

# Trophoblast Stem Cells Rescue Placental Defect in SOCS3-deficient Mice\*

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Stem cells have important clinical and experimental potentials. Trophoblast stem (TS) cells possess the ability to differentiate into trophoblast subtypes *in vitro* and contribute to the trophoblast lineage *in vivo*. Suppressor of cytokine signaling 3 (SOCS3) is a negative regulator of cytokine signaling. Targeted disruption of SOCS3 revealed embryonic lethality on E12.5; it was caused by placental defect with enhanced leukemia inhibitory factor receptor signaling. A complementation of the wild-type (WT) placenta by using tetraploid rescue technique showed that the embryonic lethality in SOCS3-deficient embryo was due to the placental defect. Here we demonstrate that TS cells supplementation rescues placental defect in SOCS3-deficient embryos. In the rescued placenta, TS cells were integrated into the placental structure, and a substantial structural improvement was observed in the labyrinthine layer that was disrupted in the SOCS3-deficient placenta. Importantly, by supplying TS cells, living SOCS3-deficient embryos were detected at term. These results indicate a functional contribution of TS cells in the placenta and their potential application.

In mammals, trophoblast cells in the placenta are essential for the growth and survival of the embryo. Trophoblast stem (TS)<sup>3</sup> cells have been established from either blastocysts or early postimplantation trophoblasts in the presence of fibroblast growth factor 4 (FGF4) (1). These cell lines differentiated into trophoblast subtypes *in vitro* and have the potential to contribute to the placenta in chimeras *in vivo*. However, it remains to be clarified whether the *in vivo* differentiated trophoblasts from TS cells are functional.

SOCS3 is an essential negative regulator of leukemia inhibitory factor receptor signaling in trophoblast differentiation (2). Targeted disruption of SOCS3 demonstrates embryonic lethality with placental defect (2, 3). In the SOCS3-deficient placenta, an excess status of trophoblast giant cell differentiation is observed. The embryonic lethality in SOCS3<sup>-/-</sup> embryos is rescued by the complementation of wild-type tetraploid embryos, thus demonstrating an essential role of SOCS3 in placental development and a non-essential role in embryo development (2). To explore the potential of TS cells in rescuing the placental defect in SOCS3-deficient mice, we attempted to prepare a chimera by using SOCS3<sup>-/-</sup> embryo and WT TS cells.

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<sup>3</sup> The abbreviations used are: TS, trophoblast stem; FGF4, fibroblast growth factor 4; SOCS3, suppressor of cytokine signaling 3; WT, wild-type; GFP, green fluorescent protein; EGFP, enhanced GFP; TGC, trophoblast giant cell.

## MATERIALS AND METHODS

**Mice**—The generation of SOCS3-disrupted mice was as described in a previous study (2). Using tail biopsies, genotyping was performed by PCR as described. Mutant phenotypes were analyzed in a mixed 129/Sve, C57Bl/6 background.

**TS Cell Injections**—TS cell lines were derived from B5/enhanced green fluorescent protein (EGFP) transgenic mice (4) (kindly supplied by Dr. J. Rossant) that ubiquitously express EGFP. These cells had already been cultured for more than 30 passages and showed a typical colony (Fig. 1, left). TS cells were maintained in the presence of FGF4 and conditioned medium from mouse embryonic fibroblast cells (1). On E2.5, morula stage embryos were collected from SOCS3 heterozygous intercrosses, the zonae were removed, and embryos were aggregated with 10–15 TS cells in drop culture. After overnight culture, the developed blastocysts were transferred to the uteri of pseudopregnant females. In the injection method, 10–15 TS cells were injected into the blastocysts from the SOCS3 heterozygous intercrosses. Blastocysts in which only the injection medium were used as the control. After injection, 8–10 blastocysts per pseudopregnant female were transferred into the uteri. Tetraploid rescue experiments were performed as described previously (2).

**Histological Analysis**—Freshly isolated placenta were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned (4  $\mu$ m). Sections were analyzed by hematoxylin/eosin staining.

**Immunohistochemistry**—Immunohistochemical staining of cells was performed according to standard protocols as described previously (5). In brief, GFP expression in sections of the placenta was identified by overnight incubation of formalin-fixed, decalcified, paraffin-embedded sections at 4 °C with a rabbit anti-GFP antibody (1:300) (Molecular Probes, Eugene, OR). The primary antibody was visualized with a biotinylated goat anti-rabbit secondary antibody (1-h incubation, room temperature, dilution of 1:200), and peroxidase-conjugated avidin (ABC kit, Vector Laboratories) by using NovaRED (Vector Laboratories) as the substrate; counterstaining was performed with Harris hematoxylin (Surgipath Medical Industries, Richmond, IL).

