Inhibition of mTORC1 Kinase Activates Smads 1 and 5 but Not Smad8 in Human Prostate Cancer Cells, Mediating Cytostatic Response to Rapamycin

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Abstract
Although hyperactivated mTOR is well recognized as being pivotal to prostate cancer growth and progression, the underlying mechanisms by which it promotes such responses remain incompletely understood. Here, we show that rapamycin activates Smads 1 and 5 in human prostate cancer cells and tissues through blocking mTORC1 kinase. Small hairpin RNA–based gene silencing and gene overexpression approaches reveal that Smads 1 and 5 mediate, whereas Smad8 represses, rapamycin-induced cell death and expression of the bone morphogenetic protein (BMP) transcriptional target Id1 in human prostate cancer cell lines. Moreover, such phospho-Smad1/5–mediated rapamycin responses were blocked by LDN-193189 (a BMPRI kinase inhibitor) or Noggin (a BMP antagonist) in LNCaP prostate cancer cells. Likewise, the mTOR kinase inhibitors Ku-0063794 and WYE-354 each enhanced phosphorylation of Smad1/5. Intriguingly, silencing raptor alone enhanced, whereas silencing rictor repressed, the phosphorylation of Smad1/5, indicating that mTORC1 represses, whereas mTORC2 activates, BMP signaling. Immunohistochemical analysis showed increased levels of phospho-Smad1/5 concomitant with suppression of phospho-S6 and survivin levels in PC3 human prostate cancer xenografts in athymic mice administered rapamycin (intraperitoneally, 5 mg/kg/d, 2–6 days). Moreover, we show that compared with prostate tumor tissue from untreated patients, levels of phospho-Smad1/5 were significantly elevated in the prostate tumor tissue of patients with high-risk prostate cancer who received 8 weeks of the rapalog everolimus as part of a neoadjuvant clinical trial before undergoing local definitive therapy by radical prostatectomy. Taken together, our data implicate Smads 1, 5 and 8 as potential prognostic markers and therapeutic targets for mTOR inhibition therapy of prostate cancer. Mol Cancer Res; 10(6): 821–33. © 2012 AACR.

Introduction
mTOR is a 298-kDa serine–threonine kinase that plays critical roles in the regulation of growth, survival, protein synthesis, metabolism, and angiogenesis. mTOR is activated predominantly through the Akt signaling pathway (1) and is hyperactivated in many human cancers through either loss of phosphatase and tensin homolog (PTEN) or activation of oncogenes such as phosphoinositide-3-kinase (PI3K) and Ras (2–4). Loss of the tumor suppressor PTEN has been implicated in the development of prostate intraepithelial neoplasia (PIN; refs. 5, 6) and castration-refractory prostate cancer (7).

Rapamycin and rapamycin-like analogues (rapalogs) are direct inhibitors of the mTOR complex 1 (mTORC1), which contains raptor and regulates protein synthesis and cell growth through phosphorylating S6K and 4E-BP1 (8). However, rapamycin does not directly inhibit mTOR complex 2 (mTORC2), which contains rictor and can activate Akt by phosphorylating Akt at Ser473 (9). Rapalogs inhibit mTORC1 through forming a complex with the immunophillin FKBP12 at a site juxtaposed to the kinase domain of mTOR (10). Rapalogs, such as everolimus (Afinitor, Novartis) and temsirolimus (Torisel, Pfizer, Inc.), are now a standard of care in patients with advanced renal cell cancer (11) and their optimal use in treating other solid tumors continues (2, 12–14). Despite some encouraging results, rapalogs have had limited therapeutic success as single agents (15, 16), a phenomenon attributed to several mechanisms including the activation of mitogen signaling through IRS-1...
Materials and Methods

Materials

Stemfactor recombinant human BMP4 (cat. no. 03-007) and LDN-193189 (Stemgent); rapamycin, (LC Labs); anti-phospho(p)-Smad3 antibody (p-Smad1/5/8, Cat. no. 9516), anti-Smad-1/5 (Cat. no. 9514), anti-Smad-1/5/8 antibody (p-Smad1/5/8, Cat. no. 9511), anti-Smad1/5 (Cat. no. 9516), anti-Smad3 (sc-8332), anti-survivin (Cat. no. 66220; Santa Cruz Biotechnology, Inc.); anti-Smad2 antibody (Cat. no. 3101), and anti-cyclin D1 (Cat. no. 2926; Cell Signaling); anti-Smad2 antibody (Cat. no. 66220; Transduction Laboratories); anti-Smad3 (sc-8332), anti-survivin (sc-10811), anti-Smad1 (sc-7965), anti-cyclin D2 (sc-593), and BMPRII (sc-130704); Santa Cruz Biotechnology, Inc.); Dulbecco’s Modified Eagle’s Media (DMEM)/F12 (1:1); characterized FBS (HyClone Inc.); insulin (BioSource International); dexamethasone (Sigma); HTS466284 (EMD Chemicals); and Ku-0063794 and WYE-354 (Selleck Chemicals). Lentiviral constructs for sh-scrambled, sh-mTOR, sh-raptor, and sh-Id1 were obtained from the laboratory of David Sabatini through Addgene, Inc.

