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## Mouse embryonic stem cells with aberrant transforming growth factor $\beta$ signalling exhibit impaired differentiation *in vitro* and *in vivo*

Accepted in revised form: 25 February 1998

**Abstract** Embryonic stem (ES) cells are resistant to transforming growth factor  $\beta$  (TGF $\beta$ ). We have shown previously that they lack type-II binding receptors (T $\beta$ RII) and in this respect resemble the inner cell mass and ectoderm cells of mouse embryos 4.5–7.5 days post coitum (dpc); they do however express type-I (alk-5) signalling receptors. Here we show that in contrast to several tumour cell lines, stable transfection of wtT $\beta$ RII is not sufficient for ES cells to become biologically sensitive to TGF $\beta$ . We analysed the expression of several downstream molecules known to be involved in TGF $\beta$  signalling (Smads) and TGF $\beta$ -mediated cell cycle regulation (cyclins D) during the differentiation of control and wtT $\beta$ RII-expressing ES cells and showed that upregulation of these molecules correlated with (i) an increase in plasminogen activator inhibitor-1 (PAI-1) synthesis and (ii) growth inhibition, following addition of TGF $\beta$ 1. These TGF $\beta$  responses were reduced in an ES cell line expressing a dominant negative (truncated) T $\beta$ RII ( $\Delta$ T $\beta$ RII). The differentiation pattern of control and wtT $\beta$ RII-expressing ES cells was indistinguishable in monolayer culture and as embryoid bodies, but in  $\Delta$ T $\beta$ RII ES cells, the capacity to form mesodermal derivatives in monolayer cultures in response to the addition of retinoic acid (RA) and removal of leukemia inhibitory factor (LIF) was lost, and only endoderm-like cells formed. The T $\beta$ RII and  $\Delta$ T $\beta$ RII ES cells were, however,

both distinguishable from control ES cells when allowed to differentiate in chimaeric embryos following aggregation with morula-stage hosts. Conceptuses containing mutant cells, recovered from pseudopregnant females at the equivalent of 9.5 dpc, exhibited highly defective yolk sac development; most strikingly, no blood vessels were present and in addition the yolk sacs with derivatives of ES cells containing wtT $\beta$ RII were blistered and lacked haematopoietic cells. The implications for understanding TGF $\beta$  signalling in early mouse development are discussed.

### Introduction

Embryonic stem (ES) cells are derived from the inner cell mass of blastocyst-stage embryos. When cultured in the presence of LIF, they retain many of the properties of their embryonic precursors, and are used as a model for studying the regulation of growth and differentiation *in vitro*. *In vivo*, upon reintroduction into a blastocyst or morula-stage embryo, they will contribute to all tissues of the developing host, including the germ line. Withdrawal of LIF is sufficient to induce differentiation of ES cells in culture into endoderm- and mesoderm-like cells, and effect accelerated by RA. We are interested in the signalling events that result in pluripotent embryonic cells forming derivatives of all three germ layers of the embryo proper and extraembryonic cell types. Member of the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily may be involved in regulating some of these early differentiation steps [1, 13, 28, 42]. TGF $\beta$  itself is a secreted growth factor that exists as three different isoforms in mammals.

TGF $\beta$  exerts a variety of biological effects depending on the target cell and its physiological setting. These include negative or positive regulation of cell proliferation, differentiation and the production and remodelling of the extracellular matrix (ECM), resulting in alterations in cellular adhesiveness and migration [2, 24, 36]. Changes in ECM composition induced by TGF $\beta$  are the net result

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of both an increase in the synthesis of its components, such as fibronectin, and in the synthesis of inhibitors of degrading enzymes, such as plasminogen activator inhibitor (PAI-1) [12, 57].

TGF $\beta$  and related proteins exert their effect by interacting with two transmembrane serine/threonine kinases, known as type-I and type-II receptors. TGF $\beta$  binds to the type-II receptor, which recruits and phosphorylates a type-I receptor to produce a heteromeric signalling complex [55]. The type-I receptor phosphorylates one or more members of a family of proteins known as Smads, mammalian homologues of genes first identified in *Drosophila* known as mothers against decapentaplegic (MAD). Smad2 and 3 appear to be specific for TGF $\beta$  and activin family members [5, 19, 21, 31, 56, 57]. The phosphorylated Smad (or Smads) then forms a stable complex with Smad4, which translocates to the nucleus, where it regulates transcriptional responses to TGF $\beta$ s. Numerous type-I and type-II receptors have been described which bind and signal for different members of the TGF $\beta$  superfamily with varying specificity [22, 49]. However, only one binding and signalling complex has been described so far for TGF $\beta$ 1, namely T $\beta$ RII with T $\beta$ RI (or alk-5)[48].

Several members of the TGF $\beta$  superfamily are secreted by the early mouse embryo, or are available to it from maternal sources. The distributions of TGF $\beta$ s and their receptors in pre- and early post-implantation mouse embryos [2, 23, 33–35, 37] suggest an important role for TGF $\beta$ s during development, but their exact function is still incompletely understood. To address this problem, we interfered with TGF $\beta$  signalling in embryos by modifying receptor expression; we thus expected to avoid the rescue of embryonic phenotypes by maternal ligand reported previously for TGF $\beta$ 1 knock-out mice [16, 17] and identify TGF $\beta$  functions at early postimplantation stages. Since T $\beta$ RII is the binding receptor for TGF $\beta$ 1, we decided to express either a truncated or wild-type (wt) T $\beta$ RII ectopically. However, in preimplantation embryos, T $\beta$ RII is already expressed in the zygote [39]; expression is lost at the 4-cell stage but is regained when the blastocyst is formed at 3.5 days of gestation, when it is expressed in the trophectoderm. First expression in the embryo proper occurs as the heart forms at 8.5 dpc [37, 38]. It was likely that simply zygote injection of the constructs would interfere with preimplantation development when the genes were highly expressed, and indeed zygote injection of  $\Delta$ T $\beta$ RII resulted in a 2-cell block in many embryos [39]. We therefore introduced the constructs into ES cells. This would permit analysis of post-implantation chimaeric embryos containing the ES cells as well as providing a means of analysing the TGF $\beta$  signalling pathway during differentiation in vitro.

We biased the morula aggregation with ES cells towards strong chimaeras so that virtually all of the embryos proper and the mesodermal component of the yolk sac were ES-derived. In contrast to control ES chimaeras, which were indistinguishable from wt embryos, both wtT $\beta$ RII- and  $\Delta$ T $\beta$ RII-expressing lines surprisingly gave

rise to distinct defects in yolk sac vasculogenesis and haematopoiesis, superficially reminiscent to that described for a proportion of the TGF $\beta$ 1 knock-out mice generated in certain genetic backgrounds [4, 8]. As a first attempt to understanding this phenotype and in the light of the known effects of TGF $\beta$  on cellular behaviour, we compared the wtT $\beta$ RII and  $\Delta$ T $\beta$ RII ES cell lines with control ES cells for their response to TGF $\beta$  during differentiation in vitro. The results showed that the ES cells expressing wtT $\beta$ RII had a strikingly enhanced ECM response to TGF $\beta$  while the  $\Delta$ T $\beta$ RII ES cells differed from controls and wtT $\beta$ RII ES cells in their capacity for differentiation. The possible molecular basis for these effects and the implications for yolk sac development are discussed.

## Methods

### Expression constructs

The expression vectors for the wt and  $\Delta$ T $\beta$ RII (Fig. 1) contain the *Eco*RI fragment from the H2-3FF plasmid (kindly provided by Dr. H.Y. Lin) [18] inserted into a vector contained the PGK-1 promoter (PGK-CAS), which was subcloned 3' from the hygromycin resistance gene from pPGK-hyg [47]. A KT3 epitope-tag was introduced in frame by polymerase chain reaction (PCR) at the carboxyl terminus of the wt and  $\Delta$ T $\beta$ RII.  $\Delta$ T $\beta$ RII was made by truncating the wtT $\beta$ RII at amino acid residue 700 in the juxta-membrane region; the entire kinase domain of the receptor is thus removed. The nucleotide sequence of the cDNA was confirmed by sequencing. The TGF $\beta$ - and activin-responsive 3TP-lux reporter construct, containing three TPA-responsive elements and region –740 to –636 of the PAI-1 promoter in front of the luciferase gene [54] was obtained from Dr. J. Massagué.

