Insulin-Like Growth Factor I Suppresses Bone Morphogenetic Protein Signaling in Prostate Cancer Cells by Activating mTOR Signaling

Reema S. Wahdan-Alaswad¹,², Kyung Song¹, Tracy L. Krebs¹, Dorjee T.N. Shola¹,⁴, Jose A. Gomez¹,², Shigemi Matsuyama¹,²,³, and David Danielpour¹,²,⁵

Abstract

Insulin-like growth factor (IGF) I and bone morphogenetic proteins (BMP) are critical regulators of prostate tumor cell growth. In this report, we offer evidence that a critical support of IGF-I in prostate cancer is mediated by its ability to suppress BMP4-induced apoptosis and Smad-mediated gene expression. Suppression of BMP4 signaling by IGF-I was reversed by chemical inhibitors of phosphoinositide 3-kinase (PI3K), Akt, or mTOR; by enforced expression of wild-type PTEN or dominant-negative PI3K; or by small hairpin RNA-mediated silencing of mTORC1/2 subunits Raptor or Rictor. Similarly, IGF-I suppressed BMP4-induced transcription of the Id1, Id2, and Id3 genes that are crucially involved in prostate tumor progression through PI3K-dependent and mTORC1/2-dependent mechanisms. Immunohistochemical analysis of non-malignant and malignant prostate tissues offered in vivo support for our model that IGF-I–mediated activation of mTOR suppresses phosphorylation of the BMP-activated Smad transcription factors. Our results offer the first evidence that IGF-I signaling through mTORC1/2 is a key homeostatic regulator of BMP4 function in prostate epithelial cells, acting at two levels to repress both the proapoptotic and pro-oncogenic signals of BMP-activated Smads. We suggest that deregulation of this homeostatic control may be pivotal to the development and progression of prostate cancer, providing important implications and new potential targets for the therapeutic intervention of this malignancy.

Introduction

Bone morphogenetic proteins (BMP) are multifunctional cytokines belonging to the transforming growth factor (TGF)β superfamily that play critical roles in osteogenesis, organogenesis, and embryogenesis, in which they control cell differentiation, proliferation, migration, and apoptosis (1–6). BMP signaling is initiated by the association of a BMP ligand (any 1 of ≥14 isoforms) with two transmembrane serine/threonine receptor kinases: BMP receptors II and I (typically BMP receptors IA and IB), the latter of which directly phosphorylate the transcription factors Smads 1, 5, and 8 (1–6). The phosphorylated Smads then couple to Smad4 and translocate to the nucleus, where they modulate the transcription of numerous genes in part by binding to BMP response elements. Whereas BMPs function as tumor suppressors in early-stage prostate cancer, they are reported to also promote progression of advanced/hormone refractory prostate cancer (7–9). However, the mechanisms underlying this functional dichotomy are poorly understood but likely involve the combined action of multiple gene changes.

Insulin-like growth factor (IGF)-I is a well-known survival factor for both normal and malignant cells in many tissues, including the prostate (10, 11), although IGF-I has been shown to also be critical in controlling the differentiation of many tissues through mechanisms that remain underexplored (12–15). The survival function of IGF-I seems to be predominantly through a signal transduction cascade involving phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR; refs. 11, 16, 17). Numerous studies collectively suggest that enhanced IGF-I signaling is critical for the development and progression of prostate cancer (11). Importantly, correlative studies have linked high plasma IGF-I levels and prostate cancer risk (18). Moreover, transgenic mice overexpressing IGF-I in the prostate basal epithelial layer develop prostate cancer (19), strongly implicating high IGF-I levels in the etiology of prostate cancer. Significantly, functional loss of PTEN, which induces the development of prostate cancer in knockout mice, leads to activation of Akt, a critical component of the survival and oncogenic function of IGF-I (11, 20).

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Recent studies show that IGF-I can inhibit TGF-β transcriptional activity by selective suppression of Smad3 activation through a PI3K/Akt-dependent mechanism (21). Further work has implicated mTOR in such regulation (22); however, the mechanism of how mTOR intercepts TGF-β signaling remains to be defined. With the use of rat and human prostate epithelial cell lines, we provide the first evidence that IGF-I suppresses BMP4-induced cell death; activation of Smads 1, 5, and/or 8; as well as induced expression of BMP4 target genes through a mechanism dependent on the PI3K, Akt, mTOR, Raptor, and Rictor signaling pathway. Particularly intriguing is our observation that this IGF-I signaling pathway clearly represses the ability of BMP4 to induce expression of inhibitor of differentiation/DNA binding (Id)1, Id2, and Id3, proteins whose overexpression promotes growth and progression of prostate cancer (23–25). Our results support that the ability of mTOR to repress BMP signaling is part of an important homeostatic switch that is deregulated in prostate cancer.

