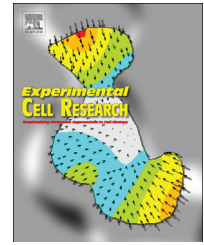


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Research Article

Overexpression of activin-A and -B in malignant mesothelioma – Attenuated Smad3 signaling responses and ERK activation promote cell migration and invasive growth



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ABSTRACT

Activin-A and activin-B, members of the TGF- β superfamily, are regulators of reproductive functions, inflammation and wound healing. These dimeric molecules regulate various cellular activities such as proliferation, migration and survival. Malignant mesothelioma is an asbestos exposure related tumor affecting mainly pleura and it usually has a dismal prognosis. Here, we demonstrate that both activin-A and -B are abundantly expressed in mesothelioma tumor tissue as well as in cultured primary and established mesothelioma cells. Migratory and invasive mesothelioma cells were also found to have attenuated activation of the Smad2/3 pathway in response to activins. Migration and invasive growth of the cells in three-dimensional matrix was prevented by inhibition of activin activity using a soluble activin receptor 2B (sActR2B-Fc). This was associated with decreased ERK activity. Furthermore, migration and invasive growth was significantly inhibited by blocking ERK phosphorylation. Mesothelioma tumors are locally invasive and our results clearly suggest that activins have a tumor-promoting function in mesothelioma through increasing expression and switching from canonical Smad3 pathway to non-canonical ERK pathway signaling. Blocking activin activity offers a new therapeutic approach for inhibition of mesothelioma invasive growth.

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Abbreviations: ACVR2, type 2 activin receptor; ALK, activin receptor-like kinase; ERK, extracellular signal-regulated kinase; FST, follistatin; FSTL3, follistatin like 3; JNK, c-Jun N-terminal kinase; TGF- β , transforming growth factor- β

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Introduction

Malignant mesothelioma is a rare but very aggressive tumor originating from mesothelial cells lining body cavities. Most commonly mesothelioma affects pleura, sometimes peritoneum and very rarely pericardium. Mesothelioma is usually related to previous asbestos exposure and develops during a latency of 20–40 years [1]. The incidence of mesothelioma is expected to increase worldwide in the near future [2,3]. Late stage of the disease at the time of diagnosis as well as only moderate effectiveness of current therapies lead to negligible survival time of the patients after diagnosis. Novel potential diagnostic markers for early detection and drug targets are needed for mesothelioma.

Activins, members of the TGF- β superfamily of growth factors, are known regulators of development and reproductive functions [4]. Currently, activins, especially activin-A, are also recognized as important regulators of immune responses, wound healing and cancer [5]. Activins enhance cell proliferation and migration as well as scar formation during wound healing [5]. Increased cell proliferation and migration are also characteristics of cancer cells. In addition, activins may also promote tumor progression indirectly by altering the tumor microenvironment through regulation of immune functions and angiogenesis. Whether activins promote or inhibit carcinogenesis depends on the cell, tissue and cancer type [5–7]. A recent study reports that activin-A promotes mesothelioma cell growth and migration through regulation of cyclin D [8].

The genes *INHBA* and *INHBB* code for the activin β -subunits, which form disulfide-linked functional dimers. Homodimerization of the β_A -subunits generates activin-A and β_B -subunits form activin-B [9]. The β -subunits can also heterodimerize and form activin-AB. The β -subunits heterodimerize also with α -subunits and form inhibins, which inhibit activin action by competing for activin receptors. Activin signaling is also regulated by soluble inhibitors follistatin (FST) and follistatin like-3 (FSTL3) [10], which inhibit activin action by binding to activins and blocking association with cell surface receptors [11].

Activins signal through activin type 1 and type 2 transmembrane serine/threonine kinase receptors. Activin action is initiated when activin binds to a type 2 activin receptor (ACVR2A or ACVR2B). This binding leads to recruitment, phosphorylation and activation of type 1 activin receptor followed by phosphorylation and activation of Smad proteins. Both activin-A and -B can activate the Smad2/3 pathway through type 2 receptors and common type 1 receptor ActR1B (activin like kinase-4, ALK-4) [12]. Activin-B can also activate Smad2/3 via ActR1C (ALK7) [13,14]. Furthermore, activin-B has been reported to activate Smad1/5/8 through BMPR1A (ALK3) [15]. The receptors that bind TGF- β -family members can also activate non-Smad proteins [16]. Activin-A can induce ERK and p38 MAP kinase phosphorylation [17], and activin-B has been reported to be able to phosphorylate SAPK/JNK and to induce keratinocyte migration [18] and wound healing [19]. Furthermore, activation of both JNK and ERK is required for activin-B induced migration in bone marrow-derived mesenchymal stem cells [20]. Activin-A and activin-B have partially overlapping expression patterns but are functionally distinct [21].

In this study, we characterized activin-A and -B expression and their receptors in mesothelioma tissue and in cultured primary and established mesothelioma cells. We aimed to clarify the role of Smad mediated and non-canonical activin signaling in mesothelioma cell migration and invasion. We report here that activins are

abundantly expressed in mesothelioma cells and that by blocking activin activity the migration and invasive growth of mesothelioma cells can be significantly reduced. Furthermore, our results suggest a new mechanism through which mesothelioma cells promote migration and invasive growth: switching activin signaling from canonical Smad3 pathway to non-canonical ERK pathway signaling.

Materials and methods

Antibodies and growth factors

Mouse monoclonal antibodies against human activin-A (mAb 18/26A) and activin-B (mAb 12/9A for IHC, 46A/F for Western blotting) subunits and follistatin (mAb 4/73C for IHC, 4/208 for Western blotting) were generated by AnshLabs LLC (Webster, TX). Rabbit monoclonal p-ERK1/2 and mouse monoclonal ERK1 were from Cell Signaling (Danvers, MA). Human recombinant activins were from R&D Systems (Gaithersburg, MD) and used at 25 ng/ml. The recombinant fusion protein containing the ectodomain of human ActR2B (sActR2B-Fc) or anti-Müllerian hormone receptor (sAMHR2-Fc) fused to the Fc domain of human IgG1 was produced as described previously [22] and used at 650 ng/ml (2D) or 10 μ g/ml (3D). Soluble receptors sequester ligands and inhibit their binding to cell surface receptors. MAP kinase kinase (MEK) inhibitor PD98059 was from Calbiochem (Merck, Darmstadt, Germany) and used at 30 μ M.

Patients and tissues specimen

A statement for the use of human tissue and pleural effusion materials was approved by the Ethics Committee of the Helsinki University Hospital, Helsinki, Finland (permit number 308/13/0301/2010). All patients gave written informed consent to participate in the study. Tissue biopsies and pleural effusion samples were obtained from patients who were undergoing diagnostic procedures and who had a clinical and/or radiological suspicion of malignant mesothelioma. All patients included in the study ($n=12$) were later diagnosed to have mesothelioma confirmed by a biopsy from the tumor tissue (Table 1).

Immunohistochemical staining

Paraffin-embedded tissue from mesothelioma tumor samples were processed and stained using the Novolink Polymer Detection System

Table 1 – Mesothelioma patient characteristics.

Patient	Type	Location	Age	Gender
JP1	Epithelial	Pleura	53	Male
JP2	Biphasic	Pleura	71	Male
JP3	Epithelial	Pleura	63	Male
JP4	Biphasic	Peritoneum	72	Female
JP5	Epithelial	Pleura	69	Female
JP6	Biphasic	Pleura	71	Female
JP7	Epithelial	Pleura	62	Male
JP8	Epithelial	Pleura	63	Male
JP9	Epithelial	Pleura	54	Male
JP10	Epithelial	Pleura	75	Male
JP11	Epithelial	Pleura	65	Male
JP12	Epithelial	Pleura	60	Male

(Novocastra, Leica Biosystems, Newcastle upon Tyne, UK) and visualized by diaminobenzidine (DAB, Vector Laboratories) has been reported previously [23]. For follistatin staining, the formalin-fixed paraffin embedded specimens were stained on Leica BOND-MAX fully automated staining system as defined in the manufacturer's staining protocol [with the Leica Bond Polymer Refine Detection-kit, Bond Epitope Retrieval Solution 2, 20 min, R.T.U. Normal Horse serum 2.5% (Vector Laboratories, Burlingame, CA) blocking]. Images of IHC sections were captured with Nikon DS-Fi1 (Nikon, Amsterdam, Netherlands).

