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The type I serine/threonine kinase receptor ActRIA (ALK2) is required for gastrulation of the mouse embryo

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SUMMARY

ActRIA (or ALK2), one of the type I receptors of the transforming growth factor-β (TGF-β) superfamily, can bind both activin and bone morphogenetic proteins (BMPs) in conjunction with the activin and BMP type II receptors, respectively. In mice, ActRIA is expressed primarily in the extraembryonic visceral endoderm before gastrulation and later in both embryonic and extraembryonic cells during gastrulation. To elucidate its function in mouse development, we disrupted the transmembrane domain of ActRIA by gene targeting. We showed that embryos homozygous for the mutation were arrested at the early gastrulation stage, displaying abnormal visceral endoderm morphology and severe disruption of mesoderm formation. To determine in which germ layer ActRIA functions during gastrulation, we performed reciprocal chimera analyses. (1) Homozygous mutant ES cells injected into wild-type blastocysts were able to contribute to all three definitive germ layers in chimeric embryos. However, a high contribution of mutant ES cells in chimeras disrupted normal development at the early somite stage. (2) Consistent with ActRIA expression in the extraembryonic cells, wild-type ES cells failed to rescue the gastrulation defect in chimeras in which the extraembryonic ectoderm and visceral endoderm were derived from homozygous mutant blastocysts. Furthermore, expression of HNF4, a key visceral endoderm-specific transcription regulatory factor, was significantly reduced in the mutant embryos. Together, our results indicate that ActRIA in extraembryonic cells plays a major role in early gastrulation, whereas ActRIA function is also required in embryonic tissues during later development in mice.

Key words: Activin, BMP, Serine/threonine kinase receptor, Gene targeting, Mesoderm formation, Gastrulation, Mouse

INTRODUCTION

Gastrulation of the mouse embryo involves extensive cell proliferation, differentiation and movement in the initially bilaminar embryonic egg cylinder, which leads to the formation of the interstitial mesoderm layer and the establishment of the embryonic body axes (Hogan et al., 1994; Tam and Behringer, 1997). In mice, gastrulation initiates at about 6.5 days post coitum (E6.5) with the formation of the primitive streak at the prospective posterior region. As gastrulation proceeds, the primitive streak extends distally, allowing neighboring primitive ectoderm (epiblast) cells to ingress through the streak to form the mesoderm layer. Although the formation of the primitive streak and various types of mesodermal tissues has been well characterized morphologically, the underlying cellular and molecular mechanisms are still poorly understood.

Studies of mesoderm induction in *Xenopus* embryos have identified several members of the TGF-β family including activins, Vg-1, BMP4 and nodal-related proteins (Xnr), as potent mesoderm-inducing factors (reviewed by Smith, 1993; Harland, 1994; Slack, 1994; Kessler and Melton, 1995; Jones et al., 1995). While BMP4 induces the ventral type of mesoderm, activin, Vg1 and Xnr (Xnr-1, Xnr-2 and Xnr-4) can induce dorsal mesoderm. Moreover, both activin and Xnr can specify different types of mesoderm in a dose-dependent manner, by induction of ventral mesoderm at low concentrations and dorsal mesoderm at high concentrations (Green and Smith, 1990; Green et al., 1992; Jones et al., 1995). Coordination of the action of different signals is probably crucial for the formation and patterning of various mesodermal tissues during gastrulation.

Genetic studies of the function of TGF-β family factors in mammalian development by gene targeting in mice have demonstrated that zygotic activins A and B are not essential for gastrulation, since embryos lacking both activin β_A and β_B genes develop to term with no gross defects in mesoderm formation and patterning (Matzuk et al., 1995). BMP4 and

nodal, however, are essential for early mouse development (Winnier et al., 1995; Zhou et al., 1993; Conlon et al., 1994), consistent with their functions in *Xenopus* embryos. BMP4 is required for epiblast cell proliferation at the egg cylinder stage, and for the formation of extraembryonic mesoderm and posterior structures, whereas nodal function has been implicated in primitive streak formation (Conlon et al., 1994).

TGF-β family members signal through membrane-bound heteromeric complexes of type I and type II serine/threonine kinase receptors and the downstream Smad family proteins (reviewed by Massagué, 1998). Upon binding to a ligand, the type II receptors phosphorylate and activate associated type I receptors, which in turn transduce the signal by phosphorylating pathway-specific Smad proteins such as Smad1 or Smad2. The phosphorylated Smad proteins are then translocated into the nucleus in association with Smad4, a common signal mediator of the TGF-β family signaling pathways, and activate transcription of downstream target genes. So far about a dozen of type I and type II receptors and at least ten members of the Smad family have been identified. However, the specificity of ligand-receptor-Smad cascades in mammalian development remains poorly defined.

ActRIA (also known as ALK-2, R1 or Tsk-7L) is a type I receptor that can bind either activin or BMP2/4 in conjunction with the corresponding type II receptors (He et al., 1993, Attisano et al., 1993; ten Dijke et al., 1993, 1994). ActRIA is widely expressed in midgestation embryos and in various tissues including brain, heart and lung in adult mice (Verschueren et al., 1995; He et al., 1993). ActRIA may also mediate the signaling of Mullerian inhibiting substance (MIS), since its transcripts colocalize with the type II receptors for MIS in the fetal Mullerian duct (Teixeira et al., 1996). Recent studies in *Xenopus* have shown that a constitutively active form of ALK2 induces the expression of ventral mesodermal markers, reminiscent of BMP2/4 activities in mesoderm formation, while expression of ALK4, an activin-specific type I receptor, is capable of transducing the activin signal to induce dorsal mesoderm (Chang et al., 1997; Armes and Smith, 1997). Moreover, overexpression of ALK2 appears to antagonize the dorsalizing effects of ALK4 (Armes and Smith, 1997). Recently, we have demonstrated that the ActRIB is required for the formation of an organized egg cylinder and the primitive streak during early mouse development (Gu et al., 1998).

To define the function of ActRIA in mammalian development, we disrupted the mouse *ActRIA* gene by homologous recombination. Our analysis of both embryos and ES cell lines homozygous for the mutation indicates that ActRIA functions in both embryonic and extraembryonic tissues to regulate mouse embryonic development.

MATERIALS AND METHODS

Histology and in situ hybridization

Decidua or embryos were fixed in Bouin's fixative, dehydrated and embedded in paraffin as described by Kaufman (1992). Serial sections were cut at 7 μm and stained with hematoxylin and eosin.

For detecting ActRIA transcripts, two ActRIA probes, one for the extracellular domain and the other for the 3′-untranslated region, were used in in situ hybridization analysis. The extracellular domain fragment spanning the region between nucleotides 504 and 742 (mustsk 7L, accession L 15436) was PCR amplified, inserted into pGEM-T and transcribed as antisense RNA from T7 promoter. The 3′-untranslated region between nucleotides 2034 and 2330 in a *Bes*YI-*Age*I fragment was inserted into pGEM-4Z and transcribed as antisense RNA from the T7 promoter. In situ hybridization with sectioned embryos was performed basically as described by Wilkinson (1992). Slides were exposed for 18-20 days and counterstained with hematoxylin.

Whole-mount in situ hybridization with digoxigenin-labeled or fluorescein-labeled antisense RNA probes for *Brachyury* (*T*) (Wilkinson et al., 1990), *Hesx-1* (Thomas and Beddington, 1996), *Bmp4* (Winnier et al., 1995) and *HNF4* (Duncan et al., 1994) was performed as described (Wilkinson, 1992). For double in situ hybridization, both digoxigenin- and fluorescein-labeled probes were used simultaneously. Alkaline phosphatase (AP)-conjugated anti-digoxigenin or anti-fluorescein antibody (Boehringer Mannheim) were used for enzyme reactions. After the first color reaction using NBT/BCIP, anti-digoxigenin antibody was heat-inactivated and replaced by anti-fluorescein antibody. The second color reaction was done using INT/BCIP (Boehringer Mannheim).

