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Activin type IB receptor signaling in prostate cancer cells promotes lymph node metastasis in a xenograft model

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ABSTRACT

Activin, a member of the transforming growth factor-β family, has been known to be a growth and differentiating factor. Despite its pluripotent effects, the roles of activin signaling in prostate cancer pathogenesis are still unclear. In this study, we established several cell lines that express a constitutive active form of activin type IB receptor (ActRIBCA) in human prostate cancer cells, ALVA41 (ALVA-ActRIBCA). There was no apparent change in the proliferation of ALVA-ActRIBCA cells *in vitro*; however, their migratory ability was significantly enhanced. In a xenograft model, histological analysis revealed that the expression of Snail, a cell-adhesion-suppressing transcription factor, was dramatically increased in ALVA-ActRIBCA tumors, indicating epithelial mesenchymal transition (EMT). Finally, mice bearing ALVA-ActRIBCA cells developed multiple lymph node metastases. In this study, we demonstrated that ActRIBCA signaling can promote cell migration in prostate cancer cells via a network of signaling molecules that work together to trigger the process of EMT, and thereby aid in the aggressiveness and progression of prostate cancers.

1. Introduction

Prostate cancer is a leading cause of illness and death among men in industrialized countries. Although androgen depletion therapy as a first line treatment is effective, eventually the tumor becomes castration-resistant and recurs with metastasis to distant organs via the lymph nodes, leading to death of the patient [1]. Thus, understanding the mechanisms governing lymph node metastasis of prostate cancer cells is required for the effective treatment of advanced cancer [2].

The transforming growth factor beta (TGF-β) superfamily is well known to play important roles not only in the determination of cell fate and growth control, but also in the pathogenesis of many cancers [3,4]. TGF-β has both tumor-suppressive and tumor-promoting functions [5]. At the advanced stage of tumor progression, TGF-β promotes processes that support tumor progression such as tumor cell invasion, dissemination, and immune evasion [6]. Consequently, the functional outcome of the TGF-β response is strongly context-dependent in cell, tissue, and cancer types. In prostate cancer, TGF-β has been shown to enhance tumorigenicity and accelerate the malignant process at an advanced stage of cancer [7,8].

Signals by the TGF-β family are transduced through a group of intracellular signal transducers, the Smad protein family [9]. Upon ligand binding, the type II receptor recruits and transphosphorylates the type I receptor, which is activated by phosphorylation; and subsequently propagates signals to the Smad pathway. Once phosphorylated, the receptor-regulated Smads (R-Smads) dissociate from the receptor complex, bind to Smad4 and enter the nucleus as a complex where they participate in transcriptional regulation of the target genes. Inhibitory Smads (I-Smad) have been shown to block the phosphorylation of R-Smad by type I receptors, or to block the heterooligomerization of R-Smad with Smad4 by binding to the type I receptor or R-Smad, thus interfering with TGF-β signaling.

Activin, a member of the TGF-β superfamily, has also been shown to regulate proliferation and differentiation of prostate cancer cells, as well as prostate gland epithelial cells [10,11]. The biological effects of activin are mediated by a complex of type I and type II receptors. Type I receptors comprise type IA and type IB, and type II receptors comprise ActRIIA and ActRIIB [12]. Although activin has been shown to phosphorylate the same subset of R-Smad proteins, Smad2 and Smad3, as TGF-β, its biological functions are clearly distinguished [11,13] In addition, the physiological role of activin in prostate cancer is less understood. In this study, we therefore investigated the signaling pathways and roles of activin in an androgen-independent human prostate cancer cell line, ALVA41. A series of transfection experiments using 3TP-Lux as

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a reporter plasmid revealed that ActRIB is a critical type I receptor in the activin signaling pathway. To further investigate the role of activin signaling in prostate cancer, cell lines expressing a constitutive active form of activin type IB receptor (ActRIBCA) in ALVA41 cells, designated ALVA-ActRIBCA cells, were established and subjected to xenograft experiments. While cell proliferation *in vitro* was not altered, the tumor growth in the xenograft model was substantially impaired by the expression of ActRIBCA. In addition, histological analysis revealed that EMT was observed concomitantly with increased expression of Snail, a cell-adhesion-suppressing transcription factor, in ALVA-ActRIBCA xenografts. Consequently, multiple lymph node metastases occurred in the mice bearing ALVA-ActRIBCA cells. Our results suggest that the activin signaling pathway might be a potential target of a new therapeutic approach to prevent the metastasis of prostate cancer.

