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Strain Dependency of TGFβ1 Function During Embryogenesis

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ABSTRACT There is incomplete penetrance to *Tgfb1* knockout phenotypes. About 50% of *Tgfb1* homozygous mutant (*Tgfb1*$^{-/-}$) and 25% of *Tgfb1* heterozygous (*Tgfb1*$^{+/-}$) embryos die during embryogenesis. In a mixed NIH/Ola × C57BL/6J/Ola × 129 background partial embryonic lethality of the *Tgfb1*$^{-/-}$ embryos occurs due to defective yolk sac vasculopoiesis and/or hematopoiesis. We show here that on a predominantly CF-1 genetic background, lack of TGFβ1 causes a pre-morula lethality in about 50% of the null embryos. This partial lethality is not reversed by transfer of *Tgfb1*$^{-/-}$ embryos to *Tgfb1*$^{+/+}$ hosts. The extent of embryonic lethality in *Tgfb1*$^{-/-}$ embryos ranges in a background dependent manner from 20% to 100%. Based on these and other studies it is clear that TGFβ1 acts at two distinct phases of embryogenesis: pre-implantation development and yolk sac vasculogenesis/hematopoiesis. The susceptibility for the pre-implantation lethality depends on a small number of genetic modifiers since a small number of backcrosses onto the high susceptibility strain C57BL/6 leads to complete penetrance of the lethality. *Mol. Reprod. Dev. 52:341–349, 1999.* © 1999 Wiley-Liss, Inc.

Key Words: pre-implantation; development; gene targeting; lethality; growth factors; knockout mouse

INTRODUCTION

Transforming growth factor β1 (TGFβ1) is the prototypic member of a large family of structurally related polypeptides that mediate important developmental and physiological processes (Akhurst et al., 1990b; Wall and Hoffmann, 1991; Shull and Doetschman 1994; Proetzel et al., 1995; Sanford et al., 1997; Kallapur et al., 1998). TGFβ1 has been detected immunocytochemically in one-cell embryos, presumably translated from maternal mRNA. Although it is barely detectable in two-cell embryos, it is easily detectable at both the message and protein levels from the four-cell embryo to the blastocyst stage (Rappolee et al., 1988; Paria et al., 1992). Specific binding of radiolabeled TGFβ1 and TGFβ2 occurs from the eight-cell to the blastocyst stage embryo (Paria et al., 1992). Finally, it is present at both protein and message levels in the post-implantation embryo (Heine et al., 1987; Lehnert and Akhurst 1988; Akhurst et al., 1990a; Gatherer et al., 1990; Pelton et al., 1991). Together, these data strongly suggest that TGFβ1 plays important roles in both pre- and post-implantation mammalian development.

In the *Tgfb1* knockout mouse there is no uniform embryonic lethality or developmental malformation since some mutants die in utero and others survive until they succumb to a multi-focal inflammatory disease (Shull et al., 1992; Kulkarni et al., 1993). These different lethalities could be explained by varying levels of compensation by other *Tgfb* family members or transplacental/breast milk transfer of TGFβ1 from a *Tgfb1*$^{+/-}$ mother to fetuses/pups (Shull et al., 1992; Letterio et al., 1994). In *Tgfb1*$^{+/-}$ crosses a deviation from the expected frequency of offspring genotypes is observed in the pups (Shull et al., 1992; Kulkarni et al., 1993). Thus, only about 50% of *Tgfb1*$^{-/-}$ and 75% of *Tgfb1*$^{+/-}$ animals are born at term, implying a partially penetrant embryonic lethal phenotype for both heterozygous and homozygous mutant animals (Shull et al., 1992; Kulkarni et al., 1993). When *Tgfb1* knockout mice were on a mixed 129 × C57BL/6J/Ola × NIH/Ola genetic background, partial embryonic lethality was observed at around d10.5 pc due to defective yolk sac vasculogenesis and/or hematopoiesis (Dickson et al., 1995). A major co-dominant modifier gene of embryonic lethality was mapped to proximal mouse chromosome 5, partly explaining the strain dependent distribution of embryonic lethality in *Tgfb1*$^{-/-}$ concepti (Bonyadi et al., 1997). In this paper, we show that in some strains a partially penetrant embryonic lethality occurs prior to the morula stage of *Tgfb1*$^{-/-}$ concepti, and that there is some haplodeficiency in *Tgfb1*$^{+/-}$ mice.

MATERIALS AND METHODS

Animals

A *Tgfb1* null allele was generated and the resulting mice were bred to homozygosity (Shull et al., 1992) (D3 embryonic stem cells from 129/SvPas strain of mice). This knockout allele was then bred for a small number

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of generations on various mouse backgrounds. The source of the animals is as follows: C57BL/6, C3H/HeSnJ, SCID(C3H/HeSnJ-SCID) and ICR all from Harlan Sprague Dawley (Indianapolis, IN), CF-1 from Charles River (Wilmington, MA), Balb/c from Taconic (Germantown, NY), and 129 *Rag2*$^{-/-}$, gift of Dr. Coffman, DNAX Laboratories, Palo Alto, CA.

Breeding

Timed matings were performed for certain experiments. In some cases females were superovulated using pregnant mare's serum (PMSG; Calbiochem, La Jolla, CA) 5 IU intraperitoneal (ip), followed 48 hr later by human chorionic gonadotropin (HCG; Sigma, St. Louis, MO) 4 IU ip. The morning of detection of a vaginal copulatory plug was regarded as day 0.5 postconception (pc).

