

## Embryo Implantation is Blocked by Intraperitoneal Injection with Anti-LIF Antibody in Mice

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**Abstract.** Leukemia inhibitory factor (LIF) is essential for embryo implantation in mice and plays an important role in other mammals including humans. Intraperitoneal (ip) injections with anti-LIF antibody (7.5 µg/g body weight, 3 times) between D3 (D1 = day of vaginal plug detection) and D4 effectively blocked embryo implantation; complete inhibition was achieved in C57BL/6J mice, and implantation was dramatically reduced in ICR mice (reduced to 27%). Normal rabbit IgG used as the control did not disturb embryo implantation. Anti-LIF antibody was localized not only in the stroma, but also in the luminal epithelium and the glandular lumen after ip injections. Growth-arrested blastocysts were recovered from the uterus without any implantation sites in both strains. Blastocysts made contact with the LE on the antimesometrial side; however, uterine stromal cells did not undergo secondary decidual reaction, and the uterine lumen was open, even at D7. Several regions of decidualization in ICR mice treated with anti-LIF antibody were smaller than those of the control, and development of blastocysts was delayed. The expression of LIF-regulated genes, such as immune-responsive gene-1 and insulin-like growth factor binding protein-3, was significantly decreased in C57BL/6J mice treated with anti-LIF antibody compared with the control, but not in ICR mice. The present study demonstrated that simple ip injections of an antibody are sufficient to block one of the important factors involved in embryo implantation in mice, and this method should also be easily applicable to the investigation of other factors involved in implantation.

**Key words:** Anti-LIF antibody, Embryo implantation, Intraperitoneal injection, Leukemia inhibitory factor, Mice

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**E**mbryo implantation is an important event in early pregnancy. Synchronization between active blastocysts and maternal tissues is essential for successful implantation. In addition to the secretory molecules regulated by progesterone and estrogen, appropriate modulation of the extracellular matrix is also essential for accommodation of the embryo [1, 2].

In mice, blastocysts nidate on the receptive endometrium in response to transient estrogen stimulation at D4 (D1, the first day of pregnancy, is defined as the day when a vaginal plug is first observed) [3]. Estrogen triggers the secretion of leukemia inhibitory factor (LIF), a member of the interleukin (IL)-6 family of cytokines (such as IL-11, oncostatin M, ciliary neurotrophic factor and cardiotrophin-1), mainly from the uterine glandular epithelium (GE) [4, 5]. Targeted mutation of the *Lif* gene in mice has been shown to result in infertility [6]. In female LIF-deficient mice, blastocysts make contact with the luminal epithelium (LE) at D4, but subsequent processes, such as closure of the uterine lumen, stromal decidualization and apoptosis of the LE at the site of attachment of the blastocyst, do not occur [7]. However, implantation can be rescued by exogenous LIF, and the process of pregnancy is restored [7]. In

artificial delayed-implantation (DI) mice (established by ovariectomy and continuous progesterone priming), exogenous LIF instead of estrogen can re-initiate the implantation process [7]. Therefore, LIF is a critical factor for the initiation of embryo implantation [8, 9]. Several mammals, including humans, require LIF for embryo implantation [10–14]. Depleted LIF secretion and/or mutations of the *LIF* gene are believed to be involved in infertility in humans [15–18]. However, women with no impairment of LIF also show infertility [19, 20].

In the murine uterus, peak LIF expression is observed primarily in the GE and LE at estrus and at D4 [21, 22]. LIF binds the LIF receptor, a heterodimer of LIF receptor β (LIFRβ) and gp130, and signals are transmitted via the Jak-Stat3 pathway [23]. The expression pattern of LIFRβ is similar to that of LIF in the GE and LE at estrus and D4 [21]. At the time of implantation, gp130 is highly expressed in the LE [21]. Both LIFRβ and gp130 are upregulated in the stroma at D5 [21]. The expressions of immune-responsive gene-1 (*Irg1*), insulin-like growth factor binding protein-3 (*IGFBP3*), co-chilin and amphiregulin are raised in response to LIF [24, 25]. LIF also affects the adhesion molecules on the LE [26] that are involved in angiogenesis [27]. Agrin, a critical factor for acetylcholine receptor aggregation at neuromuscular junctions, accumulates on the apical surface of the LE in response to LIF [28]; however, the essential molecular networks that underlie LIF signaling remain to be elucidated.