2. Materials and methods

2.1. Cell culture

The human prostate ALVA41 cancer cell line [14] was kindly provided by Dr. Naitoh (Kyushu University). ALVA41 cells were cultured in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Sera Laboratories Ltd., Sussex, UK), penicillin (100 Units/ml), and streptomycin (100 μg/ml) in a 5% CO_2 atmosphere at 37 °C. Recombinant human activin A was kindly provided by Dr. Y. Etoh (Ajinomoto Co. Inc., Central Research Laboratories, Kawasaki, Japan).

2.2. Cell proliferation assay

A cell counting Kit-8 (Dojindo Co. Ltd., Kumamoto, Japan) was used to measure cell proliferation according to the manufacturer's protocol [15].

2.3. Plasmids

Expression vectors for rat ActRIA and human ActRIB, human ActRIBCA were kindly provided by Dr. L.S. Mathews (University of Michigan, Ann Arbor, MI). A constitutively active form of ActRIB, designated ActRIBCA, was generated by substitution of threonine with glutamine at position 206 in human ActRIB cDNA [12]. Myc epitope-tagged expression constructs for Smad2 and Smad3 were kindly provided by Dr. K. Miyazono (University of Tokyo). The reporter plasmid 3TP-Lux, containing three consecutive TPA responsive elements and TGF-β responsive elements of the plasminogen activator inhibitor 1 (PAI-1) promoter, was kindly provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY). Construction of the ActRIBCA expression vector is described elsewhere [16].

2.4. qRT-PCR

To analyze the expression of the activin signaling components, sensitive qRT-PCR was performed as previously described [16].

2.5. Functional reporter assay

ALVA41 cells were used for the functional assays. A total of 1×10^5 cells/well were transfected using 2 μl/well Superfect Transfection Reagents (QIAGEN, Hilden, Germany) with 0.5 μg/well 3TP-Lux reporter construct along with indicated plasmids and 1.5 ng/well pRL-CMV (Promega Corp., Madison, WI) as an internal control. After 24 h incubation of the cells in the presence or absence of activin A (25 ng/ml) or TGF-β1 (25 ng/ml, Sigma, St. Louis, MO), luciferase activity was measured using a dual luciferase assay system (Promega Corp.) using a microLumat LB9507 luminometer. The luciferase activity was normalized for transfection efficiency using the Renilla luciferase activity from pRL-CMV.

2.6. Transwell assays

Cells (3×10^4) were suspended in 100 μl of DMEM and seeded in the upper well of a Transwell chamber (Corning costor 3422 Transwell, 8.0 μm pore, Cambridge, MA). The lower well contained 600 μl of DMEM. After 48 h incubation, the number of cells that had migrated into the lower well was counted. Each assay was performed in triplicate and repeated two times ($P < 0.05$).

2.7. Tumor xenografts

A total of 5 × 105 cells were subcutaneously injected into the backs of male nu/nu athymic mice. The volumes of tumors were measured every week. After 5 weeks, all mice were euthanized and examined. Mouse experiments were performed according to guidelines set by the animal ethics committee of Kyushu University Graduate School of Medicine. Immediately after excision of the mouse tumors and tissue, small pieces of tissue about 5–10 mm in diameter were sampled, fixed in 4% paraformaldehyde at 4 °C overnight, and prepared to make paraffin-embedded blocks. All of the resected tissues were histologically examined by hematoxylin and eosin (HE) staining.

2.8. Antibody and immunohistochemistry

Immunostaining was performed for the deparaffinized sections using primary antibodies against Snail (1:300, rabbit polyclonal; Abcam, Cambridge, UK) and S100A4 (1:100, rabbit polyclonal; Abcam). Immmunostaining was performed with diaminobendidine solution with hydrogen peroxide for 20 min.