Immunofluorescent staining

An antibody (875) was raised against a KLH-conjugated synthetic rat ActRIA peptide (amino acids 109-121, T-K-G-K-S-F-P-G-S-Q-N-F-C, which differs from the mouse sequence by an S-to-T substitution at position 117. This unique sequence, which differs from other members of the type I receptor family, was selected from the extracellular region near the transmembrane domain because of its favorable antigenecity profile analyzed by MAC Vector 5.0 (Campbell, CA). The antibodies were raised in chickens, and the IgY fraction purified with Eggstract (Promega, Madison, WI). The specificity of the antibody was documented by immunohistochemical localization of the antibody to the Mullerian duct of the 15 day rat embryo and colocalization with antibodies to the MIS type II receptor (Masiakos et al., unpublished data). In addition, western analysis of cell lysates from MIS responsive cells showed bands of appropriate molecular weight for the type I receptor. Embryos at E6.5, E7.0 or E7.5 were fixed with 4% paraformaldehyde inside decidua for 6 hours or overnight at 4°C, dehydrated, embedded in paraffin and cut at 7 μm thickness. Immunostaining was performed according to the protocol described by Hogan et al. (1994). The primary antibodies were used at a dilution of 1:200, while FITC-conjugated rabbit anti-chicken IgG (1:500) (from ICN) was used as the secondary antibody. The preimmune serum was tested for background staining.

Construction of the targeting vector

Mouse genomic DNA for the *ActRIA* gene was obtained by screening a 129/Sv genomic library (Stratagene, Lambda FIX II) with rat ActRIA cDNA probes (He et al., 1993). The DNA fragments from four isolated clones range in size from 13 kb to 18 kb. An 11.5 kb genomic DNA was subcloned into the *Xho*I site of the pGEM-11z vector in which the *Sal*I site was eliminated. A *Sal*I linker was inserted into the *Kpn*I site in exon 4, encoding the transmembrane domain. A PGK-neo-poly(A) cassette (the *Xho*I-*Sal*I fragment from pKJ-1) was then inserted into the *Sal*I site in the same transcriptional orientation as *ActRIA* to disrupt exon 4. The final targeting vector contained 7.5 kb and 4.0 kb genomic DNA 5′ and 3′ to the neo gene cassette, respectively (see Fig. 2A).

Generation of ActRIA-deficient mice and ES cell lines

The J1 ES cells were transfected with a linearized targeting vector by electroporation and selected in G418-containing medium as described (Li et al., 1992). The genotype of $G418^r$ clones was analyzed by Southern blot hybridization using a 0.6 kb *Xho*I-*Eco*RI genomic fragment as a probe (see Fig. 2A). One positive recombinant clone was obtained, from which chimeric mice were derived by microinjection of ES cells into blastocysts of BALB/c mice. Male

chimeric mice transmitting the mutant allele through their germline were bred to either 129/Sv females to establish an inbred strain or to BALB/c to obtain heterozygous F_1 progeny.

To establish *lacZ*-marked ActRIA-deficient ES cell lines, the Rosa-26 mice (Friedrich and Soriano 1991) which contain a *lacZ* transgene, were crossed to *ActRIA*$^{tm1/+}$ to obtain *ActRIA*$^{tm1/+}$ Rosa-26$^{+/-}$ offspring. Delayed blastocysts were produced by intercrosses of *ActRIA*$^{tm1/+}$ Rosa-26$^{+/-}$ mice and ES cell lines were isolated from cultured inner cell mass cells as described (Robertson 1987). The genotypes of isolated ES cell lines for both the *ActRIA* mutation and the Rosa-26 transgene were determined by Southern blot analysis (Fig. 2C) and by X-gal staining (data not shown).

Chimera analysis

One type of chimeric embryos were generated by microinjection of 10-15 *ActRIA*$^{tm1/tm1}$ mutant ES cells into wild-type blastocysts or aggregation of mutant ES cells with wild-type 8-cell-stage uncompacted morula (Nagy and Rossant, 1993). The second type of chimeras was generated by injecting about 15 wild-type R/S4 ES cells (Gu et al., 1998) into blastocysts produced by intercrosses of *ActRIA*$^{tm1/+}$ heterozygous mice. Injected embryos were then transferred into the uteri of pseudopregnant foster mothers for reimplantation. Usually, the reimplanted blastocysts are delayed in development by approximately one day. Embryos were dissected at different stages, fixed and stained with X-gal as described (Hogan et al., 1994). After X-gal staining, embryos were re-fixed in Bouin's solution or 4% paraformaldehyde, followed by dehydration, embedding and sectioning as described (Hogan et al., 1994). The ratio of the blue cells derived from injected ES cells to the non-blue cells derived from host blastocysts was estimated by examining serial sections of each embryo. The genotype of the host blastocysts was determined by Southern blot analysis of DNA isolated from the parietal yolk sac.

In vitro differentiation of ES cells

Wild-type and ActRIA-deficient ES cells were induced to differentiate by culturing ES cells in suspension as embryoid bodies (Robertson, 1987). For histological analysis, embryoid bodies were fixed in 4% paraformaldehyde for 3 hours at room temperature, dehydrated and embedded as described earlier. Samples were sectioned at 4 μm and stained with hematoxylin and eosin.

RESULTS

Expression of ActRIA in the early mouse embryo

Previous analysis of RNA prepared from individual germ layers by reverse transcription polymerase chain reaction (RT-PCR) has indicated that ActRIA is expressed in the visceral endoderm at E6.5 and, subsequently, in both visceral endoderm and mesoderm at E7.5 (Roelen et al., 1994). In this study, we analyzed the expression pattern of ActRIA in embryos from E6.5 to E8.5 by immunofluorescent staining and in situ hybridization. We found that, at E6.5 and E7.0, ActRIA protein was confined to the apical surface of the visceral endoderm, while the expression in other cells of the embryo was similar to the background (Fig. 1A,B). At E7.5, ActRIA expression was detected in extraembryonic ectoderm, chorion, amnion and allantois, in addition to the visceral endoderm (Fig. 1C). However, the amount of ActRIA protein was very low in the embryonic regions. In E8.5 embryos, ActRIA expression was detected in the head mesenchyme and in the heart (Fig. 1D,F). We also performed in situ hybridization and found that ActRIA transcripts were expressed at very low levels before gastrulation (data not shown). At E7.5, ActRIA transcripts

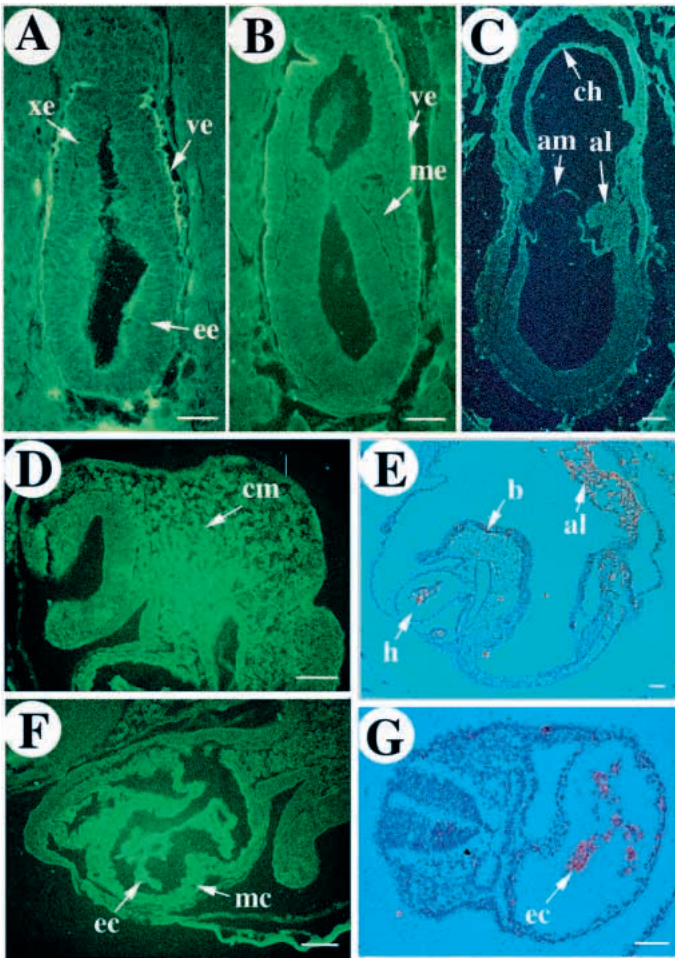

Fig. 1. Expression of ActRIA during gastrulation. Immunostaining with anti-ActRIA antibody on sections of embryos at E.6.5 (A), E7.0 (B), E7.5 (C) or E8.5 (D,F) or in situ hybridization with E8.5 embryos (E,G)**.** All embryo sections are sagittal or parasagittal, except G which is a transverse section in the heart region. In E6.5-E7.5 embryos, the apical surface of the visceral endoderm cells was positively stained. In E8.5 embryos, expression of ActRIA is detected in the heart, cephalic mesenchyme and allantois by both in situ hybridization and immunostaining. al, allantois; am, amnion; b, brain; cm. cephalic mesenchyme; ec, endocardium; ee, embryonic ectoderm; h, heart; mc, myocardium; me, mesoderm; pe, parietal endoderm; ve, visceral endoderm; xe, extraembryonic ectoderm. Bars, 50 μm.

