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小鼠新穎母源性 Eso-1 基因功能分析(第 2 年)
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行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告
□期中進度報告

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中文摘要

同源箱基因是一群具有轉錄調節能力的基因，此基因群可藉由轉譯出具有同源箱區的蛋白質(homeodomain proteins)來調控胚胎發育過程的細胞分化及決定細胞的命運。在過去的研究中，發現一個在卵細胞及胚胎幹細胞中大量表現的基因—*Eso-1*（expressed in ES cells and ovaries）。*Eso-1* 是一個專一性表達在卵細胞的母源性（maternal effect）基因，從胚胎第 13.5 天開始到成熟的卵細胞都有 *Eso-1* 的表現，*Eso-1* 在成熟的卵中具有 Cytoplasmic polyadenylation 現象，使細胞質內的 *Eso-1* mRNA 之 3'-UTR 具有 poly(A)延長的現象，推測將可以使得 *Eso-1* mRNA 更為穩定並促進轉譯作用的進行。由於卵細胞的成熟發育是一個相當複雜的過程，且目前調控的分子機制大多未明，因此研究 *Eso-1* 基因之功能將有助於我們了解卵細胞的成熟發育及早期胚胎之發育機制。在先前的研究中分析了 *Eso-1* RNA 的表現，在此我們進一步以實驗室產製的 *Eso-1* 多株抗體分析 Eso-1 蛋白質的表現，結果顯示 Eso-1 蛋白質在卵巢組織的表現量由三周開始逐漸增加，且表現於卵巢組織的卵細胞中。本研究論文以 RNAi 的策略來探討 *Eso-1* 基因的功能，利用 *Zp3* promoter（專一性在卵細胞透明環中啟動）驅動 *Eso-1* hairpin 序列，使其能轉錄出雙股 RNA 干擾 *Eso-1* 基因的表現，先轉殖入細胞發現確實能降低 Eso-1 蛋白質的表現量。接著將此 Eso-1-RNAi construct 打入小鼠受精卵中，製作出基因轉殖鼠。在此我們所得到五條不同 copy number 數的基因轉殖鼠中，基因轉殖較高的 line（高：8 copy；其他：3 copy 以下），發現有明顯的生育能力下降的狀況（正常：約 8 隻/胎；轉殖鼠：約 4 隻/胎），而其他 line 中，在生育能力方面，則沒有較明顯的變化。我們亦以 Real-time PCR 進行了 RNA 表現的分析，在 M31、M47、M48 及 M50 這四條 line，十週大轉殖基因鼠卵巢組織中，均發現 *Eso-1* mRNA 表現量下降的情況，而在四周大的小鼠中，*Eso-1* mRNA 表現量則與衛兵鼠無差異。之後更進一步分析其可能的上、下游基因，發現 *Gdf9* 表現量於各轉殖鼠中皆有下降，其可能為 *Eso-1* 的下游基因，將來可進行更進一步的分析。

英文摘要

The homeobox gene products act as transcription factors during animal developmental process. From previous results, we identified a novel homeobox-containing gene, *Eso-1*, which is preferentially expressed in embryonic stem cells and oocytes. The *Eso-1* transcripts contained cytoplasmic polyadenylation sequences whilst the length of poly (A) tails varied during oocyte maturation, indicating that *Eso-1* expression is controlled by a time-dependent translational activation and suggesting that the Eso-1 protein plays a role during oocyte maturation and early embryonic development. Here, we used RNA interference technique to explore the in vivo functions of the *Eso-1* gene. We generated transgenic mice expressing a long double-stranded Eso-1 RNA, drived by an oocyte-specific ZP3 promoter, to interfere *Eso-1* gene expression. Among five transgenic lines, only the line with high copy of transgenes showed significant decrease in female fertility than control littermates. Preliminary real-time PCR also showed decrease in *Eso-1* gene expression in adult ovaries of this transgenic mice. Moreover, we screened the ovaries' RNAs from transgenic mice and observed the decrease of *Gdf9* mRNA. Thus, *Gdf9* might be a downstream gene of the *Eso-1*. We will further analyze the relationship between decrease of *Eso-1* gene expression, oocyte maturation, and early embryonic development.

目錄：

Functional studies of a novel oocyte-specific maternal effect Eso-1 (Cphx) gene (小鼠新穎母源性 Eso-1(Cphx)基因功能分析)

前言:

脊椎動物卵細胞是一種相當特殊的細胞，也是唯一一個能啟動體細胞核使其進行孤雌生殖的一種細胞。然而，卵細胞的成熟過程相當複雜，其間包括很多基因之間的相互作用而達成。由於受限於研究材料的不易獲得，目前對於卵細胞發育成熟之相關分子機制並不清楚。根據過去的研究發現，轉錄因子在胚胎發育的過程扮演很重要的角色，尤其是那些在組織或發育上專一性表現的轉錄因子，細胞可以透過活化或抑制基因的轉錄作用而達到細胞分化的目的，並產生特定的細胞或組織型態。例如：控制眼睛發育的 Pax6 基因，可以讓果蠅在腿或翅膀上長出眼睛(Gehring W.J., 1996)。**了解卵細胞發育過程中之完整的轉錄調控機制，特別是卵細胞專一表現的轉錄因子，可能是將來決定體細胞是否能轉變成卵細胞之重要的關鍵因子**，特別是最近已有研究指出將 Oct4, Sox2, Klf4 及 c-Myc 轉殖入體細胞，可以將體細胞誘導成為與胚幹細胞相似的誘導胚幹細胞(iPS; inducible embryonic stem cell)(Takahashi and Yamanaka, 2006) ，其中 Oct4 即屬於胚幹細胞專一之轉錄因子，藉由 Klf4 及 c-Myc 改變染色質的構造後，再由 Oct4 及 Sox2 啟動下游基因表現，因而使體細胞成為與胚幹細胞相似的 iPS；除此之外，卵細胞專一表現的轉錄因子對於授精作用的成功與否及早期胚胎發育或卵巢腫瘤的形成，也扮演重要的角色。

研究目的：

同源箱基因家族（homeobox gene families）是一群轉錄調節因子的基因群組，其蛋白產物可藉由調節下游基因的表現，而調控生物個體之發育過程（De Rebertis, E.M., 1994）。

我們的研究目標在發現只在早期胚胎表現的新穎同源箱基因，並研究這些新同源箱基因的功能及對胚胎發育的影響。目前對於早期胚胎發育之分子機制未知的比已知的多，尤其是人類，主要受限於道德法律的規範問題，而老鼠卻提供了一個很好的動物模式，來研究早期胚胎發育的分子機制。即使如此，根據 cDNA library 及 EST database 的分析結果發現，在早期胚胎發育中表現的基因大部分是未知功能的基因。為了發掘參與早期胚胎發育之未知功能同源箱基因(Wang et al., 2003; Li et al.,2006)，我們根據同源箱區中之高度保留的氨基酸序列，設計退化寡核酸引子，進行 PCR 反應篩選小鼠胚胎幹細胞 cDNA 基因庫，發現了數個新的同源箱基因，其中一個與目前已知之同源箱基因之同源箱區最高只有 38%的相似性，屬於新的同源箱基因。此同源箱基因同時表現在胚胎幹細胞及卵巢組織中之卵細胞，因此我們命名為 Eso-1 (Embryonic Stem cell-Oocyte expressed genes) 。進一步研究發現 Eso-1 基因主要表現在卵細胞，由於卵細胞及濾泡組織的發育成熟是一個相當複雜的過程，目前對於調控的分子機制所知有限。因此，**研究此 Eso-1 基因之功能將有助於了解卵細胞成熟發育及早期胚胎之發育機制。**

文獻探討:

在卵細胞成熟的過程中，會合成大量的母源性基因mRNAs累積在卵細胞中，這些母源性基因產物將調控受精後胚胎早期的發育。在減數分裂期間，生殖細胞不會進行轉錄作用，當精卵結合後，在胚胎發育至一個細胞晚期到二細胞時期，才又開始胚胎時期的轉錄作用。因此，先前在卵細胞中所累積的母源性基因產物(轉錄及轉譯出之蛋白質)將會影響卵細胞轉變成為胚胎過程中的發育。以基因踢除的方式研究母源性基因，如*Mater*(Tong et al., 2000), *Zar1*(Wu et al., 2003), *Stella* (Payer et al., 2003)及*Npm2*(Burns et al., 2003)的功能，發現當這些母源性基因缺乏時，胚胎發育停留在一個或二細胞時期無法再發育。雖然目前陸陸續續有一些新的卵細胞專一表現的基因被發現，但這些基因的蛋白產物是否存在，並未證實。在卵細胞成長過程中所累積的基因轉錄產物，可能直接轉譯成為蛋白或留待之後經由cytoplasmic polyadenylationc活化轉譯作用的進行。cytoplasmic polyadenylation可使母源性基因轉錄產物加上長的poly (A) tail因而活化轉譯作用的進行。cytoplasmic polyadenylation的進行需要兩種序列的存在：HPS(hexanucleotide polyadenylation signal),即AAUAAA, 以及CPE (cytoplasmic polyadenylation element),即UUUUA$_{1\text{-}2}$U (Mendez and Richter 2001)。已知一些母源性基因如c-mos, tPA, cyclin B1 等藉由cytoplasmic polyadenylation活化轉譯作用的進行。雖然已知適時的調節轉譯作用之進行對原本卵細胞發育轉變為胚胎的發育很重要，**但對於如何活化母源性基因及調節其產物的產生的機制並不清楚，有待研究更多的母源性基因及其產物的產生。**除了轉譯的調控很重要外，活化一些卵細胞專一性表達基因的轉錄，對於卵細胞成熟及早期胚胎發育也是必須的，但目前對這些轉錄的調控機制也並不清楚，**有待研究更多的卵細胞及早期胚胎表現的轉錄因子。**

目前已知調節卵細胞基因表現的轉錄因子，如 *Zfx* (Luoh et al., 1997), Nobox (Rajkovic et al., 2004)及 *Figα* (Liang et al., 1997; Soyal et al., 2000)等，對於生殖扮演重要的角色。小鼠缺乏 Zfx 使卵細胞數目減少且降低生殖力，而同源箱基因 *Nobox* 的缺失則使母鼠不孕，另外 Figa 可調節 ZP 蛋白的合成作用，因此對於初始濾泡的形成扮演重要的角色。當 *Nobox* 基因踢除後，可使一些卵細胞中的基因的表現減少，如 *Mos, Oct4, Zar1, Gdf9* 及 *Bmp15*，因而造成初始濾泡以後之卵細胞無法繼續發育。大部份的轉錄因子根據其與 DNA 結合之特殊結構而分類，這些轉錄因子結合到特定的 DNA 序列後，可以直接活化或抑制下游基因的表現，或經由與其它蛋白質的結合而調節基因的表現。轉錄因子 *Nobox* 基因屬於同源箱基因，同源箱基因家族在生物的個體發育上扮演很重要的角色 (De Rebertis, E.M., 1994)。本研究計畫中之母源性基因 *Eso-1* 就是屬於同源箱基因，以下簡介同源箱基因之相關資料。

同源箱基因最早由果蠅中發現，當同源箱基因 Antennapedia (Antp)發生突變，會產生果蠅觸角異位現象（McGinnis, et al., 1984）。往後的研究發現參與果蠅發育之基因都含有一段高度保留之序列，稱為同源箱（homeobox），故命名之。此段高度保留區可轉譯出約 60 個胺基酸，形成 helix-turn-helix 的結構，藉此與其調控之下游基因特定 DNA 序列結合，而調節基因之表現（Gehring, et al., 1994）。同源箱基因也受一些基因調控而影響其表現。一些訊號蛋白，如 Sonic hedgehog (*SHH*), fibroblast growth factor 及 bone morphogenetic protein (*BMP*)(Chang, et al., 1998)等等，皆可調控特定的同源箱基因表現。

在不同的真核生物上，包括線蟲（Burglin, et al.,1991）、果蠅（McGinnis, et al., 1984）、小鼠（Monaghan, et al., 1991）及人類（Boncinelli, et al., 1988），甚至植物及真菌中，都存在同源箱基因，且在演化上具有保留性，可以影響胚胎時期動物之發育，掌控細胞分化及引導器官之形成。在年幼動物體的第二性徵之產生中，同源箱基因也佔有重要的調控角色（Gehring, et al., 1994）。

目前發現之同源箱基因約有四、五百個之多，且根據其高度保留之核酸序列的特徵，可將其分成三十至四十種的同源箱基因亞群（Kappen, et al., 1993）。脊椎動物之同源箱基因可以分成兩大類，第一類是族群性的，稱為 class I，如 Hox 基因群，這些基因位於相同的染色體上形成群落，在動物胚胎的型態形成上扮演很重要的角色。若 Hox 基因發生突變，將造成與果蠅相似之發育異常現象（Ramirez-Solis, et al., 1993; Gavalas, et al., 1997; Davis, et al., 1995）。第二類之同源箱基因是屬於分布在不同染色體中的其它同源箱基因，除了同源箱區的相似性外，再根據其第二個保留區可將它們再分成不同的同源箱基因次家族，如具有 paired domain 的 paired-type (PAX) (Silberstein, et al., 2002), Iroquois-type (IRX) (Cavodeassi, et al., 2001), 及 Double homeodomain (DUX) (Ding, et al., 1998)同源箱基因次家族等等。

雖然在老鼠基因上已發現大量的同源箱基因，對於早期胚胎之發育過程也累積了越來越多的證據，但對於整個胚胎發育機制還是不很清楚，尤其是著床前胚胎的發育，及更早期的包括卵細胞的成熟與發育的分子機制，更是不清楚。其原因主要是受限於研究材料的取得不易。陸陸續續有新的同源箱基因被發現，也證實同源箱基因在胚胎發育的過程確實扮演很重要的角色。同時，除了正常組織有同源箱基因表現外，一些不正常的組織或病變組織，甚至代謝異常的組織中，都發現其形成跟特定的同源箱基因之不正常表現有關 (Cillo, et al., 2001)。

本研究計劃中新穎同源箱基因與果蠅的 pair type 同源箱基因 BSH9 的同源箱區有最高的相似性，但是也只有 38% 相似性(圖一)，因此並不屬於 pair-type 同源箱基因次家族，應該屬於新的同源箱基因家族。由初步的 RT-PCR 分析此同源箱基因，其 mRNA 主要表現在胚胎幹細胞及卵巢組織中，因此將此基因命名為 *Eso-1* 基因。**經由進一步研究發現此母源性基因具有 cytoplasmic polyadenylation 現像，因此也將其命名為 *Cphx* (cytoplasmic polyadenylation homeobox gene)。**

研究方法:

我們主要以下四個方向分析 *Eso-1* 基因之功能

1. 找出全長的 cDNA 序列，並分析在不同物種上是否存在對應基因

利用 5'及 3'RACE 的結果，獲得全長的 *Eso-1* 完整 cDNA 序列，並觀察是否有不同編譯的 cDNA 序列存在。根據小鼠 *Eso-1* 基因序列，可以再進一步找出其他物種是否存在此 *Eso-1* 基因之 orthologs，它們在不同的物種發育上是否扮演相同的角色。

2 基因結構分析：

根據 *Eso-1* 基因的完整 cDNA 序列，找出相對應之 genomic DNA 序列，並推算出蛋白質的氨基酸序列。這些資料可提供將來分析基因之調控因子及詳細結構功能特徵的骨架，並以此作為建構蛋白質表現載體之模板，以產製 Eso-1 之抗體，進行免疫呈色分析；或建構 Eso-1 conditional knockout DNA construct 之參考。

3.基因表現的模式分析：

利用 RT-PCR, Northern blot, whole mount *in situ* hybridization, immunohistochemistry, 甚至 immunocytochemistry 等各種技術的結合，分析小鼠發育過程中 *Eso-1* 基因表現的分佈趨勢。由初步的結果顯示 *Eso-1* 基因專一性的表現在成熟動物體的卵巢組織。因此進一步分析卵巢組織中之濾泡及不同濾泡時期之卵細胞，及不同減數分裂時期的生殖細胞中，觀察 *Eso-1* 基因的表現，確認 *Eso-1* 基因表現的時間與部位。

4. 基因功能的分析：

由於目前除了胚胎幹細胞之外，並未發現其他的小鼠細胞株表現 *Eso-1* 基因。因此利用 RNAi 技術，產製 *Eso-1* Long dsRNA 基因轉殖鼠，可以檢測 *Eso-1* 基因的不同表現量，對小鼠卵細胞成熟過程及早期胚胎發育過程之影響，進而得知 *Eso-1* 基因在小鼠早期胚胎發育上所扮演的角色。

結果與討論:

1.新的同源箱基因：

為了得到與胚胎早期發育相關之新的同源箱基因，我們以退化性核酸引子（degenerated oligonucleotide primers）進行 PCR (Wang et al., 2003; Li, et al., 2002)，篩選小鼠之胚胎幹細胞 cDNA 基因庫中存在之同源箱基因。退化性核酸引子的設計是根據高度保留之同源箱區之辨識 helix 3 區所設計 23 base 長的核酸，PCR 的產物經由序列分析，我們得到許多個 DNA clones 帶有同源箱序列，根據這些序列推測出之胺基酸序列與 NCBI databases 做比對，發現一個全新的基因，與果蠅的 pair type 同源箱基因 *BSH9* 的同源箱區有最高的相似性，但是也只有 38% 相似性**(圖一)**，因此並不屬於 pair-type 同源箱基因次家族，應該**屬於新的同源箱基因家族。由初步的 RT-PCR 分析此同源箱基因，其 mRNA 主要表現在胚胎幹細胞及卵巢組織中，因此將此基因命名為 Eso-1 基因。**

2. 基因結構分析:

In silico cloning a novel homeobox gene:

將 PCR 所得之 DNA 片段與 NCBI databases 比對，我們發現一個胚胎幹細胞表現的 cDNA 序列(RIKEN cDNA 序列:Accession number: NM_175342)，與 Eso-1 基因具有 100% 的相似性。根據此 RIKEN cDNA 序列與 EST database 比對，發現由未受精卵到 7.5 天之小鼠胚胎及卵巢組織所建立之 EST clones 中，有些與 Eso-1 有極高之相似性，且其中相似性最高且最多之 EST clones，主要來自於卵細胞，由此推測 Eso-1 可能是早期胚胎發育表現的同源箱基因。

根據 NM_175342 序列及利用 RACE（Rapid amplification of cDNA end）-PCR 的方式從胚胎幹細胞表現的 mRNA 進行 reverse transcription (Frohman, et al., 1988)，再以 *Eso-1* 特異性的 primers 進行 PCR，藉此找出 Eso-1 的 transcription initiation site，我們得到 *Eso-1* 全長 cDNA 序列大約由 1710 個核甘酸組成(Accession number: DQ224405) (**圖二**)，將此序列與 MGSC database(http:/www.ensembl.org/Mus-musculus/blastview)比對，發現 *Eso-1* 位於小鼠 14 號染色體。根據 GT-AG 原則推測其 splice sites 可知，*Eso-1* 基因由四個 exons 組成**(圖三)**。由 exon1 到 exon4 之 genomic DNA 大約有 13.8Kb。根據 *Eso-1* cDNA 序列推測，應可轉譯出 149 個氨基酸，其中同源箱區位於 exon2 及 exon3 上(**圖三**)。

根據 cDNA 與 genomic DNA 之結構分布，我們首先設計跨 intron 的 primers (**圖三**)，分析不同發育階段及不同組織或細胞株中，*Eso-1* 基因的表現情形。以 P1 及 P4 primers 進行 RT-PCR 時，發現有兩條 PCR 產物，長度分別為 593bp 及 686bp。經由 DNA 選殖及序列分析後，發現兩者不同處為較長的 686bp 片段比 593bp 片段多包含了 intron3 (93bp)，顯示 Eso-1 基因有 alternative splicing form，而主要的表現形式仍為 1710bp，即不包含 intron3。