Neonatal/Fetal Genotyping

Tail samples from neonatal mice, yolk sac, or part of the fetus were used for genotyping. DNA was extracted using standard procedures. An aliquot of the DNA was used for PCR genotyping. An upstream primer 5′-GAG AAG AAC TGC TGT GTG CG-3′ and downstream primer 5′-GTG TCC AGG CTC CAA ATA TAG G-3′ corresponding to *Tgfb1* sequences in exon 6 and flanking the *Neo gene* insert in the *Tgfb1* null allele were used to amplify fragments of 142 bp and 1.25 kb from the wild type and null alleles, respectively. A 20 µl capillary PCR reaction of 95°C for 20 sec, 55°C for 50 sec, and 72°C for 1 min was carried out on a Corbett (A.B. Technology, Inc., Pullman, WA) cycler for 30 cycles. Reaction mix contained 1× PCR buffer, 2.5 mM dNTPs, 10 µM of primer, 0.4U *Taq* polymerase (all from Boehringer Mannheim, Indianapolis, IN) and PCR dye (0.04% cresol red in 60% sucrose).

Pre-Implantation Embryo Genotyping

After scoring the morphology, individual pre-implantation embryos were transferred to an eppendorf tube and osmotically lysed in the presence of proteinase K (20 mg/ml). This was then heat inactivated at 95°C for 10 min. The entire DNA extracted was used for PCR analysis. In some reactions, the primer set mentioned above was used. In other reactions, triple primer sets were used to increase the sensitivity. The upper and lower primers flank the *Neo* cassette's targeted insertion into exon 6. The *Neo* primer corresponds to sequences in the *Neo* insertion and is in the same orientation as the upper primer (Fig. 1). The sequences are as follows: Upper primer 5′ GAG AAG AAC TGC TGT GTG CG 3′, *Neo* primer 5′ GCC GAG AAA GTA TCC ATC AT 3′ and Lower primer 5′ CTT GCT GTA CTG TGT GTC CAG 3′. Amplification was carried out for 32–37 cycles, 95°C 20 sec, 57°C 50 sec, 72°C 1 min 30 sec. PCR buffer was the same as above. Using these primers a mutant band 550bp and wild type band of 147 bp were seen.

Embryo Transfer

For embryo transfers, two-cell stage embryos were harvested from superovulated C3H × 129 × CF-1 mice (approximately 75% C3H, 12.5% 129, and 12.5% CF-1). The recipient animals for the embryo transfer experiments were *Tgfb1*$^{+/+}$ pseudopregnant ICR female mice. These were then transferred to appropriately prepared wild type ICR female mice using standard procedures (Hogan et al., 1986).

RESULTS

TGFβ1 Is Required for Pre-Implantation Development

Timed pregnancies with 129 × CF-1 *Tgfb1*$^{+/-}$ crosses (50/50 mixture of the two strains) were carried out and the genotypic frequencies of the progeny were analyzed at various points during gestation (Table 1). In preliminary experiments, the fidelity of PCR was verified by Southern blotting of the diagnostic PCR product using an end-labeled internal cDNA probe (results not shown). To increase specificity of the PCR, sequences unique to the *Neo* gene were used as a primer for the reaction. Also, care was taken to prevent cross-contamination and PCR product carry-over contamination. At embryonic day 7.5 (E7.5) (neural fold stage) fewer than expected *Tgfb1*$^{-/-}$ were found and the morphology of the remaining *Tgfb1*$^{-/-}$ embryos was indistinguishable from their wild type litter mates. Thus, the partial embryonic lethality could be early post-implantation or pre-implantation. To distinguish between these possibilities, the ratio of various genotypes was analyzed at the blastocyst stage. As can be seen in Table 1, the ratio of *Tgfb1*$^{-/-}$ blastocysts is indistinguishable from that seen at birth. Furthermore, *Tgfb1*$^{-/-}$ blastocyst morphology was no different from that of the wild type blastocysts. Consequently, all embryonic lethalities occur before implantation on the 129 × CF-1 background.

In order to determine the precise developmental stage of the partial embryonic lethality, embryos were collected from *Tgfb1*$^{+/-}$ crosses at E2.5. In contrast to E3.5 where less than 3% of the blastocysts were abnormal or disintegrating, at E2.5, 72/261 (28%) of the embryos were disintegrating. The PCR strategy used for genotyping could not reliably distinguish between *Tgfb1*$^{+/+}$ and *Tgfb1*$^{+/-}$ embryos at this stage, presumably because of the competition between the wild type and the null allele as DNA template, in the face of limiting DNA quantities. However, the *Tgfb1*$^{-/-}$ embryos could be reliably genotyped. Therefore, embryos derived at E2.5 or earlier, were genotyped and categorized as *Tgfb1*$^{-/-}$ or *Tgfb1*$^{+/+}$ and *Tgfb1*$^{+/-}$ combined. As can be seen from Table 1, the ratio of *Tgfb1*$^{-/-}$ to total embryos was no different regardless of whether healthy E2.5 embryos, blastocysts, or postnatal offspring were being compared. We could genotype only about 40% of the disintegrating embryos, presumably because of extensive DNA degradation in the rest. The mutants represented about 31% of the disintegrating

