Hic-5 Controls BMP4 Responses in Prostate Cancer Cells through Interacting with Smads 1, 5 and 8

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Abstract

Hydrogen peroxide-inducible clone-5 (Hic-5, or androgen receptor-associated protein 55) is a transforming growth factor-β (TGF-β)-inducible LIM protein whose deregulation is implicated in the progression of prostate cancer. Here we report that Hic-5 binds to Smads 1, 5 and 8, and represses bone morphogenetic protein (BMP) signaling responses. Myc-Hic-5 but not Myc-paxillin was specifically immunoprecipitated with anti-FLAG IgG1 from lysates of HEK293 co-transfected with either Myc-Hic-5 or Myc-paxillin and FLAG-tagged Smads 1, 5 or 8. We showed that such interactions require the LIM3 domain of Hic-5 and the MH2 domain of those Smads. Anti-Hic-5 antibody specifically pulled down endogenous Smad1 in both the PC3 human prostate cell line and primary cultures of rat prostate fibroblasts, supporting that Hic-5 binds to Smad1 at the endogenous level. Bacterially expressed GST-Smads 1, 5 or 8, but not GST alone, pulled down *in vitro* transcribed and translated Hic-5, implicating that Hic-5 binds directly to Smads 1, 5 and 8. Significantly, using Hic-5 shRNA silencing and overexpression systems, we show that Hic-5 (at both the endogenous and exogenous levels) represses the ability of BMP4 to induce expression of the inhibitor of differentiation-1 (Id1) (a downstream target gene of BMP), activate the Id1 gene promoter and induce apoptosis in human and rat prostate epithelial cells. Moreover, silencing of Hic-5 in PC3 cells as well as in the WPMY-1 human prostate stroma cell line greatly enhances the levels of endogenous phospho-Smad1/5/8. Finally, we provide fluorescent microscopic imaging to support that Smad1 and Hic-5 mutually interact also at the level of their nuclear export mechanisms. Collectively, these results provide the first evidence for a physical and mutual functional interaction between Hic-5 and the BMP signaling pathway.

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Keywords

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Introduction

Bone morphogenetic proteins (BMPs) are multifunctional regulatory proteins that belong to the TGF-β superfamily and control a diverse array of normal cellular and physiological processes, including growth arrest, apoptosis and cell differentiation in various tissues (Miyazono *et al*., 2010). BMPs bind to the extracellular domain of BMP type I receptors (BMPRIs), which are serine/threonine kinase receptors (also called ALK-2, -3 and -6) that interact with the BMP type II receptors (BMPRII) or activin receptors (ActR-IIA or ActR-IIB) to form a heteromeric receptor-ligand complex. Upon receptor oligomerization, BMPRIs undergo activation by BMPRII through a process of receptor transphosphorylation. The activated BMPRIs in turn phosphorylate the transcription factors Smads 1, 5 and 8, promoting their translocation to the nucleus where they control gene expression through interacting with Smad4 and other transcription factors.

Aberrations in BMP signaling have been identified in a variety of cancers including prostate cancer (Alarmo and Kallioniemi, 2010; Corey and Vessella, 2007; Hsu *et al*., 2005). For example, the expression of BMP7 was reported to be lower in prostate carcinoma than in normal prostate tissue (Bobinac *et al*., 2005), and loss of BMP7 was found to significantly increase tumor cell invasive potential (Ye *et al*., 2007). Inactivation of BMP signaling by via mutations in BMPR-IA is found in familial juvenile polyposis (Howe *et al*., 2001). Conversely, the expression of the above mentioned BMPs were found to be elevated in late-stage prostate cancer where they have been reported to enhance tumor growth and metastasis, suggesting that BMPs also have a role in tumor promotion (Barnes *et al*., 1995; Li *et al*., 2006; Masuda *et al*., 2003). These studies nonetheless imply that aberrations of BMPs play a pivotal role in tumorigenesis.

Hydrogen Peroxide Inducible Clone-5 (Hic-5)/Androgen Receptor Associated protein 55 (ARA55) is a TGF-β1- and hydrogen peroxide (H_2O_2)-inducible LIM domain protein that shares high homology with Paxillin (Shibanuma *et al*., 2004; Thomas *et al*., 1999). Hic-5 has been shown to be involved in a diverse range of biological processes including senescence (Shibanuma *et al*., 1997), tumorigenesis (Heitzer and DeFranco, 2007; Miyoshi *et al*., 2003), steroid hormone action (Gao and Schwartz, 2005; Rahman *et al*., 2003), apoptosis (Hornigold *et al*., 2010; Mori *et al*., 2009), integrin signaling, and differentiation (Dabiri *et al*., 2008; Gao *et al*., 2007). Hic-5 shuttles between the cytosolic and the nuclear compartments, with oxidative stress promoting Hic-5's nuclear localization by inhibiting its nuclear export (Shibanuma *et al*., 2003). Once in the nucleus, Hic-5 functions either as a transcriptional co-activator in association with Sp1, PPARγ and the androgen receptor (AR) (Drori *et al*., 2005; Heitzer and DeFranco, 2006; Shibanuma *et al*., 2004; Yeh *et al*., 1999), or as a co-repressor (i.e, LEF/TCF transcription factors) (Ghogomu *et al*., 2006). Although Hic-5 is predominantly expressed in the stromal compartment of the normal prostate, its expression is lower in tumor associated stroma and such loss of Hic-5 expression is

implicated in aiding tumor progression (Heitzer and DeFranco, 2007). Although Hic-5 has been reported to be absent in normal prostate epithelium and hormone-responsive adenocarcinoma cells, it has been recently found to be expressed in in the normal and tumor prostate epithelium in androgen-depleted hosts (Li *et al*., 2010) as well as in AR-deficient prostate cancer cells (Wang *et al*., 2008). Hic-5 can promote TGF-β-induced epithelial-mesenchymal transition (EMT) in both breast and prostate cancer cells (Tumbarello and Turner, 2007; Wang *et al*., 2008) and reported to mediate apoptosis of normal prostate epithelium induced by androgen ablation (Li *et al*., 2010). Previous studies in our laboratory have shown that Hic-5 modulates TGF-β signaling by binding to and inactivating Smad3 (Wang *et al*., 2005) and an inhibitory Smad, Smad7 (Wang *et al*., 2008). Inactivation of Smad3 by Hic-5 effectively repressed TGF-β-induced apoptosis. However, inactivation of Smad7 enhanced TGF-β receptor signaling, with a net effect of the selective activation of Smad2 and non-Smad TGF-β responses, such as EMT (Wang *et al*., 2008).

In this study, we show that Hic-5 suppresses BMP4 signaling along with BMP-induced apoptosis in prostate cancer cells through binding to Smads 1, 5, and 8. Our results support that Hic-5 is a new regulator of BMP signaling.

