行政院國家科學委員會補助專題研究計畫成果報告

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※　早期發育階段豬胚表現其胚源性基因之時間的特異性　※

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□赴國外出差或研習心得報告一份

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□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

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中　華　民　國　九十一年四月十日

行政院國家科學委員會專題研究計畫期末成果報告

早期發育階段豬胚表現其胚源性基因及時間的特異性 (1~3)

計畫編號: NSC89-2313-B-002-163
87 年 8 月 1 日至 90 年 7 月 31 日
主持人: 鄭登貴 國立台灣大學 畜產學系

一、中英文摘要：

鑑於豬之受精卵自 1-細胞卵裂至 4-細胞階段係其輸卵管內完成，本研究首先針對母豬在發情後不同生理時段，探討其輸卵管上皮細胞之基因表現情形及蛋白質之分泌模式，結果證明母豬自開始發情後第 1 日至第 5 日，其輸卵管上皮細胞不僅確認 13 個基因之表現具有時間特異性，且其蛋白質之分泌模式亦頗有差異。其屬有趣者，乃該 13 個表現具有時間特異性之基因中，有 11 個基因表現之時間，適與豬胚發育跨越 4-細胞障阻階段相符合；顯示此等基因之適時表現，或係豬胚完成早期發育所不可或缺者。此外，試驗亦針對豬輸卵管中前述表現具有時間特異性之基因，分別探討其與豬胚早期發育之關係；結果除進一步證明其中 4 個源自豬輸卵管上皮細胞表現具有時間特異性之基因屬性，分別為激濾胞素接受體(FSH-r)、心房鈉尿因子接受體(ANF-r)、細胞轉型生長因子-á (TGF- á)及細胞轉型生長因子-結合蛋白-II (TGF-BP-II)外 (見第一年研究結果與討論；附件一)，亦證明培養液中添加 TGF- á (20 ng/ml)，確可有效誘發 4-細胞階段豬胚內 *TIG-1* 基因之表現(見第二年研究結果與討論；附件二)，從而確保豬胚順利發育超越其 4-細胞障阻期。試驗最後乃針對 *TIG-1* 基因探討其影響早期胚發育之分子調控機制。鑑於試驗執行期間適逢國內豬隻爆發口蹄疫事件，本校為謀徹底杜絕遭受病媒傳染，嚴禁自外引進任何豬源，前項試驗遂改以小鼠胚完成之(見第三年研究結果與討論；附件三)。試驗結果除順利選殖獲得該同源性之小鼠 *TIG-1* 基因(*mTIG-1*)外，經定序結果並確認其 cDNA 序列全長為 2,322 bp (Fig. 1)。此等小鼠研究模式之完成，除有助於瞭解哺乳動物胚胎發育之分子調控機制外，且對未來建立哺乳動物胚之體外培養系統，及應用遺傳工程技術改善母豬生殖效率等，均有極大之助裨。

Abstract:

While a large pool of maternal messenger RNAs have been accumulated within the ooplasm during meiotic maturation of the oocytes and these maternal mRNAs are thought to be important for programming the very initial development of embryos in most mammalian species, the maternal mRNAs are found to be decreased quantitatively and qualitatively along the progression during early cleavage of embryos and they are eventually replaced by those mRNAs derived from the transcriptional activation of embryonic genome. Development of porcine embryos culture in vitro was frequently arrested at 4-cell stage and this particular phenomenon is referred as "culture block". Based on the fact that porcine embryos normally completed their 4-cell development within the oviducts, some factors essentially required for early development of pig embryos must be synthesized

and secreted by the oviduct epithelial cells and these have been confirmed by our preliminary results indicating that there are significant differences in expression of gene(s) at both transcription and translation levels when comparisons were made by method of differential display reverse transcription-polymerase chain reaction (DDRT-PCR) between oviductal epithelia cells obtained respectively from sows at days 1-5 after the standing heat. Among the 13 differential fragments of cDNA detected, 11 were identified to be expresses at 96 h post-hCG injection, coincidental with the time of 4-cell block found in pig embryos. This implies that timely expression of these genes in epithelial cells of porcine oviducts is essential for the pig embryos to develop beyond the 4-cell stage. Four of the said 13 time-specific gene fragments have been confirmed to have sequences identical to those genes encoding for the receptor of porcine follicle-stimulating hormone, the receptor of porcine atrial natriuretic factor, the porcine transforming growth factor-α and the porcine transforming growth factor-β-binding protein II, respectively. However, to characterize the other 9 gene fragments, much longer length of sequences need to be raced. Therefore, in this three years of research proposal, studies will first be emphasized on characterization of these 9 time-specific gene(s) that have been previously identified to be expressed in the oviduct epithelial cells and further studies will be extended to exploring the interaction, both in vivo and in vitro, between the expression of embryonic gene(s) and those time-specific genes that have been previously identified to be expressed by oviduct epithelial cells as temporal-dependent according to the physiological status of the sow's reproductive cycle. It is anticipated that valuable data generated from these studies would be rather helpful for contributing a better understanding in the mechanism of the molecular control of early development in porcine embryos, and also would be useful in the future for establishment of an optimal condition for culture of porcine embryos in vitro. Moreover, the reproductive efficiency of sows may therefore be improved due to the further application of this knowledge in attempting to manipulate their genetic materials.

Key words: Preimplantation development, porcine embryo, embryonic mRNA, mRNA differential display

二、緣由與目的：

許多哺乳動物之胚，在體外進行培養時經常面臨一個共同問題，即胚於體外經過 1-4 次不等之卵裂作用後，發育即告停頓。此一體外發育停頓現象，稱為胚之“培養障阻(culture block)”；一般而言，小鼠胚之培養障阻常發生於 2-細胞期，而豬胚者則常發生在 4-細胞期。

鑑於豬胚之自單細胞階段卵裂至 4-細胞階段，其發育均在輸卵管內完成者，顯示在該特定生理時段，在豬輸卵管之上皮細胞內，勢必有若干重要因子之合成並分泌進入輸卵管中，且此等因子係支持豬胚早期發育所不可或缺者；本研究室有鑑於此，先後曾應用蛋白質雙向電泳及訊息核醣核酸分示法(mRNA Differential Display)，針對母豬於發情後 1-5 日之輸卵管上皮細胞，詳加探討其在該特定生理階段對於基因表現及蛋白質分泌模式之變化情形。其中蛋白質雙向電泳分析結果發現，母豬自開始發情後第 1 日至第 3 日，其輸卵管上皮細胞之蛋白質分泌模式，確有生理階段之差異性；惟鑑於彼等分泌具有生理階段差異性之蛋白質含量極微且成分複雜，殊難逕自雙向電泳結果進一步明確分析各個特定蛋白質之胺基酸序列，遂無法針對各個特定蛋白質探討

其在生理學上可能扮演之真實意義。

本研究將陸續針對前述豬輸卵管中表現具有時間特異性之各基因，分別詳加探討其與豬胚早期發育之關係。全程計畫擬分三年完成；其中第一年之研究重點，除繼續確認前述源自豬輸卵管上皮細胞表現具有時間特異性之 9 個基因片段的屬性外，並擬應用核醣核酸分示法(mRNA differential display; DD)比較在體內發育之各不同階段(1-至 8-細胞)豬胚中，胚源性基因組基因轉錄活動之差異性；第二年之研究重點，除進一步就第一年研究中所發現之各表現具有時間特異性的胚源性基因，分別完成選殖及定序工作外，並擬比較各不同階段(1-至 8-細胞)豬胚在體外培養時，培養液中添加前述源自豬輸卵管上皮細胞表現具有時間特異性之基因轉譯物，對於胚源性基因組基因轉錄活動之差異性；第三年之研究重點，則針對此等被表現具有時間特異性之輸卵管上皮細胞基因轉譯物發軔轉錄之各胚源性基因片段，分別完成其基因之選殖及定序工作。此等研究之完成，除有助於瞭解豬胚早期發育之分子調控機制外，且對未來建立豬胚之體外培養系統，及應用遺傳工程技術改善母豬生殖效率等，均有極大之助裨。

三、結果與討論：

第一年研究： 本年度之研究成果已發表於 **Mol Reprod Dev 2000 Jul;56(3):331-335**，題目為: **Identification of Genes Expressed in the Epithelium of Porcine Oviduct Containing Early Embryos at Various Stages of Development** (附件一)。茲簡述重要結果如下:

試驗應用更具高靈敏性之訊息核醣核酸分示技術，針對母豬於發情後特定時段之輸卵管上皮細胞，分析其基因表現之時間特異性；結果已證實母豬在 hCG 注射後 50-120 h 之特定時段中(該時段相對涵蓋胚之發育其自 4-至 8-細胞階段)，其輸卵管上皮細胞合計被確認有 13 個基因片段表現具有生理時段特異性。其中在 hCG 注射後 50-85 h 與 120 h 階段表現者，分別為長度 200 bp 與 250 bp 之基因片段，另有 11 個長度介於 220-900 bp 之基因片段，分別均在 hCG 注射後 96 h 階段被表現。其屬有趣者，乃該 13 個表現具有時間特異性之基因中，有 11 個基因表現之時間，適與豬胚發育跨越 4-細胞障阻階段相符合；顯示此等基因之適時表現，或係豬胚完成早期發育所不可或缺者，誠值得分別深入詳加探討其與豬胚早期發育間之關係。此外，前述 13 個基因片段經分別選殖及定序，並使用 GCG 軟體進行比結果，已確認基因屬性者有 4 個基因片段，分別為豬之激濾胞素接受體(receptor of porcine follicle stimulating factor, FSH-r)、心房鈉尿因子接受體(receptor of porcine atrial natriuretic factor, ANF-r)、細胞轉型生長因子-α (transforming growth factor-alpha, TGF-α)及細胞轉型生長因子-β-結合蛋白-II (transforming growth factor-β-binding protein II, TGF-β-BP-II)。本研究室於國科會補助之次年研究計畫中，將針對 TGF-α 探討其在早期發育階段豬胚所扮演之生理意義，並瞭解該生長因子作用於胚後對胚內基因之調控情形。

第二年研究： 本年度研究重點，除進一步就第一年研究中所發現之各表現具有時間特異性的源自輸卵管基因，分別完成選殖及定序工作外，並擬比較各不同階段(1-至 8-細胞)豬胚在體外培養時，培養液中添加前述源自豬輸卵管上皮細胞表現具有時間特異性之基因轉譯物，對於胚源性基因組基因轉錄活動之差異性；有關生殖道上皮細胞所表現之各類生長因子，對於胚早期發育之重要性，可經由其在胚之體外培養系統添加與否，分別予以比較證明之。

試驗首先以隨機引子 H-AP 7 及 錨定引子 H-T11G 之配對，分析 4-細胞階段之豬胚經培養於添加生長因子 TGF-α (20 ng/ml) 之 NCSU-23 培養液中，對其表現之 mRNA 是否可因 TGF-α 添加與否而有差異。試驗結果證明，經使用上述引子組於 mRNA Differential Display 分析後，可有效確認一條具有特異性之基因片段，係用添加生長因子 TGF-α 之添加而被表現者。此具有 TGF-α 特異性之基因片段，經應用各原先使用之相同引子組，進行各基因片段之第二次 PCR 擴增結果，更確認其長度約為 300 bp；進一步試驗將該基因片段分別構築於 TA vector 及 pGEM-T 質體上，經選殖大量回收基因片段之質體 DNA 後，將經核酸限制酵素截切分析結果，確認其長度無誤外，並分別經定序及使用 GCG 軟體進行比對；此外，試驗更以巢式聚合酵素連鎖反應 (nested PCR)及原位雜交(*in situ* hybridization)；進行重複驗證，確認豬胚之 *pTIG-1* 基因係在 4-細胞階段被 TGF-α 誘發而表現者。本研究結果已撰寫成為學術論文，題目為: **Timely Expression of *TGF-α* in Porcine Oviduct Epithelia May Further Modulate the Expression of Embryonic Gene at 4-cell stage of development** (附件二)；該文並已投稿於 Mol. Reprod. Dev. 學術性期刊，刻正進行審稿作業。

第三年研究: 本計畫於第二年執行期間適逢台灣爆發豬口蹄疫；鑑於試驗所需動物之取得及疾病控管面臨困境，遂改以使用小鼠胚做為研究模式繼續探討哺乳動物胚胎發育之分子調控機制。試驗首先針對生長因子 TGF-α 所誘發表現之 *pTIG-1* ，探討其對早期胚發育可能扮演之生物功能。為謀達成此一目標，初始試驗除先建立不同發育階段小鼠胚之 cDNA 基因庫外，並以前述豬胚之 *pTIG-1* 基因序列為探針，嘗試選殖與之同源的小鼠 *mTIG-1* 基因。試驗結果除完成成熟卵母細胞與 1-, 2-, 8-細胞及桑椹期與囊胚期胚等六個 cDNA 基因庫外，且自小鼠基因庫成功篩選並完成定序獲得完整 cDNA 序列之 *mTIG-1*，確認其全長為 2322 bp (Fig.1)；刻正針對 *mTIG-1* 進行其後續之功能性驗證工作，包括確認其在成鼠組織器官之轉錄與轉譯等表現影像 (expression profiles)及其對於下游基因之調控與生理功能之影響；此外，本研究同時並成功選殖獲得若干涉及小鼠胚發育具有階段特異性之新基因(Fig.2～4.)。前述試驗結果並已完成撰稿，題目為: **Large-Scale Identification of Genes Differentially Expressed in Mouse Pre-implantation Embryos** (附件三) ；該文且投稿於學術性期刊 "*Theriogenology*"，刻正接受審稿作業。

四、參考文獻：

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CCAAAAATCTGGTTGCCATGAAAGAAATTCTGTATGGCACAAATGAAA
AAGAGCCTCAGACAGAAGCAGTAGCTCAACTTGCTCAAGAACTCTATA
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ACCTCGCATTTCATTTCAACGATGCTTACAGGTTTCTTTTGTATATAA
TTCTTAGAATGCTCATTTCTTTTAAATGATTTAATTTGTACAGCAGAG
GAATGTTATTGTATTAGTATGTAACTATTACCTAATAAAGAGTTTTTG
CAAAAAAAAAAAAAAA--3280 bp

Fig. 1. The *mTIG-1* sequence of the full-length cDNA was obtained by cDNA library screening. Nucleotide sequence of the mouse *mZFG-1L* cDNA contain one cytoplasm polyadenylation signal (gray region) AATAAA, as well as a poly-A tail.