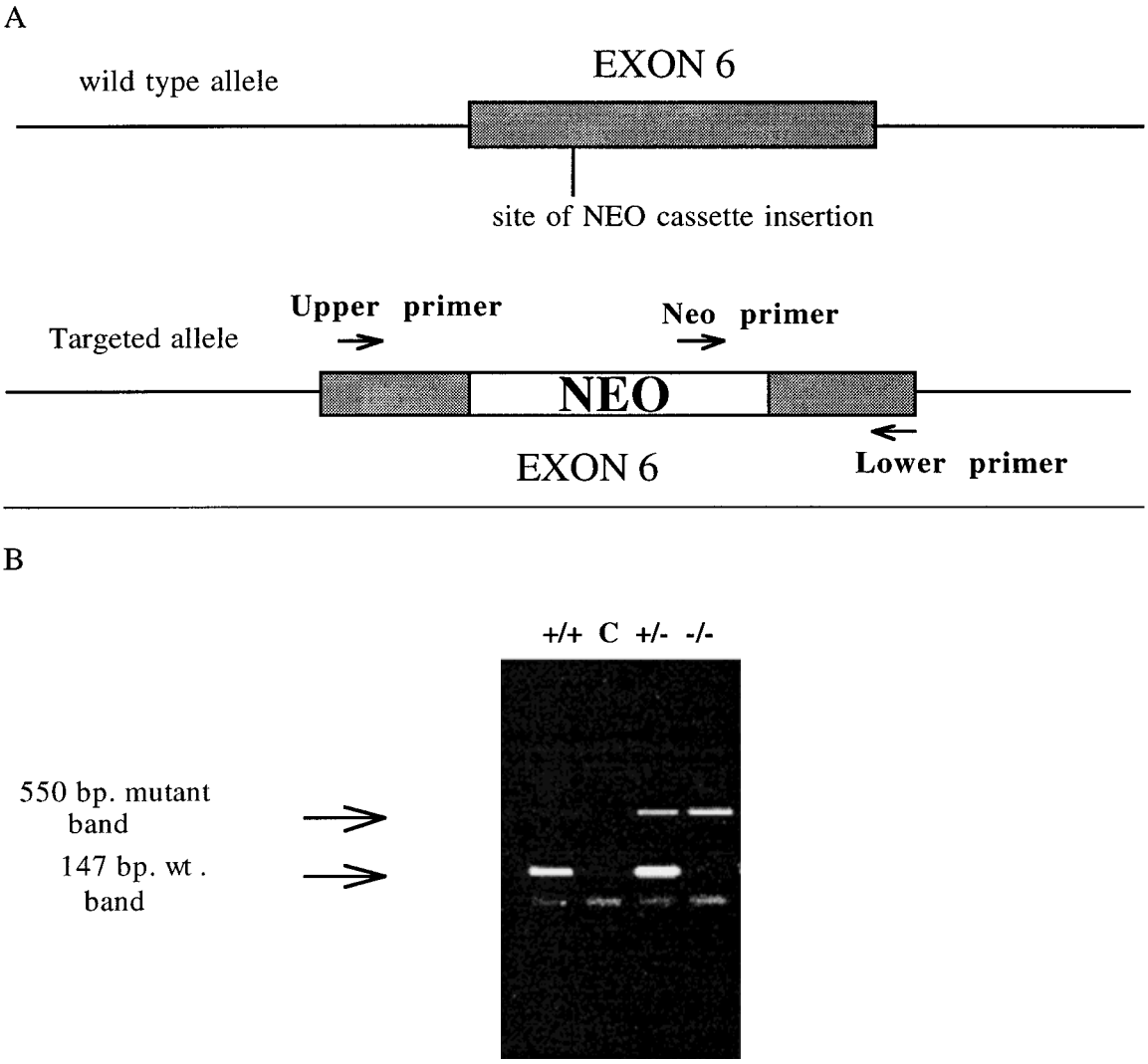

Fig. 1. Scheme for PCR *Tgfb1* genotyping. (**A**) Schematic representation of the *Tgfb1* wild type and targeted allele along with the site of *Neo* gene insertion. The position of the primers for the PCR reaction is also shown schematically. (**B**) Representative PCR gel showing the three genotypes and their diagnostic bands. With the three primers, the wild type allele amplifies a 147 bp product. In the targeted allele, two products should be produced: neo primer–lower primer (550 bp) product and upper primer–lower primer (around 1250 bp) product. In practice, however, only the 550 bp band is seen, probably because this reaction is more efficient and competes out the larger product. C = negative control. +/+, +/−, and −/− correspond to *Tgfb1* genotypes.

E2.5 embryos capable of being genotyped ($P < 0.01$, χ^2 test for disintegrating vs. healthy E2.5 embryo mutant ratio), indicating that the pre-implantation lethality had already occurred prior to the E2.5 morula stage. As can be seen in Fig. 2, E2.5 *Tgfb1*$^{-/-}$ embryo morphology ranged from normal appearing morula (Fig. 2A) to disintegrating (Fig. 2B). Most of the genotypable disintegrating *Tgfb1*$^{-/-}$ embryo had disintegration of the blastomeres (Fig. 2B). In some cases the disintegration was so extensive that blastomere morphology had been completely obliterated. In rare instances intact embryos arrested at the two- or four-cell stage could be retrieved at E3.5. Of the five such embryos that could be genotyped, three were *Tgfb1*$^{-/-}$ (Table 1). Thus, the preimplantation lethality in *Tgfb1*$^{-/-}$ embryos occurs prior to the morula stage. Consequently, TGFβ1 can be essential for embryos to develop beyond the two- or four-cell stage.

