科技部補助專題研究計畫成果報告
期末報告

Th1/Th2細胞激素對乳腺構形及功能的角色

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計 畫 主 持 人： 李宜儒

計畫參與人員： 碩士班研究生-兼任助理人員：黃俊浩
碩士班研究生-兼任助理人員：沈欣儒
碩士班研究生-兼任助理人員：姜智尹

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中 華 民 國 104 年 10 月 28 日

中 文 摘 要 ： 懷孕時乳腺結構產生巨大的變化，即在原有之乳管上衍生出腺泡。在此期間，腺泡前驅細胞（alveolar progenitor cells）會產生腺泡細胞（alveolar cells），腺泡細胞再分化因而具備合成乳汁的能力。許多荷爾蒙和生長因子皆參與調節此時期的發育。近來的文獻指出，調控第二型輔助型T細胞（Th2）的轉錄因子GATA-3，對於指定及維持乳腺腔道細胞的命運也扮演了重要的角色。這些發現雖然令人意外但是是可以理解的，因為懷孕時體內環境是偏向於Th2以防止母體對胎兒的排斥；乳腺可能是同時利用此環境以進行其發育。從另一個角度來看，感染或發炎導致體內環境轉偏向於Th1時應會阻礙乳腺的發育。基於此因，我們對Th1/Th2細胞激素在乳腺發育中的角色倍感興趣，尤其是對乳腺構形及功能之影響。在此，我們發現促發炎細胞激素TNF-alpha和IL-1alpha會抑制泌乳激素（prolactin）所刺激的beta-casein的表現。beta-casein是一乳蛋白基因，常被用來作為乳腺細胞分化的標的。這兩種細胞激素也會抑制乳腺腺泡結構的形成。相反地，單有IL-4即能刺激beta-casein的表現，其效力與泌乳激素相當。另外，長時間刺激IL-4會使乳腺腺泡明顯增大。這些細胞表現高量的proliferation cell nuclear antigen（PCNA）以及納入大量的5-ethynyl-2’-dexoyuridine（EdU），顯示了IL-4能刺激細胞的增生。基於乳腺發育時期的特定，我們推測IL-4可能在懷孕前期扮演促進細胞增生的角色，而在懷孕後期扮演促進細胞分化的角色。因此，細胞激素不僅作用於免疫系統，也能調控乳腺的發育。我們希望這些研究能使我們對乳腺的正常發育有更深入的了解，並進而找出治療乳癌的策略。

中 文 關 鍵 詞 ： 細胞激素、乳腺、分化、形態

英 文 摘 要 ： During pregnancy, mammary glands undergo massive changes in their structures with the appearance of alveoli in the existing ducts. The luminal alveolar cells are then differentiated and acquire the ability to synthesize milk. This is delicately controlled by various hormones and growth factors. Recent evidence has shown that the key transcription factor of T helper (Th)2 cells, GATA-3, specifies and maintain the luminal cell fate in mammary glands. It is well known that a Th2 bias is formed during pregnancy to prevent rejection of the fetus by the mother. Being a late-evolved organ, the mammary gland might just make use of this setting for its own development. On the other hand, tipping the Th1/Th2 balance toward a Th1 milieu caused by infection or inflammation might jeopardize proper development. We are thus interested in the role of Th1/Th2 cytokines in mammary gland development, especially in the aspects of tissue architecture and functional differentiation. Here we have found that proinflammatory cytokines TNF-alpha and IL-1alpha inhibit prolactin-induced expression of beta-casein, a milk protein gene that is often used as a marker for differentiation. These cytokines

also hamper the formation of acinar structures in mammary cells cultured on basement membrane (BM). On the contrary, IL-4 alone is able to stimulate beta-casein expression with comparable efficiency to prolactin. Prolonged treatment of IL-4 results in the enlargement of mammary acinus. These cells express higher levels of proliferation cell nuclear antigen (PCNA) and incorporated higher amounts of 5-ethynyl-2' -deoxyuridine (EdU) than untreated cells, indicating that IL-4 stimulates cell proliferation. Based on the specific features for mammary gland development, we reckon that IL-4 might promote cell proliferation during early pregnancy and facilitate differentiation during late pregnancy. Thus, cytokines regulates not only immune functions but also mammary gland development. We hope our work helps decipher the control of normal development of mammary gland and furthermore, devise strategies for cancer therapy.