Materials and Methods

Materials
Recombinant human BMP4 and TGF-β1; anti-Id1 antibody (R&D Systems, Inc.), Stemfactor Recombinant human BMP4 (Stemgent); LY294002 and rapamycin (BioMol), perifosine (Selleck Chemicals LLC); anti–phospho-Smad3 antibody (P-Smad1/3/5/8); anti–phospho-Smad1/5/8 antibody (P-Smad1/5/8), anti–phospho-Smad2 (Cell Signaling); anti-Smad2 antibody (Transduction Laboratories); anti-Smad3 and anti-Smad1 (Santa Cruz Biotechnology, Inc.); IGF-I and LR3–IGF-I (GroPep); DMEM/Ham’s F-12 (1:1); characterized fetal bovine serum (FBS) (HyClone Inc.); insulin (BioSource International); cholera toxin and dexamethasone (Sigma); pCEP4-PTEN (Dr. Ramon Parsons, Institute for Cancer Genetics, Columbia University, NY, NY, USA); DN-P13K (pSG5-p85αΔSH2) and CA-P13K (pSG5-p110αCAAX; gift from Dr. Downward, Signal Transduction Laboratory, Cancer Research UK Downstream Protein Analysis Laboratory, London, UK), and DN-Akt1 (pUSE-Myc-Akt1ΔK79M, Upstate Biotechnology, Inc.) were used.

Cell culture
The LNCaP, PC3, RWPE-1, VCaP, and DU145 cell lines were obtained from American Type Culture Collection (ATCC) and maintained in either DMEM/Ham’s F-12 containing 5% to 10% FBS or keratinocyte medium (RWPE-1). All above cell lines were authenticated by ATCC with the use of DNA profiling, cytogenetic analysis, and used within 20 passages of authentication. The NRP-152 and DP-153 cell lines were developed in our laboratory and maintained in GM2.1 and GM2, respectively, as previously described (22). The NRP-152 and DP-153 cell lines were authenticated by karyotype and isozyme analysis, and used within 20 passages of authentication. All above cell lines were confirmed to be free of mycoplasma contamination by the MycoAlert Mycoplasma Detection kit (Cambrex Bio Science).

Cell viability assay
Cell viability was assessed by trypan blue exclusion under phase-contrast microscopy as before (26). See the supplementary section for specific details.

Hoechst 33258 staining
Cells were plated in six-well dishes at a density of 3 × 10^4 to 5 × 10^4 cells per well in 2 mL of DMEM/Ham’s F-12, 1% FBS, and 15 mmol/L HEPES (pH 7.4; for LNCaP, PC3, DU145), or in GM3.1 (for HRG-152, DP-153). Cells were treated with vehicle or LR3–IGF-I (10 nmol/L) 24 hours before BMP4 (5 ng/mL) addition. After 24 to 48 hours, cells were stained with 10 μg/mL Hoechst 33258 (Sigma), and apoptotic cells were counted with the use of fluorescent microscopy. Three hundred cells were analyzed in triplicate (27).

Flow cytometry
Detached cells (1.5 × 10^5) were washed once with PBS, fixed with 90% methanol, sequentially incubated with 0.1 mg/mL of RNase A followed by 50 μg/mL of propidium iodide, and then analyzed with an EPICS XL-MCL flow cytometer. Sub-G1 cells, which have <2n DNA content, are considered to be apoptotic.

Cell number assay
Cells (3 × 10^4 to 5 × 10^4 cells/1 mL) were seeded in 12-well dishes in medium described in Hoechst staining assay. The next day, cells were pretreated with ± LR3–IGF-I (10 mmol/L) for 24 hours before ± BMP4 (5 ng/mL) treatment for up to 72 hours. Adherent cells were detached by trypsinization and enumerated with a Coulter Electronics counter.