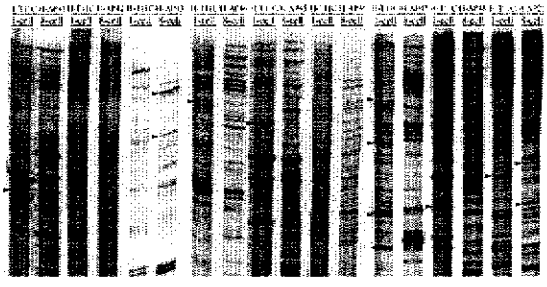

Fig.2. Differential display of mouse maternal and zygotic RNA with primers 3'-T11MC and 5'-APs. Arrowheads indicate examples of conserved cDNA bands selected due to stage-specific expression.

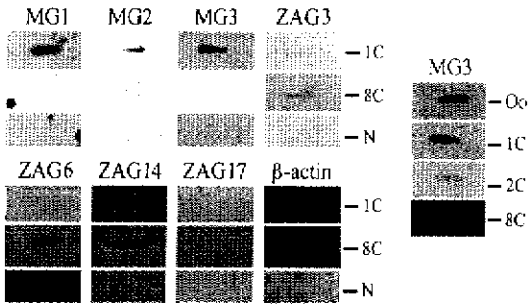

Fig. 3. Re-confirmed differentially expression of novel clones from mRNA differential display using Reverse-Northern slot blot. One of the membranes (1C) was hybridized with ^{32}P-labeled cDNA pool from fertilized oocyte. The other membrane (8C) was hybridized with ^{32}P-labeled total cDNA from 8-cell embryos. The blot was also hybridized with -actin as control at each sample.

MG1
AAGCTTCTCCGGAGTGTCTGGCGCCTGCTGACTTGCTGGTT
AAGAAGGAGCCCAAGGCAAATGGCAGCAGTCAAAGGATGAT
GCAGTGGCTGGCTACAAAGTCACCCAAAAAGGAAGTCCCTG
ACTCACCCAAAAAGGATGCATCAGGTCTACCCCAGTGGTCC
AGCCAGTTTCTCCAGAAGAGCCCATTGCCTGCTAAAAGAGG
TGCTACCAGCAGCTTCCTGGATCGATGGCTGAAGCAGGAGA
AGGAGGATGAGCCCATGGCCAAGAAGCCTAACAGCTAGGCT
CCATACCAGGCCTCAGCCTGATACAGCATTACTGTTGTCAC
CAGTTGACTGGAATTGTAACCTTTTACTTGGGAATGTGGTG
GCCCAATGTCAGCTGACCTGAAAGAACATGGCTACCGTGAG
ACAGCCCTGTGGGCCCTGCTACCCCTCCTATTTCTGCCTCC
CTGCTGGGAGCTTTCCTGTTCCCTGGTGTTCCACGAAGGAG
GCTCCTCTCAGTCTCTGGGCTCTCCTTTAGAGTTAAGGTCT
TAAAATGTATTTTGTCCTAAATAAACTGCGTTTGGTAAAAA
AAAAAAGCTT

MG2
AAGCTTCTCCGGAGTGTCTGGCGCCTGCTGACTTGCTGGTT
AAGAAGGAGCCCAAGGCAAATGGCAGCAGTCAAAGGATGAT
GCAGTGGCTGGCTACAAAGTCACCCAAAAAGGAAGTCCCTG
ACTCACCCAAAAAGGATGCATCAGGTCTACCCCAGTGGTCC
AGCCAGTTTCTCCAGAAGAGCCCATTGCCTGCTAAAAGAGG
TGCTACCAGCAGCTTCCTGGATCGATGGCTGAAGCAGGAGA
AGGAGGATGAGCCCATGGCCAAGAAGCCTAACAGCTAGGCT
CCATACCAGGCCTCAGCCTGATACAGCATTACTGTTGTCAC
CAGTTGACTGGAATTGTAACCTTTTACTTGGGAATGTGGTG
GCCCAATGTCAGCTGACCTGAAAGAACATGGCTACCGTGAG
ACAGCCCTGTGGGCCCTGCTACCCCTCCTATTTCTGCCTCC
CTGCTGGGAGCTTTCCTGTTCCCTGGTGTTCCACGAAGGAG
GCTCCTCTCAGTCTCTGGGCTCTCCTTTAGAGTTAAGGTCT
TAAAATGTATTTTGTCCTAAATAAACTGCGTTTGGTAAAAA
AAAAAAGCTT

ZAG3
AAGCTTACGGGGTACTGGTTAGTTCATAATGTTGTTCCACC
TATAGGGTTGCAGATCCCTTTAGCTCCACAAGGGGATTATA
ATACAGTGTTCTTGGTTGGGTTTGGTCTTATTAGAAATCCC
TGGTGTAATCCACAAAGAATAGAAAATAAATACATCTATTC
ATGTTGACAAATTAACCCCTCAGATATTAGTAAGAAGTAAA
CATGTATGCCATGAAACTAATTAACTCACTTAACCAGATGT
AATGATGCATTTAAAAATATATCATACAAACACTGATAATA
CCACCCCCCAAAAAAAAAAAGCTT

Fig..4. Representative cDNA fragments of MG1 (maternal gene1), MG2 and ZAG3 (zygotic activation gnee3) sequenced. The underlined sequences are primers used in differential gene display analysis. Similar sequencing results have also been available for MG3 (C44M1), ZAG6, ZAG14, and ZAG17 while data not shown here.

MOLECULAR REPRODUCTION AND DEVELOPMENT 56:331–335 (2000)

Identification of Genes Expressed in the Epithelium of Porcine Oviduct Containing Early Embryos at Various Stages of Development

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ABSTRACT As a first step toward elucidation of the action of factors secreted by the epithelium of oviduct, differential display reverse transcription-polymerase chain reaction (DDRT-PCR) was used in this study to identify transcripts of such oviductal factors in gilts carrying various stages of early embryo development post hormone-induced ovulation. A total of 13 differentially expressed transcripts were identified between 50 and 120 hr post-hCG injection (between 1- and 8-cell embryonic stages). Twelve of these transcripts were found to be initially expressed at 96 hr post-hCG injection (at 4-cell embryonic stage) and beyond. Three of such genes were shown by sequence analysis to be the porcine transforming growth factor-α, the porcine transforming growth factor-β-binding protein II and a porcine astral natriuretic factor receptor-like transcript. Only one differentially expressed gene was detected between 50–60 and 85 hr post-hCG injection, and this gene turned out to be the porcine follicle-stimulating hormone receptor. The remaining eight transcripts detected by DDRT-PCR were novel. Moreover, most of these newly expressed genes were found to be turned on at a time coincidental with that of the 4-cell block of porcine embryos cultured in vitro. Our results demonstrate that DDRT-PCR is a feasible approach for rapid identification of genes that are differentially expressed in oviductal epithelium. Some of the genes thus identified may be important for unhindered development of embryos in the oviduct. *Mol. Reprod. Dev. 56:331–335, 2000.*

Key Words: oviduct; gene expression; differential display; porcine

INTRODUCTION

In fertilization in mammals, the early embryos need to transverse the length of the oviduct before implanting into the uterus. During this pre-implantation period, the developing embryos secrete numerous growth factors and glycoproteins that cooperatively act as autocrines to support continuous cleavage of neighboring embryos (Reisch et al., 1999). Other studies have also shown that, in conjunction with the embryonic factors, the oviductal tube also provides an optimal and essential biochemical environment for proper embryo development (Heyner, 1997). Co-culture of very early embryos in the presence of oviductal cells results in viability and growth rates comparable to embryo development in vivo (Sakkas et al., 1989). Otherwise, the majority of early embryos cultured in vitro in chemically defined media soon reach the arrest phase of cell cleavage, which occurs at different cell number in different species (Gardner, 1998). In porcine, such an in vitro developmental block occurs at the 4-cell stage (Bavister, 1998). This phenomenon hints at the requirement of some yet unidentified but essential factors which are present in the oviduct in vivo for normal embryo development. It is noteworthy that the occurrence of such a block in various species roughly coincides in time at which zygotic gene activation is thought to occur (Stein et al., 1997). A number of growth factors have been identified in the oviduct by various means (Heyner, 1997). The importance of some of these growth factors for embryo development is clearly demonstrated by stimulation of embryonic cleavages in vitro in their presence (de Moraes et al., 1999). It is unclear, however, if proteins of other nature are also expressed by the epithelial cells of oviduct which are needed for a full complement of biological functions for development as well as for sperm capacitation and transportation of the developing embryo in the tube.

As a first step toward the elucidation of factors important for the functioning of the porcine oviduct, differential display reverse transcription-polymerase chain reaction (DDRT-PCR) was used in this study. In addition to some known growth factor genes, other novel genes have been identified which are expressed in the epithelial cells of porcine oviduct carrying embryos at various stages of early development.

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TABLE 1. Anchor and Arbitrary Primers Used for DDRT-PCR Analysis

Anchor primer	Arbitrary primer
A1 : Poly(T)$_{11}$-M[a]C	R1 : GATCAAGTCC
A2 : Poly(T)$_{11}$-MA	R2 : GATCTAAGGC
A3 : Poly(T)$_{11}$-MG	R3 : TACCTAAGCG
A4 : Poly(T)$_{11}$-MT	R4 : TCGATACAGG
	R5 : GATCTAACGG
	R6 : CTGCTTGATG
	R7 : TGGATTGGTC
	R8 : TACAACGAGG
	R9 : CTTTCTACCC
	R10 : TTTTGGCTCC

[a]M was A, C, or G.

MATERIALS AND METHODS

Collection of Porcine Oviducts Carrying Embryos at Various Stages of Early Development

Gilts between 12 and 15 months of age (120 kg) were used as the source of porcine oviducts. The gilts were fed 0.4% Altrenogest (Regumate®, Hoechst, UK) continuously for 18 days. On termination of the feed, 1,500 IU of PMSG was immediately administered, followed by injection of 1,000 IU of hCG. Twenty-four and thirty hours after hCG injection, respectively, two rounds of artificial insemination were carried out. Full-length oviducts that supported the development of porcine embryos at the 1-cell, 2-cell, early 4-cell, and late 4-cell stages were collected 50–60, 72, 85, and 96 hr, respectively, post-hCG injection. For the collection of 8-cell embryos within the uterine horn, full-length oviducts were collected 120 hr post-hCG injection. After several washes with Dulbecco's phosphate buffer saline (DPBS), cells of the lined epithelium layer were isolated from the oviduct by scrapping with a surgical scalpel. In separate experiments, cells were collected by digestion of the luminal epithelium with 25% trypsin/1 mM EDTA at room temperature for 1 min (Zhang et al., 1991). Microscopic observation showed that cells collected using either of these procedures were similar and that they were epithelial cells. Furthermore, DDRT-PCR patterns derived from cells obtained by both methods were also identical (data not shown). The collected epithelial cells were washed twice with DPBS followed by centrifugation for cell harvesting. Cells were finally solubilized in RNA^{zol}B (TEL-TEST, Texas) for the preparation of total RNA.