In order to rule out germ cell effects, the frequencies of genotypes in pups resulting from *Tgfb1*$^{+/+}$ × *Tgfb1*$^{+/-}$ crosses involving both sexes were analyzed. As seen in Table 2, there was no under-representation of *Tgfb1*$^{+/-}$ pups when the null allele was contributed by the father or mother. In the female *Tgfb1*$^{+/-}$ × male *Tgfb1*$^{+/+}$ cross, there is actually an overabundance of *Tgfb1*$^{+/-}$ pups. The reason for this observation is not clear. Nonetheless, these results support the hypothesis that germ cell effects do not play a role in the observed *Tgfb1*$^{+/-}$ and *Tgfb1*$^{-/-}$ lethality. Therefore, these data suggest that there is a requirement for TGFβ1 during

TABLE 1. Embryonic Lethality of *Tgfb1*$^{-/-}$ Embryos in 129 × CF-1 Strain*

Gestational age	+/+	+/−	−/−	Total
Post natal d 3–5	309	475 (1.54)	129 (0.42)	913
E 7.5d	24	31 (1.29)	10 (0.42)	65
E 3.5d (Blastocyst)	142	187 (1.32)	69 (0.49)	398
E 2.5d (Healthy morulae)	Combined	+/+ & +/− 150	21	171
E 2.5d (Disintegrating embryos)	Combined	+/+ & +/− 24	11	35
E 3.5d (Two- to four-cell stage arrested embryos)	Combined	+/+ & +/− 2	3	5

*Genotypic frequencies of concepti resulting from mating of *Tgfb1*$^{+/-}$ mice in the 129 × CF-1 strain (F_2–F_n) is shown at various gestational ages. The presence of a vaginal copulatory plug was regarded as day 0.5 p.c. (D0.5). The genotypic frequencies at E2.5 (healthy embryos), E3.5, E7.5 and D3–5 are not statistically different for the +/+:+/− and +/+:−/− ratio ($P > 0.05$, χ^2 test). Blastocyst morphology appeared grossly normal. The ratio of *Tgfb1*$^{-/-}$ to total, or *Tgfb1*$^{-/-}$ to (*Tgfb1*$^{+/+}$ and *Tgfb1*$^{+/-}$) between healthy vs. disintegrating E2.5 embryos is significant ($P < 0.01$, χ^2 test). The figures in parentheses are the ratio of the respective genotype to *Tgfb1*$^{+/+}$, which is considered to be 1, and show the deviation from the expected Mendelian ratios. Thus the partial embryonic lethality in *Tgfb1*$^{-/-}$ embryos occurs before morula stage in the 129 × CF-1 strain. 'E' refers to embryonic gestation. +/+, +/− and −/− correspond to *Tgfb1* genotypes.

pre-implantation development in about half of 129 × CF-1 mice prior to the morula stage.

Transplacental Transfer of TGFβ1 Does Not Rescue Pre-Implantation Embryos From Lethality

Since the lethality in 129 × CF-1 *Tgfb1*$^{-/-}$ embryos is only about 50% penetrant, it is possible that transfer of TGFβ1 from the mother to fetus might rescue some of the *Tgfb1*$^{-/-}$ embryos. Indeed, such a transplacental transfer of TGFβ1 has been demonstrated to occur (Letterio et al., 1994), but it is unclear whether it has a significant effect on embryogenesis of *Tgfb1*$^{-/-}$ mice. *Tgfb1*$^{+/-}$ mice express only half the amount of *Tgfb1* (by steady state mRNA analysis) as *Tgfb1*$^{+/+}$ animals in all of the tissues studied (Shull et al., 1992; Kulkarni et al., 1993). To discern the effect of supplementation of TGFβ1 during embryogenesis, two-cell embryos were obtained from crosses of *Tgfb1*$^{+/-}$ mice, transferred to *Tgfb1*$^{+/+}$ pseudopregnant females, and pups born to these were genotyped. As seen in Table 3, the ratio of *Tgfb1*$^{-/-}$ to *Tgfb1*$^{+/+}$ pups from over 100 offspring was no different than that of pups born from *Tgfb1*$^{+/-}$ crosses in natural matings within the same genetic mix. Thus, transplacental passage of TGFβ1 is not responsible for rescuing some of the embryos from the pre-implantation lethality.

TGFβ1 Is Required During Embryogenesis in a Strain-Dependent Manner

Since we observed that there is a partially penetrant embryonic lethality in *Tgfb1*$^{-/-}$ embryos, we hypothesized that this phenotype would be modified by the background genetic mouse strain. Therefore we crossed *Tgfb1*$^{+/-}$ mice onto various background strains (Table 4). In the CF-1 backcross generations and the C3H backcross generations only about 50% of the mutants and 75% of the heterozygotes are born. Successive backcrossing through these generations, does not change the ratio. On the other hand, in C57BL/6 backcross generations, a partially penetrant embryonic lethal phenotype in the F_2–F_n generations is converted to nearly complete embryonic lethality by backcrossing only one generation (N_2). In the N_2 Balb/c backcross generation, the ratio of *Tgfb1*$^{-/-}$ to *Tgfb1*$^{+/+}$ pups is intermediate between the expected Mendelian frequency and those seen after one backcross onto the CF-1 strain. It is possible that in other strains, the absence of TGFβ1 might result in uniform embryonic lethality. In the mixed NIH/Ola × C57BL/6J/Ola × 129 background, partially penetrant embryonic lethality was observed at around day 10.5 pc (D10.5) due to defective yolk sac vasculogenesis and/or hematopoiesis (Dickson et al., 1995). In CF-1 backcross generations, the partially penetrant embryonic lethality occurs before implantation. Thus, both the extent and mechanism of embryonic lethality in *Tgfb1*$^{-/-}$ embryos is background-dependent. This observation also highlights the need to analyze phenotypes resulting from gene knockout experiments in different background strains.