Results

Hic-5 binds with Smads 1, 5 and 8

Our laboratory previously reported Hic-5 binds to Smad3 at its MH2 domain, a region of high sequence homology across the BMP receptor-associated Smads (Smads 1, 5 and 8). Based on this we tested whether Hic-5 could also bind to Smads 1, 5 and 8 in co-immunoprecipitation (co-IP) assays using HEK293 cells transfected with DNA constructs expressing Myc-Hic-5, FLAG-Smads 1, 5, 8 or empty control vector. Anti-FLAG IgG pulled down Myc-Hic-5 in lysates of cells over-expressing Flag-Smads 1, 5 or 8, but not in empty vector control (Fig 1a & Fig 1b). A greater amount of Smad8 than Smads 1 and 5 was pulled down by Hic-5. The levels of Flag-Smads 1, 5 and 8 expressed in cells were similar, suggesting that Hic-5 binds to Smad8 with higher affinity than to Smads 1 or 5. In contrast to Hic-5, Myc-Paxillin, (a homolog of Hic-5) was not pulled down by Smads 1, 5 and 8 (Fig 1b). FLAG-Survivin that was used as a negative control in this experiment failed to pull down Myc-Hic-5 (data not shown), supporting that binding of Hic-5 to Smads 1, 5, and 8 was specific. In addition, purified recombinant glutathione S-transferase (GST)-Smads 1, 5 and 8 fusion proteins, but not GST alone, pulled down *in vitro* transcribed and translated Hic-5, supporting that Hic-5 can bind directly to Smads 1, 5 and 8 (Fig 1c).

We next examined the *in vitro* effects of the physical interaction of Hic-5 with endogenous Smads 1, 5 and 8 activated by BMP4 in HEK293 cells expressing Myc-Hic-5 or empty vector. Following 24 h of BMP4 stimulation, we showed that Myc-Hic-5 pulled down phospho-Smads 1, 5 or 8 (Fig 1d). We detected the same level of endogenous Smad1 (with anti-Smad1 IgG) in both BMP4 and vehicle treated groups (Fig 1d), indicating Smad1 activation did not alter the strength of its interaction with Hic-5.

To define the domains of Hic-5 involved in its interaction with Smads 1, 5, and 8, we transfected HEK293 cells with various truncated constructs of Myc-Hic-5 (Fig. 2a) along

with full-length FLAG-Smads 1, 5 or 8, and then conducted a co-IP assay with anti-FLAG IgG. We found that Smads 1, 5, or 8 pulled down various Myc-Hic-5 constructs that contained the LIM3 domain, whereas the construct lacking the LIM3 domain was not pulled down by Smads 1 and 5 (Fig 2b-c), similar to the binding of Hic-5 to Smad3 (Wang *et al.*, 2005), but was pulled down by Smad8 (Fig 2d). These results suggest that LIM domains 1 through 3 are crucial for the binding of Hic-5 to Smads 1 and 5; however, only LIM domains 1 and 2 are crucial for binding of Hic-5 to Smad8.

We next defined the domains of Smads 1, 5 and 8 critical for their binding to Hic-5. HEK293 cells were co-transfected with various FLAG-Smad truncations (Fig 3a) along with Myc-Hic-5 followed by co-IP. For all members of the Smad family tested (Smad 1, 5 & 8), the constructs that contain MH2 domain (full-length, MH2+ML & MH2) were able to interact with Hic-5, whereas the MH2-deleted constructs (MH1+ML) did not bind to Hic-5 (Fig 3b-d), indicating that MH2 domain mediates the interaction of Smads with Hic-5.

Hic-5 binding inhibits the biological activity of Smads 1, 5 and 8

We next tested our hypothesis that binding of Hic-5 to Smads 1, 5 and 8 represses BMP-induced Smad responses by monitoring the activation of the Id1 promoter, which has a number of BMP response elements (BREs) (Katagiri *et al.*, 2002). For this we co-transfected a BMP4 responsive cell line, NRP-152 (non-tumorigenic rat prostate epithelial cell) with an Id1 promoter-luciferase construct in the presence or absence of a wild-type Hic-5 expression construct, and used Id1 promoter activity as a readout of Smad-dependent BMP transcriptional response. In parallel, cells were also co-transfected with an expression construct for Smad6, a known inhibitor of BMP receptor receptor, as an additional positive control. BMP4 (10 ng/ml) activated this promoter construct by over 15-fold, and overexpresssion of Hic-5 suppressed such activation by >2-fold (Fig 4a). Consistent with promoter activity, endogenous levels of Id1 mRNA and protein, which were robustly induced by BMP4 stimulation, were also suppressed >2-fold by Hic-5 (Fig 4b). We next examined whether Hic-5 could block the ability of transiently transfected Smads 1, 5 or 8 to activate the Id1 promoter. Hic-5 significantly suppressed activation of the Id1 promoter by each of those Smads (Fig 4c). These results demonstrate that Hic-5 blocks the activation of the Id1 promoter induced by overexpression of Smads 1, 5 or 8 alone.

The same Hic-5 truncations that were shown to bind to Smads 1, 5 and 8 were further examined for their ability to inhibit BMP4-induced Id1 promoter activity. For this experiment, NRP-152 cells were transfected with each of the four Hic-5 constructs (Fig 2a) along with the Id1 promoter-luciferase construct, followed by BMP4 (10 ng/ml) treatment for 24 h. The WT, LIM-only, and LIM4-deleted Hic-5 constructs suppressed BMP4-induced Id1 promoter response, whereas the LIM3-deleted Hic-5 construct did not (Fig 4d), indicating that regions of Hic-5 that are crucial for binding to Smads 1 and 5 are also crucial to Hic-5's ability to suppress BMP-4-induced Id1 promoter activity.

The effect of the endogenous Hic-5 on the activity of Smads 1, 5 and 8 was next investigated in the DU145 and PC3 human prostate cancer cell lines which express detectable levels of Hic-5 (Wang *et al.*, 2008). We evaluated the impact of silencing endogenous Hic-5 on activation of the Id1 promoter by BMP4, using Hic-5 silenced cell

lines previously developed (Wang *et al.*, 2008). Hic-5 silenced DU145 and PC3 cells (Fig 5a, Supplementary Fig 1), along with their sh-lacZ controls, were transfected with the Id1 promoter-luciferase construct and then treated with BMP4 (10 ng/ml) or vehicle (control) for 24 h. Depletion of endogenous Hic-5 enhanced BMP4-induced Id1 promoter activity by more than 37% and 27% in DU145 and PC3, respectively, consistent with the results of our Hic-5 overexpression experiments. Moreover, silencing endogenous Hic-5 also enhanced Id1 promoter activity without exogenous BMP4 treatment, indicative of a possible BMP autocrine loop. We tested this autocrine response with a BMPRI kinase inhibitor LDN-193189 (Cuny *et al.*, 2008). Pre-treatment with LDN-193189 suppressed Id1 promoter activity in both cell lines and made them refractory to the activation of the Id1 promoter induced by silencing Hic-5 (Fig 5b). In parallel, we measured changes in the expression of the Id1 protein and levels of phospho-Smad1/5/8 by Western blot analysis of PC3-sh-LacZ and PC3-sh-Hic-5 cells treated with LDN-193189 and a specific inhibitor of TβRI kinase, TKDI (Fig. 5c). Silencing Hic-5 elevated expression of Id1 and promoted the phosphorylation of Smad1/5/8. Treatment with LDN-193189, but not with TKDI, completely repressed the levels of Id1 and phospho-Smad1/5/8 induced by loss of Hic-5. These results support that endogenous Hic-5 represses the activity of autocrine BMP.

