中山醫學大學生物化學研究所博士論文

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血癌抑制因子對著床前期小鼠胚胎發育之研究

Studies of the leukemia inhibitory factor on preimplantation mouse embryo development

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血癌抑制因子	leukemia inhibitory factor	LIF
著床前期	preimplantation stages	-
內細胞團	inner cell mass	ICM
滋養外胚層細胞	trophectoderm	TE
囊胚	blastocyst	-
孵化	hatching	-
反意寡核苷酸	antisense oligonucleotide	-
顯微注射	microinjection	-

中文摘要

胚胎能否順利著床取決於著床前期胚胎和子宮內膜的發育狀況，血癌抑制因子(leukemia inhibitory factor)是著床及成功懷孕所必須的因子，但是血癌抑制因子在著床前期胚胎發育所扮演的角色並不十分清楚。本論文針對血癌抑制因子對胚胎發育的調控做研究，首先評估在正常培養狀況下，小鼠囊胚期胚胎型態與著床率之關聯，將在體外培養形成的囊胚依囊胚腔形成的大小以及是否孵化分成三組。分別植入假懷孕母鼠的子宮內，比較胚胎型態，包括囊胚直徑、囊胚細胞數目等與著床率的關聯性，我們的結果指出，囊胚腔較小則內細胞團(inner cell mass)、滋養外胚層細胞(trophectoderm)及內細胞團與滋養外胚層細胞比值皆明顯下降，導致胚胎著床率也下降，已孵化的胚胎會有最高的著床率。藉由體外培養及胚胎植入模式的建立繼續探討於雙原核時期顯微注射血癌抑制因子反意寡核苷酸(antisense)抑制胚胎內血癌抑制因子的表現，並觀察其對著床前期鼠胚發育的影響。注射 0.25 fmol 血癌抑制因子反意寡核苷酸後，胚胎的發育不受影響，注射 0.5、1.0 fmol 及 2.0 fmol 血癌抑制因子反意寡核苷酸後，胚胎繼續發育至桑甚胚期及囊胚期的比例則有意義的降低，注射 4.0 fmol 血癌抑制因子反意寡核苷酸則胚胎無法發育到四細胞期以上。測定胚胎中血癌抑制因子的免疫活性則有隨著血癌抑制因子反意寡核苷酸注入濃度升高而下降的趨勢。注射 2.0 fmol 血癌抑制因子反意寡核苷酸後，測量囊胚的直徑、胚胎細胞總數目、內細胞團(inner cell mass, ICM)及滋養外胚層(trophectoderm, TE) 的細胞數目以及內細胞團滋養外胚層比值皆有意義的降低，將注射 1.0 及 2.0 fmol 血癌抑制因子反意寡核苷酸後未孵化的囊胚植入的子宮中，各組囊胚著床率皆顯著的降低。添加 50 ng/ml 的血癌抑制因子於培養液時可以有效的改善注入血癌抑制因子反意寡核苷酸後胚胎受損的情形，本實驗結果指出血癌抑制因子在著床前期的胚胎正常發育上扮演相當重要的角色。

關鍵字: 血癌抑制因子、囊胚、內細胞團、滋養外胚層細胞、著床前期

英文摘要

Good embryo development and receptive endometrium are essential factors for embryo implantation. Leukemia inhibitory factor (LIF) is an essential factor for implantation and establishment of pregnancy. However, its role in the development of preimplantation embryos remains controversial. The aim of this study is to assess the effects of LIF on development of preimplantation mouse embryo. We observe the relationship between the blastocyst morphology and the implantation rate for mice. Mouse blastocysts were then classified into 3 grades: grade I, small blastocysts; grade II, large blastocysts; grade III, hatching blastocysts. Although there was no significantly different in the implantation rates between the grade III and grade II, grade I was significantly decreased as compared with grade III. Grade I and grade II was also significantly decreased in both the diameter of blastocysts and cell number of inner cell mass (ICM) and trophectoderm (TE) as compared with grade III. These findings indicated that the expanded and haching blastocyst selections for embryo transfer in *in vitro* culture were evaluated with the high implantation rate. We successfully established the model of *in vitro* culture and blastocyst transfer for following experiments of LIF. Changes in preimplantation embryos were determined after microinjection of LIF antisense oligonucleotide at the two-pronucleus stage. 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 ICM or 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 preimplantation stages.

Key words:leukemia inhibitory factor (LIF), blastocyst, inner cell mass (ICM), trophectoderm (TE), preimplantation stages.

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第一節 哺乳類動物著床前期胚胎發育的過程

哺乳類動物會因為種(species)的不同而有不同的懷孕期(gestation period)，就小鼠而言，不同的品系(strain)懷孕期長短亦不同。根據 Theiler(1972; 1983)以 C57BL/6J 與 CBA 小鼠雜交(hybride)得到的第一代子代所做的研究，可以將著床前期(preimplantation) 之胚胎(embryo)發育分為五個階段：第一階段為交配(postcoitum)後 0 至 1 天(0-1 day p.c.)，此階段之胚胎為卵細胞(oocyte)與精子(sperm)受精後，形成單一細胞之授精卵，精卵授精時啟動了卵細胞的第二次減數分裂(second meiotic division)，並且排出第二極體(second polar body) 。核膜(nuclear membrane)將來自父系和母系的染色體(chromosome)分別包圍起來而形成半套體(haploid)的雄原核(male pronucleus)及雌原核(female pronucleus)，雙原核發生融合後，接著進行 DNA 複製及染色體重組等有絲分裂(mitosis division)之過程(Maro et al., 1984; Schatten et al., 1985)。此時的胚胎位於輸卵管(oviduct)的壺腹區域(ampullary region)。

第二階段為交配後 1 天，胚胎發育為二細胞期，受精卵進行第一次分裂(cleavage)需 24 小時，其後約 10-12 小時分裂一次（McLaren et al., 1982），二細胞期胚胎發育至中期時需要依賴大量來自母體卵子生成(oogenesis)時所遺留下來的蛋白和 mRNA 合

成，雙套染色體之轉錄活性被誘發也啟動胚源性基因(embryonic genes)的表達(Schultz, 1993)。來自母體卵子生成時製造的蛋白在這個階段仍然存在，但是來自母體的 mRNA 已開始快速的降解(degrade)(Kidder, 1992)，這時的胚胎已逐漸移向輸卵管中，逐漸遠離壺腹區域。

第三階段為交配後 2 天，胚胎發育為 4 至 16 個細胞期，胚葉細胞(blastomere)在分裂過程中，其表面結構亦產生變化，早期胚葉細胞表面平均分佈著微絨毛(microvilli)，發育到 8 細胞期時，鄰近之細胞有類似細胞突起(lamellipodia-like)之結構相互接觸，胚葉細胞在分裂過程中，其表面結構亦產生變化，使細胞間更加緊密，此時胚胎之型態 有極大的改變稱為致密化(compation) (Ducibella and Anderon, 1975; Pedersen, 1986)，通常此時的胚胎已漸漸移向子宮和輸卵管的交接處。

第四階段為交配後 3 天，這個階段的胚胎由桑甚胚(morula)進入囊胚(blastocyst)早期，囊胚的形成為胚細胞首次分化，此階段囊胚腔(blastocoel)開始形成，已可分辨內細胞團(inner cell mass)及滋養外胚層細胞(trophectoderm cell)，在囊胚生長的過程，囊胚腔逐漸擴張增大(expanding)，外胚滋養層細胞變薄，且表面覆滿微絨毛，排列十分緊密，胚葉細胞彼此形成一連結複合物，並構

成一個密封的外圍，稱為滋養外胚層細胞，滋養外胚層細胞將形成滋養葉細胞(trophoblast)、胎盤組織(placenta tissue)以及胚體外之外胚層(extraembryonic ectoderm)，並負責囊胚內外的主動運輸，及轉移營養之功能 (McLaren et al., 1982)。而內細胞團細胞較小以膜間聯繫連結，內細胞團細胞則主要形成胎體(somites)、卵黃囊(yolk sac)、尿膜(amnion)(Gardner 1983)，此時期的胚胎通常已位於子宮腔。

第五階段為交配後 4 天，胚胎在此時期已進入囊胚後期，大部分之囊胚已脫離囊胚外包覆的透明帶(zona pellucida)，行成孵化之囊胚(hatching blastocyst)，胚胎孵化的過程是藉由滋養葉細胞合成類似胰蛋白酶(trypsin-like)的酵素，水解透明帶中的醣蛋白基質(glycoprotein matrix)，孵化過程可以在體外單獨進行，若在活體的子宮腔發生，來自子宮腔的酵素也扮演重要的角色(Wassarman et al., 1984)，當囊胚完全孵化時，胚胎便進入著床的階段。

第二節 影響哺乳動物著床前期胚胎發育之因子

著床前期的胚胎可以在沒有血流的供應下在生殖道中自由漂流，並且進行代謝、生長及分化。直到形成囊胚後才開始和母體的子宮有交流，因此，有學者認為外源性的生長因子對著床前期的胚胎是不需要的，在胚胎體外培養的過程僅需提供簡單的鹽

類、焦葡萄酸(pyruvate)及白蛋白(albumin)(Whitten and Biggers, 1968; Devreker et al., 1998)。隨著基因剔除(gene knockout)的應用才確定當老鼠缺乏某些基因時胚胎無法正常發育，如小鼠缺乏群落刺激因子-1(colony stimulating factor-1, CSF-1)或顆粒球巨噬群落刺激因子(granulocyte-macrophage colony stimulating factor, GM-CSF)時，囊胚的內細胞團細胞數目會降低(Pollard, 1992; Robertson et al., 2001)，缺乏血癌抑制因子(leukemia inhibitory factor, LIF) (Stewart et al., 1992)及上皮生長因子接受器(epidermal growth factor receptor, EGF-R)會導致著床失敗(Threadgill et al., 1995)。

生殖道及著床前期胚胎皆會分泌諸多的胜肽(peptide)生長因子及細胞動力素，藉由自體分泌(autocrine)、旁體分泌(paracrine)與內分泌的作用方式促進胚胎的生長(Kane et al., 1997; Hardy and Spanos, 2002)，這些生長因子的接受器蛋白位於胚胎細胞表面包括：類胰島素生長因子家族(insulin like growth factor family, IGF family)、纖維母細胞生長因子家族(fibroblast epidermal growth factor family, FGF family)、乙型血小板衍生因子(platelet derived growth factor β, PDGF β)、及胰島素(insulin)、血管內皮生長因子(vascular endothelial growth factor, VEGF)等，這些生長因子在胚胎

不同的發育階段表現以影響胚胎的生長，腫瘤生長因子家族(tumor growth factor family, TGF)由胚胎所分泌接受器位於生殖道，胚胎及生殖道皆會分泌上皮生長因子家族(epidermal growth factor family, EGF family)、乙型血小板衍生因子(platelet derived growth factor family, PDGF β)及血癌抑制因子，同時胚胎也具有該因子的接受器。第一型類胰島素生長因子家族只在八細胞期表現，但是其接受器從一個細胞時期至囊胚期皆可以在胚胎細胞偵測(Svalander et al., 1991; Boehm et al., 1990; Hardy and Spanos, 2002)。

Slager 等人(1991)利用組織免疫染色法，發現乙型轉型生長因子(transforming growth factor β, TGFβ)只特異的表現在滋養外胚層，在內細胞團則不存在，而甲型轉型生長因子(transforming growth factor α, TGFα)存在於內細胞團及滋養外胚層靠近內細胞團的部分(Derdik et al., 1992)，此基因發生缺陷時，囊胚會發生很高比例的細胞凋亡(Brison and Schultz, 1998)。其他促進囊胚形成有關的因子包括胰島素(insulin)(Harvey and Kaye, 1990)、類胰島素生長因子(Palma et al., 1997)、甲型轉型生長因子(Derdik, 1992)、血癌抑制因子(Lavranos et al., 1995; Fry et al, 1992)、群落刺激因子-1(Pampfer et al., 1991)或顆粒球巨細胞群落刺激因子(de Moraes

and Hansen, 1997; Robertson et al., 2001)。

此外，細胞動力素介白質-1(interlin-1, IL-1)及介白質-6(interlin-6, IL-6)也在著床前期的胚胎中被偵測到(Zolti et al., 1991; Simon et al., 1993; Sharkey et al., 1995), 並參與著床之功能。

第三節 哺乳類動物胚胎著床之機制

著床時子宮的基質細胞(stroma cell)會因應囊胚或人工刺激，釋放組織胺(histamine)、前列腺素(prostaglandis)、白三烯(leukotrienes)及血小板活化素(platelet activating factor) (Hearn et al., 1991; McMaster et al., 1993)，使纖維母間質細胞(Fibroblastic stromal cell)轉化為蛻膜細胞(decidual cell)，子宮內膜(endometrium)的蛻膜化在荷爾蒙的調節上是經由雌性素(estrogen)和黃體素(progesterone)誘發轉變而成的。在人類這種改變是由黃體激素(progesterone)所主導，始於月經黃體期後期(late luteal phase)。在小鼠，黃體素和雌性素可加強生殖道中胚胎著床前的發育(Dey et al., 1980)，除此之外，著床前胚胎尚須要額外的旁泌因子才能完全發育及分化 (paracrine factor) (Bischof et al., 1998)。

著床前子宮與胚胎同步發育，胚胎發育至囊胚時子宮轉變為可著床狀態(receptive state)，胚胎僅在一個極短的時間內有機會附著到子宮內膜上，這個時期稱為著床窗口(implantation

window)(Psychoyos, 1995; Klentzeris, 1997)，HOX (homeobox)基因、細胞動力素(cytokines)、抑鈣素（ calcitonin）、及細胞粘黏分子(cell adhesion molecules) 等都是子宮內膜是否接受胚胎著床的重要生物標記(Cavagna and Mantese, 2003)，小鼠子宮接受性僅在懷孕、假懷孕(pseudopregnancy)或適當給予黃體素或雌性素的有限時期發生(Paria, 1993; Sakoff and Murdoch, 1995)。當胚胎從輸卵管進入子宮，囊胚孵化後，胚胎滋養層細胞即自透明帶移行出來準備附著於子宮內膜上皮細胞(endometrial epithelium cell) 。

壹、影響著床的免疫因子

胚胎著床過程與免疫反應類似，胚胎著床的免疫抗排斥反應和黏膜系統不同，女性生殖道中，分泌免疫球蛋白 A (immunologlobulin A)的漿細胞(plasma cell)位於子宮頸內(endocervix)、子宮頸外(ectocervix)、陰道(vagina)及輸卵管(fallopian tube)(Kutteh and Mestecky, 1994)，子宮內膜只偵測得到極少的漿細胞，子宮腔存在極少量的典型免疫球蛋白 A (Arends et al., 1983; Buckley and Fox, 1989)，胚胎著床的免疫反應缺乏典型的黏液免疫系統(mucosal immune system)，胚胎於蛻

膜所進行的著床免疫反應大部分是由白血球(leukocyte)所分泌的因子來完成。

為了防止入侵的滋養層細胞擴張，蛻膜上的巨大顆粒淋巴球(LGL, large granular lymphocytes)具有介白質-2(interleukin 2, IL-2)的接受體，會受到介白質-2 誘導後活化(Umehara and Bloom, 1990)，表現出自然殺手細胞(nature killer cell)的型態去毒殺典型自然殺手細胞的標的物(Robertson et al., 1996)，由於囊胚的外胚滋養層細胞有部分遺傳自父系，以免疫學之觀點應視為半同種異抗原(semi-allograft antigen)，但是胎兒細胞並沒有表現第一型人類白血球抗原(human leukocyte antigent, HLA)，這一類的抗原會被自然殺手細胞分泌的 CD3、CD16 或 CD68 所溶解，因此傳統移植器官的排斥現象並不存在(Sawicki et al, 2001)，因此半異基因型的胚胎逃過免疫系統的排斥是藉著唯一與母體子宮蛻膜和血液接觸的滋養層細胞(cytotrophoblast)形成無法辨識的非典型人類白血球抗原 HLA-G (non classical human leukocyte antigent -G) (Kovats et al., 1990, Loke and King, 2000)而降低自然殺手細胞分泌的 CD8 毒殺，滋養層細胞並分泌 indoleamine 2,3-dioxygenase (IDO), 這是參與色氨酸(tryptophan)代謝的酵素，可以分解色氨酸並且抑制 T-細胞的活性(Tatsumi et al.,

2000)，幫助胚胎逃過免疫系統的排斥，在胚胎侵入子宮內膜上皮細胞(endometrial epithelial cell)時，滋養層細胞會分泌 Fas ligand 誘發 T-細胞經由 Fas 路徑(Fas pathway)凋亡(apoptosis)(Kayisli et al, 2003)使胚胎順利進入蛻膜化的子宮內膜(decidualized endometrium; deciduas)，完成著床。

貳、影響著床附著與粘黏的因子

胚胎與子宮內膜藉著分子生物訊息的分泌與傳遞進行著床作用，許多因子在這個過程中表現以幫助著床。在大鼠(Psychoyos, 1973)和人類(Martel et al., 1987; Psychoyos and Martel, 1990)的研究發現，接觸與附著的過程與子宮內膜上皮細胞上伴隨著內噬作用(endocytosis)及巨噬作用(pinocytosis)的吸液足(pinopodes)結構有關，此結構的表現受到黃體素的調控，功能尚不十分清楚。Psychoyos 及 Nikas (1994) 認為吸液足可以幫助囊胚附著於子宮腔上皮細胞，當吸液足形成時，表示子宮已經呈現接受囊胚的狀態(Martel et al., 1991)。此外，在附著的過程中 MUC-1, trophinin/tastin 及 integrin 等與細胞粘黏(adhesion)有關的分子直接參與胚胎與子宮粘黏的作用。

高度醣化(glycosylation) 的 MUC-1 蛋白 MUC-1，在人類和小鼠著床期的子宮上皮細胞皆有表現，其功能與啟動胚胎和

子宮的接觸有關(Surveyor et al., 1995; Hey et al., 1994)；trophinin/tastin 只在著床時期的子宮表現，trophinin/tastin 影響囊胚和子宮腔接觸的位置(Fukuda et al., 1995)； 此外，integrin 家族的蛋白也與著床有密切之關係，integrins 屬於穿膜醣蛋白(transmembrane glycoproteins)，至少有十四種 integrin 的次單位被發現存在於人類的子宮內膜(Reddy and Meherjy, 1999)，有五種 integrin 醣蛋白($\alpha_2\beta_1$, $\alpha_v\beta_3$, $\alpha_4\beta_1$, α_6, β_4,)在著床時期的子宮內膜上皮細胞表現，人類胚胎滋養層細胞分泌的 fibronectin、vitronetic 會分別與 intergin $\alpha_4\beta_1$、$\alpha_v\beta_3$,接合，參與囊胚與子宮內膜的接觸作用，(Lessey et al., 1996; Bowen and Hunt, 2000)。

雖然對囊胚接觸子宮內膜的著床過程的調節機轉仍未完全明瞭，但有相當多的證據顯示胚胎藉著分子生物訊息的分泌與傳遞與母體之子宮內膜進行交互作用，最近的研究指出子宮內膜和胚胎的作用，胚胎就像一顆包在糖漿的網球一樣，在子宮內膜滾動，而胚胎本身表面分泌 L-選擇素(L-selectin)蛋白，與子宮內膜的醣蛋白作用，而產生類似強力黏著劑的作用，使胚胎著床(Genbacev et al., 2003)。

參、影響囊胚穿入子宮內膜的因子

細胞動力素(cytokine) 介白質藉由自體內分泌(autocrine)與旁體內分泌(paracrine)之作用直接參與胚胎著床的調節與進行。子宮內膜表現的細胞動力素有介白質 1 至 4 (IL-1~4)、介白質 6 (IL6)、介白質 7 (IL7)、介白質 10 (IL10)，而在這當中又以介白質-1 及及介白質-6 系統為主，介白質-1 直接參與細胞的增生與分化。介白質-1 包含三個主要成份：介白質-1 類似體(IL-1 isoforms α and β)、介白質-1 接受器擷抗劑(IL-1 receptor antagonist)和介白質-1 接受器。

介白質-1 類似體藉由與介白質-1 接受器之結合，產生訊息傳導(Dinarello, 1988)。無論是胚胎或子宮內膜所分泌的介白質-1，對於子宮內膜之介白質-1 系統具有自體分泌之調節機轉。第一型介白質-1 接受體(interleukin-1 receptor type I, IL-1RI)，與介白質-1 結合為胚胎與子宮內膜接觸(attachment)所必須的因子(Simon, 1999)，介白質-1 及介白質-6 會刺激胚胎產生 integrin α1 及α2 (Das et al., 2002)，介白質-1α於著床階段在子宮內膜的基質細胞大量表現並與胚胎所產生的瘦體激素(leptin)共同活化第九型金屬蛋白酶(matrix metalloproteinase 9; MMP9)及 integrin 介白質-1，幫助囊胚穿入(invasion)子宮內膜之基底膜 (Huang et al., 1998; Simon et al., 1998; Gonzalez et al.,

2001)，胚胎滋養層細胞分泌第九型金屬蛋白酶水解細胞外基質(extracellular matrix, ECM)中的膠原蛋白(collagen)幫助胚胎滋養層細胞侵入子宮蛻膜。

根據體外的試驗得知，滋養層細胞粘著於 laminin 上成扁平狀並保持固著，與 fibronectin 結合時細胞變長且有假足(Shigemoto, 1999)，胚胎滋養層細胞上的 integrin 與細胞外基質上的 fibronectin 及 laminin 結合，則得到一個牽引的力量，這種粘附作用與胚胎後續移入子宮內膜對成功的著床是相當重要的，當胚胎滋養層細胞對子宮內膜過度入侵時，第三型金屬蛋白酶抑制劑(TIMP3, tissue inhibitor of metalloproteinase 3)就會抑制金屬蛋白酶的活性，避免胚胎過度入侵(Lecoet al., 1996; Huang et al., 1998)，藉著子宮內膜之 MMP/TIMP 系統及 integrin 家族與其接受體的交互作用，使滋養層細胞順利穿透基底膜成功著床。

