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作品名稱 **肺癌浸潤之樹突細胞分泌 Resistin 透過活化 WHSC1/Twist 途徑促進肺癌惡化**

得獎獎項 **一等獎**

美國 ISEF 正選代表:美國第 64 屆國際科技展覽會青少年科學獎

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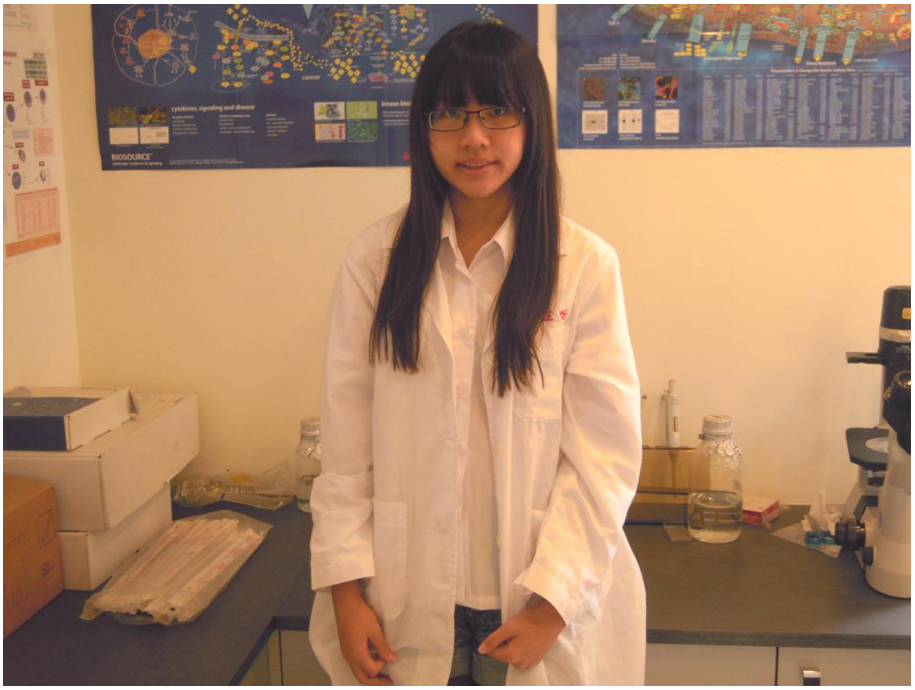
關 鍵 字　Resistin 、 WHSC1 、 Twist

作者簡介



我是郭芷忻，目前就讀高雄師範大學附屬高級中學。因父母皆從事科學研究相關工作，從小便時常進入研究室學習一些實驗技術，例如細胞培養、DNA 抽取及電泳、蛋白質定量和 Western blot 等，去年便開始進行此次科展作品。進行過程亦利用課餘時間參與教育部舉辦之實驗室安全衛生研習營、生資中心所舉辦 2012 Cell Culture Workshop、高雄醫學大學附設中和紀念醫院所舉辦的實驗動物中心使用說明會，以及 2012 人體試驗實務研討會。透過實驗室的研究經驗和各項研習營的參與，不僅奠定探討生物醫學的研究技術和基本知識，亦增加對挫折的忍受力及培養邏輯思考的能力。希望將來可以出國深造，開拓自己的視野。

摘要

本研究首度發現人類肺癌A549細胞會促進其所浸潤的樹突細胞分泌Resistin，而更深入地探究獲悉 Resistin 會透過活化 WHSC1/Twist 途徑促進肺癌 A549 細胞惡化，此惡化過程包括誘導癌細胞上皮間質轉化(epithelial-to-mesenchymal transition; EMT)及提升癌細胞的移行(migration)和入侵(invasion)能力。為確認 Resistin 在臨床的重要性，透過肺癌病患檢體分析發現，相較於健康捐贈者，肺癌病人的血清可測的較高濃度的 Resistin；更甚之，比較非腫瘤組織部位之 CD11c+樹突細胞，浸潤於腫瘤組織部位之 CD11c+樹突細胞會呈現高量的 Resistin。接續探討 Resistin 對肺癌細胞的影響機制，實驗結果發現 Resistin 會增加 A549 細胞表現 histone methyltransferase WHSC1 的表現，而 WHSC1 在 Twist 啟動子的 H3 組蛋白 lysine 36 位置進行 dimethylation 修飾，並降低 H3 組蛋白 lysine 27 位置的 trimethylation 進而促進 Twist 的表現，促使 A549 細胞進行 EMT 和增加癌細胞移行和入侵。因此，Resistin 可作為肺癌診斷分子及藥物發展的重要標靶。

Abstract

The interaction between tumors and the microenvironment leads to a vicious cycle which strengthens both immune suppression and cancer progression. The present study demonstrates for the first time that tumor associated dendritic cells (TADCs) can be a source of resistin, which is responsible for increasing lung cancer migration and invasion and the epithelial-to-mesenchymal transition (EMT). In addition, large amounts of resistin in the conditioned medium (CM) of TADCs increased cell migration and invasion as well as osteolytic bone metastases properties in A549 cells. Neutralization of resistin from TADC-CM prevented the advanced malignancy-inducing features of TADC-CM. Significantly elevated levels of resistin have been observed in tumor-infiltrating CD11c+ DCs in human lung cancer samples and patients' sera. Induction of lung cancer progression by TADC-derived resistin is associated with an increased expression of Wolf-Hirschhorn syndrome candidate 1 (WHSC1), a histone methyltransferase. Resistin-induced WHSC1 increased the dimethylation of histone 3 at lysine 36 and decreased the trimethylation of histone 3 at lysine 27 on the promoter of Twist, resulting in an enhancement of the expression of Twist. Knockdown of WHSC1 by siRNA transfection significantly decreased resistin-mediated cancer progression by decreasing the upregulation of Twist, suggesting that WHSC1 plays a critical role in the regulation of Twist by epigenetic modification. These findings suggest that TADC-derived resistin may be a novel candidate in conferring the ability for lung cancer to develop.

壹、研究背景

一、肺癌現況及臨床問題

在台灣，根據行政院衛生署的統計，自從 1982 年開始，癌症一直是國人十大死因的榜首，其中 10-15%更因肺癌而致死。肺癌目前仍居癌死亡率的首位，並且有逐年增加的趨勢(行政院衛生署 2012 年網站資料)。由於初期的肺癌並不容易診斷，因此大多數的病人在被診斷出肺癌時，常發現已合併遠處器官轉移。雖然近年來醫學已有相當進展，但是肺癌的治療成果還是有很大的改善空間，且現今仍無法有效的防範復發，以及復發後癌細胞產生抗藥性的問題。肺癌可分為小細胞肺癌(small cell lung cancer)和非小細胞肺癌(non-small cell lung cancer)兩大類，後者主要包括腺癌、鱗狀細胞癌與大細胞癌三種類型(O'Connor, 2011; Demicheli et al, 2012)。在台灣，小細胞肺癌佔肺癌病例的少部分，約 12~15%；相對的，非小細胞癌則佔多數，約 85~88%。小細胞肺癌生長速度較快，而且容易侵犯並轉移到他處 (Mok, 2011; Siegel et al., 2011)。但是，小細胞肺癌對化學及放射線治療較具敏感性，因此在臨床治療以系統性化學治療為主，但是其具有極高的復發率，且復發後之癌細胞對化療藥物會產生極高的抗性。相對於小細胞肺癌，非小細胞肺癌早期無明顯症狀，因此在病人被診斷出時多屬晚期，只有少數(約四分之一)病人適於接受外科治療。但由於非小細胞癌在手術後有極高的復發率且容易經由血行而轉移至他處；更甚之，無法以外科手術處理之非小細胞肺癌對化學藥物及放射線治療大多不敏感(Mok, 2011; Siegel et al., 2011)。整體而言，肺癌病人的預後不佳，5 年的存活率僅約 10% (O'Connor, 2011)。所以，尋求一個能預防肺癌的發生且安全又有效的治療藥、或是能預防手術後再發的有效藥物乃當務之要。

二、癌症病人之免疫問題

樹突細胞(Dendritic cell, DC)是由骨髓所衍生出的細胞 (bone marrow-derived cell)，為具專一的抗原呈現細胞(antigen-presenting cells, APCs)；也被認為是決定 T 細胞的 priming/tolerance 主要的因子之一(Apetoh et al., 2011)。當樹突細胞受到外來或內在抗原的刺激，位於末梢組織的樹突細胞會經過一連串包括分化(differentiation)、成熟(maturation)和活化(activation)的複雜過程後產生各項功能，如抗原的捕捉(capture)和呈現(present)、移行至發炎部位及淋巴組織、並分泌各種細胞激素活化 $CD4^+$和 $CD8^+$ T 細胞(Chaput et al., 2008)進而殲滅外來或不正常抗原。在發炎的情況下，樹突細胞可成熟活化而啟動 T 細胞的免疫反應。相對的，若樹突細胞處在於非發炎性的環境，則樹突細胞會啟動抑制性免疫的反應(Chaput et al., 2008)。樹突細胞對於維持腫瘤的免疫特性扮演相當重要的角色，腫瘤細胞含有許多可讓宿主辨識的抗原，經樹突細胞辨識、加工後，可呈現給 T 細胞活化腫瘤專一的 T 細胞免疫(tumor-specific T cell response)。然而，在腫瘤發展的過程中，腫瘤不但會營造一個特殊的微環境(microenvironment)使樹突細胞無法成熟和活化；更甚之，腫瘤會藉由分泌一些可溶性分子(如 prostaglandin E_2 、IL-6、IL-10、M-CSF 和 vascular endothelial growth factor 等)抑制樹突細胞的分化、成熟和活化。此時，樹突細胞不但不能活化 T 細胞，反而可能促進樹突細胞活化免疫抑制性的T細胞(如 Regulatory T cell; Treg) (Kuo et al., 2011; Ma et al., 2011; Steer et al., 2010)。再者，先前高雄醫學大學醫學院醫學系黃明賢教授也發現肺癌細胞會促進腫瘤所浸潤之樹突細胞分泌大量的生長因子包括 HB-EGF 和 amphiregulin 促進腫瘤的生長、提升癌細胞爬行和入侵能力(Hsu et al., 2011; Kuo et al., 2012)；而在老鼠的實驗也證實若能抑制這些樹突細胞所分泌的促腫瘤發展因子，則能有效緩解腫瘤細胞在老鼠的發展及增加老鼠存活率(Hsu et al., 2011)。但是，是否有其他因子的參與，以及其所調控的機制仍屬未知。深入

探究腫瘤細胞和樹突細胞間的交互作用，包括了解腫瘤所浸潤的樹突細胞如何參與調控腫瘤的惡化以及透過分泌何種 cytokine 或可溶性分子，將有助於了解免疫機制在腫瘤發展的角色，並且有利於相關預防策略及治療藥物的開發。

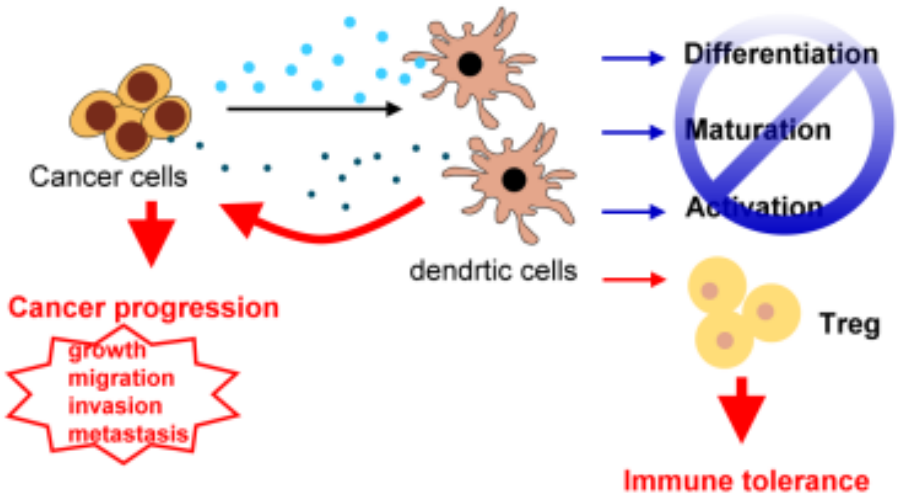


圖1　肺癌細胞與樹突細胞間之交互作用

三、癌症上皮-間質轉化(Epithelial-mesenchymal transition, EMT)及轉移 (metastasis)過程

癌症相關死亡因素中，高達 90%是因轉移所致(Turk et al., 2011; Siegel et al., 2011)。癌細胞的轉移(metastasis)是一複雜的過程，當癌細胞大量不正常增生時，會促進癌細胞周遭血管新生，以供給大量的養分給癌細胞，接著這些原位癌細胞(Primary tumor)會開始改變，並啟動複雜的訊號途徑，促進其開始由原位轉移至其他器官(Gao et al., 2012)。由於肺癌源自於上皮細胞(epithelial cells)，因此細胞間會有許多緊密結合(tight junction)，而呈現低移行(migration)和低入侵(invasion)的表現型(phenotype)。一旦腫瘤細胞開始要進行轉移，細胞會進行上皮-間質轉化(Epithelial-mesenchymal transition, EMT)－即癌細胞會減

少細胞間交互作用的黏著分子(E-cadherin)，細胞型態由上皮規則型態轉為具有高移行和高入侵之紡錘型的間質細胞型態(mesenchymal type)；並使癌細胞能脫離原位癌母細胞群(Gao et al., 2012)，產生較高的移動力。同時，這些轉化過的癌細胞會開始表現大量的基質金屬蛋白水解酶(Matrix metalloproteinases, MMPs)分解以 collagen 與 laminin 等蛋白所組成的基底膜(basement membrane) (Heinrich et al., 2012)，並使癌細胞可進一步穿透富含 collagen 與 fibronectin 的細胞外基質(Extracellular matrix, ECIM) (Orlichenko et al., 2008)，並利用滲入(intravasation)方式進入到體內循環系統，透過淋巴與血行轉移至他處，形成新的次發性腫瘤(Secondary tumor) (Soini, 2012)。

EMT 對於細胞的型態和功能具多重影響。上皮細胞間透過 adherens junction、desmosomes、tight junctions 和 gap junction 緊密結合。另外，上皮細胞期還有 aligned apical-basal polarity 的特性，只能於 basement membrane 表面進行側向移動，無法入侵至 basement membrane 下層的細胞基質(Soini, 2012)。相對的，間質細胞外觀如紡錘體，細胞間少有緊密的接觸面，而以交點式相接觸(focal adhesion)。再者，間質型態細胞通常表現大量可分解細胞基質的酵素，因此使間質細胞有高度移行和入侵能力，可以破壞 ECM 進而滲入血行或淋巴循環系統(Soini, 2012)。EMT 過程可根據三種特性的轉化定義：首先，細胞型態從上皮細胞的鵝卵石型態開始伸出突觸轉化成間質細胞的紡錘體形態；再者，上皮細胞間接觸蛋白與細胞骨架蛋白會轉換，上皮細胞之接觸蛋白如 E-cadherin 和 Claudin-3 等) 表現會減少，相對地，間質標誌(mesenchymal markers)如 N-cadherin、vimentin 和 Fibronectin 等表現會增加；最後，上皮細胞會由低移行和低入侵的表現型，轉換為高移行和高入侵的表現型(Soini, 2012)。