### Cell cultures

E14 ES cells [11] were cultured on a monolayer of mitomycin C-inactivated SNL feeder cells in Dulbecco's Modified Eagles Medium (DMEM; GibcoBRL, Breda, the Netherlands) conditioned by Buffalo Rat Liver cells (BRL) [44], supplemented with 20% fetal calf serum (FCS; Integro) and 0.1 mM  $\beta$ -mercaptoethanol (Sigma, Zwijndrecht, the Netherlands). For differentiation assays in monolayer, ES cells were grown for at least two passages on gelatinised wells without feeders. At day 0, BRL-conditioned medium was replaced by Minimal Essential Medium (MEM) with 20% FCS or Chemically Defined Medium (CDM) [13], with or without 1 unit Leukemia Inhibitory Factor (LIF; GibcoBRL, Breda, the Netherlands) per ml and/or RA ( $10^{-6}$  M; Sigma) as required, and grown for the time indicated [25]. To form embryoid bodies, ES cells were seeded at 40,000 cells/ml in bacteriological dishes for 3 days. The aggregates were plated on gelatinised coverslips in either MEM with 20% dextran-coated charcoal (DCC)-treated FCS [42] or CDM, and used for immunofluorescence. EPI-7 cells were cultured as described previously [25, 26].

### Electroporation and selection of T $\beta$ RII-expressing ES cells

For electroporation, 15  $\mu$ g ClaI-linearized vector was added to cells ( $7 \times 10^6$  cells) in a single cell suspension in 0.7 ml Ca $^{2+}$ -free phosphate-buffered saline (PBS). The construct was electroporated using a Bio-Rad Gene Pulser (500 mF, 230 V). Cells were then incubated at room temperature for 10 min, plated on to 35-mm dishes, and allowed to recover for 24 h prior to hygromycin selection (150  $\mu$ g/ml; Calbiochem, San Diego, USA). Medium was re-

freshed every 2 days; 8 days after electroporation the resistant ES clones were individually picked and culture under hygromycin selection. Two independent clones were characterized in detail for each construct. Data presented are from one clone in each case since results were identical with clones expressing constructs at similar levels.

#### TGF $\beta$ iodination and binding studies

TGF $\beta$  was iodinated using a modified chloramine-T method and used in binding studies as described by de Winter et al. [6]. Briefly,  $^{125}\text{I}$ -TGF $\beta$ 1 (specific activity  $\approx 40 \mu\text{Ci}/\mu\text{g}$ ) was diluted to  $\approx 1.0 \times 10^6$  cpm/2 ml in Hepes-buffered DF (pH 7.5) containing 0.2% bovine serum albumin and added to stably transfected E14 ES cells. Cells were incubated for 2 h at 4 $^\circ$  C. Bound TGF $\beta$ 1 was cross-linked by a 30-min incubation with 1 mM bis-sulfosuccinimidyl suberate (Pierce, Rockford, USA) at 4 $^\circ$  C. The cells were lysed in 1% Triton X-100, 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1 mM phenylmethyl sulfonyl furate (PMSF), 0.5  $\mu\text{g}$  aprotinin/ml, and 0.5  $\mu\text{g}$  leupeptin/ml and centrifuged for 10 min at 4 $^\circ$  C. Labelled TGF $\beta$  receptor complexes were precipitated from the supernatant of these lysates by overnight incubation with specific anti-tag antibodies or wheat germ agarose at 4 $^\circ$  C. The immunoprecipitates were recovered by a 30- to 60-min incubation at 4 $^\circ$  C with protein G-Sepharose, and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

#### Transcriptional response assay

Wild type ES cells or ES cells stably transfected with wt T $\beta$ RII or  $\Delta$ T $\beta$ RII were cultured in 6-well tissue culture plates for 1–8 days and transiently with the 3TP-lux reporter construct in the presence of a LacZ reporter construct alone or in combination with the indicated cDNAs by calcium phosphate precipitation. After transfection, cells were cultured for 16 h in the presence or absence of 10 ng TGF $\beta$ 1, 50 ng Activin A or 50 ng bone morphogenetic protein-7 (BMP7)/ml culture medium (MEM+20% DCC-FCS). Luciferase activity was measured as previously described [6] and corrected for LacZ activity.

#### DNA synthesis

ES cells were seeded at 11,000 cells/ml in 24-well plates in BRL conditioned medium supplemented with 20% FCS. After 16 h, the medium was replaced with MEM with 20% FCS, in the presence or absence of RA ( $10^{-6}$  M) as required. TGF $\beta$  was added to the culture on day 7, for 24 h with  $^3\text{H}$ -thymidine (0.5  $\mu\text{Ci}/\text{ml}$ ; Amersham, Den Bosch, the Netherlands) present for the last 2 h. Cultures were aspirated, washed four times with PBS, treated with methanol for 15 min, air dried, and dissolved in 0.1 N NaOH and scintillation counted. Inhibition was expressed as a percentage relative to control cultures in the absence of TGF $\beta$ . EPI-7 cells were used as a control for the TGF $\beta$  inhibition assay [50].

#### Cell labelling and immunoprecipitation

Quantification of PAI-1 protein was performed as described by Ignatz et al. [12]. Briefly, ES cells were seeded at  $1.5 \times 10^6$  cells/well

in 6-well plates and grown for 30 h. For the last 6 h, the cells were cultured in the presence or absence of 10 ng/ml TGF $\beta$ 1, and metabolically labelled with 50  $\mu\text{Ci}/\text{ml}$  Trans-label for the last 2 h. For differentiated cultures,  $5.0 \times 10^5$  cells/well were plated in 6-well plates in the absence of LIF, with or without RA ( $10^6$  M) as required, for 8 days. PAI-1 protein was recovered by scraping plates with sample buffer after extraction of the cytosolic and nuclear proteins. Smad2 and Smad3 proteins were recovered by immunoprecipitation as described by Nakao et al. [31]. The samples were analysed by SDS-PAGE (8%) and autoradiography.

#### RNA isolation and Northern blot analysis

ES cells were collected in 500  $\mu\text{l}$  Ultraspec solution (Biotex) for RNA isolation. RNA was isolated as described by Roelen et al. [37]. Northern blots containing 20  $\mu\text{g}$  mRNA were prepared and hybridized essentially as described [27] with  $^{32}\text{P}$ -radiolabelled PCR products amplified from differentiated ES cells with primers for cyclin D1:5'-TCTACTGACAACCTCTATCCG-3' and 5'-TAGCAGGAGAGGAAGTTGTTGG-3'; and cyclin D2:5'-AGACCTTCATCGCTCTGTGC-3' and 5'-TAGCAGATGACGAA CAGCC-3'. Blots were subsequently hybridized with a human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe as a control for RNA loading.

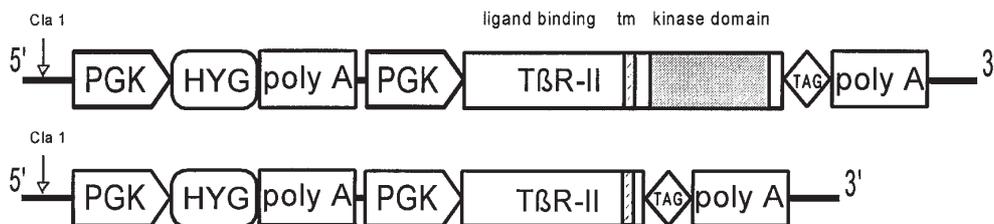
#### Antibodies

Hybridoma supernatant to chicken myosin heavy chain (MF-20) [3] was a gift from Dr. D.A. Fishman (Cornell University, NY). Rabbit polyclonal antibody against *Brachyury T* ( $\alpha$ -T) [15] was a gift from Dr. B. Herrmann, Max-Planck Institute, Freiburg, Germany. Mouse monoclonal antibody against SSEA-1 was a gift from Dr. D. Solter, Max-Planck Institute, Freiburg, Germany. Polyclonal antibodies against Smad2 and Smad3 were a gift from Dr. P. ten Dijke, Ludwig Institute, Uppsala, Sweden. Rabbit polyclonal antibody against T $\beta$ RII ( $\alpha$ -T $\beta$ RII) was purchased from Upstate Biotechnology, Lake Placid, NY. Rat polyclonal antibody against TROMA-1 was a gift from Dr. R. Kemler, Max-Planck-Institute, Freiburg, Germany. Monoclonal antibody against thrombomodulin was a gift from Dr. S.J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN).

