行政院國家科學委員會專題研究計畫 期末報告

第二群塵虫滿過敏原與呼吸道上皮細胞之上皮-間葉細胞轉換以及氣喘發展的分子機制研究(第 3 年)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 99-2320-B-040-003-MY3
執 行 期 間 ：101 年 08 月 01 日至 102 年 07 月 31 日
執 行 單 位 ：中山醫學大學生化暨生物科技研究所

計 畫 主 持 人：高紹軒

計畫參與人員：碩士班研究生-兼任助理人員：江俊毅
博士班研究生-兼任助理人員：葉期瑋
博士班研究生-兼任助理人員：林君香

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，1 年後可公開查詢

中 華 民 國 102 年 10 月 02 日

中 文 摘 要 ： 塵蟎為台灣以及亞熱帶地區最常見引起氣喘等呼吸道疾病的主要過敏物種。目前已知的塵蟎過敏原，有兩群主要過敏原(major allergen): 具有蛋白酶活性的第一群塵蟎過敏原與沒有蛋白酶活性的第二群塵蟎過敏原，過去的研究文獻顯示這兩群過敏原的盛行率皆超過 85%。在致病機轉方面，第一群塵蟎過敏原透過其蛋白酶活性破壞呼吸道上皮細胞的連結進入體內並誘發相關的發炎與過敏反應。有趣的是第二群塵蟎過敏原雖然不具有蛋白酶活性，但仍可以穿過呼吸道上皮的阻隔進入人體當中。此外，近年來的研究證據發現第二群塵蟎過敏原可以結合並活化第四型類鐸受體(Toll-like receptor 4)，進而誘發先天性免疫反應(innate immunity)。然而第二群塵蟎過敏原究竟是透過第一群塵蟎過敏原的協助穿透上皮細胞還是自己也可以通過上皮細胞，以及是透過何種機制則仍不清楚。因此，本研究第二群塵蟎過敏原對呼吸道上皮細胞所產生的影響，針對細胞間連結與上皮-間質轉換(epithelial-mesenchymal transition)的分子機轉，進行更深入的探討。

本研究利用 E. coli 表現具有 GST-tag 的歐洲室塵蟎第二群過敏原 Der p 2 重組蛋白(DP2)，以正常人類支氣管上皮細胞株 BEAS-2B 與正常人類支氣管初代細胞 HBEpiC 建立細胞模式實驗，以及利用致敏小鼠模式的動物實驗，探討 DP2 對呼吸道上皮細胞所產生的影響。我們的研究顯示 DP2 會抑制上皮細胞 E-cadherin 的蛋白表現，同時增加 vimentin 與 alpha-smooth muscle actin (α-SMA)的蛋白表現。此外，DP2 也會增加重要的轉錄因子 Snail 與 Slug 的表現，並促進其核轉位(nuclear translocation)。更進一步的實驗結果發現，前述的蛋白表現改變是透過 Erk1/2 與 Akt/GSK3β 的訊息路徑所導致的。除蛋白表現的改變之外，實驗結果也證實 DP2 確實會提高呼吸道上皮細胞 BEAS-2B 以及 HBEpiC 的遷移與侵襲能力。

綜合上述研究結果，我們首次發現無蛋白酶活性的 DP2 具有誘發上皮細胞呼吸道上皮細胞產生 EMT 的現象與提升其遷移與侵襲能力，並證實 Erk1/2 與 Akt/GSK3β 的訊息路徑參與此一現象是透過活化，這些 DP2 誘發的訊息增加 Snail 與 Slug 的表現，並促進其核轉位(nuclear translocation)。據此，我們推測第二群塵蟎過敏原可利用此一機制造成上皮細胞屏障的隙漏(leaking)，進入體內誘發相關的致敏反應。

中文關鍵詞： 第二群塵蟎過敏原, 呼吸道上皮細胞, 上皮-間葉轉換, 氣喘

英 文 摘 要 ： Non-proteolytic group 2 allergen, Der p 2 (DP2) is known as a major allergen derived from house dust

mite (HDM) Dermatophagoides pteronissinus. Paracellular epithelial barrier, being composed of a number of tight junction molecules, plays pivotal roles in resistance of pathogen invading. Although group-1 HDM allergens have been demonstrated to destroy epithelial linkage with their protease activity, the effects of DP2 on epithelial integrity are rarely investigated.
In the present study, we aimed to investigate the effects of DP2 on epithelial integrity with focus on expression of epithelial-mesenchymal transition (EMT), and the mechanism regulating the expression of the EMT markers. Human normal bronchial epithelial cell BEAS-2B and human primary bronchial epithelial cell HBEpiC were used as in vitro cell model. Acute sensitized Balb/c mice with DP2 was used as in vivo animal model. Gene expression was determined by using real-time quantitative PCR. Cell motility was accessed by wound healing and transwell migration assay. Immunoblot and immunohistochemistry was conducted for relative protein quantitation and involvement of signaling cascades.
In vitro analysis revealed that DP2 regulated expression of EMT markers, including E-cadherin, vimentin and alpha-smooth muscle actin (α-SMA), and promoted motility of the airway epithelial cells. DP2 also triggered signaling cascades including activation of Akt/extracellular response-regulated kinase (Erk)1/2, inhibition of glycogen synthase kinase-3 β (GSK3 β), and translocation of Snail/Slug. In vivo analysis also demonstrated that E-cadherin level was significantly decreased in the lung tissues of DP2-sensitized mice.
In conclusion, these findings demonstrate that the DP2 alters expression of EMT markers and enhances cell motility, attributing to Akt/GSK3 β signaling and activation of Snail and Slug, and the consequent initiation of EMT. It is suggested that DP2 alone can modulate epithelial integrity via induction of EMT.