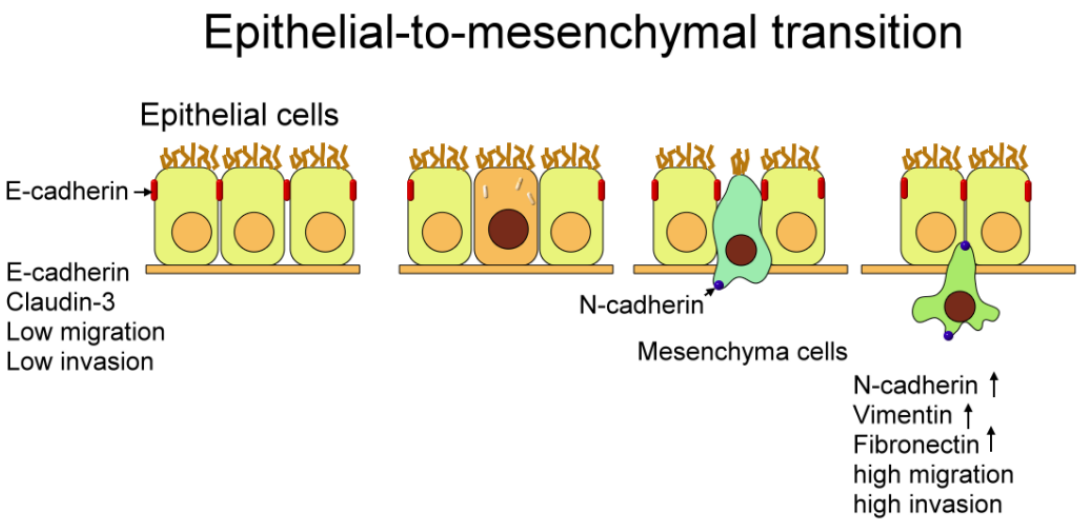


圖 2　腫瘤細胞經過 EMT 後增加移行和入侵的能力

四、Resistin

Resistin 屬於新的脂肪素家族蛋白(adipokine family protein)，目前已被證實參與多種人類疾病的發生，如肥胖、糖尿病、胃腸道疾病和癌症等(Tiaka et al., 2011; McTernan et al., 2002)。雖然 Resistin 被分類在 adipokine，但是除了脂肪組織，其他組織包括各種血球細胞(PBMC、macrophagy 和 bone marrow)、肌肉細胞、脾臟及胰臟細胞皆會分泌 Resistin (Tiaka et al., 2011; Filková et al., 2012)。目前多數研究認為脂肪組織並非人體 Resistin 最主要的來源，相對的，發炎性的非脂肪組織(inflammation-occupied non-adipose tissues)被認為是血清中 Resistin 最主要的來源(Filková et al., 2012)。單核球(mononuclear cells)被證實可分泌大量的 Resistin，且 Resistin 的表現會進一步促進多種發炎性物質包括 IL-1β、IL-6、IL-8 和 IL-12 的分泌造成局部的發炎反應。在乳癌、血癌和大腸直腸癌病人的研究也發現血清中 Resistin 的濃度有增加的趨勢(Sun et al., 2010; Gonullu et al., 2010)。 除此之外，Resistin 也被發現可透過 PI3K/AKT 途徑增加前列腺癌細胞株 PC-3 和 DU145 的增生(Kim et al., 2011)。另外，Resistin 亦可藉由改變 TIMP-2/MMP-2 的平衡而促進 trophoblast-like cells 的入侵能力

(Di et al., 2006)。在骨骼的代謝方面，Resistin 也被發現可透過增加 IL-6 表現及降低 RANKL/OPN 的比例而促進 osteoclast 的分化而造成骨溶蝕的現象(Filková et al., 2009)。到目前為止，Resistin 對於肺癌的作用情形仍屬未知。

五、表基因(epigenetic)修飾及 histone methyltransferase WHSC1

真核細胞的 DNA 纏繞在的由 4 個組蛋白(histone)所形成的核心核蛋白形成一特殊結構和核小體(nucleosome) 。核小體可進一步和非組蛋白結合形成所謂的染色質(chromatin)。染色質結構上的緊密程度，以及位於染色質上的非組蛋白因子(non-histone proteins)，如轉錄因子(transcription factor)等調控 DNA 的表現、複製、轉錄和修復。其中，透過對組蛋白或 DNA 做一些化學性修飾可影響染色質結構或 DNA 帶電性，進而影響 DNA 的轉錄與否。此一藉由不改變 DNA 序列但仍可影響 DNA 表現的調控機制被定義為表基因調控(epigenetic regulation) (Nelson et al., 2006)。核心組蛋白由四個 3 種組蛋白複合成，包括 H2A、H2B、H3 和 H4。而 H3 和 H4 在蛋白質的 N 端投射出的尾端是最常被進行化學性修飾進而影響基因表現的地方。目前研究證實對於 H3 和 H4 組蛋白可進行十六種的化學式修飾，包括乙醯化(acetylation)、甲基化(methylation)、磷酸化(phosphorylation)和泛素化(ubiquitination) 等。甲基化修飾的位置通常在 H3 或 H4 蛋白的精胺酸(arginine；簡稱 R)或離胺酸(lysine；簡稱 K)；在 Lysine 可進行單甲基化 (monomethyl-)、雙甲基化(dimethyl-)和三甲基化(trimethyl-)修飾；在 arginine 則是進行單甲基化、對稱性和不對稱性的 dimethylation (Xu et al., 2008)。Lysine 的 methylation 修飾依照甲基化程度和胺基酸位置對於基因的表現會產生不同的影響：如在 H3K4 (H3 組蛋白的位於位置 4 的 lysine)、H3K36 和 H3K79 進行 methylation 會促進基因的表現；相對的，在 H3K9 、H3K27 和 H4K20 的 methylation 則與抑制基因表現有關(Xu et

al., 2008)。目前研究已經證實不正常的 epigenetic 調控和癌症的發展息息相關，同時，以調控表基因相關分子作為治療標靶也成癌症治療的新方向。

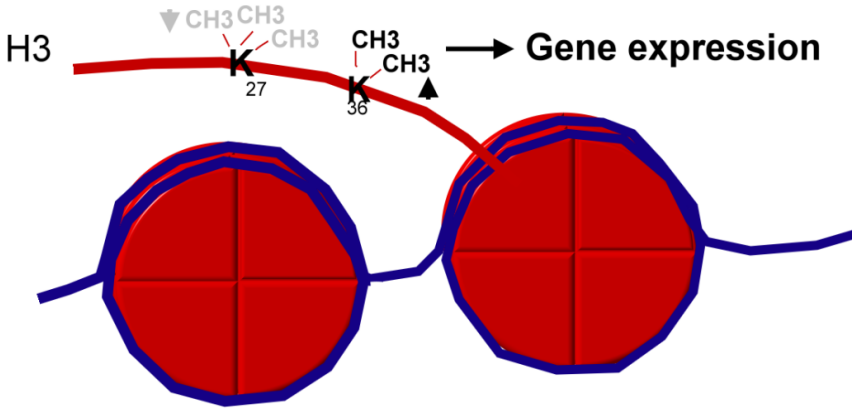


圖 3　增加 H3K36 dimethylation 和降低 H3k27 trimethylation 促進標的基因表現

WHSC1 (亦稱為 NSD2 或 MMSET)基因位於 4p16.3 染色體，其所轉錄之蛋白為 WHSC1 蛋白，為一種 histone lysine methyltransferase (Stec et al., 1998)。*WHSC1* 基因的缺失會造成遺傳疾病 Wolf-Hirschhorn 症候，導致胚胎的過度增生和畸形胎(Sharathkumar et al., 2007)。目前 WHSC1 的受質蛋白仍未完全清楚，但是有多個研究證實 WHSC1 可進行 H3K36 的雙甲基化修飾 (Nimura et al., 2009)，調控著細胞的生長和貼附。乳癌、腦癌和多發性骨髓瘤等其 WHSC1 蛋白有過度表現的情形，且當 WHSC1 表現愈高時病人的預後就愈差 (Yang et al., 2012; Toyokawa et al., 2011)。因此，WHSC1 被推測可能參與癌細胞的癌化或促進癌細胞的惡化，但其真正的角色及相關調控機制則尚待研究。

貳、研究動機及目的

在台灣，每年因為肺癌死亡的人數高居不下，肺癌可謂是人類頭號殺手。儘管現行已有多種標靶藥物，但是對多數病人並不適用，特別是對轉移性肺癌更是束手無策。因此，尋找出一個有效預防的生物標誌或新的治療標靶是現行首要之

事。免疫治療一直被認是最有效安全的治療方式，但由於現行科學對於腫瘤和免疫細胞間交互作用存在許多未知而陷入瓶頸。徹底瞭解腫瘤與免疫細胞特別是樹突細胞間的相互影響，將有助於發展免疫治療及肺癌的生物標記及治療標靶。根據高雄醫學大學醫學院醫學系黃明賢教授實驗室初期研究結果顯示，人類肺癌A549 細胞會促進其所浸潤的樹突細胞分泌 Resistin。透過肺癌病患檢體分析發現，相較於健康捐贈者，肺癌病人的血清可測得較高濃度的 Resistin；更甚之，比較非腫瘤組織部位之樹突細胞，浸潤於腫瘤組織部位之樹突細胞會呈現高量的 Resistin。因此本研究進一步分析樹突細胞是否透過分泌 Resistin 促進肺癌細胞的惡化。

一、研究目標

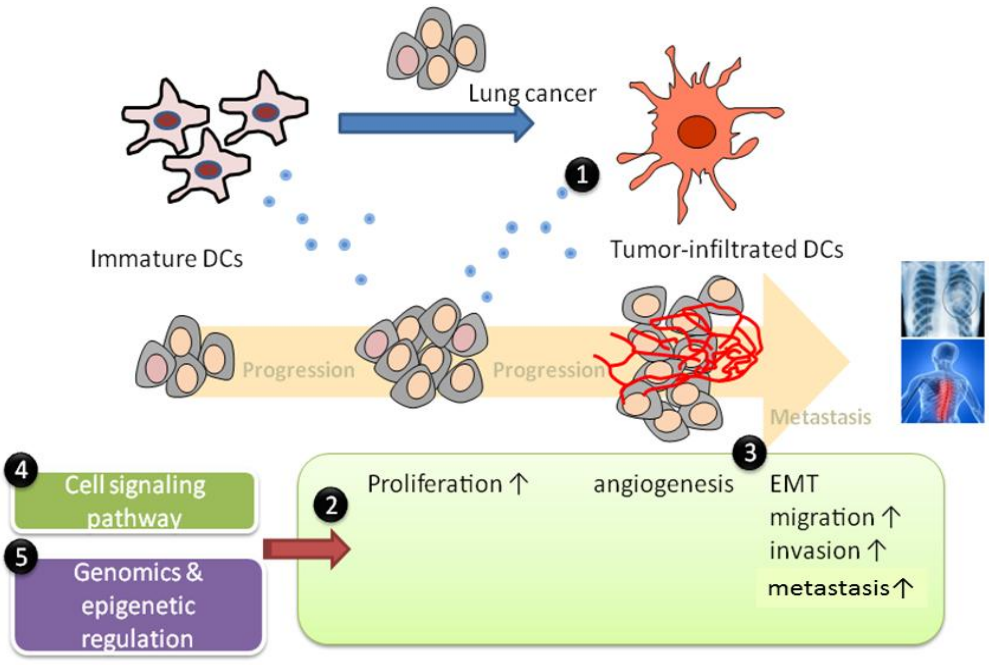


圖 4

1. 腫瘤浸潤之樹突細胞是否分泌高量 Resistin
2. 腫瘤浸潤之樹突細胞是否透過 Resistin 促進腫瘤的生長及存活

3. 腫瘤浸潤之樹突細胞是否透過 Resistin 增加腫瘤的移行、入侵和 EMT
4. Resistin 透過何種機制調節癌細胞而促進腫瘤的惡化
5. Resistin 是否透過 epigenetic regulation 方式影響癌細胞，而促進腫瘤惡化

二、研究流程統整圖

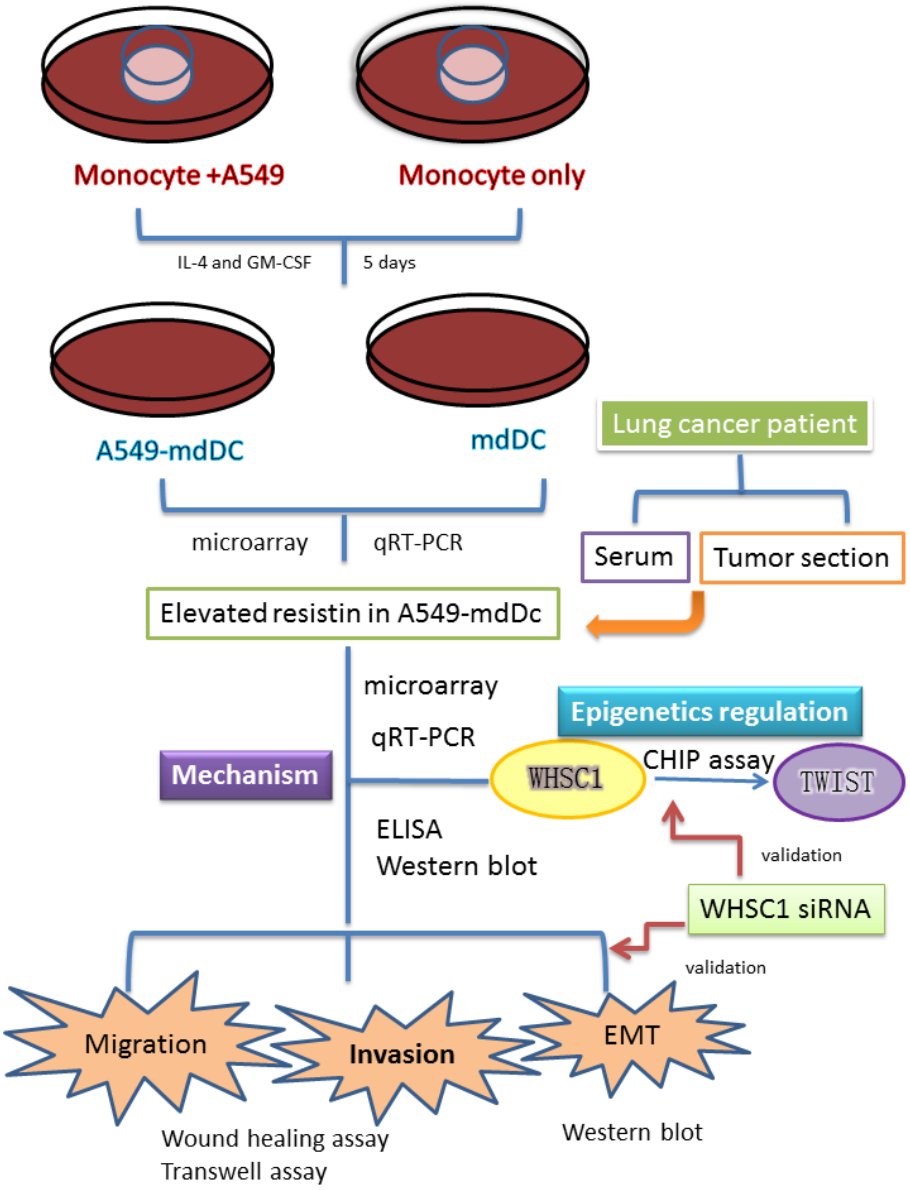


圖 5

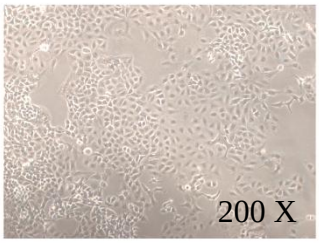
參、實驗材料及方法

表 1 材料

品項	來源
Kaighn's modification (F12K) medium Fetal bovine serum (FBS) 10X trypsin Antibiotics RPMI 1640 Lipofectamine RNAiMAX	Invitrogen, Carlsbad, CA
Ficoll-Hypaque gradient	GE Healthcare Bio-Sciences, Little Chalfont, UK
$CD14^+$ monoclonal antibody-conjugated *magnetic beads*	*MACS* MicroBeads, *Miltenyi Biotec*
Cytokines (GM-CSF, M-CSF, RANKL IL-4, Resitin) Rresistin ELISA kit	R&D Systems, Minneapolis, MN
WST-1 reagent E-cadherin antibody	Clontech Laboratories Inc., Mountain View, CA, USA
QCM™ 24-well Cell Migration Assay QCM™ 24-well Cell Invasion System H3K27 timethylation antibody H3K36 dimethylation antibody N-cadherin antibody GAPDH antibody 8 和 3 μM transwell insert	Millipore, Bedford, UK
oligo (dT) primer Reverse transcriptase	Takara, Shiga, Japan
2xSYBR Green PCR Master Mix	Applied Biosystems, Foster City, CA, USA
Agarose ChIP Kit m-Per lysis buffer	Pierce, Rockford, IL
WHSC1 antibody	Abcam, Cambridge, UK
Fibronectin antibody Control siRNA WHSC1 siRNA	Sigma Chemical Co, Deisenhofen, Germany
Vimentin antibody Claudin-3 antibody	Cell Signaling Tech, Danvers, MA