Id1 promoter assay
Cells were plated overnight at a density of either 1.0 × 10^5 cells/1 mL/well or 2.0 × 10^5 cells/2 mL/well in 12-well or 6-well dishes, respectively, transfected as before (21, 22, 28) with the human Id1 promoter construct (pGL2-Id-1; 1-2 kb) and 20 ng of cytomegalovirus-renilla reporter constructs. Transfection reagents were washed off 3 hours later, and cells were allowed to recover overnight in the low serum conditions and then pretreated with LY294002 (10 μmol/L), perifosine (5 μmol/L), rapamycin (200 nmol/L), or vehicle 2 hours before ± LR3–IGF-I (10 mmol/L, 24 h) followed by ± BMP4 (5 ng/mL, 24 h). Luciferase activity was measured with the Promega Dual-Luciferase Assay kit and ML300 Microtiter Plate Luminometer.

Western blot cell viability assay, reverse transcriptase-PCR (RT-PCR), PCR primers, real-time quantitative PCR, adenovirus, lentivirus, immunohistochemistry, and microarray preparation
(See supplementary information).

Results
Responsiveness of prostatic epithelial cell lines to the TGF-β superfamily ligands
Previous work from our laboratory showed that epithelial cell lines (NRP-152, NRP-154) derived from the preneoplastic...
prostate of the Lobund/Wistar rat are exquisitely sensitive to the induction of apoptosis by TGF-β (29). We examined the general responsiveness of NRP-152 cells versus a metastasis-derived PTEN-null human prostate cell line, PC3, to various members of the TGF-β superfamily [TGF-β1, activins (A, B, or AB), BMP4, Müllerian inhibiting substance, Nodal, or Cripto] by their ability to phosphorylate various Smads, as assessed by Western blot with the use of various phospho-Smad antibodies (Fig. 1A; Supplementary Fig. S1). Due to lack of complete isoform specificity of the antibodies available for phospho-Smads 1, 3, 5, and 8, we used an anti-phospho-Smad1/5/3/8 (antibody 1), which recognizes two specific bands [phospho-Smads 1, 5, and 8 (top), and phospho-Smad3 (bottom)], and an anti–phospho-Smad1/5/8 specific antibody (antibody 2; Fig. 1A). In both cell lines TGF-β1 and activin B specifically activated Smads 2 and 3, but not Smads 1, 5, or 8; and BMP4 specifically activated Smads 1, 5, and/or 8 (for simplicity, designated Smad1/5/8), but not Smads 2 or 3. We were unable to detect activation of Smads by Müllerian inhibiting substance, Nodal, or Cripto in either cell line under these conditions. NRP-152 and PC3 cells are thus the most sensitive to TGF-β1 and BMP4 (at the indicated concentrations) among the TGF-β superfamily ligands examined.

Figure 1. Biological activity of TGF-β superfamily ligands on prostate epithelial cell lines. A, NRP-152 cells were treated with ± TGF-β1 (0-10 ng/mL), activin (A, B, AB; 10 ng/mL), BMP4 (0-10 ng/mL), or Müllerian inhibiting substance (10 ng/mL), Nodal (10 ng/mL), and Cripto (10 ng/mL) for 24 hours and analyzed for Smad activation by Western blot with the use of antibodies against the two c-terminal serines of phospho-Smads 1, 3, 5, 8 (Ab #1), phospho-Smad1/5/8 (Ab #2), and phospho-Smad2. B, NRP-152, DP-153, LNCaP, PC3, and DU145 cells treated with BMP4 (0-20 ng/mL) for 72 hours, and total adherent cells were enumerated with a Coulter Electronics counter. Values, averages of triplicate determinations ± SE.
We next assessed the ability of BMP4 to affect growth of a panel of nontumorigenic (NRP-152, DP-153) and tumorigenic (LNCaP and VCaP) prostate epithelial cell lines (see Materials and Methods; Fig. 1B). All the above cell lines were to various degrees growth suppressed by BMP4, with greater cytostatic activity occurring in the nontumorigenic (NRP-152, DP-153) and androgen-responsive tumorigenic (LNCaP) cell lines than in the androgen refractory tumor lines (PC3 and DU145). Thus,

![Graphical representation of assay results](image_url)
BMP4 seems to be more cytostatic on premalignant or early-stage prostate cancer cells than on late-stage ones.