Cell culture

Immortalized normal mesothelial cells (Met5A) [24] and mesothelioma cell lines (211H, H28, H2452, H2052) were from ATCC. Primary mesothelioma cells (JP cells) were obtained from pleural effusion samples from malignant mesothelioma patients as described in Tamminen et al. [25]. Cells were cultured in RPMI-1640 media (Sigma, St Luis, MO) supplemented with 10% fetal bovine serum (Gibco, Paisley, UK) and antibiotics (Gibco, Grand Island NY). Cells were stimulated with activin-A (25 ng/ml), activin-B (25 ng/ml) or sActR2B-Fc (650 ng/ml) for 90 min under serum-free conditions.

Transient transfection and luciferase assay

Cells to be transfected were seeded in 96-well plates. The cells were co-transfected with promoter constructs (CAGA)₁₂-luc (Smad3 responsive), ARE-luc/Fast-1 (Smad2 responsive) or (Bre)₂-luc (Smad1/5 responsive), kindly provided by Dr. Peter ten Dijke (Leiden University Medical Center, the Netherlands), together with pRL-TK (Renilla Luciferase control, Promega, Madison, WI) plasmid using Fugene HD transfection reagent (Roche, Basel, Switzerland). The transfection, cell lysis and the measurement of the luciferase activity were performed as described in Tamminen et al. [26]. The cells were stimulated for 24 h under serum-free conditions where indicated.

RNA isolation and quantitative RT-PCR

Total cellular RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany) and reverse transcribed to cDNA using iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The quantitative RT-PCR was performed as previously described [26].

SDS-PAGE and Western blotting

Cells were lysed and protein concentrations were measured as described earlier [26]. Equal amounts of protein were separated by SDS-PAGE using 4–20% gradient Tris-glycine gels (Bio-Rad). Conditioned media from mesothelioma cell cultures were collected during 2 days, concentrated using Amicon Ultra 10K centrifugal filters (Millipore, Merck, Darmstadt, Germany) and separated by SDS-PAGE. The transfer of the proteins to Protran nitrocellulose membranes (Whatman, Springfield Mill, UK), Western blotting and final detection of the proteins were performed as described previously [26]. Relative band densities were quantified using ImageJ software (National Institutes of Health, Bethesda, MA, USA).

Cell migration assays

The migratory capacity of the cell lines was analyzed using Incucyte ZOOM 2013A kinetic live cell imaging system (Essen Bioscience, Hertfordshire, UK) according to the manufacturer's instructions. Briefly, the cells were plated on 96-well plates and the next day a wound was applied using a woundmaker (Essen Bioscience). The media was replaced with serum-free media supplemented with sActR2B-Fc where indicated. Two distinct pictures were taken of each well every 2 h for 48 h. Data were analyzed with Incucyte ZOOM 2013A software and the relative wound density was used as a measure of the wound closure. Alternatively, cells were seeded on 12-well plates and wounds were made in confluent monolayers using a P200 pipette tip. The media was replaced with serum-free media supplemented with DMSO, PD98059 or sActR2B-Fc where indicated. Two pictures per well were taken after 0, 6, 24 and 48 h using Nikon Eclipse TS 100 and Digital Sight DS-L2 (Nikon). The wound density was quantified using Image ProPlus 7.0 software (Media Cybernetics, Inc., Rockville, MD) and is shown as % area occupied by cells relative to % wound area and has been normalized to wound density data at the 0 h time point. The control treated wound density has been set to one.

3D invasive growth assay

The lower Matrigel gel (BD Biosciences, San Jose, CA) was laid on the bottom of the well on a 96-well plate and allowed to solidify at 37 °C for 45 min. Next, 3000–4000 cells per well were seeded and allowed to attach for 1 h after which the media was carefully removed and replaced with the top gel. Before seeding, the cells were pre-treated with sActR2B-Fc, sAMHR2-Fc, DMSO or PD98059 for 15 min where indicated. The top gel was allowed to solidify for 30 min after which media was added. Inhibitors were also added to Matrigel and cell culture media on top of the gel.

Fig. 1 – Activins are abundantly expressed in mesothelioma tumor tissue as well as in cultured mesothelioma cells. (A) Immunohistochemical staining of mesothelioma tumor samples using activin-A, activin-B or follistatin antibodies. See Table 2 for scoring of the staining intensity in tumors. The mRNA expression levels of activin-A (*INHBA*) and activin-B (*INHBB*) were analyzed in primary mesothelioma cells (B) and mesothelioma cell lines (C) using quantitative RT-PCR. The expression levels were normalized to the expression levels of TATA-binding protein and are shown relative to the expression levels of activin-A in Met5A cells. (D) Mesothelioma cell conditioned media were analyzed using Western blotting. The ~15 kDa activin-A monomer as well as ~55 kDa prodomain and ~30 kDa dimer of activin-B and follistatin (*FST*) are shown. Recombinant proteins used as positive controls (pos. ctrl) and molecular weight markers are shown on the right. The mRNA levels of follistatin (*FST*) and follistatin-like 3 (*FSTL3*) were analyzed in primary mesothelioma cells (E) and mesothelioma cell lines (F). The expression levels were normalized to the expression levels of TATA-binding protein and are shown relative to the expression levels of *FST* in Met5A cells. Error bars represent standard deviation ($n=3$).

Statistical analyses

Data were analyzed using PASW Statistics 21 program for Windows (SPSS, Chicago, IL). Statistical difference between two independent

groups was evaluated using nonparametric Mann–Whitney *U*-test. Statistical difference between more than two independent groups was evaluated using nonparametric Kruskal–Wallis test. A *p*-value of less than 0.05 was considered statistically significant.