肆、影響胎盤血管新生的因子

胚胎著床之目的是為了到達母體得到子宮之血液循環的氧氣及養分以繼續生長，胚胎著床也同時伴隨著血管的生成作用(angiogenesis)，當胚胎剛附著於子宮內膜時，子宮基質微血管通透性增加，血管的形成必須水溶性的介質(soluble

mediator)，黏性受質(adhesive substract)及內皮細胞表面受體(endothelial cell surface receptor)共同進行胎盤的血管新生(neoangiogenesis)。首先由血管內皮細胞生長因子 (vascular endothelial growth factor, VEGF)家族及纖維母細胞生長因子(fibroblast growth factor, FGF) 活化平滑肌細胞及微血管的外皮細胞，接著驅動微血管內皮的 integrin 和細胞外基質協同作用(Moller et al., 2001; Smyth and Patterson, 2002)，在體內的試驗中以 $\alpha_v\beta_3$、 $\alpha_v\beta_5$、 $\alpha_1\beta_1$ 或 $\alpha_2\beta_1$ 的的抗體中和這些 integrin 的作用會抑制血管內皮細胞生長因子所誘導的血管生成(Kim et al., 1993)。integrin $\alpha_v\beta_3$ 與金屬蛋白酶結合並活化血管生成而金屬蛋白酶分解細胞外基質幫助胚胎侵入子宮內膜(Bischof, 2001)，因此胚胎著床時的影響因子都是環環相扣，形成一個網狀的系統，胚胎著床必須依賴以上子宮及胚胎影響著床因子精確的的交互作用才能順利完成。

第四節 囊胚期胚胎型態之評估

在人類試管嬰兒(in vitro fertilization)的療程中，長期以來在胚胎生長至第三天時植入胚胎(embryo transfer)，人類胚胎在體外培養至第三天時為八細胞期之胚胎，通常可以生長到此時期的胚胎總數目較繼續培養至第五天囊胚期的胚胎多，但是為了提高懷

孕的機率會植入較多的胚胎於子宮腔，若植入之胚胎多數著床成功則形成多胞胎(multiple pregnancy)(Garel et al., 1997; Elster 2000)，而多胞胎懷孕的結果會造成高危險妊娠，例如胎兒神經系統缺陷比例提高、胎兒體重過、低懷孕的過程也亦誘發子癲前症(Pre-eclampsia)、孕期糖尿病(gestational diabetes)、高血壓(hypertension)、早產等(Skupski et al., 1996)。

由於人類胚胎體外培養方法的進步，將培養胚胎之培養液改為兩階段式的培養或共同培養大幅提高囊胚形成的比率(Gardner et al., 1998; Marek et al., 1999)。品質不好的胚胎通常無法生長到囊胚期，因此可以增加胚胎的選擇性，胚胎品質提高後就僅需要植入一至二個胚胎，大大的降低多胞胎的機率，此外，囊胚期胚胎植入子宮時，胚胎與子宮處於同期化(synchronization)也可以提高胚胎著床的機會(Schoolcraft et al., 1999)。由於採用囊胚期胚胎植入著床率較高(Schoolcraft et al., 2000; Shapiro et al., 2002)，所以選擇最佳品質的囊胚作為胚胎植入是必須的，因此適當的囊胚期胚胎分級相當重要。

Gardner 及 Schoolcraft 於 1999 年提出較完整囊胚期胚胎分級的報導，他們依據囊胚腔的大小將囊胚分為 6 至 1 的等級，等級 1 表示為早期囊胚(early blastocyst)，囊胚腔小於胚胎一半的體

積，等級 2 表示為中期囊胚(blastocyst)，囊胚腔開始大於胚胎一半的體積，等級 3 表示為完全囊胚(full blastocyst)，囊胚腔幾乎完全佔據胚胎的體積，等級 4 表示為膨脹囊胚(expanded blastocyst)，囊胚腔大於前面 3 級胚胎且透明帶變薄，等級 5 表示孵化的囊胚且只有一部份外胚滋養層細胞露出囊胚，等級 6 表示完全孵化的囊胚，透明帶完全脫離胚胎。囊胚內細胞團的部分則分為 A, B, C 三級，A 級表示細胞數目多且緻密，B 級表示細胞數目少且鬆散，C 級表示細胞數目極少，外胚滋養層細胞亦分為 A, B, C 三級，A 級表示多數細胞形成凝聚性的上皮細胞，B 級表示由少數細胞形成鬆散的上皮細胞，C 級表示細胞數少且為體積較大的細胞。使用這種人類囊胚分級方法篩選胚胎，再植入母體，可以得到 86.7%(59/68)的懷孕率及 69.9(95/136)的著床率(Gardner et al., 2000)。

陸續有研究者提出人類囊胚分類的方法，有學者提出直接測量內細胞團的大小及形狀來提高著床率(Kevin et al., 2001), Jeffrey 等人(2001)則分別在胚胎受精後 16 至 18 小時、25 至 27 小時及 64 至 67 小時依分裂的三個時期胚胎品質評分再相加而得到評估囊胚品質的總分以為囊胚分級的方法。

而在小鼠囊胚體外培養及著床的研究上 Lane(1997)以含有不

同的胺基酸成分的培養液培養小鼠胚胎，觀察小鼠胚胎由單一細胞生長至囊胚的發育率及胚胎孵化比例，並且測量囊胚的細胞數目，以評估不同成分的胺基酸培養液培養胚胎後對著床率及第十五天胚胎的重量的影響，結果發現囊胚的細胞數目及囊胚內細胞團的數目和胚胎移入子宮後的存活率有關，但是囊胚的形成和孵化則不適於用來評估胚胎未來的發育潛能。然而此篇作者並未將事先將欲移入子宮的胚胎型態做進一步分析，因此統計出的結果仍須再做確認，雖然已有 Gardner 及 Schoolcraft 已提出人類體外培養的囊胚在植入母體前根據囊胚腔的的擴張程度、內細胞團滋養外胚層的型態分級的可行性，但是形成囊胚的細胞數目以及著床的能力則必須進一步釐清，小鼠著床前期的胚胎在型態和人類的類似(Hardy et al, 1993)，應用小鼠的著床模式來做評估應可作為日後生殖醫學臨床的參考，此外亦則尚未有學者對囊胚之分級或著床提出相關之研究，在基因轉殖動物研究蓬勃發展之際，提供重要的動物實驗參考值也是相當重要的。

第五節 血癌抑制因子之生化特性及生理功能

壹、血癌抑制因子之生化特性

血癌抑制因子最早是由 Tomida 等人(1984)由小鼠 L929 纖維母細胞所純化出來的一種醣蛋白(glycoprotein)，因為具有抑制

小鼠骨髓白血病细胞系的骨髓瘤 M1 細胞(myeloid leukemic cell line M1)增殖的能力，並誘導小鼠骨髓瘤細胞 M1 分化成巨噬細胞， 因此被命名為血癌抑制因子，人類的血癌抑制因子基因位於第 22 對染色體 q14 的位置，血癌抑制因子屬於介白質-6 蛋白家族的成員之一，分子量為 20kD，未成熟的蛋白型態含有 202 個胺基酸，成熟的蛋白型態由 180 個胺基酸組成(Gearing et al., 1987) ，具有 6 個潛在的天門冬醯胺醣化位置 (N-glycosylation sites)，3 個雙硫鍵，3 個 exon，主要型態的 mRNA 的長度為 4kb，次要型態為 1.8kb。

血癌抑制因子的結晶體(crystal structure)結構於 1994 年被鑑定出來(Robinson et al, 1994)，主要為 4 個α螺旋(α̃ helix)分別由 2 個長環及一個短環所串聯。螺旋狀的細胞動力素被分類為短鏈(short chain)及長鏈(long chain)兩種，血癌抑制因子、介白質-2、介白質-4 及顆粒球巨噬群落刺激因皆形成單體型式(monomeric form)同屬短鏈細胞動力素(Boulay and Paul, 1992; Bazan, 1992)。血癌抑制因子具活性的接受體為血癌抑制因子接受器β及 gp130 所組成的複合體(Gearing et al., 1992; Ip et al., 1992; Taga and Kishimoto, 1992)，另有四種細胞動力素的接受器除了本身特異的接受器之外也與血癌抑制因子共用 gp130 接受

器，這四種動力素分別是抑瘤素-M (oncostatin M, OSM; Malik et al., 1989)、介白質-6、(Van Snick et al., 1988)、睫狀神經營養因數(ciliary neurotrophic factor, CNTF; Stockli et al., 1989)及介白質-11(Paul et al., 1990; Kawashima et al., 1991)。

血癌抑制因子訊息傳遞的路徑是透過與受體 gp130 結合後使得原本與 gp130 結合的 Jak 激酶(Janus Kinase)被磷酸化，已經磷酸化的 Jak 接著刺激溶於細胞質 gp130 尾端的酪氨酸(tyrosine)磷酸化，磷酸化的 gp130 再使細胞質中的轉錄作用的訊息傳遞子及活化子(Signal transducer and activator of transcription, STAT)的 SH2 domain 磷酸化，STAT 接著形成雙體進入細胞核調節下游的基因(Gerhartz, 1996; Heinrich, 1998)，也有學者提出當磷酸酪氨酸激酶與磷酸化的 gp130 結合後可能會經由 MAPK 路徑(mitogen- activated protein kinase pathway)(Stahl et al.,1995)傳遞訊息。

貳、血癌抑制因子之生理功能

血癌抑制因子是一種多功能的細胞動力素，在生物發育及維持正常生理功能中扮演重要的角色，血癌抑制因子能抑制白血病细胞的增殖，而且對多種细胞的增殖、分化、存活以及胚胎發育都具有重要的作用(Heinrich et al., 1998; Auernhammer

and Melmed 2000)，對於于神經系統的發育及修復，也是不可缺少的因子(Murphy et al., 1993; Auernhammer et al., 1998; Gadient et al., 1998)。此外，血癌抑制因子可以抑制胚胎幹細胞的分化。因此，在胚胎幹細胞的培養過程中，必需添加血癌抑制因來抑制幹細胞的分化(Niwa, 2001; Burdon et al., 2002)。

目前已知血癌抑制因子廣泛的存在各種細胞中，在心臟、肝臟、子宮內膜、中樞神經系統、腎臟、肺臟、胸腺等器官及組織也都偵測得到血癌抑制因子。血癌抑制因子和許多生理系統上的增殖、分化、細胞存活有關(Metcalf, 1992; Hilton, 1992)。

參、血癌抑制因子在生殖系統之重要性

人類及多種哺乳類動物的輸卵管及子宮內膜上皮中皆會分泌血癌抑制因子(Shen and Leder, 1992; Yang et al., 1994; Cullinan et al., 1996; Vogiagis et al., 1996)，人類血癌抑制因子會受到月經週期的調節，隨著月經週期而在子宮內膜細胞中有週期性增減的表現，此外，在受到基因突變導致缺乏血癌抑制因子的老鼠，著床無法發生(Stewart et al., 1992)。

肆、血癌抑制因子對囊胚發育的影響

在 1991 年 Robertson 等人的研究報告中發現血癌抑制因子可以加強小鼠囊胚及孵化的形成(Robertson et al.,1991)，類似

的結果在牛的胚胎也被發現(Funston et al., 1997)，而在 Fry 等人對羊的研究中則發現血癌抑制因子可以增加胚胎的著床率(Fry et al., 1992)，但是血癌抑制因子對人類胚胎體外培養的研究還相當少，Dunglison 等人(1996)將血癌抑制因子 (1,000 IU/mL) 加入由接受試管嬰兒治療夫婦捐贈的胚胎中培養，發現囊胚期胚胎的形成率從 18%增加至 44% (P<0.05)，而發育評分較好的囊胚也從 10%提高至 33% (P<0.05)。

Jurisicova 等人(1995)將 463 個臨床治療後剩餘的胚胎分成五組，這些分裂早期的胚胎，除了 164 個胚胎為控制組外，其餘四組的數目分別為 54、78、87、80；而於培養液中加入不同濃度的血癌抑制因子分別為：5、7.5、10、20 ng/mL，每天觀察每一個胚胎的胎發育狀態及型態，直到囊胚期。就總體而言，人類胚胎在體外發育至孵化囊胚的數量相少。控制組培養至第五天時只有 28%發育至早期的囊胚，到了第六、七天時有 19.5%發育至囊胚後期；而添加血癌抑制因子 5、7.5、10、20ng/mL 於培養各組，並沒有明顯促進胚胎發育成早期或後期囊胚的效果。只有在 7.5 及 10ng/mL 有稍微促進後期囊胚形成的比例，其數值分別為 38%及 36%，但是這些差異並無統計上的意義。根據以上節錄 Dunglison 及 Jurisicova 等人對血癌抑制因子影響

囊胚形成不一致的研究結果還需要未來更深入的研究。

第六節 反意寡核苷酸抑制基因表現之原理及應用

DNA 以模板股 (template)轉錄出來的 RNA 為 sense RNA，與模板股互補的那一股 DNA 稱為非模板股(non- template)，而根據 non- template 股所轉錄出來的 RNA 即為反意 RNA (antisense RNA)。使用反意寡核苷酸之目的在於抑制某特定基因產物之完成，其應用之原理為利用表現出標的基因之反意股約數十個寡核苷酸與標的基因之 RNA 序列互補而結合，如此標的基因便無法順利進行轉譯作用合成蛋白質(Green et al., 1986)。

壹、反意寡核苷酸抑制基因表現的機制

一、反意寡核苷酸直接擷抗於 mRNA 的密碼區(coding region)使得合成蛋白質的延長過程被阻斷，此外，反意寡核苷酸直接與 mRNA 上任一區域結合會引起 mRNA 構形的改變而干擾蛋白質的轉譯(Oppenheim et al., 1991)。

二、反意寡核苷酸抑制蛋白質轉錄的起始作用(initiation)，藉由與 mRNA 促進區(promoter region)或起始區(initiator region)附近的序列結合，使得核糖體的複合體(ribosomal complex)無法順利與 mRNA 結合，造成轉譯的起始作用受抑制(Kitajima et al., 1992)。

三、當轉譯作用於細胞質進行時，表示 RNA 在細胞核經由剪接(splicing)的過程成為具生物活性的型態，此時反意寡核苷酸可以直接作用於 pre-mRNA 透過抑制 polyadenylation 或阻斷穿透(transport)至細胞質的過程而達到抑制基因表現的效果。反意寡核苷酸直接與剪接連結(Splice junctions)結合會干擾 mRNA 的成熟，而造成基因表現受阻(Kulka, 1989)，

四、未與反意寡核苷酸結合的 mRNA 會被 RNase H 分解(Cerritelli, 1998)而無法表現。

貳、設計反意寡核苷酸抑制標的基因的原則

在體外試驗中，30 個核苷酸足以用於南方或北方墨點法(Southern or Northern blotting)的探針，反意寡核苷酸可以抑制基因最小的長度為 12 個核苷酸，一般建議於細胞中最適當的反意寡核苷酸長度為 14 至 25 個鹼基(Herschlag, 1991; Manche 1992)，根據以上的原理，設計反意寡核苷酸抑制標的基因的原則為：

一、若希望其抑制轉譯的起始過程可以選擇剪接後 mRNA 5’cap 端至接近轉譯起始密碼(AUG)之間的核苷酸或選擇可以抑制 nuclear processing 的過程的序列。

二、確定該序列不會自體互補。

三、此反意寡核苷酸的鳥嘌呤(guanine)的比例不可以超過36%，或連續出現 3 個以上的鳥嘌呤否則其水溶性會降低。

此外，反意寡核苷酸的碳骨架須經適當的修飾，若其骨架未經修飾直接進入體內，則半衰期可能只有五分鐘，為了避免反意寡核苷酸被水解，發展出由甲基取代氧離子的反意寡核苷酸(Tidd and Warenius, 1989)，這種甲基化的核苷酸在小鼠細胞質半衰期約6分鐘，或者以硫離子取代氧離子形成硫代磷酸酯，硫代磷酸酯修飾過的核苷酸骨架骨架在細胞質的半衰期依設計的結構不同大約為50分鐘至40小時(Schlingensiepen and et al., 1997)，硫代磷酸酯核苷酸與RNA的二級結構結合時親和力較低，此外，還有以其他各種取代的基團用於修飾反意核苷酸但是仍有專一性、穩定性及細胞毒性等因素造成研究結果不理想(Summerton and Weller, 1997)。

基於早期所設計修飾過的反意核苷酸不論在核苷酸專一性、效率以及細胞毒性上都不能完全符合實驗的需求，美國的 Gene tools 生技公司研發出一種稱為 morpholino 的反意核苷酸，其化學式為將五碳醣插入氮離子改成六環結構，而連結碳骨架

的磷酸根其中一個氧離子也置換成氮離子(Summerton and Weller, 1997)，這種結構具核切酶(nucleases)抵抗性可穩定存留於細胞內較長的時間、可避免產生出具毒性的裂解物質、作用效率高、可避免 RNase H 對與目標 RNA 相似的非目標 RNA 產生裂解作用、序列專一性為硫代磷酸酯核苷酸的 100 倍，即使在高濃度狀況下使用也不會降低其專一性，對目標 RNA 結合能力強，可侵入目標 RNA 的二級結構，達到與 RNA 穩定結合的目的，並且在細胞質及細胞核內皆有活性(Summerton and Weller, 1997)。

理論上，反意寡核苷酸可被發展和應用在中止任何基因物合成，實際上反意寡核苷酸分子已被用於治療各型的骨髓瘤(myeloma，Barille-Nion, 2003)、血管損傷(Janssens, 2003)和肺癌、淋巴癌等多種癌症(Stahel et al., 2003; Kim et al., 2004)，同時也是製藥界和臨床發展基因療法的一個方向(Biroccio et al., 2003)。

根據 Paria 等人(1992)的研究，利用 c-myc 的反意寡核苷酸抑制胚胎發育，建立了減低早期胚胎基因表現的研究模式，近年來陸續有學者應用反意寡核苷酸做為研究著床前期胚胎發育的工具來研究諸多基因在早期發育所扮演的角色，例如 E 鈣

粘附素 (E-cadherin, Ao and Erickson,1992)、鈉、鉀三磷酸腺苷酸(Na^+, K^+-ATPase, Jones et al.,1997)及第二型去氧核糖核酸拓樸異構酵素(DNA topoisomerase II, St Pierre et al., 1997)等，利用反意寡核苷酸抑制基因的表現可以解釋某些基因在著床前期胚胎表現調控的機轉。

第二章 研究動機

胚胎能否順利著床取決於著床前期胚胎和子宮內膜的發育狀況，影響著床前期胚胎發育至囊胚的因子中，血癌抑制因子是著床及成功懷孕所必須的因子，但是血癌抑制因子在著床前期胚胎發育至囊胚所扮演的角色並不十分清楚，是否應補充於體外培養的環境中也仍有爭議，本研究的目的為探討血癌抑制因子對著床前期胚胎生長發育及著床的影響。首先建立小鼠胚胎體外培養及囊胚植入的模式了解胚胎型態與著床之關聯性，進一步藉由此模式以血癌抑制因子反意寡核苷酸直接抑制血癌抑制因子血癌抑制因子在胚胎內的表現，以確定血癌抑制因子影響著床前期胚胎的生長發育及著床所扮演的角色。

第三章 材料與方法

第一節　實驗材料

第二節　實驗方法

第一節、實驗材料

壹、實驗動物

囊胚型態與著床率相關性所使用的小鼠品系皆為購自國科會動物中心的 ICR 小鼠，包括 4 至 8 週齡之供胚母鼠(embryo donor)、8 至 12 週齡之受胚母鼠(recipient)及 8 至 20 週齡之輸精管切除之公鼠(vasectomy male mice)。血癌抑制因子對著床前期胚胎發育的影響所使用的供胚小鼠品系亦購自國科會動物中心包括 C57BL/6J 母鼠及 CBA 公鼠。C57BL/6J 母鼠及 CBA 公鼠用於自行繁殖第一子代小鼠(B6CBF1)，B6CBF1 母鼠飼養至 4 至 8 週齡作為供胚母鼠(embryo donor)、B6CBF1 公鼠飼養至 8 至 12 週齡則用於與母鼠交配，此外仍以 ICR 母鼠作為受胚母鼠，8 至 20 週齡之 ICR 公鼠作為輸精管切除之雄鼠，各品系小鼠皆飼養於李茂盛婦產科診所之動物室。

貳、藥品試劑

一、下列產品購自中國化學製藥廠

動物用血清哥娜注射劑(Sergona Inj.)

動物用哥娜 500 注射劑(Gona-500 Inj.)

二、下列產品購自美國 Sigma 化學公司

液體礦物油(mineral oil; M8410)、氯化鈉(NaCl; S9888)、氯化鉀(KCl ; P5405)、磷酸二氫鉀 KH_2PO_4 (P5655)、硫酸鎂($MgSO_4 \cdot 7H_2O$; M2643)、乳酸鈉(Na Lactate 60 % syrup; L4263)、丙銅酸鈉(Na pyruvate; P5280)、葡萄糖(glucose; G7021)、碳酸氫鈉($NaHCO_3$; S4019)、水合氯化鈣($CaCl_2 \cdot 2H_2O$; C7902)、玻尿酸酶 (hyaluronidase, H3735)、戊巴比妥鈉 (sodium pentobarbital; P3761)、蛋白水解酵素(proteinase; P8811)、 M16 培養液 (M16 medium; M7292)、M2 培養液(M2 medium; M7167)、三硝基苯磺酸 (trinitrobenzenesulphonic acid, TNBS; P2297) 、 聚乙烯吡咯烷酮(polyvinylpyrrolidone, PVP; P5288)、牛血清白蛋白(bovine serum albumin, BSA; B2518)、天竺鼠血清補體(guinea pig complement serum; S1639)、甘油(glycerol, G9012)、碘化丙啶(propidium iodide, p4170)、Triton X- 100 (t-octylphenoxypolyethoxyethanol; T9284)、Tween 20(polyoxyethylenesorbitan monolaurate; P-7949)、bis-benzimide (hoechst 33258, B2883)、芝加哥天空藍 Chicago Sky Blue 6B (C8679)

三、以下藥品購自美國 Invitrogen 公司

青黴素-鏈黴素(penecillin-G streptomycin, Gibco 15140-122)、磷酸緩衝液(Dulbecco's Phosphate-buffered saline, PBS, Gibco 11500-030)、胎牛血清(Fetal bovin serum, Gibco 10270-106)、乙二胺四醋酸鹽(Ethylene Diaminetetra Acetic Acid Disodium Salt Dihydrate EDTA, Gibco 60-00-4)、小鼠血癌抑制因子重組蛋白(recombinant mouse leukemia inhibitory factor, L5158)、3,3-Diaminobenzidine Tetrahydrochloride Tablet (DAB; D5905)

四、以下藥品購自德國默克公司

酚紅(Phenol red; 7241.0025)

五、以下藥品購自日本國產化學株式會社

絕對酒精(Ethanol; 2140063)

六、以下藥品購自日本和光純藥株式會社

甲醇(methanol; Wako 137-01823)

七、以下藥品購自美國 INC 生物醫學公司

兔子抗二硝基酚抗體(rabbit anti-dinitrophenol; DNP, 61006)

八、以下藥品購自聯工化學廠股份有限公司

福馬林(formaldehyde solution ; 88725)

九、以下藥品購自澳大利亞 Chemicon 公司

血癌抑制因子抗體(anti recombinant murine LIF, 16121102-05)

十、以下藥品購自美國 Vector 公司

VECTABSTAIN ABC kit (PK-6101)

十一、以下藥品購自美國 Gene Tool 公司

fluoresceinated standard control morpholino antisense oligo (31Oct00A)、fluoresceinated LIF morpholino antisense oligo (41-17-Jan01A-K-F)

參、儀器設備

一、電動電子顯示天平(Mettler Toledo, MT AB 104; Switzerland)

二、解剖顯徽鏡 (Nikon, SMZ-2B, Japan)

三、螢光顯微鏡(Nikon, LABOPHO-2, Japan)

四、二氧化碳培養箱(Heraeus Instrument, Cytoperm 2, Germany)

五、相位差顯微鏡(Nikon, Diphot 300, Japan)

六、無菌操作台(造鑫公司, CVM-420T, R.O.C.)

