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Original Article

Signaling by TGF-betas in tubule cultures of adult rat testis

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Abstract: Although signal transduction of transforming growth factor-betas (TGF-βs) is well characterized in individual cell types, data about TGF-β signaling in a cellular context is still scarce. In this study, we used ex vivo tubule cultures from adult rat testis to investigate TGF-β signaling. We show for the first time in testicular tubules, that TGF-βs also signal via the BMP type I receptors, with ALK2 used by TGF-β1 and ALK3 and ALK6 by TGF-β2. This signal transduction is mediated via Smad3 as well as via Smad1. In contrast, BMPs (BMP2 and BMP7) do not signal via the high-affinity type I and type II TGFβ receptors, TBR1 or TBR2. Furthermore, treatment of tubule cultures with either TGF-β1 or TGF-β2 had profound significant stimulatory effects on secretion of plasminogen activator-1 (PAI-1) through utilization of TGF-β and BMP receptors. Specific inhibitors for either TBR1 or BMP receptors yielded nearly complete inhibition of TGF-β signaling. The TBR1-TBR2 signalosome was detected with Duolink upon stimulation with either TGF-β1 or TGF-β2, predominantly in spermatogenic cells of the adult rat testis, particularly in elongated spermatids. In summary, this examination of intact rat testicular tubules demonstrated for the first time that TGF-βs signal mainly through TBR1 and TBR2 but also use BMP receptors, including for secretion of PAI-1. Whereas ALK2 participates in the TGF-β1-induced TBR1-TBR2 signalosome, ALK3 and ALK6 are involved in signaling of TGF-β2. Detection of the TBR1-TBR2 signalosome in late spermiogenic cells indicates a post-meiotic activity.

Keywords: PAI-1, SMAD, TGF-betas, BMPs, TGF-beta signalosome

Introduction

The TGF-beta superfamily includes TGF-β ligands (TGF-β1, -β2, -β3), bone morphogenetic proteins (BMPs), activins/inhibins, Müllerian inhibiting substance (MIS/AMH), the growth and differentiation factors (GDFs), Nodal, myostatin as well as lefty A and B [1]. TGF-β family ligands have essential functions in reproduction, early development, tissue homeostasis, angiogenesis, wound healing, fibrosis, immune response, autoimmune disease and cancer [1, 2]. Several of these ligands are important for male fertility, functioning in specific processes required for testis growth and spermatogenesis [3].

The three TGF-β isoforms (TGF-β1-3) are the most frequently studied factors among the TGF-β family. They have distinct expression profiles and perform discrete roles [1]. TGF-β signaling is initiated by binding of a dimeric ligand to the receptors [1, 2]. The activated complex consists of two type II and two type I receptors, but in the case of TGF-β2, TBR3 (betaglycan) is also required for high-affinity binding. After complex formation, TBR1 directly phosphorylates Smad2/3, which is subsequently transferred to the nucleus together with Smad4 to target specific promoters with distinct transcription factors to regulate gene expression [2, 4].

It has recently become evident that the TGF-β and BMP receptors interact [5, 6]. In addition to the canonical activation of Smad2 or Smad3 by the TGF-βs, Smad1 and/or Smad5 were also

phosphorylated in a variety of cell types, including breast cancer cells [7-10], bone cells [11], pulmonary artery endothelial cells [12], several epithelial cells [13] and cell lines, e.g. hepatocellular carcinoma cells [14]. Often, this effect was mediated primarily by ALK5 (TBR1), the TGF-β receptor type I [9, 12-14]. However, TGF-β-dependent Smad1/5 activation was most often transient [14] and mixed Smad2/3+ Smad1/5 complexes were frequent in embryonic but only rarely observed in adult mouse tissue [10]. The physiological relevance of this was demonstrated by the requirement for TGF-β-induced phosphorylation of Smad1/5 for the pro-migratory TGF-β switch in mammary epithelial cells [8], anchorage-independent growth of epithelial cells [13], and protection against pulmonary artery endothelial cell apoptosis [12]. In contrast, TGF-β-dependent Smad2/3 activation by BMPs has been only very rarely investigated. Two studies reported that for BMP2 stimulation of Smad3 signaling only ALK3 was required [15, 16]. Another study found BMP2 upregulation of PAI-1, which is a TGF-β-regulated gene [17].

Of relevance to testicular biology, TGF-βs are central to testis cord formation [3, 18-20] and cord maintenance [21] during development. For example, TGF-β signaling via ALK5 is required for testis cord formation during the critical testis-determining period [22].

Moreover, the tight junctions of immature Sertoli cells can be structurally and functionally impaired by TGF-β2 and especially TGF-β3 [23, 24]. In spermatogenesis, TGF-β signaling components are present in spermatogonia but their levels generally decrease dramatically with entry into meiosis [3]. However, TGF-β1 was able to at least partly inhibit some steps in the differentiating pathway of pachytene spermatocytes into round spermatids [25], indicating a role for this signaling pathway in meiotic and haploid germ cells. At earlier stages, TGF-βs can induce apoptosis of gonocytes [26], and immature germ cells [19, 27]. TGF-β1 also exerts some repressive actions on steroidogenesis [28], on differentiation of adult Leydig stem cells [29]. Furthermore, steroidogenesis is reduced in TBR3 knockout mice [20].