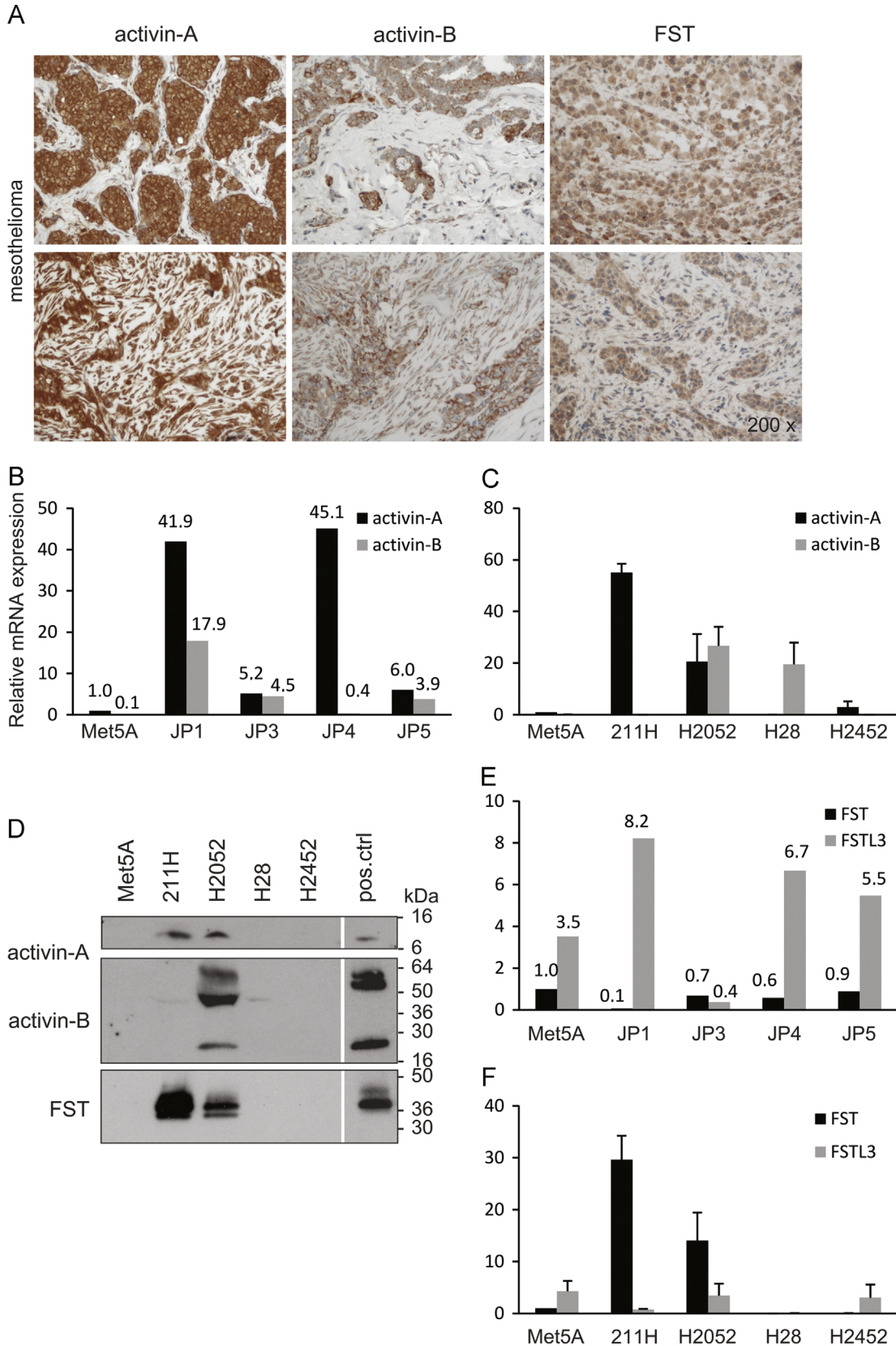


Table 2 – Scoring of activin-A, activin-B and follistatin immunoreactivity in mesothelioma patient samples.

Patient	Activin-A	Activin-B	Follistatin
JP2	+++	+++	++
JP3	+	+	++
JP5	+++	++	+
JP6	+++	+	++
JP7	+++	++	++
JP8	+++	+	++
JP9	++++	+++	+++
JP10	++	+	++
JP11	++++	++	+
JP12	++++	++	++

Extent of staining (score): no staining (0), weak staining (+), moderate staining (++), strong staining (+++) and very strong staining (++++).

Results

Activin-A and -B are highly expressed in malignant mesothelioma

Activins are known regulators of inflammation and tissue repair processes [5,27]. Activin expression has also been linked to malignant and fibrotic conditions [7]. To analyze the expression of activin-A and -B in mesothelioma, tissue biopsies from patients were stained with specific antibodies against inhibin β_A and β_B subunits. Immunoreactivity for both activins was abundantly detected in mesothelioma tumors (Fig. 1A). All tumor samples ($n=10$) were positive for both activins. In 8 out of 10 samples the immunoreactivity of activin-A was stronger compared to the immunoreactivity of activin-B (Table 2). The immunoreactivity localized into the mesothelioma tumor cells, while the stroma was mainly negative. Capillary endothelium in the stroma was found positive. In addition, normal and reactive pleura showed immunoreactivity for both activins (not shown). Tumor cells were also positive for follistatin immunoreactivity, which was observed in a more heterogeneous pattern (Table 2). In addition to tumor cells, follistatin was detected in the stroma, endothelium and strongly in normal and reactive pleura (not shown).

We have previously described isolation and culture of primary mesothelioma cells from patients' pleural effusion samples [25]. The cells retain gene expression characteristics of the tumor at early passage cultures. The mRNA expression levels of activin-A (*INHBA*) and -B (*INHBB*) in primary cells were analyzed using quantitative RT-PCR. Met5A cells, immortalized but non-tumorigenic mesothelial cells that were used as controls, expressed both activin-A and -B. The levels of activin-A were about 10-fold higher than activin-B levels (Fig. 1B). All primary mesothelioma cells expressed considerably higher levels of activins when compared to Met5A cells (Fig. 1B). Primary cells that overexpressed mainly activin-A were isolated from a patient with biphasic mesothelioma (JP4), while the other primary cells were from patients with epithelioid type mesothelioma. We also analyzed activin expression levels in established mesothelioma cell lines (211H, H2052, H28, H2452) and found that there was more heterogeneity in the expression levels of activin subunits compared to primary cells (Fig. 1C). In general, activin mRNA expression levels were significantly higher, especially in 211H and H2052 cell lines, than in Met5A cells. Interestingly, similar to what was found in JP4

primary cells we observed specific upregulation of the activin-A subunit in the 211H cell line, which is also isolated from a patient with biphasic mesothelioma. Secretion of activin subunits into the culture media was analyzed by Western blot analysis and found to correlate with the mRNA expression data (Fig. 1D).

Follistatin and follistatin like-3 (FSTL-3) are extracellular inhibitors of TGF- β family growth factors. They can bind to all activins and inhibit their biological functions [10]. The mRNA expression levels of FST and FSTL3 in mesothelioma cells were analyzed next. In primary mesothelioma cells FST expression levels were downregulated, in some cells (JP1 and JP4) more clearly than in others. FSTL3 mRNA expression levels were elevated in 3 out of 4 primary mesothelioma cells (Fig. 1E). In the established mesothelioma cell lines FST and FSTL3 expression levels showed more variability. FST levels were downregulated in H28 and H2452 cell lines, while upregulated in 211H and H2052 cell lines (Fig. 1F). FSTL3 mRNA expression levels tended to decrease, which is in contrast to what was observed in the primary cells. Secretion of FST into the culture media was analyzed by Western blot analysis and found to correlate with the mRNA expression data (Fig. 1D).

ALK7 and ACVR2A are overexpressed in mesothelioma cells

The cells' sensitivity to activins and other TGF- β superfamily growth factors is defined by cell surface receptor expression and the specificity of the signal is defined by the type 1 receptor [12]. Both activin-A and -B activate Smad2/3 pathway through ALK4 [12]. In addition, activin-B can activate Smad2/3 pathway through ALK7 [13,14] and Smad1/5/8 pathway through ALK3 [15]. The activin receptor mRNA expression levels were analyzed using quantitative RT-PCR. Met5A cells expressed activin type 1 receptors ALK3 and ALK4 and very low levels of ALK7 (Fig. 2A). Overall, ALK3 and ALK4 mRNA expression levels were not consistently altered in primary mesothelioma cells or in established mesothelioma cell lines (Fig. 2A and C). However, ALK7 receptor expression was highly increased in primary mesothelioma cells (Fig. 2B). In addition, all the established mesothelioma cell lines showed higher levels of ALK7 when compared to Met5A cells (Fig. 2D).

Met5A cells expressed both activin type 2 receptors ACVR2A and ACVR2B. The levels of receptor 2B were about 3-fold higher than receptor 2A expression levels (Fig. 2E and F). This is in contrast to what was observed in all primary mesothelioma cells. The 2A receptor expression levels increased significantly, while receptor 2B levels decreased (~13-fold higher level of receptor 2A compared to receptor 2B on average) (Fig. 2E), suggesting that there is a switch in the predominant type 2 receptor in mesothelioma cells. This was also noted in 3 out of 4 established mesothelioma cell lines (Fig. 2F). The H28 cell line was the only one showing elevated levels of both type 2 receptors.