We next tested the potential off-target effect our sh-Hic-5 construct by overexpressing a Hic-5 construct with synonymous mutations in the Hic-5 shRNA sequence to render it resistant to silencing sh-Hic-5 without altering the amino acid sequence (Supplementary Fig. 3). Overexpression of this mutant construct reversed the ability of sh-Hic-5 to enhance basal and BMP4-induced Id1 promoter activity with >2-fold reversal relative to the wild-type Hic-5 construct (Fig 5d), indicating that enhancement the BMP response by sh-RNA is not an off-target response. Together, these results strongly support our hypothesis that Hic-5 inhibits BMP-induced Smad activity.

Hic-5 inhibits BMP4-induced apoptosis of prostate epithelial cells

We next investigated whether Hic-5 could reverse BMP4-induced growth inhibition or apoptosis of prostate epithelial cells. First, NRP-152 cells overexpressing Hic-5 by retroviral transduction of pLPCX-Hic-5 versus empty vector (pLPCX), and Hic-5 silenced cells (DU145-sh-Hic-5) versus control (DU145-sh-LacZ) were treated with BMP4 (10 ng/ml) or vehicle, and cell numbers was enumerated after 72 h to measure significant changes in cell growth using a Coulter Counter. As shown in Fig. 6a, growth of BMP4 treated NRP-152 cells overexpressing Hic-5 was 27% greater than empty vector control NRP-152 cells. Similar results were observed with LNCaP cells overexpressing Hic-5 (data not shown). Similarly, growth of DU145-sh-Hic-5 cells was reduced by 37% compared to DU145-sh-LacZ control following BMP4 treatment, clearly supporting that Hic-5 reverses growth inhibition by BMP in prostate epithelial cells. We thus evaluated the impact of Hic-5 on BMP4-induced apoptosis in NRP-152 prostate cells. NRP-152-pLPCX cells were more sensitive than NRP-152-pLPCX-Hic-5 cells to BMP4-induced apoptosis, as assessed by sub-G1 levels (Flow Cytometry), by nuclear condensation (Hoechst staining), and by cell viability (Trypan Blue dye-exclusion assay) (Fig 6b, Supplementary Fig 2).

We next investigated whether endogenous Hic-5 can also block the induction of apoptosis by BMP4. For this, nuclear condensation and cell viability of DU145-sh-LacZ versus DU-145-sh-Hic-5 cells were assayed following 72 h of treatment with BMP4 (10 ng/ml) or vehicle. The number of apoptotic cells was 28% greater in Hic-5 silenced cells relative to the sh-LacZ controls (Fig 6c), consistent with our Hic-5 overexpression data. Furthermore, we tested the specificity of this shRNA on BMP4-induced apoptosis of DU145 cells through rescuing the response with retroviral transduction of mutant Hic-5 (containing a synonymous mutation within the shRNA sequence) (Supplementary Fig 3). Expression of mutant Hic-5 reversed the ability of sh-Hic-5 to enhance the induction of apoptosis by BMP4 (Fig 6d). We also examined whether Hic-5 is a general inhibitor of apoptosis. For this we assessed whether silencing Hic-5 could enhance docetaxel-induced apoptosis. We found that silencing Hic-5 did not enhance docetaxal-induced apoptosis of DU145 cells, in contrast to a significant enhancement in BMP-4 induced apoptosis, compared side-by-side with docetaxal (Fig 6e). Taken together, our findings support that endogenous Hic-5 selectively reverses a number of BMP4-induced responses in prostate cancer cells.

Smad1 mediates BMP4-induced apoptosis

Studies conducted in embryonic cells have shown that BMP4 induces apoptosis via activation of Smad1 (Gambaro *et al*., 2006; Trousse *et al*., 2001). We thus examined whether Smad1 or another Smad was critical for BMP4-induced apoptosis in DU145 cells, to better explore the potential mechanism by which Hic-5 represses BMP4-induced apoptosis. Smads 1, 5 and 8 were each effectively silenced in these cells by lentiviral-mediated shRNA expression (Fig 7a). The Smad-silenced cells were treated with BMP4 (10 ng/ml) for 72 h, and evaluated for changes in apoptosis as before. The Smad1 silenced cells were essentially resistant to BMP4-induced apoptosis, as assessed by both assays, with respect to sh-lacZ control (Fig 7b), whereas the sh-Smad5 or sh-Smad8 variants were just as sensitive to BMP4-induced apoptosis as were the sh-LacZ cells. These results suggest that Smad1 but not Smads 5 or 8 are crucial for the induction of apoptosis. Together with results in Figs. 1- 6, these data suggest that Hic-5 blocks BMP4-induced apoptosis through binding to and interfering with the activity of Smad1.

Endogenous interaction and co-localization of Hic-5 with Smad1

We explored the physical interaction of Hic-5 with Smads 1, 5, or 8 in PC3 cells and primary cultures of rat dorsal prostate fibroblasts (RDPF) under conditions in which Hic-5 is predominantly in the cytosolic compartment, as well as under conditions of oxidative stress (i.e., 1 mM, H_2O_2 for 1 h), which keeps Hic-5 in the nucleus through interrupting the interaction of CRM1 with the nuclear export signal sequence of Hic-5 (Shibanuma *et al*., 2003). We immunoprecipitated Hic-5 from lysates of the above cells using anti-Hic-5 IgG1 MoAb immobilized on agarose beads, and analyzed the immunoprecipitated material for Smads 1, 5 and 8 by Western blot. When the cells were put under oxidative stress, Smad1 was pulled down with anti-Hic-5 IgG1 beads but not with non-immune IgG1 beads (Fig 8a), supporting that endogenous Hic-5 and Smad1 physically associate under oxidative stress, conditions simulating the tumor microenvironment (Federico *et al*., 2007; Przybyszewski and Rzeszowska-Wolny, 2009). For reasons not yet clear, we were unable to specifically detect the physical interaction of Hic-5 with Smads 5 or 8 under these conditions.