In this study, we aimed to elucidate the nature of TGF-β signaling in *ex vivo* testicular tubule cultures with a special emphasis on the TGF-β and BMP interactome, because both pathways contribute to many testicular functions and this potential relationship has not been analyzed in the testis to date. Our results provide specific new information about the signaling moieties that are activated during TGF-β signaling in the intact seminiferous tubule, and we provide evidence that BMP pathways may influence TGF-β signaling outcomes.

Materials and methods

Preparation of seminiferous tubules

All animal experiments were done according to the guidelines of the local committee for animal experimentation (Giessen, Germany). Adult male Sprague Dawley rats ((Crl: CD (SD) IGS; Charles River, Cologne, Germany) weighing 150-200 g were anesthetized with 5% isoflurane (Abbott, Wiesbaden, Germany). After decapitation, testes were removed, washed in 70% ethanol and briefly rinsed in sterile PBS. The entire process of tubule isolation was performed on ice. The tunica albuginea was removed and the tissue cut into 2-3 mm^3 pieces in Petri dishes containing ice-cold tubule medium [DMEM (PAA, Pasching, Austria) supplemented with 10% fetal calf serum (FCS, Gibco, Grand Island, NY USA), 2.5 mM L-glutamine and 1% penicillin/streptomycin (PAA)]. Five of these pieces were immediately placed into a Medicon™ unit (50 µm; Becton Dickinson, Heidelberg, Germany) plus 500 µl of ice-cold culture medium and processed for 50 s in the Medimachine (Becton Dickinson). The fine slurry was recovered from the Medicon with a 5-mL disposable syringe and subsequently filtered through a 500-µm Filcon (Becton Dickinson) into a sterile falcon tube. After centrifugation (800×g, 5 min, room temperature) the tubules were resuspended in tubule medium. Approximately, equal numbers of tubule were plated onto BioCoat™ Collagen I coated 24-well plates (Becton Dickinson) and maintained in a humidified incubator at 32°C in 5% CO_2.

Treatment of tubules and collection of samples

After 48 hrs at 32°C in 5% CO_2 the tubule medium was replaced by starvation medium (tubule medium with 1% FCS) for another 24 hrs followed by addition of 5 µM TBR1 inhibitor LY364947 (Sigma Aldrich, USA) or 5 µM BMPR

Table 1. Antibodies used for detection of the TBR1-TBR2 signalosome and signaling

Protein	Provider	Cat-No	Species	Clonality	Dilution/concentration
TBR1	Santa Cruz Biotechnology (Dallas, USA)	sc-33933	Goat	polyclonal	1:200
TBR2	Santa Cruz Biotechnology	sc-220	Rabbit	polyclonal	1:350
rh ALK2 chimera	Bio-techne (Wiesbaden, Germany)	637-AR	Human	rh	5 μM
rh ALK3 chimera	Bio-techne	315-BR	Human	rh	5 μM
rh ALK6 chimera	Bio-techne	505-PR	Human	rh	5 μM
rh IgG1 chimera	Bio-techne	110-HG	Human	rh	5 μM

Cat-No, Catalog-Number; rh, recombinant human.