Cell culture

The LNCaP, PC3, and DU145 cell lines were obtained from American Type Culture Collection and maintained in DMEM/F12 + 10% fetal bovine serum (FBS). The above cell lines were authenticated by American Type Culture Collection (by DNA profiling, cyto genetic analysis, flow cytometry, and immunohistochemistry) and used in experiments within 20 passages. C4-2 and C4-2B were from Dr. Leland Chung (22) and used within 20 passages; they were authenticated by morphology, expression of androgen receptor and prostate-specific antigen (PSA; by Western blot analysis), and androgen-independent growth. The NRP-152 cell line was generated in our laboratory and maintained in GM2.1 (20, 23).

Western blot analysis

Cell lysates were prepared and analyzed by Western blot analysis as before (20).

Id1 luciferase reporter assay

Cells were transfected, treated, and assayed similarly as before (21).

Reverse transcriptase and reverse transcriptase quantitative PCR

These were carried out as before (19).

Lentivirus-based gene silencing

Lentivirus-based stable or doxycyclin-inducible small hairpin RNA (shRNA) gene silencing (for Smads 1, 5, and 8 and raptor, mTOR, and Id1) were produced and generated as previously described (19, 24). Targeting sequences for these shRNA constructs are described in Supplementary information.

Rapamycin xenograft study in vivo

PC3 cells [3 × 10^6 in 0.2 mL DMEM: Matrigel (1:1, v/v)] were implanted s.c. on opposing flanks of (6-7 weeks old) Ncr:NU athymic male mice, and tumors were allowed to reach an average of about 25 mm^2 (L X W). Groups of 5 mice were administrated rapamycin (5 mg/kg, intraperitoneally (i. p.)) or vehicle (25) daily for 2 or 6 days. Tumors were fixed in formalin, embedded in paraffin, and subjected to immunohistochemical (IHC) analysis. All experiments carried out and euthanasia protocols are detailed in our Institutional Animal Care and Use Committee (IACUC) protocol #2008-0067.

Randomized phase II study of two different doses of everolimus as a neoadjuvant therapy in patients with high-risk prostate cancer undergoing radical prostatectomy

Eligible patients were randomized to receive either 5.0- or 10-mg everolimus orally every day continuously for 8 weeks. One week after the completion of treatment, all men underwent radical prostatectomy with bilateral pelvic lymph node dissection. Pre- and posttreatment prostate tumor tissue samples were fixed in formalin, embedded in paraffin, and stained for p-Smad1/5/8 or p-S6 by immunohistochemistry.

Cell viability, Hoechst staining, flow cytometry, cell number assay, PCR primers, MTT assay, retrovirus, lentivirus, real-time PCR, immunohistochemistry, microarray analysis

See Supplementary materials.
Results

Rapamycin activates Smads in human prostate cancer cells

We recently reported that IGF-I inhibits BMP4-induced phosphorylation of Smads 1, 5, and/or 8 by activating mTOR in prostate epithelial cell lines (21), suggesting that BMP signaling responses may be involved in the mechanism by which rapalogs repress growth of prostate carcinoma cells. To test this hypothesis, we first evaluated a panel of prostate epithelial cell lines (LNCaP, C4-2, C4-2B, PC3, DU145) for response to the cytostatic activity of rapamycin [by MTT and cell number assay (Fig. 1A and B) and activation of Smads (Western blot analysis with c-terminal phospho-specific antibodies; Fig. 1C)]. Rapamycin had cytostatic activity and enhanced p-Smad1 Ser463/465, p-Smad5 Ser463/465, p-Smad8 Ser426/428 (or in short, p-Smad1/5/8) in all those cell lines. However, we were unable to distinguish between the phosphorylation of Smads 1, 5, or 8 by Western blot analysis, as they comigrate and isoform-specific phospho-antibodies for those Smads were not available. Smad2 was not phosphorylated and Smad3 appeared to be phosphorylated only in PC3 cells, which was the only cell line in this group with detectable expression of Smad3. Levels of total Smads were unaltered by rapamycin, except in PC3 cells in which Smad3 was induced and Smad5 was decreased. Rapamycin did not seem to change the levels of mTOR, PTEN, or Akt1 in those cells. The magnitude of Smad phosphorylation by 200 nmol/L rapamycin appeared to correlate with that of growth suppression. Under these conditions (DMEM/F12 + 1% FBS), activation of Smads was weakest in DU145, which is the only cell line in this group that expresses PTEN, suggesting that optimal activation of Smads by rapamycin occurred under conditions where Akt is hyperactivated by the absence of PTEN. Consistent with this possibility, culturing DU145 in higher serum (5% FBS) enhanced...
the phosphorylation of Smad1/5/8 by rapamycin (Supplementary Fig. S1A).

