科技部補助專題研究計畫成果報告
期末報告

免疫相關因子對乳腺構形及功能的角色

計 畫 類 別 ：個別型計畫
計 畫 編 號 ：NSC 102-2320-B-040-021-
執 行 期 間 ：102 年 08 月 01 日至 103 年 07 月 31 日
執 行 單 位 ：中山醫學大學微生物免疫研究所

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中 華 民 國 103 年 10 月 31 日

中文摘要：微環境中的細胞外基質對組織的構形及功能具極大的影響。在乳腺組織中，上皮細胞貼附於基底膜，形成具有空腔的腺體結構。在體外，若將細胞培養於由基底膜基質所構成的 3D 環境則能重建此種結構；再加入泌乳荷爾蒙（泌乳激素、胰島素和氫皮質酮）還能進一步刺激乳腺細胞的分化。相對於此，細胞貼附在一般的塑膠盤或是第一型膠原蛋白薄層所構成的 2D 培養環境時，僅能形成單一且扁平的細胞層，並且喪失對泌乳荷爾蒙的反應以及分化的能力。基於微環境的重要性，我們比較以 2D 及 3D 方式培養時細胞的基因表現，希望能藉此找出調控乳腺形態及功能的基因。微矩陣分析的結果顯示，有兩類基因在 3D 的培養條件下呈現增加的情形；一類是與清除凋亡細胞有關的基因，另一類則具有先天免疫功能。由於培養在基底膜上的乳腺細胞會形成空腔，而空腔的形成又是因為腺泡內部的細胞進行凋亡而後再被清除所致，因此我們推測這兩類基因可能參與了乳腺內腔的清空。我們已確認先前微矩陣分析所得之結果，發現培養在基底膜的乳腺細胞 CD14、lipopolysaccharide-binding protein、complement component 3、growth arrest specific 6 及 milk-fat globule epidermal growth factor 8 的表現量較培養在塑膠盤上細胞高。此現象發生在腺泡空腔形成之前，顯示這些基因可能參與了乳腺內腔的清空。在本篇研究，我們主要是探討調控免疫相關基因表現的機制。我們首先檢測 Stat3 是否參與其中，因為 Stat3 對誘發急性期基因十分重要，而 lipopolysaccharide-binding protein、complement component 3 及其他由微矩陣分析所得之基因皆為急性期基因。結果顯示，培養在基底膜的乳腺細胞具有較高的 Stat3 磷酸化；此外，抑制 Stat3 的活性會降低免疫相關基因的表現。接著，我們使用 caspase 抑制劑 ZVAD 和 capthepsin B 抑制劑 ZFA 來釐清細胞死亡是否扮演了重要的角色。ZFA 的處理造成免疫相關基因的表現下降以及乳腺內腔的清空受阻。所以，Stat3 訊息傳遞和溶胺體相關之細胞死亡參與調控免疫相關基因表現。總結而言，內腔的形成與細胞分化在乳腺發育過程中扮演了重要的角色。當調控失當時可能會產生內腔充填（luminal filling）和不必要的發炎反應以及分化能力喪失的情形；這些反而有利於腫瘤的進展。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。

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In mammary glands, epithelial cells contact basement membrane (BM) to form glandular structures with a central lumen. In vitro culture of mammary cells can display this morphology as long as they are grown in a 3D BM matrix. Furthermore, these cells acquire functional differentiation in the presence of lactogenic hormone. By contrast, cells cultured on tissue culture plastic, a 2D environment, form monolayers. They are unresponsive to the stimulation of lactogenic hormone, and lose the ability to differentiate. Owing to the importance of the microenvironment, we compared gene expression in cells cultured on 2D plastic and 3D BM, in order to find out genes that control breast architecture and function. Two groups of gene are upregulated in cells cultured on BM. One contributes to the clearance of apoptotic cells; the other has innate immune function. We have confirmed our microarray data and showed that the expression levels of CD14, LBP, complement 3, GAS6 and MFGE8 are augmented in cells cultured on BM. This precedes lumen formation, suggesting these genes might have a role in luminal clearance of the mammary gland. Here we explore the mechanisms for upregulation of immune-related genes. The involvement of Stat3 was examined since Stat3 induces acute-phase genes, and LBP, complement 3 and some other genes identified by microarray analysis are acute phase genes. Our results show that levels of Stat3 phosphorylation are higher in cells cultured on BM, and inhibition of Stat3 blocks the expression of immune genes. We then explore the role of cell death in gene induction using pan-caspase inhibitor ZVAD and capthepsin B inhibitor ZFA. Inclusion of ZFA leads to a decrease in expression levels of immune genes and failures in luminal clearance. Thus, Stat3 signaling and lysosome-mediated cell death affect the expression of immune-related genes. Lumen formation and differentiation are key events in development. When they are dysregulated, adverse consequences such as luminal filling, undesirable inflammation and loss of differentiation take place, which facilitate tumor progression. 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： mammary gland, matrix, morphogenesis, differentiation, immune response

