adhesive molecules and secretion of growth factors (TGF-β, IL-6, VEGF), promoting multiple myeloma cell growth, survival, drug resistance, and migration (Wang *et al.*, 2007). To determine the effects of endogenous TβRIII on adhesion of multiple myeloma cells to BMSCs, we examined the effect of shRNA-mediated silencing of endogenous TβRIII expression in the U266 myeloma cell line, with an NTC shRNA as control. Reducing TβRIII expression in U266 cells increased adhesion to HS-5 stromal cells by 30–50% (Figure 4A; $p < 0.05$) relative to control cells, indicating that TβRIII inhibits heterotropic cell–cell adhesion of myeloma cells to BMSCs. To determine the contributions of specific domains of TβRIII

to adhesion, we transiently expressed TβRIII, TβRIIIΔGAG (lacking the glycosaminoglycan chains), TβRIIIΔCYTO (lacking the cytoplasmic domain) (Figure 4B), or GFP alone as control in RPMI-8226 cells and cocultured these with HS-5 stromal cells. Both TβRIII and TβRIIIΔGAG significantly decreased adhesion of multiple myeloma cells to HS-5 stromal cells by 45–60% ($p < 0.05$) and 30–50% ($p < 0.05$), respectively (Figure 4B). Interestingly, TβRIIIΔCYTO did not significantly decrease adhesion compared with control cells (Figure 4B). These results suggest that TβRIII requires its cytoplasmic domain for its inhibitory function on heterotropic adhesion.

To explore the mechanism by which TβRIII functions to inhibit adhesion of myeloma cells to BMSCs (HS-5), we tracked the movement of individual RPMI-8226-TβRIII-GFP or RPMI-8226 GFP control adenovirally infected myeloma cells when cocultured with HS-5 BMSCs using live cell imaging. We observed that the TβRIII-expressing myeloma cells appeared to interact more often but less efficiently with the BMSCs compared with control myeloma cells/BMSCs. To quantify these observations, we determined both the number of myeloma cell/BMSC interactions and the amount of time that the myeloma/BMSCs were interacting. We determined the number of TβRIII-infected multiple myeloma cells that were interacting with HS-5 BMSCs as defined by >50% multiple myeloma cell body buried underneath the BMSC body at least one time during the period of observation (Figure 4C; Supplemental Video 1) and found that 60% of total TβRIII-expressing myeloma cells initiated this contact with HS-5 BMSCs compared with 27% of GFP control myeloma cells (Figure 4C; $p < 0.05$). TβRIII-expressing multiple myeloma cells,

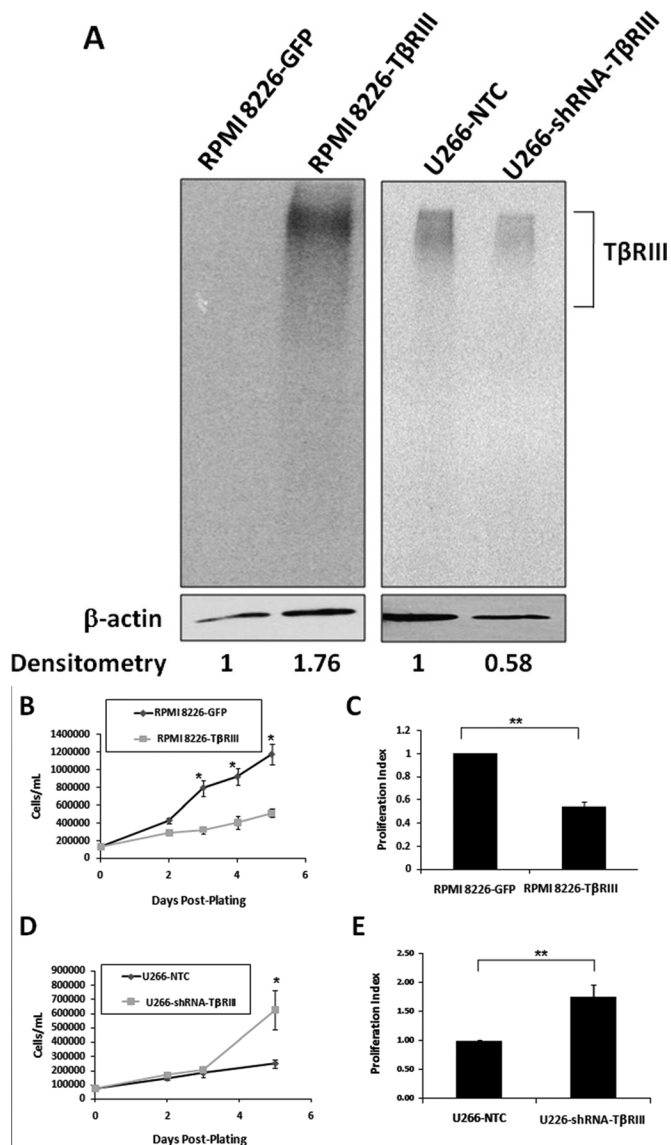


FIGURE 2: TβRIII inhibits cell growth and proliferation of human multiple myeloma cells. (A) RPMI-8226 cells were adenovirally infected with GFP vector control or GFP-TβRIII and incubated for 48 h. U266 cells were adenovirally infected with NTC or shRNA-TβRIII and incubated for 72 h. The indicated cells were then exposed to 100 pM of ¹²⁵I-TGFβ1, cross-linked, immunoprecipitated with an α-TβRIII antibody, separated by SDS-PAGE, and detected by phosphorimaging. α-β-actin is shown as loading control. Densitometry represents the mean densitometry of three independent experiments and shows TβRIII expression compared with vector control (normalized to loading control). Representative gels are shown. Quantification was performed using ImageMeter software. (B) Cells were plated at 1.0 × 10⁵ cells/ml. Cell growth was measured on the days indicated by trypan blue exclusion. Restoration of TβRIII expression inhibited the growth of RPMI-8226 cells (*p < 0.05). (C) Cells were plated at 1.0 × 10⁴ cells/ml. Proliferation was measured by [³H]thymidine incorporation. Restoration of TβRIII inhibited proliferation of RPMI-8226 cells (**p < 0.001). (D) Cells were plated at 1.0 × 10⁵ cells/ml. Cell growth was measured on the days indicated by trypan blue exclusion. Knockdown of TβRIII expression increased cell growth of U266 cells (*p < 0.05). (E) Cells were plated at 1.0 × 10⁴ cells/ml. Proliferation was measured by [³H]thymidine incorporation. Knockdown of TβRIII increased proliferation of U266 cells (**p < 0.001). All values represent means ± SEM in triplicate cultures and are representative of three independent experiments.