同時根據此 cDNA 及 genomic DNA 之結構圖，再分別設計位於 intron 及 exon 上之 primers，以胚胎幹細胞的 genomic DNA 作為模板，進行 PCR，所得到的片段做為探針，篩選 mouse genomic DNA library，藉此選殖 *Eso-1* genomic DNA。所得的 *Eso-1* genomic DNA 可用於分析 *Eso-1* 基因結構並以此作為將來分析 promoter 序列功能或建構基因剔除胚胎幹細胞及基因剔除小鼠所需之 genomic DNA 材料來源。目前已篩選到三個 phage clones，經

由 PCR 已確認它們確實包含 *Eso-1* 基因，已進一步選殖至 pBSII vector，進行 *Eso-1* genomic DNA 序列的分析與 conditional Eso-1 knockout construct 的建構。

目前已根據*Eso-1* cDNA序列，以PCR的方式複製其coding region，轉接至大腸桿菌表現載體，利用大腸桿菌生產GST或HIS_6 融合蛋白，作為生產anti-Eso-1 polyclonal antibodies之抗原。如**圖四**所示，anti-Eso-1 polyclonal antibodies可以辨識經過CMV2-Eso-1-Flag表現載體送入細胞後所產生之Eso-1-Flag fusion protein，未來將以此抗體分析卵巢組織及早期胚胎中Eso-1 蛋白的表現。

另外，由於同源箱基因本身即是一種轉錄因子，可藉由與 DNA 結合而影響下游基因的表現，因此藉由大腸桿菌表現系統所產生的融合蛋白可設計 random oligonucleotides，進行 DNA binding assay，找出 Eso-1 蛋白專一性結合之序列，再推測出可能受 *Eso-1* 基因調節之下游基因，進而推測 *Eso-1* 基因可能的功能。

3. *Eso-1* 基因的表現分布:

(A) RT-PCR and Northern blot analyses

初步以 RT-PCR 的方式分析 Eso-1 基因的表現(**圖五**)。首先分析著床後的 9.5 天胚胎到新生小鼠，及成鼠各組織中基因的表現，發現 *Eso-1* 只專一性地在胚胎幹細胞及成鼠的卵巢組織中表現，而 9.5 天到 17 天的小鼠胚胎到新生小鼠及成鼠的其他各種組織器官，都沒有表現。而在胚胎早期卵細胞時期時 *Eso-1* 表現量最高，而後慢慢的減少至囊胚期都還有 *Eso-1* 的表現，囊胚期中之 inner cell mass (data not shown)及其建立之胚胎幹(ES)細胞都有 *Eso-1* 基因的表現。但 ES cell 以 retinoic acid 誘導分化後，*Eso-1* 表現量明顯下降。有趣的是與 ES cell 特質相似的 teratocarcinoma cell line P19 中，並沒有 *Eso-1* 基因的表現。而且成熟卵細胞旁之 cumulus cell 也沒有 *Eso-1* 基因的表現。綜合 RT-PCR 及 EST database 的比對分析結果，*Eso-1* 基因主要表現於著床前胚胎、幹細胞及成鼠的卵巢組織，推測 *Eso-1* 可能與早期胚胎發育有關。同時由 Northern blot 的分析確認 *Eso-1* 基因只表現於 ES cell 及卵巢組織，分子大小約 1.7kb，和我們得到 *Eso-1* 全長 cDNA 序列相同(**圖六**)。

由以上之結果可知 *Eso-1* 基因主要表現在成熟的卵細胞，接著我們進一步分析未成熟卵細胞是否也表現 Eso-1 基因，因此取胚胎發育的第 12.5 天、13.5 天、14.5 天、17.5 天及新生小鼠，出生後第 3 天、7 天、15 天、20 天以及六週成鼠的卵巢組織及睪丸組織萃取 RNA，進行 RT-PCR。從分析得到的結果顯示(**圖七 AB**)，*Eso-1* mRNA 僅在卵巢組織中表現，而在各時期的睪丸組織中均無表現。於小鼠出生前，胚胎發育的第 12.5 天時並未偵測到 *Eso-1* mRNA 存在，但在隨後的第 13.5 天、14.5 天及 17.5 天則開始偵測到 *Eso-1* mRNA 的微量表現，出生之後一週的表現量開始迅速增加，至 20 天（第三週）表現量達到最高，成鼠之後仍持續穩定表現 *Eso-1* mRNA。顯示雖然胚胎時期卵巢開始表現 *Eso-1* 基因，但真正開始大量表現 *Eso-1* 基因卻是在出生後卵母細胞開始進行濾泡發育的時期，出生後七天幾乎沒有卵母細胞存在了(2%)，大部分卵細胞由一層濾泡細胞包圍行成 primodial follicle(80%)，推論 *Eso-1* mRNA 最早表現於卵巢早期生殖細胞，直到出生後，隨著濾泡細胞的發育，卵細胞內累積的 *Eso-1* 也逐漸的增加。

(B) Western blotting

我們同時取三週、四週、五週及六週小鼠卵巢（實驗室使用小鼠品系為FVB/NJNarl，六週母鼠即可視為成鼠），萃取卵巢組織全蛋白後，以實驗室所產製的 rabbit anti-Eso-1-polyclonal antibodies 進行西方點墨法分析 Eso-1 蛋白質表現（**圖七 C**），預測的 Eso-1 蛋白質分子量大小約 21.3 kDa，根據訊號強度，Eso-1 蛋白質表現量由三週往六週有逐漸增加的趨勢，與 RT-PCR 的表現分佈一致，推測 Eso-1 蛋白隨著卵細胞的成熟，逐漸累積所造成。

(C) whole mount in situ hybridization

因為卵巢組織中除了生殖細胞外，還包括一些體細胞形成的濾泡組織細胞，所以我們進一步以 whole mount in situ hybridization 分析卵巢組織中是否只有卵細胞表現 *Eso-1* 基因。將 4 週大的母鼠以 PMSG 及 hCG 刺激濾泡成熟後，取卵巢組織進行 whole mount *in situ* hybridization，以 *Eso-1* antisense 探針可以偵測卵細胞中有 *Eso-1* 基因表現，其他的體細胞並沒有訊號(**圖八:B,D,F**)。以未處理 4 週大及 12 週大的母鼠之卵巢進行分析，也得到相同的結果 (data not shown)。將 whole mount *in situ* hybridization 後的卵巢組織再進行切片觀察，可發現 *Eso-1* 基因專一性的表現在卵細胞(**圖八:F**)。進一步觀察可發現到 primary follicle 及 secondary follicle 中之卵細胞表現 *Eso-1* 基因，而 primodial follicle 中之訊號較淡。

(D) Immunohistochemistry

以免疫化學組織染色法同樣的發現 Eso-1 蛋白表現在卵細胞中，同時越成熟的卵細胞其表現的 Eso-1 蛋白表現量越高(**圖九**)。

(E) Immunocytochemisty

綜合以上結果可知 *Eso-1* 基因屬於卵細胞專一性表現基因，在成熟卵細胞表現量最高，而 RT-PCR 的結果顯示成熟卵細胞授精後到胚胎發育到著床前，*Eso-1* 基因的表達明顯的逐漸降低，到了囊胚期(blastocyst)後只有微量的表現，因此我們同樣的以 Eso-1 抗體進行細胞免疫染色(**圖十**)，結果顯示，Eso-1 蛋白在一個細胞時期開始微量的表現，但是在兩個細胞時期的表現量達到最高，而後慢慢的減少，在桑椹期(morula)還有微量的表現，但在囊胚期(blastocyst)則幾乎無法偵測到 Eso-1 蛋白的存在，我們因此推測 *Eso-1* 基因可能是一種母源性基因，因此當啟動合子基因的表達促使胚胎發育後，其表現量明顯的下降，推測其功能與卵細胞的成熟與早期胚胎的發育有關。

4. Eso-1 基因產物轉譯的調控:

由過去的研究發現母原性基因在卵細胞成長過程中所累積的基因轉錄產物，可能直接轉譯成為蛋白或留待之後經由 cytoplasmic polyadenylationc 活化轉譯作用的進行 cytoplasmic polyadenylation 可使母源性基因轉錄產物加上長的 poly (A) tail 因而活化轉譯作用的進行，分析 *Eso-1* cDNA 序列發現兩個可能的 CPE 序列，分別位於 AAUAAA 序列之前的 119 及 156 位置(**圖十一**)。我們以 RACE-PAT 分析生長中的卵細胞(GV)、及成熟後維持在 metaphase II 的卵細胞(MII)、受精卵(one cell)及 ES cell 中 *Eso-1* mRNA 是否進行 cytoplasmic polyadenylation。將 RACE-PAT 的 PCR 產物選殖後進行序列分析，發現 MII 的 *Eso-1* mRNA 3' UTR 中的 poly(A)長度大約有 90 到 120 個，而其它 GV 或受精卵及 ES cell *Eso-1* mRNA 中 poly(A)的長度只有 40 到 50 個，顯示 MII oocyte 中之 *Eso-1* mRNA 具有 cytoplasmic

polyadenylation 的現象。此結果暗示 *Eso-1* 基因可能與其它的母源性基因，如 Mos 等一樣，藉由 cytoplasmic polyadenylation 可活化 MII oocyte 中之 *Eso-1* mRNA 的轉譯作用，進而影響卵細胞的成熟或受精作用後早期胚胎的發育。也由於 *Eso-1* 基因具有 cytoplasmic polyadenylation 現象，因此也將其命名為 **Cphx(cytoplasmic polyadenylation homeobox gene)**。

5.基因功能的分析：

根據以上之研究結果顯示，*Eso-1* 是一個卵細胞專一性表現基因，在卵細胞成熟過程中，即從 13.5 天小鼠胚胎到成鼠的卵細胞中都有 *Eso-1* 的表現。成熟卵細胞受精後，*Eso-1* 持續表現，但二個細胞時期以後的 *Eso-1* 表現慢慢減少，至囊胚期仍維持低量的表現。而唯一表現 *Eso-1* 基因的細胞株為胚胎幹細胞，所以我們產製 RNAi 基因轉殖鼠，以改變 *Eso-1* 基因表現量的方式，檢測 *Eso-1* 基因的表現量對卵細胞成熟與早期胚胎發育的影響。

(A). dsRNAi

由於 *Eso-1* 主要表現在卵細胞。因此將以卵細胞專一性表現基因的啟動子(如 zona pellucida:ZP3)，使卵細胞專一性的表現 *Eso-1* double strand RNA (dsRNA)，以抑制卵細胞中的 *Eso-1* 基因表現。這種 long dsRNA 可以使卵細胞及早期胚產生 RNAi 的現象，但並不會造成卵細胞的 apoptosis 現象(Svoboda P, 2000)。一些卵細胞表現的基因，如 Mos 及 Msy2 等，藉由 ZP3 啟動表達 dsRNA 造成卵細胞中個別 RNA 表現量的減少大約是 90%及 80% (Stein et al., 2003; Yu et al., 2004) 。我們以相同的 ZP3 啟動子接上 *Eso-1* dsRNA，產製 ZP3-Eso-1-dsRNA 基因轉殖鼠，探討 *Eso-1* 基因表現對卵細胞的成熟與活化和胚胎發育的影響。

一開始先將 *Eso-1* coding sequence 以 Hairpin 形式構築到 pcDNA3.1（Invitrogen）載體上，製造能在細胞中表現 Eso-1 dsRNA 的 pCMV-Eso-1-dsRNA 質體(**圖十二**)。為測試 pCMV-Eso-1-dsRNA 質體可正常表現 Eso-1-dsRNA，並能抑制 Eso-1 蛋白質表現的能力，因此另外構築了能在細胞中大量表現 Eso-1-flag 融合蛋白質的 CMV2- Eso-1-flag 質體，將 pCMV- Eso-1-dsRNA 與 CMV2- Eso-1-flag 兩種質體共轉染到 HeLa 細胞之中，之後以自行製備的 anti- Eso-1 抗體及 anti-β-Actin 抗體進行西方墨點法分析，測定 Eso-1 蛋白質表現是否被 pCMV- Eso-1-dsRNA 所表現 Eso-1-dsRNA 所抑制由實驗結果可知 Eso-1 蛋白質的表現量會於與 pcDNA- Eso-1-dsRNA 質體共轉染時減少，而細胞內生性的 β-Actin 蛋白質為 internal control，證實 Eso-1 蛋白質表現量的降低確實是受到 Eso-1-dsRNA 所抑制。經過影像分析軟體 AlphaImage 測定之後，在 pCMV- Eso-1-dsRNA 2-1 這個質體抑制下，Eso-1 蛋白質表現量下降了約 25%，略高於 pCMV- Eso-1-dsRNA 3-1 僅有 17%。由這些結果可以證實表現此 pCMV- Eso-1-dsRNA 確實能夠專一性的干擾 Eso-1 蛋白質表現。

由前面的結果（**圖十二**）證明表現 pCMV- Eso-1-dsRNA 在細胞中確實能夠抑制 Eso-1 蛋白質的表現後，接著我們將 Eso-1-hairpin 片段裁切下，並接至由 *Zp3* promoter 啟動的載體 pRNAi-zp3(Svoboda et al., 2001)成為 pZp3- Eso-1-dsRNA（**圖十三**）（*Zp3* 啟動子目前已知僅在卵細胞透明環中啟動）並以此質體 DNA 產製基因轉殖小鼠，用以研究 Eso-1 蛋白質在小鼠中的功能為何。首先利用 *Bgl*II 及 *Afl*II 核酸限制酶將 pZp3- Eso-1-RNAi 線性化以及純化 Zp3- Eso-1-dsRNA DNA 片段，之後委託進階生技公司以顯微注射方式將此線性化後的 DNA 注入小鼠受精卵原核中，產製 Eso-1-dsRNA 基因轉殖小鼠。利用此種方式可使注射進入的外源 DNA 任意嵌入小鼠染色體中，並由其專一性啟動子(*Zp3* promoter)誘導轉殖基因

於小鼠的卵細胞中表現。在顯微注射 Zp3- Eso-1 片段入約略 308 顆小鼠受精卵原核，最後成功得到 106 隻新生小鼠，當小鼠 3 週大之後，分別抽取小鼠尾巴 genomic DNA 作為模板，以 primer：IF 和 IR（附表）利用 PCR 方式分析其基因型，結果在 96 隻小鼠中有 5 隻公鼠帶有 Eso-1-dsRNA 轉殖基因（M21、M31、M47、M48、M50）（**圖十四**）。將此 5 隻公鼠及其衛兵鼠取回後，藉由與 wild-type 母鼠交配繁衍其下一代。

(B)基因轉殖小鼠所帶轉殖基因套數（copy number）分析

基因轉殖鼠所帶轉殖基因套數，會影響到轉殖基因在轉殖鼠中作用的效率，因此，若能得知此五隻基因轉殖鼠所帶有的轉殖基因套數，將有助於分析轉殖基因對造成基因轉殖鼠與 wild-type 小鼠之間的外表型的差異是否相關，對此，我們對此五隻基因轉殖鼠 founder 的 genomic DNA 進行南方點墨法。Genomic DNA 經過核酸限制酶 *Pst* I 截切並以電泳膠分離至適當位置，經過轉漬與預雜交後，再以針對轉殖基因所設計並帶有放射線的探針進行雜交，之後透過清洗以及壓片，我們便可透過探針標定的位置對轉殖基因套數進行判斷（**圖十四**））。當轉殖基因套數為一套時，探針標定位置會出現在大於 2.2 kb 處，當套數為兩套時，探針標定位置，除了大於 2.2 kb，另外在 3.8 kb 處亦會出現，當套數大於兩套時，便以影像分析軟體分析訊號強度，推知所帶轉殖基因套數（**圖十四**）。由結果可知，M21 帶有轉殖基因套數最少，僅為一套，其次為 M31 及 M48 分別帶有兩套轉殖基因，M47 帶有三套轉殖基因，M50 帶有八套數的轉殖基因（**圖十四**））。

(C)基因轉殖小鼠的遺傳分析

經由多次以 Wild-type 母鼠與基因轉殖公鼠交配後，我們得到五條 line 的 F1 共 297 隻，其中帶有轉殖基因的基因轉殖小鼠為 144 隻，遺傳機率為 48%，接近孟德爾的遺傳定律（50%）（**圖十五**），由此我們可判斷帶有 Eso-1-dsRNA 此轉殖基因，對出生前小鼠胚胎而言，並無致死性，此一結果符合預期，因 Eso-1-dsRNA 並未對雄性基因轉殖鼠所生育的後代造成影響，因對雄性配子精子而言，Zp3 promoter 此時並無驅動 Eso-1-dsRNA，應當不會造成任何影響。

另外，根據統計，公鼠共 157 隻，佔 F1 中 53%，母鼠共 140 隻，佔 F1 中 47%；基因轉殖鼠中，公鼠為 84 隻，佔轉殖鼠比例為 58%，母鼠為 60 隻，佔轉殖鼠比例為 42%，；而在衛兵鼠中，公鼠為 73 隻，佔 47%，母鼠為 80 隻，佔 53%（**圖十五**）。由以上結果，公母鼠比例接近 1：1，且與是否帶有轉殖基因並無直接關係。

而在小鼠傳代的過程中，有出現小鼠於出生後未滿一週時死亡的現象，此一比例於轉殖基因鼠中略高於衛兵鼠（15%：10%），是否轉殖基因可能會造成小鼠於出生後的發育出現問題，導致帶有轉殖基因的小鼠於出生後死亡。為證明轉殖 Eso-1-dsRNA 基因是否具有胚胎致死性，故抽取所有死亡小鼠的 genomic DNA，以其為模板進行 PCR，由其結果統計出於出生後一週內死亡的小鼠中共 45 隻，其中 18 隻帶有轉殖基因，佔死掉小鼠的 40%，27 隻不帶轉殖基因，佔 60%（**圖十五**），由此結果判斷，是否帶有轉殖基因 Eso-1-dsRNA，對小鼠胎兒時期的發育，並無致死的影響。

(D)基因轉殖小鼠的生育能力分析

由於 Eso-1 為母源性同源箱基因，根據過去對母源性基因的瞭解，常於受精卵發育早

期扮演重要角色，因此，Eso-1-dsRNA 基因轉殖母鼠與其衛兵鼠母鼠相比，是否有生育能力低下或其子代胚胎發育不正常，是我們關切的重點。由於小鼠每胎子代數目關係著公鼠與母鼠的生育能力，當與母鼠交配的公鼠固定時，每隻母鼠所生育的子代數目即代表其生育能力。為比較 Eso-1-dsRNA 基因轉殖母鼠與其衛兵鼠之間的生育能力，我們採固定的五隻公鼠，分別與待測量生育能力的母鼠交配，於懷孕生產後統計每隻基因轉殖母鼠與其衛兵鼠生產子代 F2 隻數，並將其量化為圖表（**圖十六**）。由圖可知，M21、M31、M47、M48 其 F1 母鼠的生育能力相近，每胎平均生育小鼠隻數皆為八隻，與衛兵鼠每胎平均生育九隻相比較下並無明顯差異，但在帶有八套轉殖基因的 M50，其 F1 母鼠的生育能力，則分為兩個族群，分別是在 M50 前期所生的 F1，三隻母鼠共生育四胎，每胎平均生育的小鼠隻數僅為四隻，以及在 M50 較晚期所生的 F1，五隻母鼠共生育四胎，每胎平均生育小鼠隻數為八隻。推測可能在高套數轉殖基因時，Eso-1-dsRNA 表現的量才足夠將 *Eso-1* mRNA 表現量 knock down 到影響其正常功能，故僅有 M50 帶有八套轉殖基因 F1 母鼠，出現生育能力下降的現象。

此外，在已統計的 F2 中，共有 237 隻，帶有轉殖基因小鼠 113 隻，佔 F2 中 47%，而帶有轉殖基因母鼠 51 隻，佔轉殖基因鼠中 45%，與 F1 比例相似，因此，F2 轉殖基因的遺傳以及性別之間，亦無直接關係。

(E)基因轉殖小鼠的*Eso-1* mRNA表現量分析

為確定轉殖基因小鼠體內 ***Eso-1*** mRNA 表現量是否確實受到 Eso-1-dsRNA 干擾，導致 *Eso-1* mRNA 表現量降低，因而造成 M50 的 F1 母鼠生育能力下降，因此以半定量實時聚合酶連鎖反應（real-time PCR）確認轉殖鼠與衛兵鼠之間 *Eso-1* mRNA 表現量是否不同。