一、肺癌細胞及樹突細胞

1. 人類肺癌 A549 細胞培養



Human lung cancer A549 cells

圖 6

正肺癌細胞株 A549(CCL-185)購自 American Type Culture Collection (ATCC)。細胞培養於 F-12K 培養液並含 10% 胎牛血清及 1% 抗生素。細胞培養於含 5%CO_2 恆溫培養箱。每 2-3 天置換一次新鮮細胞培養液且於單層滿時進行細胞繼代 (subculture)。

2. 人類肺癌 A549 細胞條件培養液(conditioned medium)製備

將 A549 細胞(1×10^5)種於 10 cm dish，待隔天貼附後換成 8 ml F-12K 培養液。待 48 小時培養後取出上清液，經離心後分裝並於-80℃儲存。此一即定義為 A549 之條件培養液(conditioned medium)。

3. 樹突細胞分化

委託高雄醫學大學附設中和紀念醫院胸腔科洪仁宇醫師用含有抗凝固劑 EDTA (Ethylenediaminetetraacetic acid)之真空採血管，經由靜脈採血方式取得健康捐贈者之全血，帶回實驗室後，將全血以 PBS 溶液 1:1 稀釋，稀釋血液後取 35 ml 緩緩加入含有 15 ml Ficoll-paque 之離心管，1800 rpm 離心 15 分鐘後以低速停止，分離之中間層即為末梢血液單核球 (Peripheral blood mononuclear cells, PBMC)。取出 PBMC 後以 1:1 與 PBS 稀釋，1500 rpm 離心 10 分鐘，並使用含 1 %

EDTA 的 PBS 洗滌 2 次去除 Ficoll-paque。分離出 PBMC 後，加入含磁珠的抗 CD14 抗體 (MACS)，置於 4 ℃作用 20 分鐘，使抗體與單核球結合。加入 8 ml 含 0.05 % BSA 的 PBS 後離心 1500 rpm 8 分鐘。去除上清液，再加入 1 ml 0.05 % BSA 的 PBS 並與細胞混合均勻，並置入 MACS column。CD14+的單核球會與代以磁珠的 CD14 抗體結合而吸附在 MACS 磁座；所有 CD14-細胞就會藉由 PBS 的沖洗流出 Column。最後，將 MACS column 移除磁座並放置在新的 15 ml 離心管中，加入 5 ml 0.05% BSA 的 PBS 至 Column，以活塞將液體直接推出，收集 CD14+單核球細胞。分離出的 CD14+細胞培養在 RPMI1640 培養液或 A549 之條件培養液並含 20 ng/ml IL-4 和 GM-CSF，連續培養五天即成為樹突細胞腫瘤浸潤之樹突細胞。

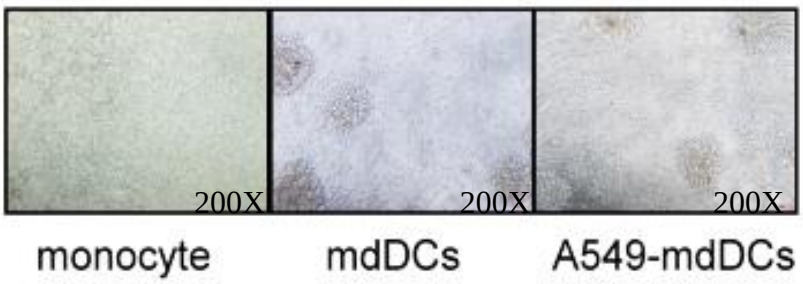


圖 7

4. 肺癌細胞浸潤之樹突細胞條件培養液製備

將正常條件下分化出之樹突細胞和腫瘤所浸潤之樹突細胞離心後再懸浮於 2 ml 的 RPMI1640，待 24 小時培養後取出上清液，經離心後分裝並於-80℃儲存。此一分別定義為正常樹突細胞和腫瘤浸潤樹突細胞之條件培養液。

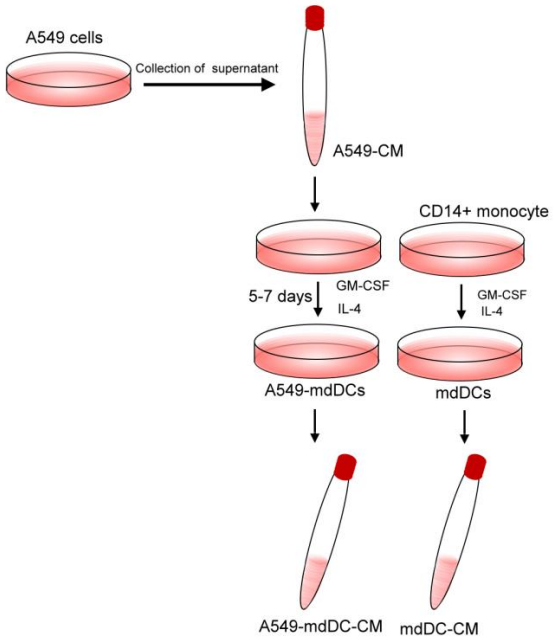


圖 8　正常樹突細胞和腫瘤浸潤樹突細胞之條件培養液之製備流程

5.　共同培養系統

A549 和 CD14+單核球共同培養系統由孔徑大小為 1 μm 之 transwell 完成。上層 insert 種入 1×10^4 之 A549 細胞，下層種植 1×10^5 之 $CD14^+$ 單核球。細胞培養液為含有 20 ng/ml IL-4 和 GM-CSF 之 RPMI 1640，連續培養 5 天。

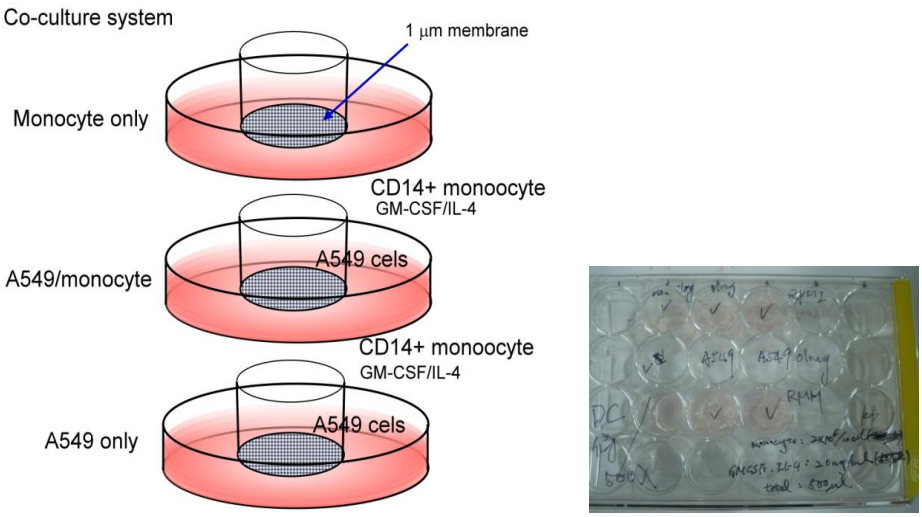


圖 9　(左邊)利用 co-culture system 培養出正常樹突細胞和腫瘤浸潤樹突細胞(右邊)實際操作情況

6. 蝕骨細胞(osteoclast)的分化

分離出 CD14+單核球的細胞培養在 RPMI1640 培養液或 A549 條件培養液並含 50 ng/ml Receptor activator of nuclear factor kappa-B ligand (RANKL)和 100 ng/ml Macrophage colony-stimulating factor (M-CSF)，每三天更換一次培養液，連續培養 21 天即成為蝕骨細胞樹突細胞。

7. 酒石酸磷酸酵素染色(tartrate-resistant acid phosphatase stain)

以 TRAP staining kit (Sigma)分析蝕骨細胞的分化情形。將 100 µl Fast Garent GBC solution 與 100 µl Sodium Nitrite solution 混和入 15 ml 離心管，靜置 2 分鐘反應成 Diazonium salt solution。加入 4.5 ml 37℃無菌水至 15 ml 離心管，並加入 100 µl 的 Diazonium salt solution、50 µl Naphthol As-BI phosphate solution、 200 µl Acetate solution 及 100 µl Tartrate solution，此一溶液為 TRAP 染色液。將 96 well plate 內的細胞培養液丟棄，以 10 %福馬林固定細胞於室溫下 3 分鐘，吸除固定液並加入 PBS 清洗 2-3 次，加入 1:1 的 Ethanol-Acetone 置於冰上 1 分鐘，再以 PBS 清洗 2-3 次，最後加入配製完成的 TRAP 染色液，放入 37℃ 培養箱中靜置 1-3 小時。染色完成，吸除 TRAP 染色液並加入 PBS 清洗，於 100X 的倒立顯微鏡下觀察蝕骨細胞型態。當細胞分化時會出現紫色到暗紅色的顆粒且成三個細胞核以上，以計數方式求得分化細胞的比例。

8. 蝕骨細胞活性分析

利用上述方式分化蝕骨細胞，但是培養盤改為含有 calcium phosphate 的 24 well plate 並讓細胞充分分化。待 21 天分化後，以含 5%漂白水清洗 24 well plate。蝕骨細胞活性則以 Pit assay 分析，記數被消蝕之孔洞做為 osteoclast 的活性指標。

二、微矩陣分析(microarray)

透過微矩陣分析快速篩選基因表現量的差異性。A549 細胞以 10 ng/ml 的 Resistin 培養 6 小時後，以 TRIzol reagent 將細胞溶解並進行 RNA 純化。取 total RNA (1μg)，利用 Agilent Quick Amp Labeling Kit (Agilent Technologies, USA)，將 RNA 反轉錄成 cDNA 後，再利用 T7 RNA poltmerase、dNTP 和 Ctanine 3-CTP (CY3)或 Cyanine 5-CTP (CY5)。進行轉錄作用，此 RNA 稱之為 cRNA。且在轉錄的過程同時標記 Cy3 或 Cy5 的螢光染劑至 cRNA。取出 Cy-labled cRNA (0.825μg)與 fragmentation buffer 混合，於 60℃環境下作用 30 分鐘，將 cRNA 斷成約 50 至 100 bp 大小的核苷酸片段，接著將 Cy-labled cRNA 片段等量置於 Agilent Whole Human Genome 4×44 oligo microarray 中，於 65℃環境下作用 17 小時。利用 Agilent microarray scanner 激發 Cy3 螢光(535 nm)或 Cy5 (625 nm)。最後以 Feature extraction 軟體 10.5 版(Agilent Technologies, USA)分析每一筆訊號資料。

三、即時定量反轉錄聚合酶鏈鎖反應(Real-time RT-PCR, qRT-PCR)

1. RNA 抽取

細胞以 1ml TRIzol reagent 將細胞溶解，吸取細胞溶液至 1.5 毫升微量離心管，再加入 200 μl phenol: chloroform: isoamyl alcohol (25:24:1)均勻混合。以 4℃、12000 g 離心 15 分鐘，促其分層：下層為有基層與介面層，含有蛋白質與 DNA；上層則為水樣層，含有 RNA。將上層液體取至新的 1.5ml 離心管，加入等體積的異丙醇 (Isopropyl alcohol)均勻混合，並於-20℃靜置 10 分鐘後， 以 4℃、12000 g 離心 10 分鐘。去除乙醇後，再將 RNA 進行自然乾燥。RNA 以 RNase

free water 溶解後再以分光光度計測波長 260 nm/280 nm 之吸光值，測得 RNA 濃度與純度。

2. 反轉錄(Reverse transcription)RNA 成 complementary DNA (cDNA)

將取出上述萃取之 RNA 4μg，加入 1 μl Oligo$(dT)_{20}$ (500μg/ml)、1 μl dNTP mixture (dATP、dGTP、dCTP、dTTP 均為 10 nM)，最後加入無菌蒸餾水使其總體積為 13 μl。放置在 PCR system 9700 96 well-plate PCR machine，於 65℃作用 5 分鐘後，迅速降溫至 4℃。取出樣本加入 4 μl 5× First-standard buffer 和 2 μl 0.1M DTT 混合均勻，以 37℃作用 2 分鐘後，最後加入 1 μl M-MLV 酵素，並於 37℃下持續反應 50 分鐘後將 cDNA 儲存於-20℃冰箱。

3. qRT-PCR

取 1μl cDNA、10 μl SYBR Advantage qPCR premix (2 倍)、0.4μl ROX Reference Dye LMP (50×)、6.6μl H_2O 與各式基因之 forward (10 mM) and reverse primer (10 mM) 1μl 均勻混合。利用 7900 ABI PCR system machine (Applied Biosystem)進行 q-RT-PCR。反應條件為：Stage I：50℃、2 分鐘、1 cycle；Stage II： 95℃、15 秒後降溫至 60℃、1 分鐘，再升溫至 95℃、15 秒。上述溫度變化結束後，可分析 Cycle threshold (Ct)，結果以 $2^{-\Delta\Delta Ct}$ 方式求得控制組及實驗組 mRNA 表現差異。

表 2

Gene name	Primer 序列(5' - 3')
GAPDH forward	GAAATCCCATCACCATCTTCCAGG
GAPDH reverse	GAGCCCCAGCCTTCTCCATG
WHSC1 forward	TTGGGAGAAATGGCAGAATC
WHSC1 reverse	ACTCCTCAAAAGACGGCAGA

四、西方墨點法(Western blot)或稱免疫墨點法(immunoblot)

1. 總蛋白質萃取

將細胞種於 10 cm dish，待細胞貼附後，加入 Resistin (1 或 10 ng/ml)培養至所需時間。以冰的 PBS 洗淨細胞，再加入 1ml PBS，利用刮勺於冰上將細胞刮取下來。以 4℃、3500rpm 離心 5 分鐘，去除 PBS。將細胞依細胞數量加入適量 cell lysis buffer (M-PER Mammalian ProteinExtraction Reagent: Protease Inhibitor Cocktail=1000 µl:10 µl)，於冰上作用 20 至 30 分鐘。在劇烈震盪 30 秒鐘後以 4℃、14000g 離心 15 分鐘。取出上清液即為總蛋白質萃取液。

2. 蛋白質定量及變性

利用 BCA Protein Assay Kit 定量蛋白質濃度。先用無菌水稀釋配製好濃度為 150、300、450、600、750 µg/ml 的 Bovine Serum Albumin (BSA) stock，各從其中取出 10 µl 至 96 well plate，最後會在各 well 中加入 140 µl Bicinchoninic acid (BCA)試劑，做為定量之標準曲線 (standard curve)。另外，則從上述蛋白質組萃取液中，取出 2 µl 至 96 well plate，並加入 148 µl BCA 試劑後，在 37℃環境下作用 30 分鐘。最後以酵素免疫分析儀測定波長 562 nm 的吸光值，即可計算出蛋白質濃度。取 50-100 µg 的總蛋白，依總體積比例 6× sample buffer (0.5 M Tris-Base (pH=6.8)、Sodium dodecyl sulfate (SDS)、goycerol、Dithiotheritol (DTT)和 Bromphenol blue) 均勻混合後，以 100℃加熱 5 分鐘使蛋白質變性完全。

3. SDS-PAGE 製備及電泳

將 6-12 % SDS-PAGE 裝置在電泳槽並加滿電泳緩衝溶液

(Tank buffer ; 25 mM Tris、192 mM Glycine、0.1 %SDS)，加入變性後的蛋白質至 well 中，先以 90V 的電壓進行 Stacking，再以 100V 電壓進行蛋白質分離。

4. 轉漬

電泳分析結束後，將 Polyvinylidene Fluoride (PVDF) 浸潤於 Transfer buffer (20 mM Tris-base、192 mM Glycin、0.1 %SDS、10 % Methanol) 中，再將 PVDF 放置 SDS-PAGE 上，上下皆各加二張 3mm Filter paper，使其形成“三明治” (sandwich) 夾層狀。再將“三明治” (sandwich) 夾層放置 Transfer buffer 中，以 100V 電壓進行 1.5 小時的轉漬。