Preparation of RNA and DDRT-PCR

Total RNA was prepared from oviductal epithelial cells as described by Chomczynski and Sacchi (1987). RNA (10 μg) was first subjected to reverse transcription (RT) using one of the four anchor primers (Table 1) and the Superscript II RT kit (Gibco BRL, NY). This is followed by PCR using the original anchor primer and one of the ten arbitrary primers (Table 1) in the presence of 2 μCi of [α-^{33}P]dCTP (Amersham, UK) for 35 thermal cycles using the following PCR conditions: 94°C for 30 sec, 32°C for 1.5 min, and 70°C for 1.5 min. The RT-PCR products were electrophoresed in 8% denaturing polyacrylamide gels of 0.2-mm thickness. After drying on a 3 MM filter, the gels were autoradiographed.

PCR, Sequence Analysis, and Ribonuclease Protection Assay

For re-amplification, bands of interest were excised and DNA was eluted from the gel by incubation of the gel slice at 70°C in deionized water. PCR was performed using aliquots of the recovered DNA fragment and the same combination of anchor and arbitrary primers. The PCR products were cloned into pGEM-T (Invitrogen, CA) and sequencing was performed using an automatic sequencer 373A (ABI, CA). Ribonuclease protection assay (RPA) was done by first generating antisense RNA probe using the MAXIscript® in vitro transcription kit (Ambion, Texas) and the cloned DDRT-PCR products in the presence of [α-^{32}P]UTP. The labeled gene-specific antisense RNA was mixed with total RNA and RPA analysis was carried out using a commercial RPA II® kit (Ambion).

RESULTS

For DDRT-PCR, 10 different arbitrary random primers and 4 anchor primers (Table 1) were used in a total of 40 different combinations. For use as templates, total RNA was prepared from epithelial cells of the porcine oviducts carrying various stages of developing embryos post-hCG injection. Thirty-four of the 40 random-anchor primer combinations produced DDRT-PCR patterns which were identical irrespective of the developmental stages of the embryos the oviducts were carrying (data not shown). Only 6 anchor and arbitrary primer combinations detected transcripts that were clearly differentially expressed. Representative DDRT-PCR patterns are shown in Fig. 1. Transcripts that were clearly differentially expressed at different conceiving stages were identified for further analysis. A total of 13 such bands were identified (Fig. 1 and Table 2). The size of these bands ranged from 225 to 637 bp.

One of the bands (A1R2/50) was detected between 50–85 and 85 hr post-hCG injection (between 1-cell to early 4-cell embryonic stages) (Fig. 1, left panel). This band was no longer detectable at 96 hr post-hCG injection (i.e., at late 4-cell stage). All other remaining differential bands were first detected 96 hr post-hCG injection (Fig. 1, central and right panels) some of which were further found to be sustained through to 120 hr post-hCG injection (8-cell embryonic stage) and beyond (Fig. 1 and Table 2).

To further characterize the differentially expressed oviductal transcripts identified, the bands were excised from the gel for further PCR re-amplification using the same primer combinations that had led to their initial identification. In all cases, such PCR re-amplification was successful and the size of the PCR products matched that of the original DDRT-PCR products (Fig. 2). The PCR products were cloned and were subjected to sequence analysis. On a comprehensive search of the

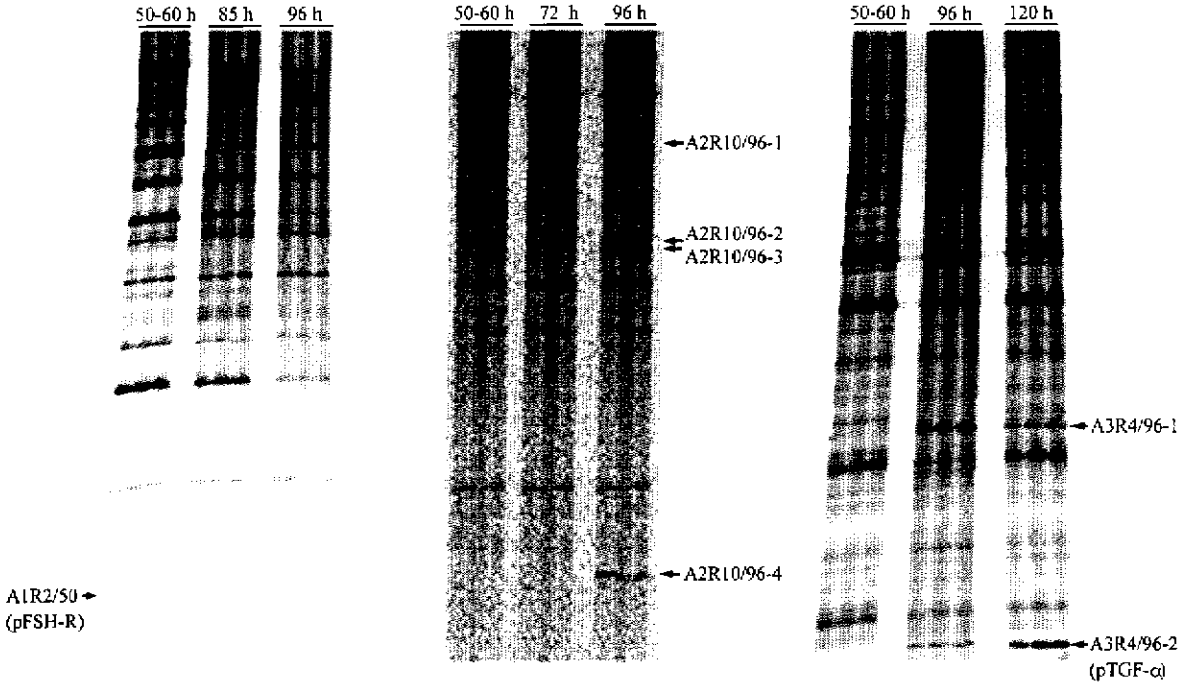

Fig. 1. Representative profiles of DDRT-PCR of RNAs isolated from epithelial cells of porcine oviducts at different stages of embryo development. The time (hr) post-hCG injection before harvesting of oviducts for collection of epithelial cells are indicated at the top of the profiles. Clearly differentially expressed transcripts are indicated by arrows and band designations.

TABLE 2. Summary of DD-RT-PCR Results From Porcine Oviductal Epithelial Cells

	Primer		Expression (post hCG injection)					
Band	Anchor	Random	50–60 hr	72 hr	85 hr	96 hr	120 hr	Gene
A1R2/50	A1	R2	+	nd[a]	+	–	nd	pFSH-R
A1R4/96-1	A1	R4	–	nd	–	+	nd	Novel
A1R4/96-3	A1	R4	–	nd	–	+	nd	Novel
A2R10/96-1	A2	R10	–	–	nd	+	nd	Novel
A2R10/96-2	A2	R10	–	–	nd	+	nd	Novel
A2R10/96-3	A2	R10	–	–	nd	+	nd	Novel
A2R10/96-4	A2	R10	–	–	nd	+	nd	Novel
A3R5/96-1	A3	R5	–	nd	–	–	+[b]	pTGFβ-BPII
A3R5/96-2	A3	R5	–	nd	–	–	+[b]	pANF-R-like
A3R4/96-1	A3	R4	–	nd	nd	+	+	Novel
A3R4/96-2	A3	R4	–	nd	nd	+	+	pTGFα
A4R8/120	A4	R8	–	nd	nd	+	+	Novel

[a]nd, not determined.
[b]RT-PCR results.

GenBank database, sequences of three of the cDNA clones matched perfectly with known porcine genes, namely the porcine follicle-stimulating hormone receptor (pFSH-R), the porcine transforming growth factor-α (pTGF-α) and the porcine transforming growth factor-β-binding protein II (pTGF-β-BPII) (Table 2). Another cDNA clone showed a high (84%) sequence homology with the porcine astral natriuretic factor receptor, and may be a novel member of this gene family. The remaining eight transcripts detected by DDRT-PCR were products of novel porcine genes.

To verify the authenticity of the DDRT-PCR-derived cDNA sequences, gene-specific primers were designed based on published and experimentally (Bauer et al., 1993) derived sequences of the three known genes and the pANF-R-like gene for direct RT-PCR detection of these transcripts. Transcripts for the pFSH-R gene were detected in the oviductal epithelial cells at 50–60 hr post-hCG injection and were absent at 96 hr and later stages (Fig. 3a). Transcripts for the pTGFβ-BPII, pTGF-α ligand and the pANF-R-like genes were detected only at and after the 96 hr post-hCG injection

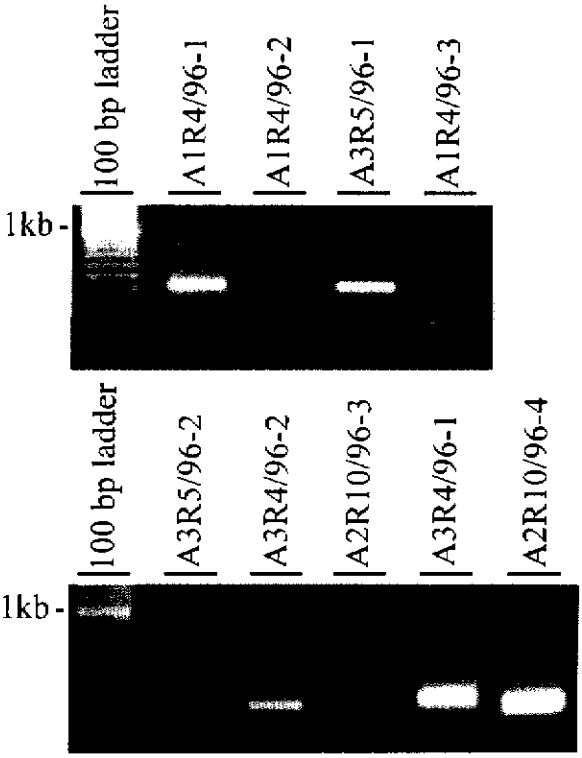

Fig. 2. PCR re-amplification of cDNA fragments eluted from the DDRT-PCR gels.

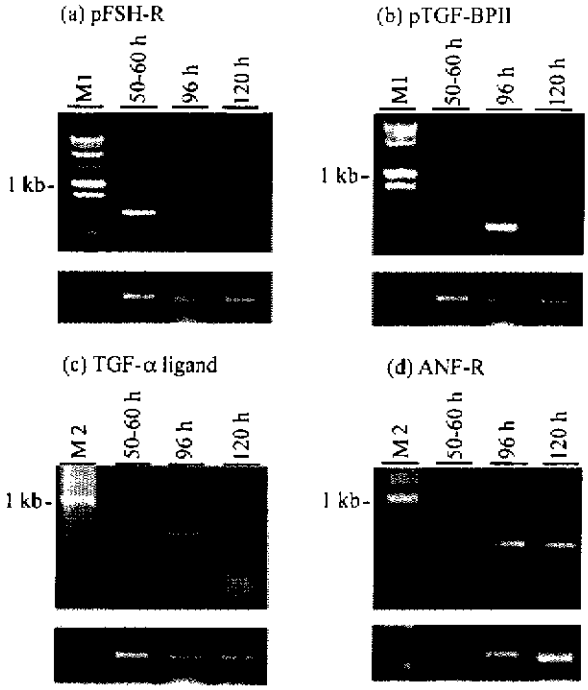

Fig. 3. RT-PCR confirmation of genes identified in DDRT-PCR. M1 indicates 1-kb size marker; M2 indicates 100-bp ladder.

(Fig. 3b,c). The direct RT-PCR results were therefore consistent with the DDRT-PCR data. The temporal expression of pFSH-R, pTGF-BPII, and pTGFα was further confirmed by ribonuclease protection assay using the cloned gene-specific fragments as probes with consistent results (Fig. 4). The data clearly indicate that these temporal-specific transcripts identified by DDRT-PCR are highly specific.

DISCUSSION

In previous work, successful approaches used for the detection and analysis of oviductal factors in a number of species include two dimensional protein analysis, in situ hybridization and RT-PCR (Liang and Pardee, 1992). Some of these studies have also concentrated on the analysis of proteins induced by ovarian steroids such as estrogen (Mahmood et al., 1998). In this study, we have successfully applied the DDRT-PCR technique to the identification of numerous transcripts that are differentially expressed in the epithelium of porcine oviduct carrying various stages of very early embryos. The authenticity of some of the genes thus derived was vigorously confirmed by direct RT-PCR and by ribonuclease protection assay. Not surprisingly, most of the stage-specific genes identified in the oviduct were found to be first expressed at the late 4-cell embryonic stage, coinciding in time with the known in vitro cleavage block of porcine embryo development in culture (Abeydeera et al., 1998). This finding suggests the possibility that some of these oviductal genes may be important to further cleavage of the passing embryos beyond the 4-cell stage both in vivo and in vitro.