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（□期中進度報告/■期末報告）

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執行期間：102 年 8 月 1 日至 103 年 7 月 31 日

執行機構及系所：中山醫學大學微生物免疫所

計畫主持人：李宜儒
共同主持人：
計畫參與人員：杜軍毅、黃俊浩、沈欣儒

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中　華　民　國 103 年 10 月 31 日

目錄

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Within the context of 3D microenvironment, extracellular matrix greatly influences tissue architecture and function. In mammary glands, epithelial cells contact basement membrane (BM) to form glandular structures with a central lumen. In vitro cultures of mammary cells can display this morphology as long as they are grown in a 3D environment made up with malleable BM matrix. Furthermore, these cells acquire functional differentiation in the presence of lactogenic hormone (prolactin, insulin and hydrocortisone). By contrast, cells cultured on tissue culture plastic or thin-layer of collagen I, a 2D environment, form monolayers. They are unresponsive to the stimulation of lactogenic hormone, and lose the ability to differentiate. Owing to the importance of the microenvironment, we compared gene expression in cells cultured on 2D plastic and 3D BM, the conditions with least and most resemblance to the in vivo state, respectively, in order to find out genes that control breast architecture and function. Two groups of gene are especially upregulated in cells cultured on BM. One contributes to the clearance of apoptotic cells; the other has innate immune function. As cells cultured on BM exhibit 3D acinar morphology with a lumen, and lumen formation requires apoptosis and the subsequent removal of apoptotic cells, we speculate that these genes are involved in luminal clearance of mammary gland. We have confirmed our microarray data and showed that the expression levels of CD14, lipopolysaccharide-binding protein, complement component 3, growth arrest-specific 6 and milk fat globule-EGF factor 8 are augmented in cells cultured on BM. This precedes lumen formation, suggesting these genes might have a role in luminal clearance of the mammary gland. Here we explore the mechanisms for upregulation of immune-related genes. The involvement of Stat3 was examined since Stat3 is a key factor to induce acute-phase genes, and LPS-binding protein, complement component 3 and some other genes identified by microarray analysis are acute phase genes. Our results show that levels of Stat3 phosphorylation are higher in cells cultured on BM, and inhibition of Stat3 blocks the expression of immune genes. We then explore the role of cell death in gene induction using pan-caspase inhibitor ZVAD and capthepsin B inhibitor ZFA. Inclusion of ZFA leads to a decrease in expression levels of immune genes and failures in luminal clearance. Thus, Stat3 signaling and lysosome-mediated cell death affect the expression of immune-related genes. Lumen formation and differentiation are key events in development. When they are dysregulated, adverse consequences such as luminal filling, undesirable inflammation and loss of differentiation take place, which facilitate tumor progression. We hope our work helps decipher the control of normal development of mammary gland and furthermore, devise strategies for cancer therapy.