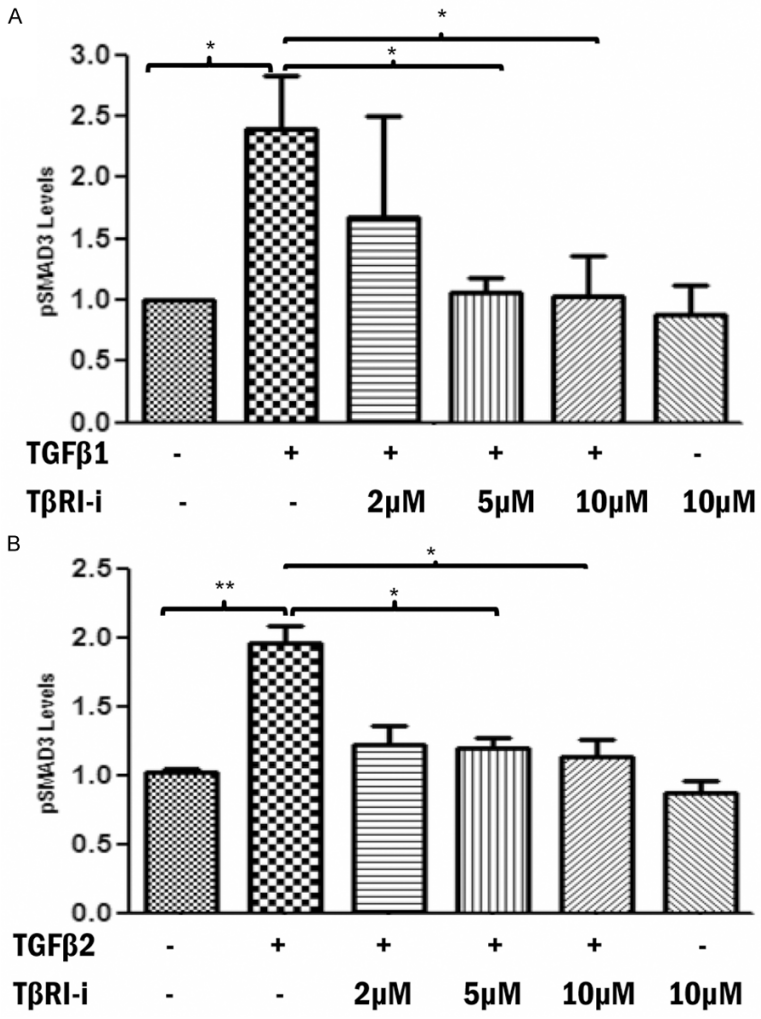

Figure 1. Treatment of tubule cultures with 10 ng/ml TGFβ1 (A) or TGFβ2 (B), respectively, significantly increased phosphorylation of Smad3. The specific TBR1 inhibitor (TβR-I) LY364947 significantly blocked TGF-β-induced phosphorylation of Smad3, reducing this nearly to control levels (**P<0.01; *P≤0.05). Each experiment was repeated three times in duplicates (A+B).

inhibitor LDN193189 (Stemgent, USA) for 2 hrs. Both inhibitors were dissolved in DMSO, which was used without the inhibitors as a vehicle control. Then, tubules were stimulated with 10 ng/ml of recombinant TGF-β1 or TGF-β2, respectively (Promocell) at 32°C for 24-48 hrs.

Withdrawn supernatants were centrifuged (5000×g, 10 min, 4°C) and the protein concentration was determined using the precision Red Advance protein assay (Cytoskeleton, USA). After adding 1 mM PMSF and 1× protease inhibitor cocktail (Sigma Aldrich, USA) the supernatants were stored at -20°C until further use. The tubules were washed with PBS and lysed in 250 μl cell lysis buffer (Cell Signaling, Germany) containing 1 mM PMSF and 1× protease inhibitor cocktail on ice according to the protocol of the manufacturer. After 10 min of incubation on ice, tubules were detached with a cell scraper and the mixture was sonicated 30 times for 1 s each with intervals of 1 s (Sonoplus mini 20, Germany) on ice. After centrifugation (13,000×g, 20 min, 4°C), the protein concentration of the supernatants was determined as described above and samples were stored at -20°C until further use.

Enzyme-linked immunosorbent assay (ELISA)

The tubule fragments were homogenized in a modified RIPA lysis buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% SDS + 1× protease inhibitor cocktail added freshly before use), then centrifuged for 15 min and