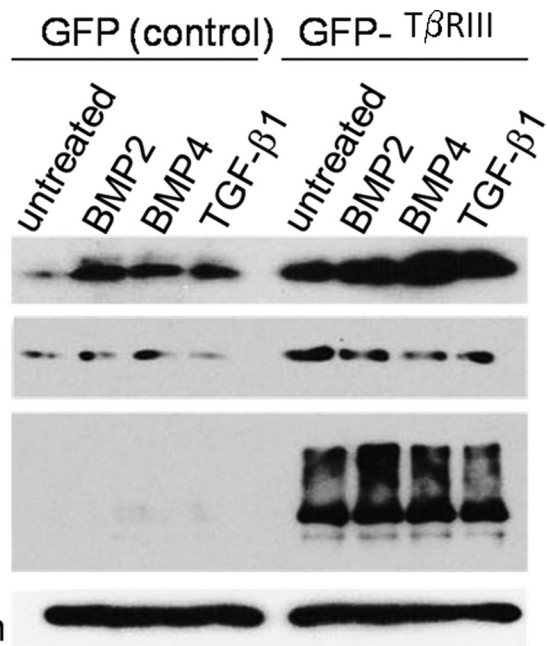


FIGURE 3: TβRIII increases cdk inhibitor expression in human multiple myeloma cells. RPMI-8226 myeloma cells were adenovirally infected with GFP vector or GFP-TβRIII. Forty-eight hours later, cells were treated with or without TGF-β superfamily ligands, BMP-2 (0–10 nM), BMP-4 (0–10 nM), and TGF-β1 (0–200 pM) for 24 h. Immunoblot was done on total lysates using an α-p21 antibody. Blot was stripped and reprobed for indicated antibodies. Note significantly increased levels of p21 and p27 (induced by ligands) in cells infected with TβRIII compared with vector control.

however, spent less time (30 min for TβRIII vs. 36 min for GFP) within a 15 μm perimeter of the BMSCs (Supplemental Video 2: Figure 4D), although this was not statistically significant. Importantly, TβRIII-expressing multiple myeloma cells spent approximately threefold less time compared with GFP control myeloma cells (8 min for TβRIII and 22 min for GFP) interacting with the HS-5 BMSCs when they were underneath the stromal cells compared with control cells (Figure 4E; p < 0.05). Taken together, these data indicate that whereas TβRIII-expressing multiple myeloma cells are proficient in establishing initial contact with the BMSCs, TβRIII-infected multiple myeloma cells were unable to sustain these interactions with BMSCs, resulting in inhibition of heterotropic cell–cell adhesion. These data collectively indicate that loss of TβRIII may contribute to the pathogenesis of multiple myeloma by enhancing adhesion and localization of multiple myeloma cells to BMSCs.

TβRIII increases homotypic aggregation of multiple myeloma cells

One potential mechanism for decreased heterotropic cell–cell adhesion to stromal cells could be increased homotypic myeloma cell–cell adhesion. To determine the effects of TβRIII on homotypic aggregation of myeloma cells, we transiently expressed TβRIII, TβRIIIΔGAG, TβRIIIΔCYTO, or GFP alone as control in RPMI-8226 multiple myeloma cells and quantified the number of aggregates at different time points. TβRIII expression significantly increased the number of aggregates at all time points (15–120 min) relative to control cells (Figure 5A; p < 0.05). Interestingly, neither TβRIIIΔGAG nor TβRIIIΔCYTO expression increased homotypic aggregation in comparison to control cells (Figure 5A). These data indicate that