七、微電腦控制拉針器(Sutter Instrument Co., P-87 Flaming/Brown Micropipette Puller, U.S.A.)

八、顯微鍛燒器(Nikon, Microforge, Japan)

九、顯微操做手臂(Narishige, Micromanipulator, Japan)

肆、其他實驗器材

35mm 培養皿、90mm 培養皿、4 孔培養皿、96 孔培養皿、酒精燈、手術針、線、鑷子、外科剪刀、30G 注射針、眼科鑷子、止血鉗、l ml 之針筒、27G 針頭、眼科夾、載玻片、蓋玻片、拋棄式培養液過濾器、玻璃毛細管、玻璃微管.(Borosilicate Glass, 外徑 1.0mm，內徑 0.75mm, Sutter Instrument Co.)、含細絲之玻璃微管(Borosilicate Glass with filament, 外徑 1.0mm，內徑 0.78mm, Sutter Instrument Co.)。

第二節、實驗方法

壹、實驗動物之飼養

實驗小鼠飼養於李茂盛婦產科診所之動物室,新購入之小鼠需適應環境二週後才開始實驗，動物室的光照週期條件為 12 小時光照(清晨六點至傍晚六點)，12 小時之黑暗週期(傍晚六點至清晨六點), 飼養溫度維持在攝氏 20-24℃之間,於飼養期間給予充分的水及飼料，使用飼料為美國 Diet Test 齧齒類動物飼料，主要成分為粗蛋白 23 %、粗脂肪 4.5%、粗纖維 6.0%、灰份 8.0%、礦物質 2.5%，其餘部分(56%)為碳水化合物。小鼠之飲用水為逆滲透膜過濾過之飲水，本動物實驗已

經動物實驗管理小組(Institutional Aminal Care and Use Committee, IACUC)核准。

貳、誘發小鼠之超級排卵與配種(Nagy et al., 2003 a)

為取得大量著床前鼠胚進行著床試驗，母鼠必須在配種前施打外源性性腺激素，本實驗以中國化學製藥廠製造的動物用血清哥娜性腺激素(gonadotropin)，仿造 FSH (follicle-stimulating hormone)之功能，刺激卵巢濾泡的成熟，並配合中國化學製藥廠製造的動物用哥娜，即人類絨毛膜性腺激素，仿造黃體促素(luteinizing hormone, LH)之功能，幫助排卵，以便於配種後獲得較多數量之受精卵。為正確掌握排卵時間，人類絨毛膜性腺激素必須於內源性之黃體促素釋出前注射。通常性腺激素與人類絨毛膜激性腺素注射時間相隔 42 至 48 小時。配種後隔天早上，若在母鼠陰道內發現由公鼠精液之膠質所形成之陰道栓(vaginal plug)，即可判定母鼠已與公鼠交配成功。本實驗於母鼠腹腔內注射血清性腺激素 5 IU，46 小時後再以腹腔注射方式注射人類絨毛膜性腺激素 5 IU，人類絨毛膜激性腺素注射完後隨即將母鼠置入公鼠籠內，準備進行交配。交配後隔天觀察有無陰道栓(圖一 A)的形成，有則表示公鼠與母鼠完成交配。

參、小鼠胚胎之收集及培養(Nagy et al., 2003 b)

收集小鼠胚胎之前須配製人類輸卵管液培養基 (Quinn 1995)，此培養基的成分為：NaCl 101.6 mM, KCl 4.69 mM, KH_2PO_4 0.37 mM, $MgSO_4 \cdot 7H_2O$ 0.2 mM, Na Lactate 60 % syrup 21.4 mM, Na pyruvate 0.33 mM, Glucose 2.78 mM, $NaHCO_3$ 2.78 mM, $CaCl_2 \cdot 2H_2O$ 2.04 mM, penecillin-G streptomycin 2000 IU/l, phenol red 1% ， 取胚前一天，將人類輸卵管液培養基以 20 μl 為一滴，滴於 35-mm 培養皿中，再覆蓋礦物油於培養基上，置於 37℃，5%二氧化碳培養箱中過夜，以調整酸鹼值，待用。

將懷孕母鼠以頸椎脫臼法犧牲，即一手置於母鼠之頸部處固定，另一手抓住尾巴往後拉，使頸椎脫臼。接著解剖母鼠取出其輸卵管，先用酒精噴灑於母鼠之腹部，以外科剪刀在腹部剪一小洞，用手將其皮膚向外拉扯，直至露出腹部肌膜，剪開腹膜，將生殖道拉出剪下輸卵管，放入人類輸卵管液培養基液滴中，將接裝有人類輸卵管液培養基注射針筒之磨鈍的 30G 注射針刺入繖部的洞口以眼科鑷子夾緊注射針與繖部，緩緩將人類輸卵管液培養基注射入輸卵管中收集單一細胞期胚胎，在解剖顯微鏡下以吸管收集鼠胚後，放入回溫之玻尿酸酶內數分鐘以去除卵丘細胞(cumulus cells)。等卵丘細胞開始脫落擴散時，即可以玻璃毛細管吸吐受精卵數次，以去除包被於鼠胚外之卵

丘細胞，再以人類輸卵管液培養基清洗受精卵後，將胚胎放入事先準備好已調整酸鹼值及溫度並覆蓋礦物油之人類輸卵管液培養液中，置入培養箱內培養，每天觀察、記錄胚胎生長狀況。

肆、小鼠囊胚之分級

本實驗採體外培養的方式得到不同型態的囊胚，由於小鼠胚胎較小且內細胞團級滋養外胚層細胞不易再做區分，所以只以囊胚腔的大小作分級，小鼠囊胚期胚胎依囊胚腔的大小及是否孵化分成三組：第一組囊胚腔小於或等於胚胎一半的體積，第二組囊胚腔大於胚胎一半的體積，第三組為剛開始孵化的囊胚(圖二) 。

伍、囊胚直徑之測量

為了評估胚胎分級後實際的大小，我們將胚胎直接至於相位差顯微鏡下，以 200 倍的倍數觀察包含透明帶的囊胚直徑，利用事先置於接目鏡內的尺測量，呈圓形的胚胎測量任選的兩段互相垂直的直徑，若胚胎呈橢圓形則測量最長及最短的直徑，每個胚胎分別測量兩次並紀錄後接著進行核差別染色。

陸、囊胚期胚胎之核差別染色(differential stain)

為了進一步評估我們對胚胎分級後，每一組囊胚的細胞總數目、內細胞團細胞數目及滋養外胚層細胞數目我們採用胚胎

免疫手術(immunosurgery)之原理對囊胚進行核的染色(Piekos et al., 1995)，以 96 孔培養皿操作，先以 pH 2.5 的 Acid Tyrods'溶液將囊胚的透明帶去除，將胚胎培養於含有 10 mM trinitrobenzene- sulphonic acid, 4 mg/ml polyvinylpyrrolidone 及 0.015%的 Triton X-100 的 M16 培養液中，置於冰上 10 分鐘後以 M2 培養液洗滌三次以上，接著將胚胎培養於含有 0.1 mg/ml anti-dinitrophenol (DNP)-bovine serum albumin 的 M2 培養液中，於 37℃作用 15 分鐘後以 M2 培養液洗滌三次以上，將胚胎培養於以 M2 培養液十倍稀釋的天竺鼠血清補體(guinea pig complement serum)中，並添加 10 μg/ml 的紅色螢光試劑 propidium iodide，於 37℃作用 15 分鐘後以磷酸緩衝液洗滌三次以上，然後將胚胎置於含有 22 μg/ml 的藍色螢光試劑 bisbenzimide 的絕對酒精中，存放於 4℃，經隔夜作用後，將胚胎固定在載玻片上，以甘油使脫水的胚胎漲大，再以蓋玻片覆蓋在胚胎上輕壓一下後，以螢光顯微鏡調整至 UV-2A 及 G-2A 兩種濾鏡觀察。滋養外胚層細胞會呈現紅色的螢光，而內細胞團會呈現藍色的螢光，分別記錄胚胎的細胞數目。

柒、公鼠輸精管結紮(Nagy et al., 2003 c)

由於進行胚移置(embryo transfer)前受胚母鼠需處於假懷

孕(pseudopregnancy)狀態，使子宮環境處於適合胚發育之階段，故需以經輸精管結紮之公鼠與母鼠交配，挑選八週齡以上配種能力強之公鼠進行輸精管結紮，公鼠於術後兩週即可用於配種。

本實驗採用之麻醉劑為戊巴比妥鹽，注射小鼠的劑量為100mg/kg，使用前秤 10 mg 直接溶於 1 ml 之生理食鹽水中備用，此種麻醉劑注射小鼠依體重每 10 克以 0.1 ml 注射公鼠。注射時以手指抓緊小鼠背頸部皮膚以固定之，以 l ml 之針筒、27G 針頭吸取藥劑，於下腹一側鼠蹊部將針頭穿刺入腹腔中，避免針頭刺到膀胱等臟器，緩緩將藥劑注入腹腔內，並於針頭抽出前停留一會以避免藥劑隨針頭抽出而流出體外。

公鼠結紮乃以手術方式進行，手術前應以濕熱方式將手術器械滅菌(autoclave)，將公鼠秤重並依體重注射麻醉劑至腹腔中，接著將公鼠平置於手術墊布、腹部朝上，以 75％酒精消毒腹部，以剪刀於腹部皮層及肌肉層切開一個約 1.5 公分之傷口，接著於腹腔壁附近尋找連接睪丸及輸精管之脂肪塊(fat pad)，並以鑷子小心將其拖出體外，以眼科夾固定部分輸精管後，以酒精燈燒紅之鑷子夾斷並移除一小斷輸精管，將結紮完成之輸精管連同睪丸及脂肪塊小心置回腹腔中，再以相同之步驟處理另一側之輸精管。完成結紮後，以手術針、線分別將肌

肉層及皮層逢合，將術後公鼠移置籠中待其恢復後再移回動物房，手術後兩週之輸精管結紮公鼠即可開始用於配種。

捌、懷孕母鼠之準備與胚移置(Nagy et al., 2003 d)

受胚母鼠必須具備母性良好且窩仔數高之特質，一般選擇混種品系(out bred)，本實驗採用目前使用最廣泛之 ICR，其母性較佳、窩仔數多且同籠之母鼠可共同哺育仔鼠。

植入囊胚的前 3 天，將母鼠作陰道抹片檢查(圖三)，選擇抹片結果為動情期的發情母鼠與輸精管結紮之公鼠交配，隔天選取具有陰道栓(圖一 B)的母鼠，將這些母鼠分籠飼養以作為假懷孕之受胚母鼠。進行胚胎移置手術時，受胚母鼠秤重後以戊巴比妥鈉進行全身麻醉，置於 90-mm 培養皿中央，並於背部以 75%酒精清潔及消毒體表，以解剖剪刀於背中線距尾部約 2 至 3 公分處之皮層切一小於 l 公分之橫向開口，滑動開口至一側卵巢上方，由體壁外可見附著於卵巢上之脂肪，將此處之體壁剪一小開口，以較鈍之鑷子拖出脂肪使卵巢、輸卵管及一小段子宮角露出後，以止血鉗夾住脂肪以固定位置，將受胚鼠移至立體顯徽鏡下，以眼科鑷將子宮角固定後、於距離子宮輸卵管交接處約 5 mm 處以 26 G 之鈍針避開血管，刺一小洞，確定針頭進子宮角內後將其抽出。

事先將囊胚移至含 5% 胎牛血清的人類輸卵管培養液中，以內徑約 200 μm 之玻璃吸管吸取少量培養液，製作兩三段氣泡，吸取 5 至 7 個囊胚。將玻璃吸管插入子宮角之小洞中，吹動吸管至囊胚後方之氣泡到達吸管尖端為止，勿將氣泡吹入，等待數秒再將吸管抽出，將生殖道置回體內，並以相同方式進行另側之移置。縫合體壁及皮層開口塗抹碘酒且置回鼠籠。評估注入血癌抑制因子反意寡核苷酸之胚胎發育至囊胚的著床能力時，為了排除受胚母鼠個體差異造成的著床結果干擾，同一隻受胚母鼠的雙邊子宮，一邊植入無意義序列注入胚胎的控制組囊胚，另一邊的子宮則植入注入血癌抑制因子反意寡核苷酸注入後而形成的囊胚。囊胚植入兩天後以將母鼠麻醉，以 1%芝加哥天空藍由母鼠尾部靜脈注射約 0.2 ml，再將母鼠腹腔的皮毛及肌肉剪開，若胚胎有著床則可以看到一小團藍色胚胎著床後的囊(sac)(圖四)，計算著床於子宮腔的胚胎數目。

玖、顯微注射法

一、反意寡核苷酸之製備

本實驗所設計之血癌抑制因子基因的反意寡核苷酸是直接委託 Gene tool (U.S.A.)公司製造標定 FITC 的抑制血癌抑制因子反意寡核苷酸，以該公司軟體設計阻礙 RNA

表現最高的一段序列為模板，抑制血癌抑制因子反意寡核苷酸的序列為: GACCTTCATTATGGGCTGGACTCTA (156~180)，另外也購入一段無意義的序列注入對照組鼠胚(nonsense Control): ATCAGGATTGGTGG CATC TTCCCAG 以排除注射時血癌抑制因子反意寡核苷酸以外的影響因素，又此二序列皆經 BLAST 基因庫的比對(附錄一、二)，前者只專一性的抑制小鼠的血癌抑制因子基因，後者在小鼠則無擷抗的基因。

二、顯微微管之製備(Nagy et al, 2003a)

本實驗所使用之顯微微管包括固定吸管(holding pipette)及顯微注射微管(microinjection pipette)，一般而言，固定吸管之外徑大小乃依操作胚之細胞質直徑而定，內徑之尺寸除依操做胚之大小調整外，與控制操作目標物吸放之正負壓調節設備有關，原則上調節正負壓設備之體積愈大者，其配合之固定吸管之內徑愈小。固定吸管的製作方法如下，調整微電腦控制拉針器的設定值為以下條件：速度指數(velocity index, Vel)為60，拉力指數(pull index, Pull)為95，熱度指數(heat index, Heat)為980, 時間指數(Time index, Time)為60，將玻璃微管兩端固定啟動拉力按

鈕將玻璃微管分拉成兩支，再使用顯微鍛燒器電烙絲前端之熱玻璃球修整，先將外徑70~100μm處切平，並小心的將切口處燒成圓滑狀，使內徑在10~l5μm間，接著將玻璃微管前端約2mm的長度至熱玻璃球下方，將電烙絲緩慢加熱使玻璃微管受熱後，彎成約35度角，分別以95%酒精及無菌之逆滲透水潤洗數次，予以滅菌鍋滅菌，烘乾後待顯微注射時固定胚胎用。注射用微管則需採用含細絲之玻璃微管為材料，調整微電腦控制拉針器的設定值為以下條件：速度指數為75，拉力指數為110，熱度指數為975, 時間指數(Time index, Time)為150，將玻璃微管兩端固定啟動拉力按鈕將玻璃微管分拉成兩支，接著將玻璃微管前端約2mm的長度至熱玻璃球下方，將電烙絲緩慢加熱使玻璃微管受熱後，彎成約35度角，分別以95%酒精及無菌之逆滲透水潤洗數次，予以高溫高壓溼熱滅菌，烘乾後待顯微注射用。

三、顯微注射

血癌抑制因子之反意寡核苷酸以生理食鹽水配置成0.5mM、0.25mM……等濃度，無意義的反意寡核苷酸序列配成0.5mM的濃度，以毛細管拉製成的口吸管吸取1ul

的反意寡核苷酸由顯微注射微管開口端注入微管內，經由玻璃微管細絲之毛細現象，會將反意寡核苷酸溶液吸至針尖，將固定吸管及顯微注射微管分別架設於顯微操作手臂兩側之固定管上，並分別調整好固定吸管及顯微注射微管的角度使其與顯微操作板呈水平狀態。在顯微操作培養皿中滴入已平衡過酸鹼值的人類輸卵管培養液，然後以礦物油覆蓋，於解剖顯微鏡下操作，將20個原核清晰的期鼠胚置於油滴下的培養液中，移至顯微操作板上，並調整固定吸管及顯微注射微管之高度。

將封閉狀態的顯微注射微管尖端，輕輕碰觸固定用微管尖端方式，注射微管管內的正壓將使反意寡核苷酸溶液流出，調整溶液的流速後，先固定吸管之位置，調整微注射針尖，使其與原核膜之焦距相同，將注射微管刺入雄原核內並維持持續正壓使反意寡核苷酸溶液注入原核中。待原核明顯漲大後(圖五) 注入的體積約1pl，將汪射針迅速抽出。釋放固定吸管上已完成注射之胚，並以相同方式進行其餘胚之注射，以注射的濃度與體積換算注入血癌抑制因子反意寡核苷酸的量為 4 fmol、2 fmol、1 fmol、0.5 fmol及0.25 fmol，注入無意義的反意寡核苷酸的量為

4.0 fmol及2.0 fmol，注射後將各組鼠胚放回二氧化碳培養箱培養，並每日定時觀察。

拾、細胞免疫染色

為確定注入血癌抑制因子反意寡核苷酸的抑制基因血癌抑制因子表現的效果，我們將注入不同濃度血癌抑制因子反意寡核苷酸後，各個發育期的鼠胚以血癌抑制因子細胞免疫染色的方法確認血癌抑制因子是否有表現。首先將胚胎以 Acidic Tyrod's 溶液溶解外層之透明帶，再以磷酸緩衝液洗滌三遍後將胚胎置於載玻片上，以 2%的福馬林固定 15 分鐘，以磷酸緩衝液洗滌三遍後，接著以 0.2%的 Triton X-100 於室溫下處理 5 分鐘，以磷酸緩衝液洗滌五遍，與 1%雙氧水作用 10 分鐘，以磷酸緩衝液洗滌三遍，以含有 10%胎牛血清的磷酸緩衝液於室溫下處理 1 小時，接著以 1mg/ml 的血癌抑制因子抗體於 4℃培養隔夜，以 TBST 溶液(Tris-HCl 50 mM, Tween 20 0.025%, pH 7.8)清洗，以含有 10%胎牛血清的磷酸緩衝液於室溫下處理 10 分鐘，再以 1mg/ml 二級抗體(goat anti-rabbit IgG)作用 1 小時，以 TBST 溶液清洗 5 遍，每遍 2 分鐘，以 Avidin- biotinylated horseradish peroxidas 處理 45 分鐘，再以 TBST 溶液清洗 5 次，每次 5 分鐘，然後以 DAB 溶液(0.05% DBA, 3%雙氧水溶於

0.05M，pH 7.4 的 Tris 中)處理 20 分鐘，再以 0.05M，pH 7.4 的 Tris 緩衝液洗滌兩次，分別以 70%、80%及 90% 的酒精處理 5 分鐘漸進脫水，然後以甘油處理，在相位差顯微鏡下觀察呈色情形。

拾壹、添加血癌抑制因子蛋白觀察胚胎發育情形

為了觀察添加血癌抑制因子蛋白於注入血癌抑制因子反意寡核苷酸胚胎發育情形，我們將2.0 fmol血癌抑制因子反意寡核苷酸注入雙原核期的鼠胚後，立刻移置已添加5 ng/ml、10 ng/ml及50 ng/ml的培養液中，將各組鼠胚放回二氧化碳培養箱培養，並每日定時觀察。

拾貳、統計方法

本研究在探討囊胚型態之胚胎著床率之統計以卡方檢定(chi-test) 為統計方法，p 值小於 0.05 時定義為有顯著差異，在胚胎直徑及核差別染色後得到的數值以平均值±平均值的標準誤(mean±SEM, standard error of the mean)表示，並先以單一變異數分析(one way ANOVA) 再以 Student's *t*-test 為統計方法，p 值小於 0.05 時定義為有顯著差異。在探討血癌抑制因子對胚胎發育影響的部分，胚胎發育率之統計以卡方檢定(chi-test)為統計方法，在胚胎直徑及核差別染色後得到的數值以平均值±標準偏差

(mean±SD, standar deviation)表示，並以 Student's *t*-test 為統計方法，胚胎著床率先以 Kruskal-Wallis test 再以 Mann-Whitneu U test 統計，p 值小於 0.05 時定義為有顯著差異。

第四章 結果

第一節　以小鼠囊胚型態分級評估其著床率

第二節　血癌抑制因子之反意寡核苷酸對著床前期鼠胚發育及著床的影響

第一節 以小鼠囊胚型態分級評估其著床率

壹、小鼠胚胎發育之結果

本實驗一共累計採用了 32 隻 ICR 品系的供胚母鼠，得到單一細胞期胚胎 650 個，繼續培養數小時後，589 個胚胎形成雙原核，也就是指精卵受精的比率為 90.6%，培養 3.5 天後行成 524 個囊胚，囊胚型成率為 89.0%，這些囊胚經過分級後：第一組囊胚腔小於或等於胚胎一半的體積的囊胚數目佔所有囊胚細胞的 40.5% (212/524)，第二組囊胚腔大於胚胎一半的體積的囊胚數目佔所有囊胚細胞的 38.5% (202/524)，第三組為剛開始孵化的囊胚佔所有囊胚的 20.1% (110/524)。

貳、囊胚期胚胎直徑之測量及核差別染色

囊胚形成並完成分級後，一部分之囊胚用於測量直徑及核差別染色，直徑的大小在第一級、第二級級第三級胚胎分別為 89.3±1.2μm、110.7±1.5μm 及 115.8±1.6μm。先以單一變異數分析三組之間具有顯著差異後，再以 Student's *t*-test 統計，第一級與第二級、第三級胚胎囊胚的直徑比較，皆具有顯著差異，第二級與第三級之間亦具有統計差異($p<0.01$)。核差別染色的結果以螢光顯微鏡觀察，滋養外胚層細胞會呈現紅色的螢光，而內細胞團會呈現藍色的螢光(圖六)。每一組分別染了 35 個囊胚，

