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RAPID COMMUNICATION

Disruption of the Mouse Thioredoxin Gene

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Thioredoxins belong to a widely distributed group of small proteins with strong reducing activities mediated by a consensus redox-active dithiol (Cys-Gly-Pro-Cys). Thioredoxin was first isolated as a hydrogen donor for enzymatic synthesis of deoxyribonucleotides by ribonucleotide reductase in *Escherichia coli.* Recent studies have revealed a variety of roles that thioredoxin plays in transcription, growth control, and immune function. In this report, we describe the phenotype of mice carrying a targeted disruption of the thioredoxin gene (*Txn*). Heterozygotes are viable, fertile, and appear normal. In contrast, homozygous mutants die shortly after implantation, and the concepti were resorbed prior to gastrulation. When preimplantation embryos were placed in culture, the inner cell mass cells of the homozygous embryos failed to proliferate. These results indicate that *Txn* expression is essential for early differentiation and morphogenesis of the mouse embryo.

INTRODUCTION

Thioredoxins are widely distributed small proteins that mediate not only redox but also nonredox reactions. Their active consensus sequence (Cys-Gly-Pro-Cys) are conserved among all species and the two Cys residues can be reversibly oxidized to participate in the redox reactions (Holmgren, 1985). Thioredoxin was originally discovered as a hydrogen donor for ribonucleotide reductase in *Escherichia coli* (Laurent *et al.*, 1964), and multiple *in vitro* substrates have been identified. Some nonredox functions of thioredoxins have also been suggested (Mark and Richardson, 1976). Recent studies have demonstrated that thioredoxin plays multiple roles in transcription, growth control, and immune function (Yodoi and Uchiyama, 1992) and is involved in the eucaryotic cell cycle, including yeast (Muller, 1991), *Drosophila* (Salz *et al.*, 1994), and *Xenopus* (Hartman *et al.*, 1993). It has been reported that thioredoxin is highly expressed in the lumen of the female genital tract (Fujii *et al.*, 1991; Kobayashi *et al.*, 1995), and it released early mouse embryos from the two-cell block *in vitro* (Natsuyama *et al.*, 1992). Here we report the generation of thioredoxin-targeted mice and their analysis in relation to early embryonic development.

MATERIALS AND METHODS

Construction of a Replacement Vector

A targeting vector (Fig. 1a) was constructed from a 129/Sv genomic thioredoxin gene (*Txn*) fragment (Matsui *et al.*, 1995). The *Bam*HI–*Xba*I (0.9 kb) and *Bam*HI (6.2 kb) fragments were placed downstream and upstream of the PGKneo cassette (Soriano *et al.*, 1991), respectively. Finally, the internal ribosomal entry site (IRES)/DTApoly(A) cassette (Yagi *et al.*, 1990) was inserted at the downstream end.

Gene Targeting in ES Cells

ES cells (1.6×10^7; D3a2 clone; Shull *et al.*, 1992) were electroporated with the linearized targeting vector. Homologous recombi-