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報告內容

一、前言

Normal tissue architecture is vital for tissues to function properly and also provides restraint to prevent tumor progression. Within the context of 3D microenvironment, extracellular matrix (ECM) is the key factor to mold tissue morphology. The mammary gland displays ductal or alveolar structure with a central lumen, and its constituent epithelial cells contact the basement membrane (BM) *in vivo*. This structure can be reestablished *in vitro* by culturing cells on pliable 3D BM matrix. Furthermore, these cells acquire functional differentiation in the presence of lactogenic hormones (prolactin, insulin and hydrocortisone). By contrast, cells cultured on 2D substratum, such as tissue culture plastic or thin-layer of collagen I (the dominant stromal matrix in mammary glands), form monolayers, and they are unable to differentiate. In light of the importance of tissue architecture, gene expression profiling was exploited to study the correlation between morphology and gene expression, with the desire to find out signature genes which link to specific functions. We have compared the gene expression profiles for mammary cells cultured on 3D and 2D, and found two types of gene are upregulated in cells cultured on 3D BM. One plays roles in the clearance of apoptotic cells, and the other contributes to innate immunity. Lots of them encode soluble proteins such as acute phase proteins and complements; thus, they might act as immune mediators.

二、研究目的

After confirming that the clearance- and immune-related genes are dominantly expressed in mammary cells cultured on BM, we then wish to unravel the underlying mechanisms for their upregulation. More specifically, the upstream signaling pathways leading to induction of these genes are of interest to be identified.

三、文獻探討

ECM in the microenvironment provides the positioning signal to establish apico-basal polarity, and instructs morphogenesis. Maintaining tissue structure is essential for normal functions. Taking the mammary gland as an example, apical secretion of milk into the lumen of alveoli and the transport of milk through ducts require correct tissue polarity and structure. Moreover, normal tissue architecture poses a barrier to block tumor progression. Conversely, losing tissue architecture facilitates the advance of malignancy. The classical example for it is about the Rous sarcoma virus and tumor formation. Injection of the virus into chicken embryos did not generate tumors. But when the embryos were dissociated and cultured in a Petri dish, transformed phenotype quickly appeared (Bissell and Hines, 2011). Another study provides a“reverse” example for it. It shows that mutant p53 disrupts mammary architecture via the mevalonate pathway. Depletion of the mutant p53 or inhibition of the pathway by statin or geranylgeranyl transferase inhibitor reverts breast cancer cells to a more normal morphology (Freed-Pastor et al., 2012). Therefore, identification of the factors jeopardizing tissue architecture might shed new light on cancer therapy. Owing to the importance of tissue architecture, gene expression profiling is used to study the correlation between morphology and gene expression, with the desire to uncover signature genes for specific functions or to predict outcome for breast cancer. A large-scale study using 25 breast cell lines show that these cells cultured on 3D BM adopt different types of morphology, referred to as: Round class, Mass class, Grape-like class and Stellate class. Interestingly, cell lines of similar morphologies share certain pattern of gene expression (Kenny

et al., 2007). Another study is to compare gene expression profiles in 2D and 3D cultures of 2 breast cell lines, which have relatively normal phenotype. The genes downregulated in 3D condition can be used as prognosis markers for breast cancer with high accuracy (Fournier et al., 2006).

Lumen formation is a key event of morphogenesis for many organs. Different mechanisms have been elucidated (Bryant and Mostov, 2008). For mammary glands and salivary glands, lumen develops by cavitation. Initially, epithelial cells aggregate to form cell clusters. As cells inside the cluster are devoid of contact with BM, they undergo anoikis (Schafer et al., 2009). p38α plays a role in anoikis during mammary morphogenesis, and inhibition of this pathway leads to luminal filling reminiscent of that observed in ductal carcinoma in situ (DCIS)(Wen et al., 2011). Cells inside the cluster also undergo autophagy because of hypoxia and nutrient deprivation (Mills et al., 2004). Apart from these types of cell death, lysosomal-mediated cell death and entosis have been detected (Kreuzaler et al., 2011; Overholtzer et al., 2007; Sargeant et al., 2014). Dead cells are then removed by neighboring epithelial cells or incoming professional phagocytes, resulting in the formation of lumen (Monks et al., 2005). As for kidney and vascular development, lumen is formed by hollowing mechanism. This does not require cell apoptosis, and is mediated by membrane separation of epithelial cells (Bryant and Mostov, 2008). Interestingly, primary endometrial epithelial cells in 3D cultures form lumen by hollowing. Glucocorticoids abrogate proper lumenogenesis by repressing TNF-α and IL-1α, resulting in multiple lumen formation. This inhibitory effect is mediated by estrogen receptor α (ERα) instead of glucocorticoid receptor (Eritja et al., 2012).