The only gene that was found to be expressed in the oviductal epithelium before the 4-cell embryonic stage has turned out to be the porcine FSH-R gene. FSH-R is encoded by a genomic structure containing 10 exons, with the longest exon 10 coding for the transmembrane domain of the receptor (Heckert et al., 1992). At different reproductive physiological stages, FSH-R transcripts of different sizes arising from differential splicing have been detected but the significance of which is unclear (O'Shaughnessy et al., 1996). FSH-R has also been detected in the Sertoli cells of the gonad and the granulosa and luteal cells of the ovary (O'Shaughnessy et al., 1996; Stanton et al., 1996). The detection of FSH-R transcripts in the epithelium of the oviduct in a very narrow time span between the 1-cell and the early 4-cell embryonic stages predicts a possible involvement of members of the FSH family in very early embryo cleavages. The exact role of FSH-R in the oviduct remains to be elucidated.

Transcripts for the porcine TGF-α and the TGF-β-BPII were detected in the oviductal epithelium at the late 4-cell embryonic stage and beyond. TGF-β-BPII associates with TGF-β and is needed for stabilization of TGF-β (Visser and Themen, 1998). Detection of TGF-β-BPII in the oviductal epithelium is thus consistent with reports of an involvement of TGF-β in early embryo development (Potchinsky et al., 1997). Paria and Dey (1990) have further shown that FSH is involved in the regulation of expression of the TGF-β gene. Thus, the

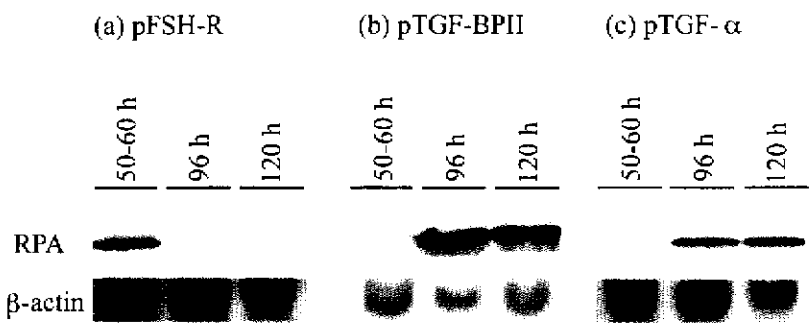

Fig. 4. Ribonuclease protection assay confirmation of genes identified in DDRT-PCR. In the RPA row, positive bands are generated as described in Materials and Methods. The RNA preparations derived from different estrous stages used for RPA analysis were also subjected to Northern hybridization using the β-actin as control as shown.

dual presence of FSH-R at an earlier stage and the TGF-β-BPII at a later stage in the oviduct provides an ideal autocrine loop for cooperative interaction between the oviductal passage and the passing and actively developing embryos. Abundant evidence has shown the involvement of TGF-α in early embryo development (Harada et al., 1997). We now confirm that TGF-α is not only secreted by the developing embryos (Brison et al., 1997) but is also supplemented by the oviduct, further indicating the importance of this growth factor for early embryo development.

We have also identified a porcine astral natriuretic factor receptor-like transcript which was first detected in our DDRT-PCR gels at 96 hr post-hCG injection and when the embryos were at the late 4-cell stage. The span of the transcript isolated showed 84% sequence homology with the pANF-R and is probably a new member of the ANF-R gene family. The atrial natriuretic hormone (ANP) is a cardiac hormone but the expression of its gene and receptors are ubiquitous (Evrard et al., 1999). ANP serves to lower blood pressure and to control electrolyte homeostasis. ANP may also act as a paracrine hormone that influences the function of other endocrine systems. However, it remains to be shown whether the original ANP-R and the oviductal ANP-R-like gene described here are closely related in structure and functions.

In conclusion, this study has clearly demonstrated the feasibility of the application of DDRT-PCR for the identification of transcripts that are differentially expressed by the porcine oviductal epithelium at various developmental stages of early embryo. Numerous novel transcripts have been identified at the late 4-cell stage of the passing embryos and thus opened a way for the elucidation of the involvement of these and other novel oviductal factors in the development of very early embryos before implantation in the uterus.

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Timely Expression of *TGF-α* in Porcine Oviduct Epithelia May Further Modulate the Expression of Embryonic Gene at 4-cell stage of development

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Abstract

The purpose of this study was to identify gene(s) that are expressed in the epithelial cells of pig oviducts at various stages after onset of their oestrus, in order to understand the regulatory mechanism of early development of pig embryos.

Total RNA samples were extracted from oviductal epithellial cells of pigs which had been induced to ovulate by administration of gonadotrophins. These pigs had been served by twice using artificial inseminations during their oestrus. Fallotomy was performed at various times at 50 - 60, 85 and 96 hours post- hCG injection. Each RNA sample was subjected to analysis by method of differential display reverse transciption-polymerase chain reaction (DDRT-PCR). Comparisons were made to specificity fragments of cDNA(s) between each sample amplified after the DDRT-PCR analysis. Experimental results showed 96-1-R5A3, which had both been found to be expressed 96 hours post-hCG injection, were identical to those gene encoding the porcine TGF- α. We, therefore, studied the roles of these growth factors on early embryogenesis using a coculture system. Treatment of porcine early 4-cell embryos coculture with TGF-α (20ng/ml) results in the stimulated one gene expression, and named them TIG-1 (TGF- α inductive gene-1) of a gene homologous to the mouse embryo Mo25 cDNA. The messenger shows homology with the porcine TGF- α gene and is potentially a molecular control factor of maternal oviduct to early mammalian embryogenesis.

Key Words: mRNA differential display, porcine embryo, preimplantation, growth factor, embryogenesis.

Introduction

The development of preimplantation mammalian embryos is interrupted at various stages when they are cultured in chemically defined media. For example, one-cell porcine embryos are often blocked at the four-cell stage has been referred as the "4-cell block". This block is reversible when fertilized eggs are co-cultured with oviduct cells, suggesting that these are providing an environment more favourable to early embryonic development and viability than culture medium. Eyestone and First (1989) suggested that the stage at which blocks occur coincides with the switch from maternal to embryonic genome control and that for the oviduct provides a critical environment. The oocyte thus accumulated a stockpile of gene transcripts and translation products that are use to direct the initial embryogenesis. Eventually they are replaced and superseded by new gene products derived from transcription from the embryonic genome. Thus, successful early embryonic development is dependent on active gene expression by early embryos. In addition, there is now a considerable amount of evidence that demonstrates that the genetic and metabolic activity of the early embryo can be stimulated by a number of factors within the maternal reproductive tract. The major function of the mammalian oviduct and its secretions to provide a biochemical environment that facilitates or is essential for establishment of progeny, including transport of gametes, capacitating of sperm, fertilization, and early cleavage-stage embryonic development (Buhi *et al.*, 1996).

The oviduct has been shown change its secretory processes, fluid volume, and concentrations of ions and organic consituents concomitant with the secretory pattern of ovarian steroids (Leese, 1988; Boice *et al.*, 1990). Several; lines of evidence suggested that oviductal and uterine cells in the maternal reproductive tract synthesize growth factors that enhance embryonic development (Buhi *et al.*, 1993). Growth factors detected in the maternal reproductive tract that have documented stimulatory effects on the conceptus include epidermal growth factor (EGF) (Morishige *et al.*, 1993), transforming growth factor-alpha (TGF- α) (Morishige et al., 1993), insulin and insulin growth factos (IGF-I and II), colony stimulating factor-1 (CSF-1)(Klentzeris, 1997), granulocyte- macrophage colony stimulating factor (GM-CSF)(Fortin et al., 1997) ,Tumer Necrosis Factor- α (Terranova et al., 1995) and leukemia inhibitory factor (LIF) (Senturk and Arici, 1998) etc. Studies with somatic cells have been documented that one of the downstream actions of growth factors is to regulate the activity of a variety of transcription factors and hence modulate gene expression (Treisman, 1994). The growth factors and their interaction with oocytes, early preimplantation embryos, or sperm, however, remain to be determined.

Peptide growth factors are known to regulate cell proliferation and differentiation during mammalian preimplantation development ; embryos express functional growth factor receptors for ligands presenting in the maternal tract and those synthesized by the embryo itself. Although embryos are capable of developing throughout the preimplantation period in vitro in the absence of the maternal tract, their developmant, is always retarded relative to development in vivo, suggesting roles for both embryo-derived and maternal growth factor (Paria and Dey, 1990). For example, exogenous TGF- α increases the rate of development and final cell number, the rate of blasrocoel expansion, and protein synthesis in the ICM for mouse embryos cultured in Whitten's medium (Whitten, 1971). TGF- α also stimulates the expression in the mouse blastocyst of a gene similar to a human HEPG2 gene (Bauer et al., 1993). Moreover,

TGF- α cognate receptor, the epidermal growth factor receptor (EGFr), are expressed from the 4-cell stage and present in both the ICM and TE of the blastocyst (Brison and Schultz, 1996). However, the mechanisms by which mitogenic and differentiating effects of TGF-a on preimplantation embryos are mediated remained unclear.

These results led us to investigate the role of TGF-α/EGFr in the regulation of porcine preimplantation development in culture. Moreover, using the mRNA differential display (Liang and Pardee, 1992), We have identified several genes whose expression is either upregulated or downregulated by TGF-α treatment.

Materials and Methods

Embryo and Tissue Collection

Fallopian tubes from sexually mature crosbred gilts (n=49) were induced to superovulate by injection of 1,500 IU PMSG followed by injection of 1,000 IU hCG. Fallotomy was performed at various times at 50-60, 85, and 96 hours post-injection. Oviductal were rinsed and transferred to a salt solution in a petri dish and they were then washed to remove fat pads and connective tissues. Fallopian tubes were flushed with 0.25% trypsin (GibcoBRL) in PBS medium. Each oviduct was closed at one end with a clip and filled with a trypsin solution and then closed at the other end and incubated at 37℃ for 10 min. The harvested cells were centrifuged at 800 rpm for 5 min in 1.5 ml tube.

To study cooperative interactions among embryos and the role of TGF- α on preimplantation embryo development. The embryos were collected in Dulbecco's Phosphate Buffer saline (DPBS; GibcoBRL) supplemented with calcium and meganesium chloride at 80 hours post-injection. Embryos were cultured with or without 20 ng/ml TGF- α in NCSU-23 medium [NaCl 108.73, KCl 4.78, CaCl2 1.70, MgSO4 · 7H2O 1.19, NaHCO3 25.07, Glucose 5.55, Glutamine 1.00 (mmole l-1)](Petters and Wells,1993), in a humidified atmosphere containing 5% CO2 at 38℃ for 3 hours. The growth factor (TGF- α) were added at the begunning of cultures.

RNA Extraction

Total RNA of epithelium were extracred using RNAzol B (Tel-Test). The RNA was treated with DNase as described by Liang et al. (1993). Early 4-cell embryos were treated with 20 ng/ml TGF-a for 3 hours, after which time RNA was isolated and subjected to mRNA differential display as previously described (Liang and Pardee, 1992). Embryonic RNA was isolated from harvested cells by acid guanidinium thiocyanate-phenol-chloroform extration (Chomczynski and Sacchi, 1987). The denaturing solution was 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0; 0.5% sarcosyl, 0.1 M 2-Mercaptoethanol. Prior to DD-RTPCR, total RNA was first treated with DNase to remove contaminating DNA.

Differential Display RT-PCR(GenHunter)

The labeled products were separated on 6% denaturing polyacrylamide gel at 55 W in IBI base runner 100. Gels were blotted without fixing and exposed to x-ray film for 2 days. The film and original gel were aligned, and bands of interest were then excised with a razorblade. Pieces of gel were placed in 1.5 ml microcentrifuge tubes and soaked in 100ul distilled water for overnight, and were boiled for at least 10 min, Spin in a microcentrifuge, and the DNA was precipitated in the presence of glycogen using 3 M sodium acetate and absolute ethanol. The samples were centrifuged to pellet and washed, and an aliquot of the DNA was reamplified with the same primer pair that was used to generate the original DDRT-PCR band.