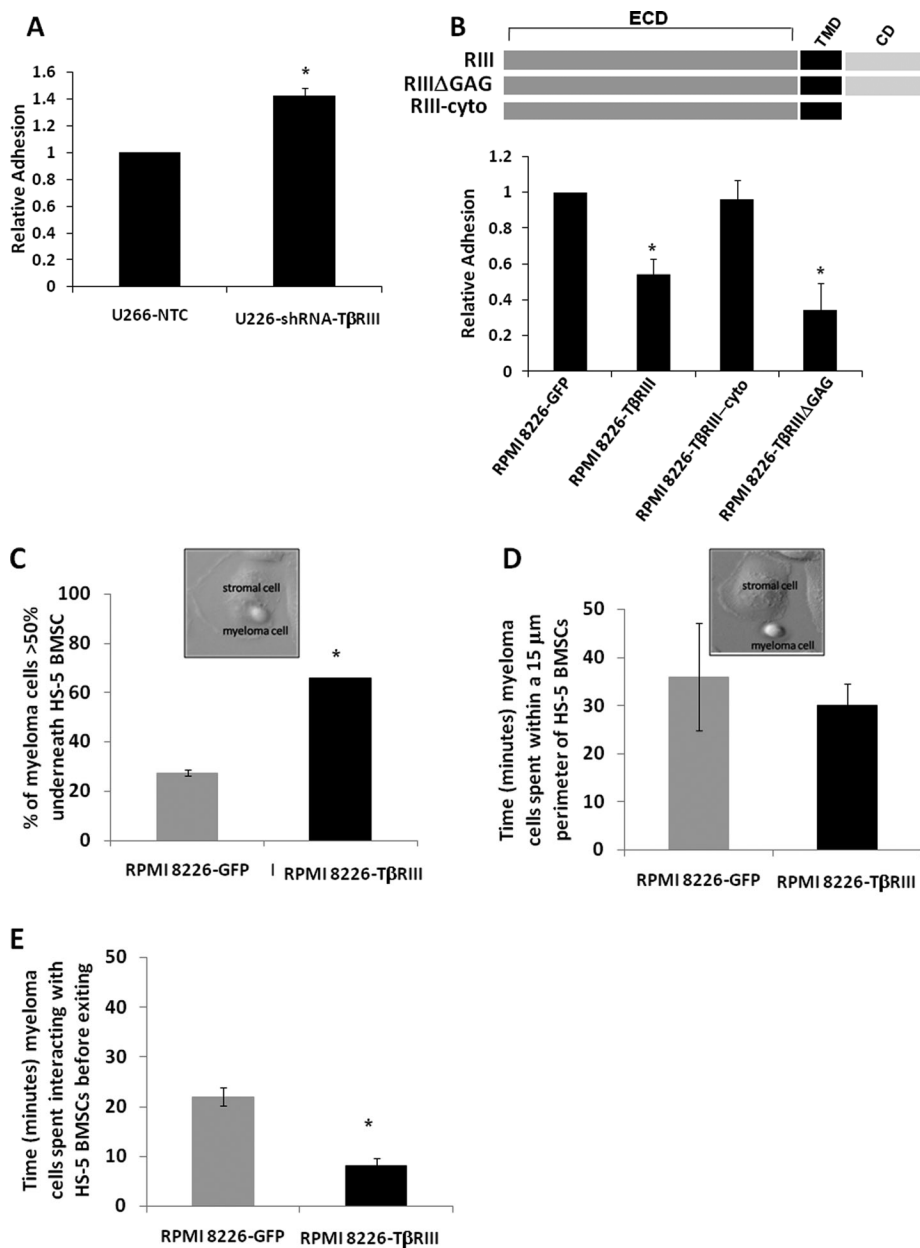


FIGURE 4: TβRIII decreases adhesion of human multiple myeloma cells to BMSCs. (A) U266 cells were adenovirally infected with NTC or shRNA-TβRIII and incubated for 72 h. Cells were then incubated in SFM with calcein-blue AM for 30 min, then resuspended in 1% BSA in RPMI and plated onto HS-5 BMSCs for 1 h (**p* < 0.05). (B) Diagram of deletions in the extracellular domain mutants of TβRIII. RPMI-8226 cells were adenovirally infected with GFP vector, GFP-TβRIII, GFP-TβRIIIΔCYTO, or GFP-TβRIIIΔGAG and incubated for 48 h. Cells were then incubated in SFM with calcein-blue AM for 30 min, resuspended in 1% BSA in RPMI, and plated onto HS-5 BMSCs for 1 h. Nonadherent cells were carefully washed off, and adherent multiple myeloma cells were measured using a fluorescence analyzer (**p* < 0.05). All values represent means ± SEM in triplicate cultures and are representative of three independent experiments. (C–E) HS-5 cells were plated on 35-mm glass-bottom dishes at a density of 10⁴ cells per well in regular culture medium and were allowed to adhere for 24 h. Cells were then washed twice in PBS. Then, 5 × 10³ of the indicated adenovirally infected (GFP vector or GFP-TβRIII) RPMI-8226 multiple myeloma cell lines suspended in SFM + 1% BSA were plated onto the BMSC-coated plates (HS-5) and placed in a temperature- and CO₂-controlled chamber. A Zeiss Axio Observer Z1 motorized microscope equipped with 20, 40, and 100× objective lenses was used for the imaging. Time-lapse recordings started ~10 min after cells were cocultured with infected RPMI-8226 myeloma cells. The images were collected at 1-min intervals for >120 min with a cooled CCD video camera. The adhesion parameters, including percent total cells and time (in minutes) that the infected RPMI-8226 myeloma cells interacted with the HS-5 BMSCs, were obtained from time-lapse movies. Cells were manually traced for each cell using Metamorph to track the adhesion characteristics of the individual cells. The adhesion parameters were

TβRIII is important in mediating homotypic cell–cell adhesion in myeloma cells and, structurally, both the cytoplasmic domain and glycosaminoglycan chains are important in mediating this effect. We next investigated the effect of shRNA-mediated silencing of TβRIII expression in the U266 myeloma cell line. Reducing TβRIII expression in U266 cells significantly decreased homotypic aggregation (Figure 5B; *p* < 0.05) relative to control cells. These data indicate that TβRIII directly promotes homotypic cell–cell adhesion of myeloma cells.

TβRIII decreases migration/chemotaxis of multiple myeloma cells

Increasing or restoring TβRIII expression in cancer models decreases cancer cell motility and invasion in vitro and angiogenesis, invasion, and metastasis in vivo (Dong *et al.*, 2007; Hempel *et al.*, 2007; Turley *et al.*, 2007; Finger *et al.*, 2008; Gordon *et al.*, 2008; Mythreye and Blobel, 2009). To determine whether TβRIII-mediated suppression of migration also occurs in a multiple myeloma cell model system, we examined transwell migration of multiple myeloma cells toward serum (10% fetal bovine serum [FBS], chemotactic gradient). We transiently expressed TβRIII in RPMI-8226 cells using TβRIII-expressing adenovirus tagged with GFP or GFP-expressing adenovirus alone as a control. Expression of TβRIII resulted in a >80% reduction in chemotactic migration relative to control cells (Figure 6A; *p* < 0.0001). To determine the structural requirements for TβRIII inhibition of migration, we

expressed as graphs using the Microsoft Excel program. The percentage of infected myeloma cells that interacted with the BMSCs was calculated as the percentage of total myeloma cells that were at least 50% underneath the HS-5 BMSC, at least once during the period of observation (C) and is represented graphically (**p* < 0.05). The time that the infected myeloma cells spent interacting with the BMSCs was calculated as both time (minutes) that the myeloma cells spent within a 15 μm perimeter of the HS-5 BMSCs (D) and the time (minutes) that the myeloma cells spent interacting with HS-5 BMSC before exiting (E) and is represented graphically (**p* < 0.05). Representative pictures above C and D show myeloma/stromal cell interactions (original magnification, 60×). For the live cell-imaging analysis, at least 35 cells were counted for each experiment during the 120-min time period. All values represent means ± SEM and are representative of at least two independent experiments.