The enhanced complex formation between Smad1 and Hic-5 under the oxidative stress (Fig. 8a) suggests oxidative stress or nuclear localized Hic-5 may be promoting the nuclear localization of Smad1. To test this possibility we first examined the localization of endogenous Hic-5 and Smad1 in PC3 cells by immunofluorescent microscopy following 1 h treatment with $\pm H_2O_2 \pm$ BMP4 (Fig. 8b). Interestingly, treatment of PC3 cells with either H_2O_2 or BMP4 alone promoted the nuclear localization of both Smad1 and Hic-5. We next examined whether the ability of H_2O_2 to promote the nuclear localization of Smad1 was dependent on Hic-5. To address this, we treated control PC3-sh-LacZ and PC3-sh-Hic-5 cells $\pm H_2O_2 \pm$ BMP4 and used immunofluorescent microscopy to assess the cellular localization of Smad1 after 1 h of treatment. In the sh-LacZ cells, either H_2O_2 or BMP4 promoted the nuclear localization of Smad1, although the sh-LacZ cells expressed higher levels of nuclear Smad1 relative to the parental cell line (Fig. 8b,c). In contrast, H_2O_2 but not BMP4 treatment failed to promote nuclear uptake of Smad1 in PC3-sh-Hic-5 relative to PC3-sh-LacZ cells (Fig. 8c). These results show that Hic-5 and Smad1 functionally interact by affecting each other's cellular localization, further supporting our model that the physical association of these growth regulatory proteins impact on their mutual biological functions.

Discussion

Here we present the first evidence that Hic-5 inhibits a number of BMP4 responses, including apoptosis and Id1 gene expression in prostate epithelial cells. We show that such suppression occurs through a physical interaction of Hic-5 with the MH2 domains of Smads 1, 5 and 8. Structural-functional analysis further support that the LIM3 domain of Hic-5 is critical for its ability to bind to Smads 1 and 5 and repress BMP responses, similar to our previous results with Smad3 (Wang *et al*., 2005). The binding of Hic-5 to Smad1 and the critical role of Smad1 in BMP4-induced apoptosis were confirmed at the endogenous level, providing evidence that Hic-5's ability to repress BMP4-induced apoptosis occurs at least in part through inactivating Smad1. Our findings here, along with previous reports that Hic-5 regulates certain TGF-β responses (Wang *et al*., 2008) suggest that Hic-5 functions as a common regulator of cellular responses by members of the TGF-β superfamily. In the case of TGF-β signaling, Hic-5 also promotes the degradation of the inhibitory Smad, Smad7, thus resulting in a net activation of non-Smad-mediated TGF-β signaling. Whether Hic-5 activates certain BMP responses similar to that of TGF-β remains to be explored. Nevertheless, the selective expression of Hic-5 in normal prostate stromal cells versus normal epithelial cells under physiological conditions is likely to play a role in the differential signaling responses by TGF-β and BMP in stromal versus epithelial cells. Similarly, the ectopic overexpression of Hic-5 in carcinomas may promote tumor progression by blocking TGF-β and BMP-induced apoptosis and by promoting EMT. Although ectopic overexpression of Hic-5 in prostate carcinoma cells can repress apoptosis by BMP, we provide preliminary evidence that expression of Hic-5 also sensitizes prostate cancer cells to the activation of Akt by insulin-like growth factor-I (IGF-I) (Supplementary Fig 4). Coincidentally, we recently demonstrated that IGF-I represses BMP4 responses through inhibiting the phosphorylation of Smads 1/5/8 (Wahdan-Alaswad *et al*., 2010). This suggests that elevated levels of Hic-5 may repress the tumor suppressor function of BMP also through enhancing the activation of Akt by IGF-I in prostate cancer cells.

Our data suggest that oxidative stress stimulates the binding of endogenous Hic-5 to Smad1, driving migration of both Hic-5 along with Smad1 to the nucleus (Fig. 8). This nuclear migration of Smad1 likely occurs through a mechanism involving Smad1's physical association with Hic-5, as silencing Hic-5 repressed the ability of H_2O_2 to induce nuclear migration of Smad1. Moreover, BMP4 treatment of PC3 cells also drives Hic-5 to the nucleus, similar to that by H_2O_2, suggesting that BMP4 may promote Hic-5's function as a transcriptional co-regulator. The nuclear entry of Hic-5 following BMP4 treatment, while likely requiring the physical association between Hic-5 and Smad1, is unlikely to be triggered simply through association of Smad1 with Hic-5, as our pull-down experiments showed that the physical binding of Hic-5 with Smad1 does not require BMP4 treatment or the phosphorylation of Smad1 (Fig. 1c,d). It is possible that such nuclear entry of Hic-5 upon BMP4 stimulation may require another BMP4 response, such as oxidative stress, as BMP4 has been previously implicated in inducing oxidative stress at least in arterial endothelial cells (Csiszar *et al*., 2008; Wong *et al*., 2010).

Smad3 was shown to be recruited by Hic-5 to the p21 promoter, enhancing Hic-5's transcriptional co-activator function on the p21 promoter (Shibanuma *et al*., 2004). Owing to similarities in the physical association of Smads 1, 5 and 8 versus Smad3 to Hic-5, it is likely that Smads 1, 5 & 8 impact Hic-5's transcriptional co-regulatory function similar to that of Smad3 (Shibanuma *et al*., 2004). The potential impact of Smads on the co-regulatory function of Hic-5 on AR signaling remains to be explored. A recent study reported that Hic-5 is expressed in prostate epithelial cells upon androgen ablation and is critical in the induction of androgen-ablation induced apoptosis (Li *et al*., 2010). Similiarly, we found that DHT inhibits the expression of endogenous Hic-5 in PC3 overexpressing AR (Supplementary Fig 5). Previous evidence supports that Hic-5 (Heitzer and DeFranco, 2007) and TGF-β (Bhowmick *et al*., 2004) also have indirect but important roles in suppressing growth of prostate epithelial cells through effects on prostate fibroblasts. Similar to prostate tumor epithelial cells, silencing Hic-5 expression in the human prostate stromal cell line, WPMY-1, elevated the phosphorylation of Smad1/5/8 (Supplementary Fig. 6). The potential influences of Smads in cooperating with Hic-5 to repress AR-induced responses (i.e., induced expression of keratinocyte growth factor) in stroma cells remain to be explored.

Although *in vitro* studies have shown that Hic-5 is ectopically expressed in androgen-independent prostate cancer cells and associated with EMT, the role of Hic-5 in enhancing prostate tumor progression remains to be confirmed *in vivo*. Hic-5 has been recently reported to be expressed in normal prostate epithelial cells and prostate carcinoma upon androgen withdrawal, and such induction has been suggested to promote androgen withdrawal-induced apoptosis (Li *et al*., 2010). However, our data on Hic-5 silencing in PC3 and DU145 cells suggest that Hic-5 represses BMP4-induced apoptosis, consistent with an oncogenic role of Hic-5, and that Hic-5 may promote the survival of prostate cancer cells that have metastasized to bone, a rich source of BMPs, by protecting them against BMP-induced apoptosis. Our immunohistochemical analysis shows that Hic-5 can be found in stage IV prostate cancer (primary tumor and bone metastasis) of non-treated patients (Supplementary Fig. 7). We speculate that anti-androgens will further enhance expression of Hic-5 in advanced prostate tumors and metastatic lesions, and that such upregulation will

contribute to the development of a castrate-resistant phenotype in which anti-androgens often accelerate disease progression (also known as the anti-androgen withdrawal syndrome) (Yoshida *et al*., 2005) (Terada *et al*., 2010). Our results thus implicate Hic-5 as a new therapeutic target for advanced prostate cancer.