5. Primary 和 secondary antibody

將轉漬完成的 PVDF membrane 放置在含 5%脫脂奶粉的 Blocking buffer，buffer 溶於 TBS-T (20 mM Tris-base、137 mM Sodium chloride、1M Hydrochloric acid、0.01 % Tween-20)，放置於 4℃冰箱 24 小時。以 Washing buffer (TBS-T) 洗滌 3 次，每次 5 分鐘。加入 1:1000 稀釋的 Primary antibody solution 及 TBS-T，均勻覆蓋在 PVDF membrane 上作用 24 小時。再以 Washing buffer 洗滌 3 次，每次 5 分鐘。將 Secondary antibody 以 1:2000 稀釋於 TBS-T 中，並均勻覆蓋在 PVDF membrane 上作用 1 小時，再洗滌 3 次，每次 5 分鐘。最後加入 Enhanced chemiluminescence (ECL) detection buffer 作用 2 分鐘，即可利用冷光螢光影像處理系統 (CHEMI-DOC XRS) 觀察蛋白質表現。

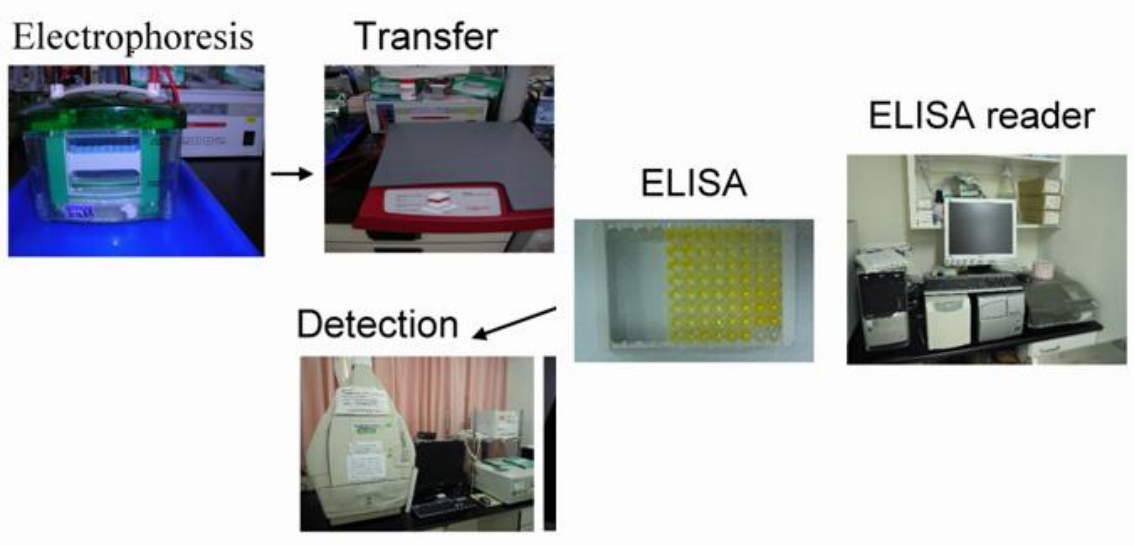


圖 10

五、酵素免疫分析法(Enzyme-linked immunosorbent assay, ELISA)

利用 R&D 之 Human Resistin ELISA Kit 檢測細胞分泌型 Resistin 蛋白質之濃度。首先，製備抗體 coating 的 96 well plate，Standard 稀釋為 2000、1000、500、250、125、62.5 和 0 pg/ml，並加入 100 µl 至 96 well plate 內。另將待測檢體混合均勻，取 100 µl 至 96 well plate 中，室溫下作用 2 小時。以 Washing buffer (0.05% Tween 20 於 PBS) 洗滌 5 次，並加入 100 µl/well 之 Detection antibody，室溫作用 2 小時，再以 Washing buffer 洗滌 5 次。並加入 100 µl/well 之 Streptavidin-HRP 並於室溫下作用 1 小時後，以 Washing buffer 洗滌 5 次。最後加入 100 µl/well 之 TMB 反應 5-30 分鐘，並加入 100 µl/well 之 0.5M H_2SO_4 終止反應。於 ELISA reader 以波長 450 nm 進行分析。

六、肺癌病人血清和腫瘤檢體及免疫螢光染色

1. 血清檢體

委託高雄醫學大學附設中和紀念醫院胸腔科洪仁宇醫師用不含抗凝固劑之真空採血管，經由靜脈採血方式取得。取得之血液靜置於室溫待凝固後以 1500 rpm 離心 10 分鐘，上清液及血清，將之分裝並儲存於-80℃。

2. 腫瘤檢體

病人肺部腫瘤及正常組織由高雄醫學大學附設中和紀念醫院組織庫提供，所有組織經高雄醫學大學附設中和紀念醫院胸腔科洪仁宇醫師協同病理科醫師進行判讀，組織皆以 OCT 方式進行包埋。

3. 免疫螢光染色

將上述檢體取出，以低溫冷凍切片機切出厚度為 3-5 μm 的組織切片，並將之貼附於 coating poly-L-lysine 的玻片上，並以含 1%BSA 之 PBS 培養 1 小時。以 PBS 清洗三次後，加入 primary antibody (anti-CD11C (1:200)和 anti-resistin (1:600) antibody)於 4℃培養 overnight。組織切片以 PBS 清洗兩遍後，加入含 anti-mouse-Dylight 488 和 anti-rabbit-Dylight 549 之 secondary antibody solution 於室溫下培養 1 小時。最後以 PBS 清洗後，以 DAPI 進行核染色。最後以共軛焦螢光顯微鏡擷取影像及分析結果。

圖 11

七、細胞增生、移行及入侵能力分析

1. 細胞增生

利用 WST-1 (4-[3-(4-iodophenyl)-2H-5-tetrazolio]-1,3benzene disulfonate, ClontechTM Laboratories) 測定。原理是利用活細胞內粒線體的 Dehydrogenase 可將四錯鹽(Tetrazolium salts)還原成橘紅色、水溶性的 Formazan，在波長 490 至 500nm 下有吸收波峰。藉由數值的差異性。可知道細胞增生的情況，顏色愈呈現橘紅色，可知道細胞增生量愈多。將 A549 細胞(3×10^3 細胞/well)種在 96 well plate，待隔天細胞完全貼附於 well 上。加入各種濃度的 Resistin 繼續培養 48 小時。移除上清液後加入 95 µl 培養基液與 5 µl WST-1 繼續培養 1 小時。以 450 nm 測試波長和 600nm 參考波長測定其光學活性(Optical density, O.D)。增生倍率%=(第 48 小時培養之 O.D/第 0 小時之培養 O.D)。

2. 以傷口癒合方式分析細胞移行(migration)

將細胞(1.2×10^5 細胞)種在 24 well plate 中。隔天細胞貼附，先利用 PBS 將多餘懸浮之細胞去除，再添加新的培養基溶液，以 200 µl tip 在 well 中間劃出一道傷口後，再以 PBS 清洗懸浮之細胞。加入不同濃度之 Resistin 後，以顯微鏡分別觀察 0 和 24 小時傷口癒合情況，並進行拍照記錄。

3. 以 transwell 暨結晶紫染色方式分析細胞移行

圖12

將 3×10^4 顆細胞數懸浮於 300 µl 無 FCS 的 F12 細胞培養液並種

於 Hanging cell culture insert (pore size 為 8.0 μm)內。陰性控制組 insert 下面之 well 添加 500μl 無 FCS 的 PF12 細胞培養液，其餘則別以 1% FBS、1ng/ml resistin/1%FBS 和 10 ng/ml resistin/1% FBS 作為誘導劑。允許細胞於 37℃含 5% CO_2 的培養箱培養爬行 48 小時後，將 insert 與 well 的培養基溶液去除，PBS 清洗 insert，並使用棉花棒將 insert 內部擦拭乾淨以清除未爬行之過去細胞。以 10% formaldehyde 的 PBS 固定細胞後，再以 20 μl 結晶紫(0.4g/l H_2O)染色，清洗晾乾後再用顯微鏡拍照記錄。

4. 以螢光分析測定細胞移行(migration)活性分析

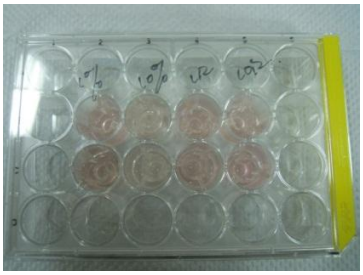


圖 13

可利用 CHEMICON QCM™ 24-well Migration Assay Kit 對移行之細胞進行定量。利用 Kit 所附之 Standing cell culture insert (pore size：8.0 μm)，同上述之步驟進行細胞移行誘導。以 PBS 清洗 insert 後，將 insert 移至乾淨的 well 內，以 225μl cell detachment solution，於 37℃環境作用 30 分鐘使移行之細胞完全脫落。加入 75 μl Lysis buffer/Dye solution (CyQUANT® GR Dyel: 4X Cell Lysis Buffer=1:75), 於室溫下反應 15 分鐘染色。染色液以 480nm 激發並測定 520nm 的發光值作為細胞移行的定量質。

5. 以螢光分析測定細胞入侵(invasion)活性分析

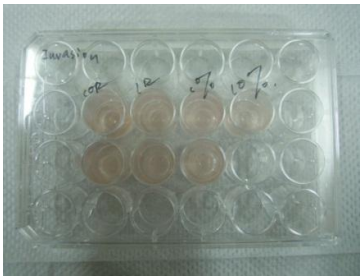


圖 14

利用 CHEMICON QCM^{TM} 24-well Invassion Assay Kit 對入侵之細胞進行定量測定。處理細胞之方如上，但是細胞數為 15×10^4 顆細胞。且 insert 再種入細胞前須經 rehydrate 步驟：添加 300 µl 無 FCS 的 F12K 培養液至 insert 內部 30 分鐘。允許細胞反映 48 小時後，同上述螢光測定方式測定細胞入侵穿透的定質量。

八、染色質免疫沉澱分析(chromatin immunoprecipitation, ChIP)

染色質免疫沉澱分析分析以 Agarose ChIP Kit (Pierce, Rockford, IL)完成。細胞加入 Resistin (1 和 10 ng/ml)培養 6 小時後移除細胞上清液，加入含 1%福馬林之 3.6 ml PBS 溶液於室溫下固定細胞十分鐘。加入 0.4 ml 10X glycerin 溶液穩混均後室溫下培養 5 分鐘。移除細胞固定液，以 PBS 清洗兩次後刮下細胞並以離心方式收集細胞。利用 cell membrane lysis buffer 將細胞膜打破後以離心方式收集細胞核。加入 Micrococcal Nuclease (ChIP Grade) (10 U/µL)於 37℃反應 15 分鐘，每五分鐘翻轉一次促進反應完全。加入 10 µL MNase Stop Solution，並置於冰上 5 分鐘終止反應。以 9000g 離心五分鐘後以 50 µL nuclear lysis buffer 打破細胞核。以離心方法收集上清液，取 5 µL 作為 Input control。其餘 45 µL 加入 0.45 ml IP Dilution Buffer 並加入適當的免疫沉澱抗體於 4℃反應 overnight。加入 20µL ChIP Grade Protein A/G Plus Agarose 於 4℃反應 2

小時後，分別以 IP wash buffer I 清洗一次、IP wash buffer II 清洗兩次和 P wash buffer III 清洗一次後加入 1X IP Elution Buffer 於 65℃加熱 45 分鐘促進 DNA 的釋放。加入 6 μL 5M NaCl 和 2 μL of 20mg/mL Proteinase K. 置免疫沉澱過之 DNA 和 input DNA 並於 65℃加熱 1 小時。每個樣品皆加入 0.75 ml DNA binding buffer 並轉移至 DNA binding column，經 Wash 步驟後以 DNA elution buffer 分離出 DNA。最後，DNA 樣品以定量反轉錄聚合酵素鏈鎖反應確立 Twist 啟動子各個區域之含量。

表3

組別	**所加之抗體**	**PCR反應之引子**
Positive control	Anti-RNA Polymerase II Antibody	GAPDH promoter
Negative control	Normal Rabbit IgG	Twist promoter
Sample	Anti-WHSC1 antibody Anti-H3K36me2 antibody Anti-H3K27me3 antibody	Twist promoter
啟動子位置	引子序列(5' - 3')	
Twist Upstream Fw	TGTAAGGGATGGACCTGAAACG	
Twist Upstream Rv	TGAACCAGAAAGGAAGTCGCC	
Twist Intron 1 Fw	GGTAAGGACCGTTTTGTCAGCG	
Twsit Intron 1 Rv	TGGAAGGATTTCAGCCCAAGG	
Twist Exon 2 Fw	CGTGGACAGTGATTCCCAGACG	
Twist Exon 2 Rv	GTGATGCCTTTCCTTTCAGTGG	

圖15

九、基因阻斷

WHSC1 的基因阻斷以 siRNA 轉殖方式完成。將細胞種於 6 cm dish，待隔天貼附後進行轉殖，使 siRNA 最終濃度為 10 nM。取 1 μl control siRNA (100μM)或 WHSC1 siRNA 至 1ml opti-MEM。加入 10 μl 的 Lipofectamine™ RNAiMAX，於室溫培養 20 分鐘。將細胞取出，加入不含抗生素的細胞培養液。最後加入轉殖試劑。於 37℃培養 6 小時後置換成一般培養液繼續培養至 48 小時後，以即時定量反轉錄聚合酵素鏈鎖反應確立基因阻斷效果。

表 4

	序列(5' - 3')
Control siRNA	GAUCAUACGUGCGAUCAGA[dT] UCUGAUCGCACGUAUGAUC[dT][dT]
WHSC1 siRNA	GAUCUUACUUCCCGGGUGU ACACCCGGGAAGUAAGAUC

十、統計分析

所有實驗皆為獨立實驗三重複。表示法為平均值±標準差。統計方法為 Student's t-test。*為 P<0.05。

肆、研究結果

一、人類肺癌細胞 A549 會刺激樹突細胞分泌 Resistin

如圖 16 A 所示，透過微矩陣分析(cDNA microarray)快速篩選比較正常條件下所分化之樹突細胞與人類肺癌 A549 細胞培養液下所分化之樹突細胞，發現存含有 A549 細胞培養液會促進樹突細胞大量表現 Resistin，其增加倍數高達 10.56 倍。再以 qRT-PCR 方式分析，確立樹突細胞培養液內含 A549 細胞

培養液，會促進其 Resistin 的 mRNA 表現增加(圖 16 B)。更甚之，利用 ELISA 再確認，CD14+ monocyte 在存有 A549 培養液下所分化之樹突細胞，其表現 Resistin 蛋白質量為正常狀態下的 2.85 倍 (p=0.0049) (圖 16C)。

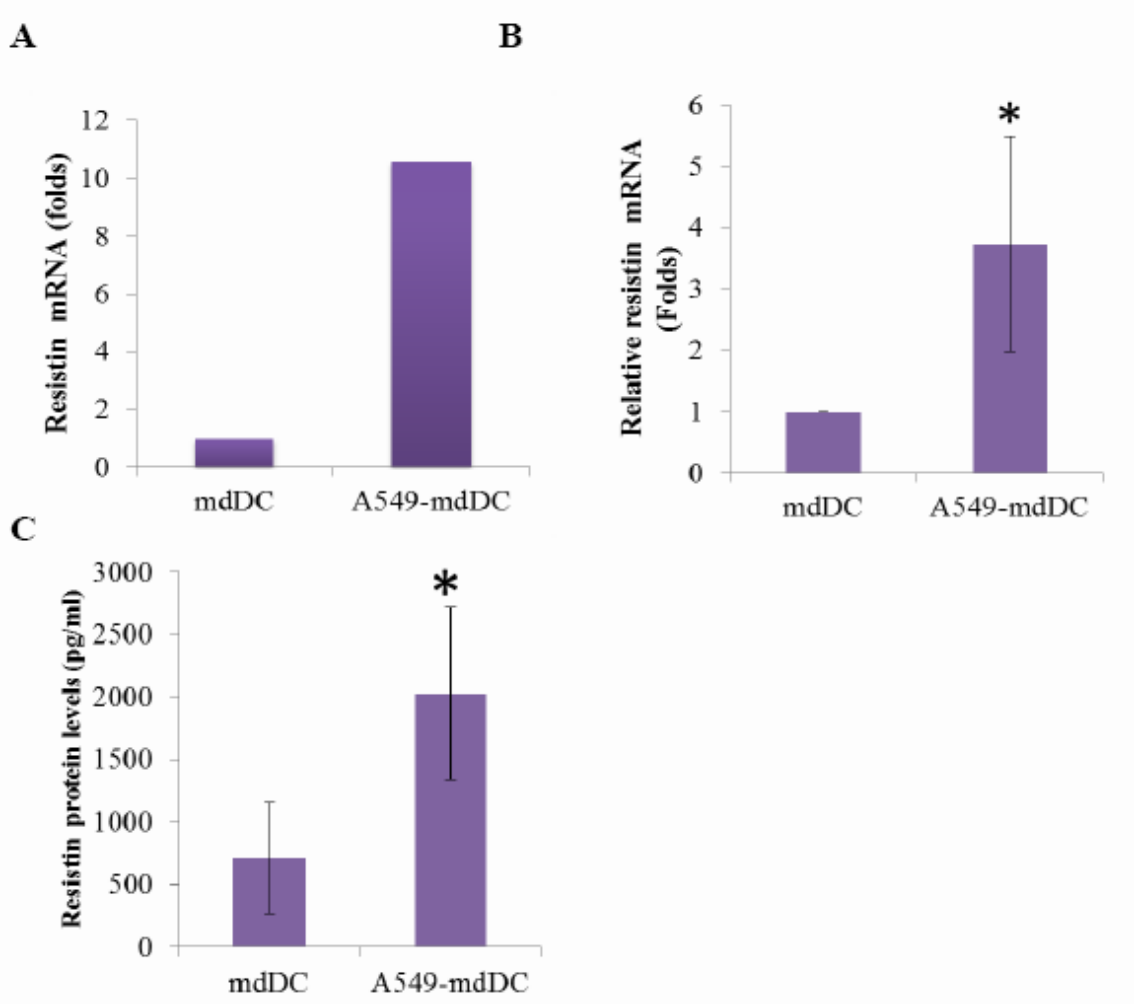


圖 16　腫瘤上清液會促進樹突細胞表現 Resistin

(A) microarray 和(B) qRT-PCR 確立人類肺癌細胞 A549 刺激樹突細胞表現 Resistin mRNA；(C) ELISA 確立 A549 刺激樹突細胞分泌 Resistin 蛋白。