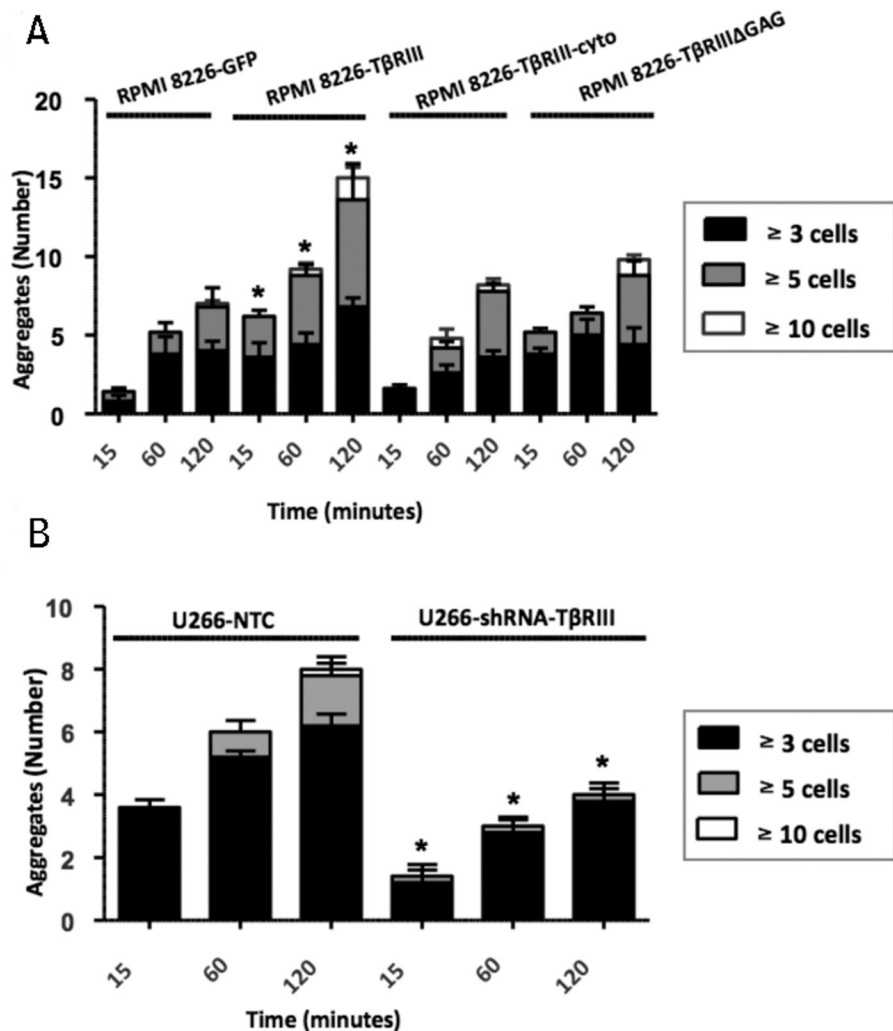


FIGURE 5: TβRIII increases homotypic aggregation of human multiple myeloma cells. (A) RPMI-8226 cells were adenovirally infected with GFP vector, GFP-TβRIII, GFP-TβRIIIΔCYTO, or GFP-TβRIIIΔGAG and incubated for 48 h. The indicated cells were dispersed with a pipette, then allowed to aggregate at the indicated time points. The numbers of aggregates in clumps of ≥3, ≥5, and ≥10 were counted (**p* < 0.05). (B) U266 cells were adenovirally infected with NTC or shRNA-TβRIII and incubated for 72 h. The indicated cells were dispersed with a pipette, then allowed to aggregate at the indicated time points. The number of aggregates in clumps of ≥3, ≥5, and ≥10 were counted (**p* < 0.05). All values represent means ± SEM in triplicate cultures and are representative of three independent experiments.

also transiently expressed TβRIIIΔGAG and TβRIIIΔCYTO, and assessed their ability to regulate migration relative to full-length TβRIII. In comparison to full-length TβRIII, which significantly suppressed migration by >80% (*p* < 0.0001), TβRIIIΔGAG and TβRIIIΔCYTO suppressed migration by ~60% (*p* < 0.0001) and 50% (*p* < 0.0001) in RPMI-8226 cells, respectively (Figure 6A), suggesting a requirement for both the cytoplasmic domain and the glycosaminoglycan chains in mediating this effect. We next investigated the effect of shRNA-mediated silencing of endogenous TβRIII expression in the U266 myeloma cell line. Reducing TβRIII expression in U266 cells increased cell migration by ~80% (Figure 6B; *p* < 0.05). Taken together, these data indicate that TβRIII directly inhibits the ability of myeloma cells to migrate.

To address the extent to which TβRIII regulates migration independent of TGF-β superfamily signaling, we next examined the effects of TβRIII on migration in the context of the TGF-β superfamily ligands, BMP-2 and TGF-β1, as well as in the presence of the small

molecule TβRI/ALK-5 inhibitor. Although BMP-2, TGF-β1, and the small molecule TβRI/ALK-5 inhibitor all inhibited myeloma cell migration, in comparison to GFP-control RPMI-8226 myeloma cells, TβRIII-expressing myeloma cells significantly inhibited migration in the presence or absence of BMP-2, TGF-β1, or the small molecule TβRI/ALK-5 inhibitor (Figure 6C). These data suggest that TβRIII largely inhibits migration independent of its ligand presentation role.