**IGF-I reverses growth suppression of prostate epithelial cells by BMP4**

Based on various published reports and our results in Fig. 1B, we speculated that the cytostatic activity of BMP4 was lost during prostate carcinogenesis by the activation of IGF-I signaling, similar to our previous report on the repression of TGF-β responses by IGF-I (21). In a time course experiment during which 72 hours of BMP4 (10 ng/mL) treatment caused a 65% loss in NRP-152 cell number (Fig. 2A), such cell death was effectively repressed by pretreatment with 2 to 10 nmol/L LR3–IGF-I (Fig. 2B), an analogue that shares similar affinity to the IGF-I receptor but is essentially unable to bind to IGF-I binding proteins.

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**Figure 3.** LR3–IGF-I abrogates BMP4-induced activation of Smad1/5/8, and Id1, Id2, and Id3 expression. A, NRP-152 cells were treated with ± LR3–IGF-I (10 nmol/L) for 24 hours followed by ± BMP4 (10 ng/mL) for 4 hours, and cell lysates were analyzed by Western blot (top), or NRP-152 cells were cotransfected with 25 ng of cytomegalovirus (CMV)-renilla reporter construct and 1 μg of Id1-luciferase reporter element 24 hours before ± LR3–IGF-I (10 nmol/L, 24 h), and then treated with ± BMP4 (10 ng/mL, 4 h). Dual-luciferase activity was then assayed, and relative values of firefly luciferase were normalized to renilla luciferase (bottom). Columns, average of triplicate determinations; bar, ± SE. B, expression of Id1, Id2, and Id3 mRNAs in NRP-152 (B) or LNCaP (C) cells treated with ± LR3–IGF-I (10 nmol/L) for 24 hours followed by ± BMP4 (10 ng/mL) for 4 hours. C, RWPE-1 and DU-145 were treated as specified in 3A, and cell lysates were analyzed by Western blot for phospho-Smad1/5/8 activation and Id1 expression. D, real-time quantitative PCR examined expression of Id1 mRNA in NRP-152 cells ± LR3–IGF-I (10 nmol/L) for 24 hours followed by ± BMP4 (10 ng/mL) for a total of 48 hours; bottom, semiquantitative PCR. Data are representative of three independent experiments.
We next characterized the ability of LR3–IGF-I to suppress the cytostatic activity of BMP4 on NRP-152 cells by measuring changes in apoptosis using three different assays (Fig. 2C). In the first method, NRP-152 cells were pretreated with ± LR3–IGF-I (10 nmol/L) for 24 hours followed by ± BMP4 (5 ng/mL) for 24 to 72 hours, and apoptosis was identified by nuclear condensation and fragmentation under fluorescent (white arrows) microscopy following Hoechst 33258 staining (Fig. 2C, left; Supplementary Fig. S2). BMP4 caused markedly increased numbers of apoptotic nuclei (~40% of cells) over control, whereas cells pretreated with LR3–IGF-I were significantly blocked in BMP4-induced apoptosis (~8% of cells). These results were consistent with changes in cell viability (trypan blue exclusion) and apoptotic fraction (sub-G1 by flow cytometric analysis) at 72 hours (Fig. 2C, middle, right). The sub-G1 fraction of the cells showed that BMP4 induced apoptosis in ~11% of the cells compared with vehicle control (~4%). LR3–IGF-I treatment blocked the percent sub-G1 fraction in each group to equal to or less than that of vehicle only (~3%). There was no significant change in the fraction of cells in G1, but there was an increase in the fraction in G2–M (Supplementary Fig. S3). Together, these studies confirm LR3–IGF-I effectively blocks the ability of BMP4 to induce apoptosis of NRP-152 cells.

We also examined the effect of LR3–IGF-I on the cytostatic effect of BMP4 in other prostate cell lines, including LNCaP, PC3, RWPE-1, VCaP, and DP-153 cells. LR3–IGF-I reversed the ability of BMP4 to suppress growth or induce cell death, as shown morphologically and by enumerating cells with the use of a Coulter counter (Fig. 2D; Supplementary Fig. S4). These results support the universality of IGF-I receptor signaling on reversing the cytostatic activity of BMP4 on prostate epithelial cells.