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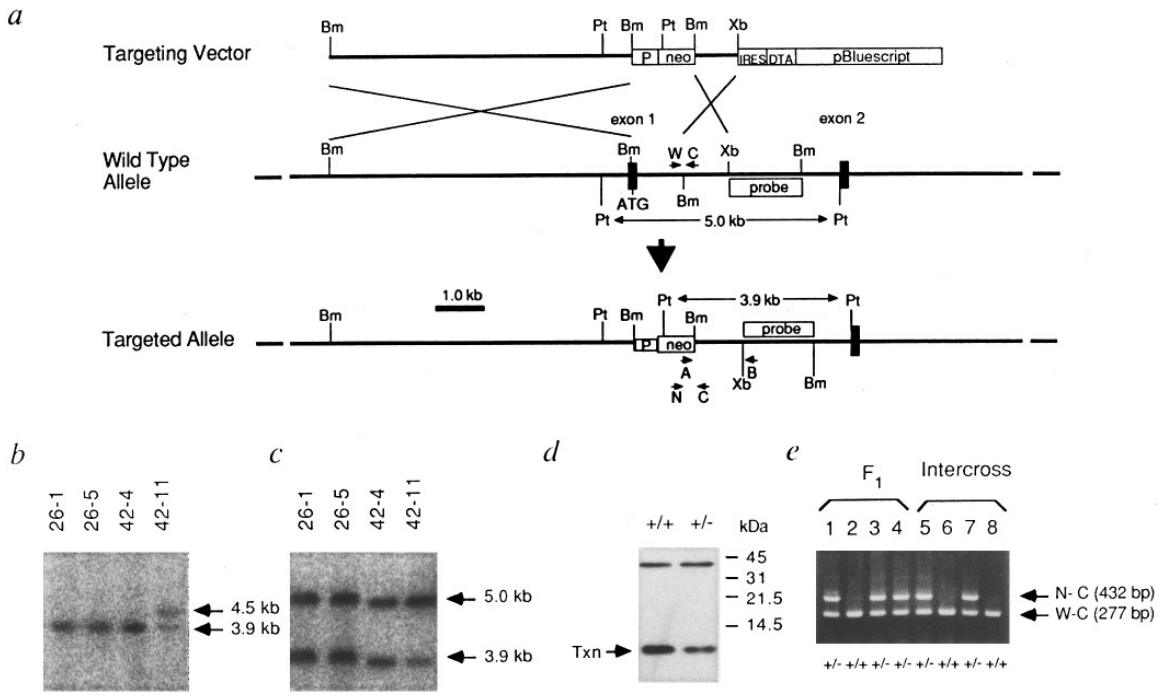

FIG. 1. Generation of *Txn* knockout mice. (a) Targeting strategy by homologous recombination in ES cells. Targeting vector pTxnKO contained the *neo* gene and the diphtheria toxin α-subunit gene (DTA; Yagi *et al.*, 1990) driven by the phosphoglycerate kinase I promoter (P). The former is for the positive selection, whereas the latter for the negative selection. Arrowheads A and B indicate the PCR primers used for homologous recombinant screening, whereas C, N, and W were for genotyping. *Pst*I sites (Pt) relevant to the identification of homologous recombinant ES cell clones are shown together with the expected sizes of the fragments hybridizable to the *Txn* and the *neo* probes. (b, c) Southern blot confirmation of the homologous recombinant ES cell clones (Shull *et al.*, 1992). (b) Hybridization with the *neo* probe showing a 3.9-kb band specific to the targeted allele. In clone 42-11, an additional 4.5-kb band was found, which was derived from a random integration of the targeting vector. (c) Hybridization with the *Txn* probe showing a 5.0-kb band derived from the wild-type full-length allele and a 3.9-kb band specific to the targeted allele. (d) Western analysis of thioredoxin expression in the parental and the *Txn*-targeted ES cells. An arrow shows the 12-kDa band corresponding to thioredoxin. An additional band of unknown identity was also shown at around 40 kDa. (e) Transmission of the targeted allele to the progeny as determined by PCR. Homozygotes are embryonic lethal. Four F_1 and four intercross offsprings are shown. One of F_1 and two of intercross pups showed a single band amplified from the wild-type allele, whereas the rest showed an additional band specific to the targeted allele.

nant candidates were screened by PCR of 35 cycles (60 sec at 94°C, 60 sec at 55°C, 60 sec at 72°C) using primers A (5′-GCCTTCTAT-CGCCTTCTTG-3′) and B (5′-AAGCACATCACAATTTTTAGC-3′) to amplify a 0.9-kb fragment. The PCR mixture consisted of 4 μM of each primer, 200 m*M* dNTPs, 2 m*M* $MgCl_2$, and 0.5 U *Taq* polymerase in Taq buffer (GIBCO) in a final volume of 20 μl. The candidate clones for homologous recombinants were further verified by Southern hybridizations (Figs. 1b and 1c). Chimeras were generated by injecting the ES cells into C57BL/6 blastocysts (Oshima *et al.*, 1995) and transferred to pseudopregnant MCH females (CLEA Japan, Tokyo).