表示法為平均值±SD。統計方法為 Student Test。*代表 p value<0.05。至少 3 次獨立試驗。

為了更加精準模擬在人體內，當肺癌細胞與樹突細胞這兩種細胞同時存在的狀態，肺癌細胞確實具刺激其分泌 Resistin 的活性，本研究利用 co-culture system 將 A549 細胞(insert)與 CD14+ monocyte (bottom well)共同培養 5 天，收

集分化出之樹突細胞以 qRT-PCR 分析 Resistin mRNA；另外，亦收集第 5 天之細胞上清液後以 ELISA 方式分析 Resistin 蛋白質。如圖 17 A 結果所示，A549 與 CD14+ monocyte 共同培養時所分化之樹突細胞會比單獨培養 CD14+ monocyte 所分化出之樹突細胞表現高量的 Resistin mRNA。再者，A549 與 CD14+ monocyte 共同培養所分化之樹突細胞，其測出的 Resistin 蛋白質會比單獨培養 CD14+monocyte 或 A549 有更高的濃度，且具有統計意義(圖 17 B)。因此，無論是以腫瘤上清液或是在共同培養系統，皆能證實樹突細胞會因肺癌細胞的刺激而分泌大量 Resistin。

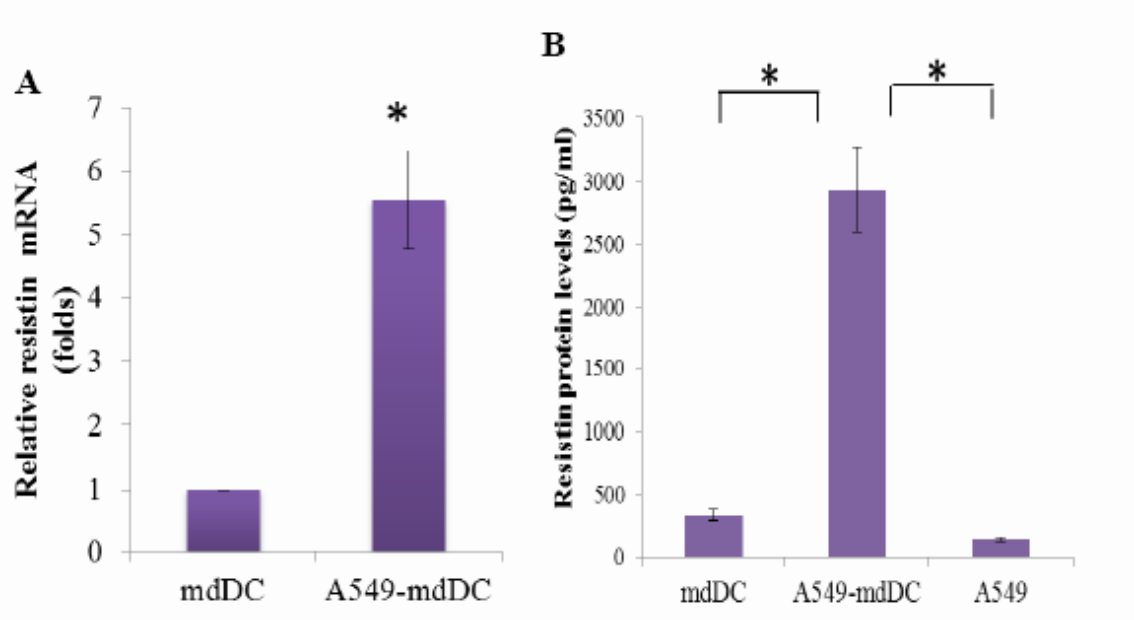


圖 17　Co-culture system 確立肺癌細胞刺激樹突細胞分泌 Resistin

(A) qRT-PCR 確立 A549 細胞刺激樹突細胞表現 Resistin mRNA；

(B) ELISA 確立肺癌 A549 細胞刺激樹突細胞分泌 Resistin protein。

表示法為平均值±SD。統計方法為 Student Test。*代表 p value<0.05。至少 3 次獨立試驗。

二、利用病人檢體證實腫瘤組織所浸潤的樹突細胞會分泌大量 Resistin

為確認 Resistin 在臨床的重要性，因此本研究更透過肺癌病患血清及肺癌

組織檢體進行分析。本研究收集 24 名健康捐贈者及 46 名肺癌患者血液進行評估。如圖 18 A 結果所示，肺癌患者的血清中 Resistin 的平均濃度為 1779.04 pg/ml；健康捐贈者血清中 Resistin 的平均濃度則為 843.86 pg/ml，顯示肺癌患者血清中含有較高濃度 Resistin (p=0.013)。由於病人血清中的高濃度 Resistin 可能不單源自於腫瘤所浸潤的樹突細胞，所以本研究進一步分析病人腫瘤檢體。如圖 18 B 所示，在非腫瘤組織(non tumor part)的樹突細胞(CD11c+，綠螢光標定) 無法偵測到 Resistin 的表現(紅螢光標定)；相對的，腫瘤組織(tumor part)所浸潤的樹突細胞則可偵測出大量的 Resistin。因此，利用血清及腫瘤組織檢體，我們更加確認腫瘤所浸潤之樹突細胞會表現大量 Resistin。

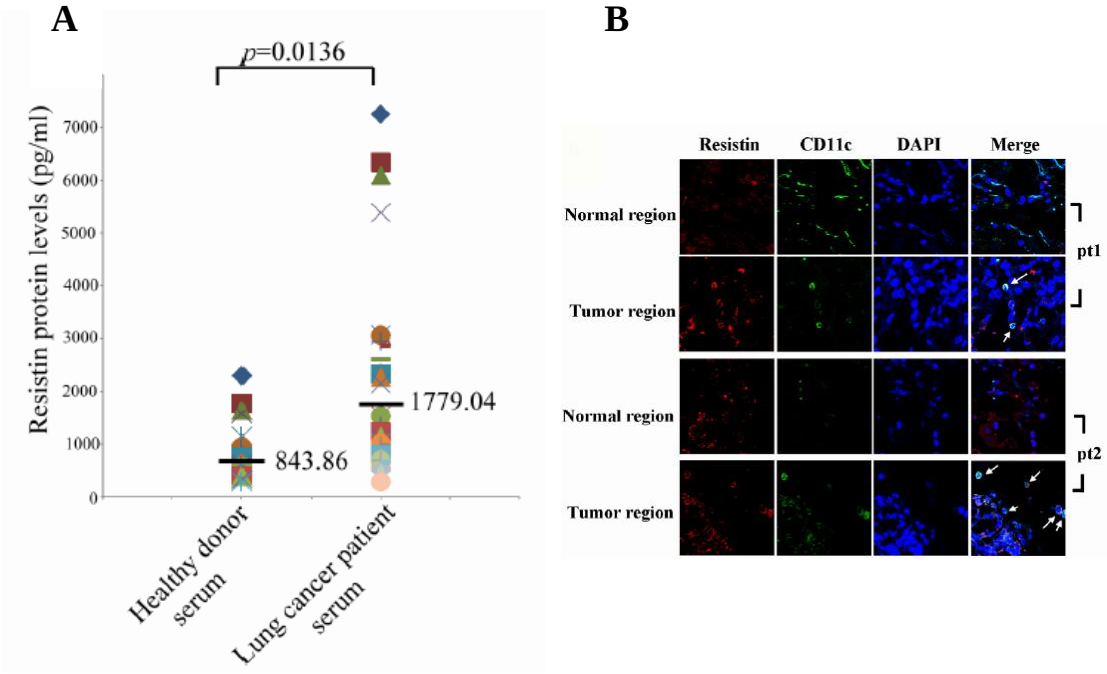


圖 18　臨床病患血清及腫瘤組織所浸潤的樹突細胞含大量 Resistin

(A) ELISA 測定健康捐贈者和肺癌病人血清中 Resistin 的濃度；

(B)免疫螢光染色方法偵測人類腫瘤組織及周邊正常肺組織的樹突細胞之 Resistin 表現 (綠螢光為 CD11c；紅螢光為 Resistin；藍螢光為細胞核)。

表示法為平均值±SD。統計方法為 Student Test。

三、Resistin 刺激肺癌細胞進行 EMT 並增加移行和入侵能力

由於 Resistin 對於肺癌的影響仍屬未知，本研究進一步探討 Resistin 對腫瘤的行為，包括細胞增生、移行、入侵和 EMT。A549 以各種濃度的 Resistin 進行培養 72 小時，細胞增生以 WST-1 方式測得。圖 19 A 結果顯示，Resistin 即使在高濃度(100 ng/ml)下對人類肺癌細胞 A549 的增生並不產生有意義的影響。然而傷口癒合試驗顯示 Resistin 在 24 小時的分析結果發現其可刺激細胞移行(圖 19 B)。利用 transwell 系統將 A549 細胞種於 insert (孔徑為 8 μM)，下層加入 1% FBS (control)、1 和 10 ng/ml Resisin/1% FBS 作為趨化劑。48 小時後以結晶紫或螢光方式分析。結果發現，無論是用螢光定量或者是結晶紫染色都確立 Resistin 會刺激細胞移行(圖 19 C 和 D)。再者，以含有 matrigel 的 insert 也確立 Resistin 有提高癌細胞 A549 的入侵能力(圖四 E)。

EMT 是腫瘤由低轉移性轉為高轉移行的重要過程。本研究結果發現，A549 細胞與 10 ng/ml Resistin 培養 24 小時後，Resistin 會降低 A549 細胞的上皮細胞標誌，如 E-cadherin 和 Claudin-3；相對的，間質細胞標誌，如 N-cadherin、Vimentin 和 Fibronectin，在 Resistin 的刺激下則其表現會增加(圖 19 F)。而調控 EMT 的重要轉錄因子 Twist 會在 Resistin 6 小時的刺激下則大量表現 (圖 19 G)。

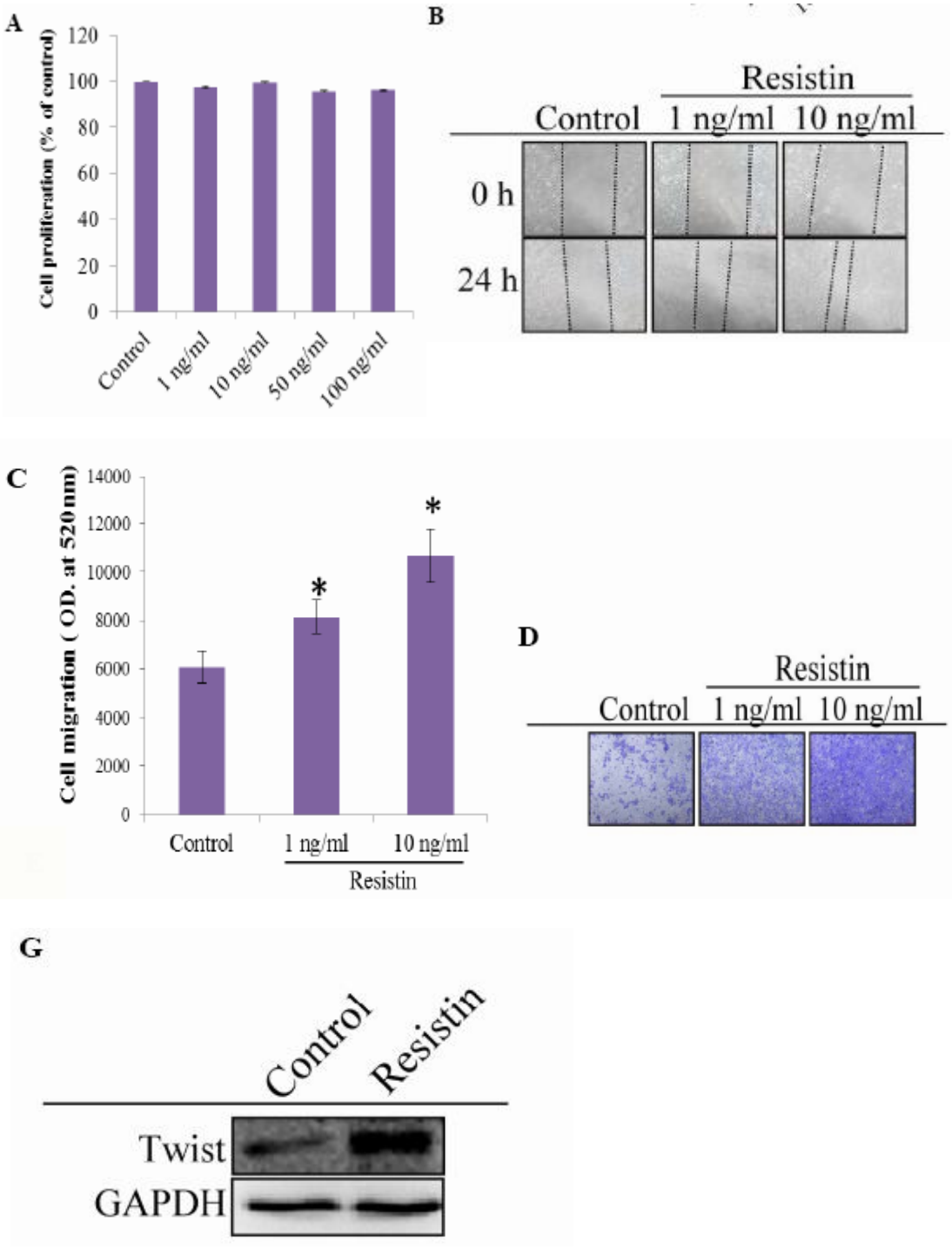


圖 19　Resistin 刺激肺癌細胞進行 EMT 並增加移行和入侵能力

(A) 細胞增生；(B) 傷口癒合試驗；(C) transwell 螢光定量；(D) transwell 螢光定量結晶紫染色實驗；(E) A549 細胞入侵能力；(F) EMT；(G) Twist 表現。

統計方法為 Student Test。*代表 p value<0.05。至少 3 次獨立試驗。

骨骼為肺癌細胞最常轉移的器官之一(Lipton, 2010)。肺癌病人之骨轉移為蝕骨性轉移，最大的特性是產生高度分化和具有高度活性的蝕骨細胞，造成病人骨質流失。因此本研究分析 Resistin 是否促進蝕骨細胞的分化。將 CD14+ monocyte 存在或不存在下 Resistin (10 ng/ml)分化 21 天。蝕骨細胞的分化以 TRAP 染色分析。細胞呈現紅色帶有三個以上細胞核即為蝕骨細胞。如圖 20 A 所示，在 Resistin 的存在下有較多的 CD14+ monocyte 分化成巨大且具有三個細胞核的蝕骨細胞(如箭頭所指)。再者，這些蝕骨細胞也具有高度骨溶蝕活性(圖 20 B; 如箭頭所指)。因此推論，Resistin 對蝕骨細胞的分化和活性皆有促進作用。