**Effect of IGF-I on activation of Smads by BMP4**

To explore the mechanism by which IGF-I intercepts BMP signaling, we assessed the ability of LR3–IGF-I to affect BMP4-induced activation of Smad1/5/8 in NRP-152 cells (Fig. 3A). We pretreated these cells with ± LR3–IGF-I (2 or 10 nmol/L) or insulin (1 μmol/L) for 24 hours, stimulated them with BMP4 (10 ng/mL) for 4 hours, and then analyzed levels of phospho-Smad1/5/8 by Western blot as in Supplementary Fig. S5. NRP-152 cells treated with BMP4 showed robust activation of Smad1/5/8, which was suppressed by 10 nmol/L LR3–IGF-I or 1 μmol/L insulin. Similar results were observed in RWPE-1 and DU-145 human prostate epithelial cell lines (Fig. 3C). To define how rapidly IGF-I suppresses BMP4-induced activation of Smad1/5/8, we pretreated NRP-152 cells with LR3–IGF-I for various times before 4 hours of treatment with BMP4 (Fig. 3A). Phosphorylation of Smad1/5/8 by BMP4 was suppressed as early as 1 hour pretreatment with LR3–IGF-I, with no change in levels of total Smad1/5/8.

**IGF-I represses transcriptional activation of Id1 by BMP4**

Given that Id proteins are transcriptionally induced by BMPs through Smad1/5/8, and IGF-I blocks this activation, we hypothesized that IGF-I suppresses the expression of the helix-loop-helix Id proteins, a well-known Smad-dependent transcriptional target of BMP (30). BMP4 rapidly (~4 h) induced Id1 protein levels in NRP-152 cells, and such induction was significantly repressed by 1 hour of IGF-I pretreatment (Fig. 3A), reflecting the general pattern of Smad phosphorylation. Additionally, we showed that 1-hour pretreatment with LR3–IGF-I also reversed BMP4-induced (5 ng/mL, 4 h) Id1 promoter activity in both NRP-152 and LNCaP cells transiently transfected with a pGL2-Id1 promoter construct containing a number of BMP response elements (BREs) [ref. 30; Fig. 3A (bottom); Supplementary Fig. S6].

Semi-quantitative RT-PCR was used to assess the ability of LR3–IGF-I to suppress BMP4-induced levels of Id1, Id2, and Id3 mRNAs in NRP-152 cells (Fig. 3B). BMP4 induced expression of all three Id mRNAs within 4, and 4-to-24 hours of pretreatment with LR3–IGF-I suppressed such induction. A similar response was observed in the LNCaP cell line for Id1 mRNA (Fig. 3B). However, for reasons not clear, the suppression of Id1 mRNA levels (Fig. 3B) was delayed relative to suppression of Id1 protein levels (Fig. 3A). Real-time quantitative PCR confirmed our semi-quantitative RT-PCR data that IGF-I effectively blocked BMP-induced Id1 mRNA expression (Fig. 3D). Overall, these data suggest that IGF-I blocks BMP4-mediated expression of Id1, Id2, and Id3 in prostate epithelial cells through a transcriptional mechanism involving suppression of the phosphorylation of Smad1/5/8.

**Role of the PI3K/Akt/mTOR pathway in mediating IGF-I suppression of BMP responses**

The PI3K/Akt pathway, which is generally hyperactivated in prostate cancer, is believed to play a prominent role in the survival function of IGF-I. We thus hypothesized that IGF-I inhibits BMP responses through a PI3K-dependent mechanism. To test this hypothesis, we cotransfected NRP-152 cells with Id1-luciferase construct along with constitutive active PI3K (CA-PI3K), dominant-negative PI3K (DN-PI3K), or empty vector control (pSG5), and then added ±10 nmol/L LR3–IGF-I for 2 hours followed by BMP4 (5 ng/mL) for 24 hours before luciferase assay (Fig. 4A, left). As anticipated, CA-PI3K suppressed BMP-induced Id1-luciferase reporter activity, whereas DN-PI3K reversed LR3–IGF-I inhibition of this BMP response. A highly selective inhibitor of PI3K, LY294002, reversed the suppressive action of LR3–IGF-I on BMP4-induced Id1 promoter activity (Fig. 4A, right). Similar results were obtained with the Akt inhibitor perifosine (Fig. 4B) or the mTOR inhibitor rapamycin (Fig. 4C). These results strongly suggest that the IGF-I suppression occurs downstream of Akt and mTOR.