在設計 real-time PCR 的引子時，需考量到 genomic DNA 會干擾 *Eso-1* mRNA 偵測的問題，故在設計引子時，以跨 intron 來避免結合到 genomic DNA 的引子為優先選擇。在這次的實驗中，引子設計在跨 intron2 兩端的 exon2 與 exon3 上。

分別取四週及十週，各條不同 line 的 F1 基因轉殖母鼠與其衛兵鼠卵巢組織。在外觀上，基因轉殖母鼠與衛兵鼠卵巢組織在大小及形狀上並無不同。取出的卵巢組織用以萃取組織 total RNAs，之後將 total RNAs 做 Reverse-transcription 後，取定量 cDNA 上 real-time PCR。將所測得的 *Eso-1* mRNA 表現量除以 *Gapdh* 表現量，便可得到單位組織所表現的 *Eso-1* mRNA。以衛兵鼠的 *Eso-1* mRNA 表現量當作 100%，比較各個不同 line 之間，*Cphx* mRNA 表現量的差異，來確定 *Eso-1*-dsRNA 是否成功 knock down 基因轉殖鼠體內 *Eso-1* mRNA 的表現量。在四週基因轉殖母鼠卵巢中，*Cphx* mRNA 表現量與衛兵鼠比較結果（**圖十七**），發現不管在 M31、M47 或是 M50 的 *Eso-1* mRNA 表現量與衛兵鼠相較之下是沒有顯著差異的，表示在四週大時，Eso-1-dsRNA 並無成功 knock down *Eso-1* mRNA 表現量。但在生育能力方面，我們確實觀察到 M50 中，F1 母鼠每胎生育子代數減少的現象，推測可能是四週小鼠卵巢內成熟卵細胞數目不多，因此 *Eso-1* mRNA 表現量也少，因此當 *Eso-1*-dsRNA knock down 後結果並不明顯。所以另外以十週成鼠卵巢組織當作樣品，進行 real-time PCR，結果顯示（**圖十七**），除了僅帶有一套轉殖基因的 M21 其 F1 *Eso-1* mRNA 表現量未下降外，M31（兩套轉殖基因）下降八成、M47（三套轉殖基因）下降四成、M48（二套轉殖基因）下降達九成以及 M50（八套轉殖基因）下降五成。

(F) *Cphx*於發育上的定位

在得到 M50 的 F1 可能因為 *Eso-1* mRNA 表現量下降，導致生育能力下降的結果，想探討是 *Eso-1* 基因表現量下降直接造成卵細胞於成熟發育方面出現問題，或是 Eso-1 透過影響其他已知的下游基因，造成受精卵無法正常發育因而影響生育能力。為得知 *Eso-1* mRNA 主要表現於卵巢中何時期的濾泡，我們重新檢視 RT-PCR 的結果，在三天到七天的卵巢中，*Eso-1* mRNA 表現量有顯著的上升，而三天到七天在卵巢的發育過程中，是原始濾泡細胞（primordial follicles）開始大量進入初級濾泡細胞（primary follicles）的時間點，因此推估 *Eso-1* mRNA 開始大量表現的時間點是在原始濾泡細胞開始進入初級濾泡細胞這段時間內。在參考許多近年來發表的卵細胞專一性表現基因後，我們決定測試 *Figlα* 與 *Gdf9* 在轉殖基因鼠中的表現情形，*Figlα* 從進入原始濾泡細胞前開始表現，而 *Gdf9* 則從進入初級濾泡細胞後開始表現，此兩基因於發育的時間點上，可能一個在 *Eso-1* 的上游、一個在下游。為證實此推論是否正確，取基因轉殖鼠與其衛兵鼠卵巢組織 cDNA，分別以 *Figlα* 及 *Gdf9* 的引子做 real-time PCR 後，得到結果（**圖十八**）為 *Gdf9* 表現量與 *Eso-1* 表現量，在基因轉殖鼠中明顯都比衛兵鼠來的減少許多，除了 M21，*Gdf9* 與 *Eso-1* 兩者的表現量都與衛兵鼠表現量相近，因此 *Gdf9* 表現的時間點，可能確實為 *Eso-1* 的下游，或是受到 *Eso-1* 所調控。而在 *Figlα* 表現量方面，在 M47 及 M48 這兩條 line 中，如同我們推測 *Figlα* 可能表現的時間點先於 *Eso-1*，因此 *Figlα* 的表現量在基因轉殖鼠與衛兵鼠之間並沒有差異，推斷為不受轉殖 Eso-1-dsRNA 基因所影響：但在 M31 及 M50 這兩條 line 卻出現預期外 *Figlα* 表現量在基因轉殖鼠中下降的現象，目前推測原因可能為轉殖 Eso-1-dsRNA 基因會導致卵巢組織中卵細胞的數目減少，因而造成 *Figlα* 表現量減少。

(G)卵巢組織中卵細胞數目是否受Eso-1-dsRNA影響

為了確認 Eso-1-dsRNA 轉殖鼠中，*Figlα* mRNA 表現量的減少是否是因為卵巢組織中卵細胞數目減少所造成，取 M50 F2 中的衛兵鼠及轉殖鼠的卵巢組織，進行包埋及切片，以蘇木紫及伊紅染色後，於顯微鏡下統計卵細胞數目（**圖十九**），將計數的卵細胞分為單層濾泡組織的 primordial 及 primary follicle，以及多層濾泡組織的 secondary 及 antral follicle，分別獨立計數兩次後，將結果整理成表（**圖十九**），結果顯示，雖然帶有轉殖基因小鼠卵巢中卵細胞平均數略少於衛兵鼠（384：442），但因此數目位於標準誤差範圍內，因此可能為個體差異所造成；此外，減少的卵細胞數不到 17%，應不至於造成 M31 及 M50 此兩條 line 中 *Figlα* mRNA 表現量減少六成以上，因此，研判 M31 及 M50 此兩條 line 中 *Figlα* mRNA 表現量大幅降低可能為其他因素所造成。

討論：

1.*Eso-1* mRNA 的表現趨勢

於小鼠發育過程中，胚胎第 13.5 天，生殖細胞進入第一次減數分裂後，開始偵測到 *Eso-1* mRNA 存在，估計於此時期 *Eso-1* 開始轉錄。但直到出生前，*Eso-1* 僅維持低量的存在，推測這段時間內卵細胞僅需極低量 *Eso-1* 即可維持正常發育；出生後生殖細胞囊（germ cell cyst）分裂，濾泡細胞（follicle cell）開始包圍每個獨立的卵細胞，約在小鼠出生後 3 天濾泡細胞完成包圍獨立的卵細胞，成為卵細胞的基本單位，此時稱為原始濾泡（primordial follicle），而於出生後 7 天，原始濾泡開始發育進入初級濾泡（primary follicle）， 約出生後第 10 天至 14 天左右初級濾泡進入次級濾泡(secondary follicle)，而分析出生後 *Eso-1* mRNA 表現量的趨勢，發現於新生小鼠開始增加，由第 3 天到第 7 天時 *Eso-1* mRNA 有明顯的增多，之後表現量則僅是稍微增加直到第 20 天，達到最大量，由此一結果推測，*Eso-1* mRNA 應為進入初級濾泡時期後開始進行大量表現，而隨著卵細胞的發育，以及 mRNA 量的累積，因此表現量由第 7 天到第 20 天持續增加，但到成鼠時期，卻發現 *Eso-1* mRNA 表現量沒有第 20 天的訊號來的多，推測可能是因為卵巢組織中的濾泡及其他細胞增生，而卵細胞數目不變，所以導致 *Eso-1* mRNA 佔 total RNAs 比例減少，因此成鼠時期的表現量看起來反而較第 20 天的少。

2.Eso-1 蛋白表現

Eso-1 蛋白質表現在卵巢組織的卵細胞中，而卵細胞蛋白質佔整個卵巢組織蛋白質的極小部份，可能是導致 Western blot 中蛋白訊號微弱的原因，但從圖我們仍然可以得知 **Eso-1** 蛋白質隨母鼠週數增加而有較高的表現量，估計可能與性成熟相關，其中包括卵巢中成熟卵細胞數目的增加，以及成熟卵細胞開始表現 **Eso-1** 蛋白質等等，將來可嘗試萃取接近一歲或生育過幾次的母鼠與六至八週母鼠的卵巢組織全蛋白，進行 Western blot，探討 **Eso-1** 蛋白表現量的增加是因為卵細胞數目的增多或是卵細胞成熟後表達量增多。

從免疫組織化學切片染色的結果中，我們可以看到 **Eso-1** 蛋白質平均表現在整個卵細胞中，但訊號並不強，因此需要提高抗體濃度以進行偵測，但提高抗體濃度會使背景訊號增加，如此一來，微量表現的細胞，便有可能被我們判斷為背景值。

3.未來研究方向

在我們所得到的轉殖鼠中，M50 這條 line 發現了生育能力下降（每胎生育隻數僅為衛兵鼠的一半），也進一步以 real-time PCR 分析，發現轉殖鼠 *Eso-1* mRNA 表現量降低，僅為衛兵鼠的五成左右。因此，我們推測生育能力的下降是與 *Eso-1* mRNA 表現量降低互相關聯，而想要更進一步瞭解，*Eso-1* mRNA 表現量降低是如何使生育能力降低，是因為在卵細胞成熟的過程需要 *Eso-1* 存在，才能正常成熟進而被卵巢排出；或是，*Eso-1* mRNA 表現量降低，降會使得受精卵在發育的過程中，因為缺少 *Eso-1* 及其相關聯下游基因，造成受精卵無法正常發育為個體。為了解決這個問題，我們將取 M50 帶有轉殖基因母鼠的受精卵進行 In vitro culture；或是注射 dsRNA 到受精卵中，觀察在胚胎著床前的時期，受精卵的發育是否正常，如果發生異常，又是發生在哪個時期，進而定位 *Eso-1* 在胚胎發育的過程中，扮演著什麼樣的角色。

另外，根據最近的文獻指出，*Figlα*（**F**actor **i**n the **g**erm**l**ine, alpha，專一性表現在生殖細胞，能調控卵及濾泡的發育與成熟）的基因剃除鼠中，也同時偵測到 *Eso-1* 基因的表現量降低，推測 *Figlα* 可能是調控 *Eso-1* 表現的上游基因。因此我們構築了 *Figlα* 蛋白表現系統，另外將 *Eso-1* promoter 區域接上 reporter gene，一同轉殖入細胞中，觀察 *Figlα* 是否直接啟動 *Eso-1 Eso-1* promoter，進而影響 reporter gene 的表現。

根據十週轉殖鼠與衛兵鼠卵巢組織中，real-time PCR 的結果，我們發現 *Eso-1* 與 *Gdf9* 的表現量是同時減少的，因此推測 *Gdf9* 可能為 *Eso-1* 時間上的下游基因，或是直接受到 *Eso-1* 的調控，為了證明此一論點，我們預計將 *Gdf9* 的 promoter 區域接上 reporter gene，與已經擁有的 *Eso-1* 蛋白表現系統共轉殖到細胞中，便可觀察 reporter gene 的改變與否，來判斷 *Gdf9* 是否直接受到 *Eso-1* 的調控。

參考文獻

Boncinelli, E., Acampora, S.R., Pannese, M.D., Esposito, M. and Someone, A. (1988) Hum.Reprod. 3, 880-886.

Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Science 296:550-553.

Burglin, T.R., Ruvkun, G., Coulson, A., Hawkins, N.C., McGhee, J.D., Schaller, D., Wittmann, C., Muller, F. and Waterston, R.H. (1991) Nature (London) 351,703-705.

Burns KH, Viveiros MM, Ren Y, Wang P, DeMayo FJ, Frail DE, Eppig JJ.,and Matzuk MM. (2003) Science 300:633-636.

Cavodeassi, F., Modolell, J. and Gomez-Skarmeta, J.L. (2001) Development 128: 2847-2855.

Chang, P.Y., Kozono, T., Chida, K., Kuroki, T., and Huh, N. (1998) Biochim Biophys. Res. Commun. 248, 749-752.

Cillo, C., Cantile, M., Faiella, A. and Boncinelli, E. (2001) J. Cell Physiol. 188: 161-169.

Davis, A.P., Witte, D.P. , Hsieh-Li, H.M., Potter , S.S. and Capecchi , M.R. (1995) Nature 375:791-794.

De Rebertis, E.M. (1994) The hemeobox in cell differenitiation and evolution. In : Guidebook to the Homeobox genes, Douboule , D. (Ed) , Oxford University Press, New York , p11-23.

Ding, H., Beckers, M.C., Plaisace, S., Marynen, P., Collen, D. and Belayew, A. (1998) Hum. Mol. Genet. 7: 1681-1694.

Frohman, M.A., Dush, M.K. and Martin, G.R. (1988) Proc. Natl. Acad. Sci. USA 85: 8998-9002.

Gavalas, A., Davenne, M., Lumsden, A.,Chambob, P. and Rijli, F.M. (1997) Development 124, 3693-3702.

Gehring, W.J., Qian, Y.Q., Bileter, M., Furukbo-ToKunaga, K., Schier, A.F., Resendez-Perez, D., Affolter, M., Otting, G. and Wuthrich, K. (1994) Cell 78, 211-223.

Gehring W.J. (1996). Genes Cells 1,11-15.

Kappen, C., Schughart, K. and Ruddle, F.H. (1993) Genomics 18, 54-70.

Li, H., Tsai, M.S., Chen, C.Y.,Lian, W.C., Chiu,Y.T., Chen, G.D.and **Wang, S.H.*** (2006) Mol. Reprod. Dev. 73, 825-833.

Liang L, Soyal SM, and Dean J. (1997) Development 124(24):4939-4947.

Luoh SW, Bain PA, Polakiewicz RD, Goodheart ML, Gardner H, Jaenisch R,and Page DC. (1997). Development 124(11):2275-2284.

McGinnis , W. , Levine , M.S. , Haefen , E. , Kuroiwa , A. and Gehring , W.J .

(1984) Nature (London) 308, 428-433.

Mendez R and Richter JD. (2001) Nat Rev Mol Cell Biol 2(7):521-529.

Monaghan, A.P., Davidson, D.R., Sime, C., Graham, E., Baldock ,R.,

Bhattacharya, S.S. and Hill, R.E. (1991) Development 112, 1053-1061.

Payer B, Saitou M, Barton SC, Thresher R, Dixon JP, Zahn D, Colledge WH, Carlton MB, Nakano T, Surani MA. (2003). Curr Biol 13(23):2110-2117.

Rajkovic A, Pangas SA, Ballow D, Suzumori N and Matzuk MM. (2004) Science 305(5687):1157-1159.

Ramirez-Solis, R., Zheng, H., Whiting, J., Krumlauf, R. and Bradley, A. (1993)

Cell 73, 279-294.

Silberstein, G.B., Dressler, G.R. and Van Horn K. (2002) Oncogene 21: 1009-1016.

Soyal SM, Amleh A, and Dean J. (2000) Development 127(21):4645-4654.

Svoboda, P., Stein, P., and Schultz, R.M. (2001) Biochem. Biophys. Res. Commun. 387, 1099-1104.

Svoboda, P. (2004) Cytogenet Genomes Res. 105, 422-434

Stein, P., Svoboda, P., and Schultz, R.M.(2003) Dev. Biol. 256:187-193.

Takahashi, K., and Yamanaka, S. (2006). Cell 126, 663–676.

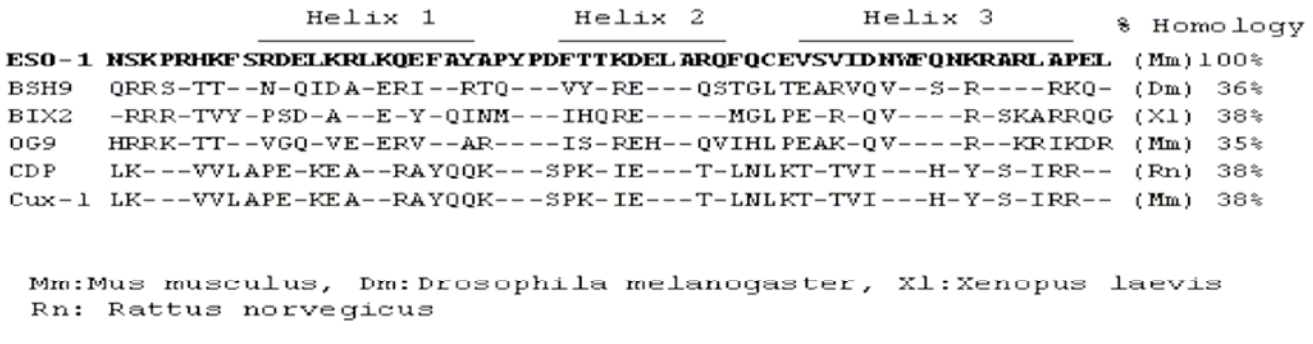
Tong ZB, Gold L, Pfeifer KE, Dorward H, Lee E, Bondy CA, Dean J, Nelson LM. (2000) Nat Genet 26(3):267-268.

Yu, J., Deng, M., Medvedev, S., Yang, J., Hecht, N.B., Schultz, R.M. (2004) Dev. Biol. 268: 195-206.

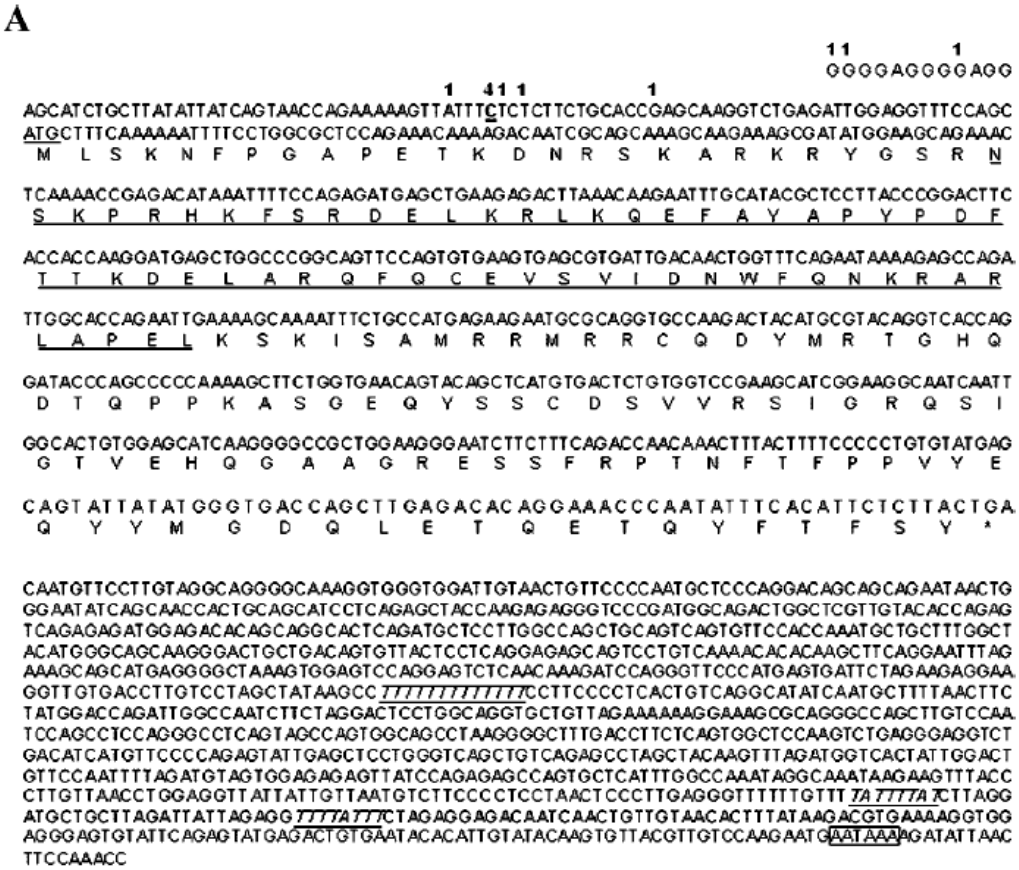
Wang, S.H., Tsai, M.S., Chiang, M.F., and Li, H. (2003) Gene Expres. Patterns, 99-103.