Anti-mouse Thioredoxin Polyclonal Antibody

An oligopeptide of 10 residues corresponding to the C-terminus of the mouse thioredoxin was conjugated to keyhole limpet hemocyanin (KLH). The antigen was suspended in saline and emulsified by mixing with an equal volume of complete Freund's adjuvant. It was injected into three to four subcutaneous dorsal sites of New Zealand white rabbits, 3–9 months old, for the primary immunization. Subsequent immunizations were performed using incomplete Freund's adjuvant. After immunization three times, the animals were bled from the auricular artery. The blood was allowed to clot and serum was collected by centrifugation. The polyclonal antibody reactive with the oligopeptide was further purified by an affinity column with the antigen peptide coupled to Affi-Gel 10 (Bio-Rad).

Western Analysis

Cells were lysed in solubilizing buffer containing 0.5% Nonidet P-40. After boiling for 5 min in the sample buffer containing 100 m*M* dithiothreitol (DTT) and 2% sodium dodecyl sulfate (SDS), 10 μg protein per lane was separated in a 15% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, MA) by electroelution. After blocking in 5% BSA and 5% skim milk, the membrane was incubated with 1 μg/ml anti-mouse thioredoxin antibody. Horseradish peroxidase-conjugated anti-rabbit

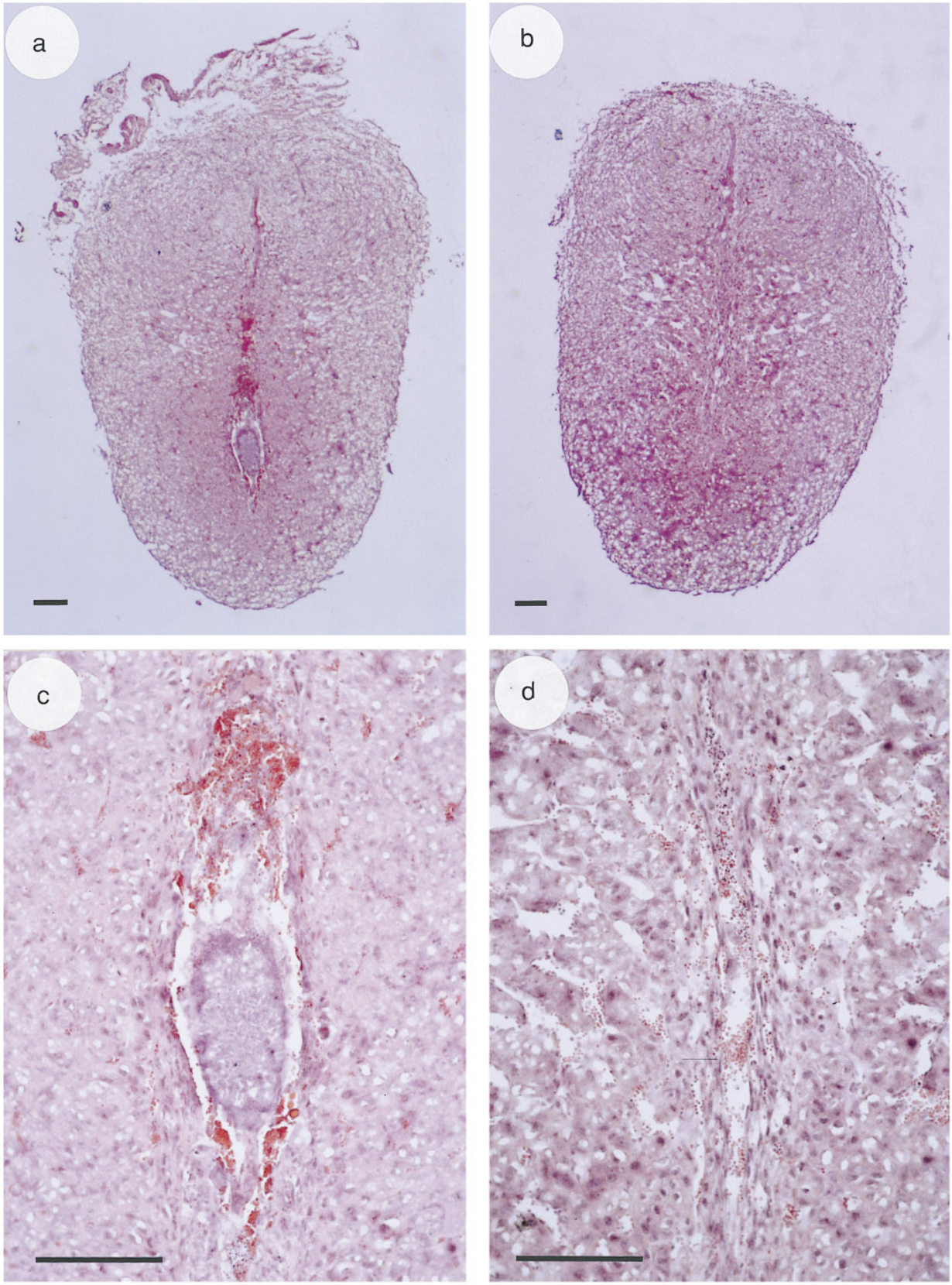

FIG. 2. Histological sections of heterozygous (a, c) and presumptive homozygous (b, d) *Txn* embryos grown *in utero*. The decidual swellings of *Txn*(+/−) intercross females were isolated at 6.5 dpc., and 10-μm sections were prepared as described under Materials and Methods. Bars, 100 μm.

TABLE 1
Genotype Analysis of *Txn*(+/−) Intercross Progeny

DNA source	Genotype +/+	+/−	−/−	Unknown	Total