DISCUSSION

The TβRIII coreceptor has a well-established role as a suppressor of cancer progression in a broad spectrum of epithelium-derived tumors (Dong *et al.*, 2007; Hempel *et al.*, 2007; Turley *et al.*, 2007; Finger *et al.*, 2008; Gordon *et al.*, 2008; Margulis *et al.*, 2008). Although the TGF-β signaling pathway has defined roles in multiple myeloma disease pathogenesis, little is known about the mechanisms that result in resistance of myeloma cells to the homeostatic functions of TGF-β. Here we investigated the role of TβRIII in multiple myeloma disease progression, demonstrating that TβRIII expression is decreased during disease progression at both the mRNA and protein level. Although the control bone marrow had a low number of plasma cells, we identified these cells with a certified hematopathologist and established that the plasma cells in normal patient specimens contained high TβRIII expression whereas plasma cell-derived myeloma cells had low TβRIII expression. TβRIII expression was also decreased/lost at the mRNA and protein level in the majority of human multiple myeloma cell lines analyzed. Thus, loss of TβRIII expression is the most frequent alteration in the TGF-β signaling pathway in multiple myeloma described to date. Importantly, restoring TβRIII expression decreased cell growth, proliferation, heterotropic adhesion, and migration in myeloma cells, whereas silencing of endogenous TβRIII expression increased cell growth, proliferation, heterotropic adhesion, and migration. On the basis of our findings, we believe that loss of TβRIII may contribute to the pathogenesis of multiple myeloma by promoting increased multiple myeloma cell growth, proliferation, migration, and adhesion to BMSCs.

How might the loss of TβRIII expression result in increased proliferation in multiple myeloma? As in other human cancers, resistance to TGF-β-mediated growth inhibition is a common event in multiple myeloma. The mechanisms of resistance to TGF-β-mediated growth inhibition in myeloma are unknown, however. In epithelial cancers, resistance to TGF-β-mediated growth inhibition may occur through reduced expression of TGF-β receptors or their signal transducers (Roberts and Wakefield, 2003; Hempel *et al.*, 2008). Based on the current data, the increased proliferation of myeloma cells could be due, at least partly, to decreased/lost TβRIII expression. Mechanistically, the antiproliferative effects of the TGF-β signaling pathway are

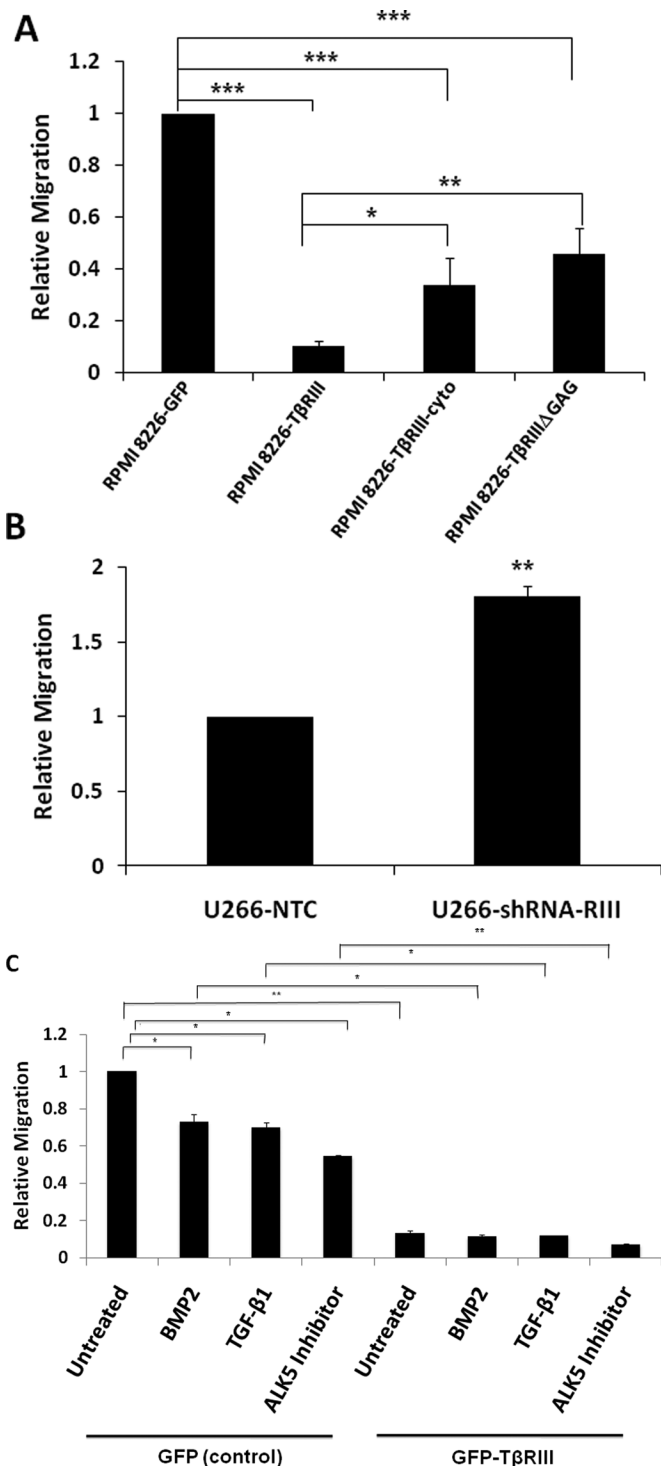


FIGURE 6: TβRIII decreases migration/chemotaxis of human multiple myeloma cells to BMSCs. (A) RPMI-8226 myeloma cells were adenovirally infected with GFP vector, GFP-TβRIII, GFP-TβRIII-cyto, or GFP-TβRIIIΔGAG and incubated for 48 h. Cells were resuspended in SFM, and 5×10^5 cells in 200 μ l were placed in the top of the transwell chamber and allowed to migrate to the bottom chamber (containing 700 μ l of 10% FBS-supplemented medium) for >12 h. Cells that migrated to the bottom chamber were then counted (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$). (B) U266 cells were adenovirally infected with NTC or shRNA-TβRIII and incubated for 72 h. Cells were resuspended in SFM, and 5×10^5 cells in 200 μ l were placed in the top of the transwell chamber and allowed to migrate to the bottom chamber (containing 700 μ l of 10% FBS-supplemented medium)