The reamplified DNA products was ligated pGEM-T easy Vector (Promega) and competent E. coli DH5a cells were transformed with the ligted vector. Clones were grown up in liquid cultures of LB broth containing 50 ug/ml ampicilin. Positive colonies were screened by PCR using colonies as a template and vector/or DD-RTPCR primers. Plasmid preparations were then prepared for several clones and sequenced using the Dry Terminator Kit (Perkin Elmer) with SP6 and T7 primers.

Double-stranded cDNA was sequenced using the ABI Taq Dyedeoxy Terminator Cycle Sequencing Kit and a model 373A sequencing system as previously described. Sequence database search analysis was carried out using the BLAST sequence alignment algorithm. After confirming that the sequences of several positive clones obtained from the same DDRT-PCR band were identical.

Northern blotting and ribonuclease protection assay (RPA)

Total cytoplasmic RNA (25 ul) obtained from fallotomy of various times at 50-60, 85, and 96 hours post-injection was size fractionated on formaldehyde-denaturing 1% agarose gels and transferred to nylon membrane. After UV crosslinking, blots were hybridized to ^{32}P-labeled probes prepared by random priming using a commercially available kit (Amersham) in 0.5 M Na_2HPO4, 1 mM EDTA, 7% SDS, and 0.1 mg/ml Herring sperm DNA at 60℃. Fallowing hybridization, the blots were washed in 0.1X SSC containing 1% SDS at 65℃ for 10-15 min until the nonspecific background radioactivity was not detected, as determined by a Geiger counter. The blot was then exposed to Kodak X-ray film at -80℃. RPA was performed using a kit (RPAII; Ambion).

In situ Hybridization

In situ hybridization used Boehringer Mannheim PCR DIG Probe synthesis/DIG Nucleic Acid Detection kit (Indianapolis, IN). Embryos were fixed on poly-L-lysine coated microsphere slides. Hybridization solution which contains 60% deionized formamide, 300 mM NaCl, 30 mM sodium citrate, 10 mM EDTA, 25 mM NaH_2PO_4 (pH 7.4), 5% dextran sulfate, and 250 ng/ul sheared salmon sperm DNA, at 37℃ for 16 hours. Detection of the hybridized DIG-labeled probe was conducted by incubating the slides in a moist chamber for 45 min with 1: 500 dilution of anti-DIG-fluorescein in blocking solution (100 mM Tris-HCl; pH 7.5, 150 mM NaCl, 0.5% (w/v) blocking reagent) and then subjected to examination by a light microscope.

GenBank searching

The cDNA sequences from the cloned plasmids for the responsive genes were analyzed using the BLAST program searching GenBank (National Center Biotechnology Information).

Results

Timely expression of TGF-a in Porcine oviductal epithelium cell

Total of 10 arbitrary primers were screened against all four T11VN primers. A Differential mRNA display is shown in figure 1, using R5A3 primer (Table.1) set and oviductal epithelium cell of different post-hCG times. Arrow indicate expressed specific genes fragments found after mRNA differential display. Experimental results showed 96-1-R5A3, which had both been found to be expressed 96 hours post-hCG injection. Individual bands were cut out and reamplified by PCR and further analyzed using agarose gel. Reamplification of each specific gene fragments was carried out with the primer set identical to that used during the mRNA differential display. The PCR products were cloned and the plasmids containing inserts were used as probes for Northern blotting or for DNA sequencing. Figure 2 summarizes Northern blotting and RPA datas. Using the BLAST program to search GenBank, reveal that the clone-96-1-R5A3 were identical to porcine TGF- α (data not show). RT-PCR analysis against mRNAs from epithelium cells of porcine oviducts using primer sets designed according to the cDNA sequence of TGF- α (data not show). The result confirm that the transcription of TGF- α gene in epithelium cells of porcine oviduct, did occur perticularly at 96 h post the hCG injection.

TGF- α modulation of gene expression in the preimplantation embryos

Total of 3 arbitrary 10-mer primers were screened against all two T11V primers but only H-AP7+H-T11G (Table.1) set have differentiation. Arrow indicate expressed specific genes fragments found after mRNA differential display (Fig.3). In porcine 4-cell embryos TGF- α induces an depentent gene, and named them TIG-1 (TGF-α inductive gene-1), within 3 hours. The mRNA differential display method circumvents this obstacle in analyzing gene expresson in the preimplantation porcine embryo, since the pattern of gene expression is represented as a amplicon that were eluted from the sequencing gel, amplified, cloned, and sequenced (Fig.4). Each contains flanking primer sequences idetical to those used in the differential display (underlined). One excised bands were successfully recovered by the boiling technique after a single 35-cycle PCR reamplification. Using the BLAST program to search GenBank, reveal that the clone-TIG-1 were identical to mouse Mo25 (Fig.5). TIG-1 clone was 89% homologus with mouse embryo Mo25 gene. However, at the extreme 3' end the homology is found at lost 331 bp sequence. Wether this results may be from PCR mispriming, species variation, or tissue specificity, differential splicing. RT product was amplified by 35-cycle of PCR with oligonucleotide primers (Table.1) specific for pMo25 cDNA (Fig.6). The pMo25 mRNA in total RNA was continuous expression at between 4-cell (add TGF- α 20 ng/ml) and blastocyste stages. In situ hybridization of porcine embryos with the DIG-1 (pMo25) probe revealed the presence of pMo25 transcripts in the early 4-cell embryo which coculture with TGF-α (20 ng/ml) (Fig.7).

Discussion

In mammals, the early embryo is transported from the oviduct to the uterus as cleavage progresses. Previous studies, using two-dimensional PAGE under reducing conditions, have revealed a family of three isoelectric and molecular weight variants of estrogen-associated and estrogen-dependent glycoproteins synthesized and secreted by porcine oviductal epithelium during proestrus, estrus, and metestrus (Buhi *et al.*, 1990; Buhi *et al.*, 1993). In this study, we have shown that TGF-α were synthesized and expressed specifically at menstrual stages in porcine oviduct epithelium, and that these growth factors might play an important role in early embryogenesis. Our result was consistent with a previous report that TGF-α was localized to epithelial cells in human and porcine oviduct (Morishige *et al.*, 1993; Kennedy *et al.*, 1994).

It may be interesting to note the fact that oviductal secretory proteins are de novo synthesized and secreted specifically at the estrous stage (Kapur and Johnson, 1985) or induced by estrogen (Verhage *et al.*, 1988). Estrogen receptor is expressed in human oviduct epithelium (Pollow *et al.*, 1981). The estrogen-dependent expression of EGF and TGF- α is demostrated in mouse uterine epithelium (Bates *et al.*, 1988); the estrogen-induced TGF- α secreton is known in mammary cancer cells (Raja *et al.*, 1991); and an estrogen-responsive element is present in the upstream region of TGF- α gene (Saeki *et al.*, 1988). Relations between these oviductal secretory proteins and TGF- α remain to be clarified. A possibility is that TGF- α might induce the secretion of oviductal proteins, and another might be that some of these secretory proteins are TGF- α (Wollenhaupt *et al.*, 1997). Although oviductal secretory proteins reported have rather large molecular weights (Buhi *et al.*, 1989), multiple TGF-αs of different molecular weights are known to be secreted (Derynck *et al.*, 1984).

The transition from maternally derived transcripts to zygotic genome derived transcripts occurs during the 4-cell stage (Telford *et al.*, 1990). Many of the zygotic transcripts represent sequences already found in the maternal RNA , most of which are derived from house-keeping genes (Giebelhaus *et al.*, 1983; Bachvarova *et al.*, 1989). However, the first differentiation and blastocyst formation process require the expression of a number of zygotic genes whose product cannot be supplied maternally. Among these are genes which are required for cytokeration network formation in the trophectoderm. and some growth factors which may function in cell proliferation and differentiation (Parie and Day, 1990). It is reported that oviduct epithelium promotes embryogensis in many mammalian species (Bavister, 1987; Gandolfi and Moor, 1987), but tubal factors involved in this process have not been identified yet. The stage-specific expression of TGF- α in porcine oviduct epithelium suggested the possible important biological roles of these growth factors.

The amino acid sequence of TGF- α has 35% identity with the epidermal growth factor (EGF) sequence, which includes the conservation of all six cycteine residues (Derynck *et al.*, 1984). Two-dimensional NMR studies provide evidence that in solution the two proteins have very similar polypeptide chain folds (Montelione *et al.*, 1989). TGF- α has been shown to compete with EGF for binding to the EGF receptor (Carpenter *et al.*, 1983). The fact that EGF receptor is expressed from early stage embryos (Wiley *et al.*, 1992) may suggest the possible involvement of these growth factors in the promotive effects of oviduct epithelial cells on the embryo development in a paracrine manner. Nonetheless, TGf- α and EGF both stimulate growth and/or protein synthesis in the preimplantation mouse embryo (Paria and Dey, 1990).

However, the mechanisms by which mitogenic and differentiating effects of TGF-a on preimplantation embryos are mediated are unclear. We, therefore, studied the roles of these growth factors on early embryogenesis using a coculture system. The principal advantage of the mRNA differential display method to analyze modulation of gene expression by growth factors in the preimplantation embryo is that the modulated genes can be identified. In this regard, We report the identification of one gene whose expression is modulated by TGF- α. Computer analysis revealed that it is an TIG-1 sequence sharing a partial homology to the mouse mMo25 gene (Miyamoto et al., 1993). TIG-1 clone was 89% homologous with mouse Mo25 gene. On the other hand, the deduced amino acid sequence of the Drosophila Mo25 cDNA had 69.3% identity with with Mo25 (Nozaki et al., 1996).

Induction of Mo25 gene by TGF- α was never observed by others. The levels of mMo25 mRNA in the embryo gradually increased during preimplantation development (Miyamoto *et al.*, 1993). These data indicate that transcription of the mMo25 gene is initiate at the 2-cell stage, and slightly decreased after 14 days of gestaton. we have also shown that pMo25 were continually expressed for late 4-cell ~ Blastocyst stage of porcine embryos (Figure 5). The role of mMo25 during development and in adult remains unclear. The hydrophibicity analysis showed that the mMo25 protein has neither a signal sequence nor a transmembrane domain, the mMo25 protein probaly localizes in the cytoplasm. The deduced amino acid structure of mMo25 indicats that the protein binds to Ca2+, similarity to the EF hand consensus sequence. (Tufty and Kretsinger, 1975; Miyamoto *et al.*, 1993). Calcium is a universally employed cytosolic messenger in eukaryotic cells. Most of the proteins that bind signaling calcium are members of the calmodulin superfamily and share two or more helix-loop-helix motifs known as EF-hands (Trave *et al.*, 1995). The EF hand proteins, like calmodulin and S100 proteins, are considered to exert Ca2+ -dependent actions in the nucleus or the cytoplasm. This protein is involved in a general signal transduction system such as proliferation and differentiation (Hidaka and Watanabe, 1993). On other hand, Ca2+ storage proteins in the endoplasmic or sarcoplasmic reticulum provide the high Ca2+ capacity of the Ca2+ store sites, which regulate intracellular Ca2+ distribution. The variety and complexity of Ca2+ signaling is resulted from the cooperative actions of specific Ca2+ binding proteins (Niki *et al.*, 1996). PI-PLC isozyme employs a variety of modules (PH domain, EF-domain, SH2 domain, SH3 domain and C2 domain) that are common in proteins involved in signal transduction to reversibly interact with membranes and protein components of the signaling pathways (Williams and Katan, 1996). This study describes biochemical properties of intracellular Ca2+ binding proteins and their proposed roles in mediating $Ca2^{+}$ signaling.

In summary, the results presented here document the TGF- α can modulate gene (pMo25) expression in the preimplantation pocine embryo, and pMo25 may play a role in signaling pathways. In addition, characterization of the products of newly identified TGF- α induced genes may shed more light on the biological actions of TGF- α.

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Figure legends

Fig. 1. Differential display using R5 and A3 primer set. Three samples from oviductal epithelium of various times at 50 - 60, 85 and 96 hours post- hCG injection. Result showed 96-1-R5A3, which had both been found to be expressed 96 hours post-hCG injection. The 96-1-R5A3 fragment was marked by arrowheads.

Fig. 2. Northern and RPA analysis using cDNA probes derived from reamplification of melecules. Detection of transcripts in RNA from oviductal epithelium of various times at 50 - 60, 85 and 96 hours post- hCG injection. Each lane contains 25 ug of epithelium RNA. Blot probed with β-actin and 96-1-R5A3.

Fig. 3. Differential display of mRNAs from early embryos using primer aet of H-AP-7 and H-T11G. Arrow indicate expressed specific gene fragment found after TGF- α treatment (in vitro coculture 3 hours), and named them TIG-1 (TGF-α inductive gene-1).