#### Immunofluorescence

ES cells grown in monolayer or as plated aggregates on gelatinised coverslips were fixed in either methanol/acetic acid (95%:5%) at  $-20^\circ$  C (MF-20;  $\alpha$ -T;  $\alpha$ -Smad2;  $\alpha$ -Smad3) or 2% paraformaldehyde ( $\alpha$ -KT3;  $\alpha$ -SSEA-1;  $\alpha$ -T $\beta$ RII;  $\alpha$ -TROMA-1;  $\alpha$ -thromb-

**Fig. 1** The plasmid construct used to introduce the wild-type type-II binding receptor for transforming growth factor beta (wtT $\beta$ RII) and dominant negative truncated T $\beta$ RII ( $\Delta$ T $\beta$ RII) into ES cells. T $\beta$ RII, carboxyterminally flanked by a KT3 epitope tag, was cloned into the PGK-CAS vector, containing the PGK-1 promoter and a polyadenylation signal. Hygromycin was used as a selection marker. The  $\Delta$ T $\beta$ RII was made by truncating the juxta-membrane region, but was otherwise similar; *tm* transmembrane domain



omodulin) for 15 min, and permeabilized with 0.1% Triton X-100 (MF-20; NF;  $\alpha$ -T;  $\alpha$ -KT3;  $\alpha$ -Smad2;  $\alpha$ -Smad3;  $\alpha$ -TROMA-1) for 4 min at room temperature (RT). The cells were blocked with 4% normal goat serum in PBS (blocking buffer) for 1 h at RT. They were then incubated with the following primary antibodies for 1 h at RT in blocking buffer: MF-20 (dilution 1:250);  $\alpha$ -T (dilution 1:250);  $\alpha$ -KT3 (dilution 1:100);  $\alpha$ -SSEA-1 (dilution 1:1000);  $\alpha$ -Smad2 (dilution 1:1000);  $\alpha$ -Smad3 (dilution 1:1000);  $\alpha$ -T $\beta$ RII (dilution 1:500);  $\alpha$ -TROMA-1 (dilution 1:10);  $\alpha$ -thrombomodulin (dilution 1:100). After extensive washing in PBS with 0.05% Tween-20 (PBS-T), the cells were incubated with FITC- or Cy3-labelled secondary antibodies, dilution 1:250 (Jackson ImmunoResearch, West Grove, Penn.) for 1 h at RT, washed thoroughly in PBS-T, dipped in H<sub>2</sub>O and mounted in Mowiol (Calbiochem, La Jolla, Calif.).

#### Aggregation chimaeras

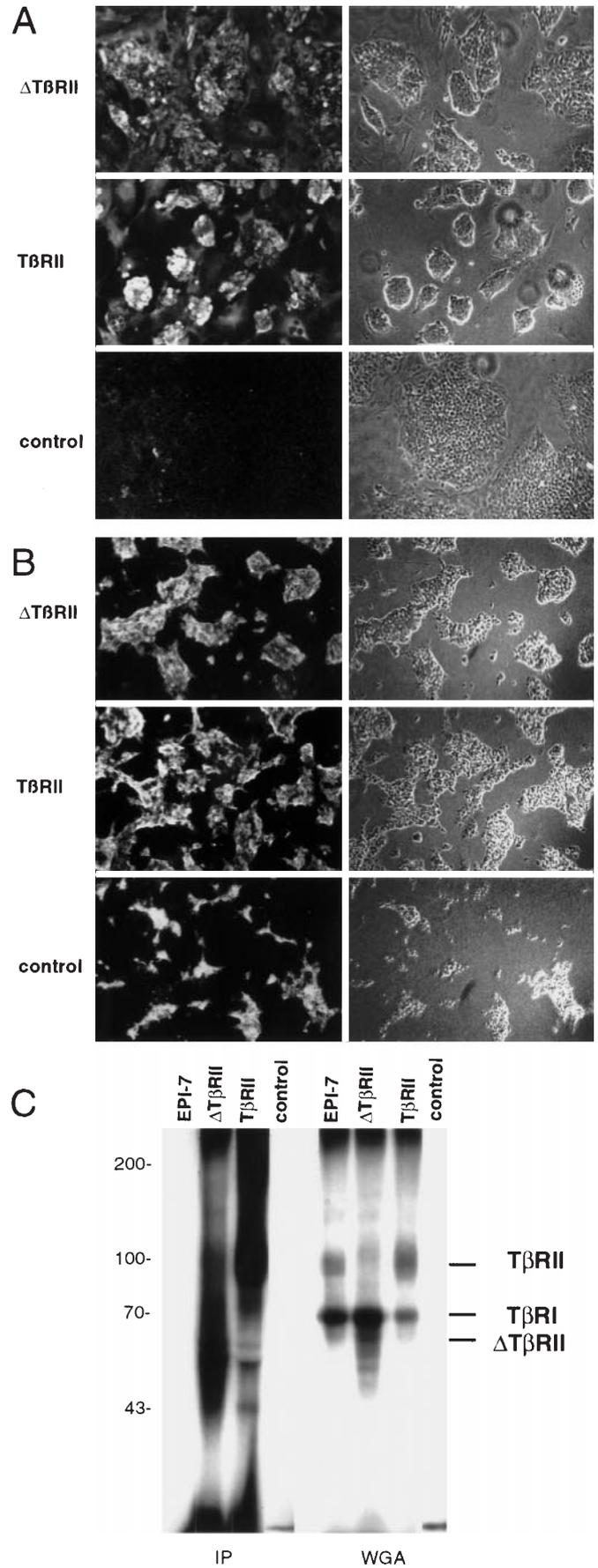
Stably transfected ES cells expressing wtT $\beta$ RII or  $\Delta$ T $\beta$ RII or empty vector were aggregated with ROSA- $\beta$ -geo-26 morulae [10] as described by Nagy and Rossant [29, 30]. Approximately 24 h after aggregation and incubation at 37°C and 5% CO<sub>2</sub>, the embryos were transferred to the uterine horn of CBA $\times$ C57B1/6 F1 females on day 2.5 of pseudopregnancy. Several independent ES clones expressing the wt T $\beta$ RII,  $\Delta$ T $\beta$ RII, or empty vector were aggregated and resulting chimaeras were analysed at 9.5 dpc. Noon of the day on which a vaginal plug was detected was designated 0.5 dpc.

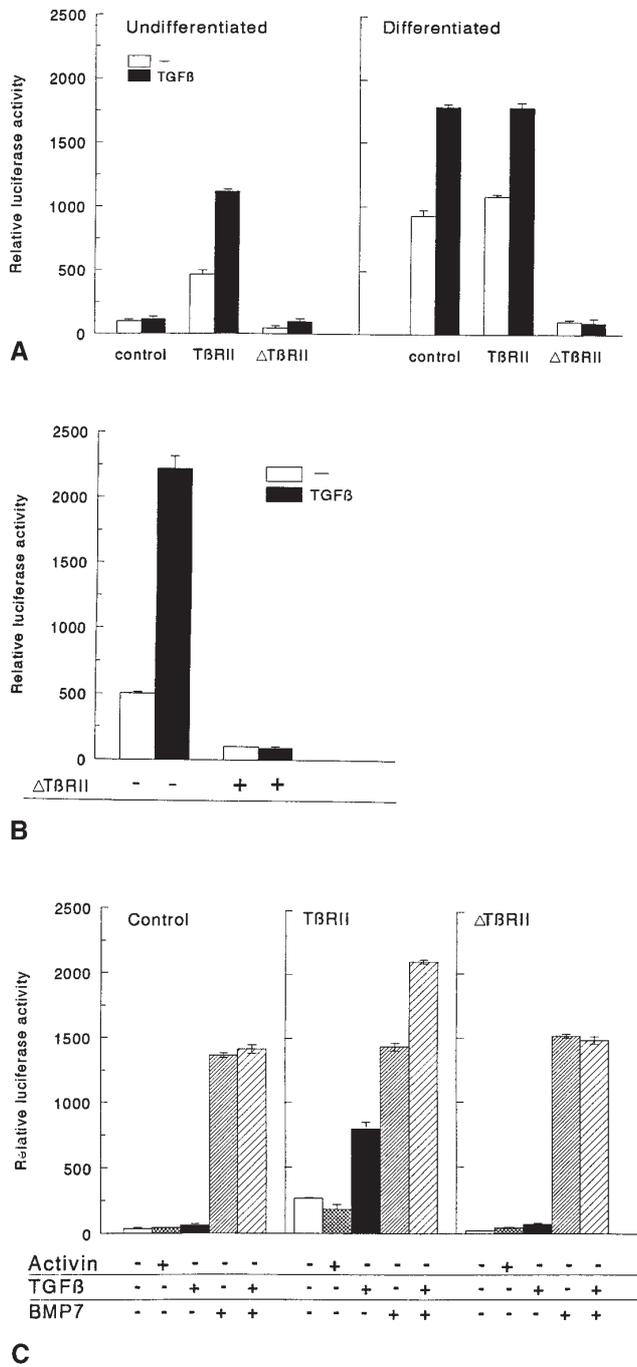
## Results

#### Expression of wt T $\beta$ RII and $\Delta$ T $\beta$ RII in ES cells

wt T $\beta$ RII,  $\Delta$ T $\beta$ RII, or empty vector, were expressed in E14 ES cells by electroporation of linearized vector (Fig. 1) and selection in medium containing hygromycin. Stably expressing clones were further selected on the basis of fluorescent plasma membrane staining with antibodies against the KT3-tag (Fig. 2a). To establish whether the receptor-expressing cell lines had remained undifferentiated, we stained the cells with anti-SSEA-1, which binds to the plasma membrane of undifferentiated embryonal carcinoma cells [45] and ES cells. As shown in Fig. 2b, the selected clones all expressed SSEA-1. To demon-