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Predicted signaling networks have been examined by various techniques that block LIF signaling, including intrauterine injection with anti-LIF antibody [29, 30], LIF inhibitors [31], Stat3 decoy [32] and Stat3 inhibitors [33]; a significant reduction in the number of implantations was noted in each case. However, it is difficult to obtain quantitative data because it is not possible to establish the precise volume of the inhibitory agent that remains in the uterine horn during each experiment. The uterine lumen of the mouse is too narrow to inject more than 10  $\mu$ l, and in some cases, the agent overflows from the vagina, even when the injection volume is less than 10  $\mu$ l. A concise and reliable method is required. Because intravascularly injected exogenous antibody is detectable in the GE and LE [34], it is assumed that simple intraperitoneal (ip) injection with anti-LIF antibody could prevent embryo implantation. This method should also be easily applicable to the investigation of other factors involved in implantation. In this study, the behavior of ip-injected anti-LIF antibody on implantation was investigated.

## Materials and Methods

### Animals

All experimental procedures were performed under avertin anesthesia (Sigma-Aldrich, St. Louis, MO, USA) according to the guidelines established by the Committee for Animal Welfare at Nagoya University (approval number: 2010042803). C57BL/6J mice (purchased from Kyudo, Saga, Japan, and bred in our laboratory) and ICR mice (Japan SLC, Shizuoka, Japan) were used in the experiments at 6 to 12 weeks of age as models of low or high fertility ( $9.5 \pm 1.3$  pups/mother for C57BL/6J,  $16.1 \pm 1.6$  for ICR mice). The first day of pregnancy (D1) was determined as the morning when a vaginal plug was observed in a female that had been housed with males the previous evening. For preparation of antibody, an 8-month old male Japanese white rabbit (Kyudo) was immunized. All animals were housed at  $23 \pm 3$  C with controlled light-dark cycles (12 h light:12 h dark) and were fed *ad libitum*.

### Preparation of antibody

A vector was constructed from pET-46 (Merck, Darmstadt, Germany) with recombinant LIF (rLIF; 179 amino acid residues containing the active sites of murine LIF, but without the signal peptide) [35, 36]. This was transformed into *E. coli* BL21 (DE3), and expression of the his-tagged rLIF was induced by treatment with 0.4 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (Wako, Osaka, Japan) for 4 h at room temperature (RT). Cells were lysed by ultrasonication, and recombinant protein was purified using His-trap FF (GE Healthcare, Little Chalfont, U.K.). Subsequently, rLIF was purified by reversed-phase chromatography (TFA/acetonitrile system) with a Proteonavi apparatus (Shiseido, Tokyo, Japan). The activity of purified rLIF was assessed by administration to DI mice, and successful implantation was observed. DI mice were prepared as previously reported [27].

IgG purified with Hitrap rProtein A FF (GE Healthcare) from rabbit serum collected before immunization was used as the control. Antibody against LIF was developed by the rabbit, which was subcutaneously injected with a mixture containing rLIF (500  $\mu$ g in 500  $\mu$ l 10 mM PBS) and 500  $\mu$ l TiterMax Gold (TiterMax,

Norcross, GA, USA). Boosters were administered by intravascular injection of the antigen on 3 consecutive days (500, 1,000 and 2,000  $\mu$ g rLIF, respectively) after 4 weeks. The antibody was purified from serum collected 1 week after the final injection.

The high reactivity of purified anti-LIF antibody against rLIF was determined by ELISA. Interaction between anti-LIF antibody and rLIF is detectable above  $10^{-6}$   $\mu$ g/ml. ELISA was performed by using the following materials: TaKaRa Peptide Coating Kit (Takara, Kyoto, Japan), polyclonal goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP; dilution 1: 75,000; Bethyl, Montgomery, TX, USA), and TMB Substrate (Bethyl).

The specificity of anti-LIF antibody against natural LIF protein in mice was confirmed by Western blot analysis. Crude protein (20  $\mu$ g) from the uterine tissues at D4 was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in an 8% gel. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (45 min) using Trans-Blot SD (Bio-Rad, Hercules, CA, USA). Blocking was performed by constant shaking in 2.5% bovine serum albumin (BSA)/Tris-buffered saline with Tween-20 (TBS-T) for 1 h at RT. Membranes were probed overnight at 4 C with anti-LIF antibody (40  $\mu$ g/ml, diluted 1:1,000 in 2.5% BSA/TBS-T). Subsequently, the membrane was treated with polyclonal swine anti-rabbit antibody conjugated with HRP (Dako, Glostrup, Denmark) diluted 1:2,000 in 1% skimmed milk/TBS-T. Signals were detected using ECL Plus reagents (GE Healthcare) and a LightCapture II system (Atto, NY, USA).