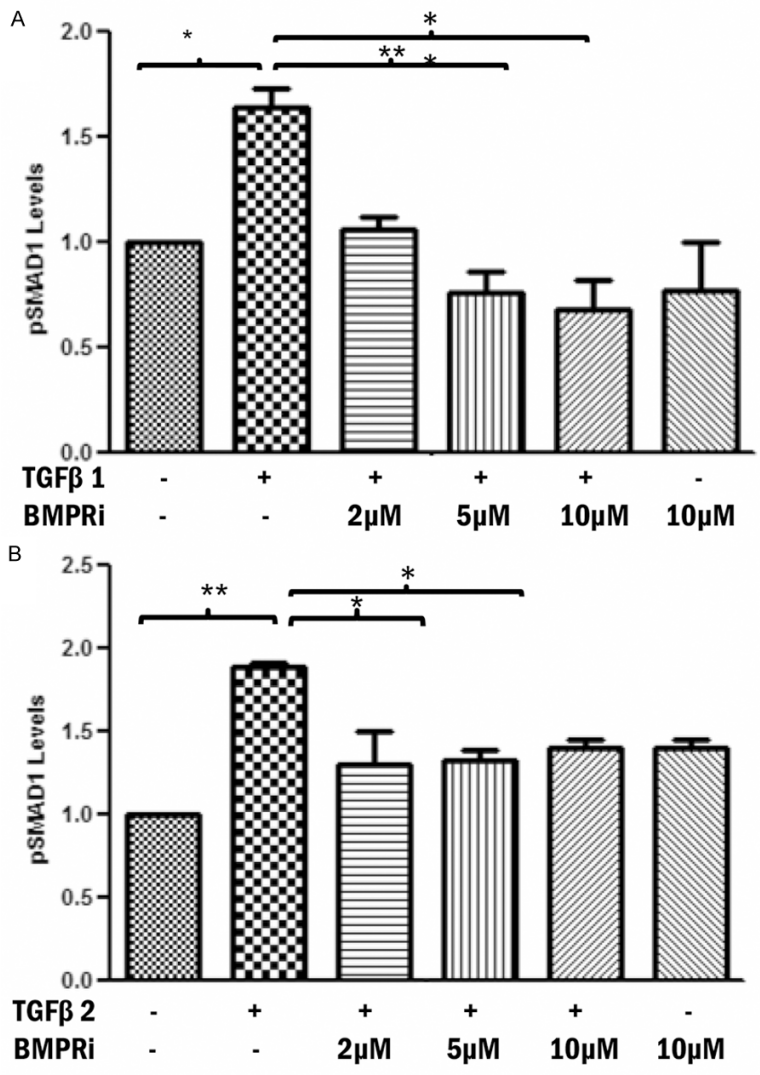

Figure 2. Treatment of tubule cultures with 10 ng/ml TGF-β1 (A) or TGF-β2 (B), respectively, significantly increased phosphorylation of Smad1. The specific BMP receptor inhibitor (BMPRI) LDN193189 significantly blocked TGF-β-induced phosphorylation of Smad1 nearly to control levels (**P<0.01; *P≤0.05). Each experiment was repeated three times in duplicates (A+B).

the supernatants were collected. Smad1 phosphorylation was detected with the phospho-Smad1 (Ser463/465) ELISA (eBioscience, USA). Phosphorylated Smad3 was quantified with the phospho-Smad3 (Ser423/425) ELISA (Abcam, Cambridge, MA).

Culture medium samples were collected and stored at -80°C. PAI-1 levels in the medium were determined using the rat PAI-1 ELISA (Cusabio Biotech, China).

Each ELISA was performed according to the manufacturer's instructions, quantitated by the Benchmark Reader infinite M200 (Tecan, Austria), and values were normalized to equal protein levels (50 mg) of each lysate.

Detection of receptor interaction with the Duolink PLA assay

Equal volumes of the tubule fragment suspension were plated onto 6-cm culture plates and incubated at 32°C and 5% CO_2 in tubule culture medium. After 24 hrs, tubule fragments were stimulated without or with 25 ng/ml TGFβ1 or TGFβ2 (dissolved in water plus 2 mg/ml BSA) for 30 min in starvation medium. Starvation medium plus BSA was used as a control. The tubule fragments were centrifuged at 500×g for 10 min at room temperature. After careful discarding the supernatant, the tubule fragments were Bouin-fixed, paraffin-embedded, cut into 5 μM sections and placed onto slides [27]. After incubating the slides overnight at 37°C sections were dewaxed with Neoclear three times for 20 min at room temperature, except for the first incubation which was done at 40°C. After a standard descending alcohol series sections were rehydrated in distilled water for 5 min with shaking. Then the slides were incubated in 10 mM citric acid DAKO antigen-retrieval buffer pH 6.1 in a steamer (Multigourmet F510, Braun, Germany) for 20 min at 94°C. After cooling down in the steamer for 20 min, slides were washed 3 times with 1× PBS for 5 min. Then samples were incubated with 3% hydrogen peroxide in methanol for 30 min, followed by three washes in 1× PBS for 5 min each and then blocked in 1.5% BSA for 20 min at room temperature. Then the primary antibodies (**Table 1**) diluted in DAKO diluent buffer were added and the slides incubated overnight at 4°C.