**Fig. 2** **A** Expression of TGF $\beta$  type II receptor in stably transfected embryonic stem (ES) cells. ES cells were stably transfected with cDNA encoding wt T $\beta$ RII (*T $\beta$ RII*), truncated T $\beta$ RII ( $\Delta$ T $\beta$ RII), or the empty vector (*control*). Several stable clones were tested for their expression of the T $\beta$ RII at the cell surface by immunofluorescence using an antibody against the C-terminal KT3-tag of the receptor constructs. **B** Phenotype of the stably transfected ES cells. To establish whether the stable clones were undifferentiated as their morphology suggested, an antibody against the membrane protein stage-specific embryonic antigen-1 (SSEA-1) was used and expression confirmed. **C** Ligand binding capacity of the stably transfected ES cells. The selected clones expressing wtT $\beta$ RII or  $\Delta$ T $\beta$ RII were tested for their ability to bind <sup>125</sup>I-TGF $\beta$ 1. As a control for the assay, EPI-7 cells were used. After TGF $\beta$  was bound and cross-linked to the cells, receptor complexes were precipitated from cell lysates with an antibody against the C-terminal tag of the receptor construct, or by wheat germ agarose (WGA) absorption and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. After autoradiography, wild-type and truncated T $\beta$ RII are visible, as is T $\beta$ RI





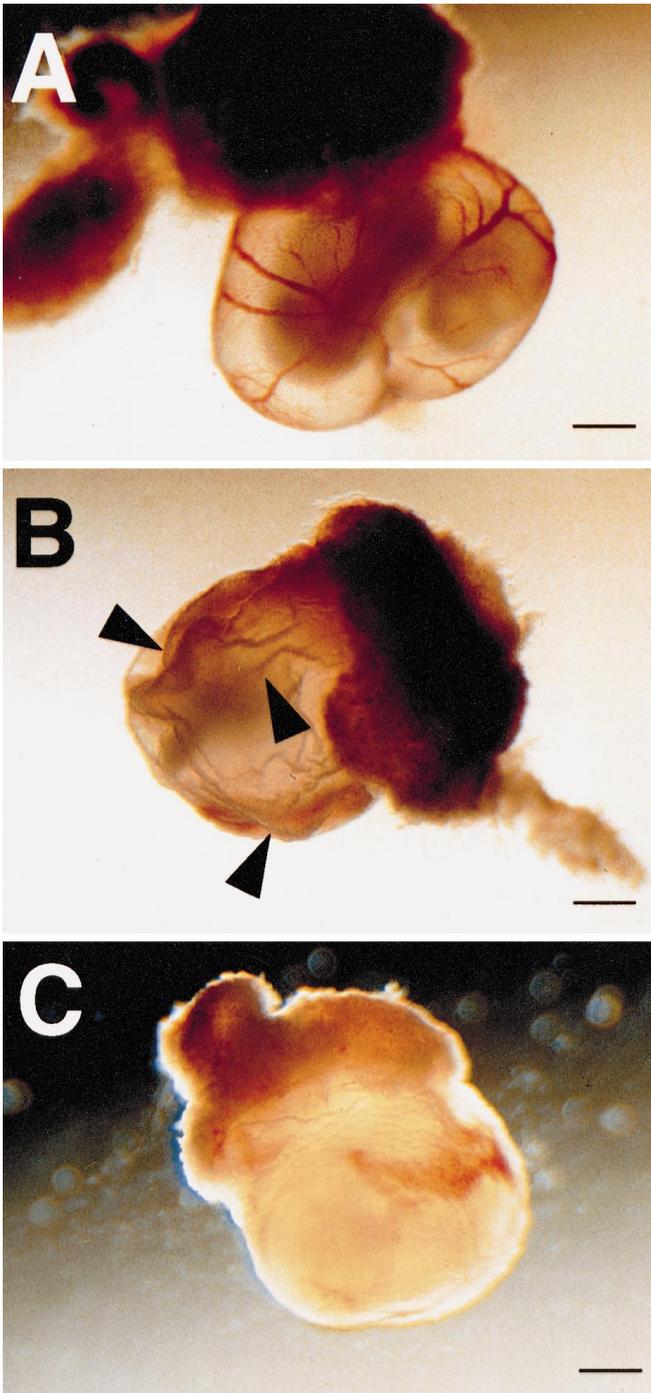
**Fig. 3** **A** Induction of the TGF $\beta$ -responsive 3TP-lux reporter construct in stably transfected ES cells. E14 ES cells transfected with empty vector (*control*), or with the cDNAs encoding wild-type or truncated T $\beta$ RII were transiently transfected with the 3TP-lux reporter in combination with a LacZ reporter construct. Cells were cultured for 18 h in the presence or absence of 10 ng/ml TGF $\beta$ 1. Induction of luciferase was measured and corrected for LacZ activity. The experiment was repeated five times with the same results. **B** Endogenous TGF $\beta$  augmented basal reporter gene activation after introduction of the wtT $\beta$ RII. ES cells stably transfected with cDNA encoding the wtT $\beta$ RII were transiently transfected with the truncated T $\beta$ RII and the 3TP-lux reporter construct in combination with LacZ. Cells were cultured for 18 h in the presence or absence of 10 ng/ml TGF $\beta$ 1 prior to determining luciferase activity. Results are shown from a representative experiment that was repeated twice with similar results. **C** Induction of the 3TP-lux reporter by

strate that these clones were now able to bind ligand,  $^{125}\text{I}$ -TGF $\beta$ 1 was chemically cross-linked to the transfected cells. Precipitation of receptor complexes with anti-tag or by wheat germ agarose (WGA) absorption showed ligand binding to the T $\beta$ RII protein at the cell surface (Fig. 2c). Quantification of cross-linked  $^{125}\text{I}$ -TGF $\beta$ 1 prior to precipitation showed that ES cells expressing wtT $\beta$ RII and  $\Delta$ T $\beta$ RII bound approximately 50% and 75% of the TGF $\beta$ 1 bound to endogenous receptors on EPI-7 control cells, respectively (data not shown). The TGF $\beta$  responsiveness of a 3TP-lux reporter construct, widely used to demonstrate cellular responsiveness to TGF $\beta$  superfamily ligands [54], was also tested in these lines. In undifferentiated cells, introduction of the wtT $\beta$ RII resulted in a low, but significant (>2-fold) induction of the 3TP-lux reporter after TGF $\beta$  addition, compared with negligible induction in ES cells containing the empty vector or  $\Delta$ T $\beta$ RII (Fig. 3a). Since the higher basal levels of reporter in the ES cells containing wtT $\beta$ RII may reflect their (autocrine) response to endogenous TGF $\beta$  [43], we transiently transfected the  $\Delta$ T $\beta$ RII cDNA into the wtT $\beta$ RII cell line. The expression of the  $\Delta$ T $\beta$ RII completely inhibited the induction of the reporter prior to TGF $\beta$  addition (Fig. 3b), confirming that the reporter had responded to endogenously produced TGF $\beta$ . After differentiation in monolayer induced by LIF removal, the 3TP-lux response was significantly lower in ES cells expressing  $\Delta$ T $\beta$ RII, compared to control ES cells now expressing the endogenous T $\beta$ RII, or to the cell lines expressing wt T $\beta$ RII (Fig. 3a). To test whether  $\Delta$ T $\beta$ RII blocked the response of the reporter construct to other members of the TGF $\beta$  superfamily, activin A and BMP7, were also added; neither induced a change in luciferase activity (Fig. 3c) making it most likely that we interfered only with the TGF $\beta$  signalling pathway.

#### Exogenous wtT $\beta$ RII and $\Delta$ T $\beta$ RII affect differentiation in vivo

Since ES cells incorporate efficiently into host embryos, we used chimaeras as a vehicle to express wtT $\beta$ RII and  $\Delta$ T $\beta$ RII ectopically. Monitoring the effect of aberrant TGF $\beta$  signalling on differentiation in vivo has the advantage that the different cell types can be identified on the basis of their position in the developing conceptus. Clumps of 10–15 ES cells expressing empty vector, wt T $\beta$ RII or  $\Delta$ T $\beta$ RII were aggregated with morula-stage host embryos from ROSA- $\beta$ -geo-26, a transgenic line expressing LacZ ubiquitously. After overnight culture, the resulting blastocysts were transferred to pseudopreg-

other members of the TGF $\beta$  superfamily. Stable transfected ES clones were transiently transfected with the 3TP-lux reporter construct in combination with a LacZ reporter construct. Cells were cultured for 18 h in the presence or absence of 10 ng/ml TGF $\beta$ 1, activin A, BMP7 or a combination. Induction of luciferase was measured and corrected for LacZ activity. The experiment was repeated three times with the same results