Mesothelioma cells have altered Smad activation in response to activins

Activin-A and -B induced Smad responses were studied in mesothelioma cells using transient transfection of Smad3 responsive [(CAGA)₁₂-luc], Smad2 responsive [ARE-luc] or Smad1/5 responsive [(Bre)₂-luc] promoter-luciferase constructs followed by overnight stimulation by activin-A or -B (see "Materials and methods" section). Basal Smad3 activity was somewhat higher in primary cells (JP5) compared to established mesothelioma cell lines or Met5A cells

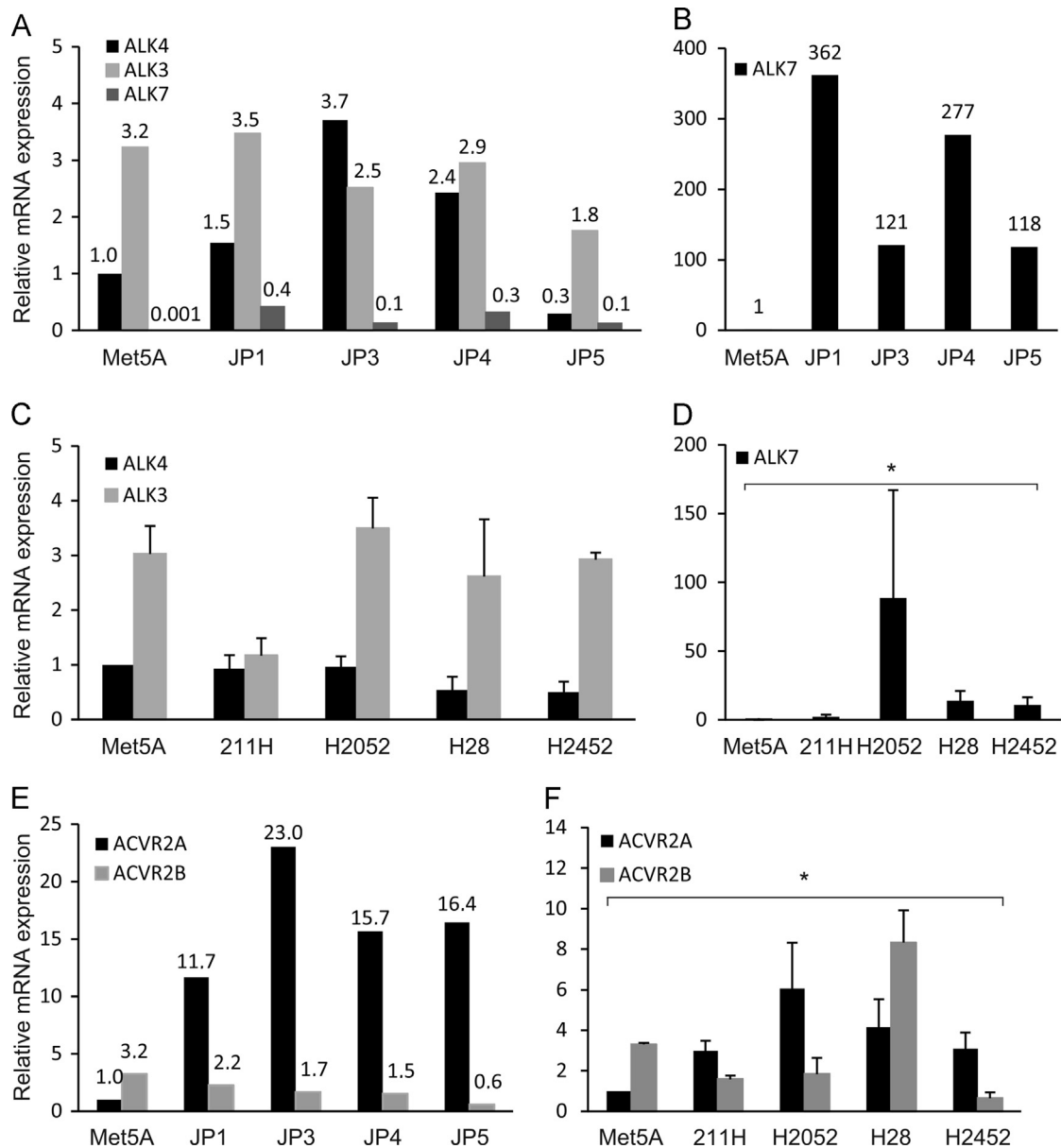


Fig. 2 – The expression of ACVR2A and ALK7 is increased in mesothelioma cells. The mRNA expression levels of activin type 1 (ALK3, ALK4 and ALK7) and type 2 (ACVR2A and ACVR2B) receptors were analyzed in primary mesothelioma cells (A, B, E) and in mesothelioma cell lines (C, D, F). The expression levels were normalized to the expression levels of TATA-binding protein and are shown relative to the expression levels of ALK4 (A, C), ALK7 (B, D) or ACVR2A (E, F) in Met5A cells. Error bars represent standard deviation ($n=2$). * $p \leq 0.025$ Kruskal–Wallis test.

(Fig. 3A). Smad3 activity increased ~ 80 -fold in activin-A and ~ 60 -fold in activin-B treated Met5A cells (Fig. 3B). The H28 mesothelioma cell line showed similar responses. In contrast, H2052 and 211H cell lines as well as JP5 primary cells had strongly attenuated activin induced Smad3 activation.

Basal Smad2 activity differed between the cells. Significantly lower basal Smad2 activity was observed in 211H and H2052 cells (Fig. 3C). Both activins induced Smad2 activity in Met5A cells. Induction of activity was also observed in 211H cells, whereas H2052 and H28 cells showed only a moderate 2-fold increase in Smad2 activity in response to activin-A and -B (Fig. 3D). In JP5 primary cells activins failed to induce Smad2 activity.

A tendency towards higher basal Smad1/5 activity levels were noted in JP5 primary cells as well as in 211H and H2052 cells compared to Met5A cells (Fig. 3E). H28 cell line showed significantly increased basal level Smad1/5 activity. Interestingly, activin-B increased Smad1/5 activity in this particular cell line, but not in any other cells tested (Fig. 3F).

Mesothelioma cells differ in migratory capacity

To analyze the migratory capacity of established mesothelioma cell lines, a scratch-wound assay was performed. Cell migration was monitored using a live-cell imaging system (see “Materials and