英文關鍵詞： Group 2-house dust mite allergen, airway epithelial cell, epithelial to mesenchymal transition, asthma

行政院國家科學委員會補助專題研究計畫 ☑ **成 果 報 告**
□ **期中進度報告**

第二群塵蟎過敏原與呼吸道上皮細胞之上皮-間葉細胞轉換以及氣喘發展的分子機制研究

Investigating effects of group 2-house dust mite allergens on epithelial-to-mesenchymal transition of airway epithelial cells and asthma development

計畫類別：☑ 個別型計畫　　□ 整合型計畫
計畫編號：NSC　　NSC 99-2320-B-040 -003 -MY3
執行期間：99 年 08 月 01 日 至 102 年 07 月 31 日

計畫主持人：高紹軒

計畫參與人員：王威鈞、葉期瑋、洪玉全、曾邵群、林君香

成果報告類型(依經費核定清單規定繳交)：□精簡報告　☑完整報告

本成果報告包括以下應繳交之附件：
□ 赴國外出差或研習心得報告一份
□ 赴大陸地區出差或研習心得報告一份
□ 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

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：中山醫學大學生化暨生物科技研究所

中 華 民 國　1 0 2　年　1 0　月　0 5　日

第二群塵蟎過敏原與呼吸道上皮細胞之上皮-間葉細胞轉換以及氣喘發展的分子機制研究

Investigating effects of group 2-house dust mite allergens on epithelial-to-mesenchymal transition of airway epithelial cells and asthma development

一、 中文摘要

塵蟎為台灣以及亞熱帶地區最常見引起氣喘等呼吸道疾病的主要過敏物種。目前已知的塵蟎過敏原，有兩群主要過敏原(major allergen): 具有蛋白酶活性的第一群塵蟎過敏原與沒有蛋白酶活性的第二群塵蟎過敏原，過去的研究文獻顯示這兩群過敏原的盛行率皆超過 85%。在致病機轉方面，第一群塵蟎過敏原透過其蛋白酶活性破壞呼吸道上皮細胞的連結進入體內並誘發相關的發炎與過敏反應。有趣的是第二群塵蟎過敏原雖然不具有蛋白酶活性，但仍可以穿過呼吸道上皮的阻隔進入人體當中。此外，近年來的研究證據發現第二群塵蟎過敏原可以結合並活化第四型類鐸受體(Toll-like receptor 4)，進而誘發先天性免疫反應(innate immunity)。然而第二群塵蟎過敏原究竟是透過第一群塵蟎過敏原的協助穿透上皮細胞還是自己也可以通過上皮細胞，以及是透過何種機制則仍不清楚。因此，本研究第二群塵蟎過敏原對呼吸道上皮細胞所產生的影響，針對細胞間連結與上皮-間質轉換(epithelial-mesenchymal transition)的分子機轉，進行更深入的探討。

本研究利用 E. coli 表現具有 GST-tag 的歐洲室塵蟎第二群過敏原 Der p 2 重組蛋白(DP2)，以正常人類支氣管上皮細胞株 BEAS-2B 與正常人類支氣管初代細胞 HBEpiC 建立細胞模式實驗，以及利用致敏小鼠模式的動物實驗，探討 DP2 對呼吸道上皮細胞所產生的影響。我們的研究顯示 DP2 會抑制上皮細胞 E-cadherin 的蛋白表現，同時增加 vimentin 與 alpha-smooth muscle actin (α-SMA)的蛋白表現。此外，DP2 也會增加重要的轉錄因子 Snail 與 Slug 的表現，並促進其核轉位(nuclear translocation)。更進一步的實驗結果發現，前述的蛋白表現改變是透過 Erk1/2 與 Akt/GSK3β 的訊息路徑所導致的。除蛋白表現的改變之外，實驗結果也證實 DP2 確實會提高呼吸道上皮細胞 BEAS-2B 以及 HBEpiC 的遷移與侵襲能力。

綜合上述研究結果，我們首次發現無蛋白酶活性的 DP2 具有誘發上皮細胞呼吸道上皮細胞產生 EMT 的現象與提升其遷移與侵襲能力，並證實 Erk1/2 與 Akt/GSK3β 的訊息路徑參與此一現象是透過活化，這些 DP2 誘發的訊息增加 Snail 與 Slug 的表現，並促進其核轉位(nuclear translocation)。據此，我們推測第二群塵蟎過敏原可利用此一機制造成上皮細胞屏障的隙漏(leaking)，進入體內誘發相關的致敏反應。

Abstract

Non-proteolytic group 2 allergen, Der p 2 (DP2) is known as a major allergen derived from house dust mite (HDM) *Dermatophagoides pteronissinus*. Paracellular epithelial barrier, being composed of a number of tight junction molecules, plays pivotal roles in resistance of pathogen invading. Although group-1 HDM allergens have been demonstrated to destroy epithelial linkage with their protease activity, the effects of DP2 on epithelial integrity are rarely investigated.

In the present study, we aimed to investigate the effects of DP2 on epithelial integrity with focus on expression of epithelial-mesenchymal transition (EMT), and the mechanism regulating the expression of the EMT markers. Human normal bronchial epithelial cell BEAS-2B and human primary bronchial epithelial cell HBEpiC were used as in vitro cell model. Acute sensitized Balb/c mice with DP2 was used as in vivo animal model. Gene expression was determined by using real-time quantitative PCR. Cell motility was accessed by wound healing and transwell migration assay. Immunoblot and immunohistochemistry was conducted for relative protein quantitation and involvement of signaling cascades.

In vitro analysis revealed that DP2 regulated expression of EMT markers, including E-cadherin, vimentin and alpha-smooth muscle actin (α-SMA), and promoted motility of the airway epithelial cells. DP2 also triggered signaling cascades including activation of Akt/extracellular response-regulated kinase (Erk)1/2, inhibition of glycogen synthase kinase-3β (GSK3β), and translocation of Snail/Slug. *In vivo* analysis also demonstrated that E-cadherin level was significantly decreased in the lung tissues of DP2-sensitized mice.

In conclusion, these findings demonstrate that the DP2 alters expression of EMT markers and enhances cell motility, attributing to Akt/GSK3β signaling and activation of Snail and Slug, and the consequent initiation of EMT. It is suggested that DP2 alone can modulate epithelial integrity via induction of EMT.