Overall, the above results suggest that the PI3K/Akt/mTOR mediate the ability of IGF-I to suppress the activation of Smad1/5/8 by BMP4 and, hence, activation of the Id1 promoter. To confirm our model, we examined the effect of LY294002, rapamycin, or perifosine on the ability of LR3–IGF-I to suppress BMP-induced Smad activation under conditions as in Fig. 4B and C, except that cells were...
treated with BMP4 for 4 hours and harvested for Western blot analysis (Fig. 4D and data not shown). Clearly, LY294002, perifosine, and rapamycin each reversed the ability of LR3–IGF-I to suppress the activation of Smads by BMP4. We also used adenoviral-mediated gene delivery to efficiently overexpress DN-PI3K or DN-Akt in NRP-152 cells. As expected, overexpression of either DN-PI3K or DN-Akt enhanced BMP-induced phospho-Smad1/5/8 levels through a PI3K/Akt/mTOR-dependent mechanism. (A) NRP-152 cells were transfected with 0.8 μg of expression constructs for control (pSG5), DN-PI3K, or CA-PI3K, and co-transfected with Id1-luciferase as described above for 24 hours followed by treatment with ± LR3–IGF-I (10 nmol/L) or vehicle for 24 hours before ± BMP4 (5 ng/mL); luciferase activity was measured after 24 hours. (A) NRP-152 cells were cotransfected with 20 ng of CMV-renilla reporter and 1 μg of Id1-luciferase constructs; 24 hours later cells were incubated with ± LY294002 (10 μmol/L) for 2 hours, followed by ± LR3–IGF-I (10 nmol/L) or vehicle for 24 hours. Cells were then treated with ± BMP4 (5 ng/mL) and luciferase activity measured after 24 hours. (B) and (C), NRP-152 cells were transfected with Id1-luciferase reporter element as described in (B) and then incubated with either ± perifosine (10 nmol/L) or ± rapamycin (200 nmol/L) for 2 hours, followed by ± LR3–IGF-I (10 nmol/L) for 24 hours. Cells were then treated with ± BMP4 and assayed for luciferase 2 hours later. (D), NRP-152 cells were pretreated with 10 μmol/L LY294002 or 200 nmol/L rapamycin for 2 hours followed by ± LR3–IGF-I (10 nmol/L) or vehicle for 24 hours, and then treated with ± BMP4 (5 ng/mL) for 4 hours. Western blot analysis was conducted for P-Smad1/5/8 (Ab #1 or Ab #2) or total Smad1/5/8 expression. Data are representative of two or three independent experiments. Columns, average of triplicate determinants; bar, ± SE.
(Supplementary Fig. S7), suggesting basal levels of PI3K and Akt suppress BMP signaling.

**Silencing expression of mTOR, raptor, or rictor reverses the ability of IGF-I to inhibit BMP signaling**

We further investigated the roles of each of the two mTOR complexes (mTORC1 and mTORC2) in BMP4 signaling by efficiently and stably silencing mTOR as well as a critical component of mTORC1 (Raptor) and mTORC2 (Rictor) complexes. For this we used specific small hairpin RNA interference delivered by a doxycycline-inducible lentiviral transduction system, as previously described (31), which knock down mTOR, Raptor, and Rictor in NRP-152 cells by >95% (Fig. 5A). The stably silenced cell lines were treated with LR3–IGF-I before BMP4 addition and analyzed as before for levels of total and phospho-Smad1/5/8. Silencing mTOR, Raptor, or Rictor reversed the ability of IGF-I to inhibit BMP4-induced phosphorylation of Smad1/5/8 (Fig. 5B and C; Supplementary Fig. S8) and the suppressive action of IGF-I on BMP-induced Id1 promoter activity (Fig. 5D). Consistent with these results, overexpression of mTOR, Raptor, and Rictor in NRP-152 cells each alone suppressed BMP-induced Id1 promoter activity (data not shown). Taken together, our results suggest that both mTORC1 and mTORC2 play a role critical in mediating the suppression of BMP responses by IGF-I in prostate epithelial cells.