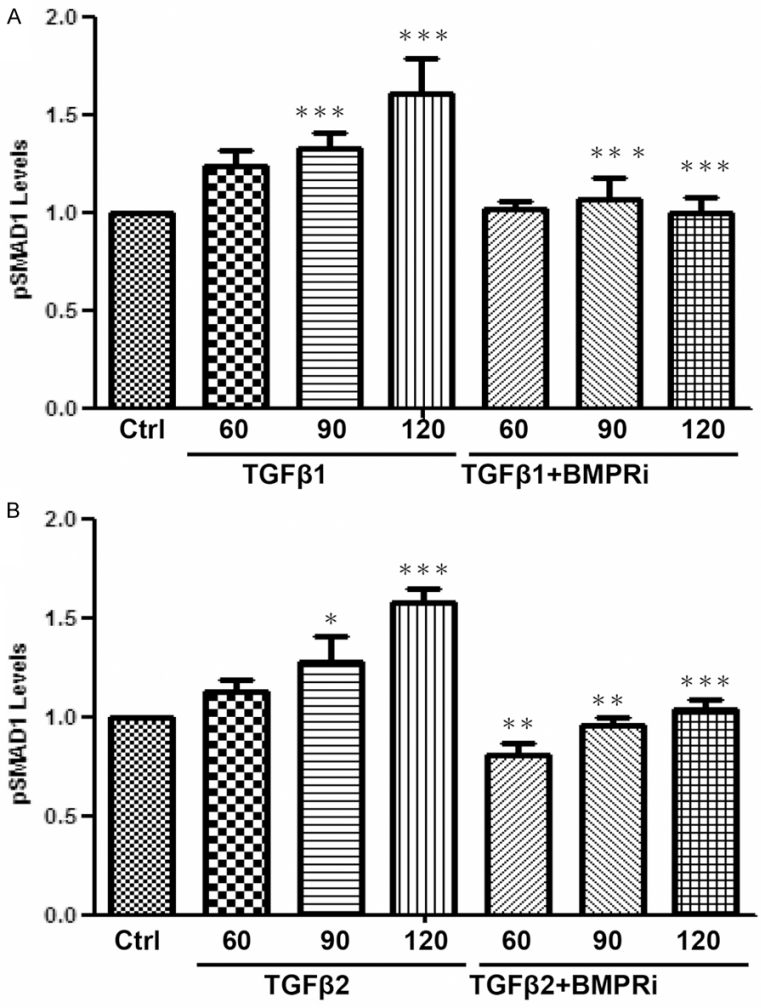

Figure 3. Treatment of tubule cultures with 10 ng/ml TGF-β1 (A) or TGF-β2 (B), respectively, significantly increased phosphorylation of Smad1 in a time-dependent manner. The specific BMP receptor inhibitor (BMPRI) LDN193189 significantly blocked TGF-β-induced phosphorylation of Smad1 nearly to control levels (***P<0.001; **P<0.01; *P≤0.05). Each experiment was repeated three times in duplicates (A+B).

The PLA assay was performed with the Detection Red kit (Olink Bioscience, Sweden) according to the manufacturer's instructions. The slides were subsequently washed in 1× PBS and incubated with mouse PLUS or goat PLUS and rabbit MINUS secondary PLA probes for 1 hr at 37°C. After a further wash with 1× PBS, the slides were incubated with the ligation solution at 37°C for 30 min and washed again with 1× PBS. Then the amplification-polymerase solution was applied to each slide and incubated for 100 min at 37°C in the dark. The slides were washed two times with 1× PBS at room temperature for 5 min each in a Coplin jar protected from light. Then, Duolink II mounting medium with DAPI was applied to cover slips and gently placed over each slide. Images were generated with an Olympus IX81 microscope (Olympus, Germany).

Statistical analysis

All experiments were repeated independently at least three times in duplicate. Values from all experiments were used for calculation of the means and their respective standard errors of the mean (SEM). The comparison of the means between groups was performed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc-test using GraphPad prism software (Version 5.0, GraphPad Inc. La Jolla, USA). *P* values of less than 0.05 were considered significant.

Results

In order to elucidate TGF-β signaling, tubule cultures were stimulated with TGF-β1 or TGF-β2, respectively. As clearly shown, both ligands significantly stimulated phosphorylation of Smad3 approximately two-fold (**Figure 1A**, **1B**). Specificity of signaling was demonstrated by incubation with the specific TBR1 kinase inhibitor LY364964 which decreased TGF-β-induced Smad3 phosphorylation to control levels (**Figure 1A**, **1B**). Of note, TGF-β1 as well as TGF-β2 also initiated phosphorylation of Smad1 significantly (**Figure 2A**, **2B**), which was decreased by the BMP receptor inhibitor LDN193189, which blocks ALK2, ALK3, and ALK6 signaling (**Figure 2A**, **2B**). The increase of Smad1 phosphorylation by TGF-β1 as well as by TGF-β2 was time-dependent (**Figure 3A**, **3B**).

To examine in more detail which BMP receptor is responsible for TGF-β-dependent phosphorylation of Smad1, we performed experiments with ALK2-, ALK3- and ALK6-specific inhibitors.