二、 研究背景

過敏 (allergy) 為免疫系統發生異常所導致的疾病，其中較為嚴重的症狀有過敏性休克與氣喘(1,2)。依據過去的研究顯示其致病機制與第二型助手型 T 細胞 T helper cell-2 (TH-2)所主導的免疫機制所產生(3)。一般認為過敏疾病的產生，是由於外來物 (過敏原) 的侵入，經抗原呈現細胞 (Antigen-presenting cells, APCs) 處理之後，活化 TH-2 細胞使得 B 細胞產生專一性的 IgE 抗體。當人體再次接觸到同一種過敏原的入侵時，過敏原會與肥大細胞 (mast cell) 或嗜鹼性白血球 (basophils) 上高親和力的 IgE 結合造成交叉連結 (cross-linking reaction)，進而使肥大細胞與嗜鹼性白血球產生去顆粒化 (degranulation) 反應而釋放出許多的發炎介質 (mediator)：如組織胺 (histamine)、白三烯素 (leukotrienes)、前列腺素 (prostaglandins) 與血小板活化因子 (platelet-activating factor, PAF) 等。這些介質會造成氣管平滑肌的收縮，黏液增加而導致氣喘等症狀(4)。

塵蟎為導致呼吸道過敏的最主要室內過敏來源，已有超過 20 種以上的塵蟎過敏原被發現、鑑定和研究(5)。其中以第一群的塵蟎過敏原最廣為人知，第二型塵蟎過敏原 (Group II-house dust mite allergen)，已知為一種主要過敏原(major allergen)，然而其致敏機制仍尚未被研究清楚。近年來的研究發現第二群歐洲室塵蟎過敏原 Der p 2 可透過 Toll-like receptor 4 的訊息途徑誘發先天性免疫反應 (6)。然而 Der p 2 是否具有影響第一道防線:呼吸道上皮細胞的完整性則仍未清楚。

我們先前的研究已建立酵母菌蛋白表現系統以及大腸桿菌蛋白表現系統，可以持續地表現與純化 Der p 2 重組蛋白(DP2)，並證實 DP2 可以調控人類肺癌細胞 A549 的 claudin-2 以及 ZO-1，以及 Akt/glycogen synthase kinase (GSK)3β 訊息傳導路徑的參與。在本研究中，我們建立兩種細胞模式與致敏動物模式，針對 DP2 是否透過誘發上皮間質轉換 EMT，進而影響呼吸道上皮細胞的完整性進行更深入的分子機制探討。

三、 研究方法

Expression and purification of DP2 and control protein GST

DP2, a fusion protein of Der p 2 with an N-terminal GST tag, and control protein GST were expressed in *E. coli* and purified using the same methods as previously described [11]. After filtrated with 0.22-μm sterile filter (Millipore, Bedford, MA), purified proteins were quantitated using BCA protein assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions and used for the following treatments.

Cell culture and treatments

Human bronchial epithelial HBEpiC cell (Cat no.3210) was purchased and cultured in the recommended cell medium from ScienCell Research Laboratories (Carlsbad, CA, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and subcultured according to resources' instructions.

For investigation of protein expression, cells at a density of 5 x 10^5/mL were incubated with DP2 at serial concentrations or GST protein at 20 μg/mL for 24 h. For investigation of kinase activation, cells at a density of 1 x 10^6/mL were incubated with DP2 at 20 μg/mL for indicated times. Induction of serine-9 phosphorylation at GSK3β (pS9-GSK3β) was achieved by treating cells with LiCl (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 5, 10 or 20 mM in DMEM. Inhibition of Erk1/2 or Akt activation was performed by pretreating cells with MEK1 inhibitor PD98059 at 20 μM or phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin at 200 nM for 30 min and following the DP2 treatments, respectively.

Subcellular fractionation

Enriched nuclear and cytosolic fraction was obtained as previously described [11]. Briefly, cells were washed with PBS and subsequently lyzed with HEPES buffer containing protease inhibitor cocktail, and then the lysates were centrifuged at 2,500 g for 10 min. The supernatant was further centrifuged at 20,000 g for 15 min at 4°C, namely cytosolic fraction. The pellets were washed with PBS, resuspended in the HEPES buffer containing additional 0.1% v/v Igepal CA-630, and centrifuged at 10,000 g for 15 min at 4 °C. The resulting supernatants were collected, namely nuclear fraction.

Immunoblotting

Cells were collected and lysed for protein extraction and the followed immunoblot as previously described [11]. Phosphorylation and level of protein was demonstrated by using antibodies against human cellular proteins, including E-cadherin, vimentin, α-SMA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphorylated Akt (p-Akt), total Akt (t-Akt), phosphorylated serine-9 of glycogen synthase kinase 3β (pS9-GSK3β), phosphorylation of Thr202/Tyr204 in Erk1/2 (p-Erk1/2), total Erk1/2 (t-Erk1/2), Snail, Slug and histone H1 (Cell signaling, Beverly, MA), and antibody against mouse E-cadherin (Novus Biologicals, Littleton, CO). The detection of antigen-antibody complex was performed by using ECL reagent (Millipore) and luminescence image system (LAS-4000; Fujifilm, Tokyo, Japan). Semi-quantitation of reacted signals was determined using Multi Gauge software version 2.2 (FujiFilm) and three independent immunoblot analyses were performed for statistical analysis.

Wound healing assay

Cells were seeded on 6-cm dishes at 1 x 10^6 cells/mL and cultured in DMEM supplemented with 10% FBS until 90% confluence, followed by starvation with serum-free DMEM for 16 h. The wound line was generated by scratching in cell monolayer using a sterile 10 μL-pipette tip, and the cells were subsequently exposed to DP2 or inhibitors in DMEM at indicated concentration for 24 h. Images of resulting culture were obtained at 0 and 24 h using a light microscopy with digital camera.

Transmigration assay

Transmigration assay was performed in 24-well Transwell plates (pore size: 8 μm; Costar, Cambridge, MA, USA) as previously described [12]. In brief, $5x10^4$ of cells were seeded on the upper chamber and treated with various concentrations of DP2 in serum-free DMEM, incorporating placement of 10% FBS/DMEM in the lower chamber. After 24-h incubation, the cells were fixed with methanol and then stained with crystal violet (Sigma-Aldrich). The cells that migrated to the lower surface of the membrane were counted. The average number of migrating cells from 5 randomly chosen fields on the lower surface of the membrane was counted. Three independent experiments were performed for statistical analysis.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA extraction of lung was performed by using RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was synthesized from total RNA by reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). qPCR was performed using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). For mRNA quantitation, FastStart Universal SYBR Green Master (Roche Applied Science, Mannheim, Germany) was used for Taqman PCR. Relative gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method [13]. The GADPH gene was used an internal endogenous control. All qPCR experiments were performed in duplicates for each sample. The correct size of the PCR products was confirmed by agarose gel electrophoresis.

Statistical analysis

Data were expressed as means ± SEMs of the three independent experiments. Statistical significance analysis was determined by using 1-way ANOVA followed by Dunnett's test for multiple comparisons with the control or the impaired 2-tailed Student *t* test. The differences were considered significant for *p* values less than 0.05.