Wu X, Viveiros MM, Eppig JJ, Bai Y, Fitzpatrick SL, and Matzuk MM. (2003) Nat Genet 33(2):187-191.

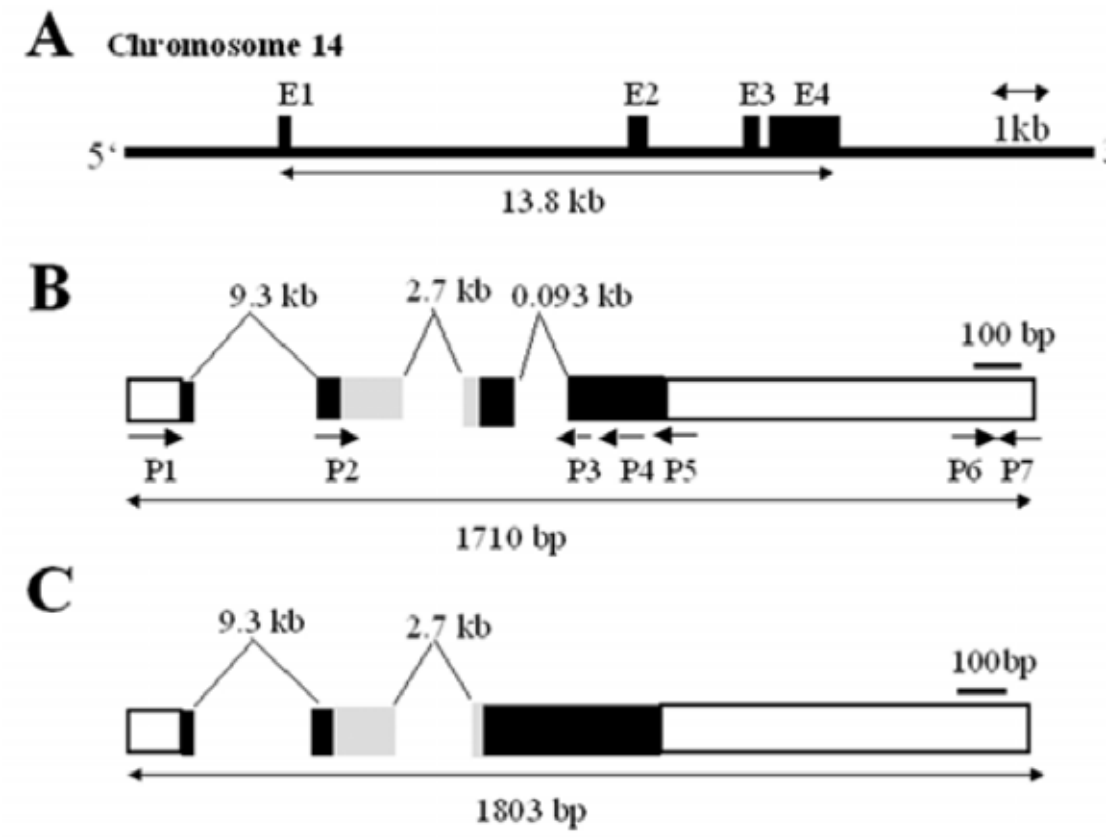
附圖：



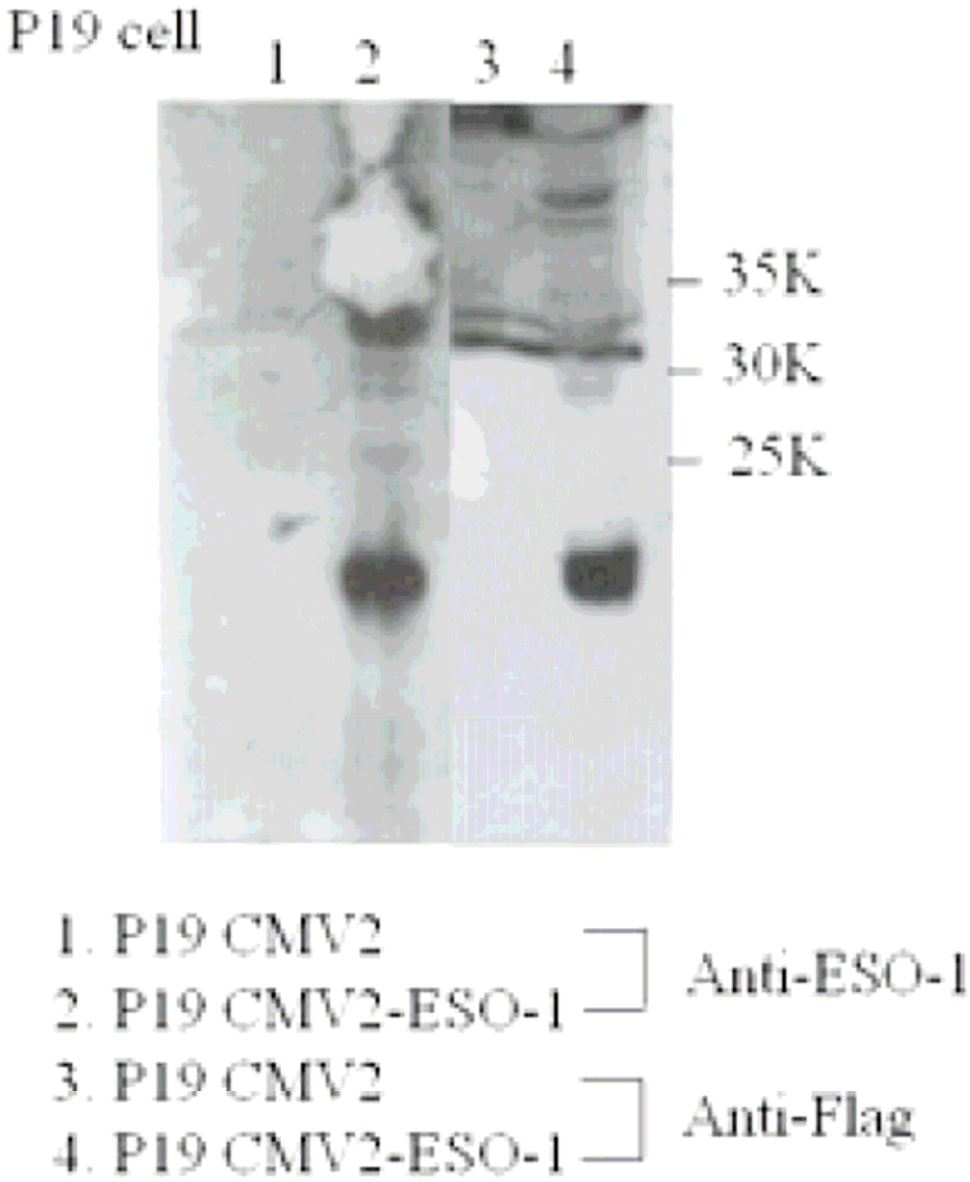
圖一、 將推測的小鼠 *Eso-1* 同源箱蛋白序列與其他同源箱蛋白做序列上的比較。橫線部份表示與小鼠 *Eso-1* 相同的序列。*Eso-1* 為根據 NCBI 資料庫上大鼠 *Eso-1* 轉錄序列（XM_577474）所推測的同源箱蛋白序列，其座落在大鼠第十六號染色體得 P16 臂部份;CUX-1 是小鼠 cut-related 的同源箱蛋白(Vanden Heuvel et al., 1996)；BIX2 是非洲爪蟾中的同源箱蛋白 BIX2（Tada et al., 1998)：CDP 是大鼠中 CCAAT displacement 蛋白（Yoon and Chikaraishi, 1994)；BSH9 是果蠅中 gooseberry 蛋白（De Graeve et al., 2004)；OG9 為小鼠的同源箱蛋白（Cinquanta et al., 2000）



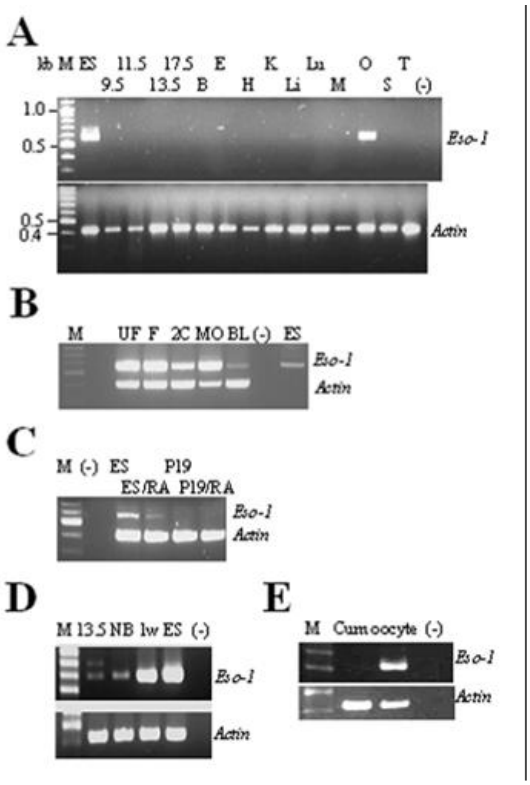
圖二、*Eso-1* 基因的DNA序列及推測的蛋白質序列。*Eso-1* 基因的cDNA序列全長以及推測其所相對應的胺基酸序列。小鼠*Eso-1* 的cDNA序列長主要為 1710bp，可以轉錄出 182 個胺基酸。轉錄起始點（ATG）為底線所標示位置，轉錄終點（TAA）以星號標記，核內的polyadenylation訊號以框線表示，而同源箱蛋白質所在位置以底線標示。在五端非轉錄區域中加底線的C為AK049114 的序列起始處，核苷酸序列上標示的數字為不同的轉譯起始所出現的頻率。另外，底線標示出三個可能在細胞質內polyadenylation區（CPE），分別是U_{13}，UUUUAUUU以及UAUUUUAU。*Eso-1* 的核苷酸序列已經在GenBank中註冊，索引號碼為DQ224405



圖三、 小鼠 *Eso-1* 基因在基因體中的組成結構。 A：*Eso-1* 基因座落在小鼠第十四號染色體 A3 臂的位置，含有四個 exon (E1-E4)。 B：黑色實心部份為蛋白轉錄區域，灰色部份代表同源箱結構，而白色框表示非轉錄區域。Introns 大小如圖所標示。箭頭所表示的位置及方向為本篇研究中所使用到的引子。*Eso-1* 主要轉錄出帶有四個 exon 的片段的 mRNA。 C：*Eso-1* 次要轉錄出帶有三個 exon 片段的 mRNA。小鼠 *Eso-1* 在基因體上分佈的大小以及轉錄片段的大小標示如上。



圖四、 將 CMV2-Eso-1 表現載體送入 P19 細胞株中進行蛋白表現，之後進行西方點墨法，分析 anti-Eso-1 抗體效果。Lane 1、3，表現 CMV2 vector only，為負控制組；lane2，4，表現帶有 *Eso-1* 基因的 CMV2-Eso-1。Lane1、2 以 anti-Eso-1 抗體進行辨識；lane3、4 以 anti-Flag 抗體進行辨識。CMV2-Eso-1 融合蛋白大小為 22.8 kDa。

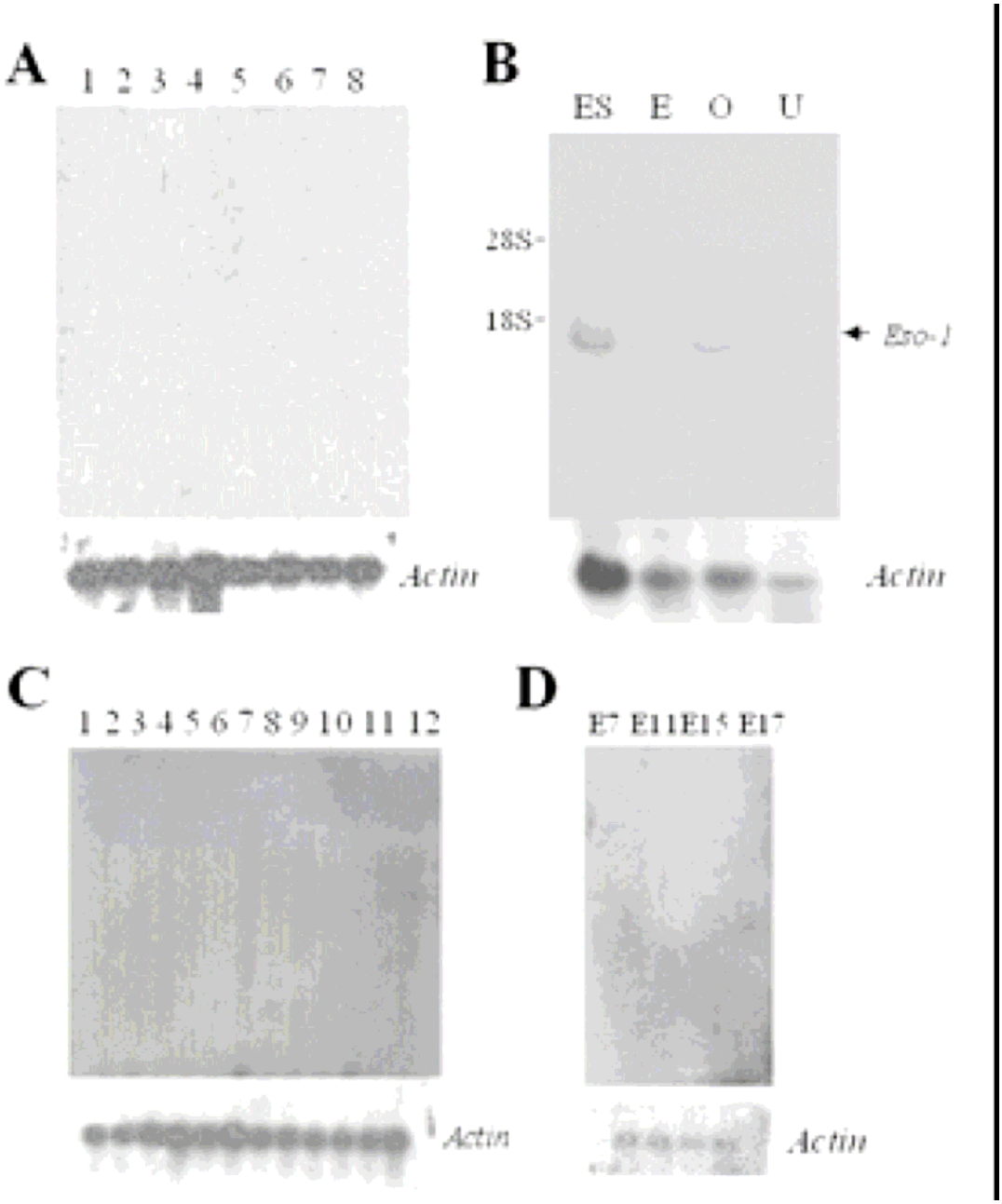


圖五、 藉由 RT-PCR 分析小鼠 *Eso-1* 在不同組織及不同細胞株中的表現情形。利用 β-actin 的 cDNA 及 mRNA 作為 RNA 製備與放大過程中的控制組。

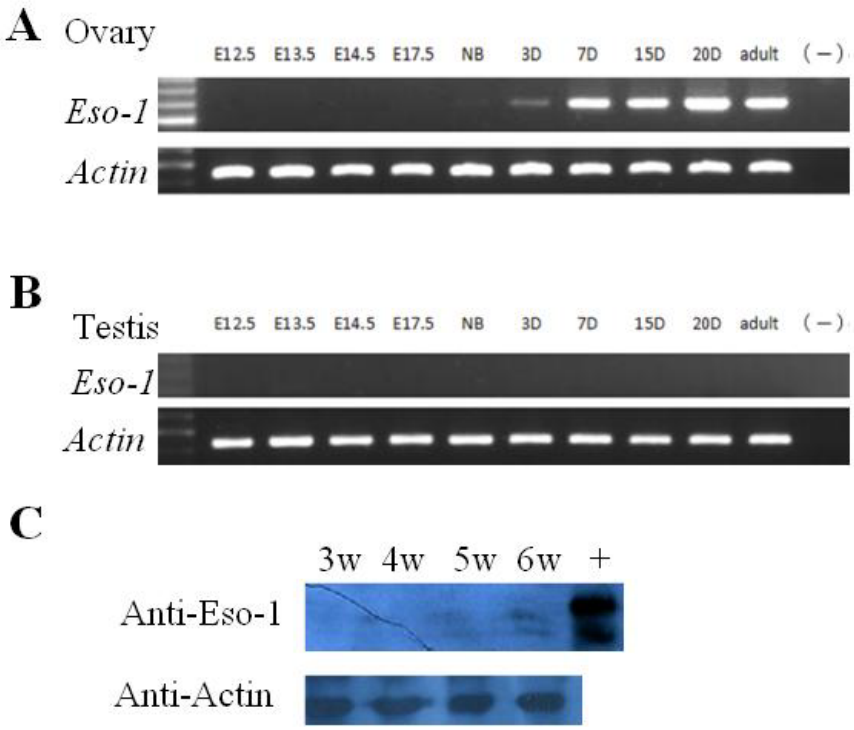
A. 在胚胎發育過程中以及不同的成鼠組織裡 *Eso-1* mRNA 的表現情形。在胚胎幹細胞及成鼠卵巢中皆可偵測到有兩種片段（主要：593bp；次要：686bp）*Eso-1* 的 cDNA 表現。B. *Eso-1* 於胚胎著床前不同時期與卵細胞的表現情形。 C. *Eso-1* 在未分化及以 RA 誘導分化後，胚胎幹細胞株及 P19 中的表現情形。 D. *Eso-1* 在不同發育時期的卵巢中表現情況，胚胎幹細胞 *Eso-1* 的表現為 positive control。 E. *Eso-1* 表現在卵細胞或是卵丘細胞（cumulus cell）。

（A）ES 為胚胎幹細胞，9.5、11.5、12.5 以及 17.5 為胚胎發育的天數。（B）UF 為未受精卵，F 未受精卵，2C 為兩細胞時期，MO 為桑椹胚期，BL 為囊胚期