Fig. 4. Putative nucleotide sequence of the porcine TIG-1 clone.

Fig. 5. Computer analysis of the TIG-1 and its partial homologous sequence mouse Mo25 (mMo25). The FASTA program was used to search GenBank and EMBL data bases and a partial sequence mMo25 to share 89% identify with the TIG-1 in 284 bp overlap.

Fig. 6. Detection of transcripts in RNA from preimplantation embryos by RT-PCR. RNA from 10 ealy 4-cell , 6 eight-cell, 5 16- ~ morular-cell, and 4 Blastocyst embryo was reverse transcribed with oligo-dT, and the RT products was amplified by 35-cycle of PCR with oligonucleotide primers for TIG-1 (pMo25).

Fig. 7. Distribution of pMo25 transcripts in early 4-cell porcine embryo with *In situ* hybridization. Arrow indicate expressed pMo25 mRNA. Early 4-cell embryos were treated with 20 ng/ml TGF- α for 3 hours.

Table 1. Primers used in mRNA differential display, Northern blotting, and RT-PCR of interested genes.

Primers used	Nucletide sequences	Remarks
R5	5'-GATCTAACGG-3'	
A3	5'-TTTTTTTTTTTMA	M=A, C, or G.
H-AP7	5'-AAGCTTAACGAGG-3'	
H-T11G	5'-AAGCTTTTTTTTTTG-3'	
pMo25 (+)	5'-CTTGAGACTCGTAAACCTG-3'	
pMo25 (-)	5'-ATAACATTCCTCTGCTGTAC-3'	
pb-actin (−)	5'-TGAACCCTAAGGCCAACCGTG-3'	
pb-actin (-)	5'-GCTCATAGCTCTTCTCCAGGG-3'	

Fig. 1

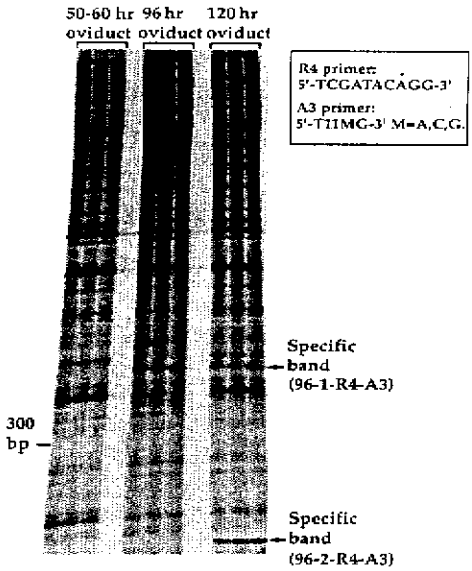
50-60 hr oviduct
96 hr oviduct
120 hr oviduct
R4 primer:
5'-TCGATACAGG-3'
A3 primer:
5'-T11MG-3' M=A,C,G.
Specific band (96-1-R4-A3)
300 bp
Specific band (96-2-R4-A3)

Fig. 2

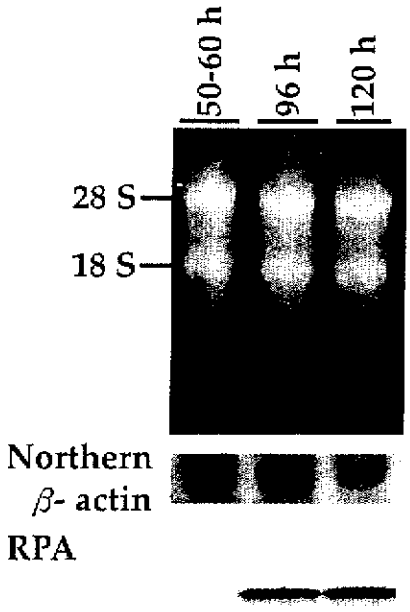

Fig. 3

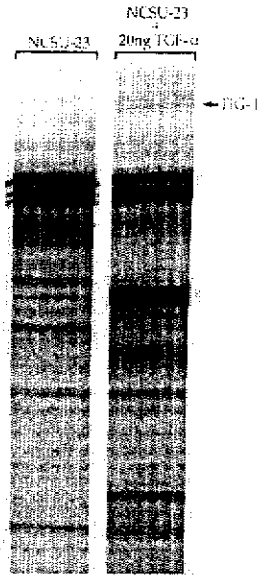

Fig. 4

AAGCTTAACGAGGTCATAAGGAAAATGTATATATGCTTTTCACAGCTTTCT
AGAATTTTTTGGACATTTGGTTTTTCTTGAGACTCGTAAACCTGGAATATG
TTGAAGGGTATTTGTTCATCTTACTTTTTGAAGGTATTTTAAAACAGTAGA
GCTAGCAACGACACCTCGCATTTCATTTCAACGATGCTTACAGGTTTCTTT
TGTATATAATTCTTAGAATGCTCATTTCTTTTAAATGATTTAATTTGTACAG
CAGAGGAATGTTATTGTATTAGTATGTAACTATTACCTAATATTGAGTTTT
TGCAAAAAAAAAAAAGCTT---326 bp

Fig.5

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gb|S51858|S51858 Mo25 gene [mice, embryos, mRNA, 2322 nt] Length = 2322

 Score =  280 bits (141), Expect = 9e-74
 Identities = 254/284 (89%), Positives = 254/284 (89%), Gaps = 7/284 (2%)

Query: 11   aggtcataaggaaaatgtatatatgctttt-cacagctttctagaacttttgacatttg 69
            ||| |||||| ||| ||||||||||||||| |||||||||||||| ||| |||||||||
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            ||||| ||||| ||| |||||||||||| || |||||||||| ||| | || ||||||||
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Query: 130  tgaaggtattttaaaacagtagagctagcaacgacacctcgcatttcatttcaacgatgc 189
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Query: 190  ttacaggtttcttttgtatataattcttagaatgctcatttcttttaaatgatttaatttt 249
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Query: 250  gtacagcagaggaatgttattgtattagtatgtaactattacct 293
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Fig. 6

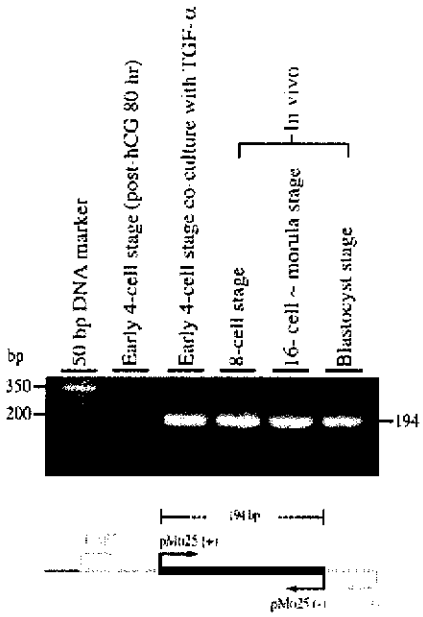
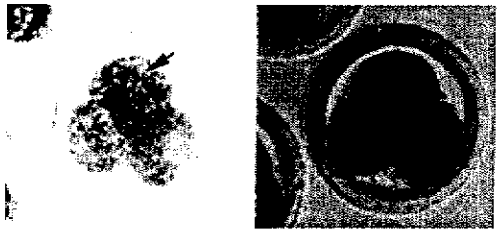

50 bp DNA marker
Early 4-cell stage (post-hCG 80 hr)
Early 4-cell stage co-culture with TGF-α
8-cell stage
16- cell ~ morula stage
Blastocyst stage
In vivo
bp
350
200
194
194 bp
pMo25 (+)
pMo25 (-)

Fig. 7



Large-Scale Identification of Genes Differentially Expressed in Mouse Pre-implantation Embryos

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Running title: Differential Expression of Mouse Genes in Pre-implantation Embryos

Key words: pre-implantation; mRNA differential display; maternal gene; zygotic activated gene

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Abstract

In this study, the expression of mouse maternal and zygotic genes were compared and identified by using the strategy based on mRNA differential display against to samples from mouse freshly ovulated oocytes and 8-cell embryos, respectively. To date, we have used 24 combinations of arbitrary with anchor primers, and obtained numerous differences in the pattern of gene expression between maternal and zygotic genes. A total of 20 differentially expressed transcripts were identified between maternal and zygotic activated genes. Thirteen of such genes were shown by sequence analysis to be the mouse *U2 snRNP*, *Suil*, *Tim23*, *polymerase gamma*, *NIPI-like protein*, *ribosomal protein L35a*, *L37a*, *L3*, *calmodulin synthase*, *GTPase activating protein*, *ATP synthase subunit F*, and human *CGI-65*, *splicing factor 3b*. The remaining seven transcripts detected by mRNA differential display were novel. The temporal expression of known and novel genes was further confirmed by reverse Northern blotting with consistent results. Our results demonstrate that mRNA differential display is a feasible approach for rapid identification of genes that are differentially expressed in pre-implantation embryos. These genes may be play important roles for unhindered the secret of how pre-implantation embryos develop.

Introduction

Progression of fertilized mammalian oocytes through early cleavage stages, blastocyst formation, and implantation depends on successfully genetic implantation, developmental programs, and triumphant interaction of the pre-implantation embryo with its environment (Osborne and Richter 1997). In this period, maternal mRNA determines the first cleavage of mouse embryo, but before the second cleavage a transition from dependence on maternal mRNA to embryonic mRNA takes place and from then the expression of embryonic genes regulate development of embryo. Although after the two-cell stage maternal mRNA is rapidly degraded, the products of these maternal messengers may be detected for much longer periods (Vassalli and Stutz, 1995). Most of these products will be used within the early cleavage divisions, at which point the pre-implantation embryo supports its own development by transcripted its genome (Brinsko *et al.*, 1995). Molecular changes of these variant transcripts have been reviewed (Seydoux, 1996; Hoffert *et al.*, 1997; Stebbins-Boaz and Richter 1997; Niemann and Wrenzycki, 2000) repeatedly and will not be discussed in detail here. However, how a maternally inheritance can direct effect the gene expression is a conundrum. In addition, how zygotic mRNAs be activated after transition from maternal mRNAs to embryonic mRNAs remains large unknown.

Detail analyses of gene expression during mammalian embryogenesis will be valuable for understanding basic cellular and molecular mechanisms of gene regulation, development of better embryo culture systems and better strategies for transgenic and cloning studies. The toughest technique in analyzing differential gene expression of pre-implantation in mouse embryo is the paucity of biological material. When the technique of mRNA differential display of eukaryotic mRNA was first published in 1992, it was presented as a fast and reliable method for comparing differential gene expression of two or more cell populations or tissues. In this study, mRNA differential display was as the first step to find some factors that is important in the development of pre-implantation embryos. In additionally some known genes and seven novel genes have been identified, and they express at various stages of early development in pre-implantation embryo.

General Materials and Methods

Embryo Collection

All reagents were purchased from Sigma Co., unless otherwise stated. Mice used for mRNA differential display and reverse Northern slot blotting analyses were the ICR strain. Embryos were obtained as described (Clarke *et al.*, 1992). Briefly, ICR were super-ovulated by an injection of 7.5 IU of pregnant mares' serum gonadotrophin (PMSG) followed 48 h later by 5 IU of human chorionic gonadotrophin (hCG) and caged individually with ICR males overnight. The oocytes and 8-cell embryos were collected from either the oviduct or the uteri at 20 and 75 hours post-hCG, respectively.

RNA Isolation of Embryos

Total RNA was isolated from mouse embryos at different development stages (oocyte and 8-cell embryos). Tree replicates of embryos collected at each stage were carried out. Total RNA preparations were modified from Chomocyznski and Sacchi (1987). Briefly, the pool of embryos was mixed by vortex in 100 µl of denature buffer (4.5 M guanidinium thiocyanate; 0.75 M sodium citrate, pH 7.0; 10 % sacosyl and -mercaptoethanol), then frozen in liquid nitrogen and stored at –80 °C. After thawing, total RNA was extracted with phenol/chloroform and precipitated with glycogen (10 mg/ml) and 2.5-volume ethanol. After drying, the total RNA was reconstituted in 12 µl of DEPC water containing Rnasin™ (Promega), and stored at –80 °C until the RT-PCR.

mRNA Differential Display

Total RNA was isolated from embryos at different stages of development and subjected to mRNA differential display as previously described (Chang *et al.*, 2000). Aliquots of embryonic total RNA were reverse-transcribed with 300 units of superscript II reverse transcriptase (Gibco/BRL) in a buffer containing 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2, 10 µM DTT, 1 µM anchor primer (Table 1; GeneHunter) and 250 µM each of dATP, dCTP, dGTP and dTTP for 70 min at 42 °C. After heating at 85 °C for 5 min to inactivate the reverse transcriptase, 2 µl of reverse-transcribed reaction mixtures were added to 18 µl of PCR labeling buffer containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 1.5 mM of MgCl2, 25 µM each of dATP, dCTP, dGTP, and dTTP, 1 µM anchor primer, 0.5 µM random primer (Table.1; GeneHunter), 10 µCi ^{33}[P]-dCTP

and 1 unit of DNA *Taq* polymerase (TaKaRa). The cycling parameters for PCR were 95 °C for 30 sec, 40 °C for 2 min and 72 °C for 45 sec, followed by 72 °C for 5 min. The radiolabelled DNAs were electrophoreses in 6% denaturing polyacryl-amide gels and after drying, the gels were exposed to BAS-1500 (FujiFilm). Two μl of the eluted cDNAs were re-amplified in a 30-μl reaction volume using the same set of primers and PCR conditions as used for the mRNA differential display.