第一組囊胚細胞數目為 15.3±0.6，滋養外胚層細胞數目為 24.0±0.8，囊胚總細胞數為 39.3±1.3。第二組囊胚，內細胞團數目為 26.7±0.5，滋養外胚層細胞數目為 59.2±1.0，囊胚總細胞數為。第三組囊胚，內細胞團數目為 32.2±1.0，滋養外胚層細胞數目為 45.2±1.7，囊胚總細胞數為 77.4±2.6，第一級、第二級與第三級囊胚的內細胞團與滋養外胚層細胞的比值分別為 64.1± 1.9、82.3± 1.4 及 73.0± 2.1。(表一)，以單一變異數分析三組之間具有顯著差異後再以 t-test 檢定這三組胚胎細胞數目的差異，第一級與第二級、第三級胚胎囊胚的內細胞團、滋養外胚層細胞的數目及內細胞團與滋養外胚層細胞的比值，第一級與第二級、第三級囊胚之間具有統計差異，第二級與第三級之間亦具有顯著差異 ($p<0.05$)。發現第二組囊胚外觀較第一組大實際染出來的細胞數目不論在內細胞團或外胚層細胞的細胞數目上也真的比第一組多，但是這些數值並無統計之意義。第三組囊胚的大小較不一致，不過內細胞團或外胚層細胞的細胞數目細胞的數目也都多於第一組和第二組，且內細胞團的細胞數目與第一組比較具有顯著差異($p<0.01$)。

參、胚胎著床率之統計

這些經過分級之後的囊胚移置到與輸精管切除之公鼠交配成功的假孕母鼠子宮腔內。兩天後計算受胚母鼠子宮腔著床的胚胎數，第一級囊胚腔小於或等於胚胎一半的體積的胚胎，一共成功植入 60 個囊胚，著床率為 56.7(34/60)，第二級較大的囊胚一共成功植入 60 個囊胚，著床率為 73.3% (44/60)，而第三組剛開始孵化的囊胚共植入 66 個胚胎，著床率為 80.3 (53/66)。以卡方檢定分析這些數值，第二組和第三組的胚胎著床率明顯高出第一組胚胎且具有統計之差異(表二)。

肆、評估囊胚孵化潛力

由前面的結果得知，欲得到最高的著床率應該置入已孵化的囊胚，因此繼續培養第一級及第二級囊胚至孵化，再培養一天後至第六天，第一級及第二級囊胚孵化比例分別是 43.3% (13/34)及 65.6%(21/32)。培養二天後至第七天，第一級及第二級囊胚孵化比例分別是 63.3% (19/34)及 68.8%(22/32)(表三)。由這個結果得知，較大的囊胚在繼續培養的第一天就孵化達 65.6%，第二天則只有一個胚胎繼續孵化，比起第一級較小的囊胚有較高且快速的孵化能力。雖然第一級的囊胚在第二天也達到 63.3% 的囊胚孵化率，但是這一類的胚胎顯然有延遲發育的現象。

第二節 血癌抑制因子之反意寡核苷酸對著床前期鼠胚發育及著床的影響

壹、血癌抑制因子反意寡核苷酸對鼠胚生長速率之影響

本研究分別注入 0.25 fmole、0.5 fmole、1.0 fmole 或 2.0 fmole 及 4.0 fmole 的血癌抑制因子反意寡核苷酸於鼠胚中，並逐日紀錄胚胎發育情形，由於實驗前胚胎皆已在相位差顯微鏡下篩選過為雙原核期胚胎，因此各組胚胎達到雙原核期的比例是 100%，本研究結果採累積方式，將各次做的結果加總，統計于表四，並以卡方檢定來統計各組的生長速率與對照組間是否有差異。

未做任何處理的空白對照組、注射溶液對照組、注射 2 fmol 及 4 fmol 無意義序列之對照組，累計的鼠胚總數為 151、171 及 146、83，達到二細胞期的比率分別為 90.7%、91.8%、92.5%及 90.4，分別注入 0.25 fmole、0.5 fmole、1.0 fmole、2.0 fmole 及 4.0 fmole 血癌抑制因子反意寡核苷酸的各組胚胎達到二細胞期的比率分別為 93.1%、90.3%，90.3%，92.1%及 96.4%，除了一小部分的胚胎停滯雙原核時期，大部分的二細胞期胚胎內的兩個細胞大小相同，於此時期胚胎的型態及外觀上與控制組比較並未出現異常，且生長速率與未做任何處理的空白對照組比較

並無差異。

當胚胎繼續發育至四細胞期時，空白對照組、注射溶液對照組與注射 2 fmol 及 4 fmol 無意義序列之對照組，達到四細胞期的比率分別為 88.7%、88.3%及 87.7%及 85.5，分別注入 0.25 fmole、0.5 fmole、1.0 fmole 及 2.0 fmole 血癌抑制因子反意寡核苷酸的各組胚胎達到四細胞期的比率分別為 85.5%、88.2%、82.1%、83.9%及 70.4%，注入血癌抑制因子反意寡核苷酸的各組胚胎達到四細胞期的能力隨著注入濃度增高而降低，注入 4.0 fmole 血癌抑制因子反意寡核苷酸已完全停滯於二細胞期不再發育了，血癌抑制因子反意寡核苷酸在四細胞期已開始對胚胎造成傷害，一部份的胚胎停滯于二細胞期，注入 2.0 fmole 血癌抑制因子反意寡核苷酸的四細胞期鼠胚小部分出現胚胎內細胞大小不一的現象。

當胚胎生長至收集胚胎後第四天時，大部分胚胎進入桑椹胚時期，空白對照組、注射溶液對照組與注射 2 fmol 及 4 fmol 無意義序列之對照組，達到桑椹胚期的比率分別為 87.4%、86.6%及 84.9%，注入溶液的控制組與無意義序列的胚胎發育至桑椹胚為止皆與控制組鼠胚的生長數率及型態上沒有差異，但是在此時期空白控制組已有約 30%生長較快之胚胎已出現囊胚。分別

注入 0.25 fmole、0.5 fmole、1.0 fmole 及 2.0 fmole 各組生長至桑椹胚期的速率分別為 84%、76.6%、72.3%及 63.8%；當注射血癌抑制因子反意寡核苷酸濃度高於 0.25fmole 時小鼠胚胎在桑甚胚時有意義的降低。2.0 fmole 的高濃度組未達桑甚胚期的胚胎除了有縐縮的情形也有一部份胚胎內出現碎片。

當胚胎生長至收集胚胎後第五天時，大部分胚胎進入囊胚時期，空白對照組、注射溶液對照組與注射 2 fmol 及 4 fmol 無意義序列之對照組，達到囊胚期的比率分別為 85.4%、79.5%及 79.5%及 78.3%，注入溶液的控制組與無意義序列的胚胎發育至分桑椹胚為止皆與控制組鼠胚的生長數率及型態上沒有差異，但是在此時期空白控制組已有約之 50%生長較快之胚胎出現孵化囊胚了。分別注入 0.25 fmole、0.5 fmole、1.0 fmole 及 2.0 fmole 各組生長至囊胚期的速率分別為 80.6%，63.5%，39.4%及 13.2% ，當注射血癌抑制因子反意寡核苷酸濃度高於 0.25 fmole 時胚胎的囊胚生成率與控制組比較有顯著差異，而注射濃度高於 0.25 fmole 血癌抑制因子反意寡核苷酸後，各組胚胎由桑椹胚發育至囊胚的比例有意義的降低。注射 2.0 fmole 血癌抑制因子反意寡核苷酸的高濃度組未達桑椹胚期的胚胎除了有縐縮的情形也有一部份胚胎內出現碎片，而此組實驗所形成的囊胚較

小，且胚胎內出現較多的空泡及碎片。第三天至第五天胚胎生長發育的型態見圖七。

貳、血癌抑制因子反意寡核苷酸注入之效果

根據螢光顯微鏡觀察本實驗所設計的反意寡核苷酸可以進入鼠胚中，且會由細胞核逐漸擴散至整個細胞，取一部份顯微注射後的胚胎每日於螢光顯微鏡下觀察並拍照，以螢光是否存在,確定注入之反意寡核苷酸,可以維持到囊胚期而不衰退(圖八)。

為了進一步確定血癌抑制因子蛋白質是否仍存在於胚胎中，我們以細胞免疫染色的方法的檢視，對照組的胚胎每一個胚胎生長階段都可以偵測到血癌抑制因子蛋白質的表現，而注入血癌抑制因子反意寡核苷酸的胚胎在血癌抑制因子免疫染色後，二細胞期即顯現出血癌抑制因子蛋白質表現量有降低的趨勢，在四細胞期、桑甚胚期及囊胚期同樣也顯現出血癌抑制因子蛋白質表現量降低(圖九),這個結果表示本篇所設計以反意寡核苷酸抑制鼠胚中血癌抑制因子基因的表現，確實可達到抑制的效果。

參、注射血癌抑制因子反意寡核苷酸後囊胚型態之改變

我們以測量囊胚期胚胎的直徑及核差別染色後(圖十)計算出細胞數目作為注射血癌抑制因子反意寡核苷酸後胚胎發育型態的評估，在 2.0 fmol 血癌抑制因子反意寡核苷酸注入後囊胚直徑的平均大小為 99.5±5.8μm，明顯小於未處理的空白對照組且在統計上具有顯著差異，2.0 fmol 血癌抑制因子反意寡核苷酸注入後囊胚的總細胞數、內細胞團及滋養外胚層細胞數目、內細胞團及滋養外胚層細胞數目的比值皆顯著的小於未處理的空白對照組($P<0.01$)。而以 1.0 fmol 血癌抑制因子反意寡核苷酸注射的胚胎發育成的囊胚除了內細胞團及滋養外胚層細胞數目的比值與未處理的空白對照組比較無顯著差異外，其餘型態評估值包括胚胎半徑、總細胞數、內細胞團及滋養外胚層細胞數目都有意義的低於未處理的空白對照組(表五)

肆、血癌抑制因子反意寡核苷酸對鼠胚著床率之影響

為了評估注入血癌抑制因子反意寡核苷酸之胚胎生長至囊胚期之胚胎的著床功能，本實驗設計直接將囊胚移置到假懷孕母鼠的子宮腔，此外，為了排除受胚母鼠個體差異造成的著床結果干擾，同一隻受胚母鼠的雙邊子宮，一邊植入無意義序列注入的控制組囊胚，另一邊的子宮則植入注入血癌抑制因子反意寡核苷酸而形成的囊胚。為了排除顯微注射反意寡核苷酸

對形成囊胚後的著床功能可能造成傷害，我們首先比較分別植入未處理的空白組囊胚和注射無意義反意寡核苷酸囊胚的著床率，未處理的空白組囊胚和注射無意義反意寡核苷酸囊胚的著床率分別為 81.7±21.3 及 77.5±7.4, 兩組之間的著床率並無顯著差異。0.5 fmole 、1.0 fmole 及 2.0 fmol 血癌抑制因子反意寡核苷酸的囊胚生成率為 62.4% (63/101) 、37.8%(76/201) 及 15.1%(46/305)三組。這三組實驗組分別對照各組受胚母鼠之另一邊子宮植入未處理的空白對照組囊胚的著床率，1.0 fmole 及 2.0 fmole 血癌抑制因子反意寡核苷酸的著床率顯著下降，表示注入 2.0 fmole 及 1.0 fmole 血癌抑制因子反意寡核苷酸這兩組雖有囊胚形成，但是胚胎的著床功能已經受損，而 0.5 fmole 血癌抑制因子反意寡核苷酸注入後生成的囊胚著床率不受影響(表六)。

伍、外源性血癌抑制因子的補充對血癌抑制因子基因受損之影響

為了確認注射血癌抑制因子反意寡核苷酸於胚胎後造成生長的速率降低確實是由血癌抑制因子受抑制的影響，本實驗在顯微注射 2.0 fmole 血癌抑制因子反意寡核苷酸後立刻添加 5、10 及 50 ng/ml 不同濃度的血癌抑制因子於培養液中，觀察胚胎生長情形。雖然顯微注射 2.0 fmole 血癌抑制因子反意寡核苷

酸之胚胎生長至囊胚的比例經添加血癌抑制因子後仍然比未處理的對照組明顯的低。但是顯微注射 2.0 fmole 血癌抑制因子反意寡核苷酸後再添加 50 ng/ml 的血癌抑制因子，囊胚形成率由 15.3%提高為 40.0% ($p<0.01$)，添加血癌抑制因子可以恢復一部分因血癌抑制因子受抑制而受損的胚胎繼續生長到囊胚的的能力(表七)。

第五章 討論

第一節 以小鼠囊胚型態分級評估其著床率

第二節 血癌抑制因子之反意寡核苷酸對著床前期鼠胚發育及著床的影響

第一節 以小鼠囊胚型態分級評估其著床率

選擇具有高著床潛能的良好胚胎在試管嬰兒輔助生殖的治療上是相當重要的，囊胚期胚胎植入子宮的優點為子宮內膜與胚胎同期化發育，此時的子宮環境較適合囊胚著床(Cruz et al., 1999)，選擇胚胎植入時前，必須先做胚胎的分級以篩選最好的胚胎植入，囊胚的分級篩選較分裂期的胚胎複雜，但是植入囊胚卻可以有效的降低多胞胎懷孕而降低多胞胎懷孕所帶來的併發症(Gardner et al., 2000; Milki et al., 2000)。人類囊胚期胚胎植入前的分級主要依據內細胞團、滋養外胚層細胞及囊胚腔大小三部份來分級(Gardner and Schoolcraft, 1999)，根據我們的實驗結果小鼠囊胚腔的形成及孵化的與否皆是影響著床是否成功的的重要因子，Balaban 等人(2000)也提出類似的結果，他們在進行試管嬰兒療程的婦女子宮中植入至少一個品質良好的囊胚或一個孵化的胚胎，可以得到非常高的懷孕率。

用顯微鏡觀察囊胚並不能很精準的判斷出的內細胞團及滋養外胚層細胞的部分，這可以利用免疫手術(immunosurgery)的方法分別將這兩種細胞染上不同顏色的螢光染劑來觀察確實的細胞數目。此外，小鼠胚胎的總數目會隨著環境而調節(Van et al., 1997 Tao and Niemann 2000)，根據 Papaioannou 及 Bert 於

1995 年發表的報告，利用小鼠的半胚作研究，發現發現小鼠胚胎的總數目會隨著環境而調節(Papaioannou and Ebert 1995)，亦有學者提出，體內生長的胚胎內細胞團的數目與體外培養的囊胚類似，體外培養的系統滋養外胚層的細胞較多(Hurst et al., 1993)，這份研究得到的總細胞數和我們在體外培養囊胚的結果類似，內細胞團與細胞總數之比例只要達到 30%以上就屬於生長發育好的胚胎(Papaioannou and Bert, 1995)，本實驗的三組胚胎都有達到 30%以上的內細胞團比例，表示這些胚胎都屬於生長發育情形良好的胚胎。

根據 Lane 及 Gardner(1997)結果發現囊胚的細胞數目及囊胚內細胞團的數目和胚胎移入子宮後的存活率有關，但是囊胚的形成和孵化則不適於用來評估胚胎未來的發育潛能，但是這位作者並未將欲移入子宮的胚胎型態做進一步分析，因此統計出的結果和我們的結果不同，小鼠的胚胎植入實驗應根據本篇的方法作分級再植入子宮，我們認為孵化在胚胎著床的角色應該比囊胚的體積及細胞數目還重要。

胚胎發育成囊胚及著床的潛能可能和胚胎本身細胞核或細胞質的基因是否表現有關，例如雌性激素會誘導蛋白水解酵素分解透明帶(Hoversland and Weitlauf, 1981)，TGF-α(Derdik et

al., 1992)、HB-EGF(Das et al., 1994; Birdsall et al., 1996)、或干擾素(INF-τ)會誘導囊胚孵化。此外，亦有學者以 CB6F1 小鼠為實驗材料，提出當囊胚在體外生長時，成功的孵化尚必須囊胚腔足夠的擴張張力及達到最小閥值(threshod)胚胎細胞數目，我們以 ICR 品系做實驗，得到這種品系的囊胚孵化最小總細胞數目為 77.4±2.6。

在本篇研究中以第三組已孵化的囊胚有最高的著床率，在同樣的品系和飼養條件下，這一類的胚胎生長速率較快，且透明帶在胚胎成功著床與否扮演很重要的角色。透明帶的主要功能是阻隔精子與卵細胞產生多重授精，幫助著床前期的胚胎在輸卵管順利運行，並且維持胚胎的形狀(Mishra and Seshagiri, 1998)。在試管嬰兒的療程若胚胎植入前，當透明帶過硬或者過厚不易孵化時利用雷射、酵素或強酸等方法將透明帶溶一個洞, 已輔助胚胎孵化達到提高著床的目的，但是以雷射及強酸的方法輔助胚胎孵化幫助的效果並不大(De Vos and Van, 2002)，使用酵素雖然較費時，卻可以增加臨床之著床率(Domitrz et al., 2000; Hwang et al., 2000)。但是若胚胎本身缺乏著床必須具備的因子如 L-selectin，即使將透明帶完全剝離也未必對著床有助益(Genbacev et al.,2003)。

在體內生長的囊胚期牛胚，其胚胎本身較緻密，內細胞團較小、數目較多。而體外培養的囊胚則細胞較大且較鬆散(Van et al., 1997)。人類胚胎要達到囊胚的比例只有 50% (Hardy, 1993; Langley et al., 2001)，本研究中小鼠胚胎體外培養達囊胚的比例為 89.0%。Khorram 等人(2000)將生長至第六天仍未孵化的胚胎再移置回母體，著床率很低。體外孵化的觀察在本研究中可見孵化較慢之胚胎繼續培養雖可以增加孵化的比例(表六)，但是，不論第一組或第二組仍然有約三分之一的胚胎停滯於囊胚期，表示這些胚胎於囊胚期繼續生長及孵化的機制可能有缺陷，胚胎延遲孵化在移置回子宮也錯過了子宮接受囊胚最佳的時機。胚胎發育不好或著床困難，可能的原因有染色體的異常(Jamieson et al., 1994)、細胞核或細胞質在卵子生成(oogenesis)時不足或變異(Moor et al., 1998)、胚胎生長的環境中荷爾蒙與生長因子的量不足或過多(Bavister, 1995)，而著床失敗也極有可能是胚胎和母體的交互作用發生障礙(Simon and Valbuena, 1999)。

本部分的研究證實了要使胚胎成功的著床，所篩選的胚胎最好是在囊胚移置時已孵化或囊胚腔已擴張的胚胎，如此便可排除孵化缺陷導致著床失敗的因素，本研究結果應可應用到人類試管嬰兒體外培養囊胚期的篩選的參考，如果盡量於體外培

養第五天植入孵化或囊胚腔已擴張的囊胚一至二個，將可增加胚胎著床率並降低多胞胎引發的高危險妊娠併發症。

第二節 血癌抑制因子之反意寡核苷酸對著床前期鼠胚發育及著床的影響

根據結果部分第一節之研究成果我們得以藉由體外培養技術及囊胚期胚胎植入的模式繼續探討血癌抑制因子對著床前期鼠胚發育及著床的影響。

先前的研究已證實利用共同培養的技術研究血癌抑制因子在體內對著床(Mitchell et al., 1994; Cai et al., 2000)或著床前期胚胎發育的影響(Mitchell et al., 2000;Tsai et al., 2000)。雖然這些實驗可以觀察到血癌抑制因子對胚胎生長的促進效果，但是這些體外試驗的方法很難判斷當血癌抑制因子基因受損時對胚胎的影響。血癌抑制因子基因剔除(knockout)的研究證實了子宮內膜血癌抑制因子對小鼠的著床是一個必須因子(Stewart et al., 1992)。但是製造基因剔除的小鼠是很繁複且耗時的工作，反意的去氧核糖核酸(DNA)及核糖核酸(RNA)對哺乳類胚胎著床前期抑制基因表現的效果已被證實是有效的(Heasman, 2002)，小鼠胚胎在原核期處理 c-myc 反意寡核苷酸可能可以抑制胚生長發育至囊胚期，其中以胚胎第一次由單一合子分裂成兩細胞期是

發育的速率決定步驟(Naz et al., 1994)。Paria 等人則於二細胞期注入 c-myc 反意寡核苷酸於鼠胚發現會導致胚胎生長停滯於八細胞期至囊胚之間(Paria et al., 1992)。如果以反意寡核苷酸破壞 Na/K 腺苷三磷酸水解酶(ATPase)基因的表現可能會阻斷囊胚的形成(Watson et al., 1999)，使用 morpholino 反意寡核苷酸處理哺乳類動物著床前期胚胎已被證實可以有效造成基因表現的降解(Siddall et al., 2002)，而以 2'-methoxyethoxy 修飾過的反意寡核苷酸也可以有效的抑制著床前期胚胎的基因表現(Kimber et al., 2003)。在本實驗中我們應用 morpholino 反意寡核苷酸抑制血癌抑制因子基因的表現，以免疫細胞染色的方法偵測未處理的空白對照組血癌抑制因子，發現血癌抑制因子蛋白的表現在雙原核時期即可偵測得到，而注入 1.0 fmole、2.0 fmole 或 4.0 fmole 的血癌抑制因子反意寡核苷酸從二細胞期到囊胚期皆會抑制血癌抑制因子蛋白的表現，這個結果表示我們的實驗設計應用血癌抑制因子反意寡核苷酸研究血癌抑制因子於著床前期小鼠胚胎發育是有效的。

在本實驗中於雙原核時期注入不同劑量的血癌抑制因子反意寡核苷酸的每一個實驗組，皆可發育到二細胞期，雖然反意寡核苷酸作用的效果是在注入後立刻開始發生，但是於雙原

核時期原本就存在於胚胎內的血癌抑制因子蛋白的量還足夠促使胚胎進入二細胞期(Schultz, 2002)。當注入血癌抑制因子反意寡核苷酸的量提高 4 fmole 時，胚胎的發育會停滯於二細胞期，這種現象應該不是血癌抑制因子反意寡核苷酸的毒性，因為根據 Summerton 及 Weller(1997)的報導，注入細胞內的反意寡核苷酸可能會產生無法預測的活性喪失，但是經由 morpholino 修飾過的寡核苷酸則可以避免在細胞內受到破壞，因此本實驗採用 morpholino 修飾過的血癌抑制因子反意寡核苷酸作為抑制血癌抑制因子基因表現的寡核苷酸所造成二細胞的停滯應主要是來自於血癌抑制因子高劑量的效果。