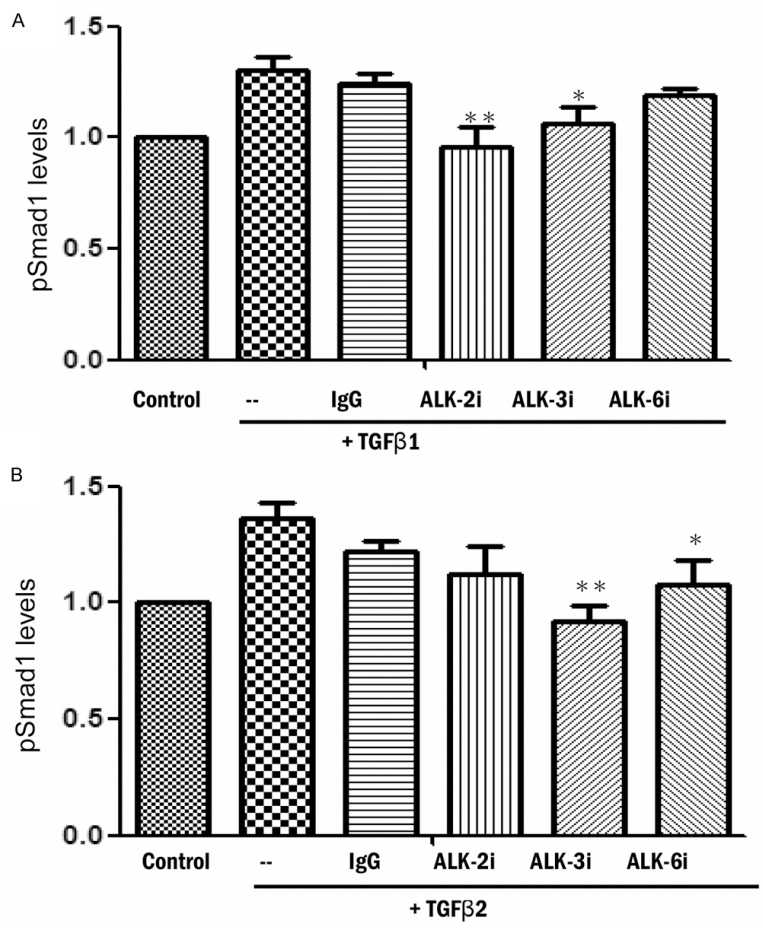

Figure 4. Treatment of tubule cultures with 10 ng/ml TGF-β1 (A) or TGF-β2 (B), respectively, significantly increased phosphorylation of Smad1. (A) The ALK2- and ALK3-specific chimeric inhibitor significantly blocked TGF-β1-induced phosphorylation of Smad1 nearly to control levels (**P<0.01; *P≤0.05). (B) The ALK3- and ALK6-specific chimeric inhibitor significantly blocked TGF-β2-induced phosphorylation of Smad1 nearly to control levels (**P<0.01; *P≤0.05). The control experiment with IgG isotypes did not show any effect. Each experiment was repeated three times in duplicates (A+B).

We observed that phosphorylation of Smad1 by TGF-β1 depended mainly upon ALK2 (**Figure 4A**), because inhibition of ALK3 and ALK6 had no significant effect. In contrast, TGF-β2 required mainly ALK3 and ALK6 to modulate phosphorylation of Smad1 (**Figure 4B**).

Because PAI-1 is a well-known target of TGF-β signalling, we asked whether this important function of the TGF-βs could also be influenced by additional signaling via the BMP receptors. We found that PAI-1 secretion was significantly increased by TGF-β1, and was completely reduced by the TBR1 inhibitor LY364964, as expected. TGF-β1-mediated PAI-1 secretion was only partially blocked by the BMP receptor inhibitor LDN193189 (**Figure 5A**), and similar results were obtained for the TGF-β2 stimulated response (**Figure 5B**).

We next aimed to determine the cell types mediating TGF-β signaling in the rat testis. We observed that the TBR1-TBR2 signalosome was especially evident in late spermatogonia and in elongated spermatids after stimulation with TGF-β1 (**Figure 6A-D**). In contrast, the TBR1-TBR2 signalosome was preferentially localized in elongated spermatids after stimulation with TGF-β2 (**Figure 6E**, **6F**). All controls were negative (**Figure 6G**, **6H**).

Discussion

The major findings of this study are: (i) TGF-β1 and TGF-β2 not only cause phosphorylation of Smad3 but also of Smad1, (ii) TGF-β1/2-dependent Smad1 phosphorylation is mediated by the kinase activity of TBR1, (iii) TGF-β1-dependent Smad1 phosphorylation is also mediated by the kinase activity of the BMP receptors ALK2 and ALK3, (iv) TGF-β2-dependent Smad1 phosphorylation is also mediated by the kinase activity of the BMP receptors ALK3 and ALK6 (v) TGF-β1/2-dependent PAI-1 protein secretion is mainly mediated by TBR1 and only partially by the BMP receptors and (vi) the TBR1-TBR2 signalosome is preferentially found in elongated spermatids upon stimulation with TGF-β1 or TGF-β2. Our results clearly showed that TGF-β1/2 signaling in testicular germ cells is not only transduced by the canonical Smad3 but also by the BMP-dependent Smad1.

In addition to canonical Smad2/Smad3 phosphorylation mediated by the TBR1-TBR2 signalosome, the non-canonical Smad1/Smad5 pathway has also been found to be switched on by TBR1 [9, 12-14]. Although the connection of the TBR1-TBR2 signalosome to the BMP recep-