mediated through regulation of the cdk proteins that drive the G1 phase of the cell cycle, namely cdk2, cdk4, and cdk6 (Massague *et al.*, 2000). In epithelial cells from skin, lung, and breast, TGF- β increases the cdk4/cdk6 inhibitor, p15Ink4b, and the cdk2 inhibitor, p27Kip1 (Hannon and Beach, 1994; Warner *et al.*, 1999). In keratinocytes and in colon and epithelial cells, TGF- β also increases a p27-related inhibitor, p21Cip1 (Elbendary *et al.*, 1994; Datto *et al.*, 1995; Li *et al.*, 1995). Here we demonstrate that TβRIII increased expression of the cdk inhibitors, p21 and p27, and that this effect was enhanced by the TGF- β ligands, TGF- β 1, BMP-2, and BMP-4. In contrast, TβRIII did not induce apoptosis in myeloma cells. Thus, TβRIII appears to regulate myeloma cell proliferation through induction of p21 and p27. Although mechanisms for TGF- β -induced transcription of p21 and p27 have largely been established, the mechanisms by which TβRIII regulates their expression are currently under investigation.

The effects of TβRIII on multiple myeloma cell adhesion are quite intriguing, particularly as multiple myeloma pathogenesis is dependent on the interaction of multiple myeloma cells with the stroma. Binding of multiple myeloma cells to BMSCs triggers expression of adhesion molecules and secretion of cytokines that are known to promote myeloma cell growth, survival, drug resistance, and migration (Teoh and Anderson, 1997). Here we demonstrate that TβRIII decreased multiple myeloma cell adhesion to BMSCs, while increasing homotypic cell-cell adhesion. These effects are specific, as we also investigated the effect of TβRIII expression on adhesion of multiple myeloma cells to extracellular matrix proteins, fibronectin, laminin, and collagen and did not find any significant difference between TβRIII-expressing multiple myeloma cells versus control multiple myeloma cells (unpublished data). How might TβRIII specifically mediate differential effects on heterotropic and homotypic adhesion? One straightforward explanation is that increased homotypic interactions would leave less surface area available for interaction with stromal cells. This may be the case, but by using live cell imaging to track the behavior of individual cells, we demonstrated that TβRIII increases the initial interaction of multiple myeloma cells with BMSCs. The durability of this interaction is decreased, however, relative to multiple myeloma cells in the absence of TβRIII. These results suggest that, although TβRIII may be functioning, in part, as an adhesion receptor, upon multiple myeloma-stroma contact, additional adhesion receptor interactions and/or signaling may regulate the duration of this interaction. A role of TβRIII-mediated signaling in regulating multiple myeloma cell adhesion is further supported by the dependence of the TβRIII cytoplasmic domain for both heterotropic and homotypic adhesion. The importance of myeloma cell-stromal cell adhesion for survival and for resistance to chemotherapeutic drugs is well documented (Uchiyama *et al.*, 1993; Hazlehurst *et al.*, 2000), suggesting that treatments designed to restore TβRIII expression/function in multiple myeloma may represent an attractive therapeutic target.

for >12 h (** $p < 0.001$). All values represent means \pm SEM in triplicate cultures and are representative of three independent experiments. (C) RPMI-8226 myeloma cells were adenovirally infected with GFP vector or GFP-TβRIII. After 48 h of infection, cells were resuspended in SFM, and 5×10^5 cells in 200 μ l were placed in the top of the transwell chamber and treated with vehicle control, 10 nM BMP-2, 200 pM TGF- β 1, or 5 μ M ALK5 inhibitor. Migration toward 700 μ l of 10% FBS-supplemented medium was allowed for 12 h, and the myeloma cells that had migrated to the bottom chamber were subsequently counted (* $p < 0.05$, ** $p < 0.001$).

Cell migration involves dynamic regulation of adhesion events. We have previously demonstrated in several epithelial tumors that T β RIII inhibits cancer cell migration, invasion, and metastasis in vitro and in vivo (Dong *et al.*, 2007; Hempel *et al.*, 2007; Turley *et al.*, 2007; Finger *et al.*, 2008; Gordon *et al.*, 2008). Mechanistically, we have demonstrated that T β RIII-mediated inhibition of migration occurs through β -arrestin2-mediated Cdc42 activation, which alters actin cytoskeleton organization to decrease directional persistence (Myhre and Blobel, 2009). Furthermore, T β RIII inhibits both random and chemotactic migration, and was independent of canonical TGF- β signaling (Myhre and Blobel, 2009). Unlike most epithelial tumors, however, which have the propensity to metastasize to distant organs, multiple myeloma malignant plasma cells often home to their native bone marrow, where they are dependent on interactions within their environment. In the present study, we demonstrate that T β RIII has inhibitory effects on chemotactic multiple myeloma cell migration. Mechanistically, the T β RIII-mediated inhibition was at least partially dependent on its cytoplasmic and glycosaminoglycan chain domains. The relative contribution of T β RIII's effects on adhesion and on signaling to Cdc42 and the actin cytoskeleton on multiple myeloma cell migration are currently under investigation.