were detected in the visceral endoderm, chorion, amnion, allantois, and embryonic ectoderm (data not shown). By E8.5, ActRIA transcripts were detected in the head mesenchyme, endocardium and extraembryonic tissues such as allantois (Fig. 1E,G), consistent with the immunostaining results.

Disruption of the *ActRIA* gene results in early embryonic lethality

To mutate the *ActRIA* gene in mouse embryonic stem (ES) cells, a targeting vector was constructed in which a neomycin-resistance gene cassette was inserted into the fourth exon encoding the transmembrane domain (Fig. 2A). The linearized targeting vector was transfected into ES cells by electroporation. Of 400 G418-resistant colonies screened by

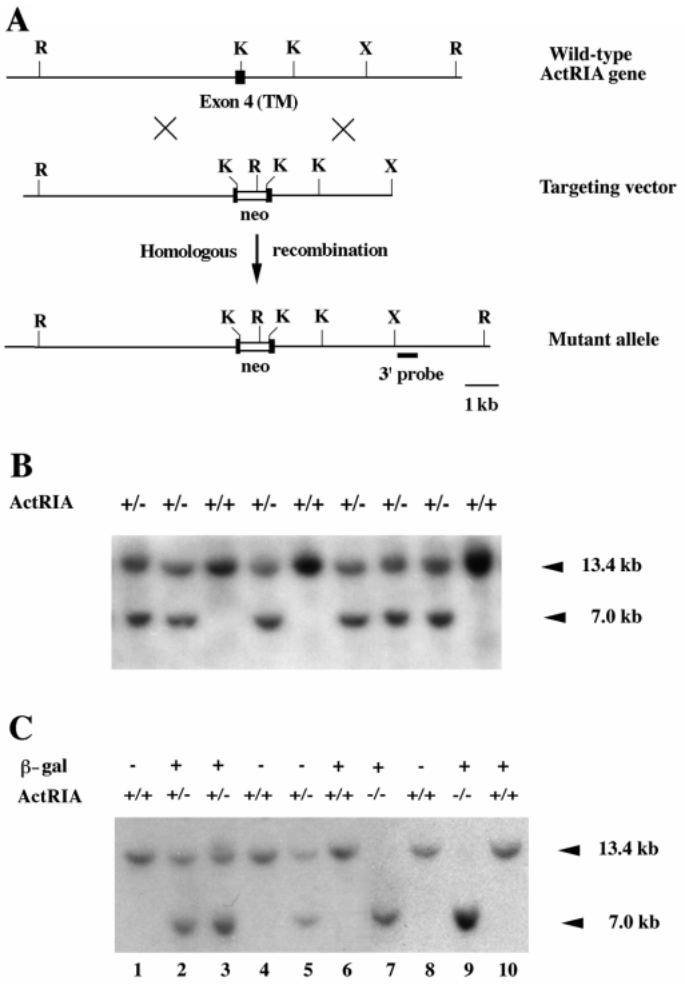

Fig. 2. Targeted disruption of the *ActRIA* gene. (A) (Top) The wild-type *ActRIA* locus with exon 4 (closed box) encoding the transmembrane domain (TM). (Middle) The targeting vector contains a neomycin resistant gene cassette (*neo*) insertion at the *Kpn*I site within exon 4. (Bottom) The mutated *ActRIA* locus. The 3′ external probe, a 0.6 kb *Xho*I-*Eco*RI fragment, for Southern blot analysis is shown. S, *Sal*I; R, *Eco*RV; K, *Kpn*I; X, *Xho*I. (B) Genotype analysis of a litter of newborn mice from heterozygous mating. Tail genomic DNA was digested with *EcoRV* and blotted with the 3′ external probe. The genotypes are marked as +/+ for wild type and +/– for heterozygote. No homozygous live-born mice were found among 223 analyzed. Arrowheads indicate 13.4 kb band (wild type) and 7.0 kb bands (mutant). (C) Genotype of ActRIA-deficient ES cells. Genomic DNA from ES cell lines was analyzed by southern hybridization. Two cell lines (7 and 9) were identified as *ActRIA*^{tm1} homozygous (–/–) mutants. The expression of β-galactosidase was determined by X-gal staining. + indicates β-galactosidase positive, and – indicates negative.

Southern blot analysis using a 3′ external probe, one clone showed correct homologous recombination between the vector DNA and the endogenous *ActRIA* locus (Fig. 2B). The mutant allele was termed *ActRIA*^{tm1} (for disruption of the transmembrane domain). Based on the genomic structure of the gene (Schmitt et al., 1995), it was predicted that any alternative splicing skipping the disrupted exon would cause a frame shift in the kinase domain, thus the inactivation of the receptor. In addition, levels of ActRIA transcripts were expected to be significantly reduced, since the *neo* gene cassette was inserted in the same transcriptional orientation as the *ActRIA* gene. While we cannot exclude the possibility that truncated ActRIA might be produced, *ActRIA*^{tm1} probably represents a severe loss-of-function mutation.

Mice heterozygous for the mutation (*ActRIA*$^{tm1/+}$) were morphologically normal and fertile. Genotyping of newborn progeny from heterozygous crosses by Southern blot analysis showed that 32% of them were wild type, 68% heterozygous and none were homozygotes (*ActRIA*$^{tm1/tm1}$) (Fig. 2B; Table 1), indicating that the disruption of ActRIA is recessive embryonic lethal. In addition, *ActRIA*$^{tm1/tm1}$ mice were never recovered from continuous breeding of heterozygous parents for more than three years, indicating that the mutant phenotype co-segregates with and is therefore resulted from the *ActRIA* mutation.

To determine when mutant embryos die in utero, embryos from *ActRIA*$^{tm1/+}$ intercrosses were dissected and analyzed at different days of gestation (Table 1). At E7.5, 29 out of 38 embryos were normal and nine were abnormal. Genotyping by PCR revealed that the normal embryos were either wild type or heterozygous for *ActRIA* mutation, while all the abnormal embryos were *ActRIA*$^{tm1/tm1}$ mutants. The *ActRIA*$^{tm1/tm1}$ embryos were much smaller than their normal littermates and lacked a morphologically discernible primitive streak (Fig. 3A). At E8.5, the mutant embryos were growth retarded and disorganized (Fig. 3B). At E9.5, about one quarter of decidua contained resorption sites and no homozygous mutants were recovered. These results suggest that the development of ActRIA-deficient embryos was arrested prior to or during gastrulation.