英文關鍵詞：cytokine, mammary gland, differentiation, morphology

報告內容

一、前言

The mammary gland is an epithelial organ. Two types of epithelium, luminal and basal cells, form a network of bi-layered structure. The luminal cells form the ducts and alveoli, and the basal cells are myoepithelial cells that contact the basement membrane (BM). Growth and development of mammary glands occur primarily after birth, especially during puberty and pregnancy. It is during pregnancy that alveolar structures are budded from the existing ducts. The luminal cells of alveoli, termed the alveolar cells, are then differentiated and acquire the ability to synthesize and secrete milk. Thus, cell lineage commitment and subsequent morphogenesis and differentiation are required for the ultimate function of mammary gland, lactation. This is delicately controlled by various hormones and growth factors. The emerging players for mammary gland development during pregnancy are T helper (Th)2 cytokines. It seems reasonable that a bias of Th2 environment facilitates mammary gland development, and at the same time, prevents rejection of the fetus in the uterus. On the other hand, tipping the Th1/Th2 balance toward a Th1 milieu caused by infection or inflammation might jeopardize proper development.

二、研究目的

We are thus interested in the role of Th1/Th2 cytokines in mammary gland development, especially in the aspects of morphogenesis and functional differentiation.

三、文獻探討

Within the immune system cytokines play central roles in determining the differentiation of naïve $CD4^+$ T cells into different lineages. The polarization of Th cells into Th1 is regulated by IL-12, which activates STAT4. IL-4/IL-13, which activate STAT6, skews Th cell differentiation to the Th2 lineage. STAT6 upregulates the expression of GATA3, which binds to crucial regulatory elements of the Th2 cytokine locus, and thus is indispensable for Th2 cell differentiation (Paul and Zhu, 2010).

The mammary gland is a ductal epithelial organ. Rapid ductal growth and branching morphogenesis are induced during puberty, whereas the development of lobuloalveolar structures along the existing ductal tree takes place during pregnancy. Towards the end of gestation, alveolar epithelial cells are terminally differentiated, and acquire the ability to synthesize milk. After weaning, mammary glands undergo involution. Extensive tissue remodeling occurs during this period of time with massive death of secretory epithelial cells. There are two types of epithelial cells in the mammary gland: the luminal epithelial cells, which form the ducts and alveoli, and the myoepithelial cells, which line the basal surface of the luminal cells. The epithelium is separated from the surrounding stroma by an intact BM. A number of cell types are present in the stroma, including adipocytes, fibroblasts, vascular endothelial cells and immune cells. The complicated program of mammary development is controlled by soluble factors such as hormones, growth factors and cytokines, as well as various cell-cell and cell-matrix interactions (Hennighausen and Robinson, 2005).

Communication between the immune system and the mammary gland contributes to the normal development of the mammary gland. During puberty, macrophages, eosinophils and mast cells are distributed around the terminal end buds, and helps ductal outgrow and branching morphogenesis. Macrophages and eosinophils also play a role during pregnancy and lactation as the blockade of their recruitment to mammary glands leads to a decrease in milk production. Lymphocytes are found in the lactating glands. Their secreted

products (ex: IgA) are in the milk, providing passive immunity for the newborn. After weaning, mammary glands undergo involution with massive cell death taking place. More macrophages come in to remove cell corpses (Atabai et al., 2007; Reed and Schwertfeger, 2010). It has been shown that lymphocytes and plasma cells are also infiltrated to the mammary gland at the later stage of involution (Stein et al., 2004).

Mammary epithelia itself possesses immune functions. Many constituents in milk such as lysozyme and xanthine oxidoreductase that are synthesized by mammary cells are known to have antimicrobial function. This can protect the newborn from infection, and also protect the mother from mastitis. In fact, the mammary gland is considered to be evolved from the innate immune system since it is virtually an appendage of the skin (Vorbach et al., 2006). Furthermore, in vitro experiments reveal that mammary cells synthesize Th2 cytokines (IL-4 and IL-13) in the course of differentiation. This is accompanied by a decrease in the production of Th1 cytokines (IL-12 and TNF-α) in KIM-2 cell line (Khaled et al., 2007). In agreement with these results, levels of Stat6 phosphorylation and GATA3 expression are elevated during pregnancy. Stat6 is the downstream signaling molecule of IL-4/IL-13, and GATA3 is the target gene of Stat6. Interestingly, Stat6-deficinet and IL-4/IL-13-doubly deficient mice display similar phenotype in mammary glands, with a delayed development of lubuloalveolar structures (Khaled et al., 2007; Watson et al., 2011).