Development of *Tgfb1*$^{-/-}$ Embryos in a *Tgfb1*$^{-/-}$ Environment

The role of transplacental transfer of TGFβ1 to the embryo can be studied by analyzing offspring developing in a *Tgfb1*$^{-/-}$ mother. Unfortunately, immunocompetent *Tgfb1*$^{-/-}$ animals succumb to a multifocal inflammatory pathology and die around weaning age (Shull et al., 1992; Kulkarni et al., 1993). To circumvent this problem we have increased the longevity of these mice by immunosuppression achieved either by chronic anti-LFA-1 antibody injection or by placing the *Tgfb1* mutation on a severe combined immunodeficiency (SCID) background (Diebold et al., 1995). The immunosuppressed *Tgfb1*$^{-/-}$ mice routinely live to adulthood. Although nearly all *Tgfb1*$^{-/-}$ females are infertile (unpublished observations), an occasional female (<1%) has become pregnant. One case involved a healthy immunodeficient (SCID) *Tgfb1*$^{-/-}$ female that had mated with a *Tgfb1*$^{+/-}$male. Neither the homozygous mutant nor heterozygous offspring displayed any evidence of cardiac or gross congenital malformations (Diebold et al., 1995). We have now extended our observations in at least two *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$ females. Their four 129 N_4 *Tgfb1*$^{-/-}$ pups also appeared normal at birth (not shown) and autopsy at 3–3.5 months

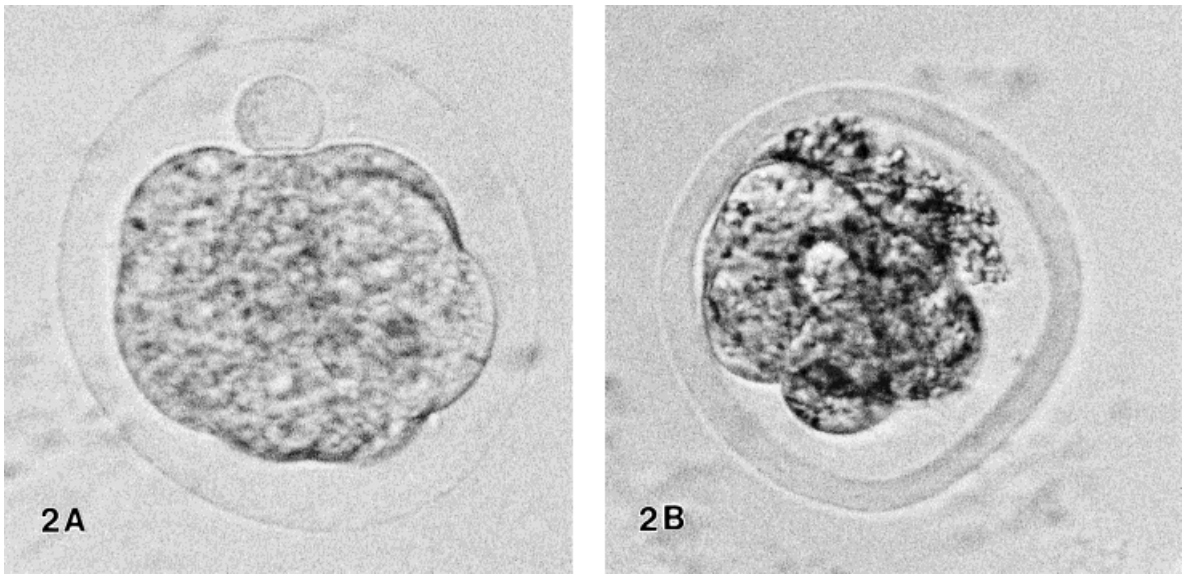

Fig. 2. Morphology of *Tgfb1*$^{-/-}$ E2.5 embryos. Representative photographs using Hoffman modulation contrast system (Nikon Diaphot microscope) of E2.5 embryos genotyped as *Tgfb1*$^{-/-}$ is shown. **A** shows a normal appearing morula whereas **B** shows a disintegrating embryo. Note that some of the blastomeres appear to be intact whereas some have disintegrated. In some embryos with extensive degeneration it was not possible to discern any blastomeres (not shown). Enlargement 630×.

TABLE 2. Pups Born to *Tgfb1*$^{+/+}$ × *Tgfb1*$^{+/-}$ Matings in 129 × CF-1 Strain*

Mating scheme	+/+	+/−	Total
Tgfb1$^{+/+}$ male × *Tgfb1*$^{+/-}$ female	99	82	181
Tgfb1$^{+/+}$ female × *Tgfb1*$^{+/-}$ male	71	102	173

*Genotypic frequencies of pups resulting from mating of *Tgfb1*$^{+/-}$ and *Tgfb1*$^{+/+}$ mice in the 129 × CF-1 (F_2–F_n) strain is shown. The frequency of *Tgfb1*$^{+/-}$ pups in male *Tgfb1*$^{+/+}$ × *Tgfb1*$^{+/-}$ female matings is not statistically different from the expected Mendelian frequency ($P > 0.05$, binomial test using normal approximation). The frequency of *Tgfb1*$^{+/-}$ pups is more than the expected Mendelian ratio in *Tgfb1*$^{+/+}$ female × *Tgfb1*$^{+/-}$ male matings ($P < 0.05$, binomial test using normal approximation). Thus there is no under-representation of *Tgfb1*$^{+/-}$ genotypes in pups at birth. +/+ and +/− correspond to *Tgfb1* genotypes.