Morphological defects in *ActRIA* mutant embryos

To determine the defects in *ActRIA*$^{tm1/tm1}$ embryos, histological analysis of normal and mutant embryos was performed. No apparent abnormality was observed in 27 embryos analyzed at the egg cylinder stage (E6.0-6.5) (Table 1), suggesting that the mutant embryos developed normally before the initiation of gastrulation. At E7.0-7.5, about 23% (11 out of 47) of embryos showed various morphological defects and were significantly smaller than their normal littermates. In the normal embryos, the primitive streak, the mesodermal layer and extraembryonic structures such as amnion, chorion and allantois are well formed (Fig. 4A,D). In the mutant embryos, however, a morphologically distinguishable primitive streak or the mesodermal layer was absent (Fig. 4B,E). The epiblast in some regions of the embryonic cylinder lost its usual columnar shape and was thickened (Fig. 4B). A constriction near the embryonic/extraembryonic junction could be observed in most mutant embryos (Fig. 4B,C). In the extraembryonic region, the extraembryonic ectoderm failed to form an organized epithelial layer, and no allantois, chorion, amnion or amniotic folds were observed (Fig. 4B,C). In addition, the proximal visceral endoderm cells were less vacuolated and abnormal as compared with the characteristic columnar shape of the visceral endoderm in normal embryos. The parietal endoderm layer in mutant embryos appeared to be normal except that the cell density was higher than that in normal embryos. Analysis of mutant embryos at E8.0-8.5 revealed that some mesoderm-like cells were formed but disorganized (Fig. 4C,F). The

Table 1. Phenotype and genotype analyses of embryos or newborn mice from intercrosses of *ActRIA$^{tm1/-}$* mice

Stage	Assay	Normal	(+/+ +/–)	Abnormal	(–/–)
Newborn	Southern	223	(71 152)	0	
E9.5	Southern/PCR	38	(13 25)	0	
E8.5	PCR	31	(9 22)	12	(12)
E7.5	PCR	29	(12 17)	9	(9)
E7.5	Histology	36		11	
E6.0-6.5	Histology	27		0	

Note: embryos subjected to histological analysis had not been genotyped.

embryonic ectoderm was folded, resulting in disorganization of the egg cylinder. In most E8.5 mutant embryos, a large number of dead cells were found inside the proamniotic cavity, indicating tissue degeneration (Fig. 4C).

Expression of early markers in *ActRIA^{tm1}* mutant embryos

To further characterize the gastrulation defects, expression of several early markers was analyzed in ActRIA-deficient embryos. *Brachyury* (*T*) is one of the earliest mesoderm markers. In normal embryos, *T* expression is first detected at E6.0 in the proximal rim of the epiblast before the initiation of gastrulation, and then in the primitive streak formed in the prospective posterior region of the epiblast (E6.5-7.0), and later in the head process and the notochord as well as in the primitive streak (after E7.5) (Wilkinson et al., 1990; Fig. 5A,B). However, *T* expression in E7.5 embryos with characteristic *ActRIA$^{tm1/tm1}$* mutant morphology was either absent (2 out of 9) or greatly reduced (7 out of 9) (Fig. 5A).

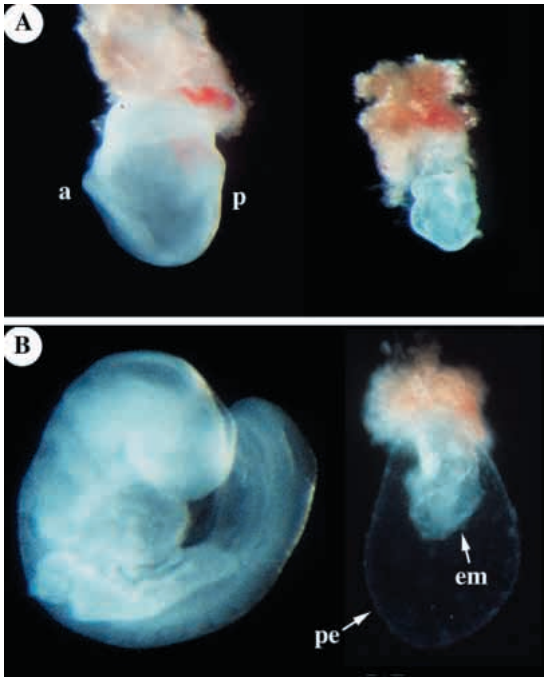

Fig. 3. Gross morphology of *ActRIA$^{tm1/tm1}$* mutant embryos. Wild-type (left) and *ActRIA$^{tm1/tm1}$* mutant (right) littermate embryos at late E7.5 (A) and late E8.5 (B). In the wild-type embryo anterior is marked as a and posterior as p. In the mutant, there is no apparent anteroposterior polarity. em, embryonic part; pe, parietal endoderm.

When *T* expression was detected in the mutant embryos, the *T* transcripts were confined to a small area near the embryonic/extraembryonic junction. *T* expression in mutant embryos at E8.5 showed a similar pattern (Fig. 5B). The restricted *T* expression in mutant embryos suggests that the initiation of gastrulation and primitive streak formation probably occurred, but gastrulation failed to progress further.

Since the anteroposterior (A-P) axis is established at the egg-cylinder stage prior to the formation of the primitive streak, it is important to test whether the A-P axis is established in the *ActRIA$^{tm1/tm1}$* mutant embryos. We examined the expression pattern of T and an anterior marker Hesx1 simultaneously by double in situ hybridization. Hesx1 is first detected in the anterior visceral endoderm (AVE) in the wild-type embryos at the pre- and early streak stages, and then in the adjacent prospective neuroectoderm after the primitive streak is formed (Thomas and Beddington, 1996) (Fig. 5B). In the mutant embryos, Hesx1 transcripts were detected on the opposite side of the embryo where *T* expression was detected, indicating that the anteroposterior axis was established in the mutant embryos (Fig. 5B).

HNF4 is a transcription factor that is expressed specifically in the visceral endoderm during early embryogenesis (Duncan et al., 1994). It has been shown that HNF4 is required for the differentiation and function of the visceral endoderm during early development. Disruption of the *HNF4* gene results in reduction or elimination of expression of multiple genes in the visceral endoderm, and developmental arrest of the mutant embryos at the early gastrulation stage (Chen et al., 1994; Duncan et al., 1997). The morphological defects of *ActRIA$^{tm1/tm1}$* mutant embryos were similar to, though appeared to be more severe than, those of HNF4-deficient embryos. HNF-4 transcripts were first detected in the primitive endoderm at the blastocyst stage and then in the visceral endoderm in the wild-type embryos (Duncan et al., 1994, Fig. 5C). In contrast, HNF-4 expression in the visceral endoderm was drastically reduced in E7.5 and E8.5 *ActRIA$^{tm1/tm1}$* embryos (Fig. 5C), suggesting that visceral endoderm differentiation might be impaired.

We also analyzed the expression pattern of Bmp4 in the *ActRIA$^{tm1/tm1}$* mutant embryos. In normal embryos, Bmp4 is initially expressed around the distal part of the extraembryonic ectoderm before gastrulation and later in the posterior region of the embryo as well as in the extraembryonic tissues such as allantois and amnion (Jones et al., 1991; Winnier et al., 1994; Fig. 5D). In the mutant embryos, Bmp4 transcripts were detected in the extraembryonic ectoderm near the epiblast, suggesting that the early Bmp4 expression pattern was not affected in *ActRIA$^{tm1/tm1}$* embryos (Fig. 5D). The BMP4 expression