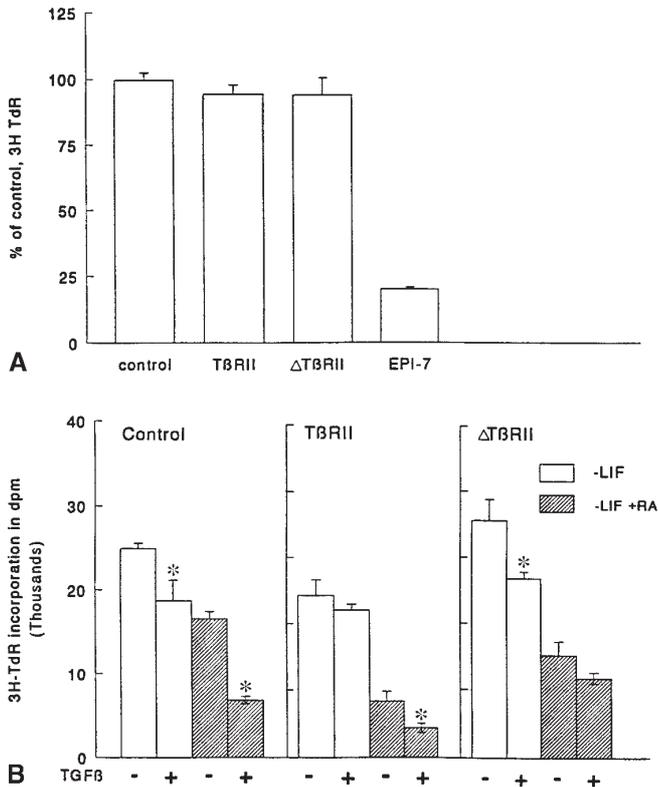
**Fig. 4A–C** In vivo differentiation of ES cell lines in chimaeric embryos. Chimaeric embryos were collected at 9.5 dpc and photographed before fixation. Conceptus resulting from morula aggregation with control (A), wt T $\beta$ RII (B) or  $\Delta$ T $\beta$ RII (C) ES cells are shown, respectively. Arrowheads indicate the blebbing of the yolk sac. Scale bar is 1 mm

nant females. The pregnancy was allowed to proceed to the equivalent of 9.5 dpc, the conceptuses were recobred, stained for LacZ and analysed. For all ES cell lines, the resulting conceptuses were either entirely blue, i.e., no ES cells had been incorporated, or the embryonic part

was unstained with the exception of some blue cells in the gut, indicating that the embryos were entirely ES derived. When the embryo remained unstained, the mesodermal component of the yolk sac was also unstained whereas the visceral endoderm of the yolk sac was always intense blue, demonstrating that the mesodermal cells were ES-derived and the endodermal component of this tissue was supplied by the host. Figure 4a shows the yolk sac of a control chimaera formed from ES cells expressing empty vector. The yolk sac is indistinguishable from a normal yolk sac at the same stage (not shown), consisting of a smooth visceral endoderm layer with an underlying network of blood-filled vessels, the endothelial cells, and primitive haematopoietic cells, both derivatives of the extraembryonic mesoderm. By contrast, in chimaeric embryos derived from  $\Delta$ T $\beta$ RII ES cells, haematopoietic cells, evident in red, are clearly present in the yolk sac, but they are contained in a vascular plexus and not within a network of large endothelial vessels (Fig. 4c). Even more strikingly, chimaeras formed from ES cells expressing wtT $\beta$ RII not only appear to lack vessels but also have virtually no red blood cells localized at the surface of the yolk sac (Fig. 4b). The yolk sacs containing ES cells with the wtT $\beta$ RII were also blebbed (arrowhead), suggesting that the endodermal and mesodermal layers had separated, or that the mesodermal layer had failed to expand at the same rate as the endodermal component and was therefore too small. Approximately 40 chimaeric embryos from two independent ES clones were analysed for each construct. All showed yolk sac defects similar to those illustrated with the exception that occasionally haematopoietic cells were present in the wtT $\beta$ RII ES chimaeras.

#### Biological responses of wtT $\beta$ RI and $\Delta$ T $\beta$ RII ES lines to TGF $\beta$ in vitro

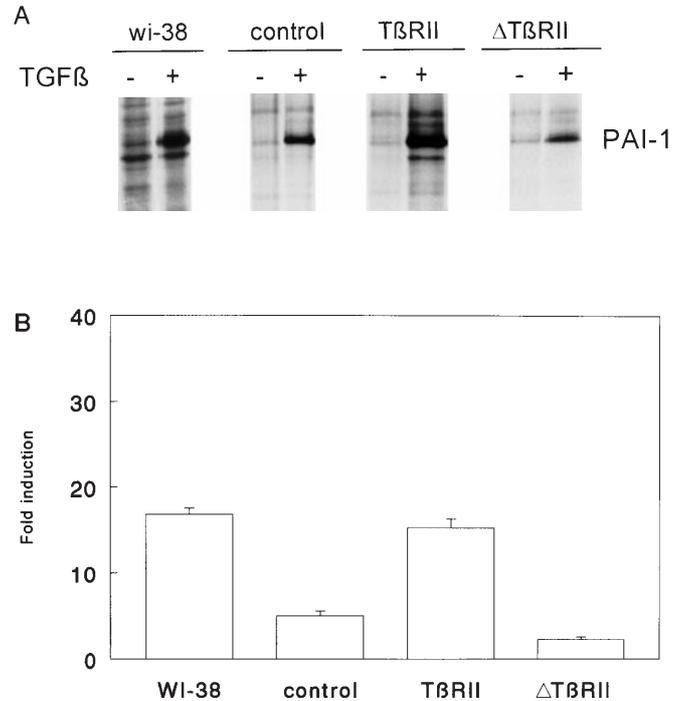
Since introduction of both cell lines into chimaeras resulted in a defect in yolk sac development, we used the ES lines expressing exogenous receptors for biochemical assays to assess their responses to TGF $\beta$  in vitro, since many of these assays require much larger cell numbers than could be obtained by isolating the mesodermal component of the yolk sac. Since TGF $\beta$  affects cell proliferation and ECM composition in many cell types, we first used two assays to search for differences in response of the transfected lines to TGF $\beta$ ; these were (i) DNA synthesis determined by  $^3$ H-thymidine incorporation [50] and (ii) synthesis of PAI-1 protein [12]. In none of the undifferentiated ES cell lines (wt T $\beta$ RII,  $\Delta$ T $\beta$ RII or control with empty vector; Fig. 5a), did introduction of the type II receptor result in growth inhibition upon TGF $\beta$  treatment, although EPI-7 cells, with similar generation times, are 80% growth-inhibited under identical conditions. After 7 days of differentiation by LIF deprivation alone, the ectopically expressed wtT $\beta$ RII mediated growth inhibition by endogenous ligand we know to be secreted by the differentiated ES cells [43], and evident



**Fig. 5A, B** TGF $\beta$ -induced growth effects in the stably transfected ES lines. **A** Undifferentiated ES cells were treated for 64 h with 10 ng TGF $\beta$ 1. During the last 2 h, the cell cultures were labelled with  $^3\text{H}$ -TdR to monitor DNA synthesis.  $^3\text{H}$ -TdR incorporation is expressed as a percentage of the untreated cells. EPI-7 cells were used as a positive control for the TGF $\beta$  inhibitory effect. The mean of triplicate samples is shown in the figure. **B** ES cells were differentiated by removal of LIF with or without addition of  $10^{-6}$  M RA and treated with TGF $\beta$ 1 at day 7 of differentiation. During the last 2 h, the cultures were labelled with  $^3\text{H}$ -TdR.  $^3\text{H}$ -TdR incorporation in TGF $\beta$ 1-treated and untreated cells was measured. The figure shows the mean  $^3\text{H}$ -TdR incorporation of triplicate samples. Bars marked with asterisks differ significantly from their matched control without TGF $\beta$ 1 ( $P < 0.01$ )

here by the lower basal levels of  $^3\text{H}$ -TdR incorporation (Fig. 5b) and the data of Fig. 3b. When these cultures were induced to differentiate by LIF deprivation in the presence of RA, addition of purified TGF $\beta$ 1 resulted in greater decreases in DNA synthesis in both the wtTβRII and control ES cells (Fig. 5b). In the differentiated ΔTβRII cell cultures, the basal level of  $^3\text{H}$ -TdR per well was higher than in control and wtTβRII lines, and the addition of purified TGF $\beta$ 1 to these cultures had hardly any effect. These results show that ES cells exhibit a greater growth inhibitory response to TGF $\beta$  after differentiation induced by LIF deprivation together with RA addition, and that this greater response is abolished by ΔTβRII.