GATA-3 is required to specify and maintain the Th2 cell fate. It also regulates luminal cell differentiation from its progenitor in mammary glands. Targeted deletion of GATA-3 leads to severe defects in ductal and lubuloalveolar development. The loss of GATA-3 during pregnancy causes lactation insufficiency and the expansion of undifferentiated cells, which accumulate in the lumen of mammary glands. Expression of GATA-3 in luminal progenitor cells results in milk protein expression even in the absence of lactogenic hormones. One target gene of GATA-3 is FOXA1, which is essential for estrogen signaling. Based on microarray profiling analysis of breast tumors, GATA-3 status correlates ER status and the prognosis (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006).

A large number of genes involved in immune function are induced during involution. These include cytokines, acute phase proteins, soluble defense factors and immunoglobulins. As the tissue remodeling of mammary glands at this stage resembles the process of wound healing, some immune mediators have been implicated in the incidence and poor prognosis of pregnancy-associated breast cancer (Asztalos et al., 2010; Schedin, 2006). Clearance of apoptotic cells, termed efferocytosis, occurs intensively during involution. It has been considered to suppress inflammation by producing IL-4, IL-13, IL-10 and TGF-β. However, a recent report has shown that efferocytosis actually promotes metastasis, which might explain the poor outcome of postpartum breast cancer (Stanford et al., 2014). Even so, there are mechanisms to counteract the inflammatory responses. Some acute phase proteins such as pentraxin 3, serum amyloids A, ceruloplasmin, uterocalin and secretory leukocyte protease inhibitor, exhibit anti-inflammatory functions (Atabai et al., 2007; Clarkson et al., 2004).

四、研究方法

Substrata and Cell Cultures

Collagen I thin gel-coated dishes were prepared by incubating plates overnight at 4 °C with collagen I at 8 μg/cm^2. The plates were washed extensively with PBS before use. Reconstituted basement membrane matrix (Matrigel) was coated onto dishes at 14 mg/ml. Primary epithelial cultures were prepared from mid-pregnant ICR mice and plated on different substrata in nutrient mixture F-12 containing 10% fetal bovine serum, 1 mg/ml fetuin, 5 ng/ml EGF, 5 μg/ml insulin and 1 μg/ml hydrocortisone. After 72 h, medium was changed to

Dulbecco's modified Eagle's medium DMEM)/nutrient mixture F-12 containing hydrocortisone, insulin and prolactin (3 μg/ml). Second passage cells were obtained by trypsinization of cells cultured on BM and replated on different substrata.

RNA Extraction and RT-PCR

Cells were lysed by TRIzol reagent, and total RNA is extracted. Reverse transcription was performed on 1 μg of total RNA using reverse transcriptase and oligo(dT) primers. The reverse transcription products were then used as templates for PCR amplification using gene-specific primers. PCR products were separated on 1.5% agarose gel and analyzed by ethidium bromide incorporation.

Immunoprecipitation and Western Blot Analysis

Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. Cell lysates containing equal amounts of protein were incubated with 1-2 μg of antibody and 20-50 μl of protein A-Sepharose beads for 2-4 h at 4°C. Immunoprecipitates or whole cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with antibody. Proteins were visualized using an ECL kit.

Immunofluorescence Microscopy

Cells were equilibrated in 25% sucrose in PBS for 1 h, fixed in cold methanol:acetone (1:1) overnight at -20°C, and then re-equilibrated in 25% sucrose in PBS at room temp for 1 h. The samples were blocked for 1 h with the blocking solution containing 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 0.05% NaN_3, 10% goat serum, 0.2% Triton X-100 and 0.05% Tween 20 and incubated with rhodamine phalloidin and Hoechst 33342. After mounting the samples on glass slides, slides are observed under a confocal microscope.

Cell Proliferation Assay

Measurement of cell proliferation was performed according to manufacturer's instructions (Click-iT Plus EdU Imaging Kits; Molecular Probes). Briefly, cells cultured on BM were pulsed with 10 μM EdU for 6 h to measure DNA synthesis. The cells were fixed in 4% paraformaldehyde, permeabilized using 0.5% Triton X-100, and blocked 1% goat serum in PBS. EdU was detected by incubating cells with fresh Click-iT Plus reaction cocktail for approximately 30 min, protected from light. The cells were washed with PBS and stained with Hoechst 33258 (4 μg/ml), protecting from light at all times.