The genomic locus for T β RIII, TGFBR3, is on the short arm of chromosome 1, 1p32, a region that is frequently deleted in human epithelium-derived cancers, including cancers of the breast, colon, endometrium, kidney, lung, ovary, stomach, and testis (Johnson *et al.*, 1995; Ragnarsson *et al.*, 1999). We have demonstrated loss of heterozygosity at the TGFBR3 locus in 37–50% of breast cancer, non-small cell lung cancer, and prostate cancer patients (Dong *et al.*, 2007; Turley *et al.*, 2007; Finger *et al.*, 2008). Additionally, the short arm of chromosome 1 has been reported to be lost in multiple myeloma patients (Taniwaki *et al.*, 1994; Shaughnessy *et al.*, 2007). We have also previously demonstrated that loss of T β RIII expression in human cancers could occur through epigenetic regulation or TGF- β 1-mediated down-regulation of TGFBR3 at the transcriptional level (Dong *et al.*, 2007; Hempel *et al.*, 2007; Turley *et al.*, 2007; Finger *et al.*, 2008; Hempel *et al.*, 2008). As multiple myeloma patients have higher concentrations of TGF- β 1, this increase may represent another mechanism for suppression of T β RIII expression. The mechanism by which T β RIII expression is decreased and whether T β RIII is a tumor suppressor gene on chromosome 1p32 in multiple myeloma remain to be defined.

In summary, the current findings define a novel role for T β RIII in the pathophysiology of multiple myeloma as an inhibitor of proliferation, heterotropic cell–cell adhesion, and migration, all largely independent of its ligand presentation role. These results provide a model to study myeloma plasma cell dissemination within the bone marrow microenvironment. Whether T β RIII plays a similar role in other hematologic malignancies and whether therapies designed to restore T β RIII function could circumvent chemotherapeutic resistance remain to be explored.

MATERIALS AND METHODS

Cell culture and reagents

Human multiple myeloma cell lines NCI-H929, OPM2, RPMI-8226, and U266 and human BMSC line HS-5 were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were maintained in RPMI-1640 medium supplemented with 10% FBS. The cells were grown in 5% CO₂ at 37°C in a humidified atmosphere. In all assays, RPMI-8226 myeloma cells were adenovirally infected with GFP vector or GFP-T β RIII and incubated for 48 h. U266 myeloma cells were adenovirally infected with NTC or shRNA-T β RIII and incubated

for 72 h before initiation of the assay. In all experiments, infected RPMI-8226 and U266 cells were sorted, gating on GFP or dsRED, using a FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ), and the adenovirally infected cells were used.

T β RIII protein analysis on bone marrow tissue array

Immunohistochemical studies were done on a paraffin-embedded tissue microarray containing myeloma and normal tissue controls with duplicate cores per case (US Biomax, Rockville, MD). Tissues were probed with a purified anti-human T β RIII-specific polyclonal antibody. Immunoreactivity and specificity of this antibody for T β RIII has been previously verified in immunohistological studies in our laboratory (Dong *et al.*, 2007). Following rehydration and blocking with 1% hydrogen peroxide, 10% goat serum/phosphate-buffered saline (PBS), and an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), tissue samples were incubated overnight at 4°C with T β RIII-specific antibody or preimmune serum at a dilution of 1:200–400 (vol:vol) in 10% goat serum/PBS. Following secondary antibody incubation with biotinylated anti-rabbit IgG at room temperature for 1 h, tissues were incubated with Vectastain ABC reagent (Vector) for 30 min, and immunoreactivity was visualized using the avidin-biotin complex immunoperoxidase system and diaminobenzidine (Vector). Slides were counterstained with hematoxylin, and immunoreactivity for T β RIII in the specimen was scored by staining intensity in a blinded manner with 0–1 = no or weak staining, 2–3 = moderate staining, and 4–5 = intense staining. Standards for each staining score were used to maintain consistent scoring across specimens.

TGF- β binding and cross-linking assay

Cells were incubated with Krebs-Ringer-HEPES (KRH) buffer (50 mM HEPES, pH 7.5, 130 mM NaCl, 5 mM MgSO₄, 1 mM CaCl₂, and 5 mM KCl) containing 0.5% bovine serum albumin (BSA) for 30 min at 37°C, and then with 100 pM [¹²⁵I]-TGF β 1 for 3 h at 4°C. [¹²⁵I]-TGF β 1 was cross-linked with 0.5 mg/ml disuccinimidyl suberate for 15 min and quenched with 20 mM glycine for 10 min. Cells were then washed with KRH buffer, lysed in RIPA buffer, and immunoprecipitated at 4°C overnight using a polyclonal antibody directed toward the extracellular domain of T β RIII (R&D Systems, Minneapolis, MN) bound to protein G sepharose beads. All samples were analyzed by SDS-PAGE and phosphorimaging analysis of dried gels.

Adenoviral constructs

All adenoviral constructs were made using the Becton Dickinson Adeno-X expression system (BD Biosciences), purified using the Adeno-X Virus Purification Kit and titered using the Adeno-X Rapid Titer Kit. The T β RIII and NTC shRNA sequences were generated by Dharmacon (Lafayette, CO).

Cell growth

Cells were plated at 1.0×10^5 cells/ml in 24-well plates. Cell growth was measured by trypan blue exclusion.

[³H]Thymidine incorporation assay

Cell sorting was done on the adenovirally infected myeloma cell lines by gating the GFP- and dsRED-expressing cells and subtracting out the uninfected cells. Adenovirally infected GFP- or dsRED-expressing cells were then concentrated using a FACSAria cell sorter (BD Biosciences) and plated at 1.0×10^4 cells/ml in 96-well, flat-bottom culture plates to a final volume of 200 μ l. After incubation for 24 h, cultures were pulsed with 1 μ Ci [³H]thymidine (Amersham Biosciences, Piscataway, NJ) for 4 h, harvested, and counted in a

MicroBeta Trilux liquid scintillation counter (EG&G Wallac, Turku, Finland) in triplicate.