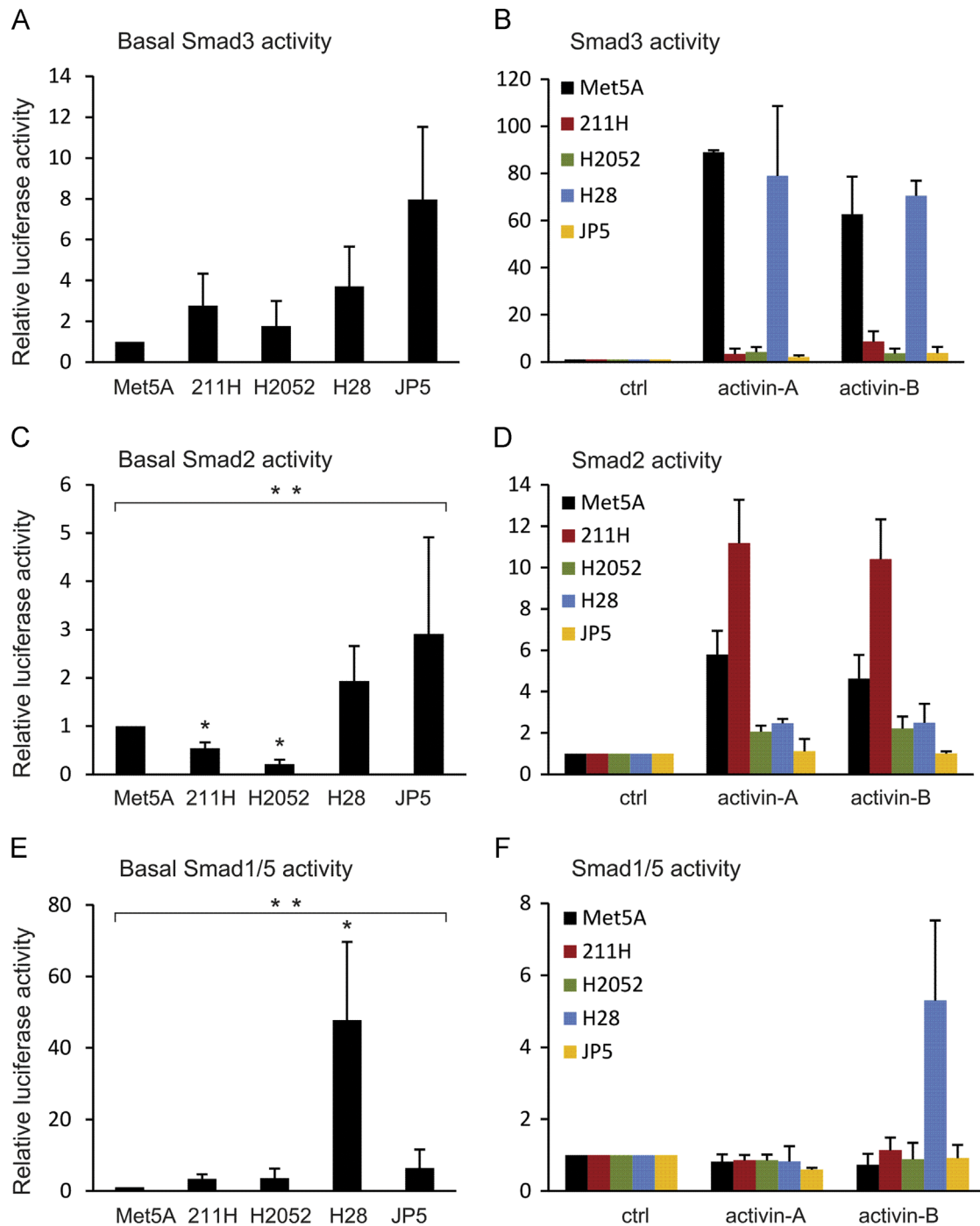


Fig. 3 – Activin induced Smad3 activation is attenuated in mesothelioma cells. Met5A and mesothelioma cells were transfected with Smad3 (A, B), Smad2 (C, D) or Smad1/5 (E, F) responsive promoters and stimulated overnight with activin-A or -B. Promoter activity is shown relative to the level in Met5A cells. (A) Basal Smad3 activity was somewhat elevated in primary mesothelioma cells (JP5, $n=2$). (B) Activin induced Smad3 activation was dramatically decreased in primary mesothelioma cells and in 211H and H2052 cell lines ($n=2$). (C) Basal Smad2 activity was downregulated significantly in 211H and H2052 mesothelioma cells [$**p=0.007$ Kruskal–Wallis test comparing all samples; $*p=0.008$ Mann–Whitney test comparing two groups (Met5A vs. 211H and Met5A vs. H2052) ($n \geq 3$)]. (D) Activin-A and -B induced robust Smad2 activation in Met5A and 211H cells whereas other cells showed attenuated Smad2 response ($n \geq 2$). (E) Basal Smad1/5 activity was significantly increased in H28 cells and slightly increased in other mesothelioma cells [$**p=0.006$ Kruskal–Wallis test ($n \geq 2$); $*p=0.007$ Mann–Whitney test comparing two groups (Met5A vs. H28) ($n \geq 3$)]. (F) Activin-B induced Smad1/5 activation only in H28 cell line ($n \geq 3$). Error bars represent standard deviation.

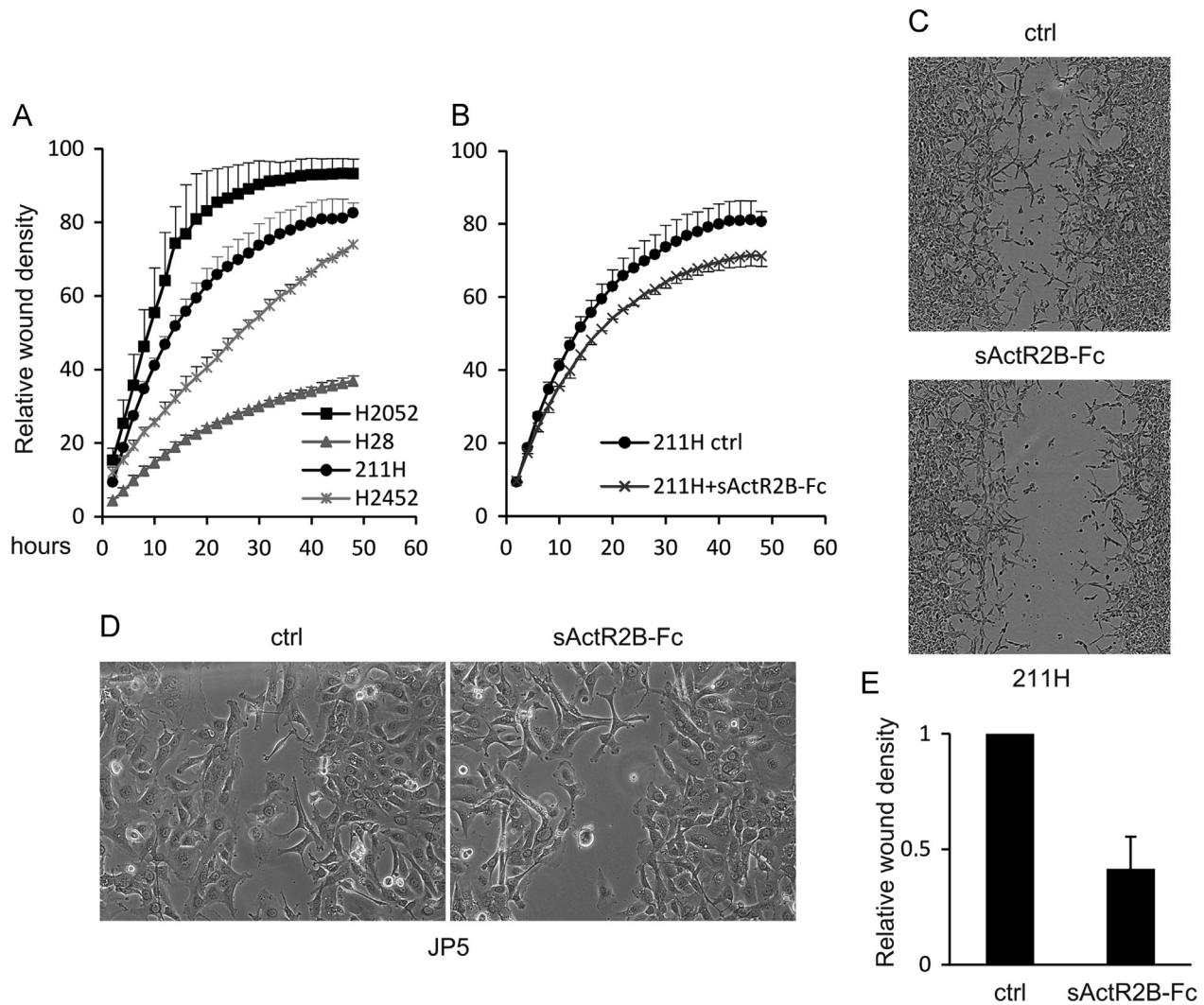


Fig. 4 – Inhibition of activin activity reduces mesothelioma cell migration. Cell migration was analyzed using a scratch-wound assay and monitored by 48-h live cell imaging (A–C) or imaging at 0, 6, 24 and 48 h time points (D, E). The results are presented as relative wound density. H2052 and 211H mesothelioma cells were found to rapidly close the wound. Inhibition of activin signaling using a soluble type 2 receptor (sActR2B-Fc) delayed wound closure in 211H cells. Error bars represent standard deviation ($n=2$). (C) Representative pictures of 211H cells at the 24 h time point are shown. (D, E) Representative pictures of JP5 primary mesothelioma cell scratch-wound assay. Inhibition of activin signaling (by sActR2B-Fc) delayed wound closure in primary cells [$p=0.1$ Mann-Whitney test, ($n=3$)]. Error bars represent standard deviation.

