行政院國家科學委員會專題研究計畫　成果報告

利用反向寡核甘酸抑制小鼠原核期胚胎之血癌抑制因子表現：探討著床前期胚胎發生之基因調控

計畫類別：個別型計畫
計畫編號：NSC93-2320-B-040-066-
執行期間：93年08月01日至94年07月31日
執行單位：中山醫學大學生化暨生物科技研究所

計畫主持人：劉哲育

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 94年10月31日

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

利用反向寡核甘酸抑制小鼠原核期胚胎之血癌抑制因子表現：探討著床前期胚胎發生之基因調控

計畫類別：■ 個別型計畫 □ 整合型計畫
計畫編號：NSC 93－2320－B－040-066
執行期間： 93 年 8 月 1 日至 94 年 7 月 31 日

計畫主持人：劉哲育
共同主持人：
計畫參與人員：

成果報告類型(依經費核定清單規定繳交)：■精簡報告 □完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：中山醫學大學生物化學研究所

中 華 民 國 94 年 10 月 31 日

一、 中文摘要及關鍵詞

關鍵詞：胚胎、血癌抑制因子、胚胎著床前期

血癌抑制因子（leukemia inhibitory factor； LIF) 即屬於這些分泌激素之一。基本上對於動物和人類的研究報告，都認為 LIF 在胚胎著床上扮演重要的角色，但 LIF 是否影響著床前期的胚胎發育尚無確切研究報告指出。本研究利用 LIF antisense 核酸注入原核期鼠胚，觀察胚胎著床前發育情形。分別注入 0.25、0.5、1.0 或 2.0 fmol 的血癌抑制因子反意寡核甘酸於鼠胚中，各組生長至囊胚期的速率分別為 80.6%，83.5%，39.4%及 13.2%；而沒有注射任何物質的對照組發育至囊胚期的速率為 85.4%，由各個實驗組和對照組在囊胚期形成速率統計比較發現中、高濃度的各組（0.5，1.0 及 2.0 fmol）具有統計上的差異，其囊胚期形成速率明顯降低。為了確認胚胎生長的速率的降低是否由血癌抑制因子對影響，本研究在顯微注射血癌抑制因子反意寡核甘酸後立刻加入血癌抑制因子於培養液中，觀察胚胎生長情形，外加血癌抑制因子後可以改善注入高濃度血癌抑制因子反意寡核甘酸抑制囊胚期鼠胚形成的現象。可見 LIF 在胚胎著床前發育的角色，應被重新評估。

二、英文摘要及關鍵詞

Keyword: *embryo*, *leukemia inhibitory factor*, *pre-implantation stages*

Leukemia inhibitory factor (LIF) is an essential factor for implantation and establishment of pregnancy. However, its role in the development of pre-implantation embryos remains controversy. In this study, changes in pre-implantation embryos were determined after microinjection of LIF antisense oligonucleotide at the two-pronucleus stage. Although no significant differences were found in the percentages between the untreated group and the 0.25-fmol treated group, the 0.5- or 1.0-fmol treated groups had significantly lower percentages of embryos developed to the morula or blastocyst stage and the 2.0-fmol treated group had significantly lower percentages of embryos developed to the four-cell, morula, or blastocyst stage. No embryos developed to the four-cell stage in the 4.0-fmol treated group. Moreover, there was a decreasing trend in the levels of LIF immunoactivity with the increasing amount of LIF antisense oligonucleotide injected. The diameter of blastocysts in the 2.0-fmol treated group was significantly smaller than that in the untreated group. The blastocysts in this group had significantly lower numbers of blastomeres and cells in the inner cell mass (ICM) or trophetoderm (TE) and ICM/TE ratio. The 1.0- or 2.0-fmol treated groups had significant lower implantation rates than their corresponding control groups. In the 2.0-fmol groups with supplementing exogenous LIF, significantly lower percentages were also observed in the four-cell, morula, and blastocyst stages. However, blastocysts treated with 50 ng/ml LIF had a significant higher percentage than those in the LIF gene impaired group without LIF supplement. These results indicate that LIF is a critical factor for the normal development of embryos at the pre-implantation stages.

三、前言

Leukemia inhibitory factor (LIF) is multifunctional cytokine. It has been considered to be an essential factor for implantation and establishment of pregnancy [1, 2]. Although LIF is mainly provided by material uterus for pre-implantation embryos [3], the expression of this cytokine is only depended on the embryo in *in-vitro* fertilization.

四、研究目的

In order to distinguish the precise role of LIF at the pre-implantation stages of embryogenesis *in vitro*, we employed antisense oligos [13] to attenuate the function of LIF gene in the *in-vitro* embryos and determined the effects of this treatment to the pre-implantation development and implantation. Moreover, effects of supplementing exogenous LIF to the LIF gene impaired embryos were also investigated.