**Figure 5.** Raptor, Rictor, and mTOR mediate the IGF-I suppression of BMP-induced Id1 promoter expression in NRP-152 prostate epithelial cells. A, Raptor, Rictor, and mTOR were effectively silenced individually as indicated at the protein level in NRP-152 cells. B and C, NRP-152-tTR-sh-LacZ, NRP-152-tTR-sh-Raptor or stably silenced NRP-152-sh-Raptor, and NRP-152-sh-mTOR cells were treated with LR3–IGF-I (10 nmol/L) 24 hours before BMP4 (5 ng/mL) for an additional 4 hours; cells were then lysed for Western blot analysis of phospho-Smads and/or total Smads. D, NRP-152-tTR-sh-LacZ (Sh-LacZ), NRP-152-tTR-sh-mTOR (Sh-mTOR), NRP-152-tTR-sh-Raptor (Sh-Raptor), and NRP-152-tTR-sh-Rictor (Sh-Rictor) stably silenced cells were transfected with Id1 promoter construct 24 hours before treatment with LR3–IGF-I (10 nmol/L). After 2 hours cells were treated with BMP4 (5 ng/mL) or vehicle, and luciferase activity was measured 24 hours later. Columns, average of triplicate determinants; bar, ± SE.
IGF-I represses numerous BMP-regulated genes

We examined the global effect of IGF-I on gene expression by BMP4 in NRP-152 cells with the use of microarray analysis with Affymetrix Rat Gene 1.0 ST Array microarrays containing 33,297 probe set IDs for known genes. The fold-change of each treatment set was compared with vehicle control. The total number of probe sets altered for each treatment is as follows (in brackets are number of changes ≥1.5-fold): BMP4 (521),

Figure 6. IGF-I–mediated inhibition of BMP-induced gene microarray analysis and in vivo examination of mTOR-mediated inhibition of Smad1/5/8 in advanced human prostate adenocarcinoma. A, microarray analysis of NRP-152 cells treated with vehicle control, LR3–IGF-I (10 nmol/L, 24 h) ± BMP4 (5 ng/mL) for a total of 48 hours and analyzed to determine fold-change relative to control; biological process was identified with Pathway Studio 5.0. B, immunohistochemistry of normal prostate hyperplasia (top) or advanced prostate adenocarcinoma stage III stained with H&E, phospho-Smad1/5/8, or phospho-S6. C, matched human prostate cancer cores (34 total cores). Left, H-score plotted ($R^2 = 0.431$ and $P < 0.0001$); bar chart, sequential core expression of P-Smad1/5/8 or P-S6. D, a schematic model of IGF-I regulation of BMP signaling and its implication in prostate cancer.
IGF-I (503), and BMP4+IGF-I (1,583). This analysis revealed that expression of 89 of the 235 BMP4-regulated (38%) genes was specifically altered by IGF-I in a manner that could not be accounted for by the effects of IGF-I alone (Supplementary Table S1). Twenty of these genes were grouped to specific biological responses with the use of Pathway Studio 5.0 software to determine pathway and molecular interaction analysis for each of the identified treatment groups (Fig. 6A). These data suggest that IGF-I represses the ability of BMP to modulate the expression of a number of genes involved in tumor growth as well as tumor suppression.

**Hyperactivation of mTOR in human prostate cancer correlates with loss of phospho-Smad1/5/8 expression**

To test our hypothesis that hyperactivation of mTOR represses the ability of BMP to phosphorylate Smad1/5/8, we conducted an immunohistochemical analysis of phospho-Smad1/5/8 and a key downstream target or mTOR (phospho-S6) with the use of matched cores from a human prostate tissue microarray (PR8011 series) obtained from US BioMax, Inc. (Fig. 6B). The H-score [percent positive stained cells x intensity of staining (0-3)] of 34 cores representative of localized prostate adenocarcinoma (27 stages II-IV) and 7 normal hyperplasia yielded a statistically significant inverse correlation between the levels of phospho-Smad1/5/8 and that of phospho-S6 ($R^2 = 0.4271; P < 0.0001$; Fig. 6C). This represents a significant in vivo test of our model that activation of mTOR reverses the activation of Smad1/5/8 by BMP.

**Discussion**

Here we report the first evidence that IGF-I signaling through a PI3K/Akt/mTOR pathway intercepts BMP responses by suppressing the c-terminal phosphorylation of Smad1/5/8. Silencing either Raptor or Rictor alone reversed this IGF-I repression, indicating critical and nonredundant roles for mTORC1 and mTORC2 (32, 33) in such regulation, the mechanism of which awaits further investigation.