methods” section). Clear differences in mesothelioma cell migration were observed (Fig. 4A). H2052 and 211H cells migrated efficiently to close the wound. These two cell lines exhibited increased mRNA and protein expression of activin-A as well as altered Smad3 signaling responses. H2452 cells were less migratory and H28 cells were able to close the wound only after extended periods of time (Fig. 4A). Migration of 211H cells reduced with addition of a soluble activin inhibitor (sActR2B-Fc), suggesting that endogenous activins contribute to 211H mesothelioma cell migration (Fig. 4B and C). The migratory capacity of JP5 primary cells was measured using a scratch-wound assay and imaging at 0, 6, 24 and 48 h time points. JP5 cells were also found to migrate rapidly. Treatment with the activin inhibitor sActR2B-Fc clearly delayed wound closure (Fig. 4D and E). Similar to what was observed in 211H cells the inhibition was clearly detectable at 24 h (Fig. 4D) and was sustained at 48 h, when

sActR2B-Fc treated wounds were approximately half as dense as the untreated control wounds (Fig. 4E).

Migratory mesothelioma cells also show invasive growth in 3D

To analyze the invasive growth of mesothelioma cell lines, they were seeded into 3D matrices and monitored for 36 h (see “Materials and methods” section). The highly migratory cell lines 211H and H2052 were able to form irregular shaped spheroids and invade through the Matrigel forming a network of sprouts (Fig. 5A), whereas H28 and H2452 cells were unable to do this. Next we analyzed whether activin signals contribute to invasive growth. The cells were pretreated with sActR2B-Fc, which binds and inhibits the action of both activin-A and -B, before they were seeded into

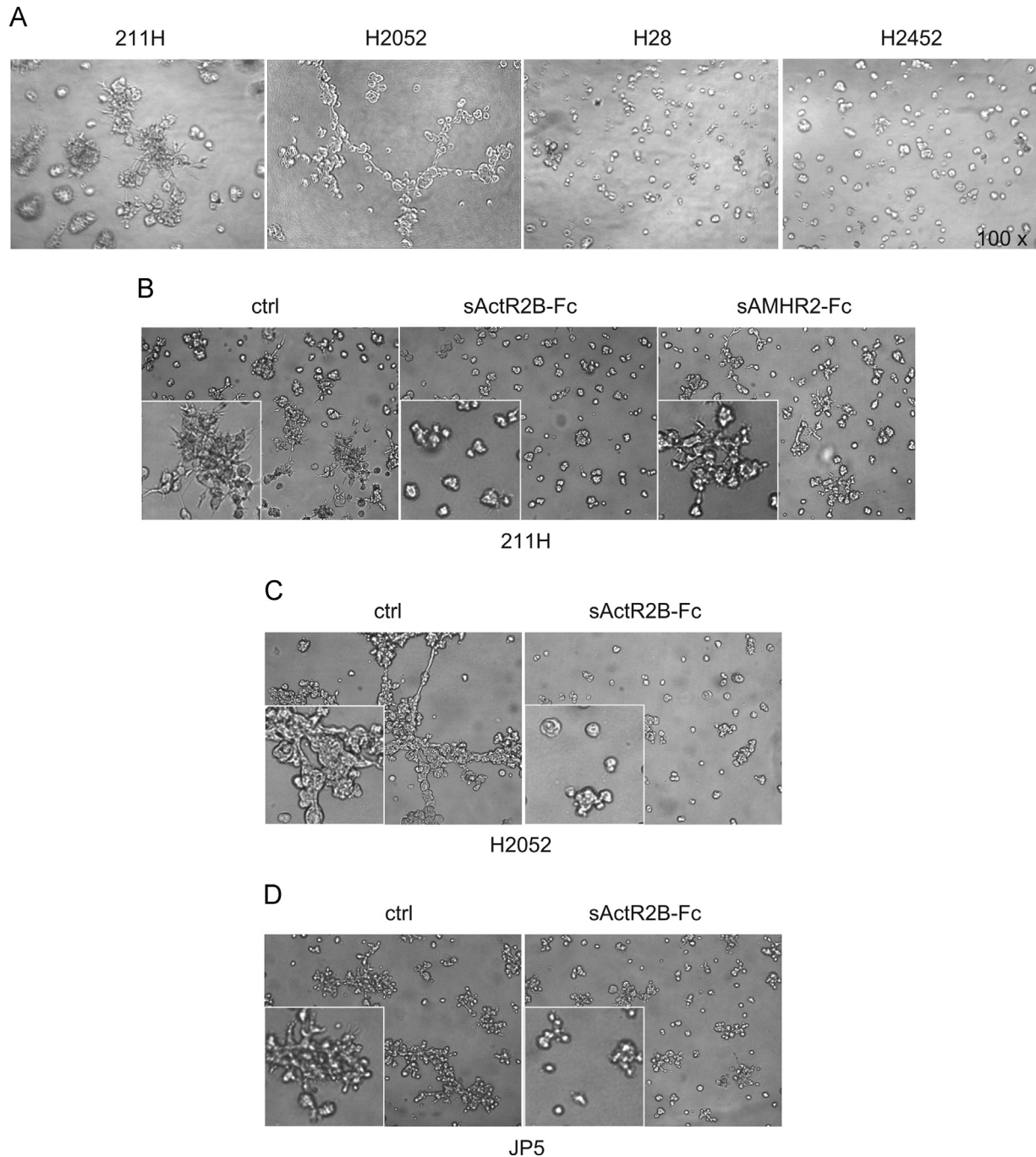


Fig. 5 – Invasive growth of mesothelioma cells was analyzed in three-dimensional matrix. (A) 211H and H2052 cells, but not H28 and H2452 cells, embedded in Matrigel were able to form irregular shaped spheroids as well as invade and sprout through the surrounding matrix. (B-D) Inhibition of activin signaling (by sActR2B-Fc) prevented invasive growth of 211H, H2052 and JP5 primary cells. Soluble anti-Müllerian hormone receptor (sAMHR2-Fc) was used as a negative control and had no effect on invasive growth. Representative pictures are shown. Original magnification 100 ×. Inset: high magnification picture.

Matrigel. Interestingly, addition of sActR2B-Fc was able to inhibit the invasive growth of 211H and H2052 mesothelioma cell lines (Fig. 5B and C) as well as JP5 primary cells (Fig. 5D). Addition of a soluble anti-Müllerian hormone receptor (sAMHR2-Fc), which was used as a control treatment, had no impact on invasive growth (Fig. 5B). These results suggest that activins contribute to the invasive growth properties of mesothelioma cells.