五、文獻探討

Since blastocyst implantation depends on maternal expression of LIF [4], LIF gene mutation may give rise to decreased availability or biological activity of LIF in the uterus and cause implantation failure [5]. Moreover, recombinant LIF in standard medium may not enhance in vitro human blastocyst formation but may play a role at later stages of human embryogenesis and during implantation [6]. LIF added to embryo culture medium has no major beneficial effect on the proportion of bovine embryos reaching the blastocyst stage [7]. However, LIF not only augments blastocyst formation and hatching in the embryos of mice [8, 9] and bovine [10] but also enhances the blastocyst formation rates of human embryos in a serum-free medium [11] or in a human tubal fluid [12]. These findings lead to the controversy of the necessity of supplementing LIF in the culture medium for *in-vitro* fertilization to emulate the conditions of material uterus. In order to distinguish the precise role of LIF at the pre-implantation stages of embryogenesis *in vitro*, we employed antisense oligos [13] to attenuate the function of LIF gene in the *in-vitro* embryos and determined the effects of this treatment to the pre-implantation development and implantation. Moreover, effects of supplementing exogenous LIF to the LIF gene impaired embryos were also investigated.

六、研究方法

Oligonucleotides

Morpholino oligonucleotides were provided by Gene Tools, LLC (Philomath, OR, USA). The LIF antisense oligonucleotide and nonsense oligonucleotide were 5′-GACCTTCATTATGGGCTGGACTCTA-3′ and 5′-CCTCTTACCTCAGTTACAATTTATA-3′, respectively. There were no sequences with significant similarity to the nonsense control. The LIF antisense oligo sequence was determined to be in the region (156-180) of murine LIF mRNA (GenBank Accession Number: NM_008501). It was formed within the translational starting target. The sequence had no more than 4 contiguous intrastrand base-pairs or 4 contiguous G:C pairs. Moreover, it did not contain over 36% guanines or more than 3 contiguous guanines for increasing water solubility. The antisense oligo was confirmed by the NCBI FASTA BLAST databases that the sequence is not corresponding to any other transcripts. The stability of these oligos were determined by injecting FITC-labeled preparations into mouse embryo and observed under a fluorescence microscope.

Preparation of embryos

Induction of superovulation was performed in virgin mice (6-8 weeks old) of the B6CBF1 strain by intraperitoneal injection of pregnant mare serum gonadotropin (5 IU) (Sigma, St. Louis, MO, USA) and HCG (5 IU) (Serono, Rome, Italy) 48 h later. Each superovulated mouse was then placed in a cage with a sexually mature male of the same strain overnight. Successful mating was determined by the presence of a copulation plug in the vagina. The mated female mice were sacrificed 20 h after HCG injection and zygotes were collected from the oviducts. The cumulus cells of the zygotes were removed by exposure to hyaluronidase (80 IU/ml) (Sigma). The zygotes were then placed into wells with fresh a human tubal fluid medium [14]. Embryos in the two-pronucleus (2PN) stage were obtained by incubating the zygotes in an atmosphere with 5% CO_2 at 37°C for 4 h.

Microinjection of oligonucleotides

In the experimental groups, embryos at the 2PN stage were injected by 1 pl with the LIF antisense oligonucleotide of 0.25 fmol, 0.5 fmol, 1.0 fmol, 2.0 fmol, or 4.0 fmol. In the positive control groups, the embryos were injected with the nonsense oligonucleotide (2 fmol or 4 fmol) or normal saline. Embryos in the negative control group remained untreated.

Injection pipettes (inner diameter 2 µm, outer diameter 5 µm) and holding pipettes (inner diameter 15 µm, outer diameter 80 µm) were produced using a micropipette puller (Sutter Instrument Co., Novato, CA, USA) and a microforge (Narishige Co., Ltd., Tokyo, Japan). Microinjections were performed under a phase-contrast microscope (Nikon, Ltd., Tokyo, Japan) with micromanipulators (Narishige). The oligonucleotide or normal saline was injected into the male pronucleus. The embryo was than incubated in an atmosphere of 5% CO_2 at 37°C and monitored daily using an optical microscope.

Immunocytochemistry

Embryos were recovered from the culture medium and freed of zona pellucida by brief exposure to acidic Tyrode solution (Hogan et al., 1994). After washing with phosphate-buffer saline (PBS) in triplicate, the embryos were placed onto microscopic slides and fixed in 2% formalin for 15 min. The embryos were washed in PBS and incubated in a blocking solution (10% fetal calf serum, 0.5% Tween 20, 0.02% sodium azide in PBS) for 1 h. After incubating with an affinity-purified rabbit antipeptide antibody preparation specific to LIF (1 µg/ml) (Chemicon, Victoria, Australia) at 4ºC overnight, the embryos were washed with the blocking solution for 10 min.