Reverse-Northern slot blotting

To identify the cDNAs that were differential expressed in pre-implantation, we used the reverse Northern slot blotting technique (Zhang *et al.*, 1996). For reverse Northern slot blotting experiments, ampicilline resistant colonies were randomly picked from each plate and lysed by boiling in 50 μl of lysis buffer (0.1 % Tween 20 in TE buffer, pH 8.5). The cloned cDNA fragments were amplified using primers flanking the cloning site of the vector. Each PCR product was slot-blotted onto nylon membranes by microfiltration system. The membranes were UV-cross-linked and probed with total [^{32}P]cDNA. The [^{32}P]cDNAprobes were prepared by reverse transcription of total RNA obtained from 80 blastomeres, in the presence of [α-^{32}P]dCTP (3000 Ci/mmol).

Results

In classical mRNA differential display, reverse transcription is primed with either an oligo-dT or an arbitrary primer, then an arbitrary primer (13 mers; H-AP1~8) is used in conjunction with the reverse transcription primer to amplify the cDNA fragment. The cDNA fragments are then separated on a polyacrylamide gel. Differences in gene expression are visualized by the presence or absence of bands on the gel, and quantitative differences in the intensity of these bands. On the basis of method, a portion of a differential display gel comparing oocytes and 8-cell embryos RNA is shown in figure 1 and 2. Comparison of banding patterns revealed difference in gene expression. These bands represent cDNA fragments up to 19 genes that showed in development regulation of ovulated oocyte and pre-implantation embryos. Theses bands were eluted from the differential display gel and subjected to a second time PCR, using the same primers as before. These genes of differential expression were re-amplification and sub-cloned into the plasmid vector pGEMT-easy. We first selected the abundant cDNAs present in three from oocytes and sixteen from 8-cell stage. Representation of transcripts MG1, MG2, and MG3 were stable during the oogenesis but transcripts ZAG1 to ZAG18 undergo a sudden increase at 8-cell stage, we refer to maternal genes (MG) and zygotic activated genes (ZAG), respectively. On a comprehensive search of the GenBank database, sequences of 13 cDNA clones matched perfectly with mouse *U2 snRNP*, *Suil*, *Tim23*, *polymerase gamma*, *NIPI-like protein*, *ribosomal protein L35a*, *L37a*, *L3*, *calmodulin synthase*, *GTPase activating protein* and *ATP synthase subunit F* (Table.2). Another two cDNA clones showed high (88% and 93%) sequence homology with human *CGI-65* and *splicing factor 3b*. The remaining 7 transcripts detected by mRNA differential display were products of novel mouse genes.

The major drawback of the mRNA differential display is that it leads to clones many false positives; therefore it needs a high throughput method to screen the true differentially expressed cDNAs. In order to develop a new version for analyzing mRNA, we combined advantages from previous described method to point that the reverse-Northern slot blotting is fast, cheap, and reliable. Reverse Northern analysis showed that MG1, MG2 and MG3 clones obtained from the oocyte exhibited higher signal when hybridized with total cDNA from oocyte than when hybridized with total cDNA from 8-cell embryos (Figure.3). This result indicates that these clones correspond

to these genes that were expressed at oocyte maturation, therefore, these genes that represent maternal genes. The novel clones of ZAG3, ZAG6 and ZAG17, whereas, was showed as zygotic activation genes. Transcript ZAG14 was failed to detected any signal at period of maternal-embryonic transition. The nucleotide sequences of novel clones are showed in figure 4. Clones of differential expression have flanking primer sequences identically to those used in the differential display and have putative nuclear polyadenylation signals or contain cytoplasmic polyadenylation elements (CPE).

Discussion

In this study, we have successfully applied the mRNA differential display technique to the identification of numerous transcripts that were differentially expressed in the very early development of oocytes or the embryos. The authenticity of the genes were vigorously confirmed by reverse Northern slot blotting. No surprisingly, most of the temporal-specific genes were identified in pre-implantation embryos.

Three of these mRNAs, ZAG11, ZAG12, and ZAG13, are related to protein biosynthesis and encode ribosomal proteins L35a (MMY16430), L37a (MM_009084), and L3 (7305440), respectively. The zygotic activation of genes encoding ribosomal proteins at 8-cell and late stage was in perfect agreement with the observation of active synthesis of ribosomal proteins at the morula stage (Karp *et al.*, 1974). This increase in transcription at the 8-16 cell stage has already been described for ribosomal protein S21 (Henrion *et al.*, 1997). It immediately precedes ribosomal RNA syntheses; moreover, it gained at the morula stage in the rabbit embryo and the increase in the number of cytoplasmic ribosomes also observed at morula stage (Manes, 1971). This accumulation of new ribosomes was concomitant to a dramatic increase in the rate of incorporation of amino acids into proteins between the 8-16 cell and morula stages. In mouse embryo, ribosomal mRNAs were transcribed at ZGA and association with their translation closely, which was not the case in some other eukaryotic cells where translation control of these RNAs plays a central role in regulating ribosome biosynthesis (Taylor and Piko, 1992). Transcript ZAG1 showed homologies with mouse U2 snRNP (AF230356). The abundance and localization of snRNAs and snRNPs involved in processing and splicing of pre-mRNA has been studied during early mouse embryogenesis. The amount of U1, U2, U4, U5 and U6 RNA remained relatively constant between the postovulatory oocyte and 2-cell stage but increases three- to ten-times increased in quantity between the 2-cell and blastocyst stages (Dean *et al.*, 1989). Localization of ZAG1 was examined by *in situ* hybridization with U1, U2 and U6 riboprobes and immunofluorescence microscopy using a monoclonal antibody to snRNP antigens. Transcript ZAG7 was homologous to human splicing factor 3b subunit 1 (SF3b; NM_012433). Human SAP 49, a subunit of the multimeric splicing factor 3b, contains two RNA recognition motifs (RRMs) and binds another SF3b subunit called SAP 145, whose yeast homologue is CUS1. Igel *et al.* (1998)

found mutations that alter putative RNA-binding residues of HSH49 RRM were lethal *in vivo*. These results demonstrated, for the first time, that an essential snRNP and splicing factor is regulated in a complex forming during pre-implantation embryo, but functions remain unknown at this period. The snRNAs, snRNPs and SFs were primarily localized to the germinal vesicle in the pre-ovulatory oocyte but were released and diluted within the cytoplasm of oocyte during germinal vesicle breakdown and meiotic maturation. They subsequently re-localized to both pronuclei following fertilization and the nuclei of the 2-cell embryo following the first cleavage division. Since the amount of snRNA and SFs are constant during the first cleavage, the small amount of pre-mRNA that was synthesized at the time of transcriptional activation in the 2-cell embryo may be spliced and processed by snRNPs and SFs of maternal origin as well as *Drosophila* (Meyer *et al.*, 1998). The nucleotide sequence of ZAG2 was 97% identical to the recently discovered translation initiation factor Suil of mouse (AF129888), suggesting that the two proteins are homologs and have similar functions as well as family. The human *Suil* gene product is a 113 amino-acid polypeptide similar to proteins known from yeast, rice, mosquito, and Methanococcus (Fields and Adams, 1994). The functions of these putative proteins are unknown, but their homology to Suil suggests that they represent an important component of the initiation complex in eukaryotic translation. Transcript ZAG4 displayed high homology to human (88%) CGI-65 (AF151823) cDNA sequence. While, the function of these proteins remains unknown, their transcripts common expressed human, mouse, and *C-elegans*. Transcript ZAG-5 showed homologies with the mouse translocate Tim23 (AB021122). Import of nuclear-encoded mitochondrial preproteins was mediated by a general translocase in the outer membrane, the TOM complex, and by two distinct translocases in the mitochondrial inner membrane, TIM23 complex and TIM22 complex. Both TIM complexes cooperate with the TOM complex but facilitate import of different precursor proteins. Precursors with a N-terminal pre-sequence are imported via the TIM23 complex, whereas mitochondrial carrier proteins require the TIM22 complex for inserting preproteins into inner membrane (Bauer *et al.*, 1999). Lohret *et al.* (1997) results showed that Tim23 is required for normal MCC activity and raise the possibility of that precursors are translocated across the inner membrane through the pores of multiple conductance channels. Besides, the functions of transcripts MG1, MG2, ZAG3, ZAG6, ZAG8, ZAG14, and ZAG17 remained unknown since no sequence homology or EST clones was found.

In conclusion, this study has clearly demonstrated the feasibility of the application of mRNA differential display for identification of transcripts that are differentially expressed by mouse embryos at various developmental stages. Numerous differentially transcripts have been identified between maternal and zygotic genes and thus established a way for elucidating of involvement of novel genes very early stage of embryos before implantation. However, the potential roles of novel clones and several undeniable genes in mouse embryonic physiology require elusive. With the fragments of these clones in hand, our next step is to characterize these clones during oogenesis and embryogenesis processes.

Acknowledgments

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Table 1. Primers for mRNA differential display analysis

Primer names	Sequences (5'-3')
H-T_{11}G	AAGCTTTTTTTTTTG
H-T_{11}A	AAGCTTTTTTTTTTA
H-T_{11}C	AAGCTTTTTTTTTTA
H-AP1	AAGCTTACGGGGT
H-AP2	AAGCTTTGCACCG
H-AP3	AAGCTTGAAGCGG
H-AP4	AAGCTTCTCCGGA
H-AP5	AAGCTTGGCTGAC
H-AP6	AAGCTTCGGTCCT
H-AP7	AAGCTTATGCCCG
H-AP8	AAGCTTGCGGTGA

Table 2. Isolation and Identification of Genes Differentially Expressed in Mouse Early Embryogenesis

Clone no.	Size (nt)	Flanking primers	Poly(A) Major: AATAAA	Character and nucleotide homology
MG1	574	Yes	Major	Human EST: BE3800895, 522/522 nt (100%)
MG2	584	Yes	Major	Mouse EST: AW546958, 562/562 nt (100%)
MG3	504	Yes	Major	No candidate matched
ZAG1	494	Yes	Major	Mouse U2 snRNP: AF230356, 362/362 nt (100%)
ZAG2	335	Yes	Major	Mouse Suil: AF129888, 255/261 nt (100%)
ZAG3	311	Yes	Major	No candidate matched
ZAG4	622	Yes	Major	Human CGI-65: AF151823, 201/266 nt (88%)
ZAG5	587	Yes	Major	Mouse Tim23: AB021122, 570/574 nt (99%)
ZAG6	308	Yes	Major	No candidate matched
ZAG7	486	Yes	Major	Human splicing factor 3b: NM_012433, 153/163 nt (93%)
ZAG8	272	Yes	Minor TTTATT	Mouse EST: AV374359, 209/216 (96%)
ZAG9	371	Yes	Major	Mouse DNA Polymerase Gamma, AI42960, 304/304 (100%)
ZAG10	309	Yes	Minor ATTAAA	Mouse NIPI-like protein NIPIL (A3), MMU67328, 267/297 nt (89%)
ZAG11	335	Yes	Major	Mouse ribosomal protein L35a: MMY16430, 316/316 (100%)
ZAG12	290	Yes	Major	Mouse ribosomal protein L37a: MM_009084, 268/268 (100%)
ZAG13	361	Yes	Major	Mouse ribosomal protein L3: 7305440, 361/361 (100%)
ZAG14	478	Yes	Unknown	Mouse EST: 8832512, 478/478 nt (100%)
ZAG15	408	Yes	Major	Mouse calmodulin synthesis: 192364, 408/408 nt (100%)
ZAG16	580	Yes	Major	Mouse Rac GTPase-activating protein: AF079974, 580/580 nt (100%)
ZAG17	378	Yes	Unknown	Mouse EST: AU046182, 378/378 nt (100%)
ZAG18	320	Yes	Major	Mouse ATP synthase subunit F: 7949004, 320/320 nt (100%)