五、結果與討論

TNF-α and IL-1α exert detrimental effects on β-casein expression and morphogenesis in mammary cells cultured on BM.

We started with the examination of the effect of proinflammatory cytokines, TNF-α or IL-1α, on mammary structure and function. Primary mouse mammary epithelial cells cultured on BM were pretreated with 50 or 100 ng/ml of TNF-α or IL-1α for 1d, and stimulated with prolactin for another day. These cytokines inhibited prolactin-induced β-casein expression, and TNF-α was more potent than IL-1α (Fig. 1A). To monitor the effect of cytokines on the 3D structures, mammary cells cultured on BM were treated without or with TNF-α or IL-1α for 2 d. They were then stained with Hoechst 33258 and rhodamin-phalloidin, and

visualized under confocal microscope. Without cytokine treatment, mammary cells formed proper acinar structure with a single lumen. The strong staining of phalloidin dictated the localization of large amounts of F-actin, particularly on the apical surface of an acinus. However, lumen disappeared and tissue polarity was lost in cells treated with TNF-α or IL-1α (Fig. 1B). Thus, proinflammatory cytokines TNF-α and IL-1α exert impact on mammary structure and function.

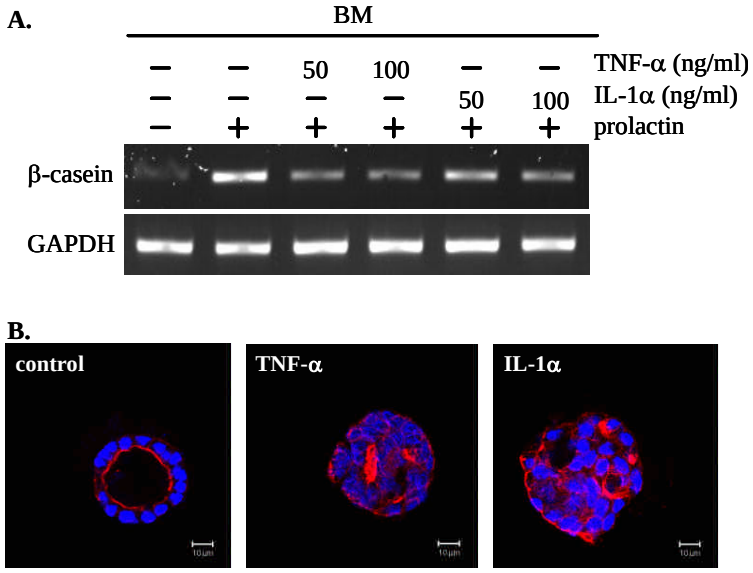

Figure 1. TNF-α and IL-1α hamper β-casein expression and the formation of alveolar structure in primary mouse mammary epithelial cells cultured on BM. (A) Mammary cells cultured on BM were pretreated with 50 or 100 ng/ml TNF-α or IL-1α for 1 h, and then stimulated with prolactin (3 mg/ml) for 1 d. Total cell lysates were analyzed by immunoblotting with antibodies to phospho-Stat3 and ERK. Total RNA was reverse transcribed and PCR-amplified with primers for β-casein and GAPDH. (B) Mammary cells cultured on BM were untreated, or treated with 50 ng/ml TNF-α or IL-1α for 2 d. Cells were fixed and stained with Hoechst 33342 (blue) and rhodamin-phalloidin (red), and visualized under confocal microscope. Scale bar: 10 μm.

Transcription factor NF-κB influences numerous cellular responses, such as inflammation, proliferation, apoptosis and survival. It also regulates mammary gland development. There is evidence that NF-κB is involved in transcription of the β-casein gene (Zhang et al., 2004). Activation of NF-κB during lactation by inducing the expression of active I-κB kinase 2 (IKK2) results in a decrease in β-casein expression. Injection of lipopolysaccharide into mammary glands to simulate mastitis activates NF-κB and diminishes β-casein expression (Connelly et al., 2010). Furthermore, levels of NF-κB activity are nearly undetectable during lactation, implying the undesirable role of NF-κB in lactation (Brantley et al., 2000). Given that TNF-α and IL-1α signaling activate the NF-κB pathway, our results are, to some extents, in agreement with these findings.