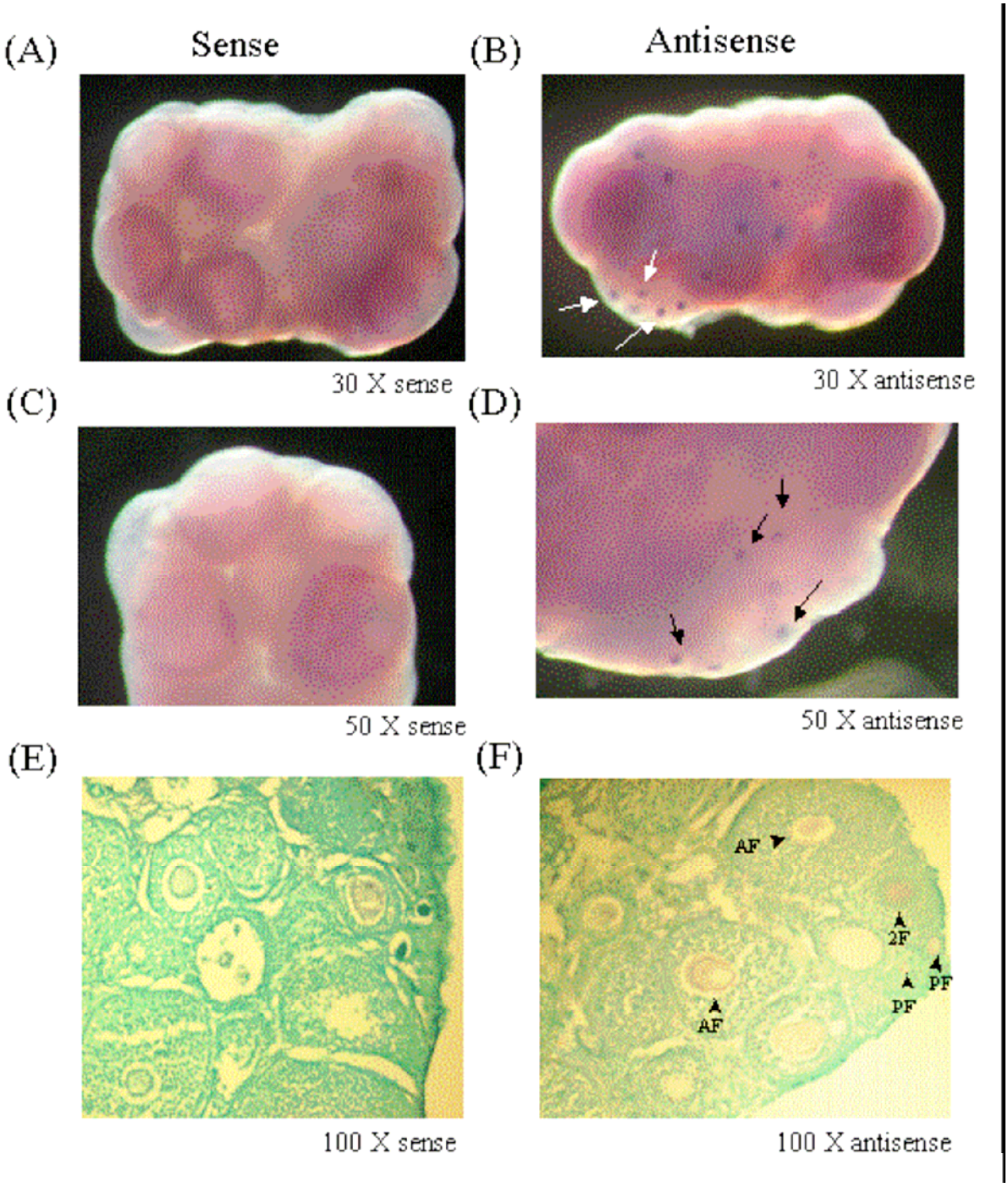
（C）ES/RA 為以 1μM RA 處理兩天；P19 為胚胎畸胎瘤細胞，P19/RA 為以 1μM RA 處理兩天。（D）自胚胎第 13.5 天取出的卵巢組織（13.5），新生小鼠（NB），一週大小鼠（1w）；negative control（-）。



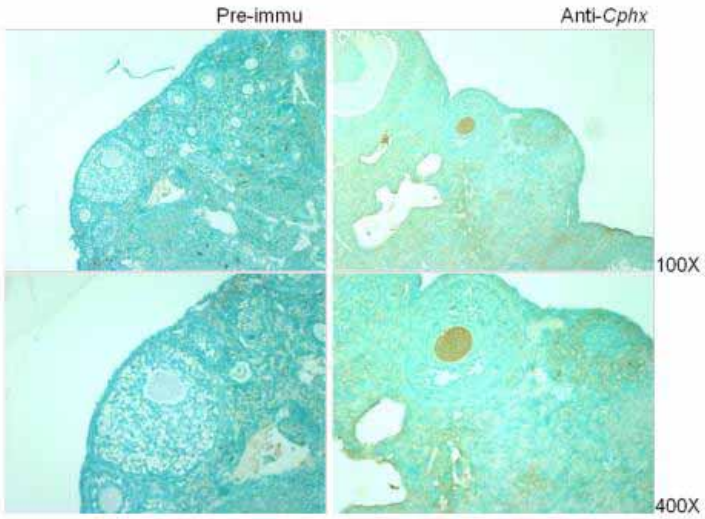
圖六、 以北方點墨法分析 *Eso-1* 在各組織及各細胞株中表現的情形。A. 以北方點墨法分析成鼠全組織 RNA，觀察 *Eso-1* 表現。B. 分析 *Eso-1* mRNA 在成鼠生殖組織及胚胎幹細胞中的表現情形。C. 分析 *Eso-1* mRNA 在各細胞株中的表現狀況。D. 分析 *Eso-1* mRNA 在不同時期胚胎中的表現情形。B, E：附睪；O：卵巢；U：子宮。D, E7：胚胎第 7 天；E11：胚胎第 11 天；E15：胚胎第 15 天；E17：胚胎第 17 天。Actin 皆為 loading control。



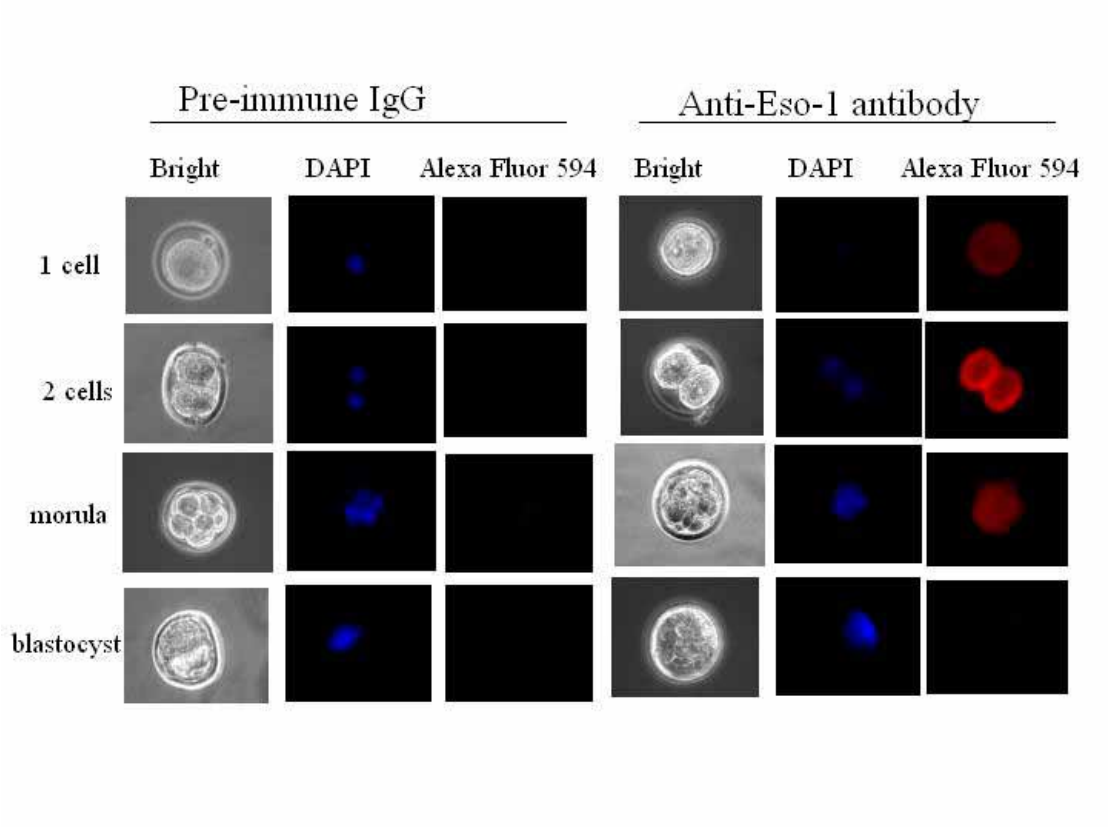
圖七、以 RT-PCR 及 Western blotting 分析 *Eso*-1 mRNA 及蛋白在小鼠不同發育時期的組織中的表現情形。(A) 以 RT-PCR 偵測卵巢 (ovary) 及睪丸 (testis) 中，*Eso*-1 mRNA 的表現，E12.5：胚胎發育第 12.5 天，以此類推；NB：新生小鼠；3D 出生後第三天小鼠，以此類推；adult：超過六週大小鼠。(B) 以實驗室產製 rabbit anti- Eso-1 polyclone antibody 進行西方點墨法，取三到六週小鼠卵巢組織全蛋白，偵測 Eso-1 蛋白表現趨勢，(＋)：為純化的 Eso-1-6*His 融合蛋白。



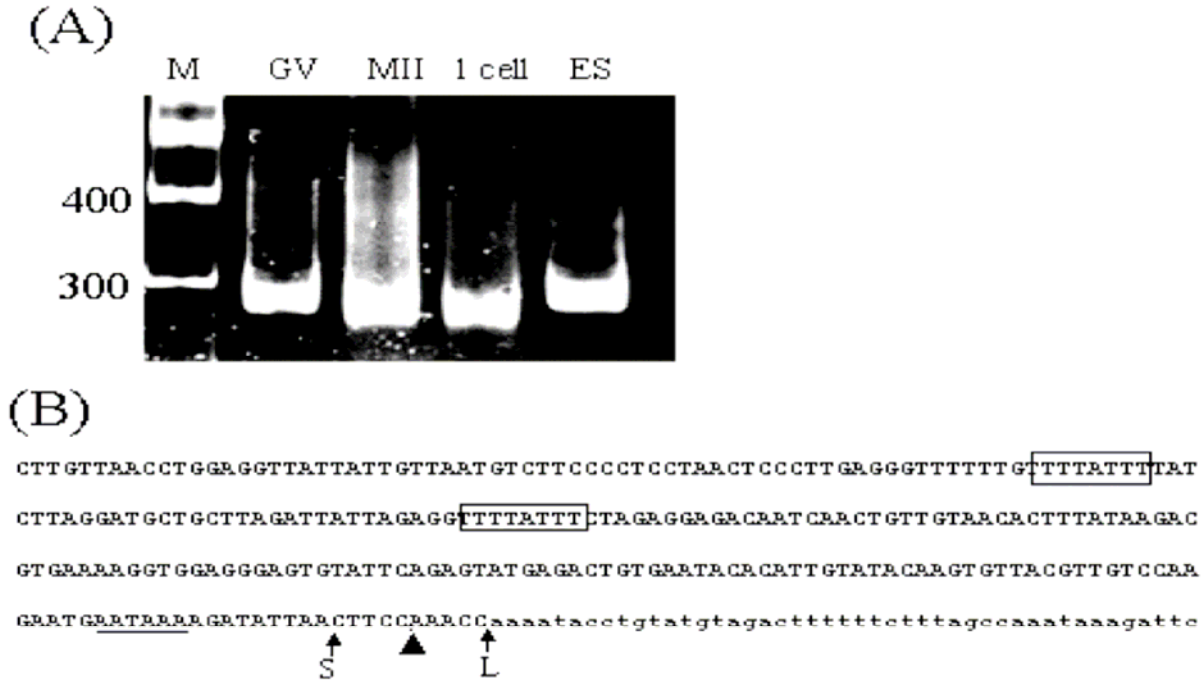
圖八、 透過全組織原位雜交法分析 *Eso-1* 的表現。取 4 周大母鼠卵巢組織與 sence probe（A）和 antisence probe（B）進行雜交反應。C 及 D：取 A 與 B 的卵巢組織進行切片染色。箭號及箭頭表示有表現 *Eso-1* 的組織。(D) PF：原始卵細胞（primordial oocytes）及初級卵細胞（primary oocytes）；2F：次級卵細胞（secondary oocytes）；AF，腔竇卵細胞（antral oocytes）。



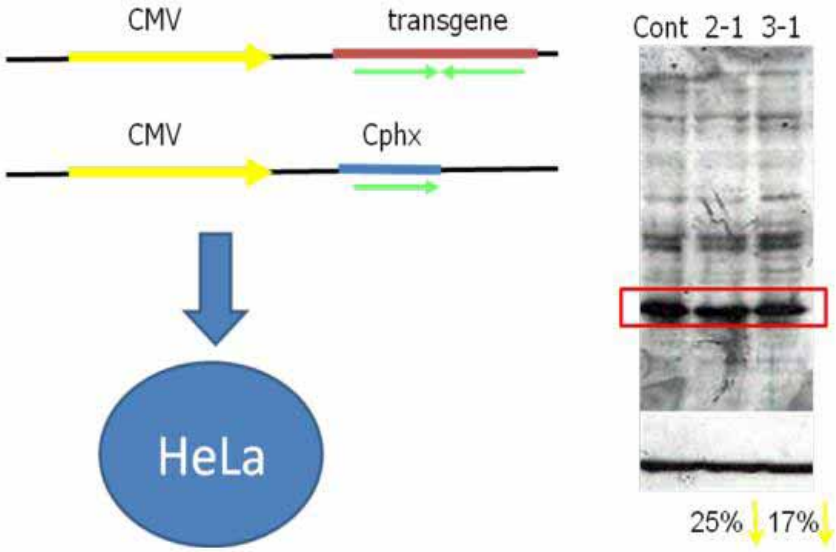
圖九、以免疫組織化學染色法確認 Cphx(Eso-1)在卵巢組織中的表現位置。Pre-immu：利用 protein-A 純化過後，rabbit normal serum 作為抗體當作對照組。Anti-Cphx：利用經過純化的 rabbit anti-Cphx 抗體，偵測 Cphx 在卵巢中的表現位置。箭頭所指為抗體偵測到 Cphx 蛋白表現位置。



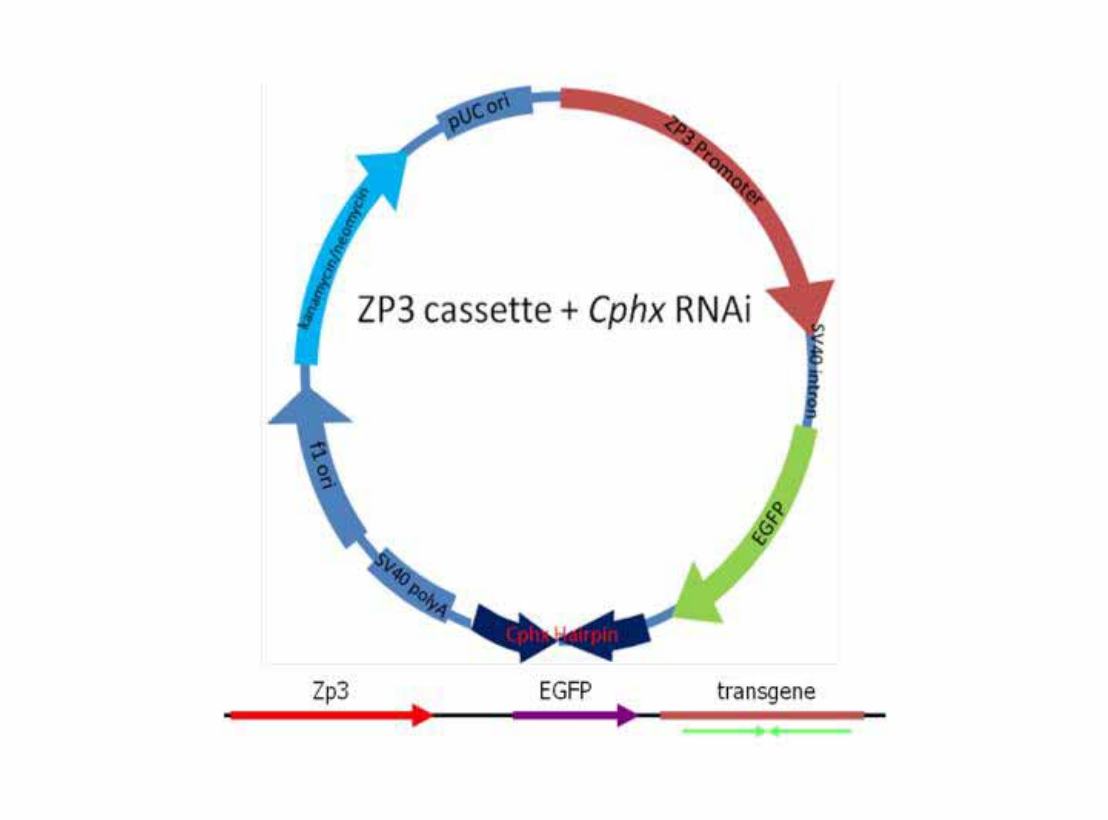
圖十、以免疫螢光染色觀察 *Eso-1* 在著床前胚胎內表現的情形，胚胎細胞各時期如圖所示，1 cell 表受精卵一個細胞時期；2 cell 表兩個細胞時期；morula 表桑椹期；blastocyst 表囊胚期。圖左是以未經過免疫的白兔血清進行染色；圖右則是以白兔 anti-*Eso-1* 抗體進行染色。胚胎中染色體 DNA 以 DAPI 進行染色，圖中呈現藍色；胚胎中 ESO-1 蛋白質以 FITC 呈色，圖中呈現紅色。



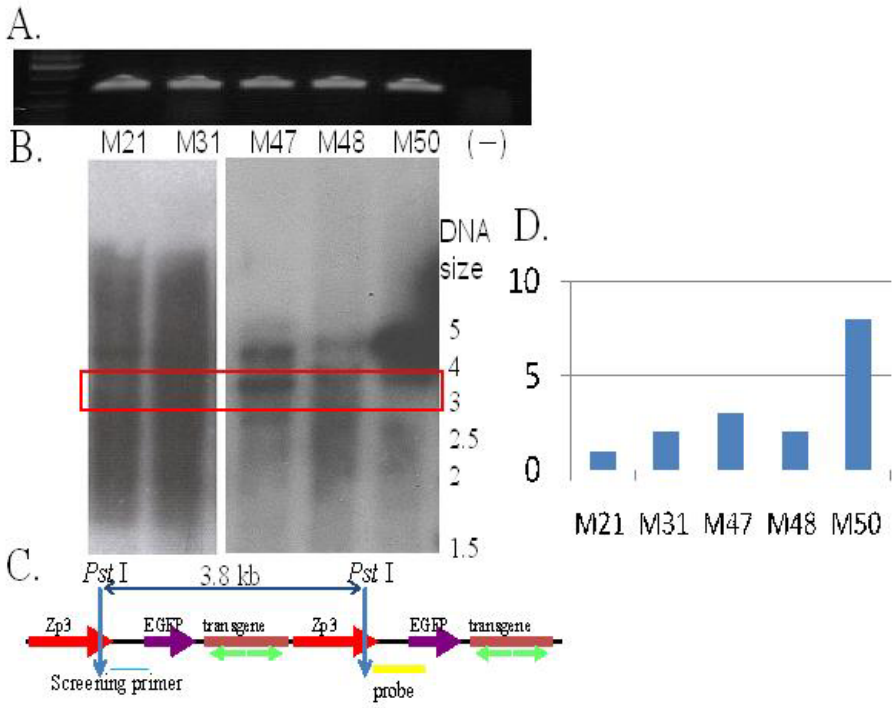
圖十一、 藉由 RACE-PAT 分析未成熟、成熟以及受精後的卵細胞中，其 *Eso-1* 轉綠的情形。A：M，Marker；GV：發育中的卵細胞；MII：處於 metaphaseII 的卵細胞；1 cell：受精卵；ES：胚胎幹細胞。 B：*Eso-1* cDNA 序列中，三端的非轉譯區及下游 polyadenylation 延長現象的切割位置。底線標示區為核內 polyadenylation 位置。箭頭所指的位置為兩種 polyadenylation 不同的切割區（S 及 L)。三角形所標示區域為 AK049114 序列的終點。S：short cleavage site；L：long cleavage site