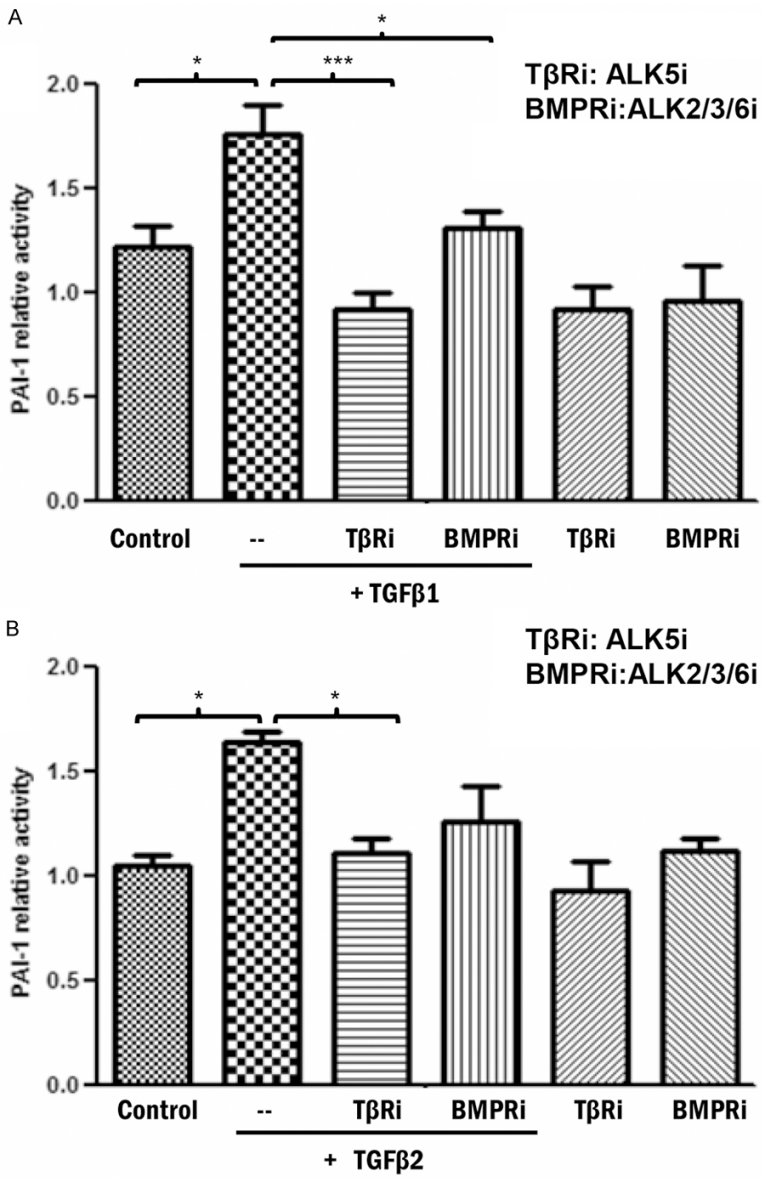

Figure 5. Treatment of tubule cultures with 10 ng/ml TGF-β1 (A) or 10 ng/ml TGF-β2 (B), respectively, significantly increased secretion of PAI-1. (A) The specific TBR1 inhibitor (TβRi) LY364947 and BMP receptor inhibitor (BMPRi) LDN193189 blocked TGF-β1-induced secretion of PAI-1 totally or only partially, respectively. (B) The specific TBR1 inhibitor (TβRi) LY364947 and BMP receptor inhibitor (BMPRi) LDN193189 blocked TGFβ2-induced secretion of PAI-1 totally or only partially, respectively. Control experiments with the inhibitors alone did not show any effects. Each experiment was repeated three times in duplicates (A+B).

tors was not investigated [9, 12-14], it was proposed that the unconventional Smad phosphorylation might occur through mixed ALK2/ALK3-ALK5 receptor complexes [13]. Furthermore, interaction of TBR2 and ALK3 [30] and between TBR2 and ALK2 [31] were described.

In this study, we observed that in addition to TBR1, activation of BMP receptors appears also to be necessary for phosphorylation of Smad1. Our interpretation is that after ligand-dependent activation of TBR1, the BMP receptors phosphorylate Smad1. Thus, we suggest that TGF-β-induced phosphorylation of Smad1 is indirectly mediated via the BMP receptors but requires TBR1. Accordingly, similar to Daly *et al*. [13] we propose that lateral signaling might occur through mixed type 1 receptor complexes.

Our finding show that, in testicular tubules the classical outcome of TGF-β signaling, PAI-1 secretion, depends primarily on TBR1 and only partially upon BMP receptors engagement. In B16 melanoma cells, BMP2 was also demonstrated to stimulate PAI-1 expression via ALK3 [17]. In a different study of transformation of the chicken atrioventricular cushion, TGF-β1 was shown to bind to both chicken ALK5 and ALK2, but only ALK5 and not ALK2 was found to stimulate PAI-1 [32]. Thus, ALK2/ALK3-dependent stimulation of PAI-1 might be cellular context dependent or requires a high expression level of the receptors, as already proposed [17].

What is the possible function of the TBR1-TBR2 signalosome in the testis? Overexpression or upregulation of the TGF-βs seems to be detrimental to male germ cells, since this can at least partly inhibit some steps in the differentiating pathway of pachytene spermatocytes into round spermatids [25], induce apoptosis of germ cells [27], or disturb the blood-testis barrier inside the tubules [24]. Thus, our finding that the TBR1-TBR2 signalosome was preferentially located in elongated spermatids upon stimulation with TGF-β1 or TGF-β2 points to a major post-meiotic role of the TGF-βs which warrants further investigation.