在本研究中我們觀察到注入 1.0 或 2.0 fmole 血癌抑制因子反意寡核苷酸於胚胎後，會導致胚胎的發育由桑椹胚期進入囊胚期生長速率有意義的降低，曾有學者提出原核時期胚胎的型態與囊胚的發育有關(Zollner et al., 2002)。著床前期胚胎在不同的生長階段會有各階段特異基因的表現， Lex[Galbeta1-4(Fucalpha1-3)GlcNAc]醣蛋白在胚胎八細胞期開始被偵測到，其功能與胚胎進入桑椹胚期時各個胚葉細胞的聚合有關，另一種被命名為 Ley[Fucalpha1-2Galbeta1-4(Fucalpha1-3) GlcNAc]的蛋白則高度表現於囊胚期胚胎的細胞表面，其功能與胚胎能否順

利附著於子宮腔有關，而以原位雜交法偵測 Lex 和 Ley 的 mRNA 發現此二種基因在桑椹胚期進入囊胚期時基因的表現量最高 (Liu et al., 1999)。牛胚中有一種 Na/K 腺苷三磷酸的抑制因子，桑椹胚期的表現量是囊胚期時基因的表現量的九倍，若以反意寡核苷酸抑制該基因的表現則胚胎無法形成囊胚(Watson et al., 1999)，還有一些基因，同樣具有階段性的特異表現，如葡萄糖轉運因子(glucose transporter)GLUT3、上皮生長因子(epidermal growth factor, EGF)及其接受器等，都在進入桑椹胚期前後有大量的表現(Pantaleon et al., 1997; Terada et al., 1997)。血癌抑制因子可能與這些基因協同表現控制囊胚的形成。

著床前期的胚胎出現凋亡(apoptosis)時的現象和體細胞一樣會有生長停滯、細胞膜受損、核碎片、DNA 碎片(Brison and Schultz, 1998; Jurisicova et al.,1998; Matwee et al., 2000)。胚胎內產生細胞碎片時，這些碎片可能會被排除到胚胎細胞和透明帶之間或是被包在囊胚腔內，這種現象通常導因於細胞核或染色體的異常，使得細胞週期無法順利運作也無法正常分裂(Hardy, 1993; Munné et al., 1995; Ruangvutilert et al., 2000)。當胚胎注射 2.0 fmole 血癌抑制因子反意寡核苷酸未達桑椹胚期的胚胎除了有縐縮的情形也有一部份胚胎內出現碎片，而此組實驗所形成

的囊胚較小，且囊胚內出現較多的空泡及碎片。而直接以螢光染料染胚胎的核，可以看到一部份的核已有碎裂的情形，由這些現象的觀察，我們認為血癌抑制因子反意寡核苷酸可能會造成胚胎進入凋亡的狀況。此外，有報導指出血癌抑制因子可以抑制瘦體激素(leptin)在胚胎發育過程中所誘導的細胞凋亡(Fedorcsak et al., 2003)，血癌抑制因子的表現或許也和這一類誘導細胞凋亡的因子的作用有關。

胚胎發育到第五天形成囊胚期胚胎時的內細胞團、滋養外胚層細胞的數目及內細胞團與滋養外胚層細胞的比值對小鼠著床前期胚胎發育是一個很重要的指標(Tarin et al., 2002)，這些指標也會隨著不同的培養條件而有所改變，當培養溫度身升高時內細胞團細胞會變得比滋養外胚層細胞敏感(Amano et al., 2000)，滋養外胚層細胞及囊胚細胞總數目在含有 5%二氧化碳的大氣空氣中培養會比在 5%二氧化碳、5%氧氣及 90%氮氣下培養來的多(Machaty et al., 1998)，改變胰島素及葡萄糖於培養液中的含量也會改變內細胞團與滋養外胚層細胞的數目(De Hertogh, et al., 1991)。細胞動力素的添加也會造成內細胞團細胞數目的改變，例如顆粒球-巨噬細胞群落刺激因子會造成囊胚形成率增加兩倍(Sjoblom et al., 1999)，不論在體內或是體外培養腫

瘤壞死因子(tumor necrosis factor alpha)皆會造成內細胞團細胞數目減少(Wuu et al., 1999)。在我們的研究中發現注入 1.0 fmole 及 2.0 fmole 血癌抑制因子反意寡核苷酸於胚胎中會造成內細胞團細胞及滋養外胚層細胞的數目皆減少，2.0 fmole 血癌抑制因子反意寡核苷酸處理胚胎後在囊胚的大小和內細胞團細胞及滋養外胚層細胞數目的比值上也有意義的降低，根據我們第一部分的結果，較小的胚胎形態和較少的細胞數目都會降低著床率。較少的內細胞團及滋養外胚層細胞的數目及內細胞團及滋養外胚層細胞的比值也在 ped 基因受損或去甲基化的的胚胎中被發現(McElhinny et al., 1998; Kang et al., 2002)，血癌抑制因子反意寡核苷酸造成囊胚型態的改變可能是反意寡核苷酸與 mRNA 的 sense 股作用而阻斷轉譯的作用造成囊胚型態的改變。

在本實驗中採用體外培養的方法得到的結果,和其他諸多體外培養的研究中皆證實血癌抑制因子是在著床前期的胚胎發育中是一個非常重要的因子(Mitchell et al., 1994; Marquant-Le et al., 1994; Dunglison et al., 1996; Hsieh et al., 2000; Tsai et al., 2000)然而血癌抑制因子基因剔除小鼠在體內血癌抑制因子完全缺乏的情況下仍能發育到囊胚階段(Stewart et al., 1992)，順利發育至囊胚的現象有可能是因為生殖道中的細胞產生的其他生長因子

所致，諸多生殖道中的生長因子及細胞動力素都會促進囊胚的形成(Hardy and Spanos 2002) ，例如：胚胎本身不表現第一型類胰島素生長因子(insulin-like growth factor I)而由輸卵管產生後存在於輸卵管液及子宮腔液中(Lighten et al., 1997)，添加第一型類胰島素生長因子於培養液中可以增加胚胎發育至囊胚的比例(Spanos et al.,2000)，此外，肝制凝素結合上皮生長因子(heparin binding-epidermal growth factor, HB-EGF) 及巨噬細胞群落刺激因子都由生殖道產生(Bridsal et al., 1996; Zhao et al., 1999)並且也可以促進著床前期的胚胎發育(Martin et al., 1998; Sjoblom et al., 1999)。

注入血癌抑制因子反意寡核苷酸於雙原核期的鼠胚對胚胎生長發育的影響主要存在於桑椹胚期至囊胚期的階段，處理 0.5 至 2.0 fmole 的血癌抑制因子反意寡核苷酸各組之間形成桑椹胚期的發育率並無顯著的差異，但比較處理 1.0 或 2.0 fmole 的血癌抑制因子反意寡核苷酸形成囊胚了比例則有統計意義，這些影響皆有劑量的依存性。將注入 2.0 fmole 的血癌抑制因子反意寡核苷酸的胚胎與 50 ng/ml 的血癌抑制因子蛋白共同培養與注入 2.0 fmole 的血癌抑制因子反意寡核苷酸未補充血癌抑制因子蛋白的胚胎比較可以顯著的恢復囊胚的形成，這個結果和

Tsai 等人(2000)及 Hsieh(2000)等人報導過以血癌抑制因子對著床前期胚胎共同培養後影響胚胎發育的結果類似，也是影響桑椹胚期至囊胚期的階段。然而注入 2.0 fmole 的血癌抑制因子反意寡核苷酸補充血癌抑制因子共同培養後的結果仍然顯著的低於未處理的空白對照組，我們所添加的劑量或許不足以使注入 2.0 fmole 的血癌抑制因子反意寡核苷酸的胚胎恢復至正常狀態，抑或是當胚胎被注入血癌抑制因子反意寡核苷酸後從二細胞期至桑椹胚的胚胎已發生不可逆的改變，而無法回復正常的發育狀態。有研究指出注入各種 DNA 片段於單一細胞的小鼠胚胎中，會導致胚胎生長停滯，而這些胚胎的核存在著大量變異的 DNA(Blangy et al., 1995)。本實驗中所設計的血癌抑制因子反意寡核苷酸包含了一段會與轉錄因子(transcription factor)YY1 結合的區域 CATT(A/T)，這個區域會與諸多的細胞動力素如巨噬細胞群落刺激因子、干擾素的基因促進區(promotor)結合(Yej et al., 1994; Weill et al., 2003)，雖然血癌抑制因子反意寡核苷酸這個區域可能經由非特異性的結合而影響胚胎的發育，但是由細胞免疫染色的結果可以看出 1.0、2.0 或 4.0 fmol 血癌抑制因子反意寡核苷酸注入胚胎後，血癌抑制因子蛋白的表現在二細胞期已經受到改變了，我們曾嘗試於二細胞期補充血癌抑制因子於

培養液中，但是也無法改善血癌抑制因子反意寡核苷酸注入胚胎發育至囊胚期的情況。本研究的發現確定了一個事實，血癌抑制因子對胚胎著床前期的正常發育是一個重要的因子。

第六章 未來研究方向

未來主要研究方向為探討血癌抑制因子受抑制時有那些基因表現同時受到影響。根據前述的結果本實驗將血癌抑制因子反意核苷酸注入原核期鼠胚發現在 1.0 fmole、2.0 fmole 的劑量下大部分的胚胎皆停滯於桑甚胚期，而且即使是發育到囊胚也不一定會著床，這個血癌抑制因子基因是否與其他基因的交互作用而影響胚胎進一步發育至囊胚期，是很值得深入研究的。

後續的研究重點將以基因晶片直接搜尋與血癌抑制因子相互作用的基因群，以確知原核期胚胎受血癌抑制因子之反意寡核苷酸抑制後，停滯於桑甚胚期及囊胚基因變化的情形。

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表一、以核差別染色法染分級後的三組囊胚之內細胞團及滋養外胚層細胞的結果

Table 1. Differential staining in the inner cell mass (ICM) and trophectoderm (TE) of the blastocysts from the three grades.

	Grade		
	I	II	III
No. of blastocysts measured	35	35	35
Diameter of blastocysts (μm)	89.3±1.2[a,*,†]	110.7 ±1.5*	115.8 ±1.6
No. of blastomeres	39.3±1.3*,†	59.2±1.0*	77.4±2.6
No of cells in ICM	15.3±0.6*,†	26.7±0.5*	32.2±1.0
No of cells in TE	24.0±0.8*,†	32.5±0.6*	45.2±1.7
Ratio of ICM/TE cells (%)	64.1±1.9*,†	82.3±1.4*	73.0± 2.1

[a]Mean ± SEM

*Compared with grade III by Student's *t-test*: $P<0.01$.

†Compared with grade II by Student's *t-test*: $P<0.01$.

表二、囊胚分級後的三組胚胎著床率

Table 2. The implantation rate of the blastocysts from three grades.

	Grade		
	I	II	III
No. of recipients	6	6	6
No. of blastocysts transfer	60	60	66
Implantation rate (%)	56.7*	73.3	80.3

*Compared with grade III by X^2 test, $P<0.01$.

表三、第一級與第二級囊胚培養培養至第六天及第七天的孵化百分比

Table 3. Percent of hatching rate in Grade I and II Blastocyst cultures on days 6 and7.

	Day 6	Day 7
Grade I (n=34)	43.3*	63.3
Grade II (n=32)	65.6	68.8

*Compared with grade II by X^2 test, $P<0.05$.

表四、於小鼠雙原核期顯微注射血癌抑制因子反意寡核苷酸後小鼠胚胎於著床前期各階段發育的百分比

TABLE 4. Percentages (%) of murine embryos developing into different preimplantation 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[a] (*n*=145)	1.0[a] (*n*=155)	2.0[a] (*n*=152)	4.0[a] (*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[b]	0[b]
Morula	87.4	86.6	84.9	83.1	84.0	76.6[c]	72.3[b]	63.8[b]	0[b]
Blastocyst	85.4	79.5	79.5	78.3	80.6	63.5[b]	39.4[b]	13.2[b]	0[b]

[a] Compared with the untreated group.

[b] $P < 0.01$.

[c] $P < 0.05$

表五、於小鼠雙原核期顯微注射血癌抑制因子反意寡核苷酸後小鼠囊胚內細胞團及滋養外胚層細胞數目的改變

TABLE 5. 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[a] (2 fmol) (n=55)	1.0[a] (n=142)	2.0[a] (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)[b]	114.2 ± 10.2	111.7 ± 8.7	111.5 ± 9.8	99.5 ± 5.8 [c]
No. of blastomeres	51.5 ± 8.5	47.6 ± 9.5	36.5 ± 10.6[c]	26.3 ± 8.7 [c]
No of cells in ICM	21.6 ± 4.2	18.6 ± 3.6[d]	14.5 ± 3.7[c]	9.1 ± 4.1 [c]
No of cells in TE	29.8 ± 6.2	29.0 ± 8.3	22.1 ± 8.3 [c]	17.3 ± 6.2 [c]
Ratio of ICM/TE cells (%)	74.9 ± 18.9	68.2 ± 21.5	71.2 ± 23.5	55.9 ± 23.9 [c]

[a] Compared with the untreated group

[b] Mean ± Standard deviation.

[c] $P < 0.05$

[d] $P < 0.01$.

表六、於小鼠雙原核期顯微注射血癌抑制因子反意寡核苷酸後囊胚著床率的變化

TABLE 6. 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 recipients	Treatment[a]	Blastulation rate (%)	No. of blastocysts transferred	% of blastocysts implanted[b]
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[c]
		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[d]
		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.

[c] $P < 0.01$,

[d] $P < 0.001$.

表七、於小鼠雙原核期顯微注射 2 fmole 血癌抑制因子反意寡核苷酸再補充血癌抑制因子後胚胎於著床前期各階段發育的百分比

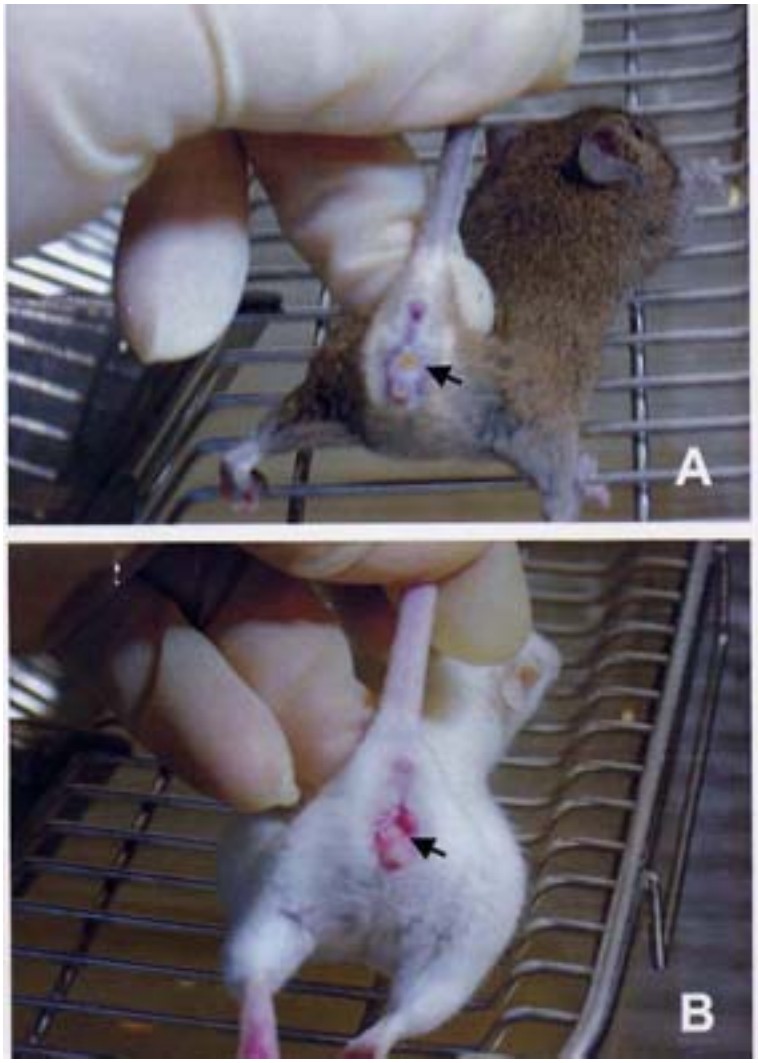
TABLE 7. Percentages (%) of murine embryos developing into different preimplantation stages after microinjection of 2.0 fmol LIF antisense oligonucleotide at the two-pronucleus stage and supplemented with LIF

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

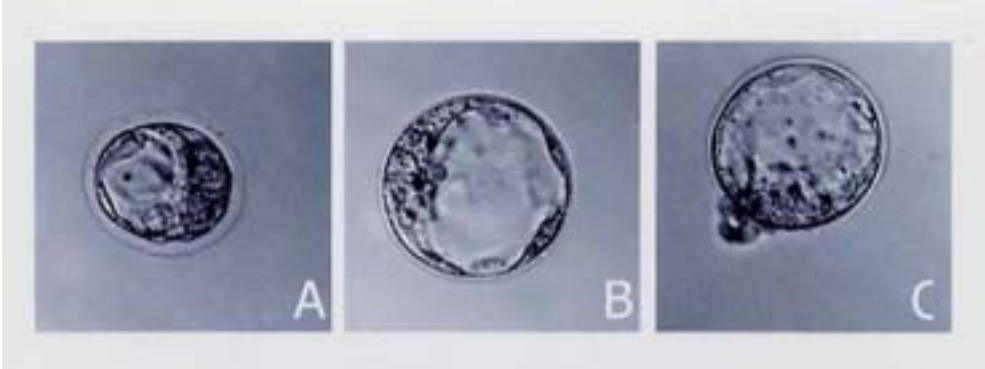
[a]Compared with the untreated group.

[b]Compared with the group without LIF supplement: $P < 0.05$.

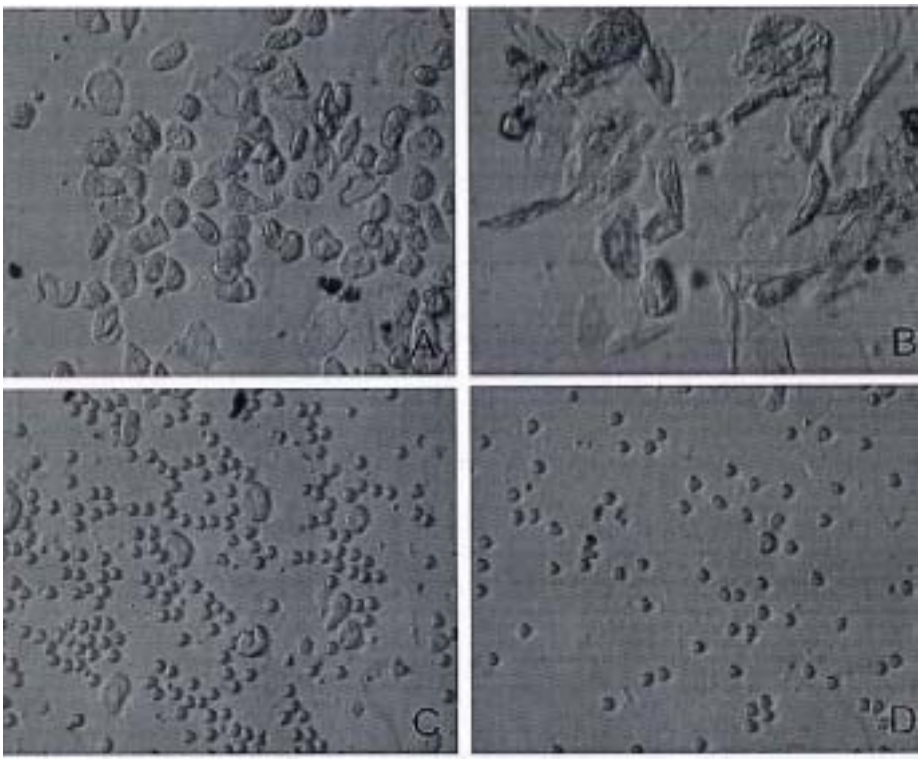
[c]$P < 0.01$.