In endometrial cells, glucocorticoids affect lumen formation by inhibiting the expression of TNF-α and IL-1α (Eritja et al., 2012), consisting with their well-known role in immunosuppression. In mammary glands, liganded glucocorticoid receptor binds to the β-casein promoter and promotes transcription with the collaboration of other transcription factors such as Stat5 and C/EBP. Another function of glucocorticoids is to maintain tight junction integrity (Woo et al., 1996). It is of interest to find out whether TNF-α and IL-1α

block mammary morphogenesis and functional differentiation by overriding the effect of glucocorticoids.

IL-4 is as effective as prolactin in stimulating β-casein expression in cells cultured on BM.

We then turned to study the effect of IL-4, one of the Th2 cytokines, on β-casein expression. IL-4 alone stimulated β-casein expression, and the extents of induction are comparable to those in response to prolactin (Fig. 2A). As optimal transcription of the β-casein gene requires activation of Stat5, we examined whether IL-4 influenced Stat5 tyrosine phosphorylation. Our results showed that IL-4 stimulated Stat6 tyrosine phosphorylation without any effect on Stat5 tyrosine phosphoryulation (Fig 2B). These results suggest that Stat6 controls gene transcription in a similar way to Stat5. Thus, they might compensate for each other or cooperate to fulfill some functions during mammary gland development.

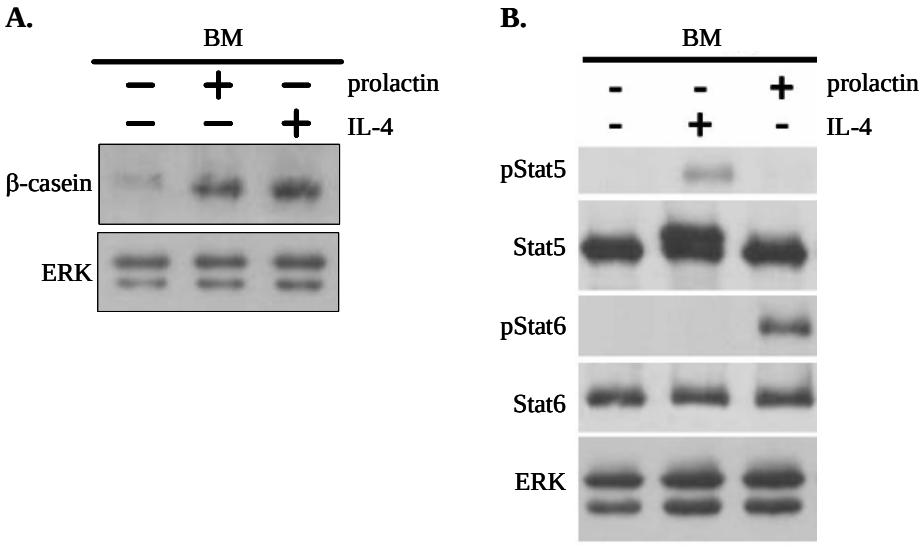

Figure 2. IL-4 stimulates Stat6 tyrosine phosphorylation and β-casein expression in mammary cells cultured on BM. (A) Mammary cells cultured on BM were stimulated with prolactin or IL-4 (50 ng/ml) for 1 d. (B) Mammary cells cultured on BM were stimulated with prolactin or IL-4 (50 ng/ml) for 15 min. Total cell lysates were analyzed by immunoblotting. Levels of ERK were used as loading controls.

IL-4 treatment leads to enlargement of mammary acinus accompanied with incomplete luminal clearance.

A striking feature for IL-4-treated cells is the enlargement of mammary acinus (Fig. 3A). To reveal the inner structure of mammary acini, cells cultured on BM were stimulated with IL-4 for 2 d, and subjected to immunofluorescence microscopy. Untreated cells displayed alveolar morphology with a hollow cavity. Lumen formation was incomplete in IL-4-treated acini. Some parts of acinus were cleared and exhibited strong staining of F-actin on the apical surface, while other regions were filled with cells. The extents of luminal clearance seemed to be correlated with the size of aninus (Fig. 3B). We believe lumen formation is delayed in the acini treated with IL-4. Owing to their large size, it should take longer to clear the lumen. On the other hand, slower clearance might be as a result of resistance to cell death conferred by IL-4.