The clearance of apoptotic cells, also termed efferocytosis, requires a crosstalk between the dying cells and phagocytes. Apoptotic cells release “find-me” signals such as lysophosphatidylcholine, CX3CL1, ATP/UTP, S19 ribosomal dimmers, endothelial monocyte-activating polypeptide II, and TGF-β to attract phagocytes. They also display “eat-me” signals. A major “eat-me” signal is phosphatidylserine (PS) which can be bound by a panel of soluble molecules, such as β2-glycoprotein I (β2-GPI), growth arrest specific 6 (GAS6), annexin I, milk-fat globule epidermal growth factor 8 (MFGE8), transglutaminase 2 (TG2), thrombospondin-1 (TSP-1) and serum-derived protein S. These molecules are then recognized by receptors on phagocytes, including β2-GPI receptor, Mer, PS receptor (PSR), αvβ3 integrin and CD36. Another type of the “eat-me” signal is oxidized low-density lipoprotein, which interacts with an array of scavenger receptors on phagocytes. The innate immunity also plays a part in efferocytosis. Complements and pentraxins can bridge apoptotic cells and phagocytes by interacting with apoptotic-cell-associated molecular pattern (ACAMP) on dying cells and CD14/calreticulin/CD91complex on phagocytes. Engagement and clustering of these receptors on phagocytes stimulate the engulfment and the subsequent immunosupression (Chao et al., 2012; Ravichandran and Lorenz, 2007).

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 the lactating mammary glands as well. Their secreted products (ex: IgA) are transported to milk, providing passive immunity for the newborn. After weaning, mammary glands undergo involution with massive apoptosis taking place. More macrophages come in to hasten the removal of apoptotic cells (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) (Khaled et al., 2007).

Gene expression profiling of mammary gland development reveals that a large number of genes involved in immune function are induced during involution. These include cytokines, acute phase proteins, soluble defense factors and immunoglobulins. Stat3 and CCAAT/enhancer binding protein δ (C/EBPδ), two key transcription factors for induction of acute phase genes, are also upregulated (Clarkson et al., 2004; Stein et al., 2004). Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response, and polarizes macrophages towards the M1 phenotype (Hughes et al., 2012). Even so, the role for induction of these immune-related genes during involution is not completely clear. As dramatic tissue remodeling in mammary glands occurs after weaning, it has been suggested that these genes are involved in a wound healing-like process. The increased expression of cytokines and immune mediators at this stage is thus implicated in the incidence and poor prognosis of pregnancy-associated breast cancer (Asztalos et al., 2010; Schedin, 2006). On the other hand, clearance of apoptotic cells leads to immunosuppression by producing TGF-β and IL-10. Some acute phase proteins such as pentraxin 3, serum amyloids A, ceruloplasmin, uterocalin and secretory leukocyte protease inhibitor, also exhibit anti-inflammatory functions. This can counteract the inflammatory responses to avoid overt inflammation (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-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.

五、結果與討論

Stat3 is involved in the upregulation of immune-related genes in mammary cells cultured on BM.

Many immune-related genes that are upregulated in primary mammary cells cultured on BM, as revealed by microarray data, are acute phase proteins, such as lipopolysaccharide-binding protein (LPS), complement component 3 (C3), haptoglobin and serum amyloid A. Stat3 is the key factor to induce acute phase genes. It is thus interesting to find out if Stat3 activity is regulated by matrix. Mammary cells were cultured on plastic or BM, and total cell lysates were analyzed by immunoblotting. Levels of Erk were used as loading controls. Our results showed that extents of Stat3 tyrosine phosphorylation were higher in cells cultured on BM (figure 1), suggesting that upregulation of acute phase genes or even other immune-related genes might be ascribed to the higher Stat3 activity.

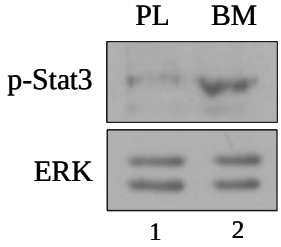

Figure 1. Stat3 activity is higher in mammary cells cultured on BM. Primary mammary epithelial cells were cultured on plastic (PL) or BM. Total cell lysates were analyzed by immunoblotting with antibodies to phospho-Stat3 and ERK. Levels of ERK were used as loading controls.