### Immunohistochemistry

Mice treated with anti-LIF antibody or vehicle were perfused with paraformaldehyde (4% in 0.1 M PB) on the evening at D4. Uteri were embedded in paraffin, and 4- $\mu$ m sections were prepared. Uterine sections were deparaffinized, treated with 0.3% H<sub>2</sub>O<sub>2</sub>/methanol for 30 min, blocked with 1% BSA/PBS-T for 1 h at RT and incubated with biotinylated goat anti-rabbit antibody (Dako) diluted 1:200 overnight at 4 C. Avidin—biotin complexation (Vector Labs, Burlingame, CA, USA) was performed for 40 min at RT, and sections were visualized with 3,3'-diaminobenzidine (DAB; Dako).

### Blockage of embryo implantation

Pregnant mice were intraperitoneally injected with antibody (7.5  $\mu$ g/g body weight in 50  $\mu$ l 10 mM PBS) based on measurements taken at 1200 and 2200 h on D3 and 1000 h on D4. The same amount of normal rabbit IgG was injected as the control. Mice were sacrificed on D7, and the number of implantation sites was counted. Macroscopic changes in the pregnant uterus were observed, and the number of corpora lutea was counted using a stereomicroscope. Statistical analysis was performed by the Welch's *t*-test. The uterus was flushed with 10 mM PBS to collect blastocysts when no implantation site was identified. Uteri were fixed with phosphate-buffered formalin and embedded in paraffin. Sections (4  $\mu$ m) were made with a Microtome (Leica Microsystems, Tokyo, Japan) and stained with hematoxylin-eosin (HE).

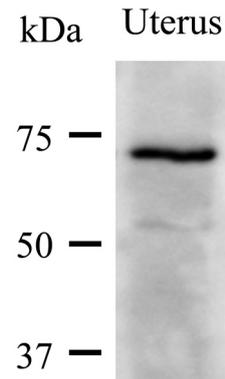
### Semi-quantitative PCR

Uterine LEs from mice treated with anti-LIF antibody (or normal rabbit IgG) were collected by an established method [37] on

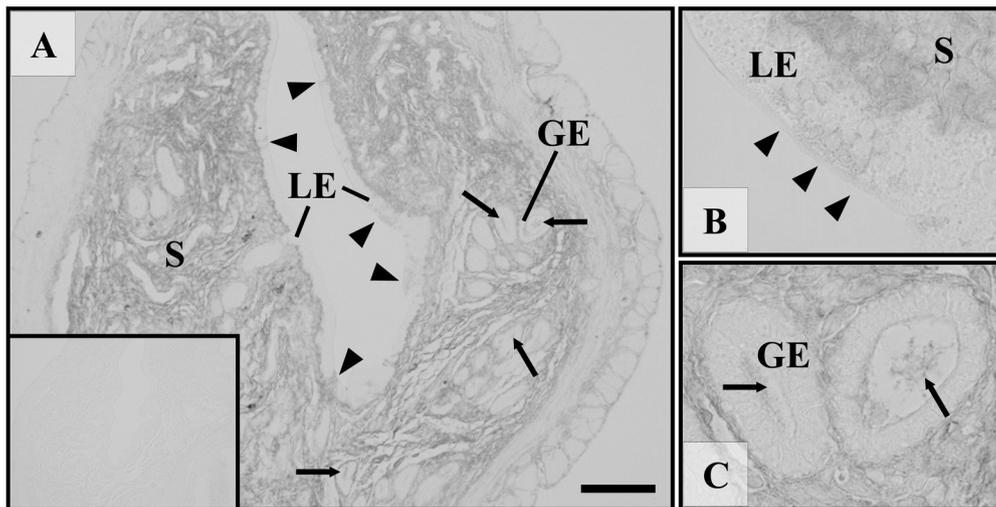
the evening at D4. In brief, LEs were incubated with 1% trypsin (Nacalai Tesque, Kyoto, Japan) in calcium- and magnesium-free Hanks' balanced salt solution (Life Technologies, Carlsbad, CA, USA) for 90 min at 4 C and 60 min at RT. The sheet-shaped LEs were collected by pipette and steadily washed with 10 mM PBS. Samples were stored at  $-80$  C. Total RNA was extracted by Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized from 1  $\mu$ g total RNA with an oligo-dT primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Real-time PCR was performed using a StepOnePlus system (Life Technologies) with SYBR Green (Life Technologies). The thermal profile was an initial denaturation step (95 C, 10 min) followed by 40 cycles of denaturation (95 C, 15 sec), annealing and extension (60 C, 1 min). The primers (*Irg1* and *IGFbp3*) used in this study have been described in a previous report [24]. Primers for cochlin were designed using Primer Express (Life Technologies): forward primer, 5'-gcaaaacctgctacaactcagtgga-3'; reverse primer, 5'-ggcggaaattgctatctcctcaa-3'. Relative expression values of mRNAs from all samples except one sample (value 1) from the control were calculated regardless of the control or anti-LIF antibody treated group. The average value from the control including value 1 and those from the anti-LIF antibody treated group were compared. Statistical analysis was performed by Welch's *t*-test or Student's *t*-test.