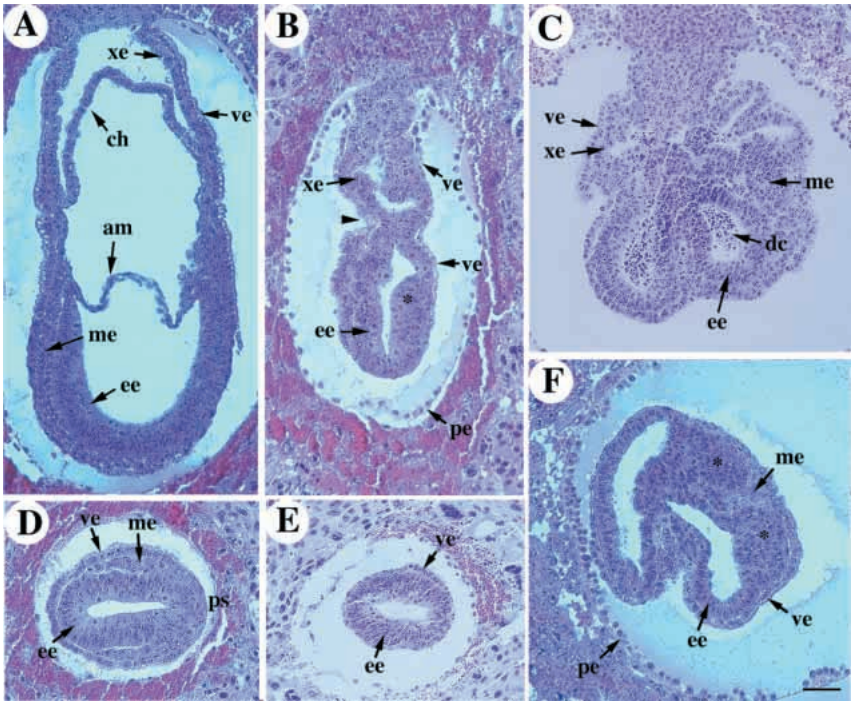

Fig. 4. Histological analysis of *ActRIA*$^{tm1/tm1}$ mutant embryos. Sagittal (A-C) or transverse sections (D-F) of wild-type (A,D) and mutant embryos (B,C,E,F) at early E7.5 (A,B,D,E), or E8.5 (C,F). Transverse sections near the embryonic and extraembryonic junction were selected. Note the thickened epiblast epithelium (*) in mutant embryos at E7.5 (B) and E8.5 (F), a constriction near the extraembryonic and embryonic junction (B; arrowhead) and dead cells (dc) in the proamniotic cavity in the E8.5 mutant embryo (C). am, amnion; ch, chorion; ee, embryonic ectoderm; me, mesoderm; pe, parietal endoderm; ps, primitive streak; ve, visceral endoderm; xe, extraembryonic ectoderm. Embryos in A-F are of the same magnification. Bar, 50 μm.

pattern was also consistent with the developmental arrest of *ActRIA*$^{tm1/tm1}$ embryos at the early gastrulation stage.

Development of chimeras derived from *ActRIA*$^{tm1/tm1}$ ES cells

ActRIA-deficient ES cells were genetically marked with a *lacZ* transgene inherited from Rosa 26 mice (Friedrich and Soriano, 1991) and used to test whether *ActRIA* mutation could affect cell proliferation and differentiation in a cell autonomous manner. Since the Rosa 26 *lacZ* transgene is expressed ubiquitously during early embryogenesis (Gu et al., 1998), it can serve as a cell lineage marker to trace tissue distribution of the mutant cells in developing embryos. The *lacZ*$^{+}$ ES cell lines were established from inner cell mass cells of delayed blastocysts produced by intercrosses of *ActRIA*$^{tm1/+}$*Rosa-26*$^{+/-}$ mice. Of ten lines established, two were confirmed to be *lacZ*$^{+}$ *ActRIA*$^{tm1/tm1}$ (Fig. 2C). Both cell lines were used to generate chimeric embryos and similar results were obtained.

We generated chimeric embryos either by injection of 10-15 *ActRIA*$^{tm1/tm1}$ ES cells into each wild-type blastocyst or by

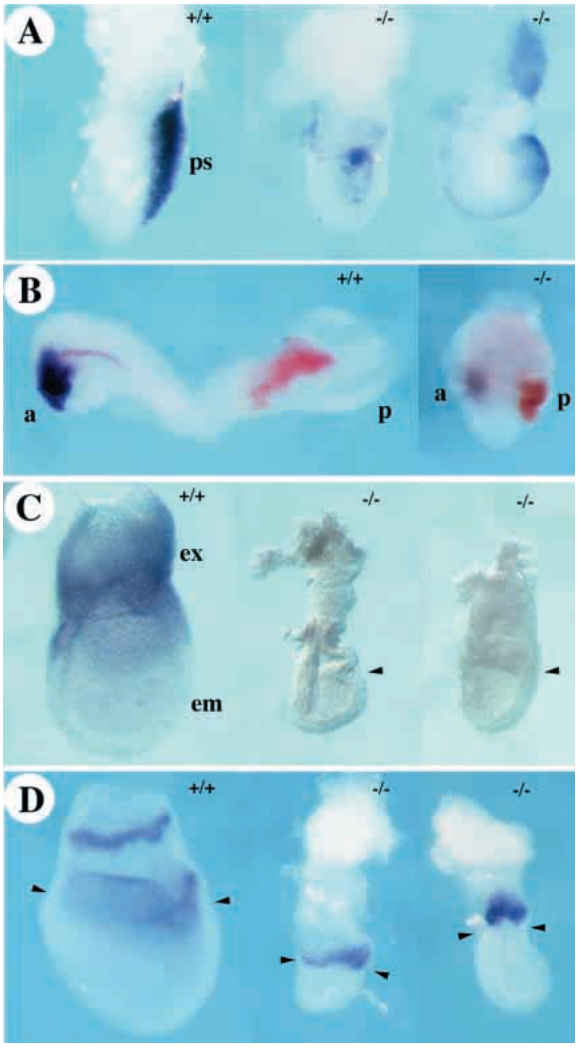

Fig. 5. Expression of early markers in *ActRIA*$^{tm1/tm1}$ mutant embryos. Expression of Brachyury (T), Hesx-1, Bmp4 or HNF4 in wild-type and *ActRIA* mutant embryos. (A) At E7.5, T is expressed along the entire primitive streak in the wild-type embryo, but only in a small patch of cells at the proximal regions of the epiblast in the *ActRIA* mutant embryos. (B) Expression of Hesx-1 (blue) in the head and T (brown) in the notochord and tail in an E8.5 wild-type embryo. In the mutant littermate, these two markers are expressed on the opposite sides of the egg cylinder. (C) HNF4 is expressed in the visceral endoderm of wild-type embryos, but not in the mutant littermates at E7.5. (D) Bmp4 is expressed at the posterior and extraembryonic regions in the wild type, and in the extraembryonic region in the *ActRIA*^{tm1} mutant embryos at E7.5. +/+, wild type; –/–, *ActRIA*$^{tm1/tm1}$ mutant; a, anterior; em, embryonic region; ex, extraembryonic region; p, posterior; ps, primitive streak. Arrowheads mark the embryonic/extraembryonic junctions.

aggregation of *ActRIA*$^{tm1/tm1}$ ES cells with wild-type embryos. Chimeric embryos were dissected at embryonic stages from E8.5 to E11.5 and stained with X-gal. Comparing with non-injected littermates, most chimeras with low or medium contribution of *ActRIA*$^{tm1/tm1}$ ES were morphologically normal. Although about two thirds of chimeric embryos with strong X-gal staining appeared to be morphologically normal from E8.5 to E10.5 (Fig. 6A,B), the remaining one third showed various abnormalities, including smaller body size, delay in development (e.g. not turned at E9.5), abnormal head structures, and shortening of the posterior and trunk regions (Fig. 6C). Most chimeras with high contribution of ES cells were growth retarded and died at E11.5 (data not shown).

Previous studies have shown that ES cells injected into blastocysts contribute predominantly to the embryonic lineage (Beddington and Robertson, 1989). As expected, the *ActRIA*$^{tm1/tm1}$ cells contributed extensively to almost all tissues in chimeric embryos, but were absent in the extraembryonic ectoderm-derived tissues, the parietal yolk sac and the endoderm component of the visceral yolk sac. Histological analysis of several most extensively stained E8.5 chimeric embryos revealed that *ActRIA*$^{tm1/tm1}$ cells contributed to embryonic tissues of all three definitive germ layers, including mesoderm-derived tissues such as the cephalic mesenchyme, heart, somites and the mesoderm component of the visceral yolk sac (Fig. 6D-F). By counting the number of mutant and wild-type cells in serial sections of several normal E8.5 chimeric embryos, we estimated that mutant cells accounted for about 80% of the cells in chimeric embryos. However, most abnormal chimeras at E9.5 and E10.5 had a higher contribution of mutant cells (data not shown). These results indicate that a very extensive contribution of *ActRIA*$^{tm1/tm1}$ cells in chimeric embryos could disrupt normal development (Fig. 6C), whereas the presence of sufficient number of wild-type cells could rescue such defects**.**

Gastrulation defects in chimeras consisting of *ActRIA*$^{tm1/tm1}$ extraembryonic cells

The detection of ActRIA protein in the extraembryonic cells prior to and during gastrulation suggests that the function of ActRIA in those cells is probably essential for gastrulation. This hypothesis is supported by the observation that wild-type extraembryonic tissues could support chimeras with extensive contribution of *ActRIA*$^{tm1/tm1}$ cells to develop normally at the early gastrulation stage. To further test this hypothesis, a second type of chimeric embryos were generated by injecting wild-type R/S4 ES cells (Gu et al., 1998), containing the Rosa 26 *lacZ* transgene, into blastocysts produced by mating male and female *ActRIA*$^{tm1/+}$ mice. If the ActRIA function is required in the extraembryonic cells, chimeras deriving from *ActRIA*$^{tm1/tm1}$ blastocysts would be expected to show gastrulation defects similar to those seen in *ActRIA*$^{tm1/tm1}$ mutant embryos.