ERK activity contributes to mesothelioma cell migration and invasive growth

Activins can also induce non-canonical signaling pathways. These include the ERK and JNK MAP kinase pathways, which have been shown to contribute to cell migration [17,20]. ERK phosphorylation, *i.e.*, activation was analyzed by Western blotting after

treatment of cells with activin-A or -B for 90 min. In Met5A control cells activins did not induce ERK phosphorylation, instead, pERK levels seemed to decrease. In contrast, in H2052 and JP5 primary cells activins induced robust ERK phosphorylation (Fig. 6A). Activin-A seemed to induce a stronger response than activin-B. To determine whether endogenous activins support ERK activation, mesothelioma cells were treated with the soluble activin inhibitor sActR2B-Fc. In all migratory and invasive mesothelioma cells (211H, H2052 and JP5) sActR2B-Fc treatment reduced ERK phosphorylation levels significantly (Fig. 6B and C). Due to a limited possibility to do experiments with early passage primary cells ($n=2$), the decrease in JP5 cells did not, however, reach statistical significance. In the other cell lines (Met5A, H28 or H2452) treatment with sActR2B-Fc did not lead to similar reduction in ERK phosphorylation (not shown). No significant changes in JNK1/2 phosphorylation in response to activin stimulation were detected (not shown).

To analyze the involvement of ERK activity in mesothelioma cell migration a scratch-wound assay was performed in the presence or absence of an inhibitor of the upstream kinase MEK (PD98059). In JP5 primary cells as well as in 211H and H2052 cells wound closure was considerably delayed by the MEK inhibitor (Fig. 6D and E). Compared to DMSO treated control cells, in MEK inhibitor treated 211H and JP5 primary cells a significantly delayed wound closure was observed at 48 h. In H2052 cells the delay in wound closure was significant already at 6 h when the wounds were approximately half as dense as the control wounds (Fig. 6E). Unlike in the DMSO treated control, H2052 wounds remained open in the presence of the MEK inhibitor at 24 h (Fig. 6D). Activin-A increased H2052 cell migration. After six hours, the wounds were approximately twice as dense as the control wounds (Fig. 6F and G). Treatment with the MEK inhibitor significantly delayed the wound closure also in activin-A stimulated cells (Fig. 6F and G).

The contribution of ERK pathway activity to the invasive growth of mesothelioma cells was analyzed next. Cells were pretreated with the MEK inhibitor or DMSO, after which the cells were seeded into 3D matrices. Inhibition of ERK pathway activity significantly inhibited invasive growth of 211H and JP5 primary cells (Fig. 6H). This was similar to what was observed with sActR2B-Fc. In addition, H2052 cells showed a similar trend of decreased ability for invasive growth (not shown). However, this observation was not so clear due to the sensitivity of H2052 cells to DMSO control treatment in a three-dimensional setting. Taken together, our data suggest that inhibition of ERK activity efficiently prevents mesothelioma cell invasive growth.

Discussion

TGF- β family growth factors have an important role during development and adult tissue homeostasis. There is also ample evidence linking aberrant growth factor activity to cancer progression [28]. TGF- β activity has been linked to mesothelioma, but the role of activins is less well understood. There is one study showing overexpression of activin-A in mesothelioma and suggesting a function in the regulation of mesothelioma cell proliferation [8]. Here, we provide evidence that activin-A and activin-B are important regulators of mesothelioma cell migration and invasive growth properties. By blocking activin activity using a soluble inhibitor, we can efficiently

inhibit invasive growth in a three-dimensional culture system. Mesothelioma cells showed a clear difference in signaling responses to activins, which may be a key mechanism for the invasive growth inducing activity. Similar to what has been described for TGF- β [29,30], we speculate that increased activin production and altered signaling responses in malignant cells mediate a switch in their function from a tumor suppressor to a tumor promoter. Interestingly, suppression of Smad activity and concomitant increase in ERK activation has been suggested to mediate the switch in TGF- β function during tumor progression [31].

All mesothelioma tumors showed consistently high immunoreactivity for activin-A, which is in agreement with a previous report [8]. In addition, activin-B immunoreactivity was observed in all tumors. Immunoreactivity for the activin inhibitor follistatin, on the other hand, was more variable and not consistently altered in tumors. We have analyzed primary mesothelioma tumor cells isolated from pleural effusion samples [25] and found very consistent changes in the activin signaling pathway. Established mesothelioma cell lines showed clearly more heterogeneity, which suggests that it is important to use cell lines with similar characteristics as the primary tumors in mechanistic studies. Primary mesothelioma cells and migratory and invasive mesothelioma cell lines showed increased expression of activin-A, activin-B, ALK7 and ACVR2A, as well as ERK activation and attenuated Smad2/3 signaling responses. These alterations were clearly associated with a migratory and invasive growth phenotype, which could be blocked using a soluble activin inhibitor (sActR2B-Fc) or MEK inhibitor.

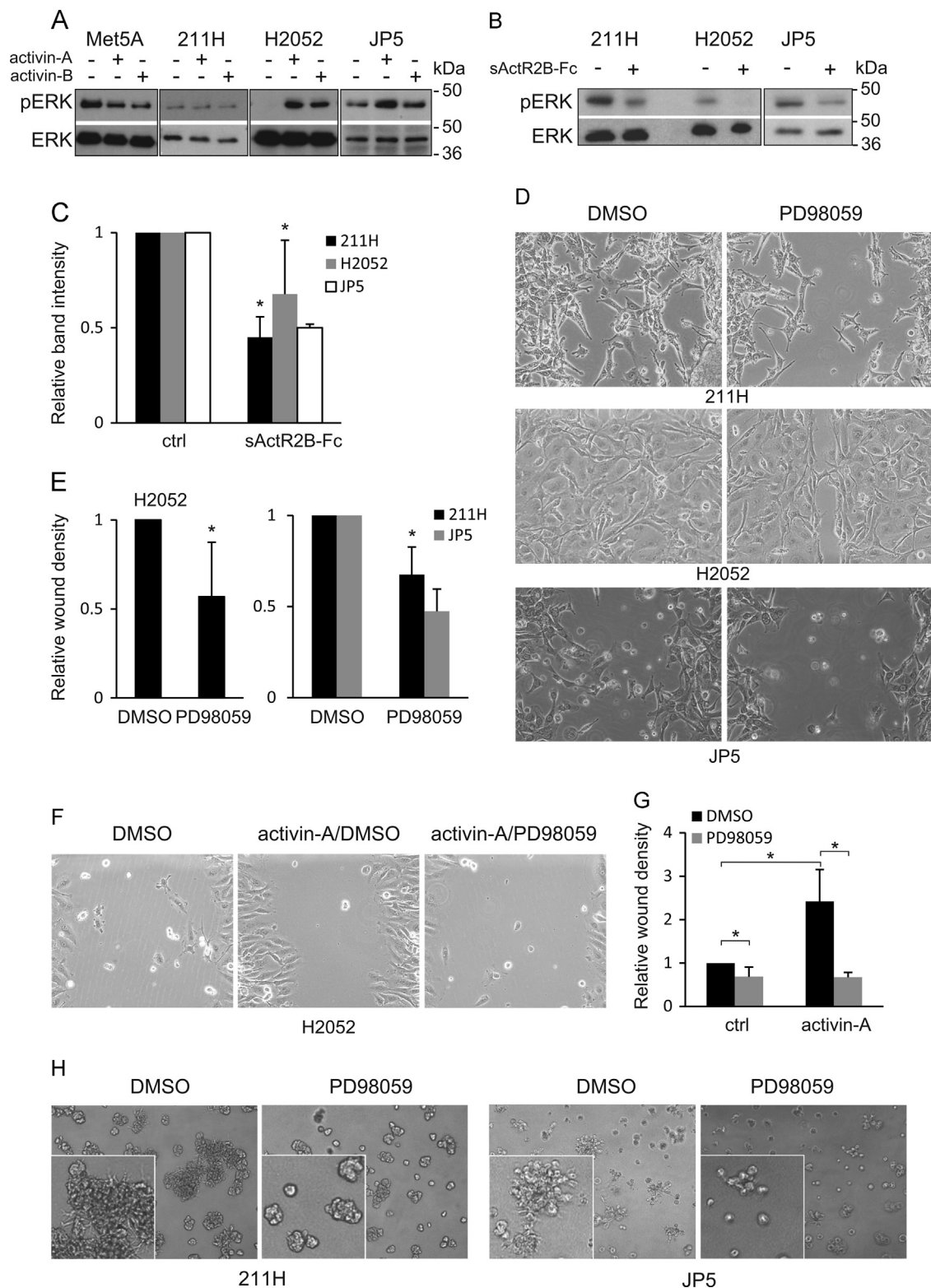
Activins can use different receptor heterodimers to mediate signaling. We found a significant upregulation of ALK7 receptor in mesothelioma. As ALK7 is an activin-B preferring receptor coexpression of ACVR2 and ALK7 should provide sensitivity to activin-B. In addition to activin-B also Nodal is a ligand for ALK7 [32]. Nodal is a developmental morphogen, which has been linked to cancer stem cell phenotype, epithelial-to-mesenchymal transition and invasion in other malignancies [33]. The possible role of Nodal in mesothelioma remains to be elucidated. Another interesting finding was a switch from a predominant expression of type 2 receptor ACVR2B to ACVR2A. This may lead to changes in activin mediated intracellular signaling and function as an adaptation mechanism required for cells to escape growth/invasion limiting actions of activins. Since activin type 2 receptors are involved in a variety of receptor signaling complexes, the switch of the predominantly expressed receptor may also alter responses to other TGF- β family ligands. We observed a clear association of type 2 receptor expression profile with loss of Smad3 responsiveness and migratory and invasive phenotype. H28 mesothelioma cells did not show this receptor switch, were sensitive to Smad3 activation and did not show high migratory or invasive growth phenotype. H28 cells have been shown to lack β -catenin [34], which is not a typical feature of mesothelioma [35]. This suggests that H28 is a unique mesothelioma cell line.