4-Week old	19	31	0	0	50
12.5 dpc	6	8	0	4[a]	18
8.5 dpc	4	17	0	9[a]	30
3.5 dpc	6	10	0	20[b]	36
Total	38	68	0	34	140
Backcross	23			3[a]	26

Note. dpc, days postcoitum.
[a] Resorptions.
[b] Eight were abnormal in appearance at 3.5 dpc. Six were in the blastocyst stage but failed to hatch or to attach to the feeder layer. Two showed poor trophoblast outgrowths. Four ICMs produced no PCR products upon genotyping; backcross offspring (either wild type or heterozygous) were not genotyped.

IgG (Amersham, UK) was used as the secondary antibody. The bands reactive with the antibodies were visualized by the horseradish peroxidase–ECL method (Amersham, UK).

Genotyping

DNA from adult tail and from embryos at 12.5 and 8.5 days postcoitum (dpc) was extracted by phenol and chloroform from proteinase K-digested samples. For DNA preparations of embryos at 6.5 dpc, ICM was scraped from frozen sections and digested with proteinase K. PCR amplifications were performed using three primers in a single reaction mixture for 30 cycles (60 sec at 94°C, 60 sec at 55°C, 60 sec at 72°C); primer C (5′-ACACCGCTCCTATTGGAATG-3′), primer N (5′-GCACGTACTCGGATGGAAG-3′), and primer W (5′-TGGAACCAGAGCCTGTTACA-3′). PCR conditions are described above.

Histological Analyses

Decidual swellings dissected at 6.5 dpc were fixed overnight in 4% formaldehyde in phosphate-buffered saline and embedded in O.C.T. compound (Miles, U.S.A.). The frozen sections were prepared at 10 μm thickness and stained with hematoxylin and eosin.

Culture of Preimplantation Embryos

Embryos were flushed from uteri at 3.5 dpc. They were cultured on a mouse embryonic fibroblast feeder layer in Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and incubated at 37°C in 5% CO_2 in air.