Figure legends

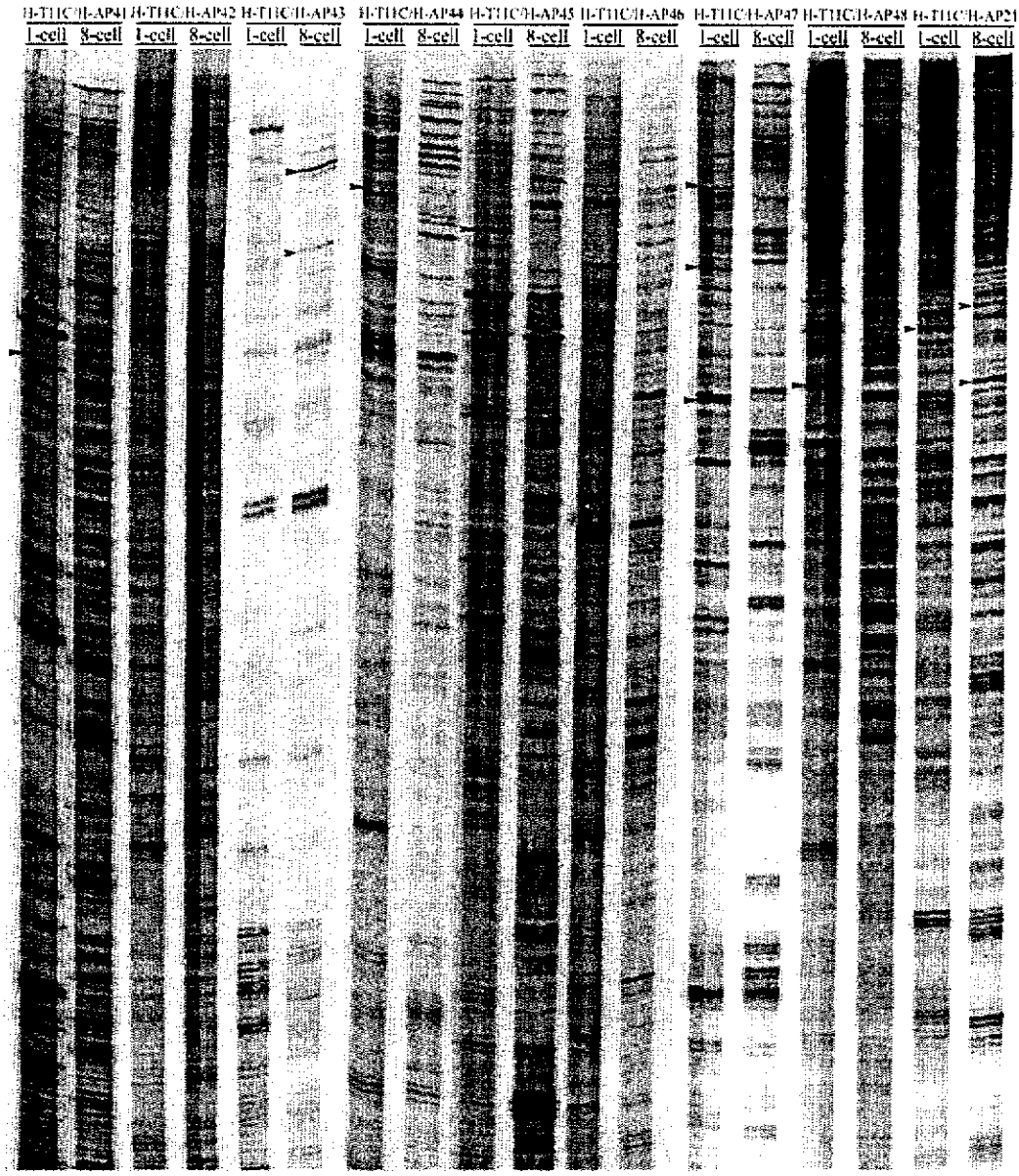
Fig. 1. Differential display of mouse maternal and zygotic RNA with primers 3'-T11MC and 5'-APs. Duplicate PCR reactions were prepared from each reverse transcription reaction and were run in adjacent acrylamide gel (6%) lanes. Arrowheads indicate examples of conserved cDNA bands selected due to stage-specific expression. Use a razor blade to cut the bands and elute the fragments from the dried gel, add 100 ul ddH_2O, and heat at 90 °C for 10 min. Spin for 5 min to pellet, and remove the supernatant to fresh tube. Precipitate and 5 ul eluted fragment for re-amplification in 50 ul reaction tube.

Fig. 2. Differential display of mouse maternal and zygotic RNA with primers 3'-T11MA and 5'-APs. Arrowheads indicate examples of conserved cDNA bands selected due to stage-specific expression.

Fig. 3. Re-confirmed differentially expression of novel clones from mRNA differential display using Reverse-Northern slot blot. One of the membranes (1C) was hybridized with ^{32}P-labeled cDNA pool from fertilized oocyte. The other membrane (8C) was hybridized with ^{32}P-labeled total cDNA from 8-cell embryos. The blot was also hybridized with ^{32}P -actin as control at each sample.

Fig. 4. Nucleotide sequences of MG1 (maternal gene1), MG2, MG3 (C44M1), ZAG3 (zygotic activation gnee3), ZAG6, ZAG14, and ZAG17 cDNA fragments. The underlined sequences are primers used in differential gene display analysis.

Fig. 1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8-cell
H-T1IC/H-AP42
1-cell
8-cell
H-T1IC/H-AP43
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H-T1IC/H-AP44
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Fig. 2

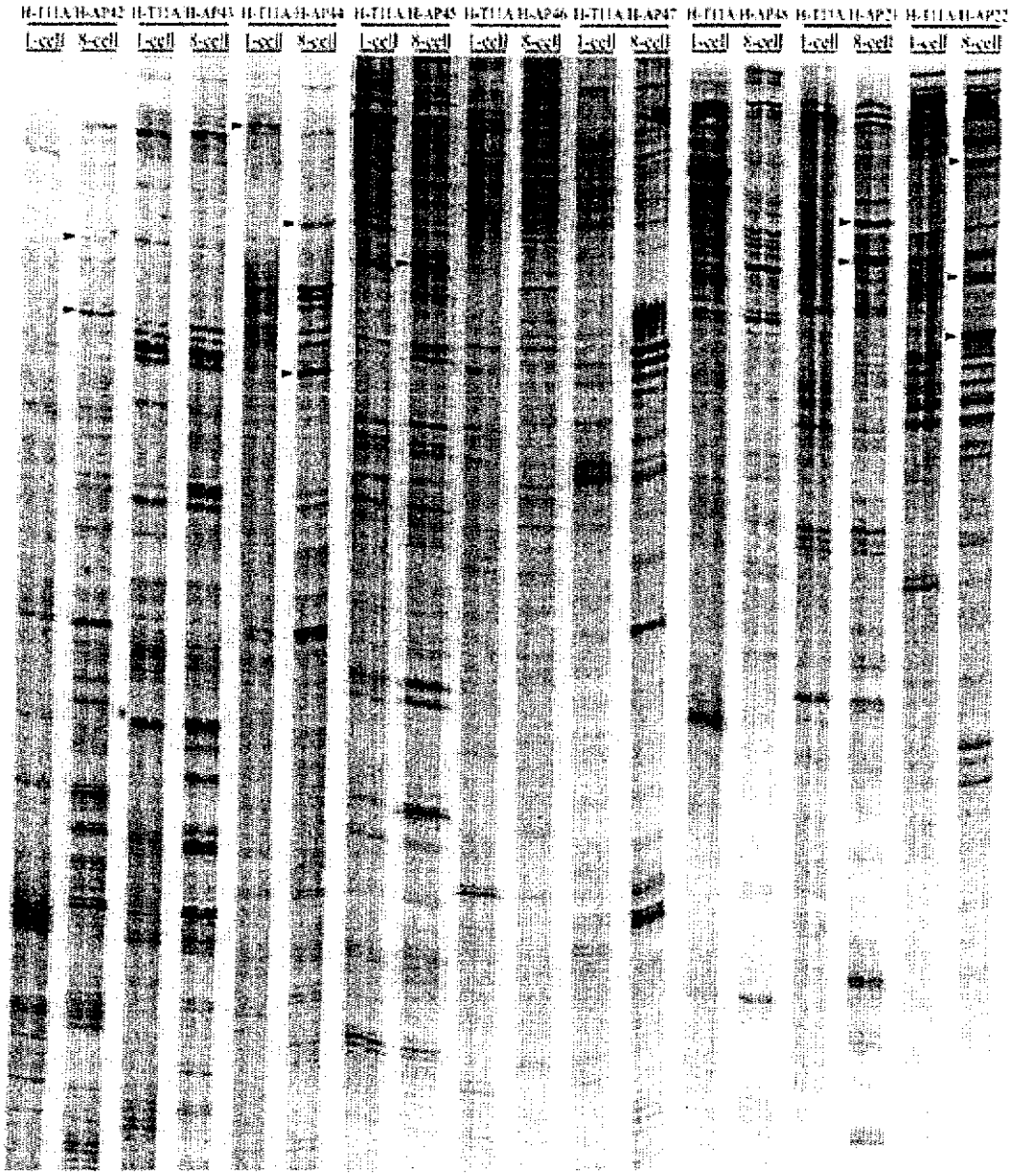

Fig. 3

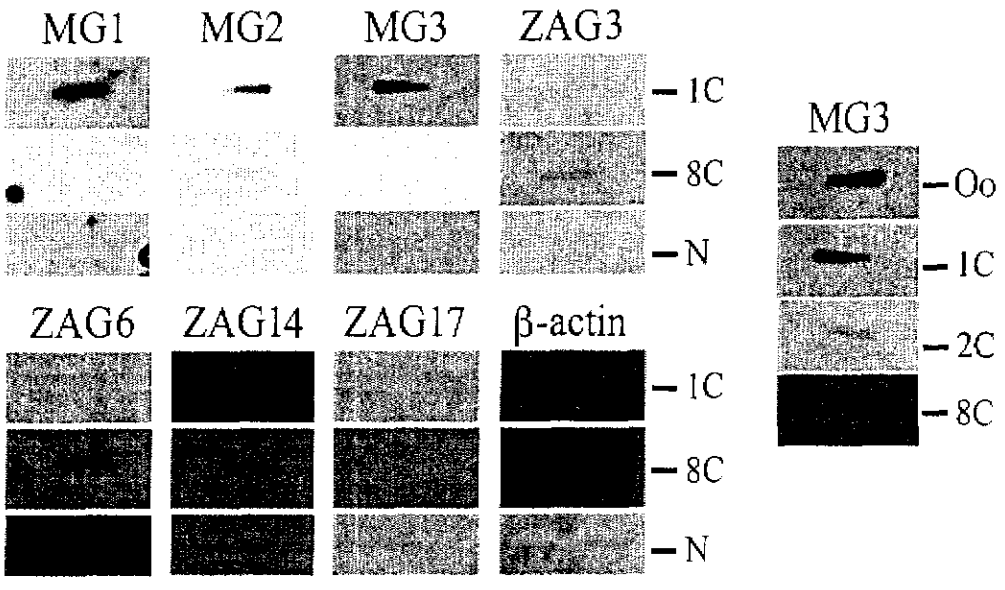
MG1
MG2
MG3
ZAG3
1C
8C
N
ZAG6
ZAG14
ZAG17
β-actin
1C
8C
N
MG3
Oo
1C
2C
8C

Fig. 4

MG1
AAGCTTCTCCGGAGTGTCTGGCGCCTGCTGACTTGCTGGTTAAGAAGGAGCCCAAGGCAAATGGCAGCAG
TCAAAGGATGATGCAGTGGCTGGCTACAAAGTCACCCAAAAAGGAAGTCCCTGACTCACCCAAAAAGGAT
GCATCAGGTCTACCCCAGTGGTCCAGCCAGTTTCTCCAGAAGAGCCCATTGCCTGCTAAAAGAGGTGCTA
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CTAGGCTCCATACCAGGCCTCAGCCTGATACAGCATTACTGTTGTCACCAGTTGACTGGAATTGTAACCT
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AGGCTCCTCTCAGTCTCTGGGCTCTCCTTTAGAGTTAAGGTCTTAAAATGTATTTTGTCCTAAATAAACT
GCGTTTGGTAAAAAAAAAAAGCTT
MG2
AAGCTTCTCCGGAGTGTCTGGCGCCTGCTGACTTGCTGGTTAAGAAGGAGCCCAAGGCAAATGGCAGCAG
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