四、 結果與討論

DP2 altered expression of EMT markers in HBEpiC cell

To investigate initiation of EMT on airway epithelial cells in response to DP 2, HBEpiC cells were stimulated with DP2 and levels of epithelial marker E-cadherin and mesenchymal makers vimentin and α-SMA were determined by immunoblot analysis. As shown in Figure 1A (upper panel), HBEpiC cells exposed to DP2 (5, 10, 20 μg/mL) resulted in a significant and dose-dependent reduction of E-cadherin level as compared to that in the cells exposed to GST (20 μg/mL). The levels of E-cadherin in HBEpiC cells treated with DP2 were reduced to 65.4 ± 2.6% of that treated with the GST (P <0.005). In addition, exposure to DP2 led to an increased protein level of mesenchymal markers vimentin and α-SMA in HBEpiC cells. Protein levels of vimentin and α-SMA in HBEpiC cells treated with DP2 were increased up to 223.4 ± 13.6% and 168.8 ± 14.2% of that treated with GST (P <0.005) (Figure 1A, lower panel).

In addition to protein levels, mRNA expression of the tested EMT markers in HBEpiC cells was further determined. As shown in Figure 1B, mRNA levels of E-cadherin were decreased in a dose-dependent manner in HBEpiC cells stimulated with DP2. Consistent with changes of protein levels, mRNA levels of vimentin and α-SMA were dose-dependently elevated in HBEpiC cells in response to DP2 treatments (Figure 1C and 1D). The level of E-cadherin mRNA in HBEpiC cells treated with DP2 was reduced to 0.29 ± 0.10-fold as compared to the cells treated with GST (P <0.01), and the levels of vimentin and α-SMA mRNA in HBEpiC cells treated with DP2 was increased up to 11.52 ± 1.24-fold and 5.82 ± 1.43-fold as compared to the cell treated with GST, respectively (P <0.01).

DP2 induced accumulation and nuclear translocation of Snail and Slug in HBEpiC cells

Transcription factors Snail (Snai1) and Slug (Snai2) are known as the critical E-cadherin repressors and the EMT inducers [14]. Since DP2 was found to regulate both the protein and mRNA expression levels of EMT markers, effects of DP2 on expression levels and cellular location of Snail and Slug in HBEpiC cells was further investigated. As shown in Figure 2A, DP2 treatments led to significant elevations in protein level of Snail and Slug. In addition, DP2 treatments further induced nuclear translocation of Snail and Slug in HBEpiC cells (Figure 2B), and the levels of nuclear Snail and Slug reached their

maximum in 90 min after DP2 treatment (20 μg/mL).

DP2 regulated levels of E-cadherin, vimentin and α-SMA in HBEpiC cells via Erk1/2 and Akt activation

Erk1/2 and Akt signaling have been reported to be involved in expression and activation of Snail and Slug [15, 16]. Therefore, we next investigated the effects of DP2 on activation of Erk1/2 and Akt in HBEpiC cells. As shown in Figure 3A, Erk1/2 and Akt were significantly phosphorylated in response to DP2 treatment (20 μg/mL) and reached peak phosphorylation at 30 min and 20 min after the treatments, respectively.

Involvement of Erk1/2 and Akt activation in DP2-triggered nuclear translocation of Snail and Slug and alteration of EMT markers was further demonstrated. As shown in Figure 3B, pretreatment of MEK inhibitor PD98059 and PI3K inhibitor Wortmannin significantly lowered nuclear levels of Snail and Slug in HBEpiC cells exposed to DP2. In addition, inhibition of Erk1/2 and Akt by PD98059 and Wortmannin restored E-cadherin level suppressed by DP2 treatment as well as diminished levels of vimentin and α-SMA elevated by DP2 treatment (Figure 3C). These findings indicated involvement of Erk1/2 and Akt activation in DP2-triggered core characteristics of EMT in HBEpiC cells.

DP2 regulated levels of E-cadherin, vimentin and α-SMA in HBEpiC cells through Akt-dependent inhibition of GSK3β

GSK3β is regarded as an important mediator that controls turnover and localization of Snail and Slug during EMT [17, 18]. Inhibition of GSK-3β leads to accumulation of Snail, downregulation of E-cadherin, and development of EMT in cultured epithelial cells [19]. Accordingly, effects of DP2 on GSK3β activation are determined. As shown in Figure 4A, DP2 significantly induced inhibitory phosphorylation of GSK3β at serine-9 (pS9-GSK3β) in a dose-dependent manner.

Activated Akt is known to inactivate GSK3β via triggering phosphorylation at serine-9. Therefore, involvement of DP2-triggered Akt and Erk1/2 activation in pS9-GSK3β was further examined. As shown in Figure 4B, DP2 significantly led to pS9-GSK3β in HBEpiC cells, which was diminished by pretreatment with PI3K inhibitor Wortmannin but not by Erk1/2 inhibitor PD98059. These findings indicated that DP2-triggered Akt

activation contributed to inhibitory phosphorylation of downstream GSK3β in HBEpiC cells.

Effects of pS9-GSK3β on alteration of EMT markers in HBEpiC cells were also demonstrated. As shown in Figure 4C, treatments of GSK3β inhibitor LiCl (5, 10, 20 mM) induced pS9-GSK3β in a dose-dependent manner, and E-cadherin levels were decreased in proportional to pS9-GSK3β levels. Conversely, levels of vimentin and α-SMA were increased in proportional to pS9-GSK3β levels.

DP2 promoted motility of HBEpiC cells via Akt/GSK3β signaling

Promoted cell motility of airway epithelial cell has been recognized as an important characteristic of EMT in airway hypersensitiveness [20]. Therefore, effects of DP2 on cell motility were investigated. As shown in Figure 5A and 5B, DP2-treated HBEpiC cells exhibited enhanced cell migration and transmigration with significance using wound healing assay and transwell migration assay, respectively (P <0.01 for 10 and 20 μg/mL DP2 as compared to GST control). Moreover, the enhanced cell migration and transmigration induced by DP2 were diminished in presence of Wortmannin but slightly affected by PD98059 (p <0.01 as compared to 20 μg/mL DP2 alone) (Figure 5C and 5D). Furthermore, effects of GSK3β on cell motility were also demonstrated. As shown in Figure 5C and 5D, motility of HBEpiC cells was enhanced by inhibition of GSK3β upon 20 mg/mL LiCl treatment (p <0.05 as compared to GST control).