To demonstrate it, two Stat3 inhibitors, Stat3 inhibitor III and VI were used. A Stat5 inhibitor was also included as a control. Stat3 and Stat5 inhibitors were effective to block IL-6-induced Stat3 phosphorylation and prolactin-induced Stat5 phosphorylation in a dose-dependent manner, respectively (figure 2). The mammary spheres formed in BM became bigger in size and rougher on the surface in response to Stat3 inhibitors (data not shown), implying an alteration in 3D structure. These inhibitors also suppressed the expression immune-related gene (figure 3). These results suggest that Stat3 is involved in the upregulation of immune gene expression and mammary morphogenesis.

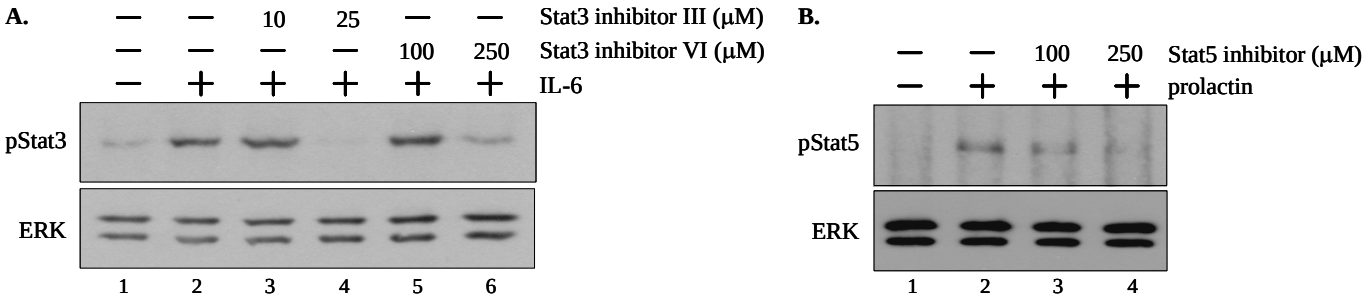

Figure 2. Stat3 and Stat5 inhibitors inhibit IL-6 and prolactin signaling, respectively. (A) Mammary cells were untreated, pretreated with 10 or 25 μM Stat3 inhibitor III, or 100 or 250 μM Stat3 inhibitor VI for 1 h, and then stimulated with 50 ng/ml IL-6 for 15 min. Total cell lysates were analyzed by immunoblotting with antibodies to phospho-Stat3 and ERK. (B) Cells were untreated or pretreated 100 or 250 μM Stat5 inhibitor for 1 h, and then stimulated with 3 μg/ml prolactin for 15 min. Total cell lysates were immunoprecipitated with Stat5 antibody followed by immunoblotting with antibody to phospho-Stat5.