Chimeras in which embryonic tissues consisting predominantly of wild-type cells were generated by injection of 15 wild-type R/S4 ES cells into each blastocyst (Gu et al., 1998). Of 24 chimeric embryos dissected at E9.5, 19 were morphologically normal, one was very small with disorganized embryonic structure and four were partially or completely degenerated. Of 30 chimeras dissected at E8.5, eight were

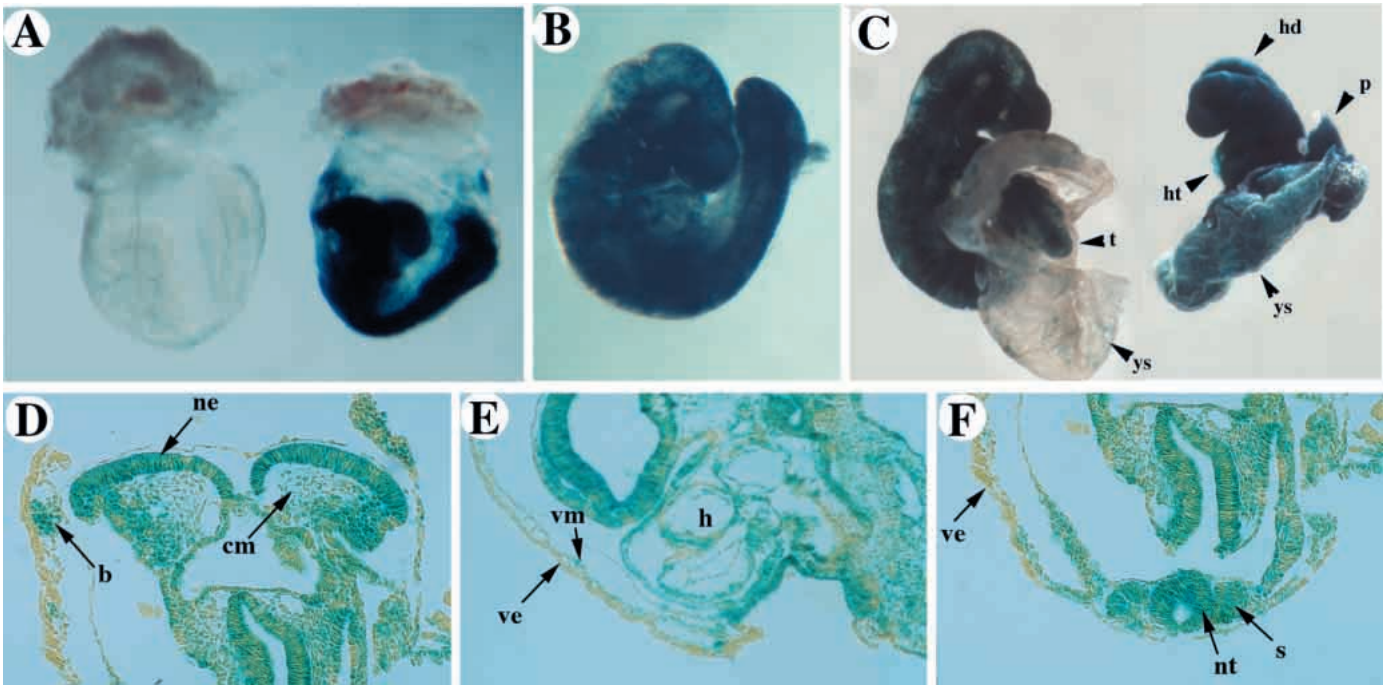

Fig. 6. Analysis of chimeric embryos derived from *ActRIA*$^{tm1/tm1}$ ES cells. (A) E8.5 wild-type control (left) and chimeric (right) littermates, and (B) an E10.5 chimera generated by microinjection. (C) E9.5 Chimeras generated by aggregation. Note the yolk sac (ys) of the abnormal embryo on the right was extensively stained by X-gal and the posterior part (p) of the embryo was shortened. hd, head; ht, heart; t, tail.
(D-F) Sections of normal chimeric embryos at E8.5 show that mutant cells contribute to neuroepithelium (ne), cephalic mesenchyme (cm) and blood cells (b) in D, visceral yolk sac mesoderm cells (vm) and heart (h) in E, somites (s) and neural tube (nt) in F. However, mutant cells were rarely found in parietal yolk sac and the endoderm component of the visceral yolk sac (ve).

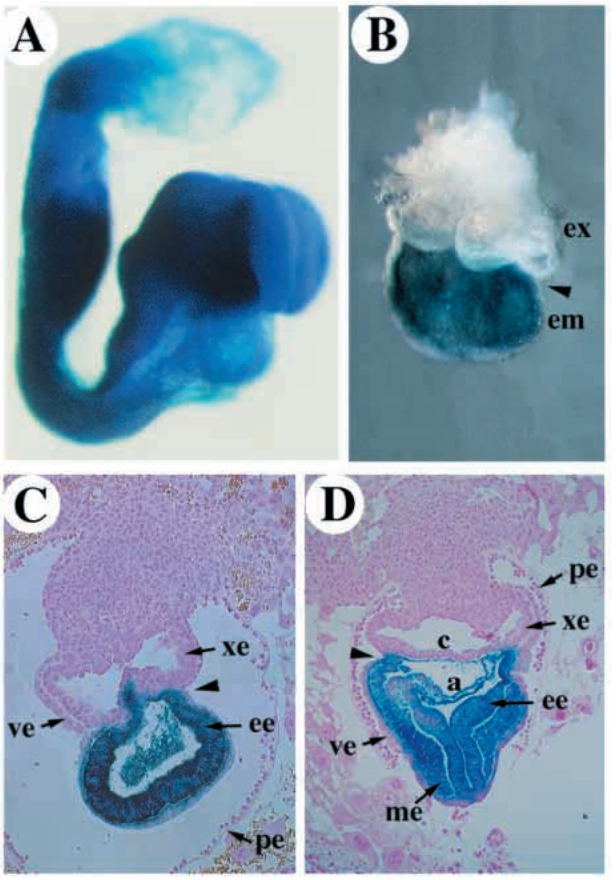

Fig. 7. Chimeric embryos derived by injection of wild-type ES cells into *ActRIA*$^{tm1/tm1}$ blastocysts. (A) Strong X-gal staining of normal E8.5 embryos whose parietal yolk sac DNA was genotyped as *ActRIA*$^{+/+}$. (B) An abnormal E8.5 embryo, whose parietal yolk sac DNA was genotyped as *ActRIA*$^{tm1/tm1}$, is arrested at early gastrulation. The embryonic ectoderm is strongly stained, but the extraembryonic tissues contains almost no blue cells. (C) A sagittal section of an abnormal E8.5 chimeric embryo which is phenotypically similar to the homozygous mutant. (D) A sagittal section of a less severe E8.5 chimeric embryo. Note a relative high contribution of wild-type blue cells in the embryonic ectoderm and mesoderm in the embryonic region. However, the formation of the extraembryonic mesoderm was significantly reduced. a, amnion; c, chorion; ee, embryonic ectoderm; me, mesoderm; ve, visceral endoderm; xe, extraembryonic ectoderm. Arrowheads mark the embryonic/extraembryonic junction in mutant embryos. Embryos in A-D are of the same magnification. Bar, 50 μm.

growth retarded. Southern analysis of DNA isolated from the parietal yolk sac, which was derived entirely from the donor blastocysts, showed that the abnormal E8.5 and E9.5 chimeras were derived from *ActRIA*$^{tm1/tm1}$ blastocysts, and all 41 E8.5 and E9.5 normal chimeras were derived from either wild-type or heterozygous blastocysts. X-gal staining demonstrated a very extensive contribution of *lacZ*$^{+}$ wild-type cells in all of the growth-retarded chimeras (Fig. 7B).