E-cadherin expression was diminished in lung tissue of DP2-sensitized mice

To investigate the influence of DP2 on E-cadherin expression in airway epithelium *in vivo*, E-cadherin in lung tissues of DP2-sensitized mice was determined by immunohistochemical analysis. Our results revealed that E-cadherin expression was significantly reduced in lung tissues of DP2-sensitized mice as compared to that of PBS- or GST-sensitized mice (Fig. 6A). Moreover, mRNA level of E-cadherin in the lung was also relatively quantitated by using qPCR. As shown in Figure 6B, mRNA expression of E-cadherin in lungs from rDP2-sensitized mice was diminished to 56.1 ± 8.4% as compared to that in lungs from PBS-treated mice (n=12 for each group, P <0.01). Collectively, these results indicated that both protein level and mRNA expression of E-cadherin was significantly lowered in lungs of mice in responses to DP2-sensitization.

圖表說明

Figure 1. **DP2 reduced E-cadherin and elevated vimentin and α-SMA expression in HBEpiC cells.**

Cells were treated with DP2 at indicated concentrations or treated with control protein GST at 20 μg/mL for 24 h (**A**) or 6 h (**B - D**), and then the cells were lyzed for immunoblotting or qPCR analysis. Quantitative analysis for immunoblotting was acquired by densitometer and level of GAPDH was used as internal control. Three independent experiments were performed for statistical analysis. *, ** and ***, p <0.05, p <0.01 and p <0.005 as compared to GST control.

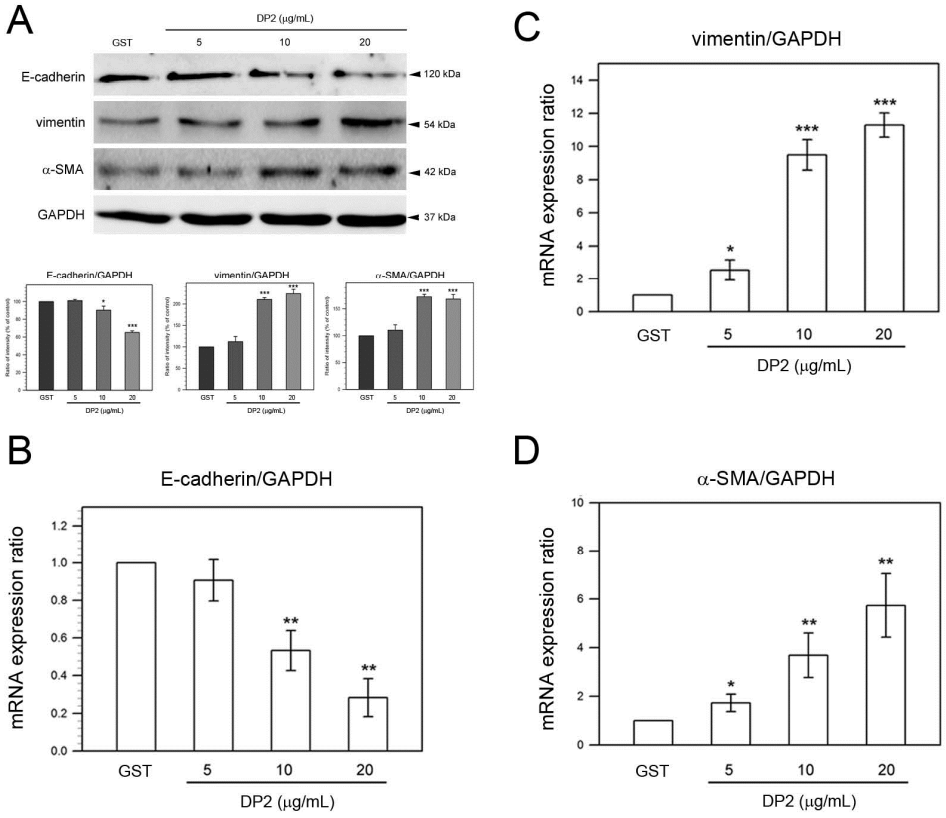


Figure 2. **DP2 elevated level of Snail and Slug and enhanced their nuclear translocation in HBEpiC cells**.

(**A**) Cells were treated with DP2 at indicated concentration for 24 h, and the level of total protein was determined by immunoblotting. (**B**) Cells were treated with 20 μg/mL DP2 for indicated time (min), and the levels of nuclear and cytosolic targets were determined by immunoblotting. Histone H1 and GAPDH was used as nuclear and cytosolic marker.

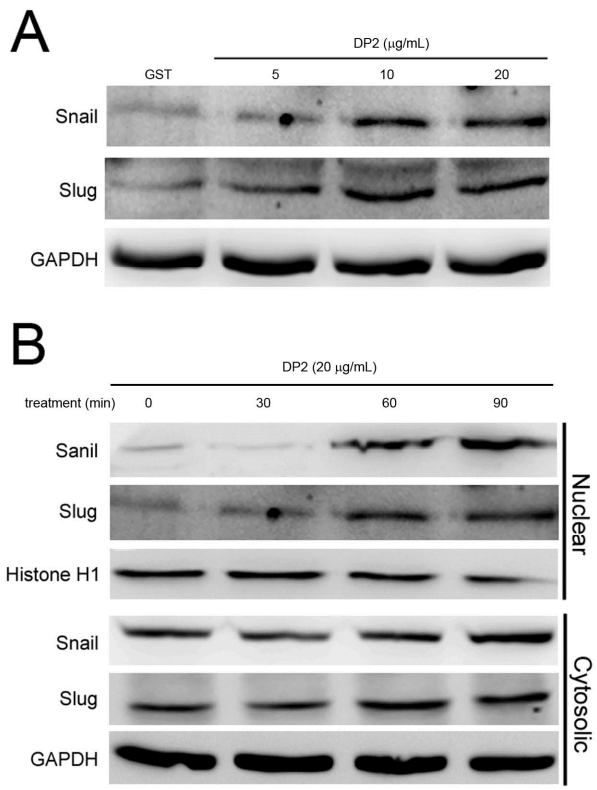


Figure 3. **Involvement of Akt and Erk1/2 activation in regulation of EMT markers in HBEpiC cells treated with DP2.**

(**A**) Cells were treated with DP2 at 20 μg/mL for 10, 20 and 30 min, and then lysed for immunodetection of p-Erk1/2, t-Erk1/2, p-Akt and t-Akt. Cells were pretreated with PD98059 or Wortmannin for 1 h, and then treated with DP2 at 20 μg/mL for 24 h. Treated cells were lysed for immunodetection of (**B**) the nuclear fraction of Snail and Slug or (**C**) E-cadherin, vimentin and α-SMA.