Methods and Materials

Cell Culture

The HEK293 cells (passage 43-52) were cultured in Dulbecco's modified Eagle's medium/ Ham's F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA, USA) containing 5% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). NRP-152 rat prostatic tumorigenic cell line (Danielpour *et al*., 1994) passage number (62-71) was cultured in GM2.1 (DMEM/F-12 containing 5% FBS, 5 mg/ml insulin and 0.1 mM dexamethasone). The PC3 (passage 64-73) and DU145 (passage 13-22) human prostatic carcinoma cell lines were cultured in DMEM/ F-12 medium containing 5% FBS. RDPF rat dorsal prostatic fibroblast primary cell (passage 9-18) was cultured in DMEM/F-12 + 5% FBS supplemented with 1 μM insulin, 10 ng/ml EGF, 10 nM DHT and 2 ng/ml bFGF. For low serum medium condition, NRP-152 cells were cultured in GM3 medium (DMEM/F12 + 1% FBS, 15 mM HEPES (pH7.4), 0.1 μM dexamethasone).

Co-IP & Western blot

HEK293 cells were plated in DMEM/F-12 + 5% FBS medium in 6-well tissue culture dishes (Falcon) at 2.5×10^5 cells/2ml/well for 48 h, and then transfected with 2 μg of plasmid DNA using a calcium phosphate precipitation method (Chipuk *et al*., 2002). Cells were lysed 36 h later, tagged proteins were co-immunoprecipitated as described previously (Wang *et al*., 2005), and subjected to 4-12% NuPAGE BisTris gel with MES buffer (Invitrogen, Carlsbad, CA).

Flow Cytometry analysis

NRP-152 cells (2×10^6) were trypsinized, washed once with PBS, fixed with 90% methanol, and incubated with 0.1 mg/ml of RNase A followed by propidium iodide (50 μg/ml) (Schimenti and Jacobberger, 1992). The cells were analyzed with an EPCS-XL MCL Flow Cytometer for sub-G1 (< 2n DNA content) to assay the apoptotic fraction.

Transfection and luciferase assay

NRP-152, DU145 and PC3 cells were plated overnight in 12-well tissue culture dishes (Falcon) with GM2.1 (NRP-152) or DMEM/F-12 + 5% FBS (PC3, DU145) medium at 1×10^5 cells/1ml/well or 2×10^5 cells/2ml/well. Adherent cells were then transfected with human Id1 promoter construct (pGL2-Id1) (1 to 2 μg) + 20 ng of CMV-renilla reporter construct using polyethylenimine (25 kDa) as described before (Yang *et al*., 2008). The cells were allowed to recover overnight in GM3 (NRP-152) or in DMEM/F-12 + 1% FBS + 15 mM HEPES (pH 7.4) (PC3, DU145), then treated with vehicle control or BMP4 (10 ng/ml) for 24 h. Dual luciferase activity was assessed as before (Song *et al*., 2003).

RNA Preparation and RT-PCR

RNA was extracted and purified with PureLink™ RNA Mini Kit (Invitrogen). M-MLV Reverse Transcriptase (Promega) was used to generate cDNA, and Taq Polymerase Master Mix (Promega) was used for the PCR amplification of rat Id1, Id2, and Id3. PCR amplifications cycle was 23 (Id1, Id2) & 40 (Id3), and condition was 95°C for 15s, 55°C for 30 s, and 72°C for 1.5 min. Products were subjected to 1% agarose gels electrophoresis and evaluated using a Biorad Molecular Imager Gel Doc XR+ System.

Reagents, ***In Vitro Transcription & Translation and GST pull-down, Lentiviral shRNA construct preparation, Adenovirus Vectors and Infection, Hoechst 33258 staining assay, Cell viability assay & cell number assay, Plasmids, Retrovirus Vectors & Infection:*** see Supplemental section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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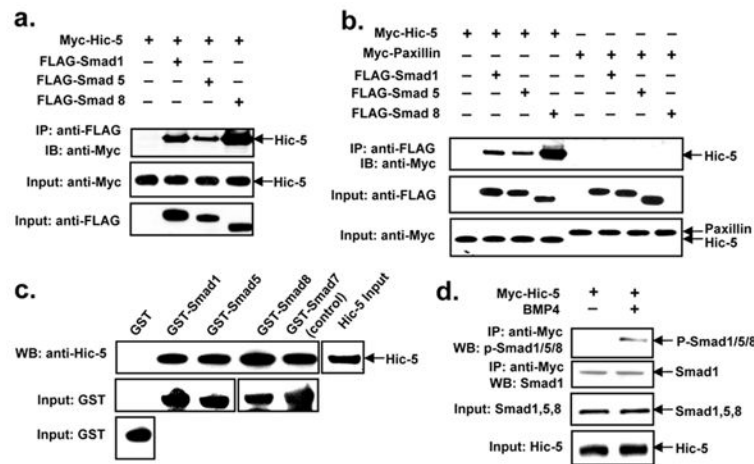
Figure 1. Binding of hydrogen peroxide-inducible clone-5 (Hic-5) to Smads 1, 5 and 8
(a) Human embryonic kidney cell line 293 (HEK293) plated in 6-well dishes were co-transfected with 1 μg of Myc-Hic-5 and 1 μg of FLAG-Smads 1 or 5 or 8 or empty vector. Cell lysates were immunoprecipitated with anti-FLAG IgG1 monoclonal antibody (M2) and both immunoprecipitated proteins and input lysates were immunoblotted with rabbit anti-Myc IgG. (b) The same co-IP experiment was conducted except Myc-Paxillin, a homologue of Hic-5, was included as a control for specificity. (c) *In vitro* transcribed and translated Myc-Hic-5 was incubated with glutathione S-transferase (GST) or GST-Smads 1 or 5 or 8 or 7 (control) fusion protein overnight at 4°C in the presence of glutathione-Sepharose resin in cold RIPA buffer. The eluate was immunoblotted with anti-Myc antibody. (d) HEK cells were transfected with Myc-Hic-5 and treated with/without BMP4 (10 ng/ml) for 24 h. Cell lysates were immunoprecipitated with anti-Myc IgG monoclonal antibody and the eluate was immunoblotted with anti-phospho-Smad1/5/8 and anti-Smad1 antibodies.
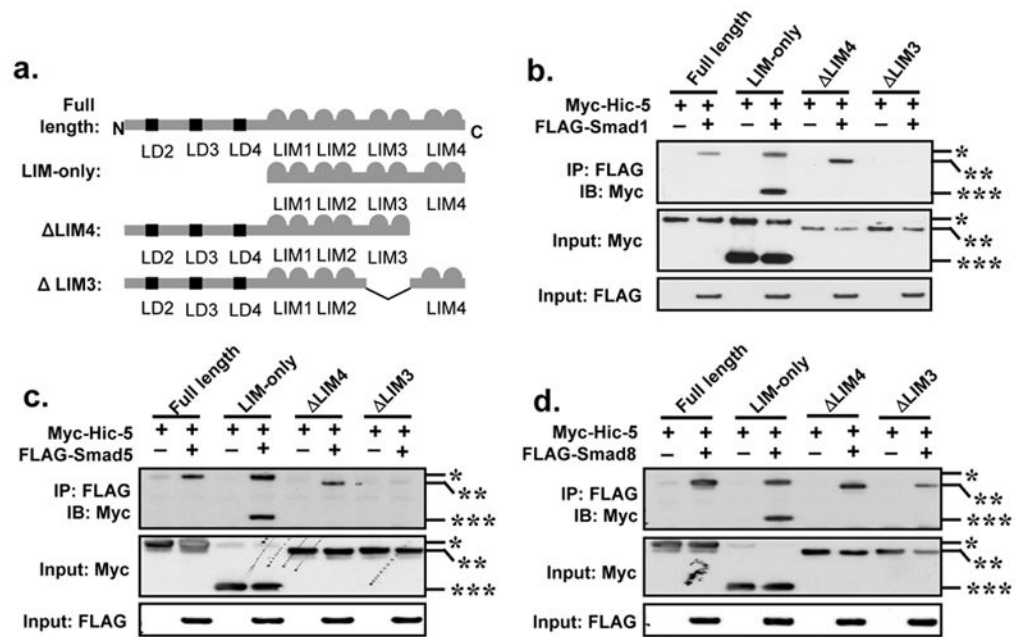
Figure 2. Characterization of Hic-5 binding domain