2.9. Western blot analysis

Fifteen microliters of protein sample (total protein, 5 μg for HA) were mixed with 15 μl Laemmli sample buffer and 1.5 μl β-mercaptoethanol before separation by an electrophoresis system. After completion of electrophoresis, samples were transferred to membrane filters. The transferred samples were probed with anti-HA antibody (1:80, 12CA5; Santa Cruz, CA) overnight at 4 °C. Chemiluminescent signals were generated by incubation with the ECL reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.10. Statistical analysis

All experiments were carried out at least three times. For the luciferase assays, each independent experiment was run in triplicate or duplicate plates and used to generate a single mean value, which was then used to generate the mean ± SD shown in the figures. All values represent the mean ± SD. Statistical significance was determined by one-factor ANOVA followed by a *post hoc* test (Fisher's protected least significant difference test).

3. Results

3.1. Androgen-independent ALVA41 prostate cancer cell line

To investigate the effects of activin on the proliferation, ALVA41 cells were cultured in the presence of fixed doses of ligands and

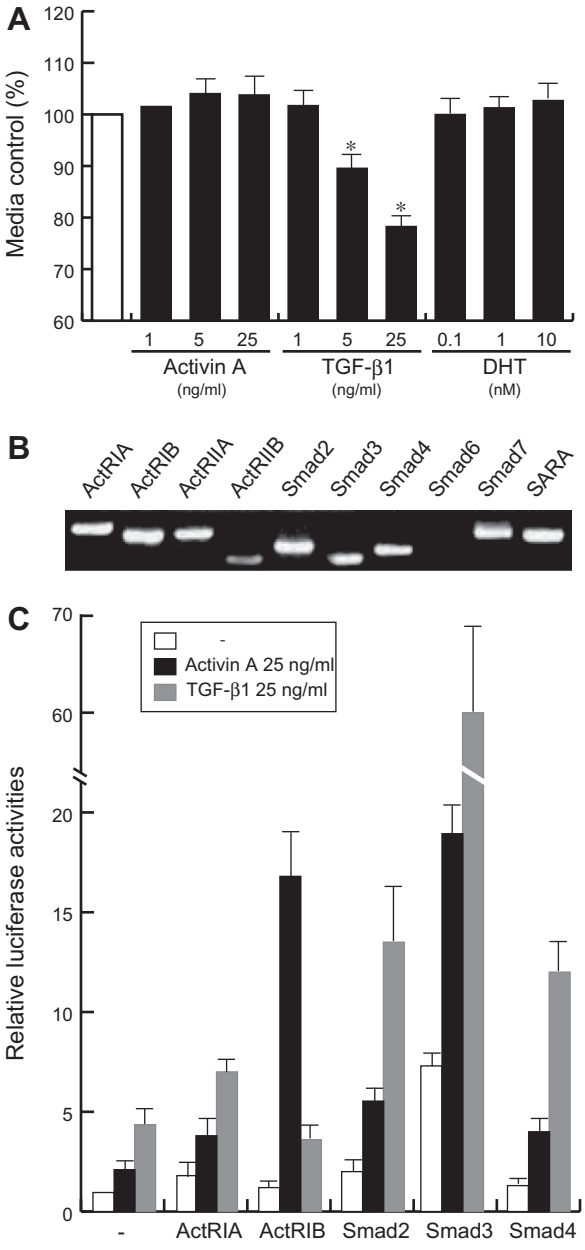

Fig. 1. Functional signaling pathways for activin and TGF-β in ALVA41 cells. (A) Effects of activinA, TGF-β and dihydrotestosterone on the proliferation of ALVA41 cells. Cells were treated with fixed doses of ligand as indicated for 96 h. The results are expressed as a percentage of the medium control value. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. $^{*}P < 0.01$. (B) qRT-PCR amplification of the known components of the activin signaling pathway. Total RNA from ALVA41 cells was prepared and qRT-PCR was performed as described in the Materials and Methods. Ethidium bromide-stained PCR products were separated on a 2% agarose gel. PCR products at 35 cycles are shown. (C) Luciferase assays were performed on the lysates from cells transfected with 3TP-Lux together with the indicated receptor or Smad in the presence or absence of activin A (25 ng/ml) or TGF-β (25 ng/ml). The fold inductions relative to the luciferase activity in the cells transfected with the reporter luciferase plasmid alone in the absence of ligand are shown. Each value indicates the mean ± SD of three experiments, with triplicate plates per point.

cell numbers were analyzed using Cell counting kit-8 as described in the Materials and Methods. While TGF-β inhibited the cell proliferation to 80% of the control level, neither activin nor dihydrotestosterone (DHT) had an effect, as shown in Fig. 1A. Thus, we concluded that ALVA41 cells are insensitive to activin and DHT with respect to the proliferation.