## Results

The specificity of anti-LIF antibody against natural LIF protein in mice was confirmed by Western blot analysis. Bands were observed at 70 and 55 kDa (Fig. 1); these bands were not present when



**Fig. 1.** Western blot analysis of uterine tissue with anti-LIF antibody. Bands are present at apparent masses of 70 and 55 kDa.



**Fig. 2.** Anti-LIF antibody detection in tissue taken on the evening of D4. Antibody is visible throughout the endometrium, in the LE (arrowheads in A and B) and on glandular luminal side (arrows in A and C). The inset (A) shows the vehicle-treated control. LE, luminal epithelium; S, stroma; GE, glandular epithelium. Scale bar, 100  $\mu$ m.

**Table 1.** The effect of anti-LIF antibody on embryo implantation

Strain	Treatment	Mice	Implantation sites	Corpora lutea
C57BL/6J	Anti-LIF antibody	6	0.0 $\pm$ 0.0*	8.2 $\pm$ 1.3
	Normal rabbit IgG	4	9.5 $\pm$ 1.3	9.8 $\pm$ 1.3
ICR	Anti-LIF antibody	9	4.4 $\pm$ 5.5*	13.7 $\pm$ 1.4
	Normal rabbit IgG	12	16.1 $\pm$ 1.6	13.4 $\pm$ 2.4

Average number  $\pm$  SD. \*,  $P < 0.01$  vs. normal rabbit IgG.

normal rabbit IgG was used; no band was observed in the absorption test using rLIF.

Anti-LIF antibody was detected throughout the endometrium 9 h after the final administration (Fig. 2). Localization of anti-LIF antibody was found in the LE (Fig. 2A and B). The few anti-LIF antibodies that were detected in GE cells were distributed in the luminal cavity (Fig. 2A and C).

The number of implantation sites was significantly decreased following injection of anti-LIF antibody, both in C57BL/6J and ICR mice (Table 1, Figs. 3 and 4). Normal rabbit IgG did not disturb embryo implantation (Figs. 3 and 4). In C57BL/6J mice treated with anti-LIF antibody, uteri were slightly reddish in color, but no visible implantation sites were detected at D7 (Figs. 3 and 4). Some blastocysts were recovered in the flushing solution from the mice treated with anti-LIF antibody (Fig. 5B). The blastocysts had already hatched from the zona pellucida, and the ectoplacental cone had formed (Fig. 5B, arrow). In uteri injected with anti-LIF antibody, blastocysts made contact with the LE at the antimesometrial side; however, uterine stromal cells did not undergo secondary decidual reaction, and the uterine lumen was open, even at D7 (Fig. 5A). In ICR mice, the average number of implantation sites in the mice with anti-LIF antibody was lower (4.4) than that for the control (16.1) (i.e., 73% reduction; Table 1, Fig. 3). Blastocysts were recovered from all mice that did not exhibit implantation sites. Development of the blastocysts in ICR mice was delayed, and the