Histological analysis of the abnormal chimeras at E8.5 revealed that the embryonic development was much delayed and impaired even though the *lacZ*$^{+}$ wild-type cells colonized almost the entire embryonic ectoderm (Fig. 7C,D). Some abnormal chimeras (n=2) showed similar defects as seen in *ActRIA*$^{tm1/tm1}$ embryos, including abnormal morphology of the visceral endoderm, thickening of the epiblast and constriction at the embryonic/extraembryonic junction (Fig. 7C), suggesting that mesoderm formation was disrupted or delayed. Other chimeras (n=6) developed further to the stage equivalent to E7.5 wild-type embryos and formed mesodermal tissues, chorion and amnion-like structures (Fig. 7D). However, the formation of the extraembryonic mesoderm was delayed. In particular, the visceral yolk sac failed to expand and the allantois was not formed (Fig. 7D). Together, these results indicate that the early gastrulation defect of *ActRIA*$^{tm1/tm1}$ embryos results from the loss of function of ActRIA in the extraembryonic cells. The development of some chimeras (6 out of 8) to a more advanced stage than *ActRIA*$^{tm1/tm1}$ embryos suggests that wild-type ES cells could partially rescue the early gastrulation defects.

In vitro differentiation of ActRIA-deficient ES Cells

When cultured in suspension, ES cells form aggregates known as embryoid bodies and undergo differentiation (Robertson, 1987). Since cellular differentiation and gene expression in embryoid bodies seem to parallel the developmental process of early development, ES cells have been used as an in vitro model for studying early developmental events (Coucouvanis and Martin, 1995; Duncan et al., 1997; Sirard et al., 1998). We also studied the differentiation of *ActRIA*$^{tm1/tm1}$ ES cells in vitro. During the first 7-8 days of culture, both wild-type and homozygous mutant cells formed embryoid bodies with a well organized inner layer of columnar ectoderm-like cells surrounded by an outer layer of vacuolated endoderm cells, resembling the columnar visceral endoderm (Fig. 8A-C). There was no discernible difference between the wild-type and mutant embryoid bodies up to this stage. During further differentiation in culture, many of the wild-type embryoid bodies grew larger and formed beating cardiac muscle (data not shown) and large cysts equivalent to the visceral yolk sac (Fig. 8D,F). However, the *ActRIA*$^{tm1/tm1}$ embryoid bodies were much smaller in size and formed neither beating muscle cells nor cystic structures (Fig. 8E,G). Histological analysis of wild-type embryoid bodies at day 17 revealed the presence of mesoderm-derived mesenchyme cells between the endoderm and ectoderm layers (Fig. 8D). In contrast, only two layers of cells, the primitive endoderm and the ectoderm, separated by an abnormally thick layer of basement membrane, were found in the *ActRIA*$^{tm1/tm1}$ embryoid bodies (Fig. 8E). These results suggest that mesoderm formation in the mutant embryoid bodies was disrupted.

DISCUSSION

Members of the TGF-β superfamily have been shown to play important roles during early development of vertebrate animals. Genetic studies by gene targeting in mice have demonstrated that nodal, BMP4 (Zhou et al., 1993; Conlon et al., 1994; Winnier et al., 1995), two TGF-β family type I receptors, Bmpr (ALK3) and ActRIB (ALK4) (Mishina et al., 1995; Gu et al., 1998), and two Smad proteins, Smad2 and Smad4 (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998; Sirard et al., 1998; Yang et al., 1998), are essential

for early mouse development. In this study, we demonstrate that yet another type I receptor ActRIA (ALK2) is also required during early development. The apparent non-redundant function of the three type I receptors indicates the complexity of TGF-β signaling in regulation of morphogenesis during early mouse development.

ActRIA-mediated signaling plays an essential role during gastrulation

The development of *ActRIA*$^{tm1/tm1}$ embryos appeared to be normal before gastrulation, but was arrested shortly after the initiation of gastrulation. The *ActRIA*$^{tm1/tm1}$ mutant embryos displayed multiple defects including constriction at the embryonic-extraembryonic junction, thickening of the embryonic ectoderm, delayed and severely impaired mesoderm formation, and the lack of extraembryonic structures such as amniotic fold or allantois. In addition, the morphology of the proximal visceral endoderm cells was abnormal in most *ActRIA*$^{tm1/tm1}$ embryos.

The defects in *ActRIA*$^{tm1/tm1}$ embryos are distinctive and less severe as compared to mouse embryos lacking either Bmpr or ActRIB receptors. In *Bmpr*$^{-/-}$ or *ActRIB*$^{-/-}$ embryos, the formation of the primitive streak and mesodermal cells is completely blocked. While Bmpr probably functions as the type I receptor for BMP4 to regulate epiblast cell proliferation and mesoderm formation (Mishina et al. 1995; Winnier et al., 1995), ActRIB appears to mediate the nodal or an activin-like signal that is essential for egg cylinder organization and primitive streak formation (Gu et al., 1998). In contrast, the formation and growth of the *ActRIA*$^{tm1/tm1}$ egg cylinder were essentially normal, and the A-P axis was established as shown by *Hesx1* and *T* expression on the opposite sides of the mutant embryos (Fig. 5). The expression of *T* and the presence of mesodermal cells suggest that the primitive streak was initially formed in most *ActRIA*$^{tm1/tm1}$ embryos. Our results thus indicate that the function of ActRIA is not required for the egg cylinder formation and growth nor for the establishment of A-P axis, but becomes essential shortly after the initiation of gastrulation.

In mice, fate mapping studies have shown that the earliest mesoderm forms in the posterior region of the primitive streak and migrates proximally into the extraembryonic region to form yolk sac mesoderm, while the embryonic mesoderm is formed by epiblast cells ingressing through the primitive streak and migrating laterally (Lawson et al., 1991; Tam and Behringer, 1997). Disruption of primitive streak formation would presumably block all mesoderm formation. Since *T* expression was restricted to the posterior region in the *ActRIA*$^{tm1/tm1}$ embryo, it suggested that the primitive streak was initially formed, but failed to elongate subsequently. Previous morphogenetic studies have shown that the primitive streak elongates through intercalation of newly recruited epiblast cells to regions proximal to the anterior aspect of the primitive streak (Lawson et al., 1991). Cell-cell interactions between the visceral endoderm and the epiblast may play a role in this process. The morphological defects in *ActRIA*$^{tm1/tm1}$ embryos, such as thickening of the epiblast epithelial layer and impaired mesoderm formation, therefore, may indicate a failure of the epiblast cells to intercalate into the early primitive streak. Alternatively, the epiblast cells may fail to ingress through the streak to form mesoderm, or the nascent mesoderm may fail to proliferate or migrate.