(a) Map of Hic-5 truncation vectors; Full-length, LIM-only (LIM1, 2, 3 & 4), LIM4 (LIM4-deleted), and LIM3 (LIM3-deleted). (b) HEK293 cells were co-transfected with 1µg of full-length or truncated Myc-Hic-5-pcDNA3 (see map in a) and 1 µg of full-length FLAG-Smad1-pcDNA3 or empty vector. Immunoprecipitation and immunoblotting were performed as in Figure 1a. (c,d) The same co-IP experiment was conducted except full-length Smad5 was used in (c) and full-length Smad8 was used in (d). * Full length, LIM-only dimer; ** LIM4, LIM3; *** LIM-only monomer.
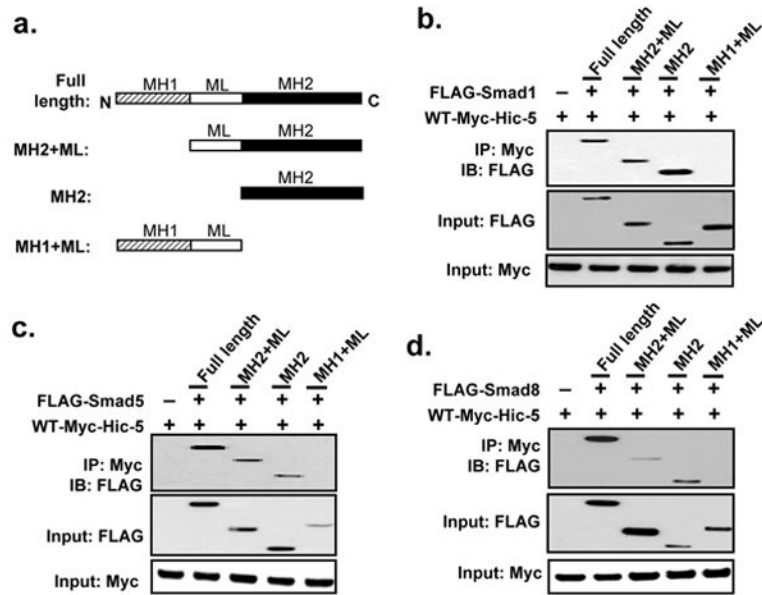
Figure 3. Characterization of Smads 1, 5 and 8 binding domain
(a) Map of Smads 1, 5 and 8 truncation vectors; Full-length, MH2+ML (MH1 domain deleted), MH2 (MH1 and ML domains deleted), and MH1+ML (MH2 domain deleted). (b) HEK293 cells were co-transfected with 1µg of full-length or truncated FLAG-Smad1 (in pcDNA3) (see map in a) and 1µg of full-length Myc-Hic-5 or empty vector. Immunoprecipitation and immunoblotting were performed in the same way as in Fig. 1a. (c,d) The same co-IP experiment was conducted except FLAG-Smad5 or its truncation vectors were used in (c) and FLAG-Smad8 or its truncation vectors were used (d).
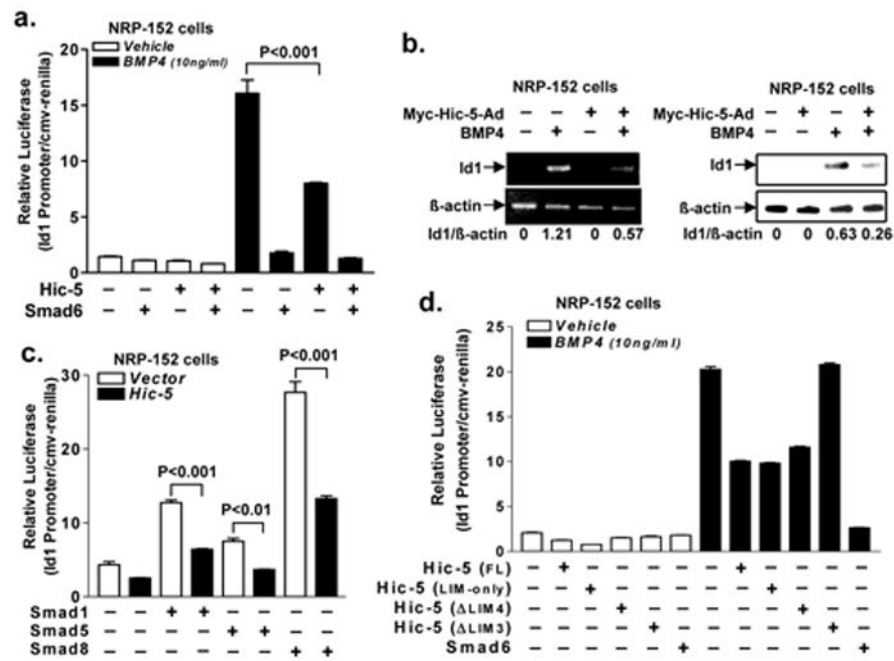
Figure 4. Assessing Hic-5's effect on Smads 1, 5 and 8 activities