3.2. Identification of ActRIB as the type I receptor for activin in ALVA41 cells

We next analyzed the expression of activin signaling components in ALVA41 cells by qRT-PCR. As shown in Fig. 1B, each product of the activin receptors and Smads amplified by qRT-PCR was detected as a single band, and the size of each fragment was found to correspond to the predicted size. The authenticity of the PCR products was further confirmed by sequencing. All of the subtypes of activin type I and II receptors, Smad2, Smad3, Smad4 and Smad anchor for receptor activation (SARA), were detected at 35 cycles of qRT-PCR, suggesting the potential for activin signal transduction in ALVA41 cells. With regard to the inhibitory Smads, Smad7 was detected at 35 cycles, whereas Smad6 could not be amplified even at 40 cycles (data not shown).

To demonstrate the functional activin signaling pathways in ALVA41 cells, luciferase reporter assays were carried out with 3TP-Lux, a TGF-β/activin-responsive reporter construct, in combination with the expression plasmids for activin receptors and Smads. When ALVA41 cells were transiently transfected with 3TP-Lux and stimulated with either activin A or TGF-β1 for 24 h before being measured for luciferase activity, activin and TGF-β increased the luciferase activity 2- and 4-fold, respectively, as shown in Fig. 1C. To address the functional type I receptor for activin, ALVA41 cells were transfected with each type I receptor along with the reporter construct as indicated in Fig. 1C. As expected, luciferase activity was further increased, up to 16-fold, by activin stimulation in the presence of ActRIB. However, cotransfection of ActRIA resulted in a slight increase of luciferase activity without further increase by additional activin stimulation. These results indicated that ActRIB is essential for activin signaling in ALVA41 cells. Both Smad2 and Smad3 increased not only basal but also ligand-stimulated luciferase activity. TGF-β stimulation in the presence of Smad3 resulted in a 60-fold induction of 3TP-Lux luciferase activity, suggesting Smad3 preferentially mediates TGF-β signaling to the 3TP-Lux reporter gene. Smad4 increased both activin and TGF-β-stimulated luciferase activity. Together, these results indicated the presence of functional activin signaling pathways and that ActRIB is essential for activin signaling in ALVA41 cells.

3.3. Establishment of ALVA-ActRIBCA cells

To address the role of activin signaling *in vivo*, we made stable transformants with the constitutively active form of activin type IB receptor in ALVA41 cells. The expression vector for ActRIBCA was constructed as described in Fig. 2A. Briefly, the expression of ActRIBCA was under the control of the EF1α promoter and a selectable marker was translated from a single fusion transcript. The IRES sequence provides cap-independent translation of a fusion protein of Neo^{r} and β-galactosidase. ALVA41 cells were transfected with the construct and cultured for 2 weeks in the presence of G418 at a concentration of 200 μg/ml. Several colonies were picked and cultured individually for another 2 weeks under the same conditions to create cell lines. We established 17 independent clones, all of which were subjected to western blotting using the anti-HA antibody and by lacZ staining to quantitate the expression levels of ActRIBCA. For further analysis, we used three lines of the stable transformants with high, moderate, and low expression, designated ALVA-ActRIBCA No.11, 17 and 8, respectively (Fig. 2A). As shown in Fig. 2B, the expression levels of ActRIBCA showed good correlation with the luciferase activities when transfected with p3TP-Lux reporter gene, indicating that ActRIBCA presents functional activities in a dose-dependent manner. Next, we analyzed the effect of ActRIB signaling on the proliferation of the cells. However, no statistical difference was found in the cell proliferation between ALVA41 and ALVA-ActRIBCA cells *in vitro* (Fig. 2C).

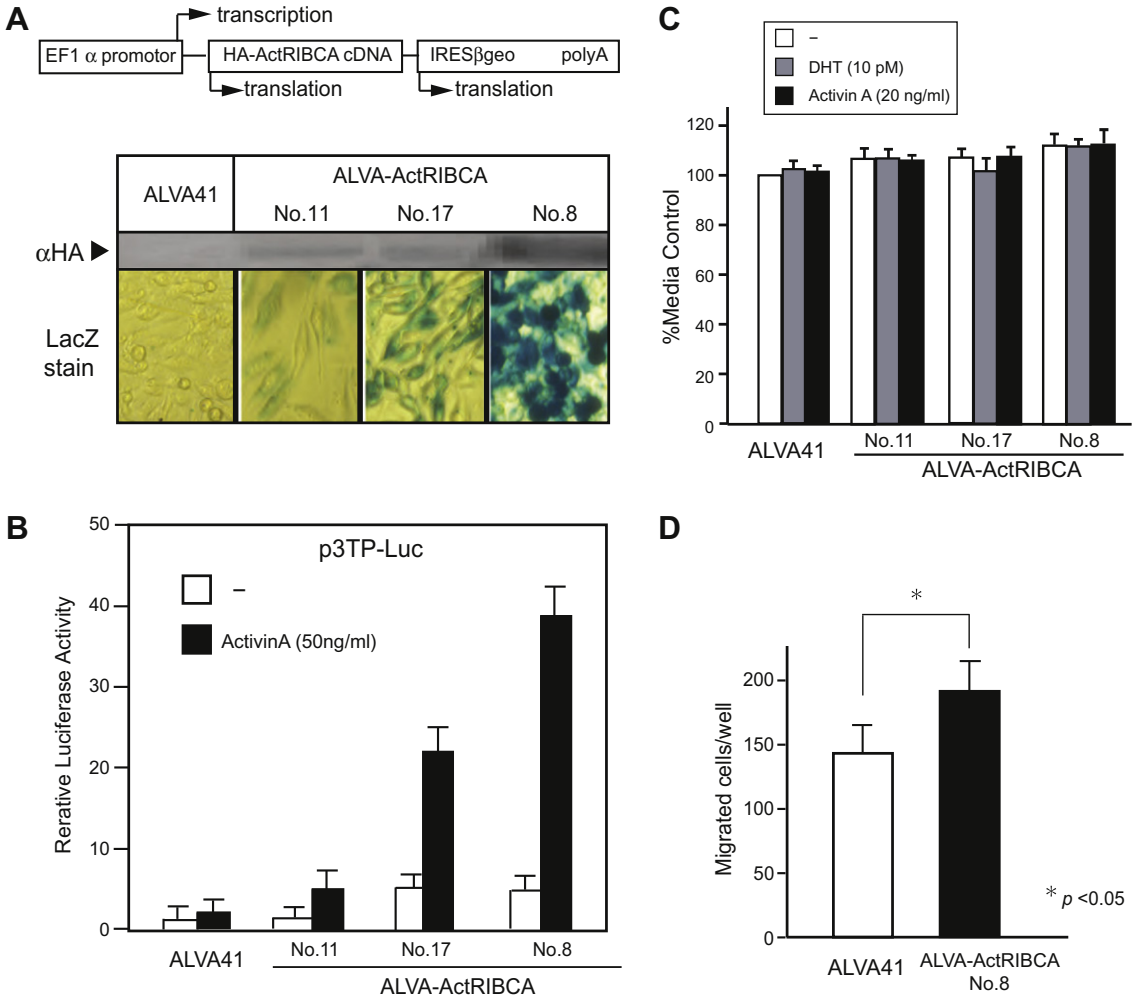

Fig. 2. Establishment of ALVA41-ActRIBCA stable transformants. (A) Construction of expression vector for constitutive active form of ActRIB. Three independent clones, numbers 11, 17, 8, were established. The expression of ActRIBCA protein was confirmed by western blotting with anti HA tag antibody and LacZ staining. (B) The luciferase activities with p3TP-Lux reporter gene were analyzed in each cell line indicated. (C) Effects of activinA and dihydrotestosterone on the proliferation of ALVA41 and ALVA-ActRIBCA cells. Cells were treated with or without ligand for 96 h. The results are expressed as a percentage of the medium control value. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. No statistical differences in the cell proliferation of each cell line was observed. (D) Transwell assays revealed that ALVA-ActRIBCA No. 8 cells demonstrated an increase in their migratory capacity compared to the parental ALVA41 cells, $^{*}P < 0.05$. Data shown is representative of at least three independent repeats. Error bars represent standard deviation.

Intriguingly, increasing evidence indicates that activin may foster the malignant progression of prostate cancer by promoting migration and dissemination [17,18]. As shown in Fig. 2D, migration assays revealed that ALVA-ActRIBCA No. 8 showed higher migration ability than the parental ALVA41 cells, suggesting that ActRIB signaling may play a role in the progression of prostate cancer.

3.4. Alteration of tumor growth and histological phenotype by ActRIBCA in a xenograft model

A mouse xenograft model was used to examine the role of ActRIB on the invasive potential *in vivo*. As shown in Fig. 3A, the tumor growth of the xenografts of ALVA-ActRIBCA cells was significantly impaired. In addition, it was noted that there was a positive correlation between levels of ActRIBCA expression and impairment of tumor growth. Representative photos of the mice and gross morphology of the tumors are shown in Fig. 3B. Compared to the xenografts from ALVA41 cells, those from the ALVA-ActR1BCA cells displayed a firm and glossy appearance (Fig. 3B). The resected xenografts were histologically examined by HE staining (Fig. 3C). Surprisingly, the histological characteristics of the tumors were greatly altered by the expression of ActRIBCA, although the morphology of the cultured cells was hardly distinguished (data not shown). As shown in Fig. 3C, histology of the xenografts from ALVA-ActRIBCA cells showed mesenchymal characteristics with a prominent ground substance matrix, indicative of EMT. This mesenchymal change was apparent in clone 8, suggesting the increased ActRIBCA expression induced EMT in a dose-dependent manner.

3.5. ActRIBCA promotes lymph node metastasis

The process of EMT is known to be crucial in embryonic development and becomes reactivated during cancer metastasis [19]. We therefore examined the metastatic potential in a xenograft model. Interestingly, all mice bearing ALVA-ActRIBCA No. 17 and No. 8 cells developed macroscopic evidence of enlarged mesenteric or iliac lymph nodes as well as additional lymph nodes in various lesions, as summarized in Fig. 4A. Lymph node metastasis was hardly observed in mouse bearing ALVA41 cells and ALVA-ActRIBCA No. 11 when dissected at 4 weeks after inoculation. Fig. 4B upper panels show macroscopic photos of mesenteric and

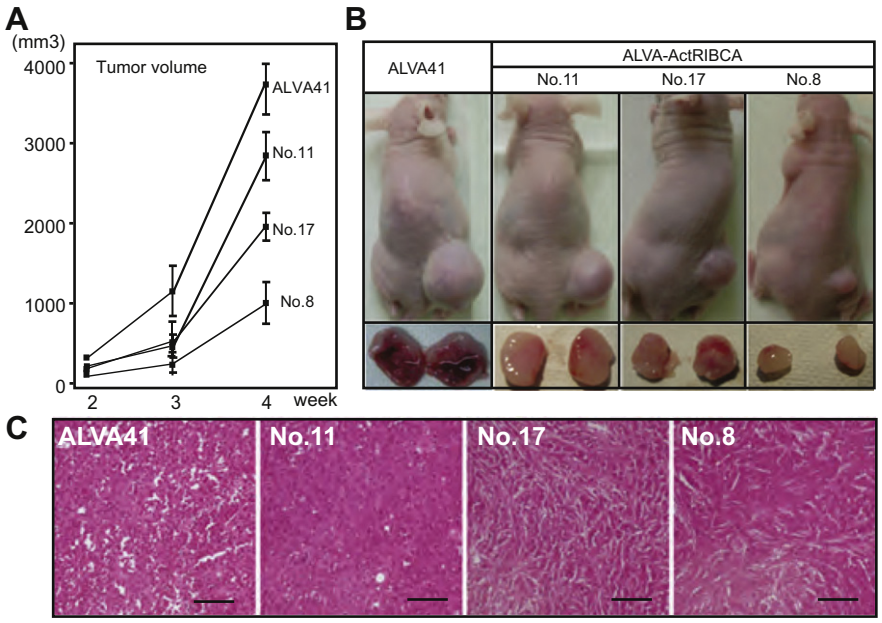

Fig. 3. Alteration of tumor growth and histological phenotype by ActRIBCA. A total of 5×10^5 cells were injected subcutaneously into the backs of nude mice and tumor volumes were measured every week. (A) The tumor growth of xenografts with ALVA-ActRIBCA cells were significantly impaired. (B) Xenografts of ALVA-ActRIBCA showed a firm and glossy appearance. (C) H&E staining of the resected xenografts. Histological alterations were found in the xenografts of mutanta cells. It is noted that mesenchymal change was prominent in ActRIBCA No. 8.

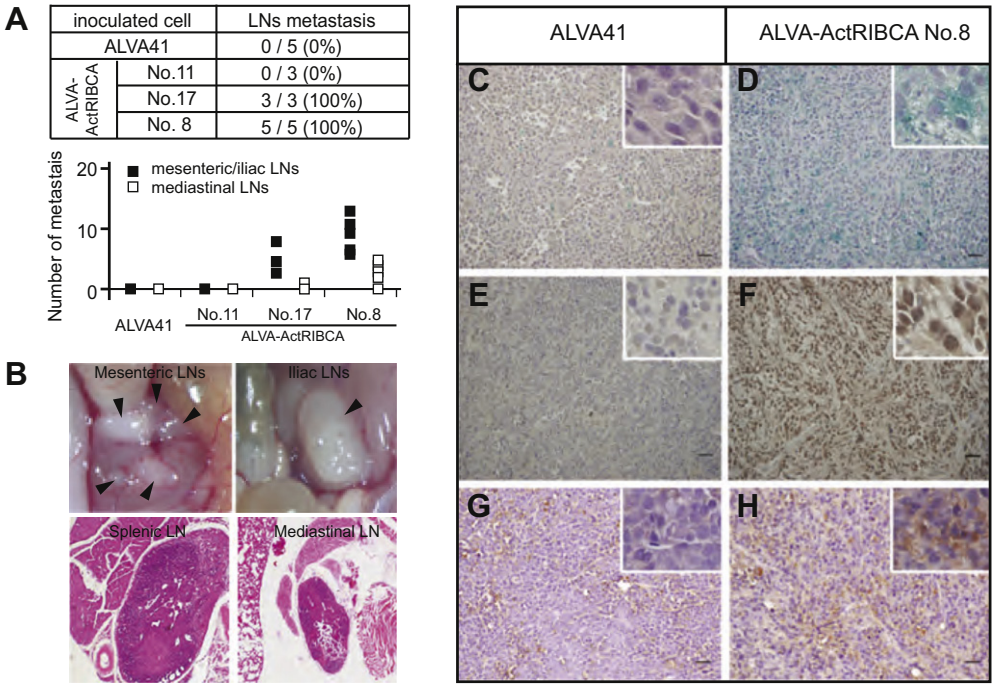

Fig. 4. Activation of ActRIB signal leads to multiple lympnode metastases. (A) The frequency of lymph node metastasis (upper table). Metastasis were observed in mouse bearing ActRIBCA, No.17 and No. 8 cells, but not in mice bearing ALVA41 cells and No. 11 clones. Numbers of metastatic nodules in individual mouse at the time of sacrifice (lower graph). (B) Photos of multiple metastases in mesenteric and iliac lymph nodes (upper panels). Arrowheads indicate metastatic nodules. H&E staining of splenic and mediastinal lymph nodes (lower panels). (C and D) LacZ staining of ALVA41 and ALVA-ActRIBCA No. 8. (E–H) Immunohistochemical staining of Snail (E and F) and S100A4 (G and H). bars, 10 μm. High magnification of each staining is shown in the right upper corner.

iliac lymph node metastases in the mice with ALVA-ActRIBCA No. 8, while the lower panels show H&E staining of definitive distant spread of prostate cancer cells within splenic and mediastinal lymph nodes. There may be a threshold mechanism in ActRIB signaling by which prostate cancer undergoes progression with lymph node metastasis.

3.6. Increased expression of Snail by ActRIBCA

As shown in Fig. 4D, cytosolic expression of lacZ was observed in ALVA-ActRIBCA No.8 tumors but not in ALVA41-derived tumors, confirming the expression of ActRIBCA in the xenografts. EMT is characterized by the loss of epithelial markers and cell–cell

contacts followed by the re-expression of mesenchymal proteins and the formation of a migratory phenotype. In particular, loss of E-cadherin is induced by transcriptional repressors such as Snail [20]. Therefore, we first studied E-cadherin expression by immunohistochemistry of paraffin-embedded primary tumors obtained from the xenograft model. However, E-cadherin expression was negative in the xenografts of either ALVA41 or ALVA-ActRIBCA cells (data not shown). However, the expression of Snail was dramatically induced by ActRIBCA, and strong nuclear expression of Snail protein was observed in ALVA-ActRIBCA xenografts as shown in Fig. 4F. In addition, another mesenchymal marker, S100A4, was significantly increased in ALVA-ActRIBCA xenografts, as shown in Fig. 4G and H, indicating EMT was induced by ActRIBCA.

4. Discussion

In this study, we investigated the role of activin signaling in the pathogenesis of prostate cancer. Stable expression of ActRIBCA in ALVA41 prostate cancer cells induces a dramatic phenotypic transition accompanied by the acquisition of migratory properties. This phenotypic change is also concomitant with an increase in the expression of Snail and S100A4 in a xenograft model. The gain-of-function experiments described here confirm that activation of ActRIB signalin is able to drive the EMT associated with the acquisition of the migratory phenotype, and provide insights into the mechanism by which activin signaling triggers this transition, namely the induction of Snail expression. Although several studies have linked activin to tumor progression and metastasis [21,22], we have demonstrated for the first time the direct involvement of Snail transcription factor in activin signaling. We also showed that expression of S100A4, a member of the S100 family of calcium-binding proteins, was upregulated in our model. S100A4 has been shown to enhance tumor invasiveness and metastasis [23]. Interestingly, Smad3 is reported to bind with S100A4 in a breast cancer cell line to cooperatively promote invasiveness [24]. Further experiments will be required to clarify whether Smad proteins act downstream to ActRIB to enhance metastasis in prostate cancer [25].

Lines of clinical investigations suggest the potential involvement of activin in the progression of various cancer types. In primary breast tumor tissues, the gene expression of activin betaA increased in invasive and bone metastatic tumor tissue [26]. Activin has been implicated in the homing mechanism of cancer metastasis. In esophageal cancer, the mRNA expression of activin betaA, which is a subunit of activin A, was associated with lymph node metastasis and clinical stage [21]. Although the ligands for ActRIB in our model are currently unknown, it is likely that activin A signals through ActRIB in prostate cancer to promote lymph node metastasis. In this study, the receptor expression level determines strength of the signals for activin in ALVA-ActRIBCA cells. In addition, there was a strong relationship between lymph node metastatic potential and strength of ActRIB expression. These results suggest the threshold mechanism in acquiring metastatic potential by activin signaling. The TGF-β family is composed of nearly 30 growth factors including TGF-βs, BMPs, activins, nodal and its related proteins [9]. In contrast, so far only seven members of type I receptors have been identified, indicating that several ligands employ the same type I receptor in the signaling pathway. Activin type IB receptor has shown to mediate nodal signaling in addition to activins. In addition, nodal has been shown to function as an autocrine regulator of proliferation and migration of prostate cancer cells [27]. It is of great interest to speculate that nodal signals through ActRIB in prostate cancer to promote lymph node metastasis.

In conclusion, we have demonstrated that ActRIB signaling can promote cell migration in prostate cancer cells via a network of signaling molecules that work together to trigger the process of EMT, and thereby aid in the aggressiveness and progression of prostate cancers such as lymph node metastases. Further studies on the role of activin signaling in prostate cancer may offer a new therapeutic approach to prevent metastasis in prostate cancer.

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