We examined the ability of rapamycin to activate Smads 1, 5, and/or 8 in a dose- and time-dependent manner in LNCaP cells. Cells were treated with rapamycin (0–1,000 nmol/L) for 24 hours or with 200 nmol/L rapamycin from 0 to 72 hours and then subjected to Western blot analysis for total and phospho-Smad1/5/8, as well as for cyclin D1 and D2 (Fig. 2A and B). Phosphorylation of Smad1/5/8 at 24 hours was induced by as low as 12.5 nmol/L rapamycin, was maximal between 100 and 200 nmol/L, and inversely correlated with changes in the cyclin D1 and D2. Rapamycin induced levels of p-Smad1/5/8 by 12 hours, with maximal induction by 72 hours. We confirmed that rapamycin activated Smad1/5/8 rather than just inducing their phosphorylation under these conditions, by transfecting cells in a parallel experiment with an Id1 promoter luciferase construct, which contains a number of BMP response elements (26) and measuring luciferase activity various times after rapamycin treatment (Fig. 2C). As anticipated, Id1 promoter was activated with 12.5 nmol/L rapamycin at 24 hours; 200 nmol/L rapamycin activated this promoter by 5-fold after 24 hours to 45-fold by 72 hours (Fig. 2D). Furthermore, rapamycin induced Id1 mRNA levels by 24 hours (Fig. 2E). Similar results were obtained in PC3 and in the non-tumorigenic rat cell line NRP-152 (Fig. 2F; Supplementary Fig. S1B and S1C); however, in those cell lines, rapamycin induced the phosphorylation of Smad1/5/8 by as early as 30 minutes. Rapamycin significantly enhanced BMP4-induced cell death (Hoechst 33258/hoe cytometry/cell number assays) and activated the Id1 promoter activity (Fig. 2G; Supplementary Figs. S2A–S2D and S3).

Rapamycin-mediated cell death requires Smad1 and Smad5 but not Smad8

We used a lentiviral delivery system for inducible expression of specific shRNAs (24) to efficiently and stably silence expression of Smads 1, 5, 1 and 5, or 8 by more than 95% in LNCaP and PC3 cells (Fig. 3A and data not shown). Using those cell lines, we assessed which of these Smads were
activated by rapamycin (200 nmol/L at 24 hours; Fig. 3B; Supplementary Fig. S4A and S4B). Our analysis revealed that silencing Smads 1 and 5, but not Smad8, repressed the levels of p-Smad1 activated by rapamycin, suggesting that rapamycin activates both Smad1 and Smad5, but not Smad8. Silencing Smads 1 or 5 also repressed activation of Smad3 by rapamycin in PC3 cells, suggesting that Smads 1 and 5 may be essential for the activation of Smad3 by rapamycin. Intriguingly, blocking Smad8 enhanced rapamycin-induced Smad activation (including Smad3 in PC3 cells), indicating that Smad8 functions as a negative regulator of the activation of Smad1 and 5 by rapamycin. Similarly, we observed that sh-Smad1 and sh-Smad5 each suppressed rapamycin-induced Id1 promoter activity, whereas sh-Smad8 enhanced such activity (Fig. 3C). Moreover, sh-Smad1 and sh-Smad5 cells were less responsive to rapamycin than were the sh-LacZ control cells, as measured by changes in cell viability (trypan blue dye exclusion), fraction of adherent cells (Coulter counter), and induction of Id1 promoter activity (Fig. 3C–E; Supplementary Fig. S5). In contrast, Smad8-silenced cells showed increased sensitivity to rapamycin with respect to the above responses. A similar pattern of Smad dependence on cell death was observed with BMP4; however, silencing either Smad1 or Smad5 alone completely reversed BMP-induced cell death (Fig. 3D). In contrast, silencing of Smads 1, 5, or 8 alone did not significantly reverse BMP4-induced Id1 promoter activity (Supplementary Fig. S5).

**Overexpression of Smad1 or Smad5 enhances rapamycin-induced Id1 promoter activation and cell death in LNCaP cells**

On the basis of the above results, we efficiently overexpressed Smad1, Smad5, and Smad8 using the pLPCX retrovirus vector in LNCaP cells (Fig. 4A, data not shown) to test whether these Smads enhance the ability of rapamycin to promote cell death and/or induce the Id1 promoter. The stable cell lines were treated with rapamycin