The function of ActRIA in both embryonic and extraembryonic cells is required for embryonic development

To understand the primary defect that causes developmental arrest of the *ActRIA*$^{tm1/tm1}$ embryos at the early gastrulation stage, it is critical to determine in which germ layer ActRIA carries out its function. Through chimera analysis, we found

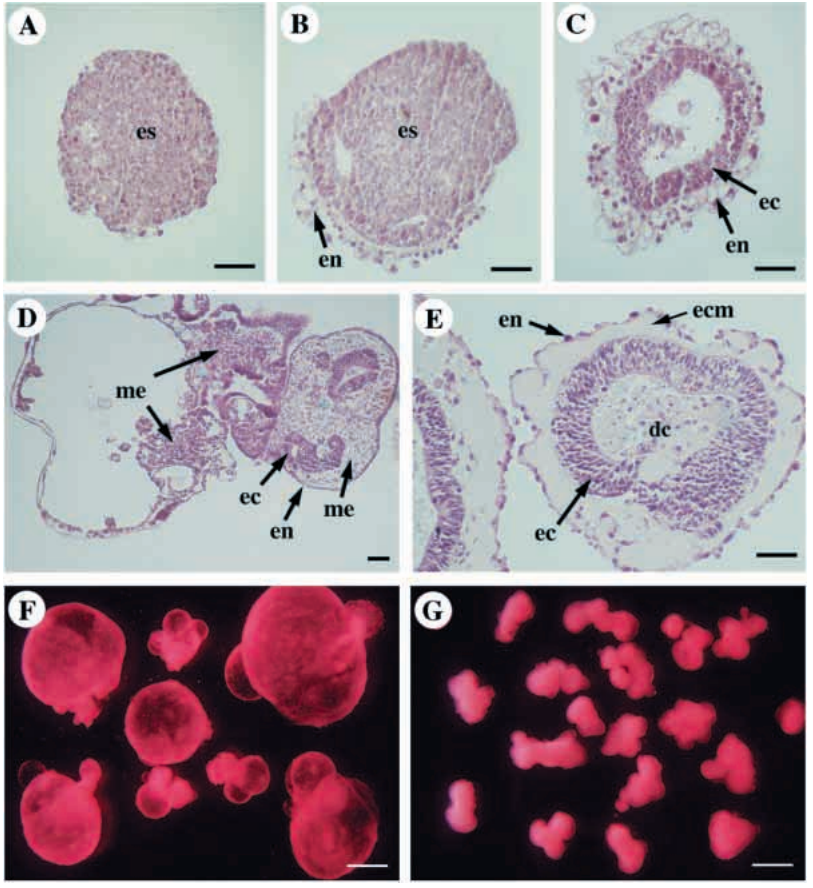

Fig. 8. In vitro differentiation of *ActRIA*$^{tm1/tm1}$ ES cells. Histology (A-E) and gross morphology (F,G) of wild-type and ActRIA mutant embryoid bodies. (A-C) The differentiation of ES cells from an ES cell aggregate to an embryoid body at day 1 (A), day 2 (B) and day 8 (C). There was no apparent difference between wild-type and *ActRIA*$^{tm1/tm1}$ embryoid bodies during these stages. (D) A day 17 wild-type embryoid body contains a large cystic structure and mesodermal cells (mesenchymal and mesenthelial) between ectoderm and endoderm. (E) A day 17 mutant embryoid body contains a thick layer of extracellular matrix, but no mesodermal cells. (F,G) Day 17 wild-type (F) and *ActRIA*$^{tm1/tm1}$ mutant (G) embryoid bodies. ecm, extracellular matrix; es, undifferentiated ES cells; en, endoderm; ec, ectoderm; me, mesoderm; dc, dead cells. Bar, 0.1 mm (A-E); 1 mm (F,G).

that mutant *ActRIA*$^{tm1/tm1}$ ES cells could contribute extensively to most embryonic tissues during gastrulation when the extraembryonic tissues were derived from wild-type blastocysts. The contribution of *ActRIA*$^{tm1/tm1}$ ES cells to various types of mesoderm tissues in chimeras (Fig. 6) indicates that ActRIA-mediated signaling is indirectly involved in mesoderm formation. In contrast, when the extraembryonic tissues were derived from *ActRIA*$^{tm1/tm1}$ blastocysts, the chimeric embryos were arrested at the early to mid-gastrulation stages even when the embryonic ectoderm was colonized extensively by the wild-type cells. It should be noted, however, that most chimeras developed to a slightly more advanced stage than *ActRIA*$^{tm1/tm1}$ embryos as shown by the presence of well-organized embryonic mesoderm and the formation of chorion- and amnion-like structures (Fig. 8D). This result suggests that ActRIA may also function in the epiblast during gastrulation. Together, our results indicate that ActRIA functions primarily, but not exclusively, in the extraembryonic cells to regulate early gastrulation.

How the ActRIA-mediated signaling pathway regulates gastrulation at the molecular and cellular level remains essentially unknown. We speculate the following possibilities. First, the ActRIA signaling pathway may regulate expression of genes that are required for visceral endoderm differentiation and function. We showed that expression of a visceral-endoderm-specific gene, *HNF4*, was dramatically reduced in the *ActRIA*$^{tm1/tm1}$ embryos. Similar to the *HNF4*$^{-/-}$ mutants, *ActRIA*$^{tm1/tm1}$ embryos are also arrested at the early streak stage with reduced *T* expression and delayed mesoderm formation, though the defects in *ActRIA*$^{tm1/tm1}$ embryos appear to be more severe. It would be interesting to investigate whether *HNF4* is a direct downstream target gene of the ActRIA signaling pathway and what other genes are regulated by ActRIA in the visceral endoderm. Secondly, ActRIA may regulate expression of extracellular matrix proteins or their receptors in the visceral endoderm, which may play an important role in mediating interactions between the visceral endoderm and the overlaying epiblast during gastrulation. Thirdly, the ActRIA-mediated signal may induce the synthesis and/or secretion of a secondary signal from the extraembryonic cells, which can in turn act upon the epiblast or newly formed mesoderm to promote gastrulation.

Several other key TGF-β family signal mediators including ActRIB, Smad2 and Smad4, have been shown to have functions in the extraembryonic cells during early mouse development. While ActRIB and Smad2 function in both epiblast and extraembryonic cells, their function in extraembryonic cells is required for the formation of the embryonic rather than the extraembryonic mesoderm (Gu et al., 1998; Nomura and Li, 1998; Waldrip et al., 1998). Similar to ActRIA, the Smad4 function was shown to be required for the differentiation and function of the visceral endoderm, and inactivation of Smad4 in the extraembryonic cells blocked embryonic growth and mesoderm formation (Sirard et al., 1998; Yang et al., 1998). It is possible that ActRIA and Smad4 may function in the same signaling pathway during early mouse development.

Is the function of ActRIA signaling pathway conserved in vertebrate development?

Which TGF-β family molecule signals through ActRIA? Since ActRIA is expressed on the apical surface of the visceral endoderm before and during gastrulation, it seems possible that ActRIA may mediate signals coming from the maternal decidual cells. It has been shown that activins (both β_A and β_B) and BMP4 are expressed in the decidual cells surrounding the embryo at E6.5 and E7.5 (Albano et al., 1994; Feijen et al., 1994; Jones et al., 1991), therefore, can presumably diffuse into the yolk sac cavity to activate ActRIA in the visceral endoderm. The expression of ActRIA also overlaps extensively with that of BMP4 in the amnion and allantois at E7.5 (Fig. 1; Jones et al., 1991). Remarkably, chimeras derived from *ActRIA*$^{tm1/tm1}$ blastocysts had a reduced amount of the extraembryonic mesoderm (Fig. 8C,D) and chimeras derived from wild-type embryos with extensive contribution of *ActRIA*$^{tm1/tm1}$ ES cells showed late gastrulation defects (Fig. 6C), reminiscent of BMP4-deficient embryos which could develop to the gastrulation stage (Winnier et al., 1995). Whether ActRIA mediates signaling of BMP4 or other BMPs during mouse development requires further investigation.

The extraembryonic and posterior mesoderm in the mouse embryo is considered to be equivalent to the 'ventral' type of mesoderm. In this regard, it appears that ActRIA plays an important role in ventral type mesoderm formation. In contrast, it has been shown that the function of ActRIB and Smad2 is essential for the formation of the primitive streak and the embryonic (or 'dorsal' type) mesoderm (Gu et al., 1998; Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998). Although it is difficult to directly compare the mouse mutant phenotypes resulting from loss-of-function mutations with those of *Xenopus* embryos overexpressing the receptors (Armes and Smith, 1997; Chang et al., 1997), the opposite effects of ActRIA and ActRIB mutations on the formation of ventral versus dorsal mesoderm in mice and frog indicate that the function of ActRIA and ActRIB in gastrulation is likely to be conserved in vertebrates.

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