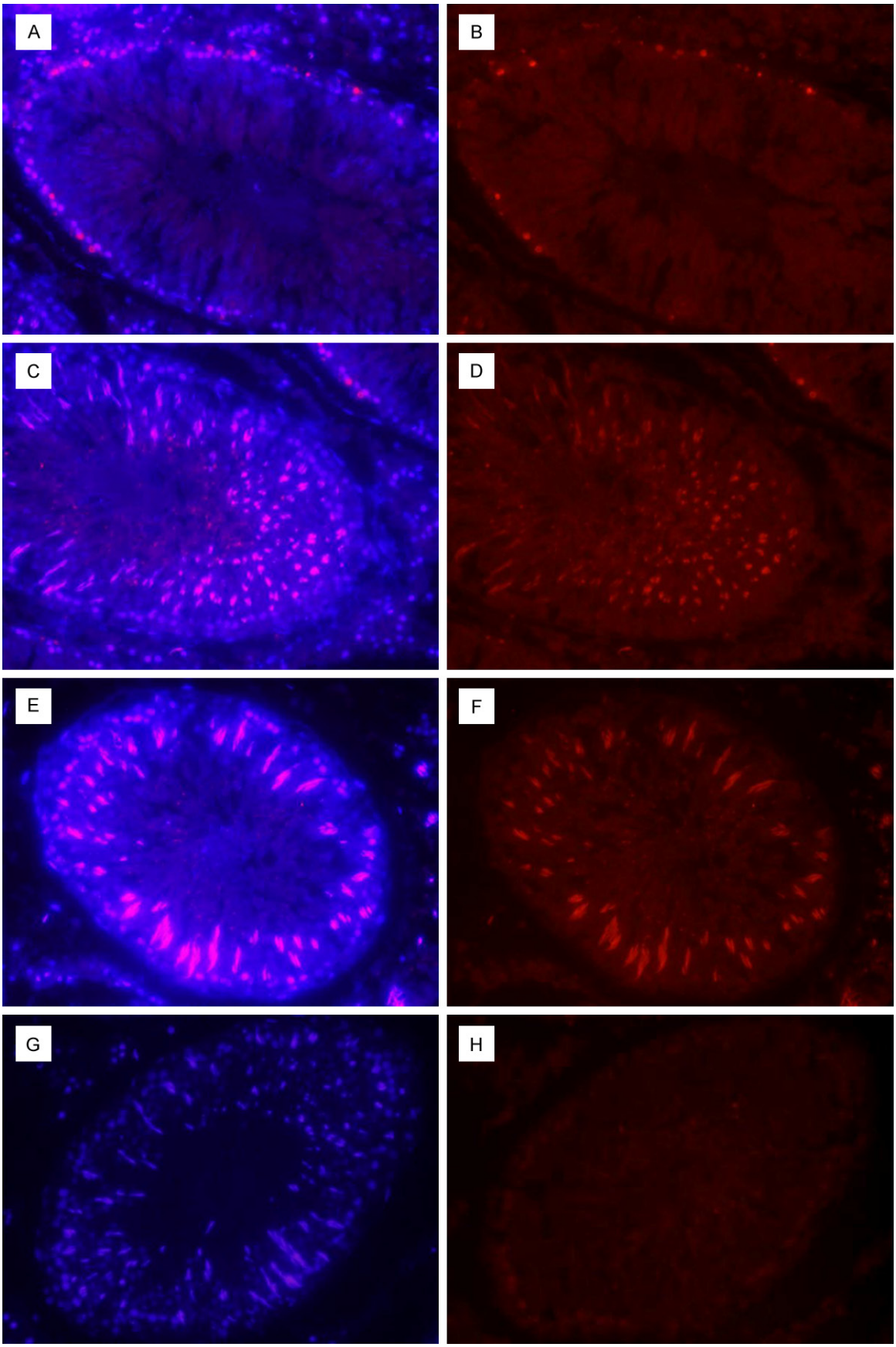
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Figure 6. Treatment of tubule culture fragments with 25 ng/ml TGF-β1 (A-D) or 25 ng/ml TGF-β2 (E, F), respectively, resulted in formation of the TBR1-TBR2 signalosome (red fluorescence) especially in late spermatogonia and elongated spermatids after stimulation with TGF-β1 (A-D). In contrast, the TBR1-TBR2 signalosome was preferentially found in elongated spermatids after stimulation with TGF-β2 (E, F). Control experiments without the antibodies against TBR1 (G, **Table 1**) or without the antibodies against TBR2 (H, **Table 1**) did not show any TBR1-TBR2 signalosomes inside the tubules.

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Disclosure of conflict of interest

None.

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