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**Figure 3.** Silencing Smad1 and Smad5 repress rapamycin-induced Smad activation, Id1 promoter activity, and cell death. A, Smad1, Smad5, and Smad8 were effectively silenced in LNCaP cells by transduction with shRNA lentiviruses. B and C, impact of silencing endogenous Smads 1, 5, 8 on rapamycin (200 nmol/L, 24 hours)-induced activation of p-Smads (B, Western blot analysis) activation of Id1 promoter (C). D and E, LNCaP cells stably expressing sh-Smad1, sh-Smad5, sh-Smad1/5, and sh-Smad8 were treated with rapamycin (200 nmol/L) for 72 hours and examined for apoptosis by Hoechst 33258 dye staining (D) or total adherent cells (enumerated by a Coulter counter; E); cells were treated with BMP4 alone or in combination with rapamycin (E) for comparative analysis. Columns, average of triplicate determinants; bar, ±SE and experiments were run in triplicate.
(200 nmol/L, overnight) and analyzed by Western blotting, Id1 promoter activity, or cell death (Fig. 4B–D; Supplementary Fig. S6A–S6C). Overexpression of Smad1 and Smad5 enhanced rapamycin-induced activation of Smads, Id1 promoter activity and cell death, whereas overexpression of pLPCX-Smad8 diminished such responses in LNCaP cells (Fig. 4). Similar results were observed in PC3 cells overexpressing Smad1, 5, and 8 (Supplementary Fig. S6). Taken together, these data support that both Smad1 and 5 play an important role in mediating rapamycin-induced cell death, whereas Smad8 functions as a negative regulator of this response.

**BMPRI kinase activity is involved in cellular responses to rapamycin**

Because rapamycin promotes the activation Smads 1 and 5, which are activated by BMP, we examined whether inhibition of BMPRI would abrogate the ability of rapamycin to activate Smad, induce Id1 luciferase promoter activity, and/or promote cell death. We examined the above activities in prostate cancer cell lines pretreated with a selective kinase inhibitor (LDN-193189) of BMP type I receptors (ALK2, ALK3, and ALK6; ref. 27) or a selective kinase inhibitor (HTS466284) of TGF-β type I receptors (ALK4, ALK5, ALK7; ref. 28). PC3 cells were treated with LDN-193189 (500 nmol/L) or HTS466284 (2 μmol/L) 2 hours before 20-hour treatment with rapamycin (200 nmol/L) and then subjected to Western blot analysis (Fig. 5A). As in Fig. 1A, rapamycin (200 nmol/L) induced expression of both p-Smad1/5/8 (top band) and p-Smad3 (bottom band). LDN-193189 repressed activation of Smad1/5/8 (top band) and also repressed p-Smad3, and the combined treatment with both inhibitors abrogated all p-Smads. The concentration of each inhibitor was chosen based on the minimal concentration that selectively and efficiently inhibited the activation of Smads by BMP4 or TGF-β.

![Figure 4](image-url)
TGF-β1 (27), suggested that activation of Smad3 by rapamycin is dependent on the kinase activity of a BMP type I receptor. Similarly, we treated the same panel of cell lines with variable concentrations of LDN-193189 (0–500 nmol/L) 2 hours before a 70-hour treatment with 200 nmol/L rapamycin, and then measured the total adherent cells using a Coulter counter (Fig. 5B; Supplementary Fig. S7A and S7B). Inhibition of BMPRI kinase activity by LDN-193189 reversed rapamycin-induced cell death in all cases.

We next examined whether LDN-193189 could block the ability of rapamycin to induce Id1 mRNA or
Id1 transcriptional regulation. Semiquantitative reverse transcriptase PCR (RT-PCR; Fig. 5C) and real-time quantitative PCR (qPCR; Fig. 5D) were used to assess the ability of BMPRI inhibitor to suppress rapamycin-induced levels of Id1 mRNA and cell death in LNCaP cells. Rapamycin induced expression of Id1 mRNA by 6-fold as determined by real-time qPCR, and LDN-193189 was able to fully suppress rapamycin-induced Id1 mRNA. Similarly, BMP4 was able to enhance Id1 mRNA levels by 20-fold, and when combined with rapamycin, this activity was heightened to 42-fold, whereas inclusion of LDN-193189 abrogated such induction. The ability of rapamycin to induce the Id1 promoter activity (Fig. 5E) or cell growth (Fig. 5F) in LNCaP cells was fully and partially reversed by LDN-193189 and HTS466284, respectively, supporting that activation of the BMPRI kinase is critical to activation of the Id1 promoter or growth suppression by rapamycin.

Next, we examined the role of Id1 induction in growth suppression by rapamycin in LNCaP and DU145 cells by silencing Id1, using previously described shRNAs (21). Cells were infected with either vehicle control pLKO.1-shGFP or pLKO.1-shId1#1 for 48 hours before a 3-day treatment with rapamycin (200 nmol/L) and/or BMP4 (5 ng/mL) and then analyzed by Western blotting for loss of Id1 and changes in both cell number and morphology (Fig. 5G; Supplementary Figs. 5C and 5E). We found that silencing Id1 alone suppressed cell growth but did not reverse rapamycin-induced cell death in either cell line. Taken together, these data support that Id1 induction does not mediate growth suppression by rapamycin.