Immunostaining was performed using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA). The embryos were incubated with biotinylated anti-rabbit IgG (1 µg/ml) for 1 h. The embryos were then incubated with avidin-biotinylated horseradish peroxidase for 1 h. After washing with TBST buffer (50 mM Tris-HCl, 0.025% Tween 20, pH 7.8) five times, the embryos were treated with 3,3′-diaminobenzidine substrate (Sigma) for 20 min. The embryos were dehydrated through graded alcohol and mounted with glycerol. Results of immunostaining were observed using a phase-contrast microscopy. The visible staining indicated the immunoreactive LIF protein sites. Corresponding non-specific binding of embryos were incubated in parallel with the antibody preneutralized with excess antigenic peptide.

Differential staining of trophetoderm and inner cell mass

Cells in the trophetoderm (TE) and inner cell mass (ICM) of the blastocysts were counted after differential staining of the nuclei using a modified method of Piekos et al. [15]. The zona-free blastocysts were incubated at 5°C in M16 medium (Sigma) containing 10 mM trinitrobenzenesulphonic acid, 4.0 mg/ml polyvinylpyrrolidone and 0.015% Triton X-100 for 10 min. After washing in M2 medium (Sigma), the blastocysts were incubated in 0.1 mg/ml anti-dinitrophenol-BSA at 37°C for 15 min and washed again with the M2 medium in triplicate. The blastocysts were then incubated in M2 medium containing a 1:10 dilution of guinea pig complement serum (Irvine, CA, USA) and 10.0 µg/ml propidium iodide (Sigma) at 37°C for 15 min and washed in Dulbecco's PBS (Gibco) in triplicate. After fixing in absolute ethanol containing 22.0 µg/ml bisbenzimide (Sigma) at 5°C overnight, individual blastocysts were mounted in glycerol on microscopic slides and compressed manually before visualizing by epi-fluorescence using the Nikon filter blocks UV-2A and G-2A.

Embryo Transfer

Recipient female mice (8-12 weeks old, ICR strain) were prepared by mating with vasectomized males of the same strain 4 days before embryo transfer. The procedures of embryo transfer were performed according to Nagy et al. [16]. In the same recipient, 5-7 blastocysts injected with LIF antisense oligonucleotide (0.5 fmol, 1.0 fmol, or 2.0 fmol) were transferred to the right uterus horn and the same number of blastocysts treated with the nonsense oligonucleotide to the left uterus horn. The mice were sacrificed 2 days after embryo transfer. Successful implantation was verified and determined by injection of Chicago Sky Blue 6B (Sigma).

Supplement of LIF to LIF antisense oligonucleotide treated Embryos

After microinjection with 2.0 fmol LIF antisense oligonucleotide, LIF (Sigma) of 5 ng/ml, 10 ng/ml, or 50 ng/ml were added to the culture medium of the treated embryos. These embryos were incubated in an atmosphere of 5% CO_2 at 37°C and monitored daily using an optical microscope.

Statistical Analysis

Rates were expressed as percentages. Differences between blastocyst formation rates were determined using the chi-square test. The numbers and dimensions of cells were expressed as mean ± standard deviations and were compared using Student's *t* test. Changes in the

implantation rates were determined using the Kruska-Wallis test followed by a Mann-Whitneu U test. $P < 0.05$ was considered to be statistically significant.

七、結果

Effects of LIF antisense oligonucleotide on pre-implantation development

Table 1 shows the percentages of murine embryos developing into different pre-implantation stage after various treatments. There were no significant differences in the percentages of embryos developing into the two-cell, four cells, morula, and blastocyst stages among the four control groups ($P > 0.05$). No significant differences were also found in the percentages between the untreated group and the group treated with 0.25 fmol of LIF antisense oligonucleotide ($P > 0.05$). In the groups treated with 0.5 fmol or 1.0 fmol of LIF antisense oligonucleotide, significantly lower percentages of embryos were found to develop to the morula or blastocyst stage ($P < 0.05$). Significantly lower percentages of embryos treated with 2.0 fmol of LIF antisense oligonucleotide developed to the four-cell, morula, or blastocyst stage. No embryos developed to the four-cell stage in the group treated with 4.0 fmol of LIF antisense oligonucleotide.

The untreated embryos reached the 4-8 cell stage on day 3, morla stage on day 4, and blastocyst stage on day 5. The groups treated with nonsense oligonucleotide had similar developmental stages to the untreated group. However, in the embryos treated with 1.0 fmol or 2 fmol of LIF antisense oligonucleotide, the number developing into the morula or blastocyst stage was greatly reduced on days 4-5. Among those treated with 4.0 fmol of LIF antisense oligonucleotide, only 2-cell stage embryos were found on days 2-5 (Fig. 1).