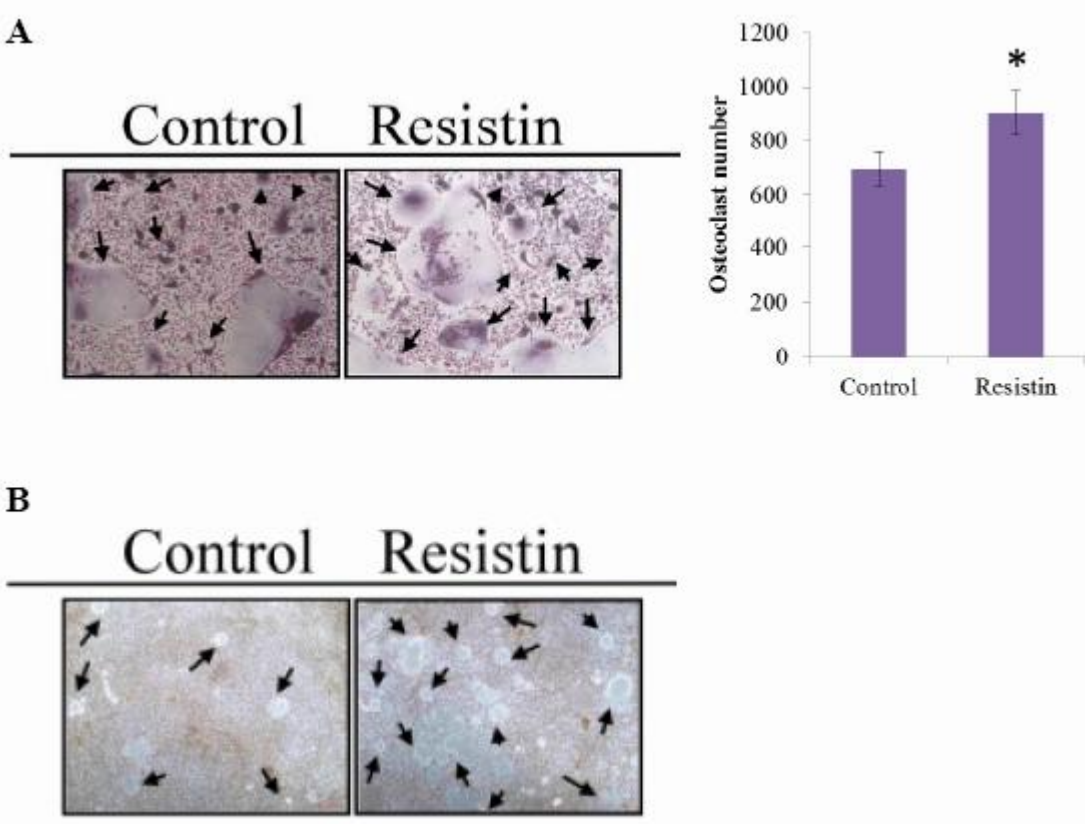


圖 20　Resistin 對蝕骨細胞的促進作用

(A) Resistin 增加蝕骨細胞的分化；

(B) Resistin 增加蝕骨細胞的活性。

表示法為平均值±SD。統計方法為 Student Test。*代表 p value<0.05。至少 3 次獨立試驗。

四、Resistin 參與樹突細胞所媒介的癌細胞惡化演進過程

依據以上的實驗結果，初步發現腫瘤所浸潤之樹突細胞會促進肺癌惡化的演進過程，且推論 Resistin 參與促進癌細胞惡化。為提供反向驗證，利用 2 μg/ml Resistin 中和抗體(Resistin neutrization antibody, Resistin AB)分析 Resistin 是否參與腫瘤所浸潤的樹突細胞造成的癌細胞惡化。A549 細胞種於 insert，下層含 20% mdDC 上清液或 A549-mdDC 上清液以及存在或不存在 Resistin 中和抗體。48 小時後以螢光方式分析。如圖 21 A 所示，腫瘤所浸潤的樹突細胞會刺激癌細胞 A549 的移行，而加入 Resistin 中和抗體能阻斷腫瘤所浸潤之樹突細胞造成的癌細胞移行。類似作用，Resistin 中和抗體也能降低腫瘤浸潤之樹突細胞所造成之癌細胞入侵促進作用(圖 21 B)。接著再進一步評估 Resistin 是否參與腫瘤所浸潤的樹突細胞所造成的 EMT 現象。當 A549 肺癌細胞以含 20% mdDC 上清液或含 A549-mdDC 上清液，並且存在或不存在 Resistin 中和抗體培養 24 小時後，EMT 分子表現以 western blot 分析。腫瘤所浸潤之樹突細胞之培養液會促進 A549 肺癌細胞表現大量的間質細胞標誌 N-cadherin 和 vimentin；相對的，腫瘤所浸潤之樹突細胞之培養液會降低上皮細胞標誌 E-cadherin 的表現，而當存在 Resistin 中和抗體時，這些腫瘤所浸潤之樹突細胞所調控 A549 肺癌細胞的間質細胞標誌與上皮細胞標誌皆可被抑制(圖 21 C)。更甚之，加入 Resistin 中和抗體也能阻斷腫瘤所浸潤之樹突細胞促進蝕骨細胞的現象(圖 21 D)。因此，透過正向與反向的驗證，證實 Resistin 參與腫瘤所浸潤之樹突細胞造成肺癌惡化的演進過程。

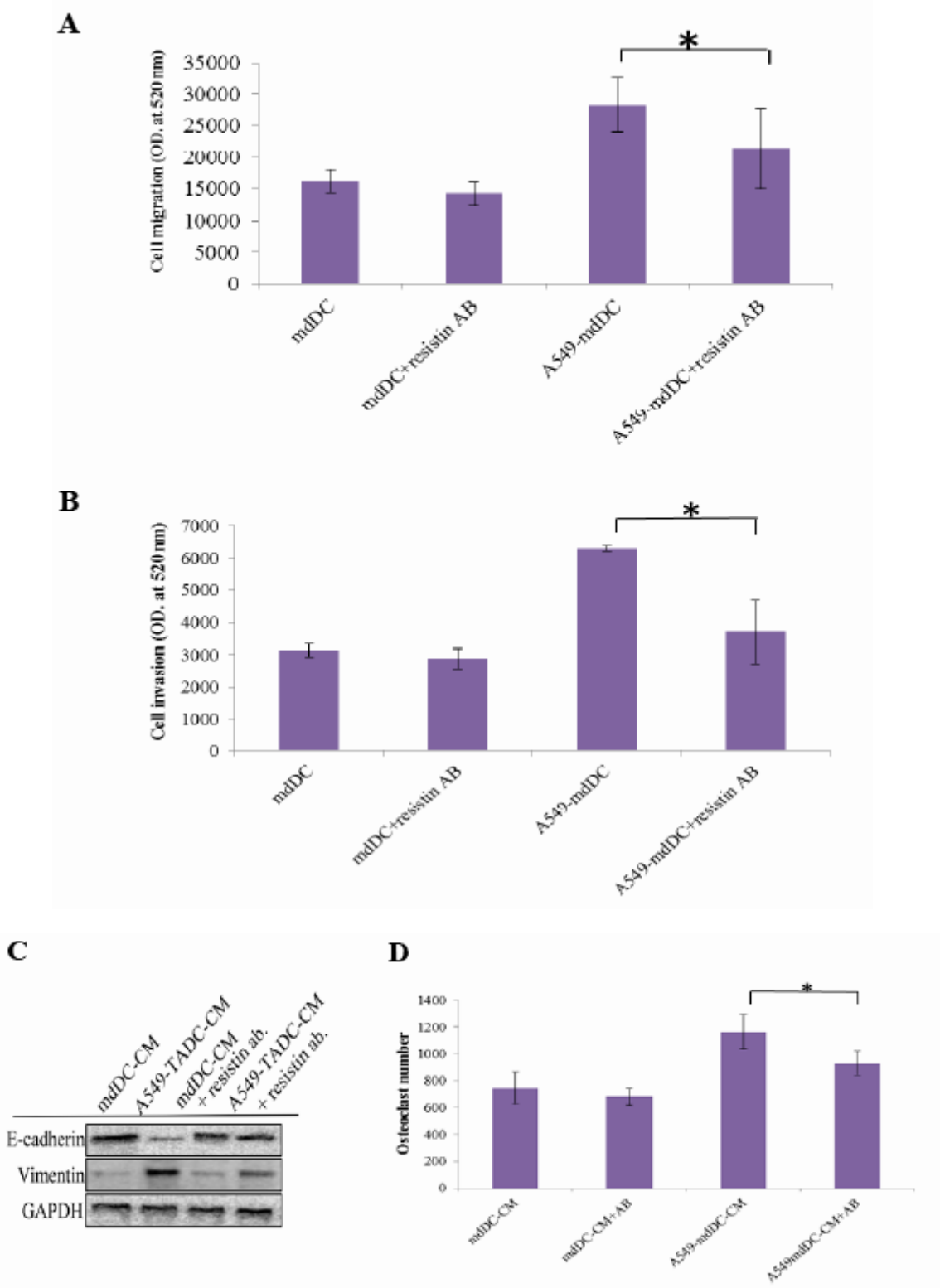


圖 21　Resistin 在腫瘤所浸潤之樹突細胞造成肺癌惡化扮演重要的角色

Resistin 中和抗體(Resistin AB)阻斷腫瘤所浸潤之樹突細胞所造成癌細胞的移行(A)、入侵(B)、EMT (C) 和促進蝕骨細胞分化(D)。

表示法為平均值±SD。統計方法為 Student Test。*代表 p value<0.05。至少 3 次獨立試驗。

五、Resistin 會增加 WHSC1 的表現

本研究為了瞭解 Resistin 如何調控癌細胞的惡化，利用 microarray 尋找 Resistin 對 A549 肺癌細胞基因改變的影響。A549 和 10 ng/ml Resistin 培養 6 小時後再以抽取其 total mRNA 並以 microarray 方式。分析如圖 22 A 所示，Resistin 在 A549 細胞會增加 36 種基因的表現，並降低 275 種基因的表現。其中 histone Methyltransferase WHSC1 會被 Resistin 所誘發，而 WHSC1 已被證實可以調控基因的表現，也有研究發現其參與細胞的癌化(Toyokawa et al., 2011)。因此本研究進一步分析 Resistin 是否透過調控 WHSC1 促進細胞的演進。以 qRT-PCR 再次確立 Resistin 會促進 A549 表現 WHSC1 的 mRNA (圖 22 B)。再者，以 Western Blot 的方式確認 Resistin 也可增加 A549 細胞表現 WHSC1 蛋白質 (圖 22 C)。因此，Resistin 會增加 WHSC1 的 mRNA 與蛋白質的表現量。

WHSC1 屬於 histone methyltransferase，已經被證實可在 histone3 lysine36 (H3K36)進行 dimethylation；同時減少 histone3 lysine27 (H3K27)的 trimethylation 而促進基因的表現(Ezpnoda et al., 2012)。因此進一步分析 Resistin 是否會改變 histone 的 methylation。如圖 22 C 所示，Resistin 會增加 A549 細胞 H3K36 dimethylation，同時將低 H3K27 的 trimethylation。

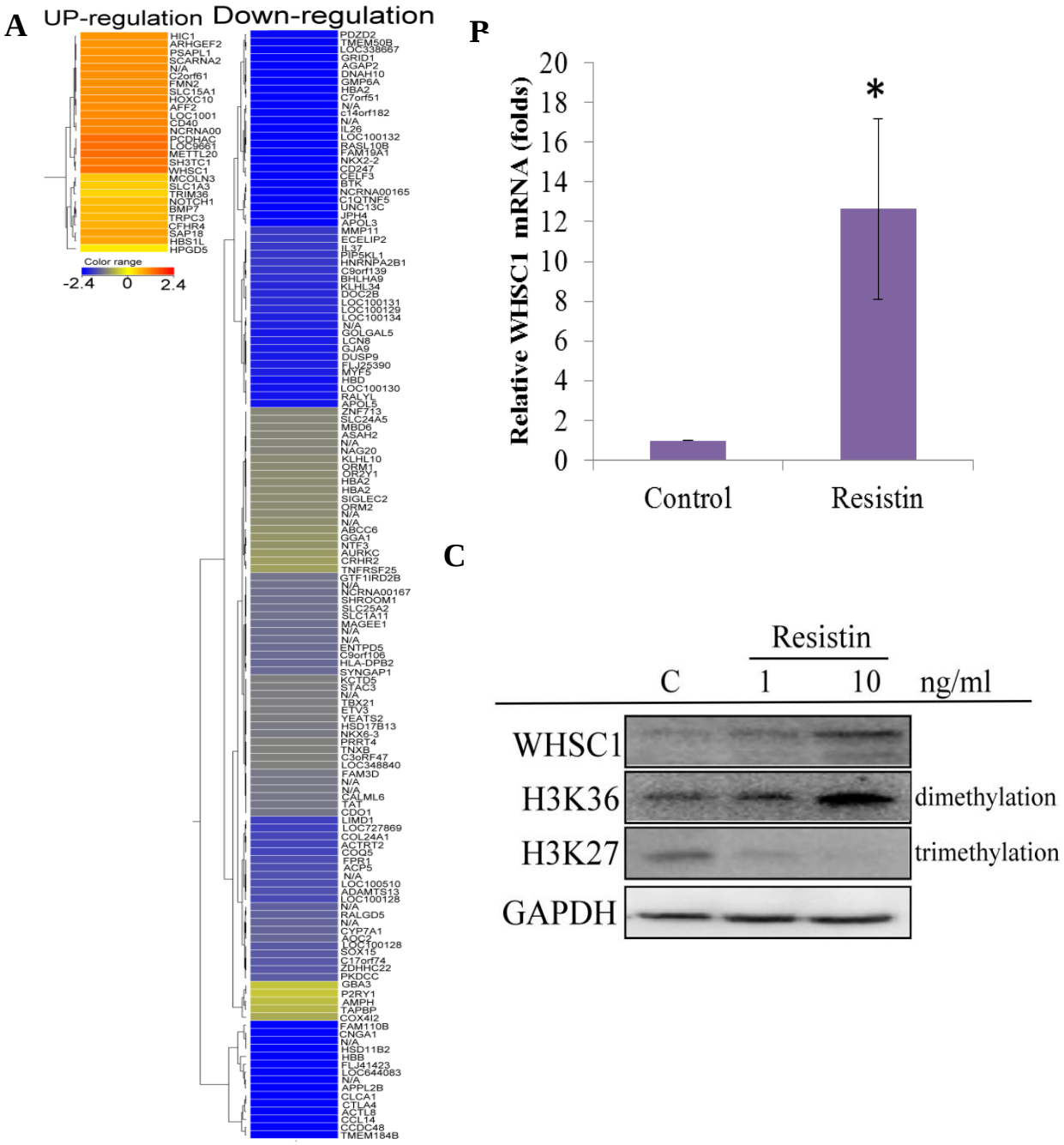


圖 22　Resistin 會增加 WHSC1 之 mRNA 與蛋白質的表現量

(A) Microarray heat map；

(B) Resistin 會促進 WHSC1 的 mRNA 表現；

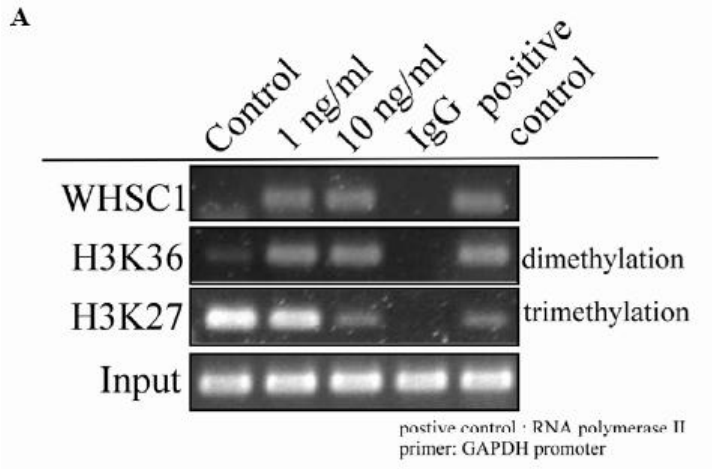
(C) Resistin 可增加 WHSC1 蛋白質表現及改變 histone 的 methylation。