Rapalogs enhance levels of p-Smad1/5/8 in human prostate cancer tissues, correlating with loss of both mTOR activity and survivin expression

Complementing our cell culture studies, we next addressed whether rapamycin would affect Smad activation in vivo. Here, PC3 cells (3 × 10<sup>6</sup> cells) were implanted s.c. in athymic nude male mice and once tumors were evident (~100 mm<sup>3</sup>), animals were administered daily with either vehicle control pLKO.1-shGFP or pLKO.1-shId1#1 for 48 hours before a 3-day treatment with rapamycin (200 nmol/mL) and/or BMP4 (5 ng/mL) or BMP4 and rapamycin (200 nmol/mL) and/or BMP4 (5 ng/mL) and then analyzed by Western blotting for loss of Id1 and changes in both cell number and morphology (Fig. 5G; Supplementary Figs. 5C and 5E). Administration of rapamycin for 2 days clearly enhanced staining for p-Smad1/5/8, p-S6, and survivin expression (Fig. 6A). Administration of rapamycin for 2 days clearly enhanced staining for p-Smad1/5/8 expression and suppressed that for p-S6 and survivin, with greater effects by 6 days. Histologic score (H-score) analysis (% positive stained cells × intensity of staining (0–3)) provided statistically significant and quantifiable changes in the pattern of expression (Fig. 6B).

To show the relevance of these findings in humans, we used radical prostatectomy tumor tissue sections from a phase II clinical trial in which patients with newly diagnosed high-risk prostate cancer were administered everolimus (5 or 10 mg/d orally) continuously for 8 weeks before undergoing radical prostatectomy. At the time of conducting these experiments, prostate cancer tissue sections from 6 patients were available. Tumor tissue specimens were evaluated for p-Smad1/5/8 and p-S6 via immunohistochemistry (Fig. 6C; Supplementary Fig. S9). We compared these results side-by-side with those from a larger (22 biopsy cores) and a comparable (localized prostate adenocarcinoma stages II–III) cohort of nontreated human prostate tumor tissues in a microarray (PR8011 series) obtained from US BioMax, Inc. H-score analysis was used to evaluate the relative expression of p-Smad1/5/8 and p-S6 in the nontreated control group (n = 22) against the everolimus treatment group (n = 6; Fig. 6D). Although the sample size (n = 6) of this ongoing clinical trial was small, statistically significant differences in relative levels of p-S6 and p-Smad1/5/8 between the everolimus-treated and nontreated groups were generated, with reduced p-S6 levels and increased p-Smad1/5/8 levels in the everolimus group compared with the nontreated group. Our data suggest that 6 of 6 (100%) of patients responded to everolimus treatment by loss of p-S6 and also showed enhanced p-Smad1/5/8 expression, supporting that suppression of mTOR by everolimus enhanced the activation of Smad1/5/8. Although levels of p-Smad1/5/8 staining were weakest in the tumor with the lowest Gleason score (4 + 3) and the highest level of p-Smad1/5/8 staining occurred in the tumor with the greatest Gleason score (5 + 4), Student t test (two-tailed) did not show any statistically significant correlation (either positive or reverse) between p-Smad1/5/8 staining versus PSA levels, PSA velocity or doubling time (during everolimus treatment), or postoperative PSA levels (Supplementary Table SI). Once this trial is completed, all specimens will be reanalyzed in similar fashion and also with the inclusion of Id1 staining and proliferative, apoptotic, and epithelial–mesenchymal transition markers.

Mechanism of Smad1/5 activation by rapamycin

Although selective for mTORC1, prolonged treatment with rapamycin is known to disrupt the function of both mTORC1 and mTORC2 (29). The recent availability of selective mTOR kinase inhibitors, such as Ku-0063794 (30) and WYE-354 (31), which target both mTORC1 and mTORC2, enabled us to test whether targeting the mTOR kinase domain would also induce p-Smad1/5. In a side-by-side comparison with optimal doses of rapamycin for Smad activation, we showed that each mTOR inhibitor activates Smad1/5 more effectively than does rapamycin (Fig. 7A). Probing the blot for p-Akt<sup>Ser473</sup> and p-S6 supports that rapamycin selectively suppresses the mTORC1 complex, whereas each of the mTOR kinase inhibitors targeted both mTORC1 and mTORC2. More interestingly, each kinase inhibitor elevated the levels of p-Akt<sup>Thr308</sup> more effectively than rapamycin, suggesting that each mTOR complex is involved in negative feedback control of PI3K and Akt.

Rapamycin appears to activate Smads through 2 distinct mechanisms: an early one occurring by 30 minutes of stimulation and another one requiring 12 hour or more (Fig. 2B and F; Supplementary Fig. S1B). To examine
whether induced secretion or activation of BMPs may explain the delayed activation of Smads by rapamycin, we cotreated LNCaP cells with Noggin, a secretory protein that binds to and neutralizes BMPs (32). Addition of Noggin robustly inhibited both rapamycin-induced and Ku-0063794–induced p-Smad1/5, although Noggin more completely inhibited p-Smad1/5 induced by BMP4 (Fig. 7B), suggesting that rapamycin-activated Smad1/5 is, at least, partially dependent on the expression and/or activation of BMP ligand.