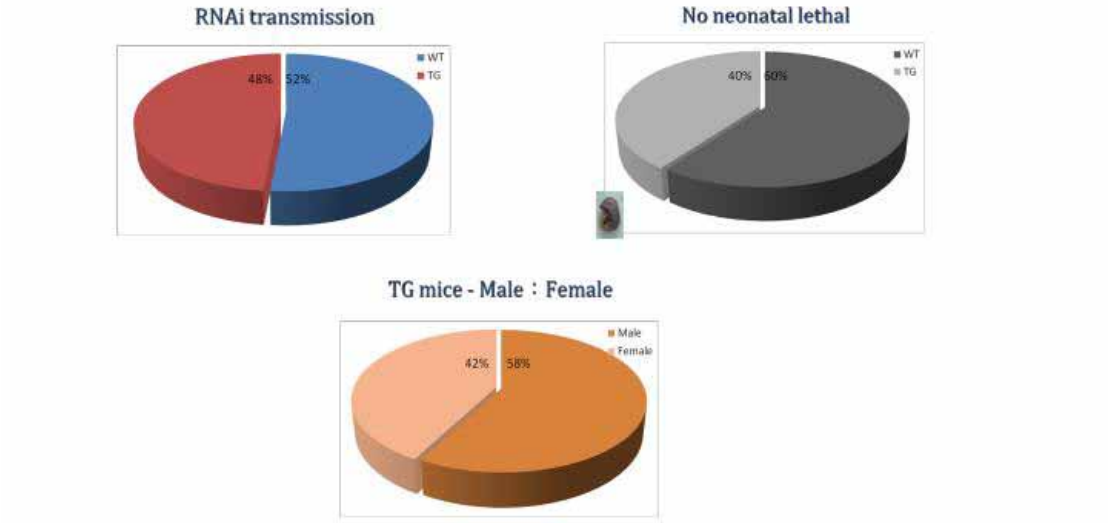
圖十二、將 Cphx (Eso-1) expression plasmid 與 Eso-1-dsRNA plasmid 共轉殖到 HeLa 細胞株中，於 36 小時後收取細胞全蛋白，以 western blot 測定 Eso-1 蛋白表現量



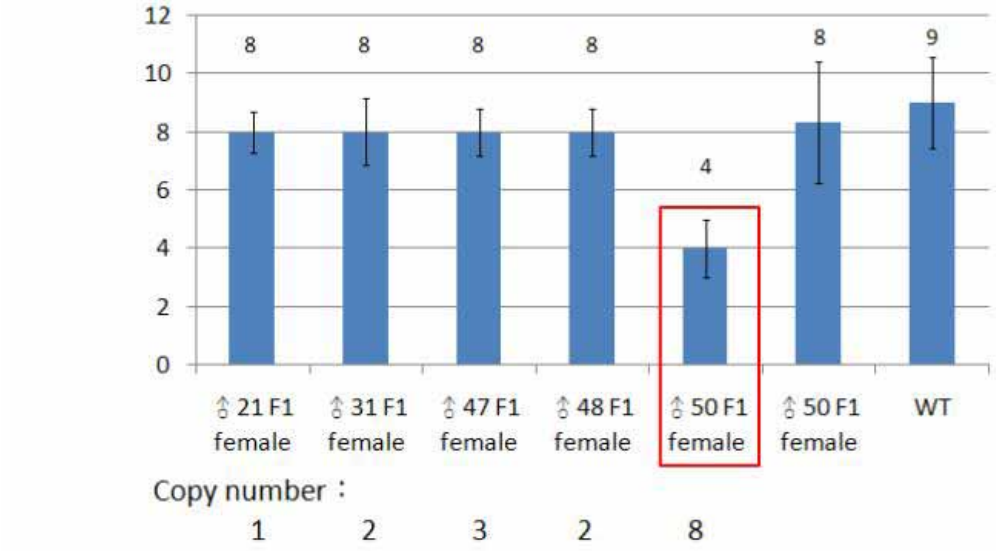
圖十三、Eso-1-dsRNA 的卵細胞專一性表現載體。*Zp3* 為一個專一性在卵細胞中表現的啟動子，以其所啟動的 Eso-1 long dsRNA 僅會在卵細胞中表現。



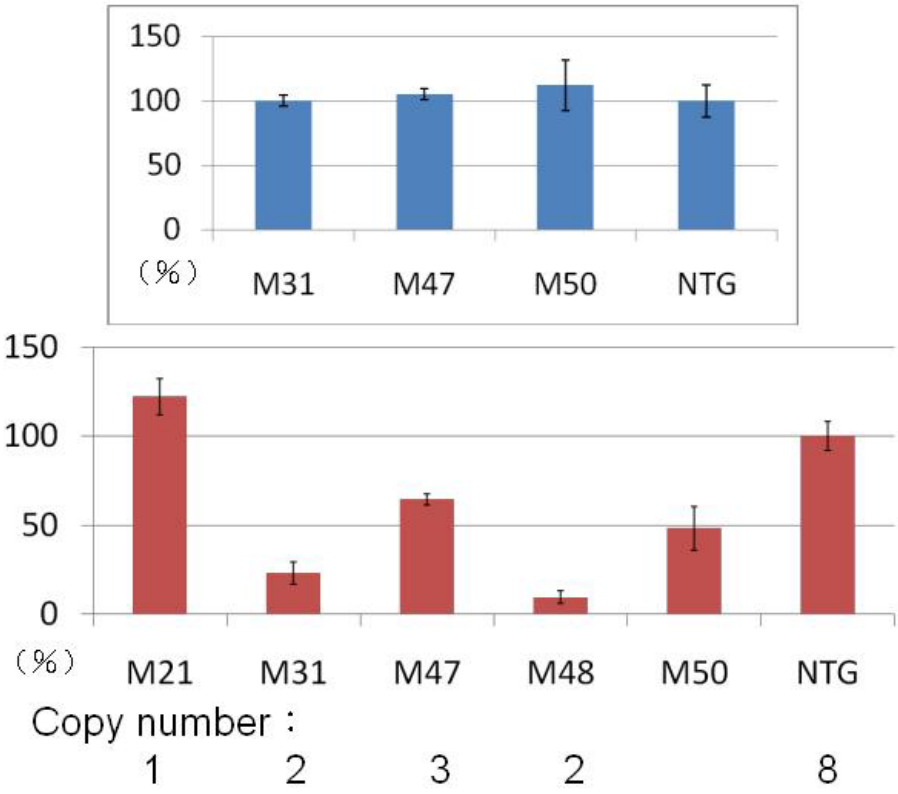
圖十四、基因轉殖鼠的 genotyping 以及 Southern blot 分析。(A) 以 IF 及 IR 引子對轉殖基因中的一段 sv40 intron 做 PCR，正常老鼠體內不會有帶有此段基因 (B) 以 sv40 intron 及 5’ 端 EGFP 作為探針，偵測基因轉殖鼠體內轉殖基因套數，紅色框線為 3.8 kb (C) 轉殖基因在小鼠 genomic DNA 上多套排列時的狀況，藍線為核酸限制酵素裁切處。(D) 為轉殖基因套數量化圖表。



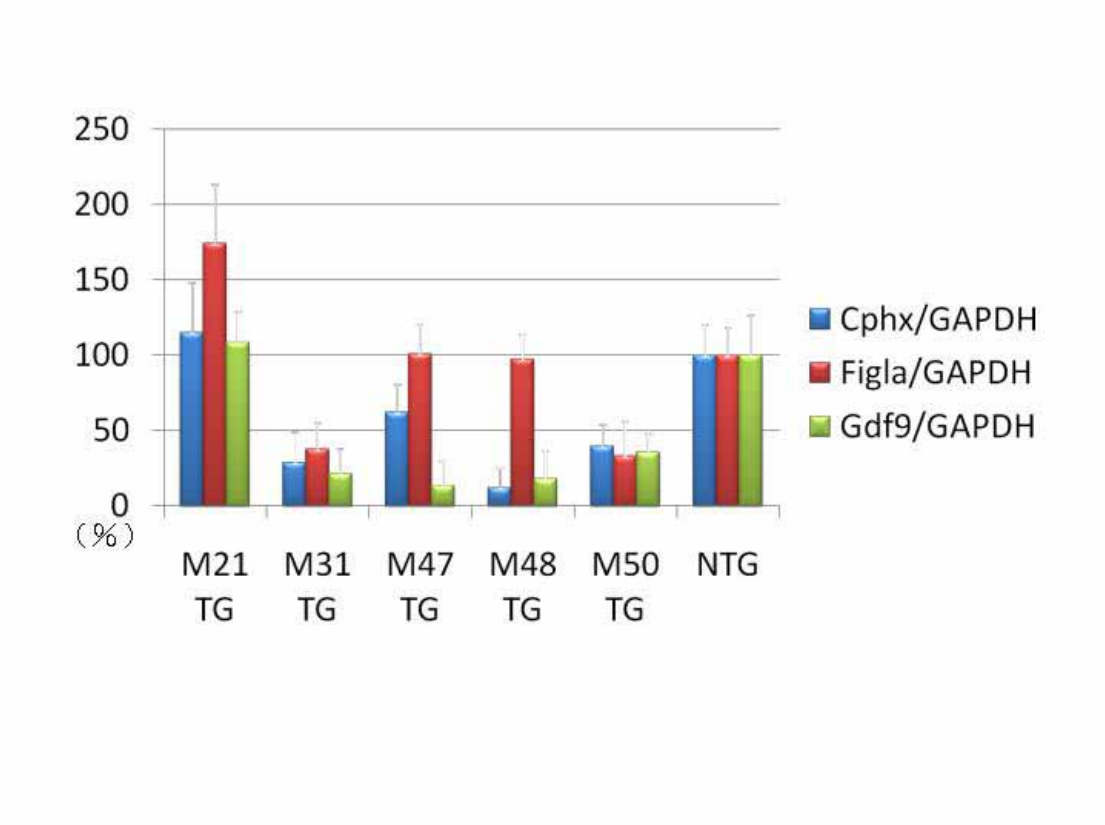
圖十五、基因轉殖小鼠子代統計圖表(A)子代中帶有轉殖基因比例。(B)帶有轉殖基因小鼠的性別比例。(C)死亡的新生小鼠是否帶有轉殖基因的比例。



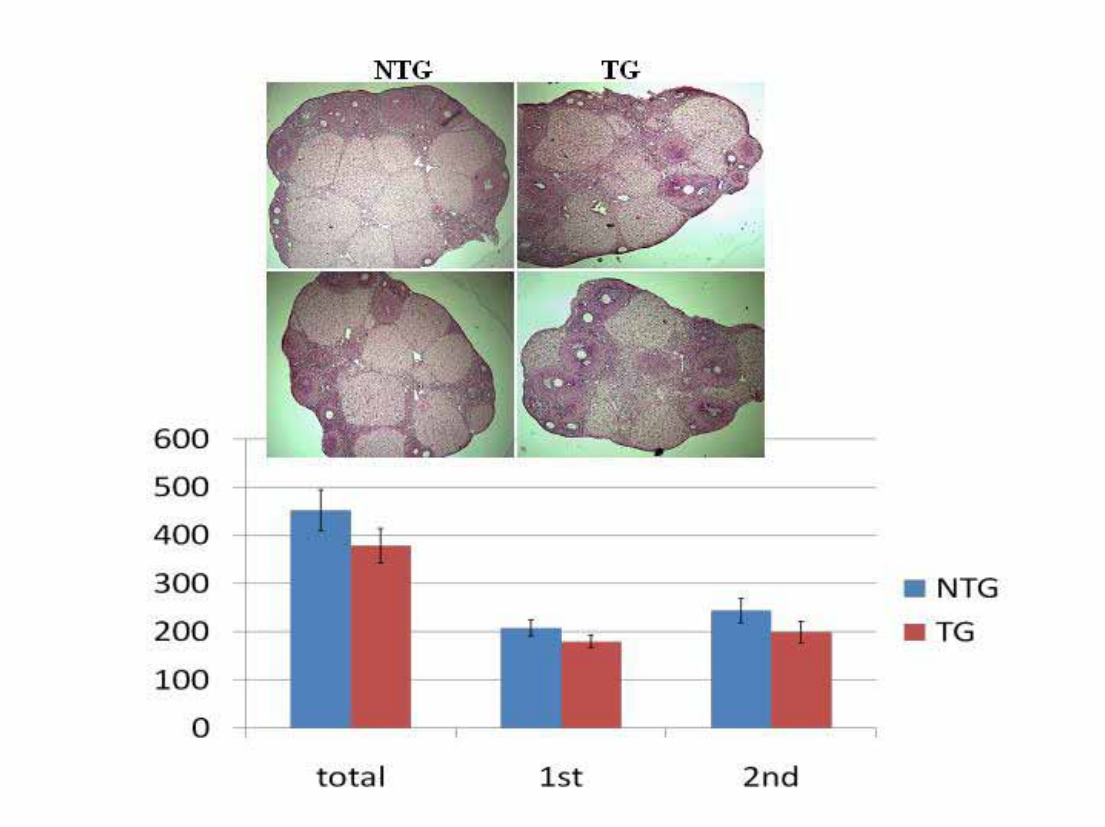
圖十六、以各條 line 中 F1 female 與固定五隻交配用公鼠進行多次交配後，所生育小鼠隻數的統計表。平均每條 line 皆為不同母鼠兩隻以上，及四次生育次數以上統計結果，M50 在早期與後期所生育的 F1，出現兩種不同的生育能力曲線，紅色框線為早期所生育 F1，其生育小鼠隻數僅為 wild-type 一半。



圖十七、四週及十週 Eso-1-dsRNA 基因轉殖鼠與衛兵鼠卵巢組織 *Eso-1* mRNA 表現量比較表 （A）四週轉殖鼠與衛兵鼠，每條 line 各取兩隻小鼠卵巢，取 total RNAs，經定量後取 2ug 進行 reverse-transcription，再進行 real-time PCR。 （B）十週轉殖鼠與衛兵鼠比較，作法同四週。



圖十八、取 10 週基因轉殖鼠及衛兵鼠卵巢組織 cDNA 進行 real-time PCR，將其表現量以 internal control 調整後，所得到的比較結果表。



圖十九、 M50 F2 的轉殖基因小鼠與衛兵鼠卵巢組織切片，以及經卵細胞計數後所得處於不同濾泡時期的卵細胞數數目整理表。

MOLECULAR REPRODUCTION AND DEVELOPMENT 73:825–833 (2006)

A Novel Maternally Transcribed Homeobox Gene, *Eso-1*, is Preferentially Expressed in Oocytes and Regulated by Cytoplasmic Polyadenylation

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ABSTRACT The homeobox gene families play important roles in the transcriptional regulation of gene expression prior to and during embryo development. To identify novel homeobox genes expressed in early embryonic development, we conducted a degenerated oligonucleotide polymerase chain reaction (PCR) to screen a mouse embryonic stem (ES) cell cDNA library. A novel homeobox-containing gene, *Eso-1*, which is preferentially expressed in ES cells and ovaries, was identified. The full-length *Eso-1* cDNA was found to be 1,710 bp with a predicted homeodomain that has no significant homology to previously reported homeodomain proteins. *Eso-1* was mapped to chromosome 14A3. Reverse transcription-polymerase chain reaction (RT-PCR) analyses showed that *Eso-1* was expressed through oogenesis and continuing to be expressed through to the blastocyst stage. De novo expression of *Eso-1* started at 13.5 days postcoitum in the ovaries, which coincides with the initiation of oogenesis. Northern blot analyses demonstrated that *Eso-1* is preferentially expressed in both ovaries and ES cells as a 1.7-kb transcript. Results from whole mount in situ hybridization revealed that *Eso-1* in oocytes showed increased expression from primordial to antral follicles. The 3′-untranslated region of *Eso-1* transcripts contained cytoplasmic polyadenylation sequences while the length of poly (A) tails changed during oocyte maturation, indicating that Eso-1 expression is controlled by time-dependent translational activation. We suggest that the novel homeodomain protein, Eso-1, plays a role during oocyte maturation and early embryonic development. *Mol. Reprod. Dev. 73: 825–833, 2006.* © 2006 Wiley-Liss, Inc.

Key Words: homeobox gene ovary; cytoplasmic polyadenylation; oocyte maturation

INTRODUCTION

Mammalian oogenesis is a complex process regulated by the expression and interaction of a multitude of genes (Song and Wessel, 2005). An oocyte must undergo several developmental transitions during which it synthesizes a unique set of proteins to support fertilization and early embryonic development. Thus, the genes expressed specifically at the oocyte stage may play important roles in fertilization, preimplantation, and folliculogenesis (Dean, 2002). However, little is known about gene expression profiles within an oocyte during oogenesis and how these oocyte-specific gene expressions are regulated. Although it has been speculated that several hundred genes participate in oogenesis, only a few have been identified so far (Ko et al., 2000). The elucidation of more oocyte-specific expressed genes will improve the understanding of the process of oogenesis and early embryo development.

During oogenesis, a large number of maternal proteins are synthesized. They accumulate in the oocytes prior to fertilization and are utilized in early embryogenesis (Song and Wessel, 2005). During meiosis, germ cell genomes are transcriptionally silenced. Following fertilization, embryonic transcription is first detected at the late one-cell zygote stage and is required for development beyond the two-cell stage (Latham et al., 1993; Schultz, 1993). Hence, the oocyte-to-embryo transition depends on the maternal transcripts and proteins accumulated during oogenesis.

The functions of only few maternal-effect genes have been characterized by gene disruption experiments, such as maternal antigens that embryos require (*Mater*)

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(Tong et al., 2000), Zygotic Arrest 1 (*Zar1*) (Wu et al., 2003), *Stella* (Payer et al., 2003), and nucleoplasmin2 (*Npm2*) (Burns et al., 2003). Early embryos lacking these maternal-effect genes exhibit developmental defects and embryonic development is arrested either at the one- or two-cell stages. Other oocyte-specific genes have been identified by in silica cloning, however, it is unclear whether the transcripts of these genes are translated (West et al., 1996; Rajkovic et al., 2002; Paillisson et al., 2005). As mouse oocytes growth, they accumulate transcripts that are either translated directly into proteins or stored for later activation by cytoplasmic polyadenylation. Cytoplasmic polyadenylation requires two cis-acting sequences in the 3′-untranslated region (3′UTR): the conserved nuclear hexanucleotide polyadenylation signal (HPS), AAUAAA, and the cytoplasmic polyadenylation element (CPE), $UUUUA_{1-2}U$ (Mendez and Richter, 2001). In general, the poly (A) tails of dormant maternal mRNAs are elongated (from 20–50 to 100–150 nucleotides) during oocyte maturation and then linked with translational activation (Richter, 1999). A number of maternal mRNAs essential for successful oogenesis and early embryogenesis in mice, such as *c-mos*, *tPA*, *cyclin B1*, and *H1oo*, have been shown to require cytoplasmic polyadenylation for translational activation (Huarte et al., 1987; Gebauer et al., 1994; Tay et al., 2000; Tanaka et al., 2001). This time-controlled translation is a general process guiding the oocyte to embryo transition (Oh et al., 2000). However, the molecular mechanisms that activate maternal-effect genes and regulate their products are largely unknown. In addition to the above translational control of maternal mRNAs, transcriptional activation of oocyte-specific gene expression is also thought to be required for completion of oogenesis and early embryonic events. However, the transcriptional regulation of oocyte-specific gene expression is still poorly understood.

Transcription factors that regulate oocyte gene expression, such as *Zfx* (zinc-finger transcription factor on the X chromosome) (Luoh et al., 1997), *Nobox* (newborn ovary homeobox-encoding gene) (Rajkovic et al., 2004), and *Figα* (germ cell-specific transcription factor), play important roles in fertility (Liang et al., 1997; Soyal et al., 2000). Mice lacking *Zfx* have a reduced number of oocytes and diminished fertility. Disruption of the transcription factor, Nobox, results in infertility in female mice, while male mice are unaffected (Rajkovic et al., 2004). Figα regulates the coordinated transcription of three ZP glycoproteins and plays an important role in primordial follicle formation (Liang et al., 1997; Soyal et al., 2000).

Homeobox gene superfamily members act as transcription factors regulating gene expressions during developmental processes (Gehring, 1987). In this study, we have identified a novel homeobox gene involved in oogenesis and early embryonic development. It is specifically expressed in oocytes and embryonic stem (ES) cells, and was cloned and named as *Eso-1*. The expression patterns of *Eso-1* suggest that it might be regulated by cytoplasmic polyadenylation during oocyte to embryo transition. Eso-1 should be a new maternal-effect transcription factor and might play an important role in oogenesis and early embryo development.