Effects of LIF antisense oligonucleotide on the expression of LIF protein at different preimplantation stages

Except the blank control, the densities of immunoreactive LIF protein sites were similar in all groups on day 1. Similar densities were also observed among the untreated embryos and those injected with 2 fmol or 4 fmol nonsense oligonucleotide from day 1 to day 5. The groups treated with 1 fmol, 2 fmol, or 4 fmol of LIF antisense oligonucleotide had apparently lower densities than the control groups from day 2 to day 5. Moreover, there was a decreasing trend in the densities with increasing amount of LIF antisense oligonucleotide injected (Fig. 2).

Effects of LIF antisense oligonucleotide on morphology of blastocyts

The diameter of blastocysts derived from embryos treated with 2.0 fmol of LIF antisense oligonucleotide was significantly smaller than that in the untreated group ($P < 0.01$). Moreover, these blastocysts also had significantly lower numbers of blastomeres and cells in ICM or TE. A significantly lower ICM/TE ratio was also found in these embryos ($P < 0.01$). Although embryos treated with 1.0 fmol of LIF antisense oligonucleotide had significantly lower numbers of blastomeres and cells in ICM or TE, no significant different was found in the ICM/TE ratio between this group and the untreated embryos (Table 2).

Effects of LIF antisense oligonucleotide on implantation rate

There was no significant between the implantation rate of the blastocysts derived from the untreated and the nonsense oligonucleotide-treated embryos and between the embryos treated with 0.5 fmol of LIF antisense oligonucleotide at the two-pronucleus stage and those treated with the nonsense oligonucleotide ($P > 0.05$). However, the embryos treated with 1.0 fmol and 2.0 fmol of LIF antisense oligonucleotide had significant lower implantation rates than their corresponding control embryos treated with the nonsense oligonucleotide ($P > 0.01$) (Table 3).

Effects of supplementing exogenous LIF to LIF gene impaired embryos

Although there was no significant difference between the percentages of untreated 2PN embryos and those treated with 2.0 fmol of LIF antisense oligonucleotide developing into the two-cell stage, significantly lower percentages were found in the treated group for the development into the four-cell, morula, and blastocyst stages ($P < 0.01$). In the groups with supplementing exogenous LIF, significantly lower percentages were also observed in these pre-implantation stages ($P < 0.01$). However, blastocysts treated with 50 ng/ml LIF had a significant higher percentage than those in the LIF gene impaired group without LIF supplement ($P < 0.05$) (Table 4).

八、討論

The co-culture technique has been used to determine the effects of LIF on implantation [17, 18] or pre-implantation development of murine embryo in vitro [8, 9]. Although the enhancing effects of LIF may be observed, it is difficult to reveal the changes in embryos with LIF-gene impairment in vitro using this technique. Knockout experiments have demonstrated that endometrial LIF is essential for in vivo murine implantation [4]. However, preparation of LIF knockout mice is a laborious task. Antisense RNA/DNA inhibition of gene expression has been documented as a feasible approach to the elucidation of mechanisms regulating the development of pre-implantation mammalian embryos [19]. Murine embryos at the pronuclear stage treated with c-*myc* antisense oligo may inhibit development to the blastocyst stage, especially at the first cleavage of zygotes to the two-cell stage [13]. Those treated at the two-cell stage may lead to developmental arrest at the eight-cell or morula stage [20]. Disruption of Na/K-ATPase gene expression by antisense oligodeoxynucleotide may abolish blastocyst formation [21]. Morpholino antisense oligonucleotides have been demonstrated to be effective tools for down-regulating gene expression during mammalian preimplantation development [22]. Moreover, the 2′-methoxyethoxy-modified antisense oligonucleotides are candidates for effective examination of roles of large numbers of genes during early embryological development [23]. In this study, we applied morpholino antisense oligonucleotide to inhibit the expression of LIF. Although LIF protein was detectable in the untreated embryo from the 2PN to the blastocyst stage by immunocytochemistry, signals of the protein expression were reduced in those treated with 1.0 fmol, 2.0 fmol, or 4.0 fmol of LIF antisense oligonucleotide from the 2-cell stage to blastocyst stage. These findings indicate that our design is useful in investigating the effects of LIF on the pre-implantation development of murine embryos in vitro.

In the four experimental groups, 2PN embryos treated with various dosages of LIF antisense oligo were able to develop to the two-cell stage. Although the inhibitory effects of the antisense oligo may commerce immediately after microinjection, the embryo may have sufficient amount of LIF for developing into the next stage. Since embryonic genome activation in mouse occurs at the two-cell stage [24], it is possible for the treated 2PN embryos to develop into the next stage. Moreover, development of embryos treated with 4.0 fmol LIF antisense oligonucleotide arrested at the two-cell stage. It has been reported that antisense oligonucleotides may lead to unpredictable activities within cells and morpholino modification of the oligos may avoid these adverse effects [25]. Since we employed morpholino oligos in this study, the arrest of the developing embryos at the two-cell stage may be mainly due to the effects of LIF antisense oligo at the high dose.