RESULTS AND DISCUSSION

Thioredoxin is encoded by a single gene (*Txn*) on mouse chromosome 4 (Taketo *et al.*, 1994). As shown in Fig. 1a, a 1.1-kb *Bam*HI fragment of the mouse *Txn* gene (Matsui *et al.*, 1995) including the translation start codon was deleted and replaced with a phosphoglycerate kinase I promoter/*neo* cassette (PGKneo; Soriano *et al.*, 1991), by homologous recombination in embryonic stem (ES) cells (Shull *et al.*, 1992). We used a diphteria toxin α subunit gene, DT-A, as a negative selection marker. In contrast to using the herpes simplex virus thymidine kinase gene, homologous recombinants are selected against random integration clones without using a nucleoside analog mutagen such as gancyclovir. Of 96 neomycin-resistant ES cell clones, three independent clones were identified as homologous recombinant candidates by PCR and verified by Southern analysis (Figs. 1b and 1c). Thioredoxin (12 kDa) was expressed at a significant level in the parental ES cells but its level decreased to about a half in the *Txn*(+/−) ES cells (Fig. 1d). A band of 40 kDa detected in the Western blot is a nonspecific cross-reaction with a protein unrelated to thioredoxin (Nakamura *et al.*, 1994). Upon injection into C57BL/6 blastocysts, the recombinant ES clone (26-1) contributed to chimeric males which transmitted the targeted allele to the germ line (Fig. 1e). A mutant mouse line was established by backcrosses with C57BL/6, i.e., in a mixture of the 129/Sv and C57BL/6 backgrounds.

Genotyping of the progeny derived from intercrosses of the *Txn*(+/−) mice showed that 19 of 50 pups were wild type, while the remaining 31 were heterozygous (e.g., see Fig. 1e and Table 1). We examined their general condition, macroscopic organ appearance upon autopsy, behavioral activities, and body weight; they were not significantly different between the *Txn*(+/−) mice and the wild-type littermates. The absence of homozygous mutants, with heterozygous and wild-type progeny obtained at the ratio of approximately 2:1, suggested that homozygosity for the *Txn* null mutation resulted in an embryonic lethality.

To determine the time of the embryonic death, embryos from heterozygous intercrosses were genotyped at 8.5 and 12.5 days of gestation. As shown in Table 1, all of them were either wild type or heterozygous. At these stages, approximately a quarter of decidual swellings were empty and the majority of them were likely to be derived from implantations of homozygous blastocysts because few empty decidua were found in *Txn*(+/+) × (+/−) backcrosses (3 in 26; Table 1).

To investigate the morphology of implanted embryos derived from *Txn*(+/−) intercrosses, decidual swellings were sectioned at 6.5 dpc and genotyped by PCR of the DNA extracted from the embryonic tissues collected from the specimens. All embryos that showed a normal growth, i.e., elongation of the egg cylinders, were either wild type or heterozygous (Figs. 2a and 2c). In contrast, the embryos proper were not found in about a quarter of the decidua, nor could the tissues be recovered for genotyping. These embryos showed a severe resorption with a trace of residual pyknotic cells (Figs. 2b and 2d) as seen in the decidua at 8.5 and 12.5 dpc. Accordingly, a significant population of them were presumed to be *Txn*(−/−). These results suggest that *Txn*(−/−) embryos induced the decidual reaction upon im-