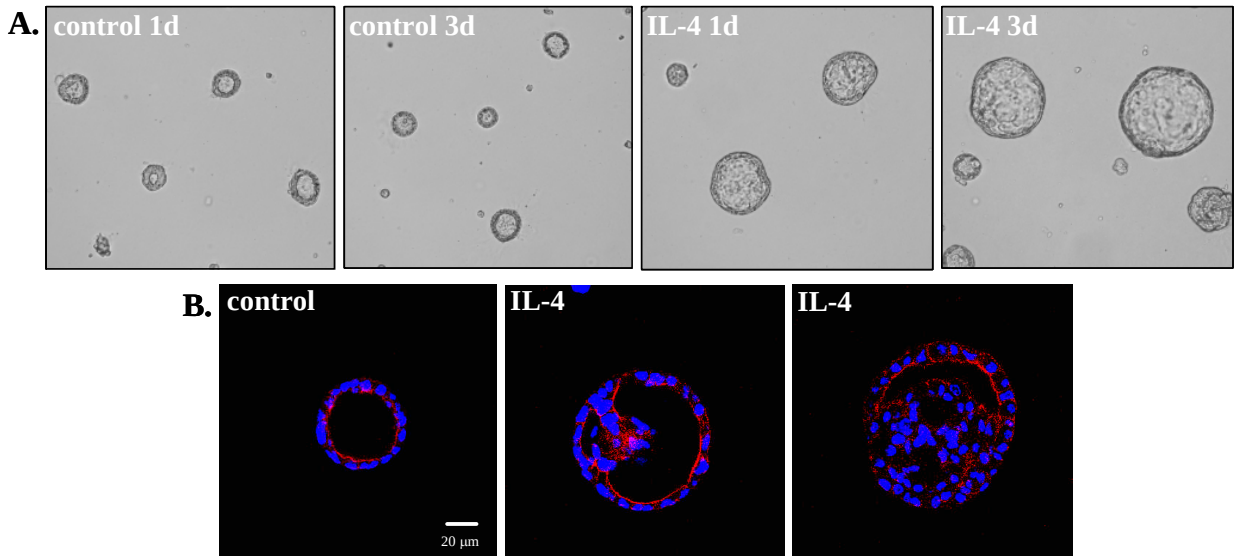

Figure 3. IL-4 treatment results in enlargement of acinus and partial clearance of acinar lumen in cells cultured on basement membrane. (A) Mammary cells cultured on BM were untreated or treated with IL-4 for 1 or 3 d. (B) Mammary cells cultured on BM were untreated or treated with IL-4 (50 ng/ml) for 2 d. Cells were then stained with Hoechst 33258 (blue) and rhodamin-phalloidin (red), and visualized under confocal microscope. Scale bar: 20 μm.

IL-4 stimulates cell proliferation in mammary cells cultured on BM.

The expansion of acinus in response to IL-4 might be ascribed to cell proliferation. To prove it, proliferation cell nuclear antigen (PCNA) expression was monitored by immunoblotting. Treatment of IL-4 for 1 d resulted in ~2-fold of increase in PCNA levels (Fig. 4A). To further demonstrate it, cells were treated with IL-4 for 18 h, and then pulsed with 5-ethynyl-2'-deoxyuridine (EdU) for 6 h to measure DNA synthesis. Higher amounts of EdU were incorporated in cells treated with IL-4 (Fig. 4B). These results show that IL-4 promotes cell proliferation of mammary cells cultured on BM. Usually primary cells cultured on BM tend to undergo cell cycle arrest, it is quite surprising to find out IL-4 has the great potency to stimulate cell proliferation. Whether this feature of IL-4 contributes cancer progression merits further investigation.