MATERIALS AND METHODS

Degenerated Polymerase Chain Reaction (PCR) Cloning of Novel Homeobox Genes

Degenerated oligonucleotides were designed based on the highly conserved helix 3 amino acid sequences of homeodomains, and then polymerase chain reaction (PCR) amplified from a murine ES cDNA library as described previously (Li et al., 2002; Wang et al., 2003). One of the novel homeodomain-containing DNA fragments were cloned, sequenced, and then the sequence used to search the EST database. The EST clones that had sequences matching this novel homeobox gene were derived from egg to blastocysts. Longer cDNA fragments of this homeobox gene were obtained by reverse transcription-polymerase chain reaction (RT-PCR) from ES RNAs using primers according to the sequences of these EST clones. The PCR products were then cloned and sequenced. The DNA sequences obtained were compared with the sequences in the GenBank, EMBL, and dbEST databases using the BLAST program. Based on the cDNA sequences, gene-specific primers were designed to screen the expression profiles of this novel gene during mouse development. The complete sequence of this novel gene was constructed using the sequences in EST databases and by 5′- and 3′-RACE (rapid amplification of cDNA ends) reactions.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Two micrograms of total RNAs from different adult mouse tissues were used for RT with the Superscript II system (Life Technologies, Carlsbad, CA). The expressions of *Eso-1* were confirmed by the following primers: P1, 5′-GCTCTCTTCTGCACCGAGCAAG-3′; P2, 5′-CC TGGCGCTCCAGAAACAAAA-3′; P5, 5′-CAGTAAGA-GAATGTGAAATATTGGGTTT-3′. These primers were designed to span an intron sequence (Fig. 1B). P1/P5 or P2/P5 primer pairs were used to amplify *Eso-1* cDNAs. As a positive control, expressions of β-*Actin* were confirmed using the following primers: Actin F: 5′-CC CTAAGGCCAACCGTGAAAAGAT-3′; Actin R: 5′-ACC GCTCGTTGCCAATAGTGATGA-3′. In the 5′-RACE reaction, primer P4 and P3 were used as gene-specific primers in the first and second round PCRs. P3, 5′-TC ACCAGAAGCTTTTGGGGGCTGGGTAT-3′; P4, 5′-GC CTTCCGATGCTTCGGACCACAGA-3′.

Animals

Mice were maintained in a specific pathogen-free environment under standard laboratory conditions and experiments conducted following the guidelines of the institutional animal committee.

RNA Isolations and Northern Blot Analyses

Total RNAs were extracted with Trizol reagent (Life Technologies) from adult mouse tissues. Twenty

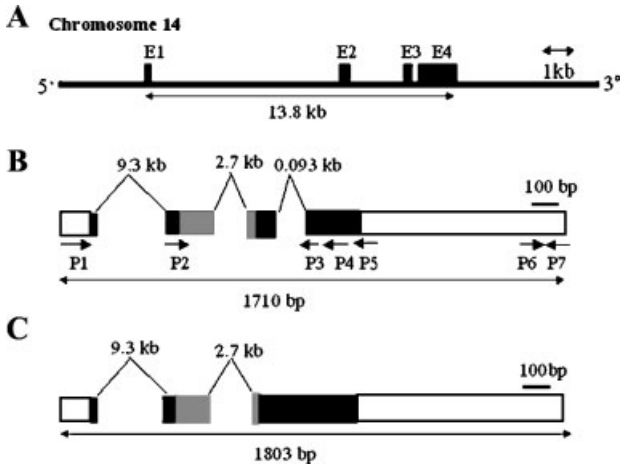

Fig. 1. Genomic structure of mouse *Eso-1* genes. **A**: *Eso-1* gene is present on chromosome 14A3 containing four exons (E1–E4). **B**: Solid regions denote protein-coding portions, gray regions denote the homeodomain, and empty regions denote the untranslated regions. The sizes of introns are indicated. Arrows label the positions and orientations of primers used in this study. The major transcript of *Eso-1* contains four exons. **C**: The minor transcript of *Eso-1* contains three exons. The lengths of mouse *Eso-1* genomic DNA and transcripts are indicated.

micrograms each of total RNA was loaded and separated on 1.5% agarose/formaldehyde gels and transferred to nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). The membranes containing poly A^+ RNAs from mouse embryos, adult mouse tissues, and mouse cell lines were purchased from BD Bioscience (Clontech, Palo Alto, CA). Blots were prehybridized in buffer (0.2% SDS, 5× SSPE, 5× Denhardt solution, 100 μg/ml sheared salmon sperm DNA, 50% formamide) for 2–4 hr at 42°C. The blots were then hybridized overnight at 42°C with ^{32}P-labeled *Eso-1* DNA fragments (53–645 bp) in the same buffer. Following hybridization, the blots were washed with buffer (2× SSC, 1% SDS) at 65°C for 20 min and then autoradiographed. The same blots were rehybridized with β-*Actin* probe after the washing out of all isotope signals.

Oocytes/Embryos Collections and RNA Isolations

Full-grown, germinal-vesicle intact oocytes (GV oocytes) were obtained from needle-punctured ovaries of 21- to 24-day-old female mice, which had been injected with pregnant mare's serum gonadotropin (PMSG, Sigma-aldrich, Louis, MO) 46–48 hr earlier. The surrounding granulose cells were separated from oocytes by pipetting. Ovulated oocytes (metaphase II-arrested oocytes) were collected from 4-week-old female mice, which had been superovulated with PMSG and human chorionic gonadotropin (hCG, Sigma-aldrich). Cumulus cells were separated from oocytes by a short incubation in M2 medium containing 500 μg/ml hyaluronidase at 37°C. The oocytes were then washed several times in fresh medium. Zygotes (one-cell embryos) were collected from the oviducts after mating. Two-cell embryos, morulas, and blastocysts were collected 36, 60, and 84 hr after mating.

RACE-PAT: A Modification of the 3′-RACE Protocol for Detecting Polyadenylation

Changes in the lengths of poly (A) tails were studied by RACE-PAT analysis; a modified reaction of 3′-RACE. First, total RNAs from oocytes were isolated as described previously (Salles et al., 1999), then total RNAs from GV oocytes (n = 100), metaphase II (MII) oocytes (n = 100), fertilized eggs (n = 100), and ES cells were reverse transcribed using an oligo (dT)/adaptor primer (5′-GCTGTCAACGATACGCTACGTAACGG-CATGACAGTGT_{18}-3′). One-twentieth (equal to five oocytes) of the obtained cDNAs were used in 25 μl PCR reactions with P6 and RACE 3′-nest primers (P6, 5′-AT GTCTTCCCCTCCTAACTCCCTTGAG-3′; RACE 3′-nest, 5′-CGCTACGTAACGGCATGACAGTG-3′). The amplified products were separated on a 6% nondenaturing TBE-polyacrylamide gel and visualized by EtBr staining.

Whole Mount In Situ Hybridization

Ovaries from 4- or 8-week-old female mice, treated with or without PMSG and hCG, were collected for whole mount in situ hybridization. After washing with phosphate buffered saline (PBS), ovaries were fixed in 4% paraformaldehyde in PBS. The cDNA fragments amplified with P1 and P5 primers were subcloned into pGem-T Easy vectors (Promega, Madison, WI). The sense and antisense riboprobes were prepared by in vitro transcriptions using SP6 and T7 RNA polymerases with digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN), respectively. Whole mount in situ hybridization of ovaries was performed as previously described (Correia and Conlon, 2001). After hybridization, the ovaries were refixed in 4% paraformaldehyde and OCT embedded. Seven-micrometer sections were cut and counterstained with methyl green for 10 min. These sections were then mounted before observation.

RESULTS

Identification of *Eso-1* by Degenerated PCR and EST Database Analyses

To identify novel homeobox-containing genes involved in early embryonic development, we conducted a degenerated PCR screening of a murine ES cell cDNA library. A PCR product containing a novel homeodomain sequence was identified. The DNA sequence of this PCR product was used for a BLAST homology search of the database at the National Center for Biotechnology Information (NCBI; http:/www.ncbi.nlm.nih.gov). Several EST clones were found that were highly homologous to this PCR product. These clones were derived from eggs to 3.5-day mouse embryos. The majority of these clones were derived from unfertilized and fertilized eggs. In addition, one clone (AK049114) from a Riken full-length-enriched ES cell cDNA library was found containing almost the same sequence as this PCR

product. Based on these database sequences, the full-length cDNA of this novel gene was cloned by RT-PCR amplification from ES RNAs and then sequenced. The sequence of this cDNA was further used for a homology search with Ensemble Genome Data Resources (Sanger Institute, http://www.ensemble.prg/Musmusculus/). The result indicated that this novel homeobox-containing gene is located on mouse chromosome 14A3 (Fig. 1A). The partial cDNA fragment from PCR amplification was also used as a probe for preliminary Northern blot analysis and to design primers for RT-PCR analyses to determine the expression pattern and transcript size of this novel homeobox gene. After preliminary Northern blot and RT-PCR analyses, this novel gene exhibited ES cell and oocyte-specific gene expressions, so we named it *Eso-1* (ES cell and oocyte expressing gene).

To authenticate and search for other 5′ ends of *Eso-1* gene, we performed 5′-RACE analysis using ES RNAs as templates. To increase sensitivity and specificity, two different *Eso-1*-specific primers, P4 and P3, were used in two successive rounds of nested PCR (Fig. 1B). Several 5′-RACE clones were subcloned and sequenced. Multiple 5′ ends of *Eso-1* transcripts were mapped upstream of the previous 5′ site of AK049114. Figure 2A indicates different transcription start sites of *Eso-1* transcripts. Full-length *Eso-1* was longer than that of AK049114. However, the 5′- and 3′-splice sites were found to be the same in all RACE clones obtained and that in AK049114. This result confirmed the existence of all predicted splice sites and the 9.3-kb, 2.7-kb, and 0.093-kb introns located in the *Eso-1* premRNA (Fig. 1B). Comparison of sequences between *Eso-1* cDNA and *Eso-1* genomic DNA from the database revealed that *Eso-1* gene is composed of four exons with the splice sites obeying the GT-AG splicing donor–acceptor rule. Using the P1 and P4 primers, two PCR products containing the 5′ ends of *Eso-1* cDNA were obtained from ES cell cDNAs (Fig. 3A). The longer PCR product (686 bp) (Fig. 1C) was consistently fainter than the shorter one (593 bp) (Fig. 1B). This reflects the relative abundance, in vivo, of the shorter transcript. After subcloning and sequencing, the sequences of these two PCR products were found to be identical from exon1 to exon4, but were different at intron3 (93 bp), which was only present in the longer transcript (Fig. 1C). This result indicates the existence of alternative or incomplete splicing of *Eso-1* transcripts.

Although *Eso-1* transcripts exhibited multiple transcription star sites at exon1, all *Eso-1* transcripts contained the same open reading frame, encoding a protein with predicted 182 amino acids containing a homeodomain (Fig. 2A). The sequence of the homeodomain of *Eso-1* gene is located in exon2 and exon3 (Fig. 1B,C). Comparison of the homeodomain of this novel gene with other homeodomain-containing gene products revealed a maximum of 38% identity with homeodomains of the *CUX-1* gene (Vanden Heuvel et al., 1996) and other known genes (Yoon and Chikaraishi, 1994; Vanden Heuvel et al., 1996; Tada et al., 1998; Cinquanta et al., 2000; De Graeve et al., 2004), indicating that *Eso-1* does not belong to any previously identified homeobox gene family (Fig. 2B).

Tissue- and Stage-Specific Expression of *Eso-1*

To determine the expression patterns of *Eso-1* during early embryonic development and within adult tissues, we performed RT-PCR analyses using RNAs from various adult tissues and different stages of embryos. *Eso-1* expressions were detected in ES cells, adult ovaries, and preimplantation embryos from egg to blastocyst stage (Fig. 3A,B). *Eso-1* expressions decreased from eggs to blastocysts, with predominant expressions in unfertilized and fertilized eggs (Fig. 3B). The expression of *Eso-1* in the blastocysts was retained within the inner cell mass (ICM), as demonstrated by whole mount in situ hybridization analysis (data not shown). This result was confirmed by detection of *Eso-1* expression in ES cells (Fig. 3), since ES cells are derived from the ICM of blastocysts and retain gene expressions and pluripotency characteristics similar to the ICM. The expressions of *Eso-1* in undifferentiated and differentiated ES cells were further determined by RT-PCR analysis. *Eso-1* was expressed at a decreased level in differentiated ES cells (1 μM retinoic acid treated for 2 days) (Fig. 3C). Furthermore, the embryonic teratocarcinoma cell line, P19, did not exhibit any *Eso-1* expression in either RT-PCR or Northern blot analysis (Fig. 3C, data not shown). To determine the expression of *Eso-1* at different stages of ovary development, we performed RT-PCR analyses using ovary RNAs from fetuses to adults. The expressions of *Eso-1* started at 13.5 days postcoitum (dpc) (Fig. 3D) and continued to the adult stages (Fig. 3A). In adult ovaries, *Eso-1* was detected only in oocytes (Fig. 3E). No *Eso-1* signal was detected in cumulus cells isolated from ovulated oocytes.

Eso-1 expressions were also confirmed as a 1.7-kb transcript specifically in ES cells and adult ovaries by Northern blot analysis (Fig. 3F). The size of the *Eso-1* transcript was in agreement with our predicted length (1,710 bp) and the ES cells was the only cell line that was detected expressing *Eso-1* (data not shown). However, following implantation, the expression of *Eso-1* was not detected by Northern blot analysis in 7- to 17-day-old mouse embryos (data not shown). We further characterized the expression of *Eso-1* in the ovaries of 4- and 8-week-old mice by whole mount in situ hybridization. Antisense probes of *Eso-1* mRNA hybridized specifically to oocytes in the ovaries (Fig. 4B). Following whole mount in situ hybridization, ovaries were fixed, sectioned, and counterstained, and the *Eso-1* mRNA signals were observed. Specific signals for *Eso-1* mRNA were clearly observed in oocytes of antral and secondary follicles, and faint *Eso-1* signals were detected in oocytes of primordial and primary follicles (Fig. 4D).

Eso-1 mRNA Undergoes CPE-Dependent Poly (A) Elongation During Oogenesis

The 3′UTR of the mouse *Eso-1* transcript contains two potential CPEs, UUUUAUUU and UAUUUUAU

A

B

	Helix 1	Helix 2	Helix 3	% Homology	
mESO-1	NSKPRHKFSR	DELKRLKQEF	AYAPYPDFTT	(Mm)	100%
rESO-1	K------- TN ----------	KCT-------	R--------R-	Q- D------	-----TPQ -- (Rn)	78%
CUX-1	LK ---VVLAP	E–KEA--RAY	QQK---SPK-	IE---T- LNL	KT-TVI--- H	-Y-S- I R R-- (Mm)	38%
BIX2	-RRR - TVY-P	SD–A- - E- Y-	Q I NM - - -HQ	RE-----MGL	PE-R- Q V---	-R-SKARRQG (Xl)	38%
CDP	LK --- VVLAP	E –KEA- -RAY	QQK - - -SPK-	IE ---T - LNL	KT-TVI --- H	-Y-S –I R R-- (Rn)	38%
BSH9	QRRS –TT - -N	- Q I DA-ERI -	-RTQ - - -VY-	RE - - - QSTGL	TEARVQV - -S	-R - - - - RKQ- (Dm)	36%
OG9	HRRK –TT - -V	GQ- VE- ERV-	- AR - - - - I S-	REH - - QVIHL	PEAK- QV - - -	-R - - KRIKDR (Mm)	35%

Fig. 2. The DNA sequences and predicted amino acid sequences of *Eso-1* gene. **A**: The full-length *Eso-1* cDNA sequence and the predicted amino acid sequence of Eso-1 protein. The major mouse *Eso-1* cDNA has a length of 1,710 bp encoding 182 amino acids. The start codon (ATG) and stop codon (TAA) are indicated by underlining and an asterisk, respectively, and the nuclear polyadenylation signal is boxed. The amino acid residues of the homeodomain are underlined. The underlined C in the 5′UTR indicates the initiation sequence of AK049114. The numbers labeled above the nucleotide sequences indicate the frequencies of different transcription initiation sites obtained. Three potential cytoplasmic polyadenylation elements (CPE), U_{13}, UUUUAUUU and UAUUUUAU are shown in underlined italics. The nucleotide sequence of *Eso-1* has been submitted to the GenBank data bank under accession number DQ224405. **B**: Comparison of the homeodomain sequences of predicted mouse Eso-1 protein with different homeodomain proteins. The dotted line represents amino acid sequences identical to those of mouse Eso-1. rEso-1, the predicted homeodomain sequence of the NCBI database predicted rat Eso-1 transcript (XM_577474) localized in rat chromosome 16p16; CUX-1, mouse cut-related homeodomain protein (Vanden Heuvel et al., 1996); BIX2, Xenopus laevis homeoprotein BIX2 (Tada et al., 1998); CDP, rat CCAAT displacement protein (Yoon and Chikaraishi, 1994); BSH9, Drosophila gooseberry protein (De Graeve et al., 2004); OG9, a mouse homeodomain protein (Cinquanta et al., 2000).

(Mendez and Richter, 2001) that are present in the 119 and 156 nucleotides upstream of the nuclear HPS (Fig. 2A). The 3′UTR of *Eso-1* transcript also contains a poly (U) stretch, U_{13}, 594 nucleotides upstream of the nuclear HPS, similar to that described for the Xenopus 'embryonic' CPE (Simon and Richter, 1994). Because of the existence of potential CPEs in the 3′UTR of *Eso-1* mRNA, we speculated that this mRNA has the capability to undergo cytoplasmic polyadenylation during oogenesis. To determine whether this is the fact, we performed a RACE-PAT analysis, a PCR-based assay using total RNAs obtained from growing oocytes (GV), oocytes that had matured to MII, fertilized eggs, and ES cells, respectively. The results from RACE-PAT showed that the lengths of 3′UTR of the *Eso-1* transcripts from MII oocytes and fertilized eggs were shorter than those from either GV oocytes or ES cells (Fig. 5A). After subcloning and sequencing, we further confirmed that *Eso-1* transcripts have more than one cleavage site for polyadenylation (Fig. 5B). The cleavage sites are about

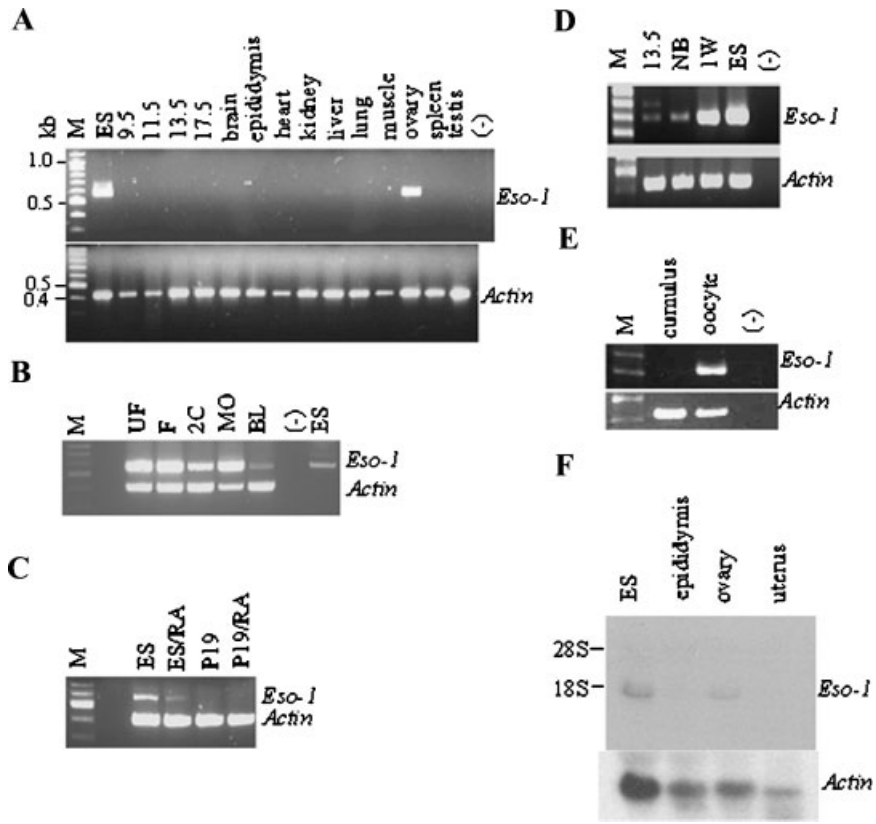

Fig. 3. Expression profiles of mouse *Eso-1* in various mouse tissues and cell lines by RT-PCR and Northern blot analyses. Amplifications of β-*Actin* cDNAs and detections of β-*Actin* mRNAs were used as controls to assess the quality and quantity of the RNA preparations. **A**: Determination of *Eso-1* mRNA expression during embryonic development and in different adult tissues. Two *Eso-1* cDNA fragments (major: 593 bp; minor: 686 bp) were detected in both ES cells and adult ovaries. **B**: Determination of *Eso-1* expression in oocytes and pre-implantation-stage embryos. **C**: Determination of *Eso-1* expression in undifferentiated and RA-differentiated ES and P19 cells. **D**: Determination of the *Eso-1* expressions in ovaries during different developmental stages. The expression of *Eso-1* in ES cells was used as a positive control. **E**: Determination of the *Eso-1* expressions in oocytes and cumulus cells. **F**: Detection of *Eso-1* mRNAs in ES cells and some adult tissues by Northern blot analysis. The 1.7-kb *Eso-1* transcript was detected as 1.7 kb in length. The abbreviations used in (A) are: ES, embryonic stem cells; 9.5-, 11.5-, 12.5-, and 17.5-day mouse embryos, respectively. The abbreviations used in (B) are: UF, unfertilized eggs; F, fertilized eggs; 2C, two cells; MO, morulas; BL, blastocysts. The abbreviations used in (C) are: ES/RA, ES cell treated with 1 μM RA for 2 days; P19, embryonic teratocarcinoma cells; P19/RA, P19 treated with 1 μM RA for 2 days. The abbreviations indicated in (D) are: ovaries from 13.5-dpc embryos (13.5), newborn mice (NB), and 1-week-old mice (1w). (−): negative control.