TABLE 3. Transfer of Two-Cell Embryos From *Tgfb1*$^{+/-}$ Matings in C3H × 129 × CF-1 Backgrounds to *Tgfb1*$^{+/+}$ Uteri*

Embryo transfer/breeding	+/+	+/−	−/−	Total
Embryo transfer				
Donor:				
[C3H × (129 × CF-1)				
F1]F1 × C3H	39	50 (1.28)	17 (0.44)	106
Natural breeding				
[C3H × (129 × CF-1)				
F1]F1 × C3H	166	294 (1.77)	50 (0.3)	510

*Comparison between frequency of genotypes of pups resulting from *Tgfb1*$^{+/-}$ crosses in natural matings vs. embryo transfer at two-cell stage into a *Tgfb1*$^{+/+}$ foster uterus is shown. Donor animals for embryo transfer experiment and those for natural breeding belong to C3H × 129 × CF-1 (approx. 75% C3H, 12.5% 129 and 12.5% CF-1). The recipient animals for embryo transfer experiment were *Tgfb1*$^{+/+}$ ICR mice. The frequency of genotypes of pups resulting from natural breeding are not statistically different from those resulting from embryo transfer for the +/+:+/− and +/+:−/− ratio ($P > 0.05$, χ^2 test). A total of 640 embryos were implanted, resulting in the birth of 106 pups (17%). +/+, +/− and −/− correspond to *Tgfb1* genotypes.

revealed no developmental or teratogenic defects except for one animal with a testicular teratocarcinoma which is common in strain 129 mice. These results combined with those from the embryo transfer experiments described earlier suggest that maternal transfer of TGFβ1 to the fetus does not explain why some embryos survive embryonic lethalities and live to weaning age.

DISCUSSION

We demonstrate here that on a predominantly CF-1 background about 50% of *Tgfb1*$^{-/-}$ embryos die before implantation, and that this is independent of maternal TGFβ1 that is known to be present in serum and other fetal tissues. Data presented here shows that TGFβ1 is required for development of embryos prior to the morula stage in CF-1 backcross mice. Furthermore, TGFβ1 appears to be essential for development of embryos past the two- or four-cell stage. This is consistent with *Tgfb1* expression (Rappolee et al., 1988; Paria et al., 1992). Development of the embryos up to this stage probably occurs due to maternal transcripts. It has been demonstrated that in mouse embryos, transcription starts at the two-cell stage and the resulting proteins are generally thought to be responsible for subsequent embryonic development (Flach et al., 1982). It has previously been demonstrated that in a predominantly C57BL/6J/Ola background, embryonic lethality occurs before or-

TABLE 4. Strain Dependency of Embryonic Lethality in *Tgfb1*$^{-/-}$ Embryos*

Strain	Viable offsprings +/+	+/−	−/−	Total
129 × CF-1 (F_2–F_n)	309	475 (1.54)	129 (0.42)	913
CF-1 (N_2)	123	230 (1.85)	46 (0.38)	399
CF-1 (N_5)	40	68 (1.7)	14 (0.35)	125
CF-1 (N_6)	57	108 (1.89)	19 (0.33)	184
129 × C57BL6 (F_2–F_n)	70	200 (2.86)	38 (0.54)	308
C57BL6 (N_2–N_6)	31	51 (1.65)	0 (0.0)	82
C3H × 129 × CF-1 SCID (F_2–F_n)	563	926 (1.64)	266 (0.47)	1755
C3H SCID (N_2)	166	294 (1.77)	50 (0.3)	510
C3H SCID (N_3)	57	81 (1.42)	14 (0.25)	152
C3H SCID (N_5)	27	60 (2.2)	13 (0.48)	100
BALB/c (N_2)	57	107 (1.88)	44 (0.77)	208

*Genotypic frequencies of pups resulting from mating *Tgfb1*$^{+/-}$ mice in various strains is shown. The targeted *Tgfb1*$^{-/-}$ allele was in 129 mouse strain (D3 embryonic stem cell). F1 hybrids were generated as follows: 129 × CF-1, 129 × C57BL/6 and 129 × Balb/c. These F_1 mice were backcrossed in the respective strains to generate the N_2 to N_6 animals as indicated in the table. Thus, CF-1 animals at the N_2 generation are approximately 75% CF-1 and N_5 generation animals are approximately 97% CF-1. C3H line was propagated as follows: 129 × CF-1 animals were crossed with C3H animals to produce F_1 C3H animals (approx. 50% C3H, 25% CF-1, 25% 129), formally designated as [C3H × (129 × CF-1)F_1]F_1 × C3H. These were then backcrossed to C3H animals to yield successive C3H backcross generations designated here as N_2–N_5. The figures in parentheses are the ratio of the number of animals of the respective genotypes to the number of *Tgfb1*$^{+/+}$ animals, with the latter being set at 1.0. This ratio shows the deviation from the expected Mendelian ratios. +/+, +/− and −/− correspond to *Tgfb1* genotypes.

ganogenesis (prior to E9.5) (Bonyadi et al., 1997), and that on the mixed NIH/Ola × C57BL/6J/Ola × 129 background about 50% of *Tgfb1*$^{-/-}$ embryos die at the yolk sac stage from defects in the vasculature and hematopoietic precursors (Dickson et al., 1995). Since we show here that in a predominantly CF-1 background about half of the *Tgfb1*$^{-/-}$ embryos succumb to a pre-implantation lethality, we suspect that the pre-organogenesis lethality shown by Bonyadi and coworkers (1997) also occurs before implantation. The yolk sac lethality correlates with studies showing high levels of *Tgfb1* expression in yolk sac hemangioblast cells (Akhurst et al., 1990a), the likely precursors of endothelial and hematopoietic cells (Choi et al., 1998).