In this study, we observed the inhibitory effects of LIF antisense oligonucleotide to the treated embryos and microinjection of 1.0 or 2.0 fmol led to a significant reduction in the percentage of embryos to develop from the morula into blastocyst. It has been report that PN stage morphology is related to blastocyst development [26]. There is a number of stage-specific genes expressed at the different stages of the pre-implantation embryos. Lex (Galbeta1-4(Fucalpha1-3)GlcNAc) and Ley (Fucalpha1-2Galbeta1-4(Fucalpha1-3)GlcNAc) are stage-specific embryonic antigens. The former is first detected on the blastomeres of the 8-cell stage embryo and correlates with the onset of blastomere compaction. The latter is highly expressed on the surface of the blastocyst and has been shown to be involved in blastocyst attachment in the mouse. By in situ hybridization, mRNAs of these two enzymes were detected only in the morula and blastocyst embryos [27]. Other genes such as the glucose transporter GLUT3, growth factor, EGF (epidermal growth factor), EFG receptor, are also detected during and after the morular stage [28, 29]. It is possible that LIF may collaborate with these genes to regulate the blastocyst formation. However, LIF has been found to prevent leptin-induced apoptosis in embryo development [30]. Therefore, LIF may also associate with some other factors to modulate the embryo growth.

Numbers of cells in the TE and ICM and the ICM/TE ratio in day 5 blastocysts are important predictive variables of in-vitro fertilization and pre-implantation embryonic development in the mouse [31]. These variables may change under different culturing conditions. The ICM at the blastocyst stage has been demonstrated to be more sensitive to high temperature than the TE [32]. The numbers of TE and total nuclei are higher in embryos cultured in an atmosphere with 5% CO_2 in air than in those developed under 5% CO_2 : 5% O_2 : 90% N_2 [33]. Changes in the

concentrations of insulin or glucose in the culture medium has been reported to affect the numbers of cells in the ICM and TE [34]. The number of cells in the ICM may be altered by addition of cytokines in the culture medium. The incidence of blastulation in human embryos may increase by twofold in the presence of granulocyte-macrophage colony-stimulating factor [35]. The specific impact of tumor necrosis factor alpha on the ICM of blastocysts has also been reported [36]. In this study, we found that the number of cells in the ICM and TE was significantly decreased in the groups treated with 1.0 or 2.0 fmol of LIF antisense oligonucleotide. Moreover, in the group treated with 2.0 fmol of the oligo, the blastomeres had a significantly smaller size and a significantly lower ICM/TE ratio. These findings indicate that microinjection of the oligo to the embryos at the 2PN stage may have significant influence to the morphological characteristics of the blastocysts, which in turn decreases the rate of implantation [37]. Since changes in the number of cells in the ICM or TE as well as the ICM/TE ratio has been observed in the blastocysts with impairments at the gene level [38, 39], the LIF antisense oligonucleotide may block the translation of the selected mRNAs (the sense strand) and lead to the morphological changes in the blastocysts. Although this region may affect the development of the pre-implantation embryos through its unspecified reactions, the expression of LIF in 2-cell treated embryos have been altered. We have also found that supplement LIF to the treated embryos at the 2-cell stage did not improve the percentage of embryos developing into the blastocyst stage (unpublished data). These findings confirmed the fact that LIF is a critical factor for the normal development of embryos at the pre-implantation stages.