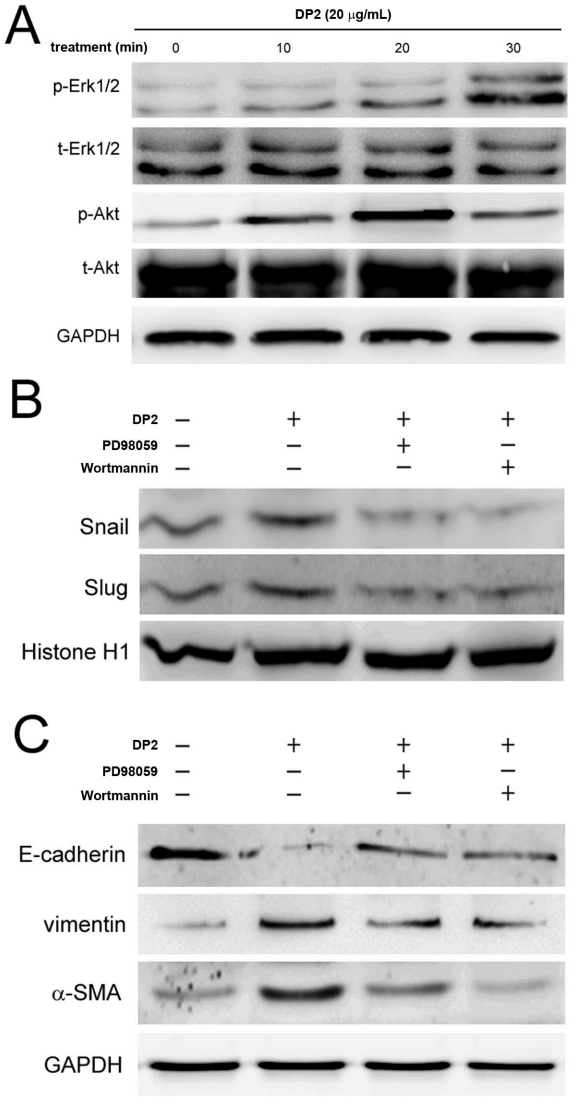


Figure 4. **DP2 regulated expression of EMT markers via Akt-dependent inhibition of GSK-3β.**

(**A**) Cells were treated with DP2 at 20 μg/mL for 10, 20 and 30 min, and then lysed for immunodetection of pS9-GSK-3β. (**B**) Cells were pretreated with PD98059 or Wortmannin for 1 h, and then treated with DP2 at 20 μg/mL for 24 h. The treated cells were lysed for immunodetection of p-Erk1/2, p-Akt and pS9-GSK-3β. (**C**) Cells were treated with LiCl at indicated concentration for 24 h, and then lyzed for immunodetection of pS9-GSK-3β and the indicated EMT markers. Level of GAPDH was used as internal control.

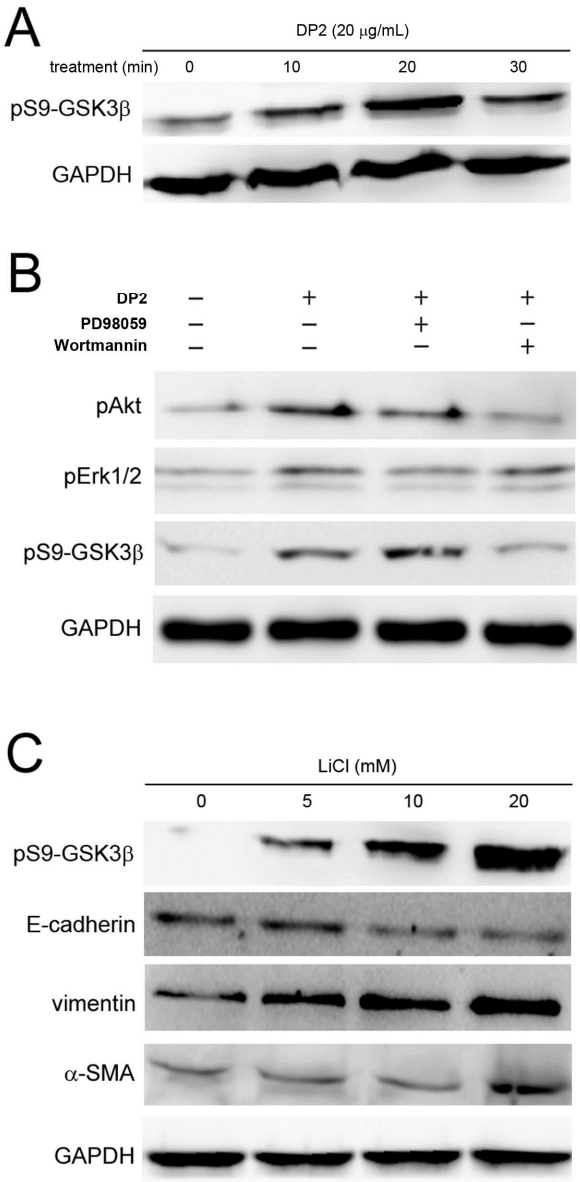


Figure 5. DP2 promoted cell motility of HBEpiC cells through Akt/GSK-3β and Erk1/2 signaling.

Cells treated with DP2 at indicated concentration for 24 h were used for (**A**) wound healing assay or (**B**) transwell migration assay. Cells pretreated with individual inhibitor for 1 h and then treated with DP2 at 20 μg/mL for 24 h, or treated with 20 mg/mL LiCl, were used for (**C**) wound healing assay or (**D**) transwell migration assay. Three independent experiments were performed for statistical analysis. #, p <0.05 as compared to GST control. **, p <0.01 as compared to DMSO control.