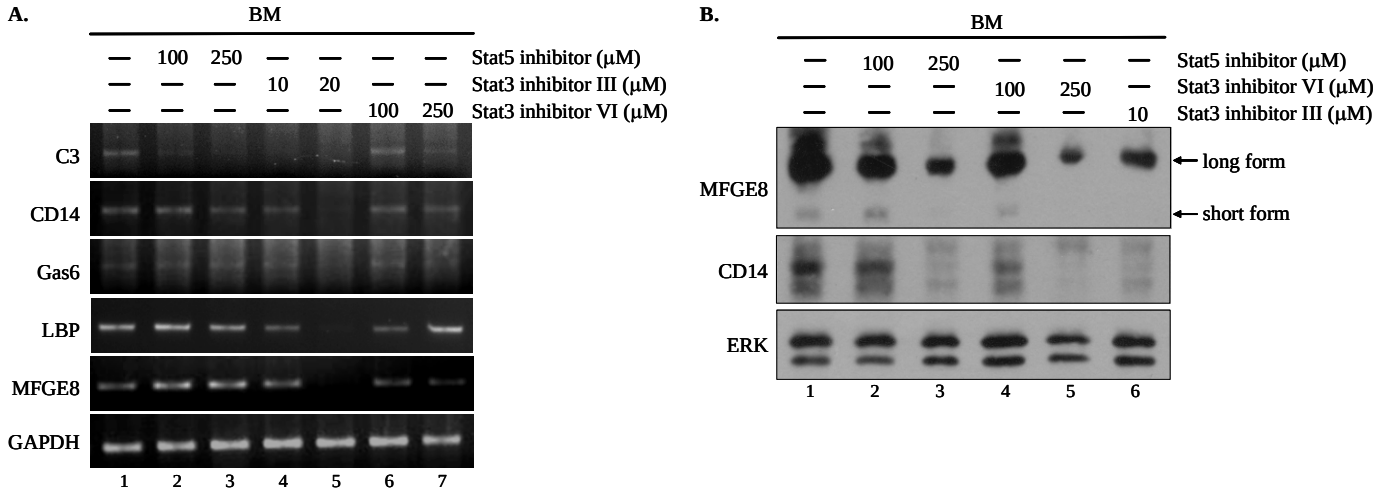

Figure 3. Stat3 inhibitors block the expression of immune-related genes. Mammary cells cultured on BM were pretreated with Stat3 or Stat5 inhibitors for 1 h before exposure to prolactin for 1 d. (A) Total RNA was reverse transcribed and PCR-amplified with primers for C3, CD14 GAS6, LBP, MFGE8 and GAPDH. (B) Total cell lysates were analyzed by immunoblotting with antibodies to MFGE8, CD14 and ERK.

Cathepsin B inhibitor ZFA inhibits the expression of immune-related genes and luminal clearance.

In mammary glands, Stat3 is essential for the initiation of involution.(Hughes and Watson, 2012). It promotes lysosome-mediated cell death by inducing the expression and leakiness of cathepsins (Kreuzaler et al., 2011; Sargeant et al., 2014). However, cell apoptosis and subsequent clearance of the corpses were also reported to be required for lumen formation of mammary acini (Debnath et al., 2002). We thus examined the effect of the pan-caspase inhibitor ZVAD and cathepsin B inhibitor ZFA on acinar morphology and immune gene expression. ZFA caused the enlargement of acini, a feature that was also observed in cells treated with Stat3 inhibitor (data not shown). Furthermore, luminal clearance was substantially impaired, resulting in luminal filling. With the treatment of ZVAD, the lumen was partially cleared, and the apico-basal polarity was moderately established (figure 4). Regarding immune-related genes, ZFA downregulated their expression, whereas ZVAD exerted no effect (figures 5). These data suggest that lysosome-mediated cell death is necessary for lumen formation of mammary acini, and a block of this event leads to luminal filling. It is likely that cell apoptosis play a minor or no part in it, which is consistent with the results of a recent report (Akhtar and Streuli, 2013). However, this contradicts with the earlier observation in that apoptosis is required (Debnath et al., 2002). Cell-type difference might cause this discrepancy as primary mammary epithelial cells were used in our and Akhtar's work, but MCF-10A was used in the studies by Debnath et al. MCF-10A cells are not entirely luminal epithelial cells since they exhibits some features of myoepithelial cells.

Figure 4. Lumen clearance was partially inhibited by ZVAD but completely blocked by ZFA. Mammary cells cultured on BM were untreated or treated with 100 μM ZVAD or 50 μM ZFA for 2 d. Cells were fixed and stained with Hoechst 33342 (blue) and rhodamin-phalloidin (red), and visualized under confocal microscope. The strong staining of phalloidin dictated the localization of large amounts of F-actin, particularly on the apical surface of an acinus. Without treatment of inhibitors, the acinus had a single and cleared lumen (control).