九、參考文獻

1. Robb L, Dimitriadis E, Li R, Salamonsen LA. Leukemia inhibitory factor and interleukin-11: cytokines with key roles in implantation. J Reprod Immunol 2002; 57:129-141.
2. Lass A, Weiser W, Munafo A, Loumaye E. Leukemia inhibitory factor in human reproduction. Fertil Steril 2001; 76:1091-1096.
3. Vogiagis D, Salamonsen LA. Review: The role of leukaemia inhibitory factor in the establishment of pregnancy. J Endocrinol 1999; 160:181-190.
4. Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. Nature 1992; 359:76-79.
5. Giess R, Tanasescu I, Steck T, Sendtner M. Leukaemia inhibitory factor gene mutations in infertile women. Mol Hum Reprod 1999; 5:581-586.
6. Jurisicova A, Ben-Chetrit, A, Varmuza SL, Casper RF. Recombinant human leukemia inhibitory factor does not enhance in vitro human blastocyst formation. Fertil Steril 1995; 64:999-1002.
7. Sirisathien S, Hernandez-Fonseca HJ, Bosch P, Hollet BR, Lott JD, Brackett BG. Effect of leukemia inhibitory factor on bovine embryos produced in vitro under chemically defined conditions. Theriogenology 2003; 59: 1751-1763.
8. Mitchell MH, Swanson RJ, Hodgen GD, Oehninger S. Enhancement of in vitro murine embryo development by recombinant leukemia inhibitory factor. J. Soc. Gynecol. Investig. 1994; 1:215-219.
9. Tsai HD, Chang CC, Hsieh YY, Hsu LW, Chang SC, Lo HY. Effect of different concentrations of recombinant leukemia inhibitory factor on different development stage of mouse embryo in vitro. J Assist Reprod Genet 2000; 17:352-355.
10. Marquant-Le GB, Humbolt P, Guillon N, Thibier M. Murine LIF improves the development of IVF cultured bovine morulae. J Reprod Fertil Abstr 1993; 12:61.
11. Dunglison GF, Barlow DH, Sargent IL. Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. Hum Reprod 1996; 11:191-196.
12. Hsieh YY, Tsai HD, Chang CC, Hsu LW, Chang SC, Lo HY. Prolonged culture of human cryopreserved embryos with recombinant human leukemia inhibitory factor. J Assist Reprod Genet 2000; 17:131-134.
13. Naz RK, Kumar G, Minhas BS. Expression and role of c-*myc* protooncogene in murine preimplantation embryonic development. J Assist Reprod Genet 1994; 11:208-216.

14. Quinn P. Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. J Assist Reprod Genet 1995; 12:97-105.
15. Piekos MW, Frasor J, Mack S, Binor Z, Soltes B, Molo MW, Radwanska E, Rawlins RG. Evaluation of co-culture and alternative culture systems for promoting in-vitro development of mouse embryos. Hum Reprod 1995; 10:1486-1491.
16. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Manipulating the Mouse Embryo: A Laboratory Manual, 3nd ed. New York: Cold Spring Harbor Laboratory Press; 2003.
17. Cai LQ, Cao YJ, Duan EK. Effects of leukaemia inhibitory factor on embryo implantation in the mouse. Cytokine 2000; 12:1676-1682.
18. Mitchell MH, Swanson RJ, Oehninger S. In vivo effect of leukemia inhibitory factor (LIF) and an anti-LIF polyclonal antibody on murine embryo and fetal development following exposure at the time of transcervical blastocyst transfer. Biol Reprod 2002; 67:460-464.
19. Heasman J. Morpholino oligos: making sense of antisense? Dev Biol 2002; 243:209-214.
20. Paria BC, Dey SK, Andrews GK. Antisense c-*myc* effects on preimplantation mouse embryo development. Proc Natl Acad Sci USA 1992; 89:10051-10055.
21. Watson AJ, Westhusin ME, De Sousa PA, Betts DH, Barcroft LC. Gene expression regulating blastocyst formation. Theriogenology 1999; 1:117-133.
22. Siddall LS, Barcroft LC, Watson AJ. Targeting gene expression in the preimplantation mouse embryo using morpholino antisense oligonucleotides. Mol Reprod Dev 2002; 63:413-421.
23. Kimber WL, Puri N, Borgmeyer C, Ritter D, Sharov A, Seidman M, Ko MS. Efficacy of 2-methoxyethoxy-modified antisense oligonucleotides for the study of mouse preimplantation development. Reprod Biomed Online 2003; 6:318-322.
24. Schultz RM. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. Hum Reprod Update 2002; 8:323-331.
25. Summerton J, Weller D. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 1997; 7:187-195.
26. Zollner U, Zollner KP, Hartl G, Diet J, Steck T. The use of a detailed zygote score after IVF/ICSI to obtain good quality blastocysts: the German experience. Hum Reprod 2002; 17:1327-1333.
27. Liu N, Jin C, Zhu ZM, Zhang J, Tao H, Ge C, Yang S, Zhang S. Stage-specific expression of alpha1, 2-fucosyltransferase and alpha1, 3-fucosyltransferase (FT) during mouse embryogenesis. Eur J Biochem 1999; 265:258-263.
28. Pantaleon M, Harvey MB, Pascoe WS, James DE, Kaye PL. Glucose transporter GLUT3: ontogeny, targeting, and role in the mouse blastocyst. Proc Natl Acad Sci USA 1997; 94:3795-3800.
29. Terada A, Minoura H, Toyoda N. Effects of epidermal growth factor on preimplantation mouse embryos. J Assist Reprod Genet 1997; 14:404-411.
30. Fedorcsak P, Storeng R. Effects of leptin and leukemia inhibitory factor on preimplantation development and STAT3 signaling of mouse embryos in vitro. Biol Reprod 2003; 69:1531-1538.
31. Tarin JJ, Gomez-Piquer V, Perez-Albala S, Hermenegildo C, Cano A. Predictive variables of in vitro fertilization and pre-implantation embryo development in the mouse. Mol Reprod Dev 2002; 63:38-46.
32. Amano T, Nakamura K, Tani T, Kato Y, Tsunoda Y. Production of mice derived entirely from embryonic stem cells after injecting the cells into heat treated blastocysts. Theriogenology 2000; 53: 1449-1458.
33. Machaty Z, Day BN, Prather RS. Development of early porcine embryos in vitro and in vivo. Biol Reprod 1998; 59:451-455.
34. De Hertogh R, Vanderheyden I, Pampfer S, Robin D, Dufrasne E, Delcourt J. Stimulatory and inhibitory effects of glucose and insulin on rat blastocyst development in vitro. Diabetes 1991; 40: 641-647.
35. Sjoblom C, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor promotes human blastocyst development in vitro. Hum Reprod 1999; 14:3069-3076.