表示法為平均值±SD。統計方法為 Student Test。*代表 p value<0.05。

至少 3 次獨立試驗。

六、Resistin 可藉由 WHSC1 對 Twist 進行 epigenetic regulation 而增加 Twist 的表現

由於 WHSC1 屬於 histone methyltransferase，且 Resistin 可以增加 Twist 的表現。因此，進一步分析是否 Resistin 可促使 WHSC1 結合至 Twist 基因上的 promoter 並促進 H3K36 dimethylation 和抑制 H3K27 trimethylation 進而提升 Twist 的表現。A549 細胞與 1 或 10 ng/ml Resistin 培養 6 小時後，經 formaldehyde 固定後利用染色質免疫沉澱分析(chromatin immunoprecipitation, ChIP)分析。結果發現 Resistin 可增加 WHSC1 結合之 Twist 的 promoter (圖 23 A 和 B)。再者，Twist promoter 上的 H3K36 也有高度的 dimethylation (圖 23 A 和 C)；相對的 H3K27 的 trimethylation 則明顯降低(圖 23　A 和 D)。因此推論 Resistin 可藉由 WHSC1 對 Twist 進行 epigenetic regulation 而增加 Twist 的表現。



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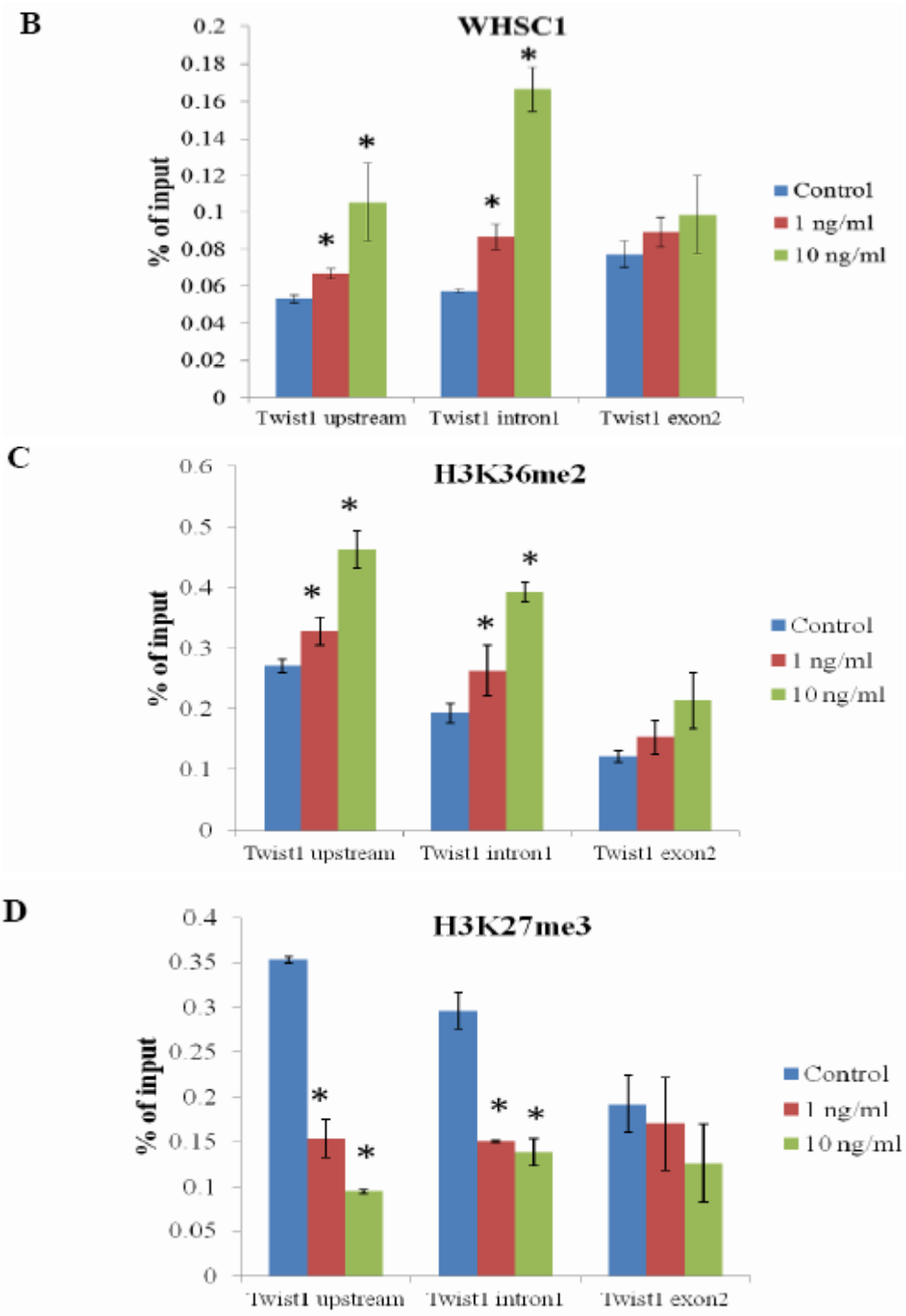
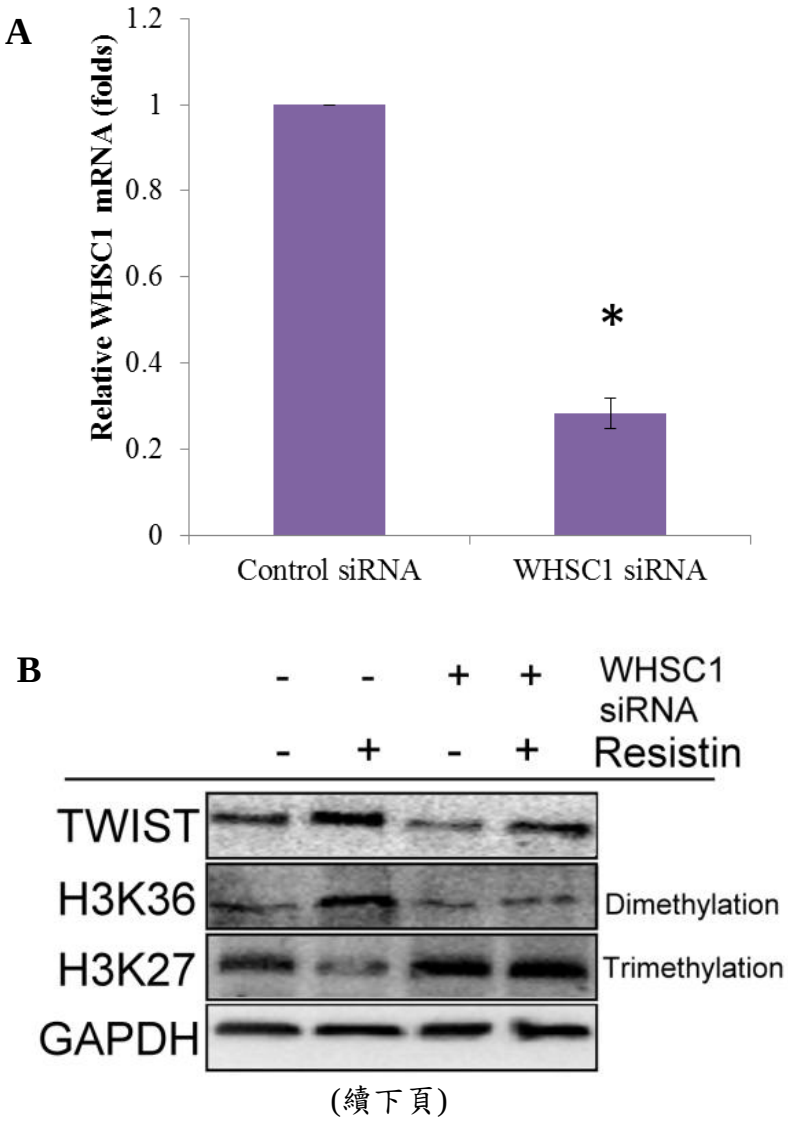


圖 23　以 ChIP 分析確立 Resistin 促進 WHSC1 結合至 Twist promoter 並加 H3K36 dimethylation 和降低 H3K27 trimethylation

(A)以 agarose 方式分析 Twist 基因的 promoter upstream 上 histone methylation 和 WSHC1 的結合(B) WHSC1 結合至 Twist promoter；(C) Twist promoter 的 H3K36 dimethylation；(D) Twist promoter 的 H3K27 trimethylation。表示法為平均值±SD。統計方法為 Student Test。*代表 p value<0.05。至少 3 次獨立試驗。

為了確認 WHSC1 參與 Resistin 促進癌細胞惡化的角色，接續以 siRNA 轉殖方式阻斷 WHSC1 的表現，藉此反向驗證是否 WHSC1 在 Resistin 所媒介 Twist 的表現以及影響癌細胞移行和入侵的角色。A549 細胞轉殖 control siRNA (10 nM)或 WHSC1 siRNA(10 nM)後，分析 Resistin 對細胞移行、入侵和 Twsit 蛋白表現。如圖 24 A 所示，WHSC1 siRNA 轉殖可產生高達 75%的阻斷效果。當 WHSC1 被阻斷時，Resistin 造成 Twist 大量表現的情形會被抑制；同時，H3K36 的 dimethylation 也會被降低。更甚之，本來被 Resistin 所抑制的 H3K27 trimethylation 的作用則會被逆轉(圖 24 B)。再者，當 WHSC1 被阻斷時，Resistin 所促進 A549 細胞移行與入侵能力則會被抑制(圖 24 C 與 24 D)。



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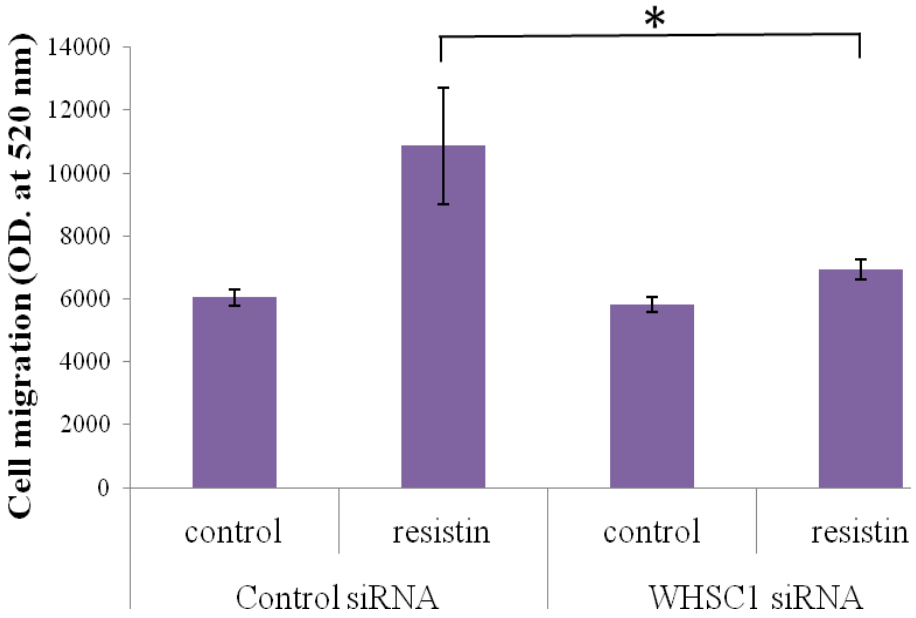

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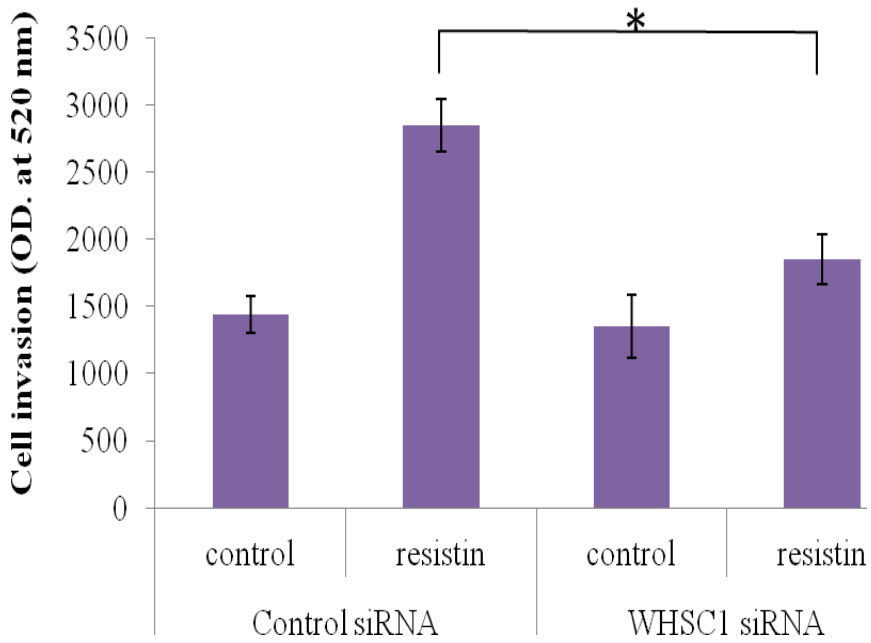

圖 24　Resistin 透過 WHSC1 媒介的 epigenetic regulation 增加 Twist 表現

(A) WHSC1 siRNA 降低 WHSC1 表現；(B) Twist 表現和 histone methylation；

(C) A549 細胞移行情況；(D) A549 細胞入侵能力。

表示法為平均值±SD。統計方法為 Student Test。*代表 p value<0.05。至少 3 次獨立試驗。

伍、討論

本研究首度發現肺腫瘤浸潤之樹突細胞可藉由分泌大量 Resistin 進而促進癌細胞的移行和入侵，而誘發腫瘤轉移；除此，Resistin 亦能刺激蝕骨細胞分化作用。Resistin 不僅促進腫瘤本身的致癌效應，亦可能還參與肺癌末期所造成之蝕骨性轉移。因此，Resistin 可作為肺癌診斷分子及藥物發展的重要標靶。

Resistin 是近年來被發現由脂肪細胞所分泌出的激素之一(Steppan et al., 2001)。在老鼠， Resistin 主要是由脂肪組織所分泌；但在人類，脂肪組織反而不是 Resistin 的主要來源。相對的，胰臟細胞、骨髓細胞(bone marrow cells)、巨噬細胞(macrophagy)和周邊血液單核球(PBMC)皆被證實具有分泌 Resistin 的能力(Tiaka et al., 2011)。Resistin 被認為和肥胖症及糖尿病的發生有關，目前也有一些文獻發現在乳癌、大腸癌和前列腺癌的病人血清測到高濃度的 Resistin (Sălăgeanu et al., 2011; Nakajima et al., 2009; Kim et al., 2011)。在前列腺癌，Resistin 可藉由活化致癌性的細胞訊息傳遞路徑 PI3K/AKT 促進腫瘤細胞的生長(Zhang et al., 2010)。另外，在 trophoblast-like cells，Resistin 會透過金屬蛋白酶而破壞細胞基質造成癌細胞入侵(Di Simone et al., 2006)。本研究首度發現肺癌細胞所浸潤之樹突細胞會表現大量的 Resistin，而 Resistin 會增加肺癌細胞表現間質標誌(包括 N-cadherin，vimentin 和 fibronection)，並降低上皮標誌(如 E-cadherin 和 claudin-3)，而進行 EMT，促使肺癌細胞變為高爬行和高入侵的表現型(phenotype)。透過 Resistin 的中和抗體可以明顯地阻斷腫瘤浸潤樹突細胞所造成的肺癌細胞移行和入侵，更進一步證明 Resistin 在腫瘤與其浸潤之樹突細胞間的惡性循環中扮演主要媒介者的角色。再者，透過肺癌病患檢體分析發現，相較於健康捐贈者，肺癌病人的血清可測得較高濃度的 Resistin；更甚之，比較腫瘤和非腫瘤組織部位之樹突細胞，浸潤於腫瘤組織部位之樹突細胞會呈現高量的 Resistin。因此，證實 Resistin 在肺癌的惡化過程扮演重要的角色，對於發展治療肺癌的藥物過程上是極具潛能的標的。