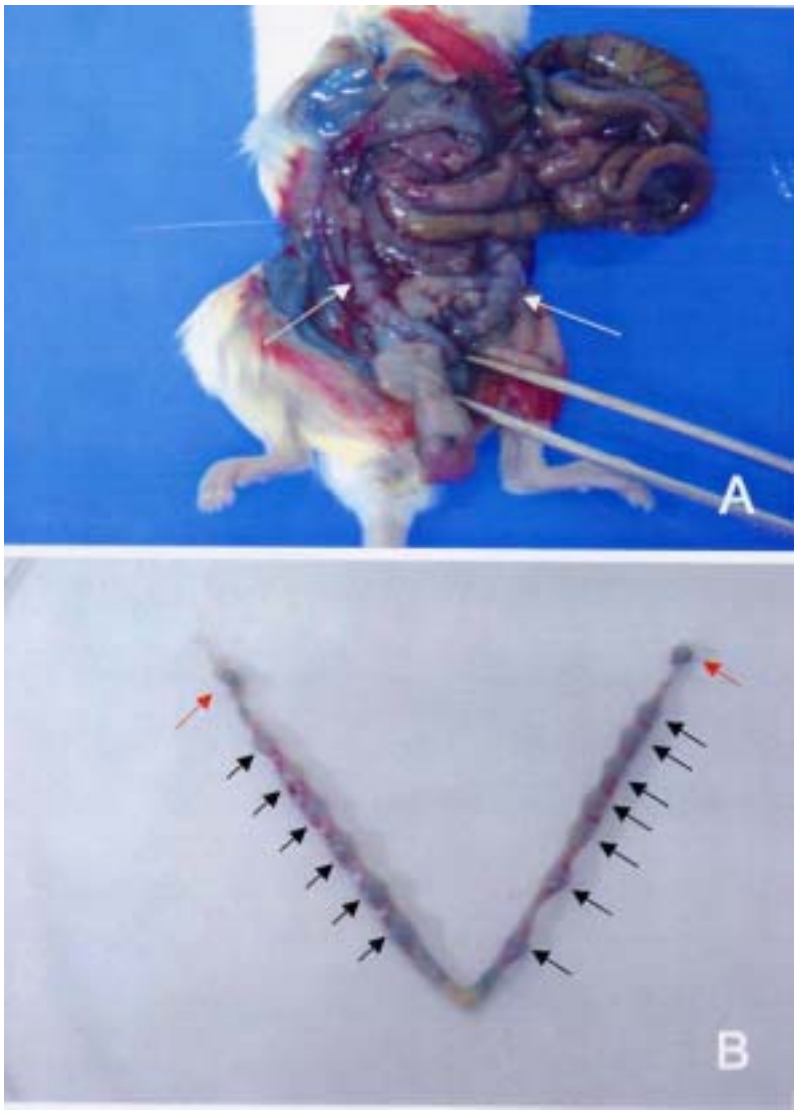
圖一、小鼠交配後之陰道栓圖，A 圖箭頭所指處之白色栓塞為 B6CBF1 小鼠交配後之陰道栓，B 圖箭頭所指處之白色栓塞為 ICR 小鼠交配後之陰道栓。



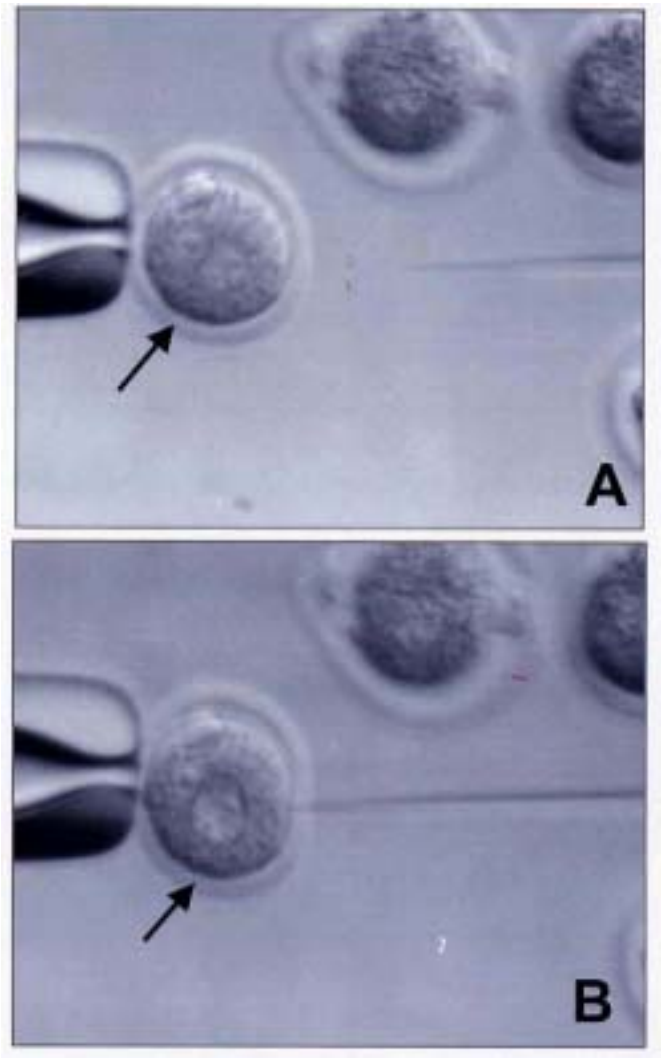
圖二、囊胚的分級圖例，A 圖為第一級囊胚，B 圖為第二級囊胚 C 圖為第三級囊胚。(200 倍)



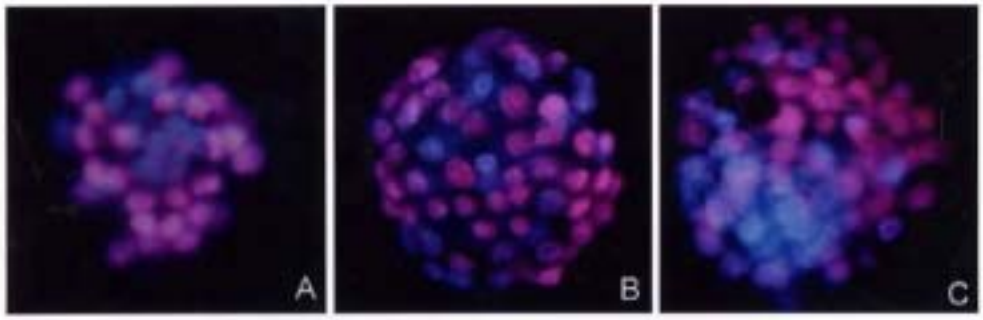
圖三、小鼠陰道抹片之圖例。圖 A 為動情前期(proestrus stage)抹片，主要為有核上皮細胞(nucleated epithelial cells)，圖 B 為動情期(estrus stage)抹片圖，細胞成為角化上皮細胞，或角化上皮細胞形成乾酪質(cheesy)的型態，圖 C 為動情間期(metestrus stage)，圖 D 為無動情期(diestrus stage)抹片圖，大部分細胞皆為白血球。(200 倍)



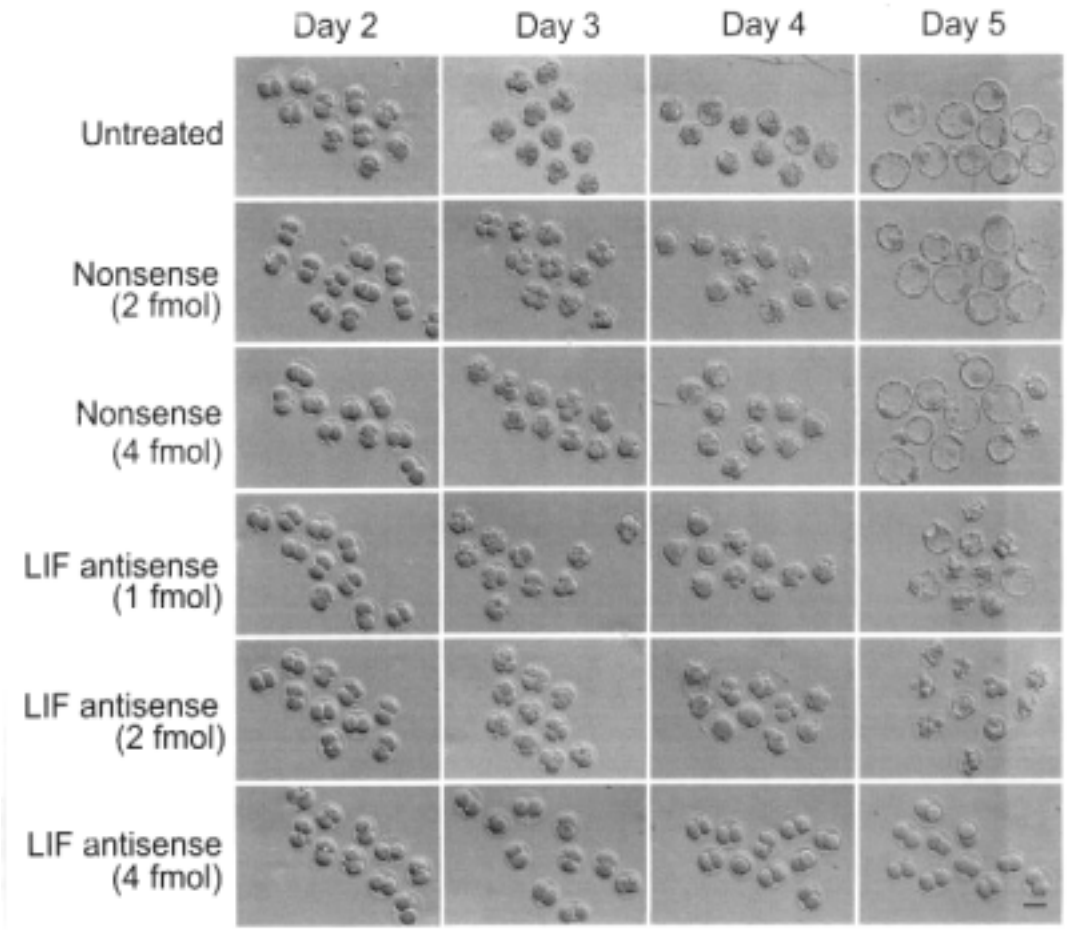
圖四、小鼠子宮著床囊之圖例，A 圖為懷孕母鼠的腹腔，白色箭頭所指為子宮，B 圖為胚胎已著床的子宮，紅色箭頭所指為卵巢，黑色箭頭所指處為著床囊。



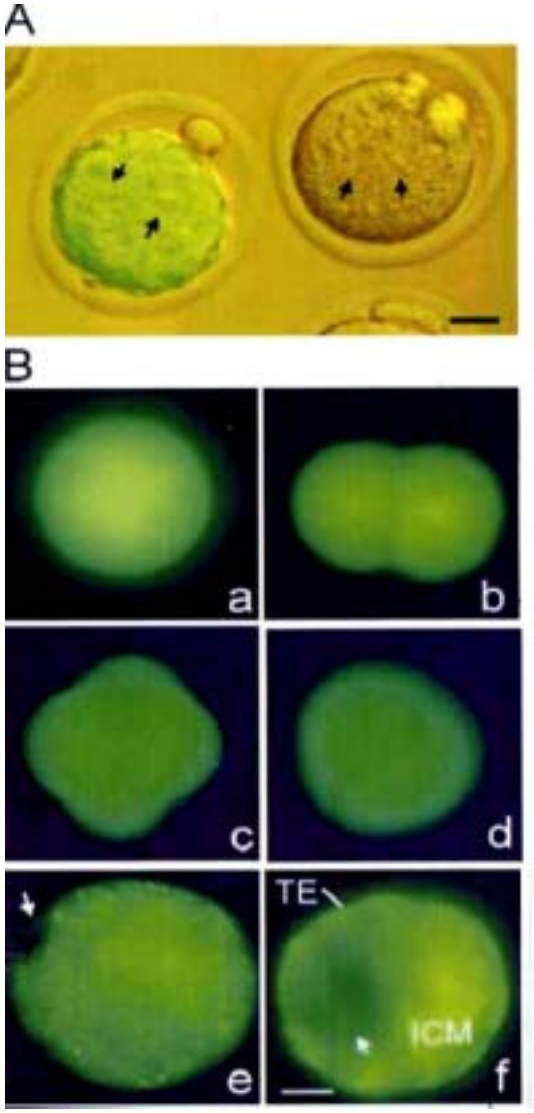
圖五、顯微注射前後原核漲大的情形，圖 A 為顯微注射前，箭頭所指處為原核，圖 B 為顯微注射後，箭頭所指處為原核，經注入反意寡核苷酸而漲大。(200 倍)



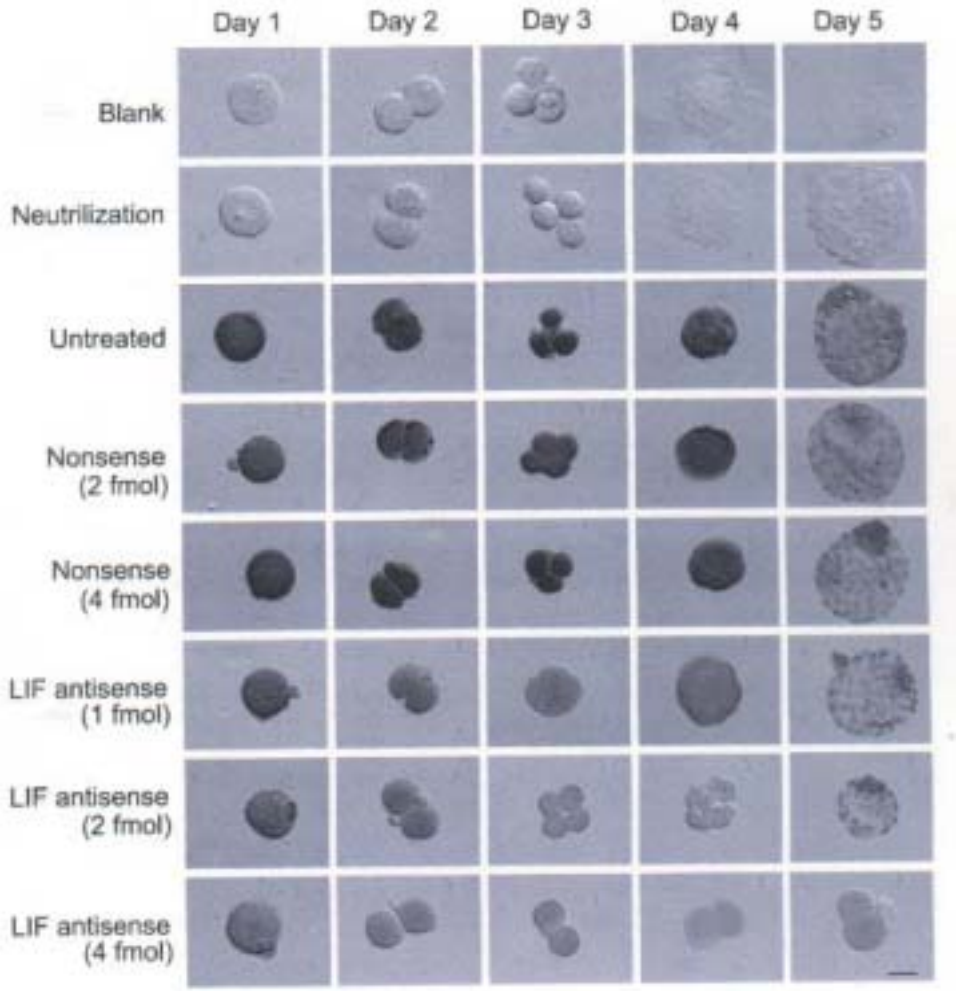
圖六、囊胚核差別染色之圖例，A 圖為第一級囊胚，B 圖為第二級囊胚，C 圖為第三級囊胚，粉紅色的細胞為滋養外胚層細胞，藍色的細胞為內細胞團細胞。(400 倍)



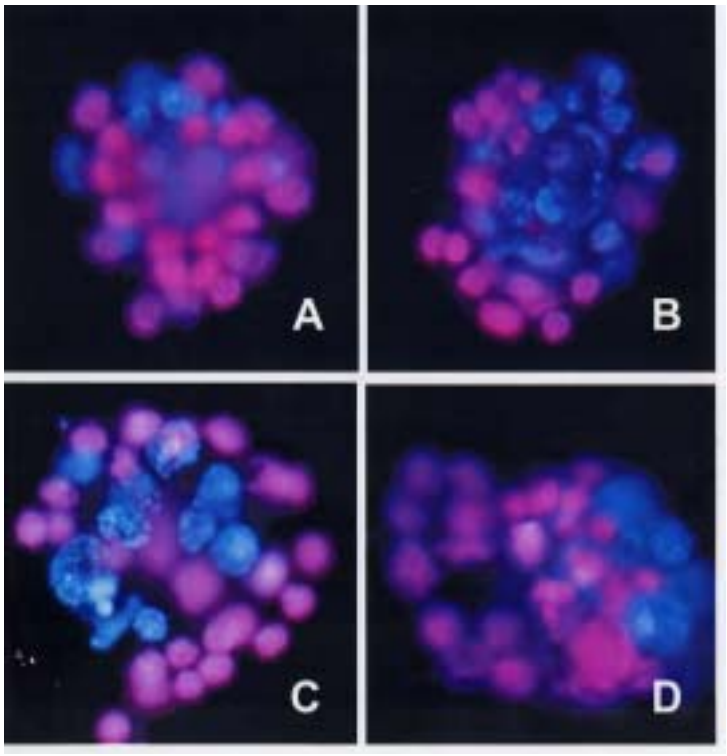
圖七、雙原核期鼠胚注入 1.0 fmol, 2.0 fmol 或 4 fmol 血癌抑制因子反意寡核苷酸後胚胎於第二至第五天發育的型態，控制組注入的是未處理的胚胎及注入 2.0 fmol 或 4 fmol 無意義寡核苷酸，右下方的黑線代表長度為 50 μm。(200 倍)



圖八、雙原核期鼠胚注入 1.0 fmole 血癌抑制因子反意寡核苷酸後追蹤螢光強度於著床前期不同階段改變的情形，A 圖左方胚胎為已注入反意寡核苷酸後的情形，右方胚胎則尚未注射，箭頭所指為原核的位置(200 倍)，B 圖為螢光表現圖，a 為雙原核期，b 為二細胞期，c 為四細胞期，d 為桑椹胚期，e 為囊胚早期，頭所指為囊胚腔 f 為囊胚中期箭頭所指為囊胚腔,ICM 標示處為內細胞團 TE 為滋養外胚層。(400 倍)



圖九、分析注入 1.0 , 2.0 及 4.0 fmole 血癌抑制因子反意寡核苷酸於雙原核期鼠胚第一天至第五天細胞免疫染色的結果，控制組為則未處理的胚胎及注入 2.0 fmol 或 4 fmol 無意義寡核苷酸，空白的對照組未加抗體，中和的對照組則加過量血癌抑制因子反應，右下方的黑線代表長度為 50 μm。(200 倍)



圖十、注入血癌抑制因子反意寡核苷酸於雙原核期鼠胚後發育至囊胚以核差別染色的之結果，A 圖為未處理的囊胚，B 圖為注入 2.0 fmol 無意義寡核苷酸的囊胚果，C 圖為注入 1.0 fmole 血癌抑制因子反意寡核苷酸的囊胚，D 圖為注入 2.0 fmole 血癌抑制因子反意寡核苷酸的囊胚，粉紅色的細胞為滋養外胚層細胞，藍色的細胞為內細胞團細胞。(400 倍)

附錄一

於 BLAST 基因庫比對血癌抑制因子反意寡核甘酸序列 [5’-gaccttcattatgggctggactcta-3’] 對應的基因的結果

BLASTN 2.2.5 [Nov-16-2002]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1041904086-029618-7928

Query=

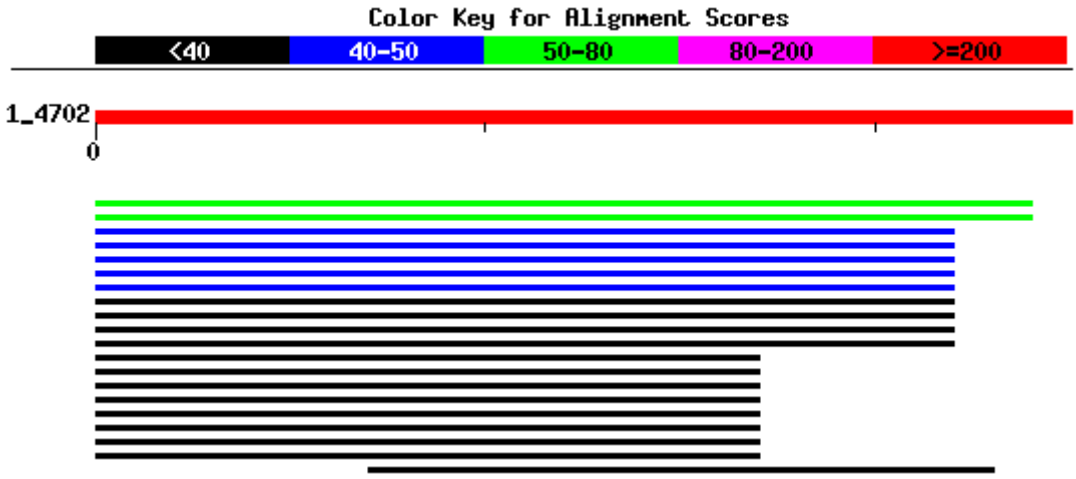
(25 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)

1,561,220 sequences; 7,640,351,724 total letters

Taxonomy reports

Distribution of 20 Blast Hits on the Query Sequence



Sequences producing significant alignments:	Score (bits)	E Value
gi|6678693|ref|NM_008501.1| Mus musculus leukemia inhibitor...	50	5e-05
gi|191877|gb|M63419.1|MUSALIFA Mouse leukemia inhibitory fa...	50	5e-05
gi|52905|emb|X12810.1|MMLIF Murine mRNA for myeloid leukaem...	46	7e-04
gi|3169667|gb|AF065918.1|AF065918 Mus musculus strain SJL/J...	46	7e-04
gi|3169665|gb|AF065917.1|AF065917 Mus musculus strain B10.S...	46	7e-04
gi|2959709|gb|AF048827.1|AF048827 Mustela vison leukemia in...	46	7e-04
gi|21627981|emb|AL731658.20| Mouse DNA sequence from clone ...	46	7e-04
gi|11061695|emb|AJ296176.1|SSC296176 Sus scrofa LIF gene fo...	38	0.17
gi|2182767|gb|U63311.1|BTU63311 Bos taurus leukemia inhibit...	38	0.17
gi|1753092|gb|U65394.1|BTU65394 Bos taurus leukemia inhibit...	38	0.17
gi|1944220|dbj|D50337.1|BOVLIFA Bovine DNA for leukemia inh...	38	0.17
gi|6006018|ref|NM_002309.2| Homo sapiens leukemia inhibitor...	36	0.69
gi|22068644|ref|XM_170988.1| Homo sapiens hypothetical prot...	36	0.69
gi|15559759|gb|BC014233.1|BC014233 Homo sapiens, clone MGC:...	36	0.69
gi|14249832|gb|BC008289.1|BC008289 Homo sapiens, clone IMAG...	36	0.69
gi|2935613|gb|AC004264.1|AC004264 Homo sapiens PAC clone RP...	36	0.69
gi|34361|emb|X13967.1|HSLIF Human mRNA for leukaemia inhibi...	36	0.69
gi|341606|gb|M27052.1|HUMDSF01 Human differentiation-stimul...	36	0.69
gi|178414|gb|M63420.1|HUMALIFA Human leukemia inhibitory fa...	36	0.69
gi|8489776|gb|AF259781.1| Ovis aries microsatellite MNS-110...	34	2.7

附錄二

於 BLAST 基因庫比對反意寡核甘酸標準控制(無意義)序列

[5’- cctcttacctcagttacaatttata -3’] 對應的基因的結果

BLASTN 2.2.5 [Nov-16-2002]

Reference:

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1041904700-04783-30605

Query=

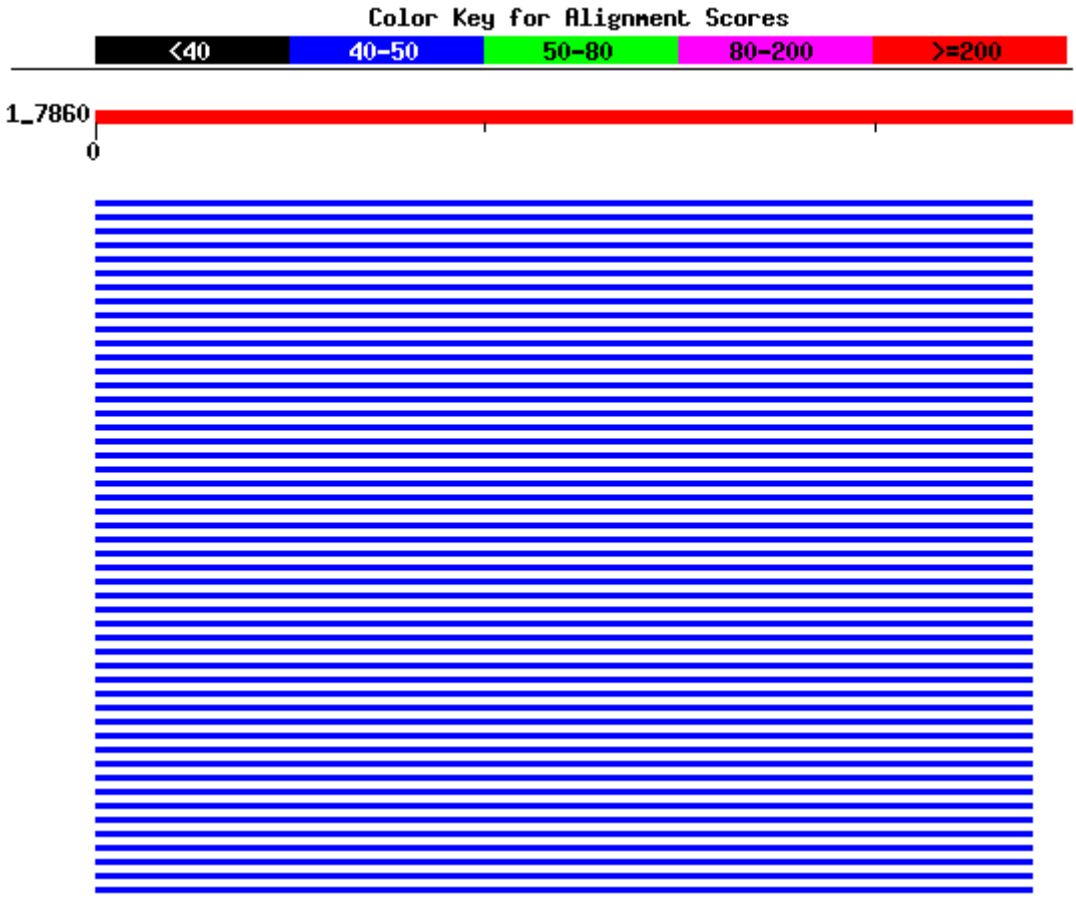
(25 letters)

Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences)

1,561,220 sequences; 7,640,351,724 total letters

Taxonomy reports

Distribution of 62 Blast Hits on the Query Sequence



Sequences producing significant alignments:		Score (bits)	E Value
gi|26892089|gb|AY163866.1|	Homo sapiens beta globin chain v...	42	0.011
gi|25706333|gb|AC129505.8|	Homo sapiens chromosome 11, clon...	42	0.011
gi|24211329|gb|AC104389.7|	Homo sapiens chromosome 11, clon...	42	0.011
gi|22758817|gb|AY128651.1|	Homo sapiens beta-globin beta th...	42	0.011
gi|22758816|gb|AY128650.1|	Homo sapiens beta-globin beta th...	42	0.011
gi|22094826|gb|AF527577.1|	Homo sapiens beta globin mutant ...	42	0.011
gi|18418632|gb|AF083883.1|	Homo sapiens mutant beta-globin ...	42	0.011
gi|18266749|ref|NG_000007.2|	Homo sapiens genomic beta glob...	42	0.011
gi|15148786|gb|AF396260.1|AF396260	Cloning vector pAAV-MCS,...	42	0.011
gi|14994098|gb|AF369966.1|AF369966	Cloning vector pCMV-MCS,...	42	0.011
gi|455025|gb|U01317.1|HUMHBB	Human beta globin region on ch...	42	0.011
gi|12083482|gb|AY013302.1|	Homo sapiens beta-globin (HBB) g...	42	0.011
gi|12083480|gb|AY013301.1|	Homo sapiens mutant beta-globin ...	42	0.011

gi|12083478|gb|AY013300.1| Homo sapiens mutant beta-globin ... 42 0.011
gi|38226|emb|X02345.1|PTGLB1 P.troglodytes beta-globin gene... 42 0.011
gi|38042|emb|X05665.1|MCBGLOG M.cynomolgus beta-globin gene... 42 0.011
gi|4929546|gb|AF083884.1|AF083884 Homo sapiens mutant beta-... 42 0.011
gi|4837722|gb|AF059180.1|AF059180 Homo sapiens mutant beta-... 42 0.011
gi|29440|emb|V00499.1|HSBGL3 Human germ line gene for beta-... 42 0.011
gi|2253431|gb|AF007546.1|AF007546 Homo sapiens beta-globin ... 42 0.011
gi|1066760|gb|L48931.1|HUMHBB45FS Homo sapiens beta-globin ... 42 0.011
gi|1066757|gb|L48221.1|HUMHBBW37X Homo sapiens beta-globin ... 42 0.011
gi|1066778|gb|L48217.1|HUMHBBPAD5 Homo sapiens beta-globin ... 42 0.011
gi|1066775|gb|L48216.1|HUMHBBPA6G Homo sapiens beta-globin ... 42 0.011
gi|1066766|gb|L48213.1|HUMHBB5E90 Homo sapiens beta-globin ... 42 0.011
gi|1066769|gb|L48214.1|HUMHBB5E88 Homo sapiens beta-globin ... 42 0.011
gi|1066772|gb|L48215.1|HUMHBB5E28 Homo sapiens beta-globin ... 42 0.011
gi|1066754|gb|L48220.1|HUMHBB37FS Homo sapiens beta-globin ... 42 0.011
gi|1066783|gb|L48219.1|HUMHBB21FS Homo sapiens beta-globin ... 42 0.011
gi|1066763|gb|L48932.1|HUMHBB114P Homo sapiens beta-globin ... 42 0.011
gi|1066781|gb|L48218.1|HUMHBB10FS Homo sapiens beta-globin ... 42 0.011
gi|183829|gb|M36640.1|HUMHBBAAZ Human beta hemoglobin gene ... 42 0.011
gi|183815|gb|M34059.1|HUMHBB222 Human beta-globin gene from... 42 0.011
gi|183814|gb|M34058.1|HUMHBB221 Human beta-globin gene from... 42 0.011
gi|432469|gb|L26478.1|HUMBETGLOR Human haplotype D3 beta-gl... 42 0.011
gi|432468|gb|L26477.1|HUMBETGLOP Human haplotype D2 beta-gl... 42 0.011
gi|432467|gb|L26476.1|HUMBETGLOO Human haplotype D1 beta-gl... 42 0.011
gi|432466|gb|L26475.1|HUMBETGLON Human haplotype C2 beta-gl... 42 0.011
gi|432465|gb|L26474.1|HUMBETGLOM Human haplotype C3 beta-gl... 42 0.011
gi|432464|gb|L26473.1|HUMBETGLOL Human haplotype C1 beta-gl... 42 0.011
gi|432463|gb|L26472.1|HUMBETGLOK Human haplotype B6 beta-gl... 42 0.011
gi|432462|gb|L26471.1|HUMBETGLOJ Human haplotype B5 beta-gl... 42 0.011
gi|432461|gb|L26470.1|HUMBETGLOI Human haplotype B4 beta-gl... 42 0.011
gi|432460|gb|L26469.1|HUMBETGLOH Human haplotype B3 beta-gl... 42 0.011
gi|432459|gb|L26468.1|HUMBETGLOG Human haplotype B2 beta-gl... 42 0.011
gi|432458|gb|L26467.1|HUMBETGLOF Human haplotype B1 beta-gl... 42 0.011
gi|432457|gb|L26466.1|HUMBETGLOE Human haplotype A4 beta-gl... 42 0.011
gi|432456|gb|L26465.1|HUMBETGLOD Human haplotype A3 beta-gl... 42 0.011
gi|432455|gb|L26464.1|HUMBETGLOC Human haplotype A2 beta-gl... 42 0.011
gi|432454|gb|L26463.1|HUMBETGLOB Human haplotype A1 beta-gl... 42 0.011
gi|432453|gb|L26462.1|HUMBETGLOA Human haplotype C4 beta-gl... 42 0.011

gi|22873|emb|X61109.1|GGBGLOBIN	G.gorilla beta-globin gene	38	0.17
gi|21327455|gb|AC092700.2|	Homo sapiens chromosome 8, clone...	36	0.69
gi|19526139|gb|AC110774.3|	Homo sapiens BAC clone RP11-254A...	36	0.69
gi|17530764|gb|AC091195.6|	Homo sapiens chromosome 8, clone...	36	0.69
gi|17047091|gb|AC084837.4|	Homo sapiens chromosome 8, clone...	36	0.69
gi|14329065|gb|AC009122.8|AC009122	Homo sapiens chromosome ...	36	0.69
gi|4678258|emb|AL049657.1|ATF6I7	Arabidopsis thaliana DNA c...	36	0.69
gi|7269318|emb|AL161562.2|ATCHRIV62	Arabidopsis thaliana DN...	36	0.69
gi|4220510|emb|AL035356.1|ATF22K18	Arabidopsis thaliana DNA...	36	0.69
gi|21536165|gb|AC104099.5|	Mus musculus clone RP24-372J8, c...	34	2.7
gi|21436707|emb|AL646098.9|	Mouse DNA sequence from clone R...	34	2.7

附錄三、五年內已發表或接受之期刊論文(1998-2004)：

1. Lee, M.S., Chang, C.C., **Cheng, T.C.,** Chen, C.I. and Huang, C.C. The cytoplasm transfer offer a new dimension to art. J. Reprod. Infertil. 7:1-6, 1998.
2. Chuang H.Y., **Cheng, T.C.,** Huang, C.C. and Lee, M.S. Doppler ultrasound Assessment of Ascendent uttering artery perfusion in IVF programe. J. Reprod. Infertil 8:174-180, 1999.
3. Huang, C.C., **Cheng, T.C.,** Chang, Chang, H.H, Chen, C.I., Liu, J. and Lee, M.S. Birth after the injection of sperm and the cytoplasm of tripronucleate zygotes into metaphase II oocytes in patients with repeated implantation failure after assisted fertilization procedures. Fertil. and Steril. 72:702-706, 1999.
4. Chang, W.C., Hsieh, Y.Y., **Cheng, T.C.,** Chang, C.C., Tsai, H.D. and Lee, M.S. Effect of Methylglyoxal on Mouse Embryogenesis. Chang Gung Med. J. 24:251-257, 2001.
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附錄四、 論文接受函：Evaluation of mouse blastocyst implantation rate by morphology grading.

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December 30, 2003

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Dear Dr. Liu,

Your revised manuscript entitled "Evaluation of Mouse Blastocyst Implantation Rate by Morphology Grading" (CJP#9248R) has been received by the Editorial Office. We believe that you have successfully revised it according to the reviewer's suggestions. This is to acknowledge you that this manuscript has been accepted for publication in the Chinese Journal of Physiology. You will receive the galley proof soon.

Thank you very much for your support to the Chinese Journal of Physiology and we look forward to your continuous support in the future.

Sincerely Yours,

Paulus S. Wang

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Evaluation of Mouse Blastocyst Implantation Rate by Morphology Grading

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Running title: Morphology grade of blastocyst and implantation rate

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Abstract

The aim of our study was to observe the relationship between the blastocyst morphology and the implantation rate for mice. Mouse embryos obtained from the superovulated-ICR mice were cultured in vitro from 1-cell zygotes to blastocysts. The blastocysts were then classified into 3 grades: grade I, small blastocysts; grade II, large blastocysts; grade III, hatching blastocysts. They were independently transferred into the uterus of recipient females mated with vasectomized male mice at 96 hours after the zygotes were cultured in vitro. The successful implantation was checked by injection of Chicago Sky Blue 6B on the second day after embryo transfer. Although there was no significant difference in the implantation rates between the grade III and grade II blastocysts, grade I was significantly decreased as compared with grade III. Grade I and grade II was also significantly decreased in both the diameter of blastocysts and cell number of inner cell mass (ICM) and trophectoderm (TE) as compared with grade III. These findings indicated that the expanded and haching blastocyst selections for embryo transfer in *in vitro* fertilization were evaluated with the high implantation rate.

Key Words: Blastocyst, Implantation rate, Inner cell mass (ICM), Trophectoderm (TE)

Introduction

In human in-vitro fertilization (IVF) treatment programs, culture and transfer of blastocysts has been reported to generate high pregnancy and implantation rates (2, 8, 14). Many infertility centers use this treatment procedure for some or all of their IVF patients. Through the selection of smaller numbers of more competent embryos, blastocyst transfer may help reduce the frequency of multiple births resulting from IVF (6). To assess the blastocyst stage embryo characteristics that are indicative of viability will further develop the ability to distinguish those embryos more suitable for implantation.

It has been reported that blastocyst formation and hatching could not be used to assess subsequent developmental potential (12). Therefore, these findings constitute a paradox to our previous knowledge. Gardner and Schoolcraft (7) developed trichotomous qualitative assessments based on blastocyst expansion, inner cell mass (ICM) and trophectoderm (TE) to grade human blastocysts before transfer. However, they did not evaluate the number of cells in embryos. Blastocysts with relatively large and slightly oval ICM were more likely to implant than other blastocysts (21). The number of TE and ICM cells allocated in normally fertilized human blastocysts appeared to be similar to that in mice (9). Quantitative measurements of the ICM were highly indicative of blastocyst implantation potential. It is important to analyze ICM

and TE for evaluation of blastocysts.

In this study, we produced an analysis method to measure the number of blastocyst cells and determine implantation potential by using a mouse embryo test system. Mouse embryos have been studied extensively as models for mammalian embryogenesis. Because mouse embryos can be grown in culture, they are also used as a routine "test system" for human IVF programs. We have studied the suitable quality for mouse blastocysts to uterus transfer from *in vitro* cultures. The aim of this research was to study the relationship between the morphology of blastocyst embryos and the implantation rate in mice.

Materials and methods

Mouse embryo collection

Six- to eight-week-old virgin ICR mice were superovulated with an intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma St. Louis, MO, USA), followed by 5 IU of human chorionic gonadotrophin (hCG; Serono, Rome, Italy) 48 hours later. After hCG injection, each female mouse was placed in an individual cage overnight with a mature ICR male mouse with proven fertility. The next morning, successful mating was confirmed by the presence of a vaginal copulation plug. Finding a vaginal plug was designated day 1 of the pregnancy. Approximately 20 hours after hCG injection, mice were sacrificed and zygotes were

obtained from the oviducts using needles. The zygote cumulus cells were removed by exposure to 80 unit/ml hyaluronidase (Sigma) for several minutes until all of them fell off. These zygotes were placed into wells with fresh modified human tubal fluid (Basal-HTF) medium (20) and incubated at 37°C and 5% CO_2 for about 4 hours. Two-pronucleus (2PN) embryos could be observed clearly selected under 40X phase-contrast microscopy. These 2PN embryos were transferred into dishes with pre-equilibrated Basal-HTF medium and incubated at 37°C and 5% CO_2 in air.

Blastocyst grading

Six hundred and fifty zygotes were produced from 32 female mice. The pronucleus fertilization rate was 90.6% (589/650). The total number of blastocysts was 524. Blastocysts were classified based on degree of expansion and hatching status, as follows: grade I, an small blastocyst with a blastocoel equal or less than half the embryo volume; grade II, large blastocyst with a blastocoel greater than half the embryo volume or blastocyst with a blastocoel completely filling the embryo. Hatching embryos were designated as grade III (Figure I). The grade I percentage was 40.5% (212/524). Grade II was 38.5% (202/524) and 20.1% (110/524) of the blastocysts developed to the hatching stage.

Differential staining

Some blastocysts were chosen from each grade randomly before embryo transfer

and stained. Cells in the TE and ICM of the blastocysts were counted after differential nuclei staining using a modified method of Piekos et al (19). Embryos were submitted to zona removal using Tyrods' solution (pH 2.2). 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 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 phoaphate buffered saline (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 using epi-fluorescence using Nikon filter blocks UV-2A and G-2A. Blue nuclei were considered as originating from the inner cells and red-to-pink fluorescing nuclei as belonging to the outer cells.

Mouse 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 embryo

transfer procedures were performed according to Nagy et al. (16). Graded blastocysts were transferred to the top of the uterus by transfer pipettes. We transferred about 7 to 12 blastocysts to each recipient. The mice were sacrificed 2 days after embryo transfer. Successful implantation was verified and determined by injection of Chicago Sky Blue 6B (Sigma).

Statistical Analysis

Rates were expressed as percentages. The numbers and diameters of cells were expressed as mean ± SEM and were statisticed using one way ANOVA followed by Student's *t* test for multiple comparison. Differences between rates were determined using the chi-square test. Differences were considered significant if $P < 0.05$.

Results

Effects of Size and Differential Staining of blastocyts

The diameter of blastocysts of grade I was significantly lower than grade II and III; the grade II was also significantly lower than grade III. We evaluated the quality of these three grades of blastocysts by differential stain (figure 2). The total ICM and trophectodermal cell numbers were calculated (Table 1). Thirty-five blastocysts were stained in grade I, the ICM number was 15.3±0.6 and the TE number was 24.0±0.8. Blastocysts had lower numbers of blastomeres in ICM or TE in grade I. Significant different was found in the cell number of blastomeres, ICM and TE and ICM/TE ratio

between grade I and grade II or III and between grade II and grade III.

Implantation Rate of Blastocysts

We assessed the implantation ability of blastocysts among these three groups by transferring embryos to uterus of foster-mothers. The implantation rates in grade I, II and III were 56.7% (34/60), 73.3% (44/61) and 80.3% (53/66), respectively (Table 2). The implantation rate in the grade III was significantly higher than that in the grade I.

Potential Hatching Rate of Blastocysts

The ability of potential hatching in blastocysts was evaluated. The partial embryos in grades I and II were cultured on day 6 and day 7 after the zygotes were chosen. The hatching rate in grade I was 43.3% (13/34) and grade II was 65.6%(21/32) on day 6 (Table 3). The potential ability of embryo hatching in grade II was higher than that in grade I on day 6. However, there was no significant different between both grades on day7.

Discussion:

Selecting good embryos with high implantation potential is one of the most important factors in the assisted reproduction field. The benefits of blastocyst transfer include suitable synchronization between the endometrium and the embryo and selection of embryos with a higher implantation potential (2). Scoring blastocysts is more complex than scoring embryos in the cleavage stage for increased embryo

transfer selection. Blastocyst culture and transfer have previously been shown to be effective in decreasing multiple gestations and thereby avoid the complications associated with such pregnancies (6, 14). Human blastocyst grading can be quantitated using a three-part scoring system that selects blastocysts for transfer (7). In our study, the quality of mouse blastocysts assessment also affected implantation. Our data indicated that the crucial factors in successful implantation may include blastocoel and hatching formation in our results (Table 2). Similar result was reported by Balaban *et al* (1). They reported that transfer of at least one good quality blastocyst or one hatching blastocyst into women undergoing the IVF cycle was associated with very high implantation and pregnancy rates.

Microscopic observations frequently do not allow the proportion of ICM and TE cells to be determined in a precise manner. This can be achieved by differential ICM and TE blastocyst staining during immunosurgery, using two different fluorescent dyes. The total number of blastocysts was regulated using different conditions, such as half embryo, isolated blastomeres and growth environment (17,22,23). The total cell number of blastocysts and the ICM ratio in our study were similar to that in a previous study (11). An acceptable ICM to total cell development ratio is thirty percent. The three grades in our study showed good blastocyst development. In conclusion, the morphology of blastocysts was good indicators for embryos transferred in the mouse

model.

It was reported that there was a significant positive correlation between number of blastocyst cells and number of ICM cells and subsequent fetal development but morphology as assessed by blastocyst formation and hatching was not correlated with subsequent fetal development (12). However they didn't re-classified quality of blastocysts in different treatments before fransfer to pseudopregnant recipients. The detail variation of blastocysts in each treatment after may disregard in previous peports . We think that to assess fetus development from blastoctst may be grading again before blastocyst transfer as this study. The role of hatching for implantation was more important than the cell number or blastocyst volume in this study.

The potential of embryos developed into blastocysts and implanted may relate to factors in the embryos themselves, such as their gene expression in the nucleus or cytoplasm. One major view was that the disruption of the zona pellucida in an estrogen-sensitized uterus was accomplished through the action of a uterine protease or pronase (10). Previous studies documented the induction of blastocyst hatching by TGF-α (3) and HB-EGF (4) or interferon (INF-τ) (13). When embryos were grown in vitro, successful hatching was dependent on blastocyst expansion and was based on a minium number of embryonic cells. The blastocysts continued their growth and, after having reached a certain threshod in the mean number of embryonic cells, underwent

blastocyst in CB6F1 mice (15). Our data showed that successful hatching in vitro also dependent on a sufficiently high number of embryonic cells as previous study.

More than thirty percent of the blastocysts in grade I and II could not achieve the hatching stage. Some of the embryos achieved full to expanded blastocysts but failed to hatch (Table 3). Embryos that fail to hatch by day 6 may have a lower implantation potential. Embryo transfer can be delayed to day 6 after oocyte insemination, at which time a small percentage of embryos will hatch, and delayed blastocyst growth or hatching did not improve implantation (5). Embryo and uterus cross-reaction is needed in a successful implantation process (18). Therefore, we suggested that predominately hatching or large volume blastocysts may be used for embryo transfer to produce high implantation rates.

In conclusion, we were able to achieve high implantation rates using hatching and expanded blastocysts. Which genes express failure or delay at the implantation stage are yet unknown. The blastocyst genes and hatching environment will be important topics for future research.

Acknowledgements:

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Table 1. Differential staining in the inner cell mass (ICM) and trophectoderm (TE) of the blastocysts from the three grades.

	Grade		
	I	II	III
No. of blastocysts measured	35	35	35
Diameter of blastocysts (μm)	89.3± 1.2[a,*,†]	110.7 ± 1.5*	115.8 ± 1.6
No. of blastomeres	39.3±1.3*,†	59.2±1.0*	77.4±2.6
No. of cells in ICM	15.3±0.6*,†	26.7±0.5*	32.2±1.0
No.of cells in TE	24.0±0.8*, †	32.5±0.6*	45.2±1.7
Ratio of ICM/TE cells (%)	64.1±1.9*,†	82.3±1.4*	73.0± 2.1

[a]Mean ± SEM

*Compared with grade III by Student's *t-test*: $P<0.01$.

†Compared with grade II by Student's *t-test*: $P<0.01$.

Table 2. The implantation rate of the blastocysts from three grades.