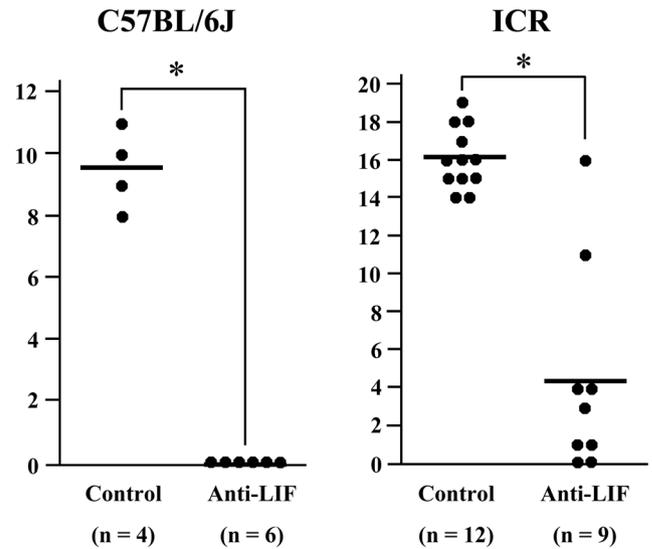


Fig. 3. Hindrance of embryo implantation by anti-LIF antibody differs between mouse strains. Horizontal lines show average values in each group. The asterisks