We examined the role of each mTOR complex in controlling phosphorylation of Smad1/5 in LNCaP cells by infecting them with lentiviruses carrying shRNAs selective for mTOR, raptor, or rictor. sh-Raptor significantly elevated levels of p-Smad1/5, whereas sh-rictor robustly repressed expression of p-Smad1/5 (Fig. 7C). In contrast, sh-mTOR minimally altered levels of p-Smad1/5, consistent with the opposing effects of mTORC1 and mTORC2 on activation of p-Smad1/5. Moreover, sh-rictor repressed the induction of p-Smad1/5 by rapamycin (Fig. 7C).

The potential mechanism by which mTORC1 represses Smad1/5 was next investigated. Affymetrix microarray analysis of gene expression changes ≥1.3-fold in LNCaP cells following a 24-hour treatment with 200 nmol/L rapamycin revealed a 1.5-fold induction of BMPRII mRNA (confirmed by RT-PCR, Supplementary Fig. S10). However, rapamycin did not alter the expression of other mRNAs coding for proteins known to be involved in activation of Smads (data not shown). Western blot analysis confirmed a small increase in BMPRII protein levels by 200 nmol/L rapamycin but not by Ku-006379 or WYE-354 (Fig. 7A), suggesting that elevated expression of p-Smad1/5.

Figure 6. Rapalogs enhance p-Smad1/5/8 expression in vivo in PC3 xenografts and in tumors of patients with newly diagnosed localized prostate cancer. A and B, expression of p-Smad1/5/8, p-S6, and survivin were assessed by immunohistochemistry of PC3 tumor xenografts implanted in (6–7 weeks old) Ncr:NU athymic male mice that received either vehicle control (n = 5) or rapamycin (Rapa) treatment for 48 hours (n = 5) or 6 days (n = 5) as described in Materials and Methods (A); staining results were quantified by measuring H-score [% positive stained cells × staining intensity (0–3)] of matched sections (B). C and D, expression of p-Smad1/5/8 and p-S6 (by immunohistochemistry) in prostate tumor sections from patients with high-risk prostate cancer on a phase II clinical trial who were treated with everolimus (5 mg or 10 mg/d for 8 weeks) as neoadjuvant therapy in patients (n = 6) and compared with nontreated control patients with prostate adenocarcinoma stage II–II (n = 22; C); H-score [% positive stained cells × staining intensity (0–3)] of nontreated control matched cores were compared with everolimus (RAD001) clinical trial matched sections (D).
Figure 7. Mechanism of Smad1/5 activation by rapamycin. A, LNCaP cells were treated with rapamycin (Rap; 100 and 200 nmol/L) or various concentrations of selective mTOR kinase inhibitors, Ku-0063794 or WYE-354 for 24 hours before Western blot analysis for p-Smad1/5/8, p-Smad1/5, p-S6, p-Akt1 (Ser473), p-Akt1 (Thr308), and BMPRII. DMSO, dimethyl sulfoxide. B, activation of p-Smad1/5 by rapamycin or Ku-0063794 in LNCaP cells is antagonized by Noggin, a BMP antagonist. Cells were treated with Noggin or vehicle 1 hour before a 24-hour treatment with 200 nmol/L rapamycin or 800 nmol/L Ku-0063794. C, silencing raptor activates Smad1/5 whereas silencing rictor represses the levels of p-Smad1/5. LNCaP cells were infected with lentivirus expressing sh-scrambled, sh-mTOR, sh-raptor or sh-rictor for 24 hours and after an additional 48 hours for effective silencing, cells were subjected to Western blot analysis. D, sh-rictor represses the activation of p-Smad1/5 by rapamycin. Cells silenced as shown in (C) were treated with or without rapamycin (200 nmol/L, 24 hours) before harvesting for Western blot analysis. E, BMPRII and Smads 1, 5 and 8 co-immunoprecipitate (IP) mTOR. HEK293 cells were transfected with empty vector (pCDNA3), Flag-Smad1, Flag-Smad5, Flag-Smad8, or Flag-BMPRII along with Myc-mTOR, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer 24 hours after transfection, and mTOR was co-immunoprecipitated with anti-Flag IgG1 (M2). IB, immunoblotting. F, a unifying model illustrating how mTORC1 and mTORC2 control BMP signaling. Also illustrated are recently described compensatory mechanisms of mTORC1 on activation of receptor tyrosine kinases (RTK), Akt, and the androgen receptor (AR).
BMPRII does not promote elevation of p-Smad1/5/8 by the mTORC1 kinase inhibitors. An alternative possibility that mTORC1 directly intercepts the BMP signaling pathway was considered by exploring whether mTOR could directly bind to BMPRII or to Smads 1, 5, or 8. For this, we transfected HEK293 cells with Myc-mTOR along with expression constructs for Flag-Smad1, Flag-Smad5, Flag-Smad8, Flag-BMPRII, or pCDNA3 empty vector control (Fig. 7E). Anti-Flag M2 pulled down Myc-mTOR in cells transfected with Flag-Smads 1, 5, 8 or BMPRII, but not with an empty vector. The greatest pull-down occurred with BMPRII and Smad8. These data suggest that mTORC1 represses BMP signaling by a physical association with BMPRII as well as with Smads 1, 5 and/or 8.