9 bp (S site) and 19 bp (L site) downstream of the nuclear HPS. The *Eso-1* transcripts using the L site for polyadenylation (L variants) were detected in transcripts from all of GV oocytes, MII oocytes, fertilized eggs, and ES cells, with poly (A) lengths of about 20–45 nucleotides. However, transcripts using the S site for polyadenylation (S variants) were only detected in MII oocytes and fertilized eggs. In MII oocytes, the maximum lengths of poly (A) detected in S variants were about 90–105 nucleotides, which were much longer than the lengths of poly (A) (20–45 nucleotides) in L variants. These results indicate the existence of cytoplasmic polyadenylation of *Eso-1* mRNAs isolated from MII oocytes.

DISCUSSION

In the present study, we report a novel ES cell and oocyte-specific homeobox-containing gene, *Eso-1*. The mouse *Eso-1* was isolated by degenerated PCR screening of a murine ES cell cDNA library. To determine the full-length *Eso-1* cDNA, we performed both 5′- and 3′-RACE reactions based on sequences obtained from the mouse EST database. The *Eso-1* mRNA was first detected in the ovaries of 13.5-dpc fetuses and continued to be expressed during oogenesis. The time point of 13.5 dpc corresponds to the time while germ cells enter meiosis in the female gonad, suggesting that the Eso-1 protein may have a similar role in ovarian development and folliculogenesis as Figα (Soyal et al., 2000). Furthermore, the lengths of poly (A) tails of *Eso-1* transcripts were increased in MII oocytes, suggesting that *Eso-1* translation was activated during oocyte maturation. From its expression patterns and the existence of cytoplasmic polyadenylation, *Eso-1* should be a novel maternal-effect gene. In addition, the predicted Eso-1 protein is a homeodomain protein, which may bind and regulate downstream gene expressions. Our results also suggest that Eso-1 protein might be important for folliculogenesis, oogenesis, and zygotic transcriptional activations of preimplantation embryos.

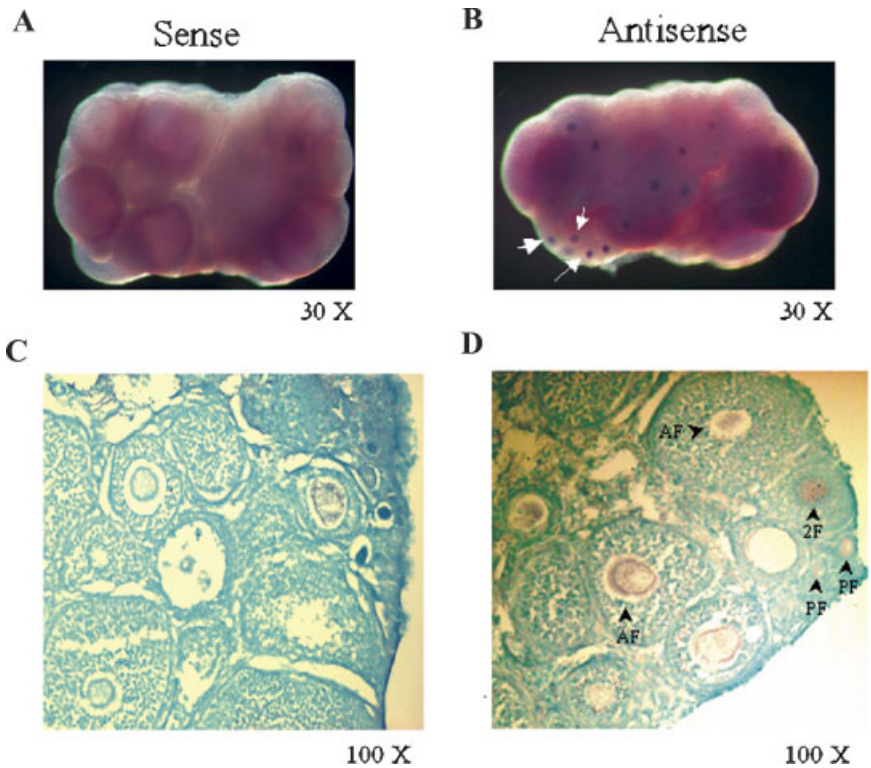

Fig. 4. *Eso-1* expressions in ovaries were analyzed by whole mount in situ hybridization. Four-week-old female mouse ovaries were hybridized with sense probe (**A**) or antisense probe (**B**). **C** and **D**: Ovary samples from (A) and (B) were sectioned and counterstained, respectively. Arrows or arrowheads indicate the expressions of *Eso-1* transcripts. The abbreviations used in (D) are PF, primordial, and primary oocytes; 2F, secondary oocytes; AF, antral oocytes. [See color version online at www. interscience.wiley.com.]

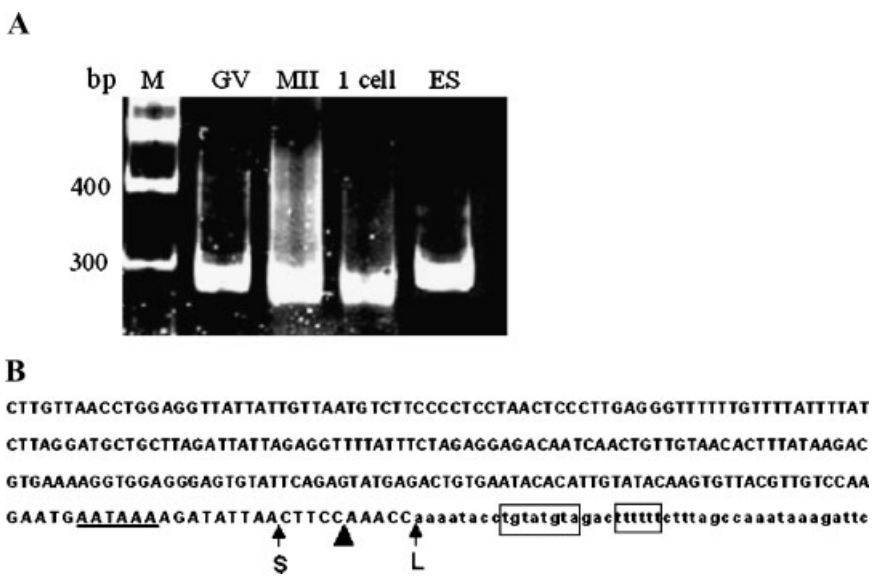

Fig. 5. RACE-PAT analysis of *Eso-1* transcripts in oocytes before or after maturation and after fertilization. **A**: M, molecular size markers (bp); GV, growing oocytes; MII, metaphase II oocytes; 1 cell, fertilization eggs; ES, embryonic stem cells. **B**: The cDNA sequences of 3′UTR of *Eso-1* transcript and the sequences of downstream cleavage sites of polyadenylation. The nuclear polyadenylation signal is underlined. The arrows indicate the identified two cleavage sites of polyadenylation (S and L). The arrowhead indicates the end of AK049114. The sequences in large font represent cDNA sequences and those in small font represent the sequences downstream L cleavage site. The cDNA sequences of U-rich stretch and predicted U/G-rich element are boxed. S, short cleavage site; L, long cleavage site.

In general, polyadenylation is performed in the nucleus by a large set of proteins and is dependent on at least two elements in the prem-RNA molecules, the upstream nuclear HPS and the downstream U- or U/G-rich element. The cleavage site on prem-RNA is always located 11–24 bp downstream of HPS and 10–30 nucleotides upstream of U- or U/G-rich element (Chen et al., 1995). In this study, the *Eso-1* transcripts contain heterogeneous cleavage sites (Fig. 5B). One cleavage site (L site) is 19 nucleotides downstream of the HPS and 20 nucleotides upstream of the U-rich element. However, the other cleavage site (S site) is only 9 nucleotides downstream of the HPS. The S variants of *Eso-1* transcripts can only be detected in MII oocytes and fertilized eggs (Fig. 5A) and contain a long poly (A) tail (with 90–105 A residues). During stages of MII oocytes and fertilized eggs, transcription is silenced and the translations of maternal transcripts are associated with cytoplasmic polyadenylation. We suggest that the S variants of *Eso-1* transcripts are derived from the L variants through an additional cleavage and repolyadenylation by cytoplasmic polyadenylation. Thus, the polyadenylation site of S variants (S site) is upstream of the nuclear polyadenylation site (L site) found in L variants.

Cytoplasmic polyadenylations of maternal mRNAs are a general finding in mouse oocytes and early embryos. However, the distances between potential CPEs and the nuclear HPS are important for effective cytoplasmic polyadenylation. In mice, the maximal distance ranges from 95 to 120 nucleotides (Oh et al., 2000). In *Eso-1* transcripts, only one potential CPE (UUUUAUUU), located 119 nucleotides upstream of the nuclear HPS, may be important for cytoplasmic polyadenylation. Furthermore, *Eso-1* transcripts contain another potential CPE, a polyuridine stretch (U_{13}), similar to the Xenopus embryonic CPE (Simon and Richter, 1994). The Xenopus embryonic CPE can prevent premature polyadenylation of maternal messages in frogs' eggs. In mammals, some maternal-effect genes, such as *Spin* and *Ptp4a1*, also contain a polyuridine stretch (Oh et al., 2000). The exact functions of these polyuridine stretches in 3′UTR in mammals are needed to be further characterized.

On the other hand, the predicted Eso-1 protein contains a homeodomain (Fig. 2A). Homeodomain proteins can bind specific DNA sequences and regulate their transcriptions in a time- or tissue-specific manner (Gehring et al., 1994). Hence, Eso-1 protein may act similarly to other oocyte-specific homeodomain proteins, such as Nobox, which functions as a transcriptional regulator during oocyte and follicle growth. Those genes that are preferentially expressed in postnatal oocytes, such as *Mos*, *Oct4*, *Zar1*, *Gdf9*, and *Bmp15*, are downregulated in *Nobox* null ovaries (Rajkovic et al., 2004). The translation of Eso-1 homeodomain protein might be regulated by cytoplasmic polyadenylation in MII oocytes, and then the expression of Eso-1 protein is suggested to be required for transcriptional activation of early embryonic genome. The in vivo expressions of Eso-1 protein will be further characterized by immunohistochemical analysis, and the RNA interference and/or gene knockout analyses will help to identify the physiological functions of this novel protein.

In addition, it is interesting that two *Eso-1*-specific RT-PCR products, one fainter than the other, were consistently observed in ES cells (Fig. 3). After subcloning and sequencing, the intron3 of *Eso-1* genomic DNA was found to be included in the long transcript but not in the short one (Fig. 1B,C). The intron3 inclusion produces an elongated protein with additional 31 amino acid residues in the middle region but does not change the in-frame stop codon. Because more than 41% of multi-exonal genes in mice have multiple splicing forms (Zavolan et al., 2003), the inclusion of *Eso-1* intron3 may be a result of incomplete splicing or alternative splicing. Furthermore, a large number of splice variants are obtained from transcriptional regulators, such as zinc finger transcription factors (Ravasi et al., 2003). Splicing variations may play a key role in the complexity of mammalian development. Since Eso-1 is a putative homeodomain protein, future identification of functions of these two Eso-1 variants should give insights into the mechanisms that regulate gene expressions during oogenesis and early embryo development.

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REFERENCES

Burns KH, Viveiros MM, Ren Y, Wang P, DeMayo FJ, Frail DE, Eppig JJ, Matzuk MM. 2003. Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. Science 300:633–636.

Chen F, MacDonald CC, Wilusz J. 1995. Cleavage site determinants in the mammalian polyadenylation signal. Nucleic Acids Res 23:2614–2620.

Cinquanta M, Rovescalli AC, Kozak CA, Nirenberg M. 2000. Mouse Sebox homeobox gene expression in skin, brain, oocytes, and two-cell embryos. Proc Natl Acad Sci USA 97:8904–8909.

Correia KM, Conlon RA. 2001. Whole-mount in situ hybridization to mouse embryos. Methods 23:335–338.

De Graeve F, Jagla T, Daponte JP, Rickert C, Dastugue B, Urban J, Jagla K. 2004. The ladybird homeobox genes are essential for the specification of a subpopulation of neural cells. Dev Biol 270:122–134.

Dean J. 2002. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. J Reprod Immunol 53:171–180.

Gebauer F, Xu W, Cooper GM, Richter JD. 1994. Translational control by cytoplasmic polyadenylation of c-mos mRNA is necessary for oocyte maturation in the mouse. EMBO J 13:5712–5720.

Gehring WJ. 1987. Homeo boxes in the study of development. Science 236:1245–1252.

Gehring WJ, Qian YQ, Billeter M, Furukubo-Tokunaga K, Schier AF, Resendez-Perez D, Affolter M, Otting G, Wuthrich K. 1994. Homeodomain-DNA recognition. Cell 78:211–223.

Huarte J, Belin D, Vassalli A, Strickland S, Vassalli JD. 1987. Meiotic maturation of mouse oocytes triggers the translation and polyadenylation of dormant tissue-type plasminogen activator mRNA. Genes Dev 1:1201–1211.

Ko MS, Kitchen JR, Wang X, Threat TA, Hasegawa A, Sun T, Grahovac MJ, Kargul GJ, Lim MK, Cui Y, Sano Y, Tanaka T, Liang Y, Mason S, Paonessa PD, Sauls AD, DePalma GE, Sharara R, Rowe LB, Eppig J, Morrell C, Doi H. 2000. Large-scale cDNA analysis reveals phased gene expression patterns during preimplantation mouse development. Development 127:1737–1749.

Latham KE, Beddington RS, Solter D, Garrels JI. 1993. Quantitative analysis of protein synthesis in mouse embryos. II: Differentiation of endoderm, mesoderm, and ectoderm. Mol Reprod Dev 35:140–150.

Li H, Huang CJ, Choo KB. 2002. Expression of homeobox genes in cervical cancer. Gynecol Oncol 84:216–221.

Liang L, Soyal SM, Dean J. 1997. FIGalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. Development 124:4939–4947.

Luoh SW, Bain PA, Polakiewicz RD, Goodheart ML, Gardner H, Jaenisch R, Page DC. 1997. Zfx mutation results in small animal size and reduced germ cell number in male and female mice. Development 124:2275–2284.

Mendez R, Richter JD. 2001. Translational control by CPEB: A means to the end. Nat Rev Mol Cell Biol 2:521–529.

Oh B, Hwang S, McLaughlin J, Solter D, Knowles BB. 2000. Timely translation during the mouse oocyte-to-embryo transition. Development 127:3795–3803.

Paillisson A, Dade S, Callebaut I, Bontoux M, Dalbies-Tran R, Vaiman D, Monget P. 2005. Identification, characterization and metagenome analysis of oocyte-specific genes organized in clusters in the mouse genome. BMC Genomics 6:76.

Payer B, Saitou M, Barton SC, Thresher R, Dixon JP, Zahn D, Colledge WH, Carlton MB, Nakano T, Surani MA. 2003. Stella is a maternal effect gene required for normal early development in mice. Curr Biol 13:2110–2117.

Rajkovic A, Yan C, Yan W, Klysik M, Matzuk MM. 2002. Obox, a family of homeobox genes preferentially expressed in germ cells. Genomics 79:711–717.

Rajkovic A, Pangas SA, Ballow D, Suzumori N, Matzuk MM. 2004. NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. Science 305:1157–1159.

Ravasi T, Huber T, Zavolan M, Forrest A, Gaasterland T, Grimmond S, Hume DA. 2003. Systematic characterization of the zinc-finger-containing proteins in the mouse transcriptome. Genome Res 13:1430–1442.

Richter JD. 1999. Cytoplasmic polyadenylation in development and beyond. Microbiol Mol Biol Rev 63:446–456.

Salles FJ, Richards WG, Strickland S. 1999. Assaying the polyadenylation state of mRNAs. Methods 17:38–45.

Schultz RM. 1993. Regulation of zygotic gene activation in the mouse. Bioessays 15:531–538.

Simon R, Richter JD. 1994. Further analysis of cytoplasmic polyadenylation in Xenopus embryos and identification of embryonic cytoplasmic polyadenylation element-binding proteins. Mol Cell Biol 14:7867–7875.

Song JL, Wessel GM. 2005. How to make an egg: Transcriptional regulation in oocytes. Differentiation 73:1–17.

Soyal SM, Amleh A, Dean J. 2000. FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. Development 127:4645–4654.

Tada M, Casey ES, Fairclough L, Smith JC. 1998. Bix1, a direct target of Xenopus T-box genes, causes formation of ventral mesoderm and endoderm. Development 125:3997–4006.

Tanaka M, Hennebold JD, Macfarlane J, Adashi EY. 2001. A mammalian oocyte-specific linker histone gene H1oo: Homology with the genes for the oocyte-specific cleavage stage histone (cs-H1) of sea urchin and the B4/H1M histone of the frog. Development 128:655–664.

Tay J, Hodgman R, Richter JD. 2000. The control of cyclin B1 mRNA translation during mouse oocyte maturation. Dev Biol 221: 1–9.

Tong ZB, Gold L, Pfeifer KE, Dorward H, Lee E, Bondy CA, Dean J, Nelson LM. 2000. Mater, a maternal effect gene required for early embryonic development in mice. Nat Genet 26:267–268.

Vanden Heuvel GB, Bodmer R, McConnell KR, Nagami GT, Igarashi P. 1996. Expression of a cut-related homeobox gene in developing and polycystic mouse kidney. Kidney Int 50:453–461.

Wang SH, Tsai MS, Chiang MF, Li H. 2003. A novel NK-type homeobox gene, ENK (early embryo specific NK), preferentially expressed in embryonic stem cells. Gene Expr Patterns 3:99–103.

West MF, Verrotti AC, Salles FJ, Tsirka SE, Strickland S. 1996. Isolation and characterization of two novel, cytoplasmically polyadenylated, oocyte-specific, mouse maternal RNAs. Dev Biol 175:132–141.

Wu X, Viveiros MM, Eppig JJ, Bai Y, Fitzpatrick SL, Matzuk MM. 2003. Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. Nat Genet 33:187–191.

Yoon SO, Chikaraishi DM. 1994. Isolation of two E-box binding factors that interact with the rat tyrosine hydroxylase enhancer. J Biol Chem 269:18453–18462.

Zavolan M, Kondo S, Schonbach C, Adachi J, Hume DA, Hayashizaki Y, Gaasterland T. 2003. Impact of alternative initiation, splicing, and termination on the diversity of the mRNA transcripts encoded by the mouse transcriptome. Genome Res 13: 1290–1300.