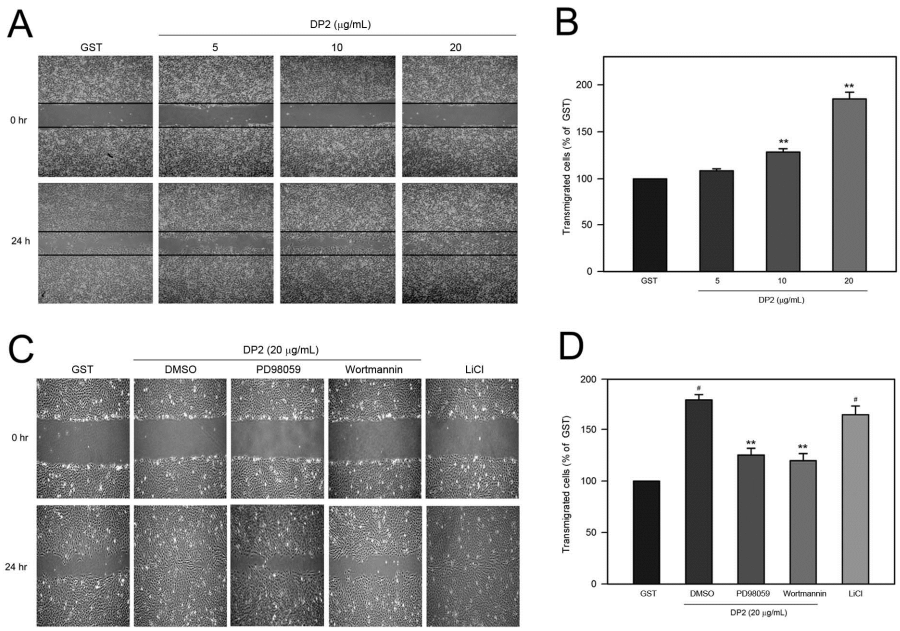
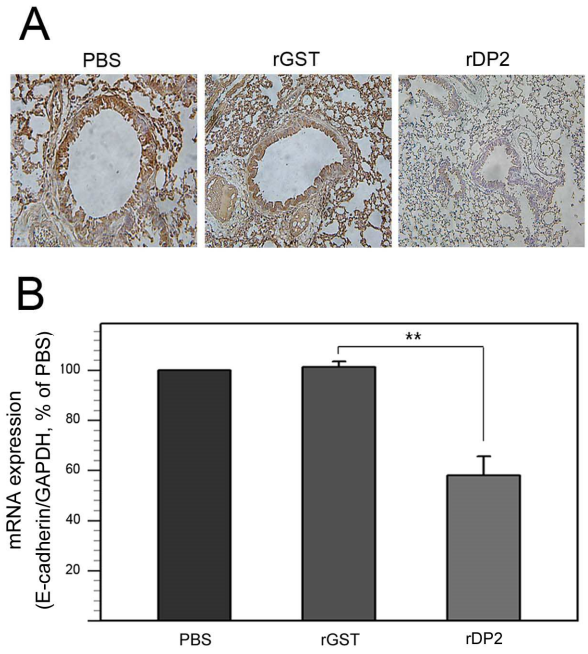


Figure 6. **Reduced level of E-cadherin in lung tissues from rDP2-sensitized mice.** (**A**) Lung tissues from rDP2-sensitized mice were analyzed by IHC staining using mAb against mouse E-cadherin. (**B**) Total RNA was extracted from lung tissues of rDP2-sensitized mice and mRNA expression of E-cadherin was quantitated by using qPCR. **, p <0.01 as compared to rGST control.



五、參考文獻

1 Thomas, W. R., Smith, W. A., Hales, B. J., Mills, K. L. and O'Brien, R. M. (2002) Characterization and immunobiology of house dust mite allergens. Int Arch Allergy Immunol. **129**, 1-18

2 Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R. S., Madan, R., Thorne, P. S., Wills-Karp, M., Gioannini, T. L., Weiss, J. P. and Karp, C. L. (2009) Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. Nature. **457**, 585-588

3 Osterlund, C., Gronlund, H., Polovic, N., Sundstrom, S., Gafvelin, G. and Bucht, A. (2009) The non-proteolytic house dust mite allergen Der p 2 induce NF-kappaB and MAPK dependent activation of bronchial epithelial cells. Clin Exp Allergy. **39**, 1199-1208

4 de Boer, W. I., Sharma, H. S., Baelemans, S. M., Hoogsteden, H. C., Lambrecht, B. N. and Braunstahl, G. J. (2008) Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. Canadian journal of physiology and pharmacology. **86**, 105-112

5 Tillie-Leblond, I., Gosset, P. and Tonnel, A. B. (2005) Inflammatory events in severe acute asthma. Allergy. **60**, 23-29

6 Zavadil, J. and Bottinger, E. P. (2005) TGF-beta and epithelial-to-mesenchymal transitions. Oncogene. **24**, 5764-5774

7 Hay, E. D. (2005) The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. Developmental dynamics : an official publication of the American Association of Anatomists. **233**, 706-720

8 Kim, K. K., Wei, Y., Szekeres, C., Kugler, M. C., Wolters, P. J., Hill, M. L., Frank, J. A., Brumwell, A. N., Wheeler, S. E., Kreidberg, J. A. and Chapman, H. A. (2009) Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. J Clin Invest. **119**, 213-224

9 Holgate, S. T., Davies, D. E., Lackie, P. M., Wilson, S. J., Puddicombe, S. M. and Lordan, J. L. (2000) Epithelial-mesenchymal interactions in the pathogenesis of asthma. J Allergy Clin Immunol. **105**, 193-204

10 Heijink, I. H., Postma, D. S., Noordhoek, J. A., Broekema, M. and Kapus, A. (2010) House dust mite-promoted epithelial-to-mesenchymal transition in human bronchial epithelium. Am J Respir Cell Mol Biol. **42**, 69-79

11 Wang, W. C., Tsai, J. J., Kuo, C. Y., Chen, H. M. and Kao, S. H. (2011) Non-proteolytic house dust mite allergen, Der p 2, upregulated expression of tight junction molecule claudin-2 associated with Akt/GSK-3beta/beta-catenin signaling pathway. Journal of cellular biochemistry. **112**, 1544-1551