Discussion

The discovery of rapamycin as an anticancer agent provided enormous impetus to identify its target, mTOR, which was later shown to be a key regulator of protein synthesis, cell metabolism, and cell growth. mTOR is activated by mitogenic signals through the receptor tyrosine kinase/Pi3K/Akt pathway (33). Importantly, Akt and mTOR are hyperactivated in many cancers including prostate cancer; principally through loss of PTEN and activation of Pi3K (34, 35). However, the underlying molecular mechanism(s) by which mTOR promotes the pathogenesis of prostate cancer remains incompletely understood. Despite strong evidence for hyperactivated mTOR in tumor growth, most cancers show limited growth suppression by rapalogs (16), attributed largely to reversal of the negative feedback of mTORC1 on IRS-1 and enhanced activation of Akt (1, 17). While mTORC2 has been shown to be critical to the development of prostate tumors in PTEN knockout mice, mTORC2 is not critical for growth of already established human prostate tumor cells (36).

We suggest that components of the BMP and TGF-β signaling pathways, particularly, the expression of Smads 1, 5, and 3 may be critical to the antitumor activity of mTOR inhibitors. Our recent report that IGF-I abrogates BMP4 signaling through activating mTOR (21) provided the first functional connection between activation of mTOR and subsequent loss of the tumor suppressor function of BMP4 in prostate cancer cells and suggested that suppressing mTOR signaling may restore tumor suppression by BMP4. Our current study here extends those findings and provides the first direct evidence that rapalogs induce the activation of Smads 1 and/or 5 in human prostate cancer cells in culture, in tumor xenografts, and in human prostate cancer tissues. Our cell culture studies support that such Smad activation is also associated with suppression of the function of mTORC1 and requires Smad1, Smad5; and the kinase activity of a BMP type I receptor (ALK2, ALK3, and ALK6); however, Smad8 represses such activation. Moreover, we show that Smad1, Smad5, and the BMP type I receptor play critical roles in the ability of rapamycin to suppress growth or induce apoptosis, whereas Smad8 reverses rapamycin-induced growth suppression. Furthermore, we show that the mTOR kinase inhibitors, Ku-0063794 and WYE-354, are more effective than rapamycin in activating Smad1/5. While our silencing data show that mTORC1 and mTORC2 have opposing roles in the activation of Smad1/5, use of novel mTORC1/2 dual kinase inhibitors thereby suggests that the suppression of mTORC1 kinase is critical to activation of Smad1/5.

Here, we also provide some insight toward understanding the mechanism by which rapamycin activates Smad1 and Smad5, as illustrated by our model in Fig. 7F. Use of Noggin, a selective BMP antagonist, suggests that mTOR kinase inhibitors activate Smad1/5, at least partly, through a BMP ligand–dependent mechanism. Affymetrix gene expression analysis shows that rapamycin elevated levels of BMPRII but not BMP mRNAs or other known activators of BMP signaling; however, the inability of 2 selective mTOR kinase inhibitors to elevate BMPRII suggests that BMPRII may not be a primary target of the inactivation of mTOR. The inability of Noggin to completely reverse the activation of Smads by rapamycin or Ku-0063794 supports that suppression of mTOR activates BMP receptors through both BMP ligand–dependent and -independent mechanisms. While relative to BMP4, rapamycin (or Ku-0063794) does not as robustly activate Smad1/5 (Fig. 7B) or the Id1 promoter (Figs. 4C and 5C–E), rapamycin significantly (about 2-fold) enhanced the Id1 promoter activity in presence of 4 ng/mL BMP4 (Fig. 5D and E), a level saturating for activation of the Id1 promoter. These results suggest that rapamycin activates BMP receptor signaling through a mechanism that also is not entirely dependent on the induced expression or activation of a BMP ligand, as the net effect of rapamycin + BMP4 would at best be additive. Our data showing that BMPRII co-immunoprecipitates with mTOR (Fig. 7E) suggest an alternative mechanism in which rapamycin activates Smad1/5 by reversing a more direct suppression of BMPRII by mTORC1 (Fig. 7F). While we show that antagonizing BMPRII kinase with LDN-193189 reverses the cytostatic action of rapamycin, supporting that BMP receptor signaling mediates or is critical to such cytostatic response by rapamycin, the signal(s) downstream BMP receptors that mediate such a response remains to be defined.