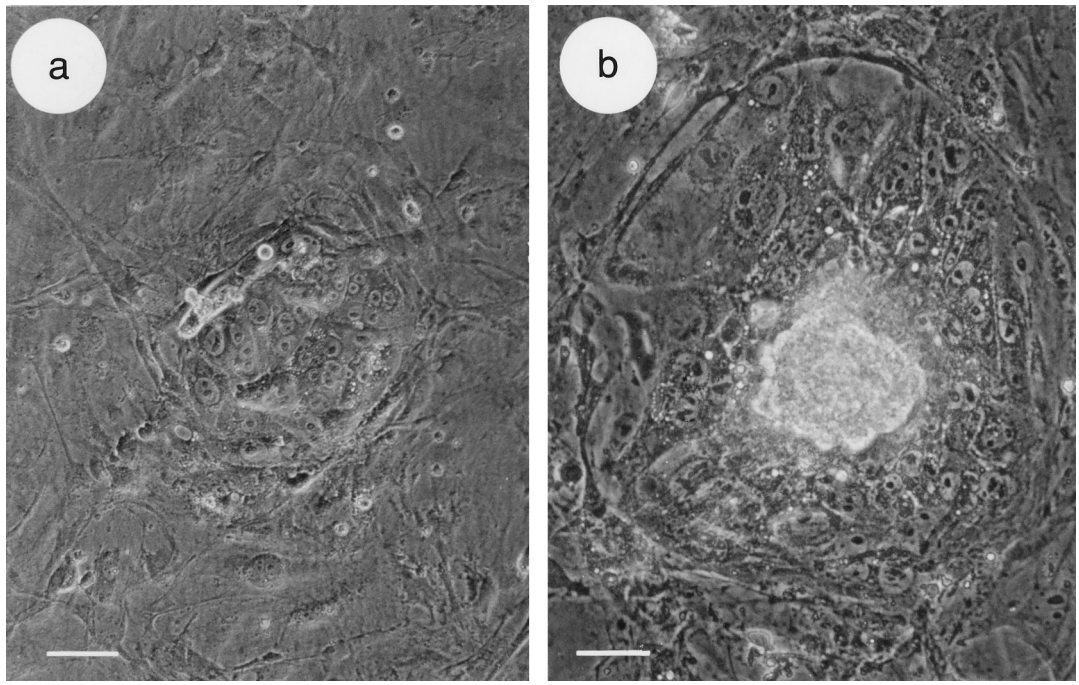

FIG. 3. Outgrowths of presumptive *Txn*(−/−) and wild-type embryos on feeder layers *in vitro.* (a) A trophectoderm outgrowth without an ICM cell proliferation after 5 days in culture, which was presumed to be *Txn*(−/−). (b) A trophectoderm outgrowth with an ICM cell proliferation after 4 days in culture. This ICM was genotyped to be the wild type. Bars, 100 μm.

plantation, but the embryos proper died as early as the egg cylinder formation stage.

To investigate this phenomenon further, 36 preimplantation embryos were collected by *Txn*(+/−) intercrosses at 3.5 dpc. Eight of them already showed a variety of retarded morphology. When 28 embryos at morula or blastocyst stage were placed into an *in vitro* culture, 6 did not hatch out of zona pellucida or their trophoblasts could not attach to the feeder layer as outgrowths. Two embryos showed poor trophoblast outgrowths, but ICM cells could not proliferate (Fig. 3a). The remaining 20 embryos showed normal outgrowths and their ICM cells proliferated well (Fig. 3b); these embryos were found to be either wild type or heterozygous (Table 1).

It appears that the *Txn*(−/−) embryos showed a variety of defects *in vitro,* ranging from the failure to hatch from the zona to form trophoblast outgrowths. Moreover, the ICM cells could not proliferate although the trophoblasts appeared to induce the decidual reaction. These results strongly suggest that the growth of *Txn*(−/−) embryos is affected at the peri-implantation stage.

Many mutants have been reported to die immediately after implantation (Vankin and Caspari, 1979; Spiegelman *et al.,* 1976; Lewis *et al.,* 1976; Spyropoulos and Capecchi, 1994; Haegel *et al.,* 1995), where the decidual reaction in the uterus is characteristic of blastocyst implantation (Copp, 1995). Among these, β-catenin null mutant embryos develop to the egg cylinder stage but die at the gastrulation stage (Haegel *et al.,* 1995). Such early lethalities as degeneration at the egg cylinder formation stage are usually due to failures in establishing the normal connection between the trophoblast and the decidua (Vankin and Caspari, 1979; Spiegelman *et al.,* 1976; Lewis *et al.,* 1976). One of the mutants that cause a peri-implantation lethality is *evx1* knockout (Spyropoulos and Capecchi, 1994). Presumptive *evx1* (−/−) embryos elicit a decidual response and invade the uterine wall, but fail to differentiate extraembryonic tissues or to form the egg cylinder; a similar phenotype to that of the *Txn*(−/−) embryos. However, the *evx1*(−/−) blastocysts appear normal and, when placed in culture, the trophoblast outgrowth and ICM cell proliferation occur almost normally. In contrast, the *in vitro* outgrowth appears to be defective in the *Txn*(−/−) embryos.