represent the significant differences between antibody-treated mice and controls within the same strain ( $P < 0.01$ ).

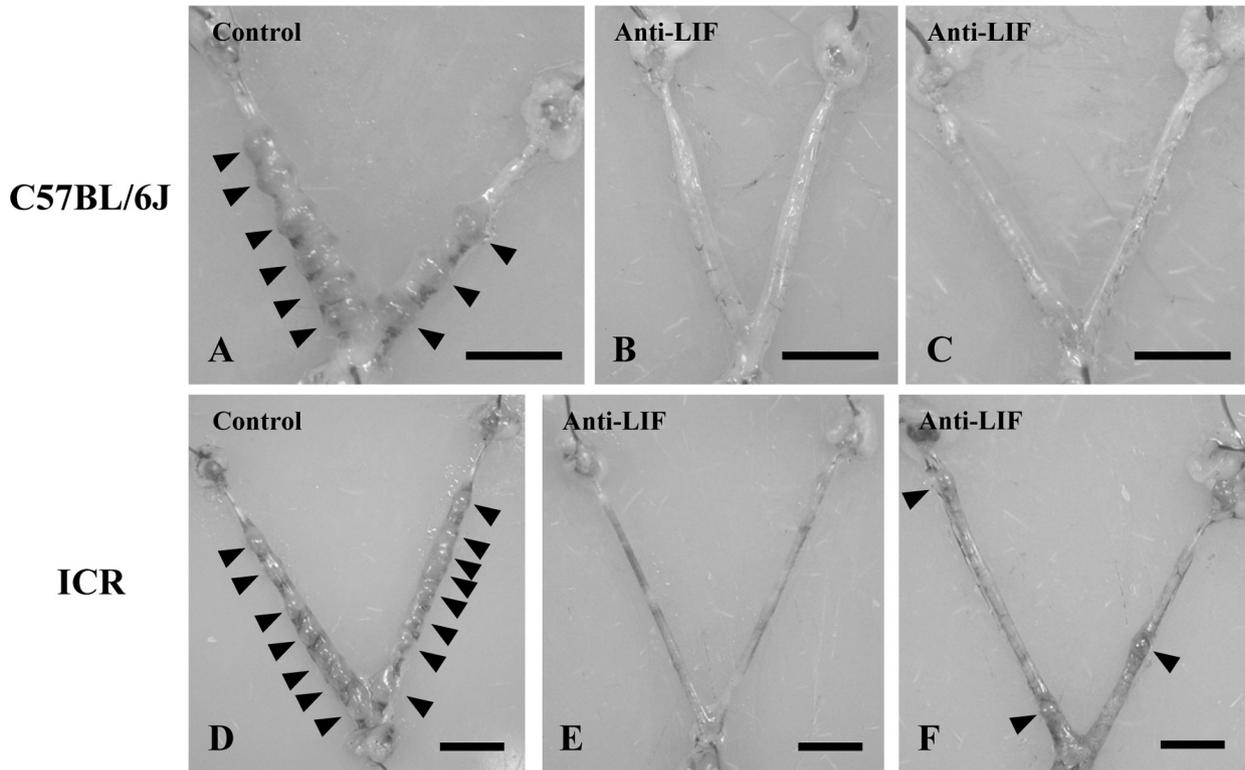
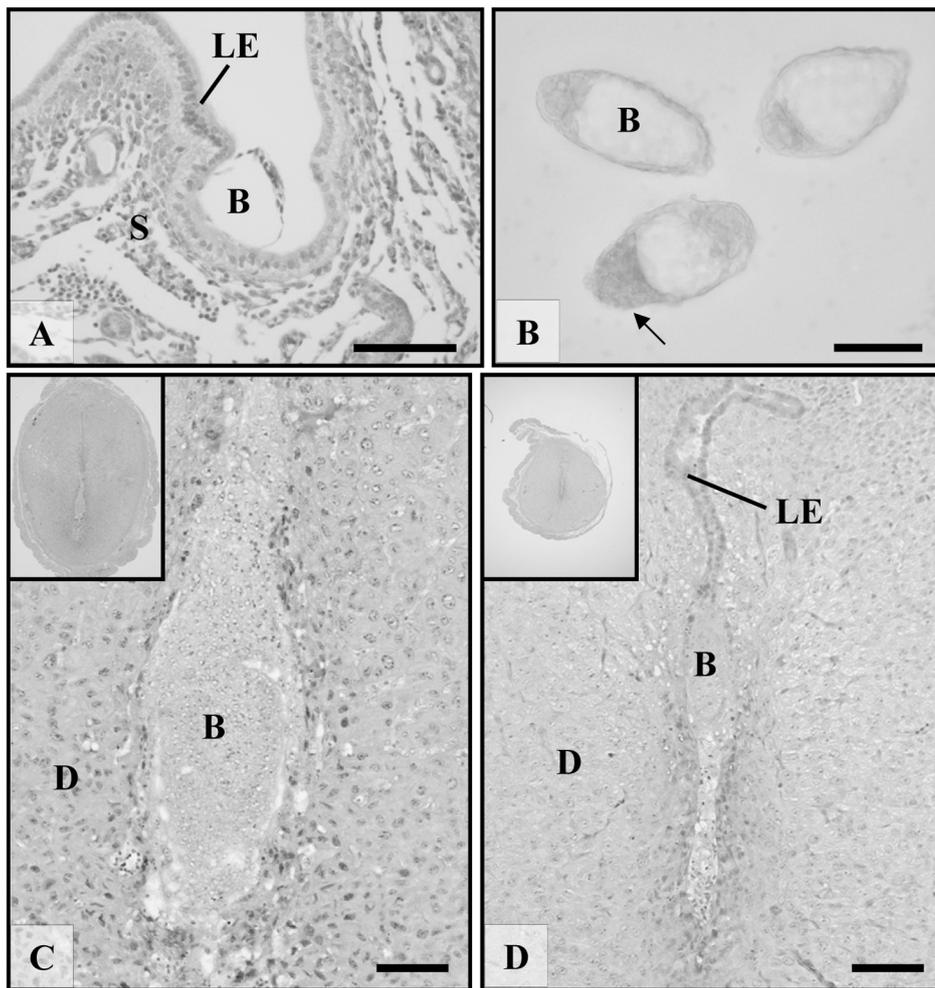