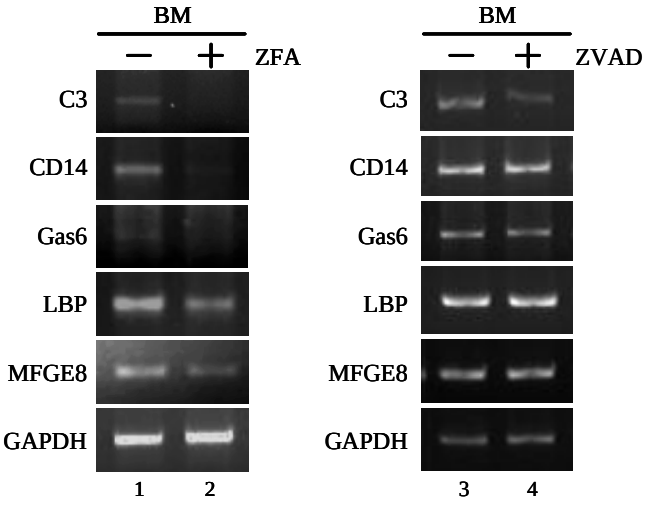

Figure 5. ZFA inhibits the expression of immune-related genes. Mammary cells cultured on BM were untreated or treated with 100 μM ZVAD or 50 μM ZFA for 2 d. Total RNA was reverse transcribed and PCR-amplified with primers for C3, CD14 GAS6, LBP, MFGE8 and GAPDH.

參考文獻

Akhtar, N., and C.H. Streuli. 2013. An integrin-ILK-microtubule network orients cell polarity and lumen formation in glandular epithelium. *Nat Cell Biol*. 15:17-27.

Asztalos, S., P.H. Gann, M.K. Hayes, L. Nonn, C.A. Beam, Y. Dai, E.L. Wiley, and D.A. Tonetti. 2010. Gene expression patterns in the human breast after pregnancy. *Cancer Prev Res (Phila)*. 3:301-311.

Atabai, K., D. Sheppard, and Z. Werb. 2007. Roles of the innate immune system in mammary gland remodeling during involution. *J Mammary Gland Biol Neoplasia*. 12:37-45.

Bissell, M.J., and W.C. Hines. 2011. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nat Med*. 17:320-329.

Bryant, D.M., and K.E. Mostov. 2008. From cells to organs: building polarized tissue. *Nat Rev Mol Cell Biol*. 9:887-901.

Chao, M.P., R. Majeti, and I.L. Weissman. 2012. Programmed cell removal: a new obstacle in the road to developing cancer. *Nat Rev Cancer*. 12:58-67.

Clarkson, R.W., M.T. Wayland, J. Lee, T. Freeman, and C.J. Watson. 2004. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res*. 6:R92-109.

Debnath, J., K.R. Mills, N.L. Collins, M.J. Reginato, S.K. Muthuswamy, and J.S. Brugge. 2002. The role of apoptosis in creating and maintaining luminal space within normal and oncogene-expressing mammary acini. *Cell*. 111:29-40.

Eritja, N., C. Mirantes, D. Llobet, G. Masip, X. Matias-Guiu, and X. Dolcet. 2012. ERalpha-mediated repression of pro-inflammatory cytokine expression by glucocorticoids reveals a crucial role for TNFalpha and IL1alpha in lumen formation and maintenance. *J Cell Sci*. 125:1929-1944.

Fournier, M.V., K.J. Martin, P.A. Kenny, K. Xhaja, I. Bosch, P. Yaswen, and M.J. Bissell. 2006. Gene expression signature in organized and growth-arrested mammary acini predicts good outcome in breast cancer. *Cancer Res*. 66:7095-7102.

Freed-Pastor, W.A., H. Mizuno, X. Zhao, A. Langerod, S.H. Moon, R. Rodriguez-Barrueco, A. Barsotti, A. Chicas, W. Li, A. Polotskaia, M.J. Bissell, T.F. Osborne, B. Tian, S.W. Lowe, J.M. Silva, A.L. Borresen-Dale, A.J. Levine, J. Bargonetti, and C. Prives. 2012. Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. *Cell*. 148:244-258.

Hughes, K., and C.J. Watson. 2012. The spectrum of STAT functions in mammary gland development. *Jakstat*. 1:151-158.

Hughes, K., J.A. Wickenden, J.E. Allen, and C.J. Watson. 2012. Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression. *J Pathol*. 227:106-117.

Kenny, P.A., G.Y. Lee, C.A. Myers, R.M. Neve, J.R. Semeiks, P.T. Spellman, K. Lorenz, E.H. Lee, M.H. Barcellos-Hoff, O.W. Petersen, J.W. Gray, and M.J. Bissell. 2007. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Mol Oncol*. 1:84-96.