It is unclear why approximately 50% of *Tgfb1*$^{-/-}$ embryos develop normally through gestation. At least two explanations are possible: (1) maternal rescue (Letterio et al., 1994; Dickson et al., 1995) and (2) genetic modifiers (Bonyadi et al., 1997). Maternal rescue could be inferred by studying development of *Tgfb1*$^{-/-}$ embryos in a *Tgfb1*$^{-/-}$ mother or by transfer of embryos into a foster *Tgfb1*$^{+/+}$ mother. Although *Tgfb1*$^{-/-}$ males and females have greatly impaired fertility, five pregnancies that resulted in live births have been reported (Letterio et al., 1994; Dickson et al., 1995; Diebold et al., 1995; this report). In the latter two cases, where the females were immunodeficient (*Tgfb1*$^{-/-}$ *Scid*$^{-/-}$ and *Tgfb1*$^{-/-}$ *Rag2*$^{-/-}$), did not suffer from a multifocal inflammatory disease, and were not treated with dexamethasone, neither homozygous mutant nor heterozygous pups had any detectable congenital malformations at birth or at autopsy (18 days to 3.5 months). In another approach, *Tgfb1*$^{-/-}$ embryos were transferred to *Tgfb1*$^{+/+}$ foster mothers in whom there was no deficiency in maternal TGFβ1. This did not result in an alteration in the extent of *Tgfb1*$^{-/-}$ embryonic lethality (Table 3). In similar experiments in which the null *Tgfb1* mutation was on a different background (NIH/Ola × C57BL/6J/Ola × 129) (Dickson et al., 1995), transfer of two-cell stage embryos from *Tgfb1*$^{+/-}$ crosses to a *Tgfb1*$^{+/+}$ foster mother also did not reduce the degree of embryonic lethality in the *Tgfb1*$^{-/-}$ embryos. Thus, the results from both embryo transfer experiments and *Tgfb1*$^{-/-}$ pregnancies do not support a significant role for maternal TGFβ1 in rescuing the partially penetrant *Tgfb1*$^{-/-}$ embryonic lethalities.

There are several possible explanations for the seemingly contradictory results between the studies of Letterio et al. (1994) and Dickson et al. (1995) showing a maternal transfer effect in preventing congenital defects in *Tgfb1*$^{-/-}$ offspring from *Tgfb1*$^{-/-}$ mothers, and our studies (Diebold et al., 1995; this report) which do not. First, some strains may develop congenital defects in the absence of maternal rescue. It may be noteworthy that in the two experiments in which developmental defects were found, the homozygous mutant mother was a NIH/Ola × C57BL/6J/Ola × 129 background mixture, whereas in the other two cases the genetic background of the pregnant mothers were different, namely, a mixture of 129, C3H, and CF-1 (Diebold et al., 1995) or predominantly 129 (this report). Second, maternal inflammatory cytokines could lead to teratogenic effects. Cytokines have been recognized to play an important role during pregnancy. Injection of TNF-α, IFN-γ, and IL-2 in pregnant mice increased fetal resorptins in a gestational age- and dose-dependent manner (Chaouat et al., 1990). Conversely, injection of IL-3, GM-CSF, and IL-10 reduced resorption rates in the abortion-prone CBA/J × DBA/2 mating combination (Chaouat et al., 1990, 1995). The osteopetrotic CSF-1 null mutant mouse *csfm*^{op}/*csfm*^{op} has impaired fertility (Pollard et al., 1991). T_H1 cytokines such as IL-2, TNF and IFN-γ can lead to fetal compromise and demise whereas some other cytokines are beneficial to pregnancy (Wegmann et al., 1993; Robertson et al., 1994). TGFβ1 is known to be a potent regulator of local cytokine production (Tsunawaki et al., 1988) and the multifocal inflammation in the *Tgfb1* null animals causes a systemic upregulation of T_H1 cytokines (Shull et al., 1992). These studies suggest the possibility of cytokine-induced teratogenicity in the fetuses of TGFβ1 null mothers. Third, dexamethasone could have teratogenic effects (Pratt et al., 1984; Abbott et al., 1992). What is clear from these studies is that at least on some genetic backgrounds there is no evidence that maternal transfer of TGFβ1 rescues *Tgfb1*$^{-/-}$ offspring from a

pre-implantation embryonic lethality or from congenital birth defects.