Fig. 4. Intraperitoneal injections of anti-LIF antibody interfere with embryo implantation at D7 in C57BL/6J mice (B, C) and ICR mice (E, F). Mice treated with normal rabbit IgG exhibited normal embryo implantation patterns (A, D). Arrowheads show the implantation sites. Scale bar, 5 mm.



**Fig. 5.** Blastocyst development. A: Dormant blastocyst at the surface of the LE on the antisymetrial side of the uterus. No apoptotic figures are recognized in the LE at the attachment site of the blastocyst. B: Blastocysts recovered from the uterus. The ectoplacental cone has formed (arrow). C: Control and D: anti-LIF-treated mice. The decidual zone is smaller in the latter; development of the blastocyst is delayed by anti-LIF antibody. B, blastocyst; LE, luminal epithelium; S, stroma; D, decidua. Scale bar, 100  $\mu$ m.

extent of decidualization in the endometrium was lower than that of the control in some implantation sites (Fig. 5C and D).

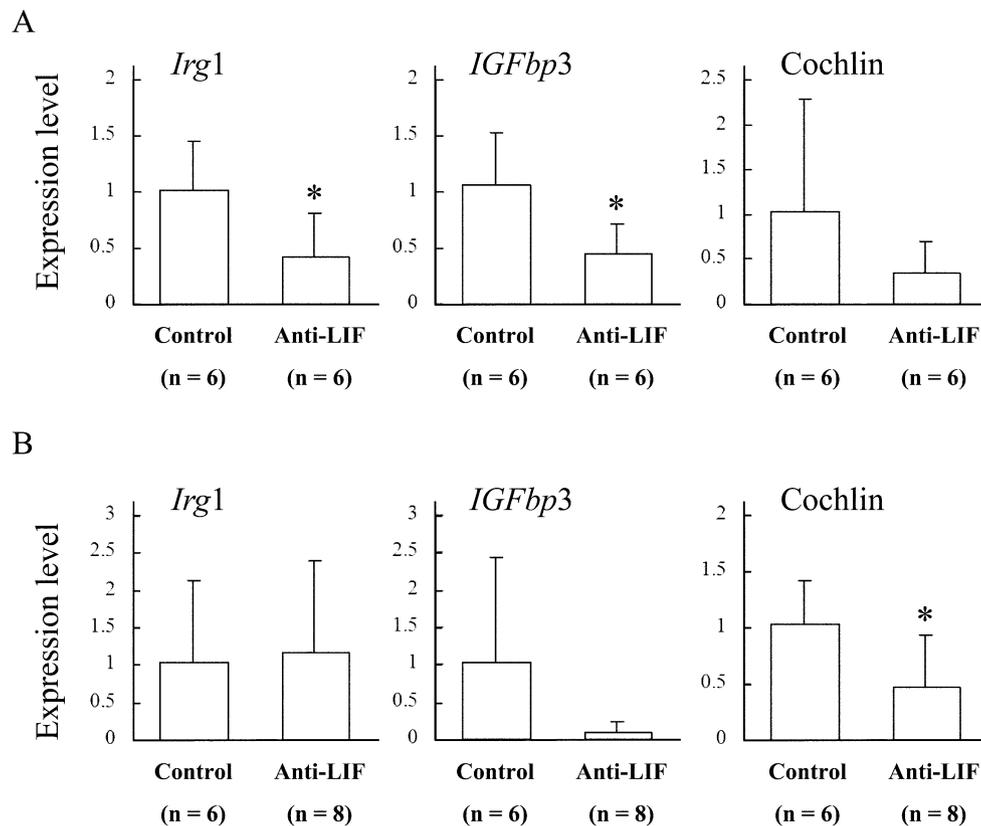
The effect of anti-LIF antibody on LIF signaling *in vivo* was confirmed by semi-quantitative RT-PCR for 3 LIF-regulated genes: *Irg1*, *IGFbp3* [24] and cochlin [25]. The expressions of *Irg1* and *IGFbp3* mRNA were significantly decreased in C57BL/6J mice treated with anti-LIF antibody compared with the control ( $P < 0.05$ ; Fig. 6A). No significant difference was observed in cochlin mRNA (Fig. 6A). On the other hand, in ICR mice, only cochlin mRNA was significantly decreased ( $P < 0.05$ ); the 2 other genes did not differ between the 2 groups (Fig. 6B).

### Discussion

The simple ip injections with anti-LIF antibody effectively blocked embryo implantation; complete inhibition was achieved in C57BL/6J mice. Because growth-arrested blastocysts were still present without the decidual response or closure of the uterine lumen at D7 (Fig. 6A and B), it is concluded that the implantation process ceased after administration of anti-LIF antibody. LIF is a glycoprotein whose molecular weight in different cells is in the range

20–70 kDa, depending on the degree of glycosylation [38–40]. The LIF protein has 3 binding sites for its receptor and can bind without any glycosylation or modification of three-dimensional structures [35]. The anti-LIF antibody used in this study bound to the binding sites of glycosylated native LIF protein (Fig. 1). It has been reported that ip injection of polyethylene glycosylated LIF antagonist (PEGLA) completely blocks embryo implantation in C57BL/6J mice [41]. The same result was obtained here by using anti-LIF antibody.

Immunohistochemistry was performed to detect anti-LIF antibody in the uterus to confirm that the antibody administered by ip injection reaches uterine tissues. The antibodies were distributed throughout the endometrium after ip injection, at least on the evening of D4 (Fig. 3A). Several cells in the LE were stained (Fig. 3B), but no strong reaction was found in the cytoplasm of the GE cells. Antibodies were also detected in the glandular lumen (Fig. 3C). Anti-LIF antibody in this study bound not only LIF in the stroma but also that in the LE and glandular lumen. An antibody administered by ip injection is taken into the blood stream through the peritoneum and diaphragm [42, 43], and its blood concentration is as high as that with intravenous injection after 15 h [42]. As blood



**Fig. 6.** Expression of *Irg1*, *IGFbp3* and cochlin in (A) C57BL/6J and (B) ICR mice. Significant differences in expression are evident between mice treated with anti-LIF antibody and the control for *Irg1* and *IGFbp3* in C57BL/6J mice. In ICR mice, only cochlin is significantly decreased by anti-LIF antibody. \*  $P < 0.05$ .

flow in the uterus is raised by progesterone and estrogen during the implantation period [44], it was assumed that antibodies were smoothly delivered to the uterine tissue. Inhibitors of LIF applied by the intraluminal route did not completely disrupt implantation in most strains of mice, apart from C57BL/6J [29–31]; this is partly because the potential of LIF to promote implantation in the stroma in these strains is not negligible.