To determine whether PAI-1 synthesis was also TGF $\beta$ -responsive in a differentiation-dependent manner, both undifferentiated and differentiated ES cells were treated with TGF $\beta$ 1 and labelled with  $^{35}\text{S}$  methionine and cysteine. WI-38 fibroblast cells were used as a con-



**Fig. 6A, B** Plasminogen activator inhibitor-1 (PAI-1) synthesis upon TGF $\beta$  treatment in stably transfected ES cell lines. Monolayers cultures of ES cells, stably transfected with the empty vector (control), TβRII, or ΔTβRII, were allowed to differentiate for 7 days in the presence of  $10^{-6}$  M RA and the absence of LIF. On day 8, the medium was replaced with methionine-free medium and the cells were treated with TGF $\beta$ 1 (10 ng/ml) for 6 h.  $^{35}\text{S}$  Trans-label was added to the cultures for the last 2 h. PAI-1 deposition on the 6-well plate was analysed by SDS-page and autoradiography **A** and quantified using a PhosphorImager **B**

rol since they have been shown by others to respond to TGF $\beta$  with large increased of PAI-1 synthesis [12]. No differences in PAI-1 synthesis were observed in the undifferentiated cells or in cells induced to differentiate by LIF deprivation in response to TGF $\beta$  (data not shown). PAI-1 synthesis was significantly increased by TGF $\beta$ 1 when RA was present during LIF deprivation both in control ES cells (5-fold) and in cells expressing excess TβRII (14-fold). This increase was only 2-fold in the cells expressing the ΔTβRII (Fig. 6). WI-38 cells were stimulated 17-fold by TGF $\beta$ 1 under similar conditions.

#### Morphology and marker expression by differentiated ES cell lines in vitro

Since the phenotype observed in vivo could have been the result of defective differentiation of extraembryonic mesoderm, we compared the differentiation pattern of wtTβRII and ΔTβRII ES cells with control ES cells. Differentiation to a wider spectrum of cell types than observed in monolayer culture can be induced by allowing the cells to form embryoid bodies and reattach to a substrate [20]. Over 14 days, many cell types can thus be

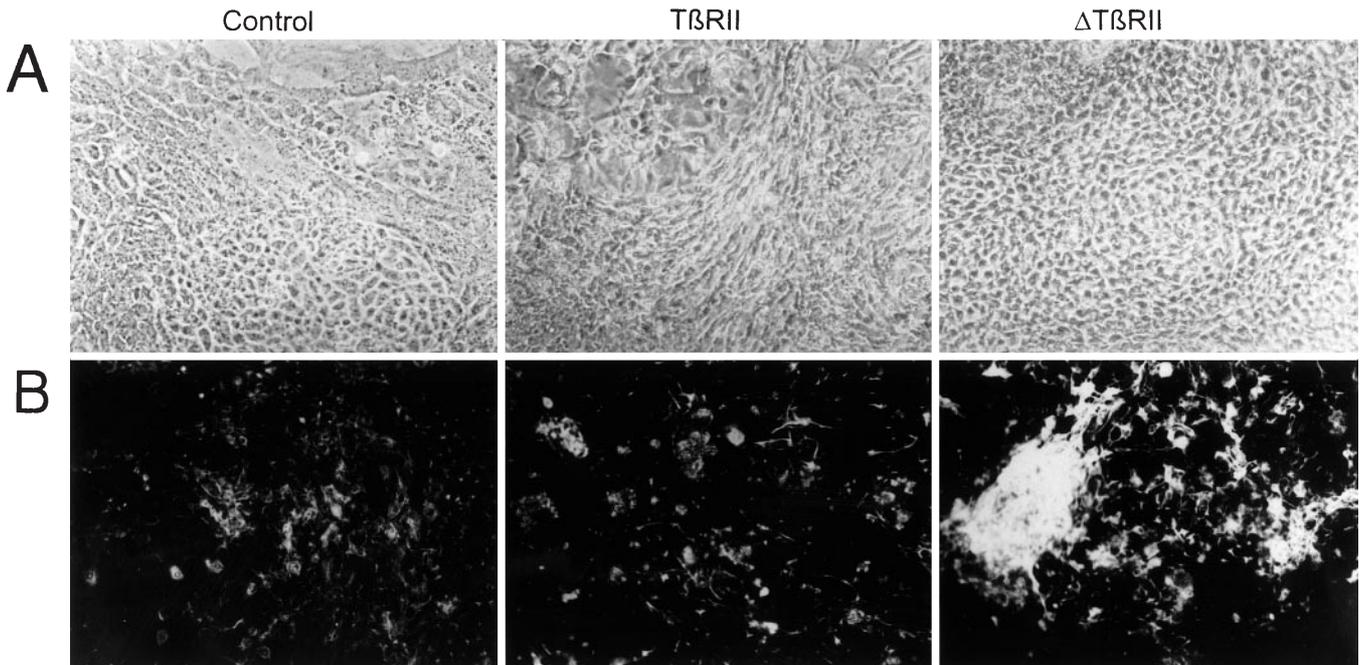
**Table 1** Properties of stably transfected embryonic stem (ES) cell lines; RA, retinoic acid; T $\beta$ RII, type-II binding receptors for transforming growth factor beta;  $\Delta$ T $\beta$ RII, dominant negative (truncated) T $\beta$ RII

Cell line	Embryoid bodies <sup>a</sup>	Monolayer <sup>a</sup>		Brach T <sup>b</sup>		MF-20 <sup>b</sup>		TROMA-1 <sup>b</sup>		Thrombomodulin <sup>b</sup>	
		-RA	+RA	-RA	+RA	-RA	+RA	-RA	+RA	-RA	+RA
Control	ect/end/mes	end/mes	end/mes	++	-	-	+	-	+	-	-
T $\beta$ R1	ect/end/mes	end/mes	end/mes	+++	-	-	+	-	+	-	-
$\Delta$ T $\beta$ RII	ect/end/mes	end/mes	end	+	-	-	-	-	+++	-	+

<sup>a</sup> Scored for morphology (ect ectoderm, end endoderm, mes mesoderm).

<sup>b</sup> -, +, ++, +++ indicates the proportion of cells in which fluorescence was detected in a monolayer culture without leukaemia in-

hibitory factor (LIF) in the absence (-RA) or presence of RA (+RA). (-, <1%; +, 5%–15%; ++, 15%–50%; +++, >50%)



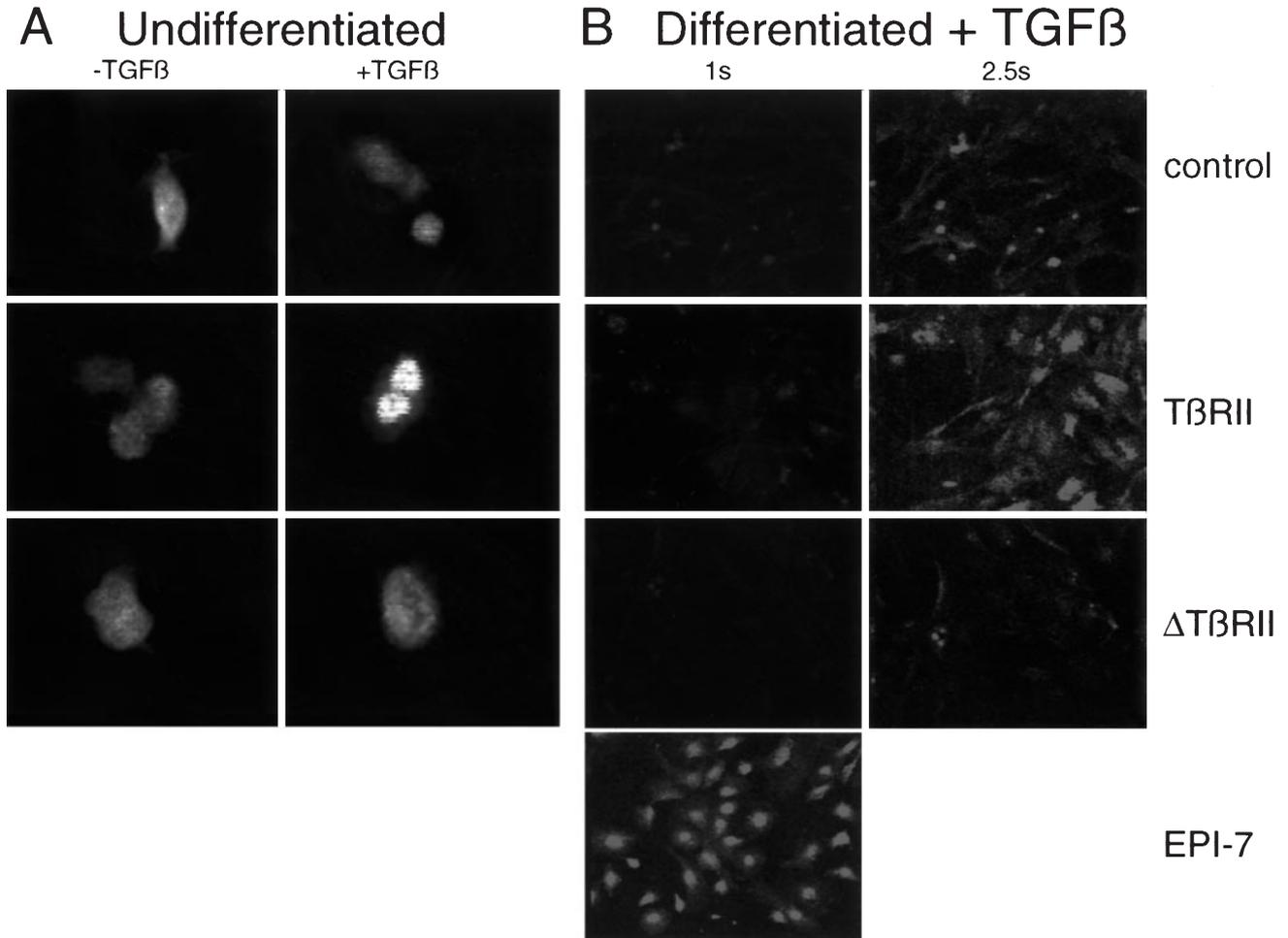
**Fig. 7A, B** Differentiation capacity of stably transfected ES cell lines. **A** Morphology of monolayer cultures of ES cell lines, differentiated for 8 days in the presence of  $10^{-6}$  M RA. **B** Staining of monolayer cultures of ES cell lines, differentiated in the presence of  $10^{-6}$  M RA for 8 days, with a marker for endodermal cells. Cells were fixed and stained with an *TROMA-1* antibody recognising endodermal cells