36. Wuu YD, Pampfer S, Becquet P, Vanderheyden I, Lee KH, De Hertogh R. Tumor necrosis factor alpha decreases the viability of mouse blastocysts in vitro and in vivo. Biol Reprod 1999; 60:479-483.
37. Cheng TC, Huang CC, Huang LS, Chen CI, Lee Sheng, Liu JY. Evaluation of mouse blastocyst implantation rate by morphology grading. Chin J Physiol. In press.
38. McElhinny AS, Kadow N, Warner CM. The expression pattern of the Qa-2 antigen in mouse preimplantation embryos and its correlation with the Ped gene phenotype. Mol Hum Reprod 1998; 4:966-971.
39. Kang YK, Park JS, Koo DB, Choi YH, Kim SU, Lee KK, Han YM. Limited demethylation leaves mosaic-type methylation states in cloned bovine pre-implantation embryos. EMBO J 2002; 21:1092-1100.

十、附表及附圖

TABLE 1. Percentages (%) of murine embryos developing into different pre-implantation stages after microinjection of LIF antisense oligonucleotide at the two-pronucleus stage

	Control				LIF antisense (fmol)				
Stage	Untreated (n=151)	NaCl (n=171)	Nonsense (2 fmol) (n=146)	Nonsense (4 fmol) (n=83)	0.25 (n=144)	0.5 (n=145)	1.0 (n=155)	2.0 (n=152)	4.0 (n=112)
Two-cell	90.7	91.8	92.5	90.4	93.1	90.3	90.3	92.1	96.4
Four-cell	88.7	88.3	87.7	85.5	88.2	82.1	83.9	70.4**	0**
Morula	87.4	86.6	84.9	83.1	84.0	76.6*	72.3**	63.8**	0**
Blastocyst	85.4	79.5	79.5	78.3	80.6	63.5**	39.4**	13.2**	0**

Compared with the untreated group: *P < 0.05, **P < 0.01.

TABLE 2. Changes in the number of cells in the inner cell mass (ICM) and trophectoderm (TE) of the blastocysts derived from murine embryos after microinjection of LIF antisense oligonucleotide at the two-pronucleus stage

	Control		LIF antisense (fmol)	
	Untreated (n=53)	Nonsense (2 fmol)	1.0 (n=142)	2.0 (n=283)
Blastulation rate (%)	83.0 (44/53)	80.0 (44/55)	39.4 (56/142)	15.9 (45/283)
No. of blastocysts measured	26	27	31	35
Diameter of blastocysts (μm)[a]	114.2 ± 10.2	111.7 ± 8.7	111.5 ± 9.8	99.5 ± 5.8**
No. of blastomeres	51.5 ± 8.5	47.6 ± 9.5	36.5 ± 10.6**	26.3 ± 8.7**
No of cells in ICM	21.6 ± 4.2	18.6 ± 3.6*	14.5 ± 3.7**	9.1 ± 4.1**
No of cells in TE	29.8 ± 6.2	29.0 ± 8.3	22.1 ± 8.3**	17.3 ± 6.2**
Ratio of ICM/TE cells (%)	74.9 ± 18.9	68.2 ± 21.5	71.2 ± 23.5	55.9 ± 23.9**

[a] Mean ± Standard deviation.
Compared with the untreated group: *P < 0.05, **P < 0.01.