The morphology of the blastocysts recovered from the uterus by flushing with PBS showed well-maintained and healthy structures (Fig. 6B). Both LIF and LIF receptor are expressed in blastocysts [45]; some investigations have demonstrated the effects of LIF and LIF inhibitors on growing blastocysts. LIF promotes the hatching and growth of blastocysts [46, 47], and anti-LIF antibody compromises the development of blastocysts *in vitro* [29]; no effect on blastocysts was observed in studies of PEGLA [41] and Stat3 inhibitors [33]. Because the blastocysts obtained both in LIF-deficient mice and LIF receptor-deficient mice had undergone implantation [6, 48], the implantation failure in this study is probably caused by disruption of maternal LIF action, and the direct effect of the anti-LIF antibody on blastocysts was likely limited.

Implantation was not completely disrupted in ICR mice, unlike that in C57BL/6J mice. Several regions of decidualization in ICR mice treated with anti-LIF antibody were smaller than those of the

control (Fig. 6C and D), and development of blastocysts was delayed. This showed that growth and invasion of some blastocysts into the endometrium were retarded because of prevention of LIF signaling in the LE by anti-LIF antibody. The difference in the number of implantation sites in ICR mice probably resulted from a high concentration of LIF in uterine tissues by continuous secretion of LIF from the GE and stroma. If an excess amount of LIF proteins that did not bind to anti-LIF antibody remained, additional administration of the antibody into ICR mice was performed. Even after 4 further injections of anti-LIF antibody (each 30  $\mu\text{g/g}$  mouse) to disturb implantation in ICR mice, some implantation sites were still present (data not shown). LIF is essential for murine implantation; however, the dependency of embryo implantation on LIF appears to differ among mouse strains. The LIF pathway appears to function in a strain-dependent manner in mice deficient in p53, which regulates expression of LIF [49]. Implantation failure is more severe in C57BL/6J background p53-deficient mice than in 129SV mice [49]. The activity of LIF was blocked by anti-LIF antibody in ICR mice, but other IL-6 family members might compensate for LIF activity and induce decidualization in these mice. Oncostatin M was localized to the implantation sites of wild-type mice at D5 and D6 [50]. Oncostatin M, ciliary neurotrophic factor and cardiotrophin-1 also have affinity for LIF receptors and main-

tain the pluripotency of murine ES cell in place of LIF [51–53]. Mice deficient in LIFR $\beta$  show multiple placental, skeletal, neural and metabolic disorders and succumb to perinatal death [48]; these effects are far more severe than those in LIF-deficient mice [6]. Other IL-6 family members can play important roles in growth and development of the embryo through the LIF receptor.

To assess the effect of the anti-LIF antibody, semi-quantitative RT-PCR was performed for LIF-regulated genes in the LE. The results for the ICR mice were similar to those in a previous study, in which the activity of LIF was suppressed by Stat3 inhibitor [33]. Among the 3 LIF-regulated genes in this study, only *Irg1* is thought to be an important mediator in LIF signaling for successful implantation because intrauterine injection of its antisense oligonucleotides disrupted embryo implantation [54]. *IGFBP3* and *cochlin* are usable as markers of LIF stimulation but are not essential for embryo implantation [24, 25]. Decline of *Irg1* mRNA was not observed in mice following intraluminal administration of Stat3 inhibitor, which partly blocks embryo implantation [33]. The downregulation of *Irg1* is most likely responsible for the failure of embryo implantation, and *Irg1* seems to be a reliable marker for embryo implantation by LIF stimulation. The expression of *Irg1* was significantly decreased in C57BL/6J mice treated with anti-LIF antibody, which show no implantation sites, but was not in ICR mice (Fig. 6A and B). The expression level of the above LIF-stimulated genes tended to differ between C57BL/6J and ICR mice in this study in spite of the same anti-LIF antibody treatment, regardless of whether that implantation sites were present or not. It is suggested that strain-dependent action should be considered when the LIF signaling in embryo implantation is examined.

The present study has demonstrated that simple ip injections of an antibody are sufficient to block the function of particular molecules involved in embryo implantation, especially where knockouts result in embryonic and/or neonatal lethality.

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