## RESULTS

To produce a chimera with SOCS3<sup>-/-</sup> embryos and TS cells, we first attempted an aggregation method (6). To visualize and monitor the TS cells, we used GFP-positive TS cells. However, the ability of the TS cells to integrate into the blastocysts was poor and the ratio of chimerism in the placenta was low (less than 5%; data not shown). We were not able to detect any SOCS3<sup>-/-</sup> embryos at term among more than 100 embryos after aggregation. Next, we attempted the injection method. Ten to fifteen TS cells were injected into blastocysts from heterozygous intercrosses (Fig. 1, right). Generally, SOCS3<sup>-/-</sup> embryos die between E11.5–13.5 and after E15.5; no SOCS3<sup>-/-</sup> embryos survived (2). We then recovered embryos at term (E18.5–19.5) and analyzed their genotypes. In the control, there were no SOCS3<sup>-/-</sup> embryos on E18.5. In contrast, 5 SOCS3<sup>-/-</sup> embryos were detected, among TS-cell injected embryos ( $p < 0.001$ , compared with the control), although, one embryo was dead at term (Table 1). The average embryo weight of SOCS3<sup>+/+</sup>, SOCS3<sup>+/-</sup>, and SOCS3<sup>-/-</sup> was  $1.57 \pm 0.22$  g,  $1.59 \pm 0.14$  g, and  $1.15 \pm 0.17$  g (mean  $\pm$  S.D. of living pups), respectively. Although, the intrauterine growth of the rescued SOCS3<sup>-/-</sup> embryos was significantly impaired, four out of five pups were alive. In contrast, in tetraploid rescue experiments, the sizes of the rescued SOCS3<sup>-/-</sup> embryo were comparable with that of <sup>+/+</sup> and <sup>+/-</sup> (data not shown) (2). Next, histological analysis of the placentas was performed (Fig. 2). In the SOCS3 deficient placenta, trophoblast giant cells (TGCs) occupied in the labyrinth, and there was no intact labyrinthine layer that is essential for the exchange of oxygen and nutrition (Fig. 2, right). In contrast, in the rescued SOCS3<sup>-/-</sup> placenta, intact labyrinthine layer was detected (Fig. 2, middle, arrows) despite the presence of aberrant TGCs in the labyrinth (Fig. 2, middle, asterisk). The degree of improvement in the rescued placenta was correlated to the embryo weight (data not shown). Immunohistochemistry using anti-GFP antibody demonstrated diffuse contribution of GFP-positive trophoblasts (Fig. 3A). Particularly, many GFP-positive cells were detected in the intact labyrinthine structure (Fig. 3A, panel a, arrow) and chorion (Fig. 3A, panel c, arrow) where the trophoblast stem cells reside (7). Interestingly, some euploid TGCs were also

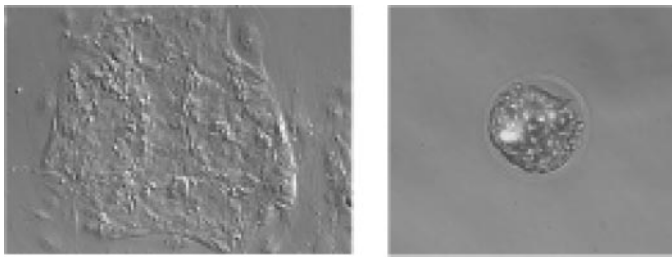


FIGURE 1. *Left*, TS cell colony in FGF4 and conditioned medium. *Right*, 10–15 EGFP-TS cells were injected into blastocysts, and GFP+ cells could be visualized under a fluorescent microscope before being transferred into the uteri of pseudopregnant females.

**TABLE 1**  
Genotyping analysis of embryos at term in TS cells rescue and tetraploid rescue experiments

WT-TS cells injected into the SOCS3<sup>-/-</sup> blastocysts significantly rescued the embryonic lethality in SOCS3<sup>-/-</sup> mice. To compare the efficiency, the ratio in tetraploid rescue experiments was also described.

	SOCS3 genotype		
	+/+	+/-	-/- (e18.5)
TS cells injection	39	59	5(1dead)
Control (vehicle injection)	47	88	0
Tetraploid rescue	21	36	8

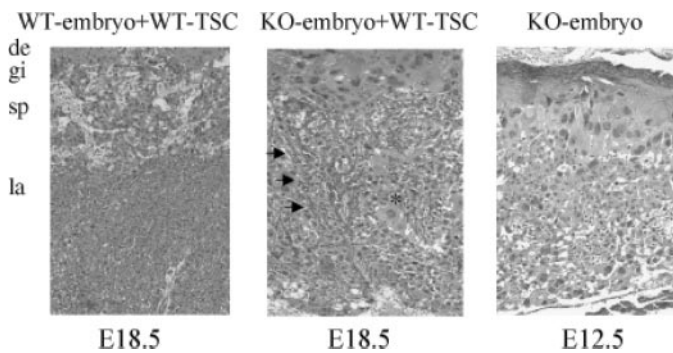


FIGURE 2. Placentas from wild-type (*left*, E18.5) and SOCS3-deficient (*right*, E12.5) embryos. The placenta from rescued SOCS3-deficient (*middle*, E18.5) embryo demonstrates partial intact labyrinthine structure (*arrowhead*) compared with SOCS3-deficient placenta (*right*). An aberrant giant cell in the labyrinth has been indicated by an *asterisk*. *de*, deciduas; *gi*, trophoblast giant cells; *sp*, spongiotrophoblast layer; *la*, labyrinthine layer.