distinguished on the basis of their morphology; these include beating cardiac muscle and various neuronal cell types [42]. The morphology of differentiated cells emerging from embryoid bodies of wt T $\beta$ RII,  $\Delta$ T $\beta$ RII or control cell lines were essentially identical (Table 1). Similarly, in monolayer culture, morphological differentiation in response to LIF deprivation was in all cases identical, although the proportion of Brachyury T-expressing (mesodermal) cells was significantly lower in the  $\Delta$ T $\beta$ RII ES cells (Table 1). However, upon addition of RA and simultaneous withdrawal of LIF the  $\Delta$ T $\beta$ RII ES cells responded very differently from the wtT $\beta$ RII and control cells. After 7 days the wtT $\beta$ RII expressing and control ES cells had formed a mixture of endoderm- and mesoderm-like cells, while  $\Delta$ T $\beta$ RII ES cells differenti-

ated primarily into endoderm (Fig. 7a) with the differentiation to MF-20 expressing (mesodermal) cells apparently blocked (Table 1). This observation was confirmed using TROMA-1, a general marker for endoderm cells (Fig. 7b) and thrombomodulin, a specific marker for parietal endoderm [52]. Differentiation characteristics of the ES cell lines are summarised in Table 1.

#### Downstream components of the TGF $\beta$ signalling cascade

Although some of the early responses to TGF $\beta$  were as would be expected for cell lines expressing (mutant) receptors, the biological responses in culture were either entirely absent (undifferentiated cells) or were only observed during differentiation of the cells, suggesting that ES cells lack one or more crucial components of the TGF $\beta$  signal pathway downstream of the receptors. In an attempt to identify these possible missing links, we determined the effects of TGF $\beta$  on the more immediate downstream signalling components that have recently been described, namely the Smads. Of the nine members of this family that have now been described, Smad2,

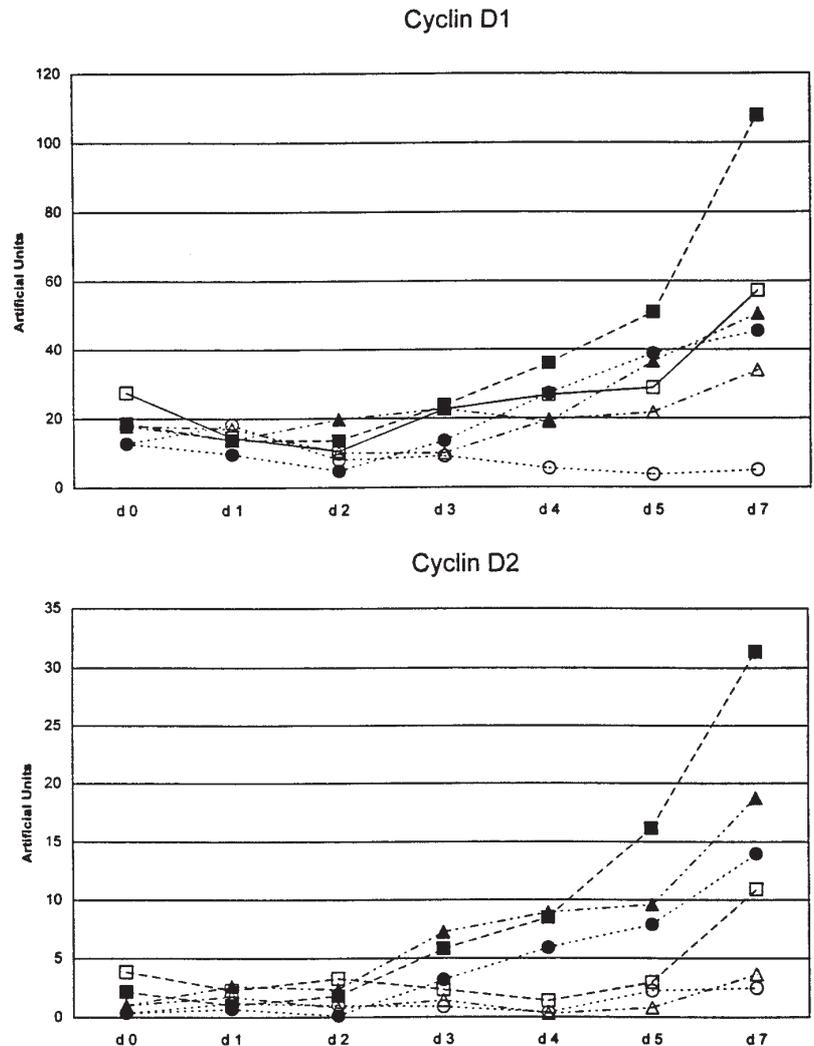


**Fig. 8A, B** Smad2 translocation in stably transfected ES cell lines. **A** Undifferentiated ES cell lines were treated with TGFβ1 for 1 h, fixed and stained with an antibody against smad2. Exposure time was 5 s, magnification  $\times 100$ . **B** ES cells were differentiated for 8 days in the presence of  $10^{-6}$  M RA, treated with TGFβ1 for 1 h, fixed and stained with an antibody against smad2. The figure shows two exposure times to indicate the difference in levels of fluorescence in comparison with EPI-7, a cell line sensitive to TGFβ.  $\times 25$

Smad3 and Smad4 have been shown to be required for TGFβ signalling [31]. Several tumour cell lines have been described that remain insensitive to TGFβ despite the presence of TGFβ type I and II receptors on the cell surface; one or more of the Smad proteins were shown in these cells to be mutated or missing [7, 9]. We assessed the different cell lines for the presence of all three Smad proteins, determined whether their expression levels were altered during differentiation and, if they were present, whether TGFβ1 induced nuclear translocation as has been described for other TGFβ-responsive cells. Smad2, Smad3 and Smad4 mRNA was detected by reverse transcriptase-PCR (RT-PCR) in controls and in ES cells expressing wt TβRII or ΔTβRII (data not shown). Northern blot analysis of total RNA from EPI-7 cells showed expression of Smads 2, 3 and 4, but only Smad4 was detected in 20 μg total RNA from ES cells [7], suggesting

that the RNA expression levels of Smads 2 and 3 are low in ES cells. These differences in expression level were also evident at the protein level from immunofluorescence using α-Smad antibodies. While no signal enhancement was required to detect Smad 2 in EPI-7 cells using a confocal laser scanning microscope, 2.5 times enhancement was required to detect it in control and wtTβRII ES lines both before differentiation and after 7 days of differentiation in the presence of RA and the absence of LIF. In undifferentiated cells, Smad2 was not translocated to the nucleus except in the cell line expressing the wtTβRII, suggesting that in these cells at least there was an intact TGFβ signalling pathway up to and including Smad translocation to the nucleus (Fig. 8a). However, this was apparently not sufficient to result in a biological response, suggesting that besides the lack of a TβRII, the amounts of phosphorylated Smad protein might also be rate limiting in undifferentiated cells or that Smad targets in the nucleus are absent. In the differentiated cultures, Smad2 protein levels were highest in cells with a mesodermal phenotype; only in these cells was nuclear translocation of Smad2 evident after TGFβ addition (Fig. 8b).