A major codominant modifier gene of *Tgfb1*$^{-/-}$ embryo lethality was mapped to proximal mouse chromosome 5, using a genome scan for non-Mendelian distribution of alleles in *Tgfb1*$^{-/-}$ neonatal animals that survive a prenatal yolk sac stage lethality (Bonyadi et al., 1997). The nature of the gene and/or its products is not yet known. This study was performed in the NIH/Ola, C57BL/6J/Ola, and F1 concepti. In the F1 concepti, the embryonic lethality was at the yolk sac stage (around E10.5); however, in the C57BL/6J/Ola background the embryonic lethality had already occurred prior to E9.5. This is consistent with our findings that in the CF-1 embryos lethality occurs before implantation. Backcrossing the *Tgfb1*$^{-/-}$ allele into C57BL/6 (N_2–N_6) converts the partial embryonic lethality to a complete lethality; whereas, when backcrossing into CF-1 (N_2–N_6) or C3H (N_2–N_5), this conversion to complete *Tgfb1*$^{-/-}$ embryo lethality is not seen. Only 20% embryonic lethality was seen in *Tgfb1*$^{-/-}$ Balb/c (N_2) embryos. This phenotype is consistent with the 10–20% embryonic lethality seen in the NIH/Ola background (Bonyadi et al., 1997). Thus, it is possible that additional modifiers of *Tgfb1* gene function might influence the phenotype in null embryos.

A limited number of preimplantation lethalities have been described in mice. Mutations in the *albino* (c) locus such as homozygous c^{25H} causes preimplantation lethality at the 2- to 6-cell cleavage stage (Lewis 1978; Nadijcka et al., 1979; Rinchik et al., 1993). Although the *albino* locus and *Tgfb1* are both on chromosome 7, the two loci are not linked. Disruption of a 2 cM region (putative *mdn* locus) on mouse chromosome 1 causes decompaction of morula in homozygous mutants (Cheng and Costantini 1993). At least two mutations in the *t* complex (t^{12} and t^{32}) on chromosome 17 affect the transition from morula to blastocyst in homozygous mutant embryos (Hillman and Hillman, 1975). Tail short mutation (*Ts*) on chromosome 11 also causes abnormal development of morulae in a recessive manner (Paterson, 1980). The *Os* mutation (oligosyndactyly) causes metaphase arrest beginning at early blastocyst stage (Paterson, 1979) (Magnuson and Epstein, 1984). Homozygous recessive mutations in the Agouti locus (lethal non agouti a^x and lethal yellow a^y) causes defective blastocyst development (Papaioannou and Mardon, 1983; Papaioannou, 1988). Targeted disruption of the DNA repair gene Rad 51 causes nonviability in ES cells in a cell-autonomous manner (Tsuzuki et al., 1996). Monosomy for several chromosomes (Epstein and Travis 1979; Magnuson et al., 1982, 1985) and an XO chromosomal defect (Burgoyne and Biggers, 1976) causes lethality of embryos between the eight-cell and blastocyst stages. The absence of TGFβ1 at least in some genetic backgrounds causes a partial lethal phenotype in preimplantation development, prior to the morula stage. Knowledge of these mutations is important since it is estimated that 25% or more of human embryos are lost very early in pregnancy, possibly masquerading as subfertility (Hakim et al., 1995; Ellish et al., 1996).

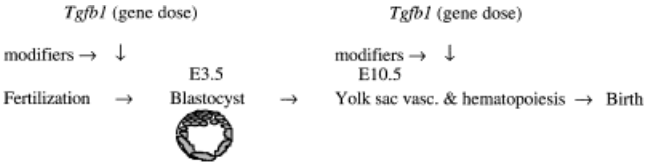

Fig. 3. Requirement for TGFβ1 during murine embryogenesis. Data presented here show the requirement of TGFβ1 during pre-implantation development of CF-1 embryos. Since the lethality of *Tgfb1*$^{-/-}$ embryos is only partially penetrant, the phenotype is likely influenced by gene modifiers. The less than expected Mendelian ratio of *Tgfb1*$^{+/-}$ pups born to *Tgfb1*$^{+/-}$ crosses suggests that the partial embryonic lethality is dependent on the gene dose. The partial embryonic lethality of *Tgfb1*$^{-/-}$ embryos and the influence of modifiers on the yolk sac vasculature and hematopoiesis were previously shown in hybrid NIH × C57BL-6 × 129 mice (Dickson et al., 1995; Bonyadi et al., 1997).

What then is the role of TGFβ1 during embryogenesis? Based on our experiments and the reported literature we propose a model for TGFβ1 function during embryogenesis (Fig. 3). TGFβ1 acts at two distinct phases during embryogenesis—pre-implantation development and yolk sac vasculogenesis/hematopoiesis. The susceptibility and the precise mechanism of lethality depend on two sets of genetic modifiers. The modifiers acting at these two phases of embryogenesis may be the same or different. Additionally, since there is about 25% lethality in *Tgfb1*$^{+/-}$ embryos, *Tgfb1* gene dosage seems to be important in preventing embryonic lethality, though a maternal contribution of TGFβ1 does not have any effect. Consequently, there is a threshold effect for TGFβ1 during critical windows of development.

Dramatic strain dependencies in mutant phenotype were also seen in the EGF-receptor (*Egfr*) and bone morphogenetic protein-4 (*Bmp4*) knockout mice (Sibilia and Wagner, 1995; Threadgill et al., 1995; Winnier et al., 1995). In *Egfr* knockout mice the cause of lethality was either peri-implantation, placental defects, or postnatal death due to multiorgan failure (Threadgill et al., 1995). Ablation of *Bmp4*, a member of the *Tgfb* super gene family, even caused variability in type of embryonic lethality within the same litter (Winnier et al., 1995). Our experiments combined with those of others (Dickson et al., 1995; Bonyadi et al., 1997) demonstrate that *Tgfb1* knockout mice also have multiple background-dependent embryonic lethalities, and point to the importance of interpreting the phenotype of a knockout mouse in the context of genetic background.

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