Khaled, W.T., E.K. Read, S.E. Nicholson, F.O. Baxter, A.J. Brennan, P.J. Came, N. Sprigg, A.N. McKenzie, and C.J. Watson. 2007. The IL-4/IL-13/Stat6 signalling pathway promotes luminal mammary epithelial cell development. *Development*. 134:2739-2750.

Kreuzaler, P.A., A.D. Staniszewska, W. Li, N. Omidvar, B. Kedjouar, J. Turkson, V. Poli, R.A. Flavell, R.W. Clarkson, and C.J. Watson. 2011. Stat3 controls lysosomal-mediated cell death in vivo. *Nat Cell Biol*. 13:303-309.

Mills, K.R., M. Reginato, J. Debnath, B. Queenan, and J.S. Brugge. 2004. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is required for induction of autophagy during lumen formation in vitro. *Proc Natl Acad Sci U S A*. 101:3438-3443.

Monks, J., D. Rosner, F.J. Geske, L. Lehman, L. Hanson, M.C. Neville, and V.A. Fadok. 2005. Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. *Cell Death Differ*. 12:107-114.

Overholtzer, M., A.A. Mailleux, G. Mouneimne, G. Normand, S.J. Schnitt, R.W. King, E.S. Cibas, and J.S. Brugge. 2007. A nonapoptotic cell death process, entosis, that occurs by cell-in-cell invasion. *Cell*. 131:966-979.

Ravichandran, K.S., and U. Lorenz. 2007. Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol*. 7:964-974.

Reed, J.R., and K.L. Schwertfeger. 2010. Immune cell location and function during post-natal mammary gland development. *J Mammary Gland Biol Neoplasia*. 15:329-339.

Sargeant, T.J., B. Lloyd-Lewis, H.K. Resemann, A. Ramos-Montoya, J. Skepper, and C.J. Watson. 2014. Stat3 controls cell death during mammary gland involution by regulating uptake of milk fat globules and lysosomal membrane permeabilization. *Nat Cell Biol*.

Schafer, Z.T., A.R. Grassian, L. Song, Z. Jiang, Z. Gerhart-Hines, H.Y. Irie, S. Gao, P. Puigserver, and J.S. Brugge. 2009. Antioxidant and oncogene rescue of metabolic defects caused by loss of matrix attachment. *Nature*. 461:109-113.

Schedin, P. 2006. Pregnancy-associated breast cancer and metastasis. *Nat Rev Cancer*. 6:281-291.

Stein, T., J.S. Morris, C.R. Davies, S.J. Weber-Hall, M.A. Duffy, V.J. Heath, A.K. Bell, R.K. Ferrier, G.P. Sandilands, and B.A. Gusterson. 2004. Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3. *Breast Cancer Res*. 6:R75-91.

Vorbach, C., M.R. Capecchi, and J.M. Penninger. 2006. Evolution of the mammary gland from the innate immune system? *Bioessays*. 28:606-616.

Wen, H.C., A. Avivar-Valderas, M.S. Sosa, N. Girnius, E.F. Farias, R.J. Davis, and J.A. Aguirre-Ghiso. 2011. p38alpha Signaling Induces Anoikis and Lumen Formation During Mammary Morphogenesis. *Sci Signal*. 4:1-13.

國科會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：

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本計畫的工作著重在釐清細胞外基質調節正常乳腺細胞構形及功能的機制，我們發現這有可能是透過影響免疫相關基因的表現所致。免疫相關基因參與凋亡細胞的清除，促進腺泡內腔的形成。此過程調控失當時可能會產生內腔充填、分化能力喪失以及不必要的發炎反應；這些情形反而有利於腫瘤的進展。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。

科技部補助計畫衍生研發成果推廣資料表

日期:2014/10/24

科技部補助計畫	計畫名稱: 免疫相關因子對乳腺構形及功能的角色
	計畫主持人: 李宜儒
	計畫編號: 102-2320-B-040-021- 學門領域: 醫學之生化及分子生物

無研發成果推廣資料

102 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：102-2320-B-040-021-
計畫名稱：免疫相關因子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	3	3	100%		
		專書	0	0	100%		
	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	1	1	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	1	1	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

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■達成目標

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□其他原因

說明：

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論文：□已發表 □未發表之文稿 ■撰寫中 □無

專利：□已獲得 □申請中 ■無

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