骨骼是肺癌常轉移的器官之一，稱為骨轉移(bone metastasis) (Patel et al., 2011)。骨轉移疼痛是使癌症病人生活品質惡化主因之一，而且腫瘤患者一旦發生骨轉移，病程進展將加快，生存期也將縮短。肺癌的骨轉移為蝕骨性(osteolytic)，其特徵包括會有大量蝕骨細胞分化，造成病人骨溶蝕作用而使骨質流失(Jimenez-Andrade et al., 2010)。因此，利用雙磷酸鹽類藥物抑制蝕骨細胞是目前延緩骨轉移的唯一策略(Coleman et al., 2010)。本研究發現腫瘤所浸潤的樹突細胞也會增進蝕骨細胞的分化，同時提升蝕骨細胞的活性，因此在肺癌所造成的蝕骨性骨轉移可能亦參與其中。利用 Resistin 之中和抗體可以降低腫瘤所浸潤之樹突細胞促進蝕骨細胞分化，如此更確認 Resistin 與肺癌病人所產生的蝕骨性骨轉移有關。因此，拮抗 Resistin 可能可成為治療肺癌病人所造成之骨轉移的新標靶。

Twist 蛋白是屬於 basic helix-loop-helix (bHLH)轉錄因子，主要促進 EMT 過程，進而提升細胞的移行和入侵(Yang et al, 2004)。Twist 在許多癌症發現有過度表現的情形，且表現的程度愈高則病人的預後就愈差(Fu et al., 2011)。Twist 可以結合到特定基因，如 E-cadherin 啟動子的 E-box 進而抑制 E-cadherin 的表現(Rosivatz et al., 2002)。本研究發現 Resistin 可以將低肺癌細胞 A549 的 E-cadherin；相對的，Resistin 會增加 Twist 的表現。因此，我們推測 Resistin 可透過增加 Twsit 的表現而抑制 E-cadherin，然後促進細胞進行 EMT，最後增加癌細胞移行和入侵的能力。

WHSC1 蛋白在胚胎發育過程會高度的表現，但在健康成人的組織中幾乎不表達(Toyokawa, 2011)。WHSC1 蛋白主要功能是對 histone 的 lysine 進行 methylation 修飾(Yang et al., 2012)。H3K36 的 dimethylation 是促進基因的表現；相反的，H3K27 trimethylation 則是抑制基因的表現(Vakoc et al., 2006)。在前列腺癌，WHSC1 被發現其可藉由增加 Twist 基因的 H3K36 dimethylation 及降低 H3K27 的 trimethylation 而促進 Twist 的表現(Ezponda et al., 2012)。本研究發現 Resistin 可增加肺癌細胞表現 WHSC1 及 Twist 蛋白，同時，Resistin 會增加肺癌細胞 H3K36 的 dimethylation 和降低 H3K27 的 trimethylation；利用 ChIP 分析更確立 WHSC1 可以結合至 Twist

基因的啟動子並增加 H3K36 dimethylation 和降低 H3K27 的 trimethylation。siRNA 基因阻斷試驗也發現當 WHSC1 被阻斷時可以有效的抑制 Resistin 對於 histone methylation 修飾和 Twist 蛋白的刺激作用；再者，Resistin 提升肺癌細胞的移行和入侵作用也明顯被抑制。因此，本研究推論 Resistin 可以藉由增加 WHSC1 蛋白質表現，透過 WHSC1 對 Twist 基因進行 H3K36 的 dimethylation 而增加 Twist 表現後，進而提升肺癌細胞的移行和入侵。

陸、結論及應用

本研究首度發現浸潤於腫瘤的樹突細胞會分泌大量的 Resistin，而 Resistin 又會促進肺癌細胞的惡化及轉移。Resistin 可藉由增加 histone methyltransferase WHSC1 透過 epigenetic regulation 調控致癌基因 Twist 的表現，使癌細胞進行 EMT，結果造成癌症的演進。對於未來臨床應用方面：

1. 本研究由基礎的研究連貫至臨床的應用，提出腫瘤和其微環境樹突細胞之間的惡性交互作用，而 Resistin 是主因之一。
2. 抗 Resistin 乃具潛能作為抑制肺癌之標的或免疫治療之策略。
3. WHSC1 的致癌角色可使 WHSC1 作為肺癌預後診斷分子或藥物開發標靶。

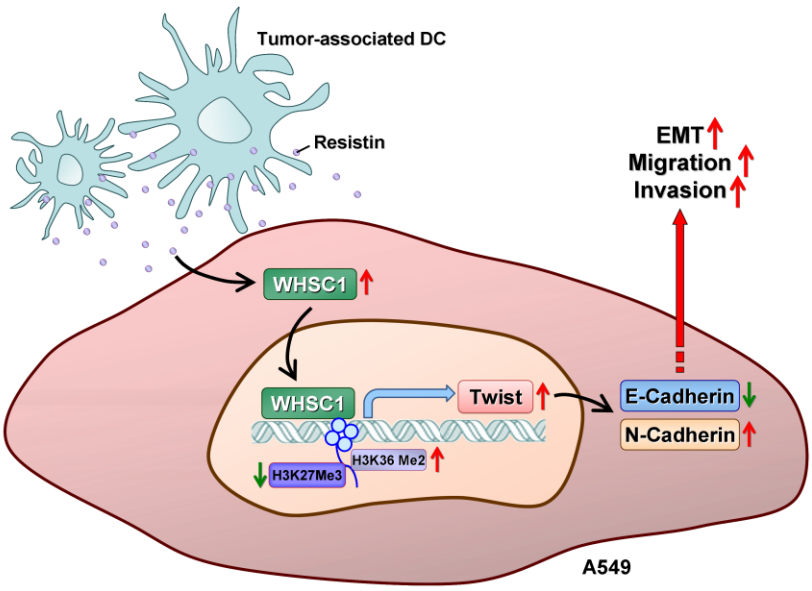

圖 25　腫瘤樹突細胞和肺癌間之交互作用

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評語

本研究探討肺癌 A549 細胞之惡化過程之分子機制，研究範圍包括 A549 之浸潤的樹突細胞分泌之 Resistin 惡化途徑 WHSC1/Twist 之基因表達等蛋白質/基因之功效關係。

本研究結構完整、邏輯清楚。研究結果證實 Resistin 活化 WHSCI/Twist 促進癌細胞惡化，進而建議 Resistin 可作為肺癌診斷分子及藥物發展標靶，研究成果亦已投稿國際期刊，作者為該論文的第一作者。

Lung tumor associated dendritic cell-derived resistin promoted cancer progression by increasing Wolf-Hirschhorn syndrome candidate 1/Twist pathway

Abstract

The interaction between tumors and their microenvironments leads to a vicious cycle which strengthens both immune suppression and cancer progression. The present study demonstrates for the first time that tumor associated dendritic cells (TADCs) can be a source of resistin, which is responsible for increasing lung cancer epithelial-to-mesenchymal transition (EMT). In addition, substantial of resistin in the condition medium (CM) of TADCs increase cell migration and invasion, as well as the osteolytic bone metastatic properties of A549 cells. Neutralization of resistin from TADC-CM prevents the advanced malignancy-inducing features of TADC-CM. Significantly elevated levels of resistin have been observed tumor-infiltrating $CD11c^{+}$ DCs in human lung cancer samples, and patients' sera. Induction of lung cancer progression by TADC-derived resistin is associated with an increased expression of Wolf-Hirschhorn syndrome candidate 1 (WHSC1), a histone methyltransferase. Resistin-induced WHSC1 increases the dimethylation of histone 3 at lysine 36 and decreases the trimethylation of histone 3 at lysine 27 on the promoter of Twist, resulting in an enhancement of the expression of Twist, the master regulation of EMT. Knockdown of WHSC1 by siRNA transfection significantly decreases resistin-mediated cancer progression by decreasing the upregulation of Twist, suggesting that WHSC1 plays a critical role in the regulation of Twist by epigenetic modification. These findings suggest that TADC-derived resistin may be a novel candidate in conferring the ability for lung cancer to develop.

Introduction

Lung cancer has been one of the most common causes of death worldwide for several decades (1, 2). Despite significant advances in treatment, improving the therapeutic outcome remains a serious problem due to the cancer's genetic variety and drug resistance (3). The microenvironment of a tumor has increasingly been recognized as a critical factor that can protect the cancer from host immunity, promote tumorigenesis, and support tumor growth and metastasis (4, 5). The cells surrounding tumors consistently release various soluble factors, such as growth and proinflammatory factors, which facilitate tumor development (6, 7). Previous studies have demonstrated that lung cancer cells promote immune tolerance through inhibiting the differentiation and maturation of dendritic cells. (8). Lung tumor associated dendritic cells (TADCs) have been shown to display an immune-suppression phenotype, and to express epidermal growth factor family growth factors, which in turn increase cancer progression (9). However, it is still not fully understood whether TADCs express other pro-tumorigenic factors responsible for lung cancer development, or precisely which molecules contribute to the interactions between the tumor cells and TADCs in the tumor microenvironment. Strategies that aim at the immune system, in combination with therapies targeting cancer cells, are valuable approach to finding optimal responses in treating this devastating disease.

Resistin, a 12.5 kDa cysteine-rich secreted protein, is found in a screen for adipocyte gene products that are reduced by the anti-diabetic drug rosiglitazone, and increased in diet-induced and genetic forms of obesity (10). Resistin is characterized by a unique structure of 10–11 cysteine residues, also known as resistin-like molecules (RELMs) (10, 11). The expression and secretion of resistin by human mononuclear cells are markedly induced by inflammatory stimuli, and levels of resistin in humans have been

shown to correlate strongly with circulating markers of inflammation of inflammatory cytokines such as IL-1b, IL-6, IL-8, IL-12, and TNF-α (11-13). Inflammation has increasingly been recognized as playing a pathogenic role in cancer (14). Recent studies have shown that higher concentrations of resistin are found in patients with esophageal squamous cell carcinoma when compared to healthy donors. In addition, resistin levels have been shown to gradually increase with tumor progression (15-17). However, the role of resistin has not been studied in lung cancer.

In the present study, items found that TADCs produce high levels of resistin, which plays a pro-tumorigenic role in the development of lung cancer. This study also investigated DCs in infiltrating tumor tissues of human patients, and the results showed that TADC-derived resistin enhanced A549 cell migration and invasion and the epithelial-to-mesenchymal transition (EMT) by the paracrine effect. This study is the first to demonstrate that TADC-derived resistin promotes lung cancer progression and may therefore be an attractive target in the development of therapeutic strategies targeting immune cells in the cancer microenvironment.

Material and Methods

Lung Cancer Cells and Conditioned Media

Human lung cancer cells A549 were obtained from the American Type Culture Collection (number CCL-185) and cultured in Kaighn's modification (F12K) medium containing 10% fetal bovine serum (FBS). To obtain the A549-conditioned medium (CM), cells were seeded at 2×10^6 cells/100 mm dish and cultivated for 24 h. The medium was replaced and the supernatants harvested after 48 h of incubation.

Serum samples from lung cancer patients

Preoperative blood samples were obtained from 46 lung cancer patients and 24 healthy donors admitted to the Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital (KMUH), Kaohsiung, Taiwan. Serum was separated by centrifugation and frozen at -80℃. Approval for this study was obtained from the Institutional Review Board of KMUH, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Isolation of $CD14^+$ monocytes and differentiation of monocyte-derived DCs (mdDCs)

Monocytes were purified from peripheral blood mononuclear cells (PBMCs) obtained from healthy consenting donors. Mononuclear cells were isolated from blood by Ficoll-Hypaque gradient (GE Healthcare Bio-Sciences, Little Chalfont, UK). $CD14^+$ monocytes were purified using $CD14^+$ monoclonal antibody-conjugated *magnetic beads* (*MACS* MicroBeads, *Miltenyi Biotec*), according to the manufacturer's protocol. mdDCs were generated by culturing $CD14^+$ monocytes in RPMI 1640 medium containing 10% FBS (Invitrogen, Carlsbad, CA) and 20 ng/mL granulocyte-macrophage colony-stimulating factor and 10 ng/mL IL-4 (R&D Systems, Minneapolis, MN) for 5

days. The medium was replaced with fresh medium containing GM-CSF and IL-4 on day 3. For maturation of the DCs, immature mdDCs were stimulated with Lipopolysaccharides (100 ng/ml) after priming with interferon-γ (INF-γ) for 3 h. A549 tumor associated mdDCs (A549-TADCs) were generated by culturing $CD14^+$ monocytes in RPMI 1640 medium containing FBS, GM-CSF and IL-4 presenting in A549-CM, then stimulated as described above. After washing, the supernatant was collected and defined as A549-TADC-conditioned medium (A549-TADC-CM). Supernatants from TADC and mdDC were collected. Resistin was quantified using a DuoSet enzyme-linked immunosorbent assay (ELISA) kit.

Analysis of cell proliferation, migration and invasion

Cell proliferation was assessed using Premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories Inc., Mountain View, CA, USA**)** according to the manufacturer's instructions. Cell migration and invasion assays were conducted using wound healing, a QCM™ 24-well Cell Migration Assay and Invasion System, as previously described (15). Resistin (1 or 10 ng/ml) was added to the bottom wells for 48 h as the chemoattractant, and the fluorescence of the invading cells was read using a fluorescence plate reader at excitation/emission wavelengths of 485/530 nm. The migration of A549 cells was also assessed using a scratch wound-healing assay.

Real-time RT-PCR (qRT-PCR) and chromatin immunoprecipitation (ChIP)

RNA isolation was performed using TRIzol reagent (Invitrogen). cDNA was prepared using an oligo (dT) primer and reverse transcriptase (Takara, Shiga, Japan) following standard protocols. Real-time PCR was performed using SYBR Green on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each PCR reaction mixture contained 200 nM of each primer, 10 μL of *2xSYBR Green PCR*

Master Mix (Applied Biosystems, Foster City, CA), and 5 µl cDNA and RNase-free water, with a total volume of 20 µl. The PCR reaction was carried out with a denaturation step at 95℃ for 10 min, then 40 cycles at 95℃ for 15 s, and then at 60℃ for 1 min. All PCRs were performed in triplicate and normalized to internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Relative expressions were presented using the $2^{-\Delta\Delta CT}$ method.

Chromatin immunoprecipitation was performed using an Agarose ChIP Kit (Pierce, Rockford, IL). Immunoprecipitated DNA was analyzed by quantitative PCR as described above, using SYBR Green dye. The primers used in this study have been described in a previous study (18).

Immunoblot

Cells were lysed on ice for 15 min using M-PER lysis reagent (Pierce, USA). Cell lysate was centrifuged at 14,000 × *g* for 15 min, and the supernatant fraction was collected for immunoblotting. Equivalent amounts of protein were resolved by SDS-PAGE (8-12%) and transferred to polyvinylidene difluoride membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 16 h. The membrane was then treated with the appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Millipore) according to the manufacturer's instructions.

Immunofluorescence

Non-cancerous lung tissue specimens obtained from human lung cancer patients were embedded in OCT **(**Tissue Freezing Medium) and frozen in liquid nitrogen. Sections (3-5 µm) were fixed with acetone at -20℃ then stained with resistin antibodies (Abcam,

UK) or co-stained with anti-CD11c antibodies. After washing with PBS containing 0.1% Tween-20, the slides were incubated with Dylight 488- or Dylight 549-conjugated secondary antibodies (Rockland, Gilbertsville, PA), with or without 4',6-diamidino-2-phenylindole (DAPI), for 1 h at room temperature. The data were analyzed with a *confocal laser scanning microscope* (Fluoview *FV1000, Olympus, Tokyo, Japan*).

Osteoclast differentiation and activity assay

PBMCs were plated and incubated overnight at 37℃. Non-adherent cells were removed by washing with PBS, and the remaining adherent cells were grown in culture medium containing mdDC-CM, A549-TADC-CM, or resistin (10 ng/ml) presenting in 200 ng/ml human M-CSF and 100 ng/ml human RANKL for 21 days. The medium was replaced every 3 days, and osteoclast formation was measured by quantifying the cells positively stained by tartrate-resistant acid phosphatase (TRAP) (Sigma-Aldrich, St. Louis, MO, USA). Osteoclasts were determined to be TRAP-positive staining multinuclear (>3 