A recent pharmacodynamic study of rapamycin in patients with intermediate- to high-risk prostate cancer (daily doses of 3 mg rapamycin administrated for 14 days) reported repression of tumoral levels of p-S6 and increased nuclear expression of p27, with no significant difference in the expression of key proliferative and apoptotic markers (p-AktSer473, Ki-67, cleaved caspase-3; ref. 37). Although p-AktSer508 levels were not assessed, it is unlikely that such resistance to tumor suppression by rapamycin was through relieving the negative feedback of mTOR via p70S6K, as rapamycin did not activate IRS-1 in those tumors. It is possible that the course of treatment was too short for detecting significant changes in the above markers. While the duration of our neoadjuvant trial was 4 times longer and our data support that everolimus robustly

www.aacrjournals.org Mol Cancer Res; 10(6) June 2012 831

Published OnlineFirst March 27, 2012; DOI: 10.1158/1541-7786.MCR-11-0615

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activates Smad1/5, there is no evidence at this time that tumor burden was suppressed. Further work will also be necessary to assess whether everolimus can improve surgical success by inhibiting inflammation and/or micrometastases.

Despite the importance of mTOR in preclinical models, the above clinical studies and others suggest that rapalogs activate a number of potential compensatory mechanisms in prostate tumors. Here, we provide 2 of such compensatory mechanisms. Our findings suggest that BMP Smads also activate other oncogenic signals (i.e., Id1; ref. 21) that may counteract the therapeutic efficacy of rapalogs and mTOR kinase inhibitors. Another likely compensatory mechanism well known to be activated by rapamycin is autophagy (38), a mechanism of cell survival activated in response to metabolic stress (39). Although autophagy initially promotes cell survival through inhibiting apoptosis, sustained autophagy by rapamycin and/or in combination with other cellular stresses may favor the induction of apoptosis (40, 41). This may provide at least part of the mechanistic basis for the enhanced therapeutic efficacy of rapalogs when combined with autophagy inducers such as P13K/Akt inhibitors (42), radiation (43), or antiandrogens (44). Our microarray expression data suggest that rapamycin activates the androgen receptor signaling pathway, as shown by the upregulation of many androgen-regulated genes (Supplementary Fig. S10B); this is consistent with a recent report that an mTOR/P13K dual kinase inhibitor activates the androgen receptor (45). Two recent articles published in Science shed light on a potential mechanism for this compensation (46, 47). Both groups identified Grb10 as an important direct substrate of mTOR kinase and showed that mTOR kinase phosphorylates Grb10 by enhancing the ability of Grb10 to bind to and repress receptor tyrosine kinases. Thus, suppressing mTOR may activate the androgen receptor through relieving the suppressive effect of Grb10 on the activation of P13K/Akt by receptor tyrosine kinases (Fig. 7F). Activated P13K/Akt has been reported to stabilize and thus activate androgen receptor (48) through FOXO3a (49). While the effort to develop mTOR kinase inhibitors was partly fueled by the incentive to minimize the compensatory feedback activity of rapamycin, we show that such mTORC1/mTORC2 kinase inhibitors are instead more effective than rapamycin in phosphorylating Akt at Ser473 (Fig. 7A).

Given substantial evidence for the oncogenic function of BMP and TGF-β signaling in a number of late-stage cancers including prostate and breast cancer (50), activation of BMP Smads may contribute to reduced therapeutic efficacy of rapalogs in androgen-refractory, metastatic prostate cancer. If so, combined therapies with rapalogs and a BMPRI kinase inhibitor (i.e., LDN-189193) may prove efficacious for such cancers. Further research is thus warranted to more fully explore the roles of BMRII and well as Smads 1, 5, and 8 as prognostic markers and therapeutic targets of rapalog- or mTOR kinase inhibitor–based neoadjuvant modalities.

Disclosure of Potential Conflicts of Interest

J.A. Garcia has received honoraria from Speakers Bureau and is a Consultant/Advisory Board member for Novartis. No potential conflicts of interests were disclosed by other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.S. Wahdan-Alaswad, K. Bane, J.A. Garcia, D. Danielpour
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.S. Wahdan-Alaswad, K. Bane, K. Song, D.T.N. Shola, J.A. Garcia, D. Danielpour
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Acknowledgments

The authors thank Tracy Krebs for technical assistance and Cristina Magi-Galluzzi for help with pathologic assessment and critique.

Grant Support

This work was supported by NIH grants R01CA102074 and R01CA134878 (D. Danielpour) and the Case Comprehensive Cancer Center P30 CA45703 (for Cytometry, Athymic Mouse, Gene Expression and Genotyping Core Facilities). R.S. Wahdan-Alaswad was supported, in part, by a pre-doctoral fellowship from Research Oncology Training Grant 5T32CA09536-15 (2009) and a National Research Service Award Individual Fellowship Application 1F31CA142311-01 (2010-2011).

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Received December 27, 2011; revised March 13, 2012; accepted March 14, 2012; published OnlineFirst March 27, 2012.

References

Inhibition of mTORC1 Activates Smads 1 and 5 in Prostate Cancer Cells


Inhibition of mTORC1 Kinase Activates Smads 1 and 5 but Not Smad8 in Human Prostate Cancer Cells, Mediating Cytostatic Response to Rapamycin

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doi:10.1158/1541-7786.MCR-11-0615

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