12 Lin, X. H., Grako, K. A., Burg, M. A. and Stallcup, W. B. (1996) NG2 proteoglycan and the actin-binding protein fascin define separate populations of actin-containing filopodia and lamellipodia during cell spreading and migration. Molecular biology of the cell. **7**, 1977-1993
13 Livak, K. J. and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. **25**, 402-408
14 Peinado, H., Olmeda, D. and Cano, A. (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nature reviews. Cancer. **7**, 415-428
15 Maseki, S., Ijichi, K., Tanaka, H., Fujii, M., Hasegawa, Y., Ogawa, T., Murakami, S., Kondo, E. and Nakanishi, H. (2012) Acquisition of EMT phenotype in the gefitinib-resistant cells of a head and neck squamous cell carcinoma cell line through Akt/GSK-3beta/snail signalling pathway. British journal of cancer. **106**, 1196-1204
16 Zhao, H. B., Wang, C., Li, R. X., Tang, C. L., Li, M. Q., Du, M. R., Hou, X. F. and Li, D. J. (2010) E-cadherin, as a negative regulator of invasive behavior of human trophoblast cells, is down-regulated by cyclosporin A via epidermal growth factor/extracellular signal-regulated protein kinase signaling pathway. Biology of reproduction. **83**, 370-376
17 Zhou, B. P., Deng, J., Xia, W., Xu, J., Li, Y. M., Gunduz, M. and Hung, M. C. (2004) Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nature cell biology. **6**, 931-940
18 Kim, J. Y., Kim, Y. M., Yang, C. H., Cho, S. K., Lee, J. W. and Cho, M. (2012) Functional regulation of Slug/Snail2 is dependent on GSK-3beta-mediated phosphorylation. The FEBS journal. **279**, 2929-2939
19 Doble, B. W. and Woodgett, J. R. (2007) Role of glycogen synthase kinase-3 in cell fate and epithelial-mesenchymal transitions. Cells, tissues, organs. **185**, 73-84
20 Johnson, J. R., Roos, A., Berg, T., Nord, M. and Fuxe, J. (2011) Chronic respiratory aeroallergen exposure in mice induces epithelial-mesenchymal transition in the large airways. PloS one. **6**, e16175
21 Trautmann, A., Kruger, K., Akdis, M., Muller-Wening, D., Akkaya, A., Brocker, E. B., Blaser, K. and Akdis, C. A. (2005) Apoptosis and loss of adhesion of bronchial epithelial cells in asthma. Int Arch Allergy Immunol. **138**, 142-150
22 Hackett, T. L., Singhera, G. K., Shaheen, F., Hayden, P., Jackson, G. R., Hegele, R. G., Van Eeden, S., Bai, T. R., Dorscheid, D. R. and Knight, D. A. (2011) Intrinsic phenotypic differences of asthmatic epithelium and its inflammatory responses to respiratory syncytial virus and air pollution. Am J Respir Cell Mol Biol. **45**, 1090-1100

23 Xiao, C., Puddicombe, S. M., Field, S., Haywood, J., Broughton-Head, V., Puxeddu, I., Haitchi, H. M., Vernon-Wilson, E., Sammut, D., Bedke, N., Cremin, C., Sones, J., Djukanovic, R., Howarth, P. H., Collins, J. E., Holgate, S. T., Monk, P. and Davies, D. E. (2011) Defective epithelial barrier function in asthma. J Allergy Clin Immunol. **128**, 549-556 e541-512

24 Nawijn, M. C., Hackett, T. L., Postma, D. S., van Oosterhout, A. J. and Heijink, I. H. (2011) E-cadherin: gatekeeper of airway mucosa and allergic sensitization. Trends in immunology. **32**, 248-255

25 Olmeda, D., Jorda, M., Peinado, H., Fabra, A. and Cano, A. (2007) Snail silencing effectively suppresses tumour growth and invasiveness. Oncogene. **26**, 1862-1874

26 Fenouille, N., Tichet, M., Dufies, M., Pottier, A., Mogha, A., Soo, J. K., Rocchi, S., Mallavialle, A., Galibert, M. D., Khammari, A., Lacour, J. P., Ballotti, R., Deckert, M. and Tartare-Deckert, S. (2012) The epithelial-mesenchymal transition (EMT) regulatory factor SLUG (SNAI2) is a downstream target of SPARC and AKT in promoting melanoma cell invasion. PloS one. **7**, e40378

27 Chiou, Y. L. and Lin, C. Y. (2009) Der p2 activates airway smooth muscle cells in a TLR2/MyD88-dependent manner to induce an inflammatory response. J Cell Physiol. **220**, 311-318

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： 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）

本研究首次證實不具蛋白水解活性的過敏原 DP2，具有調控呼吸道上皮細胞連結蛋白的表現，進而誘發上皮間-質轉換 EMT 的能力。臨床證據顯示 EMT 可能在慢性呼吸道疾病如氣喘與慢性阻塞性肺病 COPD 的致病機轉與進程惡化上扮演重要的角色，因此本研究的結果延伸我們對於第二群塵蟎過敏原在這些慢性呼吸道疾病的認知與了解，未來有機會藉此找出減緩疾病惡化的分子標的並進一步開發相關的治療策略與藥物。

無研發成果推廣資料

99 年度專題研究計畫研究成果彙整表

計畫主持人：高紹軒	計畫編號：99-2320-B-040-003-MY3
計畫名稱：第二群塵虫滿過敏原與呼吸道上皮細胞之上皮-間葉細胞轉換以及氣喘發展的分子機制研究	

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	1	1	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%		
	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	1	1	100%	人次	
		博士生	2	2	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。)	獲邀擔任宜蘭大學生物科技暨動物科學系所【102 年度轉譯醫學及農學人才培育先導型計畫】專題講員、【101 年度新竹市藥師公會繼續教育學程】專題講員、台灣調適科學會【2012 傳統中藥與環境壓力調適國際研討會】專題演講講員以及行政院退輔會台中榮民總醫院【101 年度學術專題演講】講員。參與多種國際期刊之審查委員(reviewer)與擔任編審委員(Editor, TNAG, eCAM)。

	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	

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：

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)

本研究首次證實不具蛋白水解活性的過敏原 DP2，具有調控呼吸道上皮細胞連結蛋白的表現，進而誘發上皮間-質轉換 EMT 的能力。臨床證據顯示 EMT 可能在慢性呼吸道疾病如氣喘與慢性阻塞性肺病 COPD 的致病機轉與進程惡化上扮演重要的角色，因此本研究的結果延伸我們對於第二群塵蟎過敏原在這些慢性呼吸道疾病的認知與了解，未來有機會藉此找出減緩疾病惡化的分子標的並進一步開發相關的治療策略與藥物。