BMPs are recognized to have both tumor-suppressor and tumor-promoting functions in the prostate, although the mechanisms mediating such opposing functions remain poorly defined (34, 35). Whereas various BMPs have been detected in both normal and tumor prostate tissues, BMP4 seems to be a predominant form expressed in the normal prostate relative to tumor tissue (ref. 36; Supplementary Fig. S9), in which it is shown to function as a repressor of prostatic ductal budding and branching morphogenesis (37). Evidence also support that response to BMPs is altered during prostate tumor development/progression (38). Consistent with this, BMP4 induces the apoptosis of nontumorigenic prostate epithelial cell lines (NRP-152 and DP-153), more so than tumorigenic ones (LNCaP, PC3; Fig. 2; Supplementary Fig. S4), correlating with the PTEN-negative status of the latter cell lines. Immunohistochemical analysis reveals that phospho-Smad1/5/8 is high in hyperplastic prostate tissues but lost in advanced localized prostate cancer, correlating with activation of mTOR or phospho-S6 (Fig. 6C), consistent with our in vitro data.

Functional loss of PTEN, which promotes hyperactivation of the PI3K/Akt/mTOR pathway, is well accepted to be involved in the development and progression of the majority of prostate cancers (39) but through an incompletely understood mechanism. Our data suggest that PI3K/Akt/mTOR plays an important role in loss of the tumor-suppressive function of BMP4 (apoptosis/growth arrest) in prostate cancer. Microarray expression profiling showed that IGF-I represses BMP4 to regulate expression of ~38% of the BMP4 target genes; at least two of these BMP4-inducible ones (IGF-BP5 and Gadd45α; Fig. 6A) have been shown to be associated with the control of apoptosis and growth arrest (40, 41). Thus, the oncogenic function of PI3K/Akt/mTOR may partly occur through intercepting the cytostatic functions BMP4 (through suppressing activation of Smad1/5/8; ref. 42). However, IGF-I/PI3K/Akt/mTOR pathway also represses the induction of Id1, Id3, and other tumor-promoting proteins (Figs. 5 and 6; Supplementary Fig. S10), suggesting this pathway also maintains homeostasis by repressing the oncogenic functions of BMP4. Thus, Ids and other oncogenic mediators of BMP are potential new cotargets of mTOR therapeutics.

Prostate cancer cells typically progress from a state of androgen dependence toward that of hormone independence (castrate resistance) through mechanisms under rigorous investigation (43, 44). Whereas advanced prostate cancer cells are resistant to androgens, recent studies suggest they are dependent on the androgen receptor, which is considered to become constitutively activated during tumor progression (39, 45). A number of models have been proposed for the mechanisms by which androgen receptor signaling is activated in the absence of exogenous androgens (46). Recently, BMP receptor signaling has been reported to suppress androgen receptor activity through a Smad1-dependent and mitogen-activated protein kinase–dependent mechanism involving the phosphorylation of the middle linker of Smad1 (35). The modified Smad1 then associates with the androgen receptor and suppresses gene transcription by the androgen receptor. Through this mechanism, basal levels of autocrine BMP activity (47) may help maintain the androgen-dependent phenotype of prostate tumors. Akt/mTOR signaling can significantly enhance androgen receptor activity, thus promoting “androgen independence” through mechanisms that are not clear (48). Our findings suggest that this may occur through reversing the suppressive activity of the BMP/Smad1/5/8 pathway on the androgen receptor. On the other hand, enhanced androgen receptor activity has been shown to activate mTOR (49), and results from our current study suggest that the suppressive activity of mTOR on BMP may serve to further enhance the activity of the androgen receptor. This positive feedback/signal amplification loop is likely to contribute to castration-resistant prostate cancer. In the normal or preneoplastic prostate tissue, this positive feedback loop is likely to be kept in check through the induction of BMP7 and BMP receptor II by androgens (47, 50). Taken together, our study here provides further insight on the potential mechanism by which prostate cancer cells progress toward androgen independence, with the ultimate goal of
aiding the therapeutic management of hormone refractory prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Insulin-Like Growth Factor I Suppresses Bone Morphogenetic Protein Signaling in Prostate Cancer Cells by Activating mTOR Signaling

Reema S. Wahdan-Alaswad, Kyung Song, Tracy L. Krebs, et al.

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