(a) NRP-152 cells were co-transfected with 0.2 μg of Id1 promoter-luciferase (in the pGL2 vector), 12.5 ng of pRL-CMV internal control, and 0.4 μg of Myc-Hic-5-pcDNA3, Smad6-pcDNA3 or empty vector. The next day, cells were treated with BMP4 (10 ng/ml) or vehicle control for 24 h, before measuring luciferase activity. Luciferase values represent the average of triplicate determinations ±S.E. (b) Effects of Hic-5 on endogenous Id1 mRNA and protein. NRP-152 cells were transfected with Myc-Hic-5-Ad or control vector, and treated with BMP4 or vehicle control for 24 h. Total RNA was extracted and subjected to RT-PCR with Id1 primers (left). The lysates were subjected to Western blot (right) and immunoblotted for endogenous Id1 and β-actin (control). (c) The same experiment was performed as in (a) except FLAG-Smads 1 or 5 or 8 +/- Myc-Hic-5 were overexpressed. (d) The same experiment described in (a) was conducted except Hic-5 truncation constructs (as in Figure 2d) were used. The results shown are representative of three independent experiments.
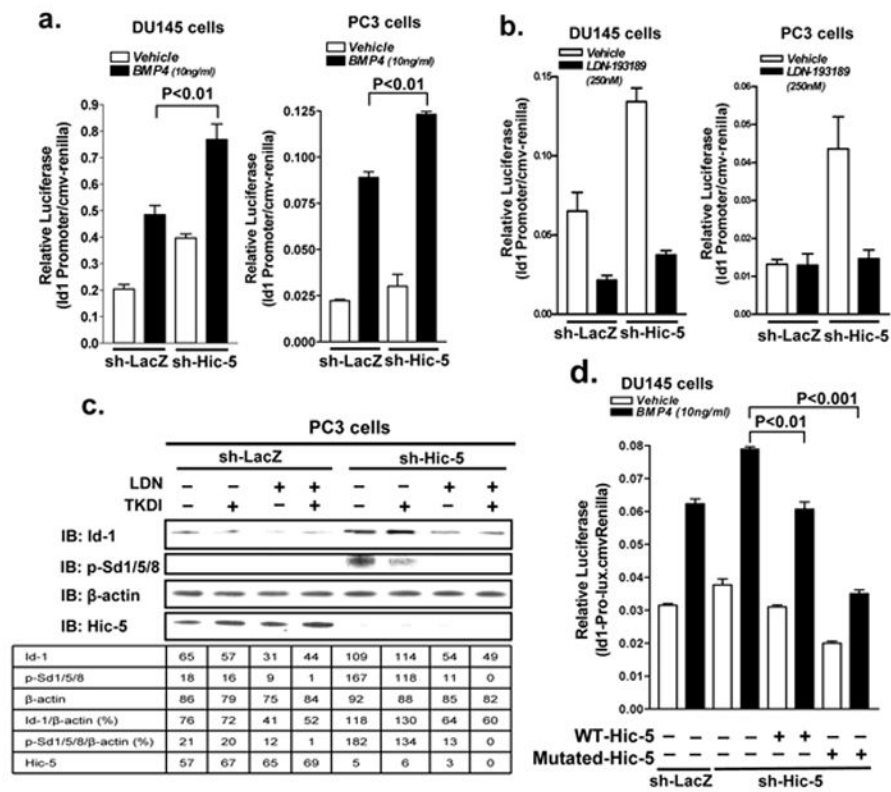
Figure 5. Endogenous Hic-5 represses autocrine BMP activity

(a) Hic-5 silenced DU145 and PC3 cells were transfected with 0.2 μg of Id1 promoter-luciferase construct followed by treatment with BMP4 (10 ng/m) or vehicle control for 24 h before harvesting for assaying luciferase activity. Hic-5 was silenced as described in published protocol (Wang et al., 2008). (b) The same experiment was conducted as in (a) except LDN-193189 (250 nM) was used instead of BMP4. (c) Hic-5 silenced PC3 and lacZ shRNA control cells were treated with LDN-193189 (250 nM) or/and TKDI (TβRI kinase inhibitor) (0.25 μM) for 48 h, before immunoblotting for Id1, phospho-Smad1/5/8, β-actin and Hic-5, and band intensities were assessed by Photoshop CS5 (Adobe, San Jose, CA, USA). (d) The same experiment was conducted as in (a) except 0.4 μg of wild-type Hic-5 expression construct, and a mutated-Hic-5 expression construct (insensitive to sh-Hic-5), were independently transfected into DU145-sh-Hic-5 cells. All of the data are representative of two independent experiments.
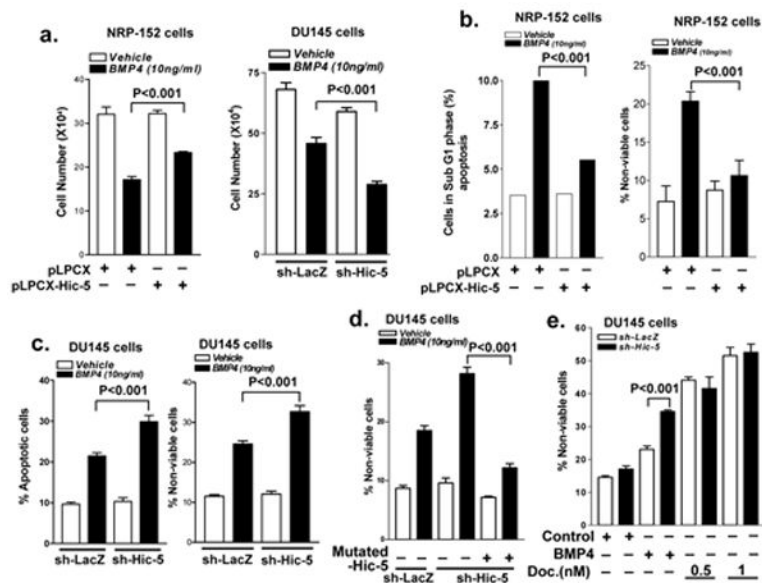
Figure 6. Effects of Hic-5 on BMP4-induced apoptosis