GFP-positive (Fig. 3A, *panel b*, *arrow*), suggesting that TS cells could differentiate into TGCs in intact localization. The ratio of the contribution of GFP-positive trophoblasts to the placentas was significantly correlated to the weight of SOCS3<sup>-/-</sup> embryos ( $r = 0.96, p < 0.05$ , data not shown). No GFP-positive cells were detected in the embryos (data not shown).

## DISCUSSION

Our results provided compelling evidence that there was a functional contribution of TS cells to the placenta *in vivo*. Although, the rescued SOCS3<sup>-/-</sup> embryos demonstrated intrauterine growth retardation, indicating partial rescue of placental function comparing with tetraploid rescue experiments, the presence of SOCS3<sup>-/-</sup> embryos at term with TS cell-injected placenta clearly indicates that the TS cells support the placental function until term as well as the histological improvement in the placental structure.

It is intriguing that the integrated TS cells differentiated properly even in SOCS3<sup>-/-</sup> placenta depending on the localization in the placenta. These results imply that the aberrant differentiation in SOCS3<sup>-/-</sup> trophoblasts was a cell-autonomous effect, and its effect did not affect the programmed differentiation property.

The embryonic lethality associated with many targeted mutations in mice has been shown to involve placental defects (7). Importantly, the associated insuffi-

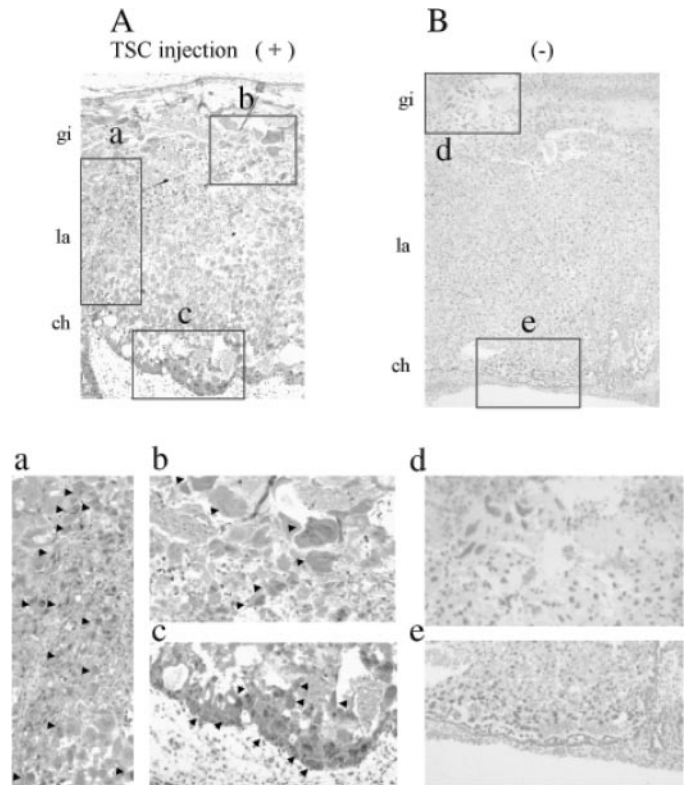


FIGURE 3. Immunohistochemistry for GFP in rescued placenta shows the integration of GFP+ trophoblast in the SOCS3-deficient placenta. *A*, GFP immunostaining in WT TS cell-injected SOCS3<sup>-/-</sup> placenta (×40). *B*, GFP immunostaining in control placenta (no TS cells injection) (×40). *Panels a–c*, the higher magnification of the squares in Fig. 3A. *Panel a*, intact labyrinthine structure. *Panel b*, GFP+ giant cells. *Panel c*, GFP+ trophoblast cells in chorion (*arrowhead* indicates GFP+ cells). Note that GFP+ cells reside particularly in the chorion and intact labyrinthine structure. *Panels d and e*, the higher magnification of the squares in Fig. 3B. *gi*, trophoblast giant cells; *la*, labyrinthine layer; *ch*, chorion.

ciencies in nutrient, gas, and waste exchange cause a secondary embryonic phenotype. In this case, tetraploid complementation rescues placental trophoblast defect and enables identification of the embryonic phenotypes that arise secondary to the defects in extraembryonic cells (8, 9). Although, the efficiency of TS cells rescue is less than that in tetraploid rescue (5 versus 12%, Table 1), TS cells rescue experiments involving simple techniques can be used for this purpose. Whether this strategy could be used for rescuing other mutations impacting placental development is an important question and further study is required in this regard. However, based on our results, and theoretical knowledge, it is strongly speculated that trophoblast stem cells could rescue other mutations with placental defect. The potential of TS cells to rescue placental defect indicates a new possible application in the treatment of placental abnormality.

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