One possible explanation for the early lethality of *Txn*(−/−) embryos is impaired DNA replication after maternal thioredoxin in the embryo is lost. Important roles of thioredoxin in DNA synthesis and normal progression through the cell cycle have been reported in lower organisms such as *E. coli* (Laurent *et al.,* 1964), yeast (Muller, 1991), as well as in *Xenopus* (Hartman *et al.,* 1993). Trophectoderm appeared more resistant to thioredoxin depletion than ICM cells as seen in the *in vitro* outgrowth and in the implantation ability of the presumptive *Txn*(−/−) embryos. Because of their rapid proliferation, ICM cells are much more sensitive to metabolic inhibitors than trophoblast cells. Exposure of blastocysts *in vitro* to X-ray or ultraviolet

irradiation interferes with the proliferation of the ICM cells but has little effect on the trophoblast outgrowth (Rowinski *et al.*, 1975). These irradiations generate reactive oxygen species (ROS) which cause damage to all major classes of biological macromolecules. Because thioredoxins scavenge ROS (Mitsui *et al.*, 1992) and act as a hydrogen donor for thioredoxin-dependent peroxide reductases in various mammalian cells (Chae *et al.*, 1994), it is possible that the failure to elaborate thioredoxin in *Txn*(−/−) mice makes the ICM cells more vulnerable to ROS induced by normal levels of metabolites and/or irradiations. In this respect, it is interesting that thioredoxin can release the two-cell block of mouse fertilized eggs *in vitro* (Natsuyama *et al.*, 1992). The ROS produced by a higher oxygen concentration than that *in vivo* (Maas *et al.*, 1976) may be responsible because a lower oxygen tension and/or an addition of superoxide dismutase (SOD) release the two-cell block (Umaoka *et al.*, 1992). A thioredoxin-related activity, ''early pregnancy factor phenomenon,'' is detected within hours after fertilization and remains during the two-thirds of pregnancy (Clarke *et al.*, 1991). Moreover, thioredoxin is expressed in the ovarian interstitial cells, ciliated cells of fallopian tubes, and endometrium (Fujii *et al.*, 1991; Kobayashi *et al.*, 1995). Such a specific distribution of thioredoxin may help protect the eggs from oxidative stress. It is worth noting that *Drosophila* thioredoxin functions as a maternal protective factor to embryos rather than an essential protein for cell viability (Salz *et al.*, 1994).

Mammalian cells elaborate many anti-oxidative systems such as SOD, catalase, glutaredoxin (Luthman *et al.*, 1979), selenoproteins (Böck *et al.*, 1991; Stadtman, 1994), and thiol-specific antioxidant (TSA; Chae *et al.*, 1994). Deficiency in catalase (Takahara, 1952) or extracellular SOD (Carlsson *et al.*, 1995) does not affect mammalian development. It may appear that such redundant mechanisms ensure the optimal redox states for various biological reactions in a fail-safe manner. We recently constructed selenocysteine $tRNA^{[Ser]Sec}$ gene (*Trsp*) knockout mice and found that homozygous mutant embryos die in the peri-implantation stage (Bösl *et al.*, to be submitted). Interestingly, however, the *Trsp*(−/−) blastocysts proceed to the trophoblast outgrowth followed by ICM cell proliferation *in vitro*. These findings, taken together, suggest that each reducing system carries out a particular function essential for ontogeny in a specific spaciotemporal manner.

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