(a) NRP-152 cells were transduced with a Myc-Hic-5 using pLPCX retroviral vector system, and stably transduced and Hic-5 silenced DU145 cells (described in Fig 5a) were treated with BMP4 (10 ng/ml) or vehicle control for 72 h, and the cells were enumeratedwith a Coulter counter. (b) NRP-152 cells transduced with Hic-5 received the same treatment as described in (a). The propidium iodide stained cells were subjected to Flow cytometry for determination of the sub-G1 fraction (left). The same NRP-152 cell treatment was performed as above except apoptotic fraction was assessed following staining with Trypan Blue (right). (c) Hic-5 silenced DU145 cells and sh-lacZ control as described in Fig 5a were treated with BMP4 (10 ng/ml) or vehicle control for 72 h, and Hoechst dye (left) or Trypan blue dye (right) were used for assessing dead cells. (d) The same experiment was conducted as in (c) except a mutated-Hic-5-pLPCX (insensitive to sh-Hic-5) was stably expressed, and dead cells were assessed with Trypan blue dye. (e) DU145-sh-Hic-5 and DU145-sh-lacZ control were treated with docetaxel (Doc.) (0.5 & 1 nM) and BMP4 (10 ng/ml), and trypan blue was used to distinguish between live and dead cells.
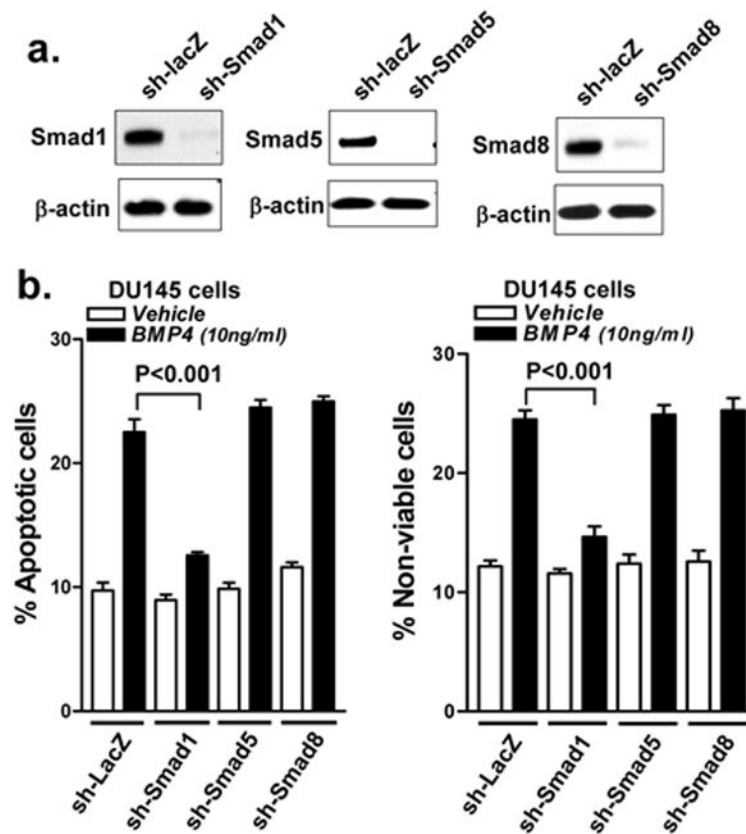
Figure 7. Identification of Smad1 as a critical mediator of BMP4-induced apoptosis
(a) Smads 1, 5 and 8 were independently silenced in DU145 cell by expression of hRNA by lentiviral transduction system; with respect to lacZ shRNA expression used as control. (b) The Smads 1, 5 & 8 silenced cells described in (a) were treated with BMP4 (10 ng/ml) for 72 h, followed by staining with Hoechst dye (left) or Trypan Blue dye (right) for enumeration of apoptotic and live cells, respectively.
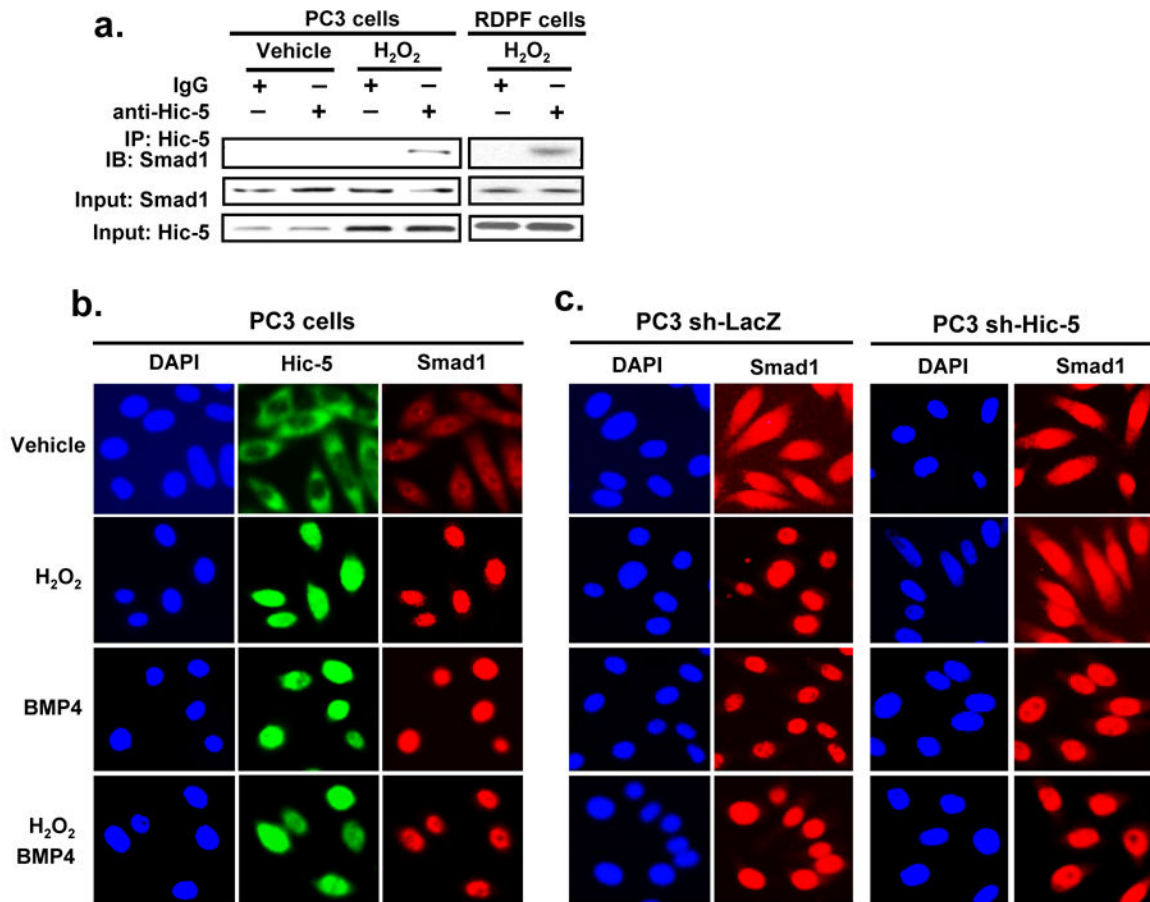
Figure 8. Localization of Hic-5 and Smad1
(a) PC3 and RDPF cells were treated with hydrogen peroxide (1 mM) or vehicle control for 1 h. Cell lysates were immunoprecipitated with anti-Hic-5 monoclonal antibody or IgG control and both immunoprecipitated proteins and input lysates were immunoblotted with rabbit anti-Smad1 IgG. (b) PC3 cells were cells were plated in DMEM/F-12 + 1% FBS overnight in an 8-well chamber slides (Lab-Tek II). The next day, cells were treated with 10 ng/ml BMP4, 1 mM H_2O_2 or vehicle for 1 h and were then subjected to immunofluorescent microscopy following binding with anti-Hic-5 mouse IgG1 and anti-Smad1 rabbit IgG. (c) PC3-sh-LacZ or PC3-sh-Hic-5 cells were cultured, treated and examined as in (b) for Smad1 and nuclei (by DAPI). All of the data shown are representative of three independent experiments.