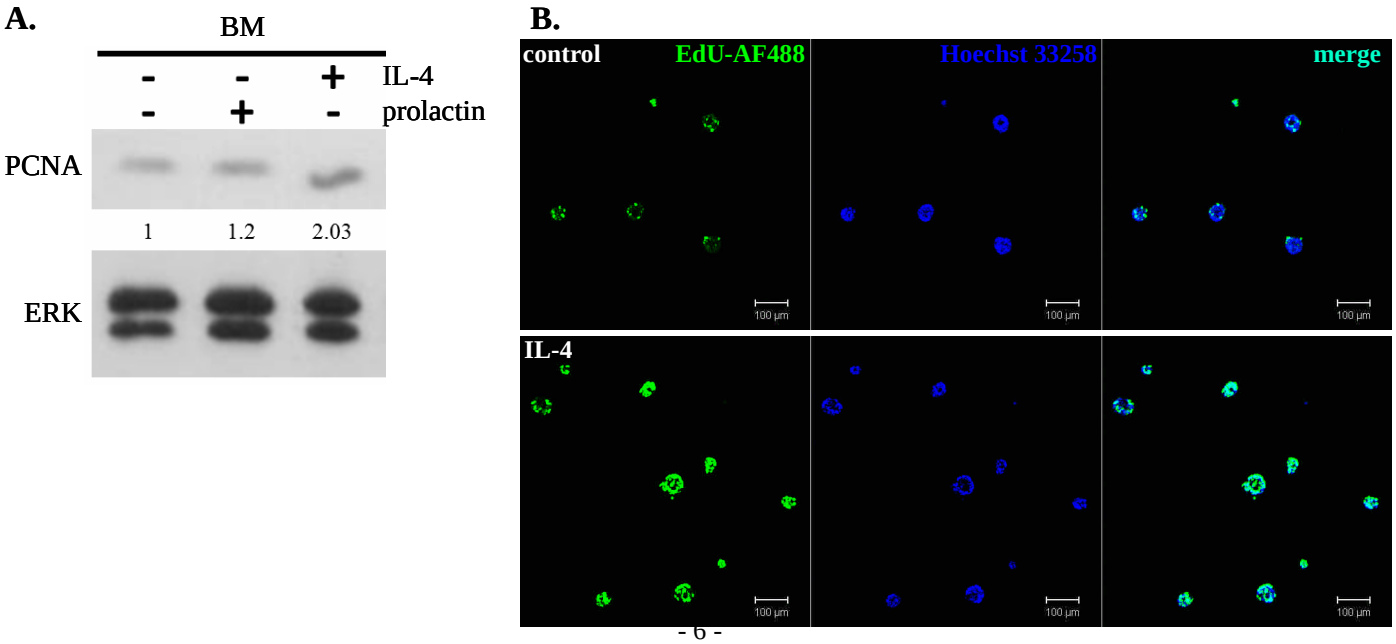

Figure 4. IL-4 stimulates cell proliferation. (A) Mammary cells cultured on BM were treated with prolactin, or IL-4 for 1 d. Total cell lysates were analyzed by immunoblotting with antibodies to PCNA and ERK. (B) Mammary cells cultured on BM were untreated or treated with IL-4 for 18 h, and then pulsed with EdU (green) for 6 h. Cell proliferation was detected by a Click-iT Plus EdU Imaging kit. Scale bar: 20 μm.

The ultimate function of mammary glands is to produce milk to nourish newborns. During pregnancy, mammary epithelial cells undergo extensive proliferation and morphogenesis, followed by functional differentiation. This leads to expansion of secretory cells to fulfill the massive production of milk during lactation. Elevation of IL-4 levels in pregnant women is known to prevent rejection of the fetus in the uterus. Here we have found that this also facilitates mammary gland development to prepare for the event after child birth, the lactation.

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科技部補助計畫衍生研發成果推廣資料表

日期:2015/10/28

科技部補助計畫	計畫名稱: Th1/Th2細胞激素對乳腺構形及功能的角色
	計畫主持人: 李宜儒
	計畫編號: 103-2320-B-040-020- 學門領域: 醫學之生化及分子生物

無研發成果推廣資料

103年度專題研究計畫研究成果彙整表

計畫主持人：李宜儒	計畫編號：103-2320-B-040-020-
計畫名稱：Th1/Th2細胞激素對乳腺構形及功能的角色	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	2	2	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	2	2	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果（無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）		無					

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形：
   論文：□已發表 □未發表之文稿 ■撰寫中 □無
   專利：□已獲得 □申請中 ■無
   技轉：□已技轉 □洽談中 ■無
   其他：（以100字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以500字為限）
   本計畫著重在探討Th1/Th2細胞激素對乳腺構形及功能之影響。我們發現促發炎細胞激素TNF-alpha和IL-1alpha抑制初代小鼠乳腺上皮細胞乳蛋白的合成以及乳腺腺泡結構的形成。反之，IL-4卻能刺激乳蛋白的表現以及細胞增生。所以，細胞激素不只調節免疫功能，亦會影響乳腺的發育。我們希望這些研究能使我們對乳腺的正常發育有更深入的了解，並進而找出治療乳癌的策略。