**Fig. 9** Cyclin D RNA expression during the differentiation of stably transfected ES cell lines. Northern blot analysis of 20  $\mu$ g total RNA for mRNAs encoding cyclin D1 and D2 was performed during differentiation of ES cell lines in the absence of LIF and with or without addition of  $10^{-6}$  M RA. d0–d7 indicates the day when differentiated cells were harvested for analysis. Samples were corrected for loading difference using glyceraldehyde phosphate dehydrogenase (GAPDH) and quantified using a Phosphor Imager. Fold induction compared with undifferentiated cells is shown in the figure. ■:  $\Delta$ T $\beta$ RII + RA; □:  $\Delta$ T $\beta$ RII; ▲: control + RA; △: control; ●: T $\beta$ RII + RA; ○: T $\beta$ RII



### Cyclin D and ES cells

An unusual property of ES cells is their notable lack of the cyclin D group before differentiation [40, 41]. This is likely to render them insensitive to exogenous control of the cell cycle and to be involved in their limited capacity to respond to growth inhibitory effects of TGF $\beta$ . The lack of cyclins in ES cells reflects a similar property of their cellular counterparts *in vivo*, namely the primitive ectoderm, which we have recently shown lack cyclins D prior to gastrulation [53]. We therefore compared expression of cyclins D1 and D2 in the three ES cell lines by Northern blot analysis and the kinetics of their upregulation during differentiation in monolayer cultures induced by LIF deprivation in the presence or absence of RA. Figure 9 shows cyclins D1 and D2 are hardly expressed in any of the cell lines prior to differentiation, that both are slightly upregulated by LIF deprivation and that RA significantly enhances this upregulation. Upregulation of cyclins D1 and D2 correlates with the ability of TGF $\beta$  to inhibit growth of ES cells under different conditions (Fig. 5b). The expression of wtT $\beta$ RII or  $\Delta$ T $\beta$ RII does not alter the expression of cyclin D mRNA

as such, although in the  $\Delta$ T $\beta$ RII ES cells, RA increases cyclin D expression much more than in the control and wtT $\beta$ RII ES cells.

### Discussion

In the present study we have addressed the possible function of TGF $\beta$ s in early embryonic differentiation. We have shown that ectopic expression of either a full length type II receptor for TGF $\beta$  or a receptor defective in its capacity to phosphorylate type I receptors causes defects in the mesodermal component of the visceral yolk sac. In both cases, the mesodermal component formed, but introduction of the wtT $\beta$ RII appeared to block the formation of red blood cells and vascularization, while the  $\Delta$ T $\beta$ RII appeared to interfere with endothelial cell differentiation, in a manner similar to that described for TGF $\beta$ 1  $-/-$  embryos [8] and the T $\beta$ RII  $-/-$  embryos [32], as might be expected when TGF $\beta$  signalling may be reduced compared with that in normal development. Together these results show that this ligand and its receptor are essential for yolk sac development. Moreover, they

demonstrate that abnormalities in yolk sac development arise whether TGF $\beta$  signalling is perturbed in a positive or negative manner, suggesting that thresholds of TGF $\beta$  activity are essential to precise regulation of yolk sac vasculature at this time of development. Detailed analysis of this yolk sac phenotype will be the subject of future study, but in the present study we have analysed TGF $\beta$  signalling during differentiation of the ES cell lines that gave rise to the phenotype in an attempt to gain insight into which of a myriad of possible TGF $\beta$  responses had been altered in the (mutant) cell lines. Most of these (biochemical) assays require more cells than could be easily isolated from (defective) yolk sacs, so studies with ES cells in culture could provide useful information for interpreting the phenotype.

We have shown that in contrast to many tumour cell lines where T $\beta$ R $\beta$ II is mutated or absent [14, 46, 51], introduction of this receptor into ES cells, where it is also absent, did not result in ligand-induced biological effects despite (1) the presence of a type I receptor, (2) ligand binding, (3) a TGF $\beta$ -responsive 3TP-lux reporter, and (4) TGF $\beta$ -mediated nuclear translocation of a downstream signalling molecule (Smad2). Only after prolonged RA treatment in the absence of LIF, when a mixture of mesenchymal and endoderm cell types is formed and the endogenous binding receptor is also expressed, did ES cells expressing ectopic receptors show an enhanced production of PAI-1 protein in response to TGF $\beta$  compared to control lines. This enhancement was reduced in cell lines expressing a mutated binding receptor lacking the cytoplasmic serine/threonine kinase domain ( $\Delta$ T $\beta$ R $\beta$ II). When exposed to RA, these cells expressing the mutated receptor were also limited in their differentiation capacity and formed predominantly endoderm, with little evidence of the mesenchymal cell types formed by control cells or those with wtT $\beta$ R $\beta$ II under the same conditions. Differentiation to mesodermal cell types therefore appeared to require an intact TGF $\beta$  ligand/receptor complex. We have recently shown that TGF $\beta$  inhibits the outgrowth of parietal endoderm from isolated inner cell masses in culture [39], a finding compatible with the results shown here. Defective differentiation, particularly to (extraembryonic) mesoderm derivatives, could contribute to the yolk sac phenotype observed with the  $\Delta$ T $\beta$ R $\beta$ II.

The expression of Smad2, Smad3 and Smad4 has been shown to be required for a biological response to TGF $\beta$  [31], but Smad2 and Smad4 alone may be sufficient to result in an optimal response of the 3TP-lux reporter [56]. Although we could detect Smad2, 3 and 4 transcripts by RT-PCR in all of the undifferentiated ES cell lines, protein levels were much lower than in a highly TGF $\beta$  sensitive control cell line, EPI-7. We propose that although present in ES cells, Smad2 and 3 levels are insufficient to result in changes in ECM protein (PAI-1) production unless the cells are induced to differentiate by RA addition and LIF withdrawal. We indeed observe under these conditions that Smad2 and 3 protein expression significantly increases in at least some differentiated derivatives. It will therefore be of importance in the future

to assess protein levels for Smads 2 and 3 in particular in the developing (ch; maeric) yolk sac to identify stages at which cells might become TGF $\beta$ -sensitive.

Only after prolonged LIF deprivation in the presence of RA did we observe substantial induction of growth inhibition by TGF $\beta$ . ES cells have been described previously as lacking cyclin D expression as well as CDK4-associated kinase activity [40, 41]. Similarly, ectoderm of the embryo proper also lacks cyclins D until the onset of gastrulation [53]. Cyclins D are the positive regulatory subunits of the cyclin-dependent kinases, so that ES cells may lack the CDK4-associated control of transit through G1 until they are induced to differentiate. Cyclin D up-regulation then reflects gain of proper control of the G1 phase, which could confer responsiveness to mitogenic or antimitogenic signals, and thereby regulate growth and differentiation. We have shown that cyclin D expression is restored by LIF deprivation and that this is further enhanced by RA addition. The TGF $\beta$  growth response was thus highly correlated with the levels of cyclins D1 and D2, which should possibly reach some threshold before becoming effective and allowing ES cells to regain exogenous control of their cell cycle. Since the lack of TGF $\beta$  growth response in the  $\Delta$ T $\beta$ R $\beta$ II cell line correlates with the absence of Smad2 translocation in this cell line, the difference in response between the T $\beta$ R $\beta$ II and  $\Delta$ T $\beta$ R $\beta$ II cells could be due to the difference in differentiation capacity, resulting in a lack of mesodermal cell types in the RA-differentiated cultures of the  $\Delta$ T $\beta$ R $\beta$ II. The higher cyclin D mRNA levels in this cell line in response to RA may suggest that the endoderm cells are terminally differentiating and accumulating in the G1/G0-phase of the cell cycle. The pattern of cyclin D expression in the yolk sac may determine the contribution of TGF $\beta$ -induced effects on cell proliferation to the yolk sac phenotype. The results shown here may suggest that the effect of  $\Delta$ T $\beta$ R $\beta$ II is primarily on differentiation rather than on proliferation, whereas the full-length receptor primarily affects proliferation and the ECM, which in turn may affect the ability of the yolk sac to retain its normal architecture.

In summary, we have determined the differentiation characteristics of three totipotent ES cell lines, transfected with control DNA, wtT $\beta$ R $\beta$ II and  $\Delta$ T $\beta$ R $\beta$ II in vitro, and related these to aspects of the phenotype that ectopic expression of these genes causes in vivo. These in vitro studies have shown that undifferentiated ES cells or cells differentiated by LIF removal are essentially refractile to TGF $\beta$  action, whereas ES cells differentiated in the additional presence of RA become sensitive to TGF $\beta$ . We have made a start towards addressing the restriction in TGF $\beta$  responses in undifferentiated ES cells. Furthermore we have shown (by use of  $\Delta$ T $\beta$ R $\beta$ II) that the TGF $\beta$  signalling pathway is essential for differentiation of mesodermal cell types in response to RA in vitro.

**Acknowledgements** We are very grateful to Dr. Peter ten Dyke for providing Smad antibodies, experimental protocols, suggestions and advice. We are also grateful to Drs. B. Defize, A.J.M. van den Eijnden – van Raaij, and B. Roelen for critical reading of

the manuscript. This work was carried out within the graduate School of Developmental Biology, Utrecht, the Netherlands and was supported by grants from EMBO (A.Z.) and the European Science Foundation (ESF) Program in Development Biology (A.Z.).

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