TABLE 3. Changes in the implantation rate of murine blastocysts derived from murine embryos after microinjection of LIF antisense oligonucleotide at the two-pronucleus stage

Group	No. of	Treatment[a]	Blastulation rate (%)	No. of	% of blastocysts
I	4	Untreated	81.1 (30/37)	22	81.7 ± 21.3
		Nonsense	80.0 (32/40)	22	77.5 ± 7.4
II	8	LIF antisense (0.5 fmol)	62.4 (63/101)	52	67.8 ± 17.6
		Nonsense	8 1.5(66/81)	52	74.7 ± 32.1
III	10	LIF antisense (1 fmol)	37.8 (76/201)	61	54.3 ± 12.2*
		Nonsense	82.5 (85/103)	61	86.1 ± 11.5
IV	8	LIF antisense (2 fmol)	15.1 (46/305)	42	32.9 ± 11.6**
		Nonsense	82.4 (70/85)	42	81.3 ± 14.0

[a] Blastocysts (5-7) treated with LIF antisense oligonucleotide were transfered to the right uterus horn of each recipient and the same number treated with 2 fmol nonsense oligonucleotide to the left uterus horn. The untreated blastocysts were transfered to the right uterus horn of each recipient.
[b] Mean ± Standard deviation.
Compared with control: *P < 0.01, **P < 0.001.

TABLE 4. Percentages (%) of murine embryos developing into different pre-implantation stages after microinjection of 2.0 fmol LIF antisense oligonucleotide at the two-pronucleus stage and supplemented with LIF

Stage	Untreated (n = 154)	LIF supplement (ng/ml)			
		0 (n = 133)	5 (n = 176)	10 (n = 181)	50 (n = 175)
Two-cell	94.2	88.0	92.6	91.2	89.7
Four-cell	91.6	75.9**	78.4**	74.6**	71.4**
Morula	89.6	60.9**	60.2**	63.0**	60.6**
Blastocyst	83.8	16.5**	15.3**	25.4**	40.0**,†

Compared with the untreated group: *P < 0.05, **P < 0.01.
Compared with the group without LIF supplement: †P < 0.05.

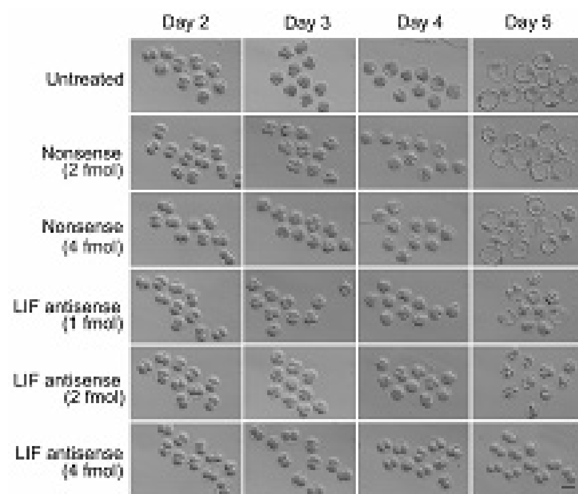

Fig. 1 Morphology of murine embryos on days 2-5 after microinjection of 1.0 fmol, 2.0 fmol or 4 fmol LIF antisense oligonucleotide. The untreated group and the 2 fmol and 4 fmol nonsense-treated groups were the controls. Bar, 50 μm.

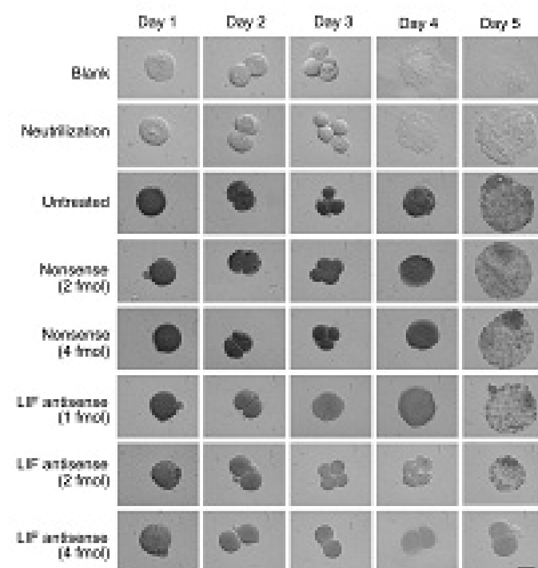

Fig. 2 Immunocytochemical analysis of LIF protein expression on days 1-5 in murine embryos. The three control groups were indicated as untreated, nonsense (2 fmol), and nonsense (4 fmol). The three LIF antisense oligonucleotide-treated groups were indicated as LIF antisense (1 fmol), LIF antisense (2 fmol), and LIF antisense (4 fmol). The blank group was embryos incubated with normal rabbit serum, and the neutrilization group in parallel with the antibody preneutralized with excess antigenic peptide. Bar, 50 μm.

十一、計畫成果自評

1. 研究內容與原計畫相符，發現 LIF 表現減少下會抑制胚胎發育，而大部分的胚胎都停滯於桑甚胚期到囊胚期之間。
2. 達成預期目標情況。
3. 研究成果之學術價值，即 LIF 在胚胎著床前發育的角色，應被重新評估。
4. 適合在學術期刊發表。