Apoptosis analysis

Dual staining with allophycocyanin conjugate (APC) annexin V and 7-aminoactinomycin D (7-AAD) was used to detect apoptosis. Flow cytometric analysis was performed using FACScan (BD Biosciences). Myeloma cells were treated with or without 50 nM bortezomib (IC50 determined by MTT assay) for 6 h as a positive control for apoptosis. Cells were then washed with PBS and stained with APC annexin V (AN) and 7-AAD according to the manufacturer's protocol (catalogue #550475; BD Biosciences, San Jose, CA). Ten thousand cells were analyzed by flow cytometry and gated for GFP+ or dsRED+ expressing cells, and then AN-/7-AAD-, AN+/7-AAD-, and AN+/7-AAD+ populations were enumerated, which correspond to live cells, early apoptotic cells, and both late apoptotic and necrotic cells, respectively. Data were analyzed using FlowJo software.

Immunoblot analysis

Myeloma cells were treated with TGF- β superfamily ligands, BMP-2 (0–10 nM), BMP-4 (0–10 nM), TGF- β 1 (0–200 pM), or vehicle for the indicated time points. In the apoptosis experiment, cells were treated with or without bortezomib as described in the previous section. Lysates were harvested with hot sample buffer and boiled for 5 min. Lysates were separated using 10 or 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Western blotting was performed using the α -p21, p27, phospho-Smad2, total Smad 2, phospho-Smad1/5, and total Smad1/5 antibodies (Cell Signaling Technology, Danvers, MA). Western blotting for T β RIII expression was performed using an antibody directed toward the extracellular domain of T β RIII (R&D Systems) and an α - β -actin antibody (Sigma, St. Louis, MO) to control for protein loading.

Adhesion assay

HS-5 cells were plated at a density of 10–20 \times 10³ per well in black, 96-microwell, flat-bottom polystyrene plates and allowed to adhere for 24 h. The indicated adenovirally infected multiple myeloma cell lines RPMI-8226 and U266 (1–2 \times 10⁵) were washed three times with PBS, resuspended in serum-free RPMI medium (SFM) with 5 μ M calcein-blue AM (BD Biosciences) for 30 min at 37°C and 5% CO₂. The cells were then washed in PBS, resuspended in SFM + 1% BSA, and plated onto the BMSC-coated, 96-well plates (HS-5) for 2 h. Nonadherent myeloma cells were removed by carefully washing twice with SFM and inverting the plates. SFM (200 μ l) was then added to each well. Adherent cells were quantified in a fluorescence multiwell plate reader (Wallac Victor2). Triplicate cultures were set up for every cell population tested.

Live cell imaging

For live cell imaging of the coculture experiments, HS-5 cells were plated on 35-mm glass-bottom dishes (MatTek, Ashland, MA) at a density of 10⁴ cells per well in regular culture medium and allowed to adhere for 24 h. Cells were then washed twice in PBS. The indicated adenovirally infected (GFP vector or GFP-T β RIII) RPMI-8226 multiple myeloma cells suspended in SFM + 1% BSA (5 \times 10³) were then plated onto the BMSC-coated plates (HS-5) and placed in a temperature- and CO₂-controlled chamber. A Zeiss (Thornwood, NY) Axio Observer Z1 motorized microscope equipped with 20, 40, and 100 \times objective lenses was used for the imaging. Time-lapse recordings were started ~10 min after cells were cocultured with in-

fecting RPMI-8226 myeloma cells. The images were collected at 1-min intervals for >120 min with a cooled charge-coupled device (CCD) video camera (Coolsnap ES high resolution CCD camera) operated by a Metamorph image analysis software (Molecular Devices, Sunnyvale, CA). The adhesion parameters, including percent total cells and time (in minutes) that the infected RPMI-8226 myeloma cells interacted with the HS-5 BMSCs, were obtained from time-lapse movies. Cells were manually traced for each cell using Metamorph to track the adhesion characteristics of the individual cells. The percentage of infected myeloma cells that interacted with the BMSCs was calculated as the percentage of total myeloma cells that were at least 50% in contact or buried underneath the HS-5 BMSC. The time that the infected myeloma cells spent interacting with the BMSCs was calculated as both time (minutes) that the myeloma cells spent within a 15 μ m perimeter of the HS-5 BMSCs and the time (minutes) that the myeloma cells spent in contact (>50%) with HS-5 BMSCs before exiting. At least 35 cells were counted for each experiment during the 120-min time period.

Aggregation assay

RPMI-8226 multiple myeloma cells were adenovirally infected with GFP vector, GFP-T β RIII, GFP-T β RIII Δ CYTO (mutant lacking the cytoplasmic domain), and GFP-T β RIII Δ GAG (mutant lacking glycosaminoglycan chains). After 48 h of incubation at 37°C and 5% CO₂, the cells were placed into a 96-microwell plate with RPMI 1640 medium supplemented with 10% FBS. U266 cells were adenovirally infected with NTC or shRNA-T β RIII and incubated for 72 h. After the myeloma cells were dispersed with a pipette, aggregation was observed at the indicated time points. The number of aggregates in clumps of \geq 3, \geq 5, and \geq 10 were counted. Triplicate cultures were set up for every cell population tested.

Migration assay

The indicated adenovirally infected RPMI-8226 and U266 myeloma cells were washed three times with PBS and resuspended in SFM at a density of 5 \times 10⁵ cells in 200 μ l in the upper chamber of a transwell filter (8 μ m pore transwell insert; Costar, Cambridge, MA) to assess cell migration/chemotaxis. As indicated, cells were treated with either no ligand, 10 nM BMP-2, 200 pM TGF- β 1, or 5 μ M ALK5 inhibitor. Cells were allowed to migrate for 12 h at 37°C toward the lower chamber containing 700 μ l of RPMI 1640 medium with 10% FBS. Cells that migrated to the bottom chamber were then counted by trypan blue exclusion. Duplicate cultures were set up for every cell population tested.

Statistical analysis

Significance of results was assessed using Student's t test for paired samples; * p < 0.05, ** p < 0.001, *** p < 0.0001. Error bars, where indicated, represent SEM (n = 3).

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