	Grade		
	I	II	III
No. of recipients	6	6	6
No. of blastocysts transfer	60	60	66
Implantation rate (%)	56.7*	73.3	80.3

*Compared with grade III by X^2 test, $P<0.01$.

Table 3. Percent of hatching rate in Grade I and II Blastocyst cultures on days 6 and7.

	Day 6	Day 7
Grade I (n=34)	43.3*	63.3
Grade II (n=32)	65.6	68.8

*Compared with grade II by X^2 test, $P<0.05$.

Legend

Figure 1.The classification of three grades of blastocysts. (A) blastocyst from grade I. (B) blastocysts from gradeII. (C) blastocysts from grade III.

Figure 2. Examples of differential staining of blastocysts.(A)blastocystfrom grade I. (B) blastocysts from grade II. (C). blastocyst from grade III. Pink cells were trophectodermal blastomeres and blue cells were ICM.

Figure 1.

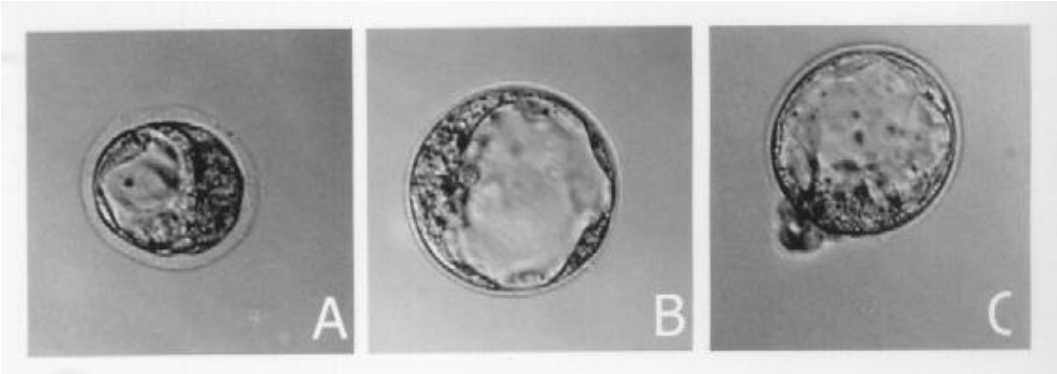
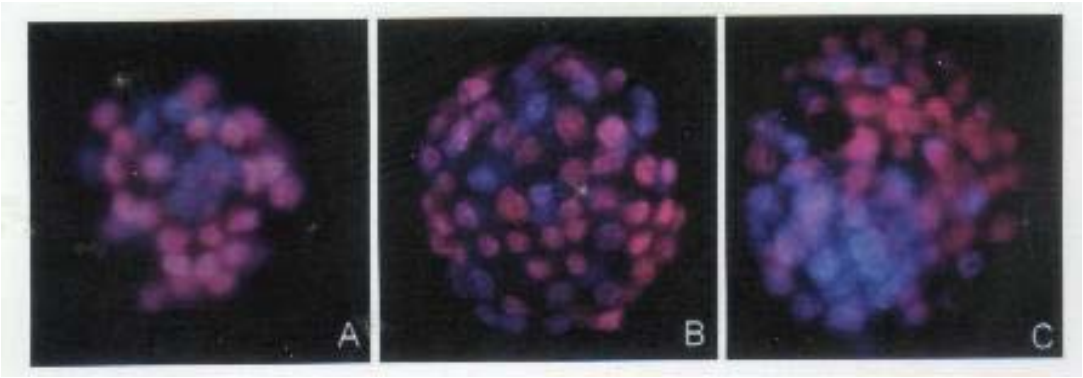


Figure 2.



附錄六、論文接受函：Leukemia inhibitory factor antisense oligonucleotide inhibits the development of murine embryos at preimplantation stages.

Liu

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RE: BIOLREPROD/2003/023283

Dear Dr. Liu:

I am pleased to inform you that your manuscript entitled "Leukemia Inhibitory Factor Antisense Oligonucleotide Inhibits the Development of Murine Embryos at Pre-Implantation Stages" has been accepted for publication in Biology of Reproduction.

As you may realize, Biology of Reproduction now publishes the peer-reviewed PDF version of all accepted papers in Biology of Reproduction-Papers in Press (PiP); a paper's appearance in BOR-PiP establishes date of publication. Your paper will also be published at a later date in traditional form in the regular print and online versions of Biology of Reproduction.

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Leukemia Inhibitory Factor Antisense Oligonucleotide Inhibits the Development of Murine Embryos at Preimplantation Stages[1]

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ABSTRACT

Leukemia inhibitory factor (LIF) is an essential factor for implantation and establishment of pregnancy. However, its role in the development of preimplantation embryos remains controversial. In this study, changes in preimplantation 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 significantly 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 preimplantation stages.

cytokines, developmental biology, early development, embryo, growth factors

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INTRODUCTION

Leukemia inhibitory factor (LIF) is a 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 maternal uterus for preimplantation embryos [3], the expression of this cytokine is only dependent on the embryo in in vitro fertilization. Because 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 maternal uterus. To distinguish the precise role of LIF at the preimplantation stages of embryogenesis in vitro, we employed antisense oligos [13] to attenuate the function of the LIF gene in the in vitro embryos and determined the effects of this treatment on the preimplantation development and implantation. Moreover, effects of supplementing exogenous LIF to the LIF gene-impaired embryos were also investigated.

MATERIALS AND METHODS

Oligonucleotides

Morpholino oligonucleotides were provided by Gene Tools, LLC (Philomath, OR). The LIF antisense oligonucleotide and non-sense oligonucleotide were 5′-GACCTTCATTATGGGCTGGACTCTA-3′ and 5′-CCTCTTACCTCAGTTACAATTTATA-3′, respectively. There were no sequences with significant similarity to the non-sense 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 four contiguous intrastrand base pairs or four contiguous G:C pairs. Moreover, it did not contain over 36% guanines or more than three contiguous guanines for increasing water solubility. The antisense oligo was confirmed by the NCBI FASTA BLAST databases that the sequence does not correspond to any other transcripts. The stability of these oligos was

*4,6 * trophectoderm * net.tw

determined by injecting fluorescein isothiocyanate-labeled preparations into mouse embryo and observing under a fluorescence microscope.

Animals

All mice were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and were housed in humidity- (40–60%)- and temperature- (22 ± 2°C)-controlled rooms and maintained on a 12L:12D photoperiod. Mice were given food and water ad libitum. All procedures were approved by the Chung-Shan Medical University Institutional Animal Care and Use Committee and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Preparation of Embryos

Induction of superovulation was performed in virgin mice (6–8 wk old) of the B6CBF1 strain (C57BL/6 × CBA) by intraperitoneal injection of eCG (5 IU) (Sigma, St. Louis, MO) 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 killed 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 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, 0.5, 1.0, 2.0, or 4.0 fmol. In the positive control groups, the embryos were injected with the non-sense oligonucleotide (2 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) 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 then 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 [15]. 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 4°C overnight, the embryos were washed with the blocking solution for 10 min.

Immunostaining was performed using the VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA). 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 phase-contrast microscopy. The visible staining indicated the immunoreactive LIF protein sites. Corresponding nonspecific binding of embryos were incubated in parallel with the antibody preneutralized with excess antigenic peptide.

Differential Staining of Trophectoderm and Inner Cell Mass

Cells in the trophectoderm (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. [16]. 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) and 10.0 μg/ml propidium iodide (Sigma) at 37°C for 15 min and washed in Dulbecco 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 epifluorescence using the Nikon filter blocks UV-2A and G-2A.

Embryo Transfer

Recipient female mice (8–12 wk 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. [17]. In the same recipient, 5–7 blastocysts injected with LIF antisense oligonucleotide (0.5, 1.0, or 2.0 fmol) were transferred to the right uterus horn and the same number of blastocysts treated with the non-sense oligonucleotide to the left uterus horn. The mice were killed 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, 10, 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 *t*-tests. Changes in the implantation rates were determined using the Kruskal-Wallis test followed by a Mann-Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of LIF Antisense Oligonucleotide on Preimplantation Development

Table 1 shows the percentages of murine embryos developing into different preimplantation stage after various treatments. There were no significant differences in the percentages of embryos developing to the two-cell, four cells, morula, and blastocyst stages among the four control groups ($P > 0.05$). No significant differences were 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 or 1.0 fmol of LIF antisense oligonucleotide, significantly lower percentages of embryos were found to develop to the morula or blastocyst stages ($P < 0.05$). Significantly lower percentages of embryos treated with 2.0 fmol of LIF antisense oligonucleotide developed to the four-cell, morula, or blastocyst stages. 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 four- to eight-cell stage on Day 3, morula stage on Day 4, and blastocyst stage on Day 5. The groups treated with non-sense oligonucleotide had similar developmental stages to the untreated group. However, in the embryos treated with 1.0 or 2 fmol of LIF antisense oligonucleotide, the number developing into the morula or blastocyst stages was greatly reduced on Days 4–5. Among those treated with 4.0 fmol of LIF antisense oligonucleotide, only two-cell-stage embryos were found on Days 2–5 (Fig. 1).

*hCG *Trophectoderm *trophectoderm * (Sigma) *Dulbecco's *Student's *Kruskal

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

	Control				LIF antisense (fmol)				
Stage	Untreated (n = 151)	NaCl (n = 171)	Non-sense (2 fmol) (n = 146)	Non-sense (4 fmol) (n = 83)	0.25 (n = 144)	0.5[a] (n = 145)	1.0[a] (n = 155)	2.0[a] (n = 152)	4.0[a] (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[b]	0[b]
Morula	87.4	86.6	84.9	83.1	84.0	76.6[c]	72.3[b]	63.8[b]	0[b]
Blastocyst	85.4	79.5	79.5	78.3	80.6	63.5[b]	39.4[b]	13.2[b]	0[b]

[a] Compared with the untreated group.
[b] $P < 0.01$.
[c] $P < 0.05$.

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 or 4 fmol non-sense oligonucleotide from Day 1 to Day 5. The groups treated with 1, 2, 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 Blastocysts

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 difference 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 difference between the implantation rate of the blastocysts derived from the untreated and the non-sense 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 non-sense oligonucleotide ($P > 0.05$). However, the embryos treated with 1.0 and 2.0 fmol of LIF antisense oligonucleotide had significantly lower implantation rates

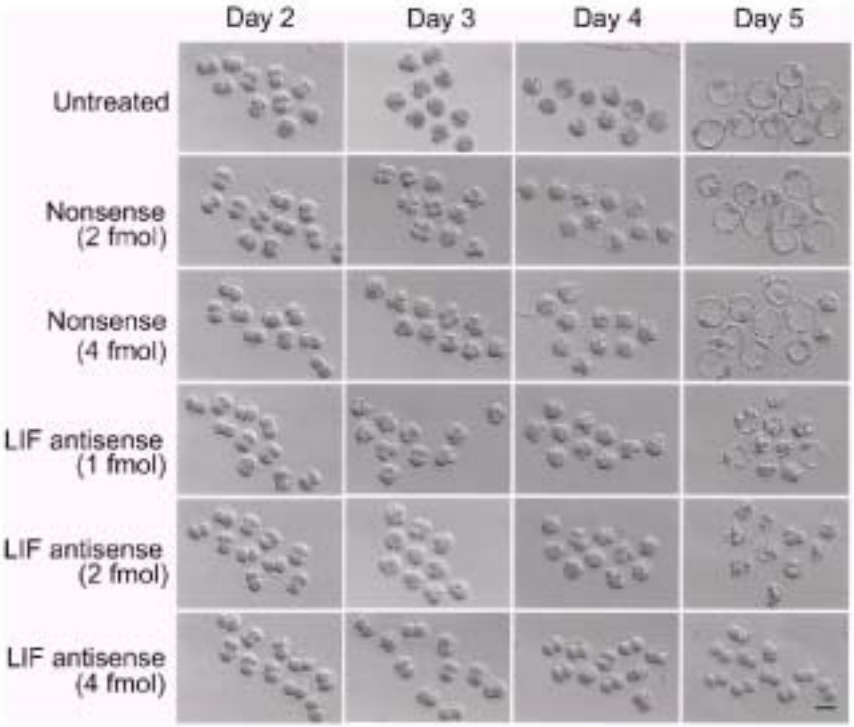

FIG. 1. Morphology of murine embryos on Days 2–5 after microinjection of 1.0, 2.0, or 4 fmol LIF antisense oligonucleotide. The untreated group and the 2-fmol and 4-fmol non-sense-treated groups were the controls. Bar = 50 μm.

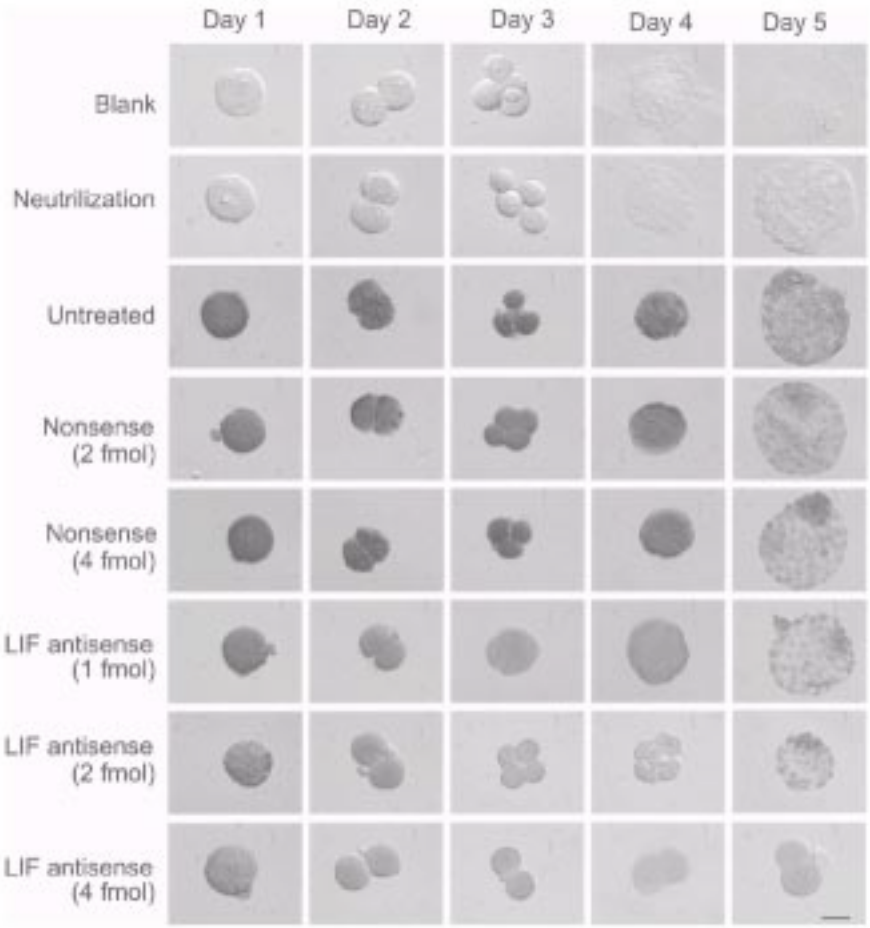

FIG. 2. Immunocytochemical analysis of LIF protein expression on Days 1–5 in murine embryos. The three control groups were indicated as untreated, non-sense (2 fmol), and non-sense (4 fmol). The three LIF antisense oligonucleotide-treated groups were indicated as LIF antisense (1 fmol), LIF antisense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

than their corresponding control embryos treated with the non-sense 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 preimplantation stages ($P < 0.01$). However, blastocysts treated with 50 ng/ml LIF had a significantly higher percentage than those in the LIF gene-impaired group without LIF supplement ($P < 0.05$) (Table 4).

DISCUSSION

The coculture technique has been used to determine the effects of LIF on implantation [18, 19] or preimplantation development of murine embryos 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 preimplantation mammalian embryos [20]. 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 [21]. Disruption of Na/K-ATPase gene expression by antisense oligodeoxynucleotide may abolish blastocyst formation [22]. Morpholino antisense oligonucleotides have been demonstrated to be effective tools for down-regulating gene expression during mammalian preimplantation development [23]. Moreover, the 2′-methoxyethoxy-modified antisense oligonucleotides are candidates for effective examination of roles of large numbers of genes during early embryological development [24]. 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, 2.0, or 4.0 fmol of LIF antisense oligonucleotide from the two-cell stage to blastocyst stage. These findings indicate that our design is useful in investigating the effects of LIF on the preimplantation development of murine embryos in vitro.

In the four experimental groups, 2PN embryos treated

TABLE 2. Changes in the number of cells in the ICM and 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)	Non-sense[a] (2 fmol) (n = 55)	1.0[a] (n = 142)	2.0[a] (n = 283)
Blastulation rate (%)	83.0 (44/53)	80.0 (44/55)	39.4 (56/142)	15.9 (45/283)
Number of blastocysts measured	26	27	31	35
Diameter of blastocysts (μm)[b]	114.2 ± 10.2	111.7 ± 8.7	111.5 ± 9.8	99.5 ± 5.8[c]
Number of blastomeres	51.5 ± 8.5	47.6 ± 9.5	36.5 ± 10.6[c]	26.3 ± 8.7[c]
Number of cells in ICM	21.6 ± 4.2	18.6 ± 3.6[d]	14.5 ± 3.7[c]	9.1 ± 4.1[c]
Number of cells in TE	29.8 ± 6.2	29.0 ± 8.3	22.1 ± 8.3[c]	17.3 ± 6.2[c]
Ratio of ICM:TE cells (%)	74.9 ± 18.9	68.2 ± 21.5	71.2 ± 23.5	55.9 ± 23.9[c]

[a] Compared with the untreated group.
[b] Mean ± SD.
[c] $P < 0.01$.
[d] $P < 0.05$.

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 commence immediately after microinjection, the embryo may have sufficient amounts of LIF for developing into the next stage. Because embryonic genome activation in mouse occurs at the two-cell stage [25], 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 [26]. Because 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 blastocysts. It has been reported that PN-stage morphology is related to blastocyst development [27]. There are a number of stage-specific genes expressed at the different stages of the preimplantation 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 eight-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 [28]. Other genes, such as the glucose transporter GLUT3, growth factor, EGF (epidermal growth factor), EGF receptor, are also detected during and after the morular stage [29, 30]. 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 [31]. 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 preimplantation embryonic development in the mouse [32]. 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 [33]. 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 [34]. Changes in the concentrations of insulin or glucose in the culture medium have been reported to affect the numbers of cells in the ICM and TE [35]. 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 2-fold in the presence of granulocyte-macrophage colony-stimulating factor [36]. The specific impact of tumor necrosis

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 recipients	Treatment[a]	Blastulation rate (%)	No. of blastocysts transferred	Percentage of blastocysts implanted[b]
I	4	Untreated	81.1 (30/37)	22	81.7 ± 21.3
		Non-sense	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
		Non-sense	81.5 (66/81)	52	74.7 ± 32.1
III	10	LIF antisense (1 fmol)	37.8 (76/201)	61	54.3 ± 12.2[c]
		Non-sense	82.5 (85/103)	61	86.1 ± 11.5
IV	8	LIF antisense (2 fmol)	15.1 (46/305)	42	32.9 ± 11.6[d]
		Non-sense	82.4 (70/85)	42	81.3 ± 14.0

[a] Blastocysts (5–7) treated with LIF antisense oligonucleotide were transferred to the right uterus horn of each recipient and the same number treated with 2 fmol non-sense oligonucleotide to the left uterus horn. The untreated blastocysts were transferred to the right uterus horn of each recipient.
[b] Mean ± SD. Compared with control.
[c] $P < 0.01$.
[d] $P < 0.001$.

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

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

[a] Compared with the untreated group: $^{a}P < 0.05$, $^{a}P < 0.01$.
[b] Compared with the group without LIF supplement: $P < 0.05$.

factor alpha on the ICM of blastocysts has also been reported [37]. In this study, we found that the numbers of cells in the ICM and TE were 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 on the morphological characteristics of the blastocysts, which in turn decreases the rate of implantation [38]. Because changes in the number of cells in the ICM or TE as well as the ICM:TE ratio have been observed in the blastocysts with impairments at the gene level [39, 40], the LIF antisense oligonucleotide may block the translation of the selected mRNAs (the sense strand) and lead to the morphological changes in the blastocysts.

The results obtained in this in vitro study were consistent with those studies on in vitro models that indicate that this protein is a critical factor for embryos at various preimplantation stages [8–12]. In contrast with our findings, embryos of knockout mouse have been shown to develop to the blastocyst stage in the absence of LIF in vivo [4]. The success in this preimplementation development may be due to the effects of the other growth factors produced by the cells of the reproductive tract. A number of growth factors and cytokines from the reproductive tract have been shown to promote blastocyst formation [41]. The insulin-like growth factor-I (IGF-I) produced by the fallopian tube is present in the oviduct and uterine fluid but is not expressed in the embryo [42]. Addition of IGF-I to the culture medium may increase the percentage of embryos developing into the blastocyst stage [43]. In addition, heparin-binding epidermal growth factor and granulocyte-macrophage colony-stimulating factor produced in the reproductive tract [44, 45] may also improve the preimplantation development [36, 46].

The effects of microinjection of LIF antisense oligonucleotide to embryo at the 2PN stage were mainly observed in the development of morula to blastocysts. Although there were no significant differences in the formation of morula among the groups treated with 0.5–2.0 fmol of the oligo, formation of blastocysts was observed to be significantly affected in the 1.0- and 2.0-fmol groups. Moreover, these effects were dose-dependent in this range. Coculture of the treated group (2.0 fmol) with 50 ng/ml of LIF was able to recover the formation of blastocysts to a significantly higher level than the group without supplement. These findings are consistent with those reported previously that LIF has beneficial effects on the preimplantation embryos, especially from morula to blastocyst stages [9, 12]. However, this level remained significantly lower than that of the untreated control. The effective concentrations of LIF supplement by coculture may not be sufficient for the normal development of these embryos. It is also possible that microinjection of LIF antisense oligonucleotide to embryos at the 2PN stage may cause irreversible changes in the embryos from two-cell to morular stages. It has been reported that microinjection of DNA fragments in including the yeast centromeric element sequence into one-cell murine embryos results in an early arrest of development with abnormal nuclei containing variable amounts of DNA [47]. The sequence of LIF antisense oligonucleotide used in this study includes a region similar to the DNA binding site CATT(A/T) of the transcription factor YY1. This binding site has been located in the sequence of the promotor of a number of cytokines [48, 49]. Although this region may affect the development of the preimplantation embryos through its unspecified reactions, the expression of LIF in two-cell treated embryos have been altered. We have also found that supplement LIF to the treated embryos at the two-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 preimplantation stages.

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*[c] *0.01

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