Activins can induce inhibition of proliferation and apoptosis [5]. Cancer cells can avoid these suppressive functions through various mechanisms. Loss of the CDKN2A/ARF locus is the most common genetic alteration in mesothelioma and leads to inactivation of the p16^{INK4a} tumor suppressor gene [36]. This may also reduce activin induced apoptotic responses [37]. Smad-mediated signaling is also crucial for activin induced growth arrest [38]. We have shown here a clear transformation in activin induced intracellular signaling cascades in mesothelioma cells. Smad3 activation, measured using a

promoter assay, was almost lost in migratory and invasive mesothelioma cells, while Smad2 activation was partially retained in some cell lines. In addition, TGF- β was still able to induce Smad3 activation (our unpublished data), suggesting an activin specific loss of responsiveness. Higher follistatin expression levels in established mesothelioma cell lines could not explain the loss of responsiveness, since activins induced a robust ERK activation in H2052 cells.

Furthermore, JP5 primary cells had low levels of follistatin expression but attenuated Smad3 activation. We have previously shown high P-Smad2 immunoreactivity in mesothelioma tumor samples [39], but P-Smad3 levels *in vivo* have not been specifically analyzed and remain to be determined.

Previously it has been observed that activin-B induces skin wound re-epithelialization through Smad3-independent mechan-



isms [40]. This is also demonstrated by accelerated wound healing in Smad3 knockout mice [41]. The ERK pathway has been found important for activin induced epithelial wound healing [40]. The migratory and invasive growth phenotype in mesothelioma cells was also clearly associated with activin induced ERK activity, since inhibition of both activin-A and -B by using a soluble type 2 receptor reduced levels of ERK phosphorylation and migration and invasive growth in 3D matrix. Similar effects were observed when ERK phosphorylation was blocked with an inhibitor of the upstream kinase MEK. ERK is one of the well-known activators of cyclin D [42]. Our results are in agreement with a report by Hoda et al. [8] suggesting that activin-A can also function through cyclin D in mesothelioma. The ERK pathway is involved in the regulation of cell cycle progression, apoptosis and differentiation. It is also commonly activated in tumors [43]. ERK is known to be highly activated in mesothelioma [44] and central to mesothelioma cell migration, proliferation as well as tumor development and chemoresistance [45]. In addition to TGF- β family ligands, receptor tyrosine kinases can contribute to ERK pathway activity in mesothelioma cells. Fibroblast growth factor receptor (FGFR)1 was recently found upregulated in mesothelioma cells and in patient samples [46]. Inhibition of growth caused by FGFR inhibition was associated with downregulation of basal ERK activity [46].

The exact molecular mechanism of the switch from Smad3 signaling to ERK pathway signaling in activin responsive mesothelioma cells remains to be elucidated. Changes in activin type 2 receptor expression profile may contribute to this switch. Downregulation of TGF- β type 2 receptor has been implicated in the loss of responsiveness to the anti-proliferative effects of TGF- β in lung cancer cells [47]. In addition to changes in receptor expression, it has been suggested that a specific isoform of PP2A is differentially recruited to TGF- β type 1 receptor in normal and malignant cells leading to differential activation of ERK [31]. Under hypoxic conditions PP2A can dephosphorylate Smad3 without affecting Smad2 [48]. Similar mechanisms may play a role in the observed alterations in activin responses.

Mesotheliomas are characterized with aggressive and locally invasive phenotypes. Understanding of the mechanisms that the tumor invasion is vital for the development of treatment strategies. Cancer cells and the tumor microenvironment both contribute to the invasive phenotype of the tumors. We have shown here that mesothelioma cells produce activin-A and -B, which both contribute to the invasive and migratory phenotype of the cancer cells. High expression levels of activins are associated with attenuation of the canonical Smad3 signaling and increase in non-canonical ERK signaling responses. Similar changes in the signaling pathways have

been associated with the function of TGF- β as a tumor promoter in cancer cells [31]. In conclusion, inhibition of activin or ERK activity, or both, represents a therapeutic approach for inhibition of invasive mesothelioma tumors. Novel inhibitors of activin signaling are under development for clinical use, which makes activins a plausible target for adjunctive therapy in mesothelioma.

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Fig. 6 – ERK activity is essential for activin induced mesothelioma cell migration and invasive growth. (A) Cells were stimulated with activin-A or -B for 90 min and analyzed for ERK phosphorylation using Western blotting. (B, C) When mesothelioma cells were treated with sActR2B-Fc (90 min) to inhibit activin signaling, ERK phosphorylation levels decreased significantly [$*p=0.029$ Mann–Whitney test ($n=4$); JP5 primary cells ($n=2$)]. (D) Representative pictures of 211H (48 h), H2052 (24 h) and JP5 (48 h) scratch-wound assay are shown. (E) Compared to DMSO treated control cells MEK-inhibitor (PD98059) significantly delayed wound closure. H2052 cell wound density was analyzed at the 6 h time point. JP5 primary and 211H cell wound density was analyzed at the 48 h time point [$*p=0.029$ Mann–Whitney test ($n=4$); JP5 primary cells, ($n=3$)]. Error bars represent standard deviation. (F) Representative pictures of H2052 scratch wound assay are shown. (G) H2052 cell wound density was analyzed at the 6 h time point. The wound density in activin-A stimulated cells was twice as high compared to the DMSO treated control cells. The MEK-inhibitor (PD98059) significantly delayed wound closure in activin-A stimulation cells [$*p=0.029$ Mann–Whitney test ($n=4$)]. (H) The effect of inhibition of ERK activity to mesothelioma cell invasive growth in three-dimensional matrix was analyzed using the MEK-inhibitor (PD98059). PD98059 prevented invasive growth of 211H and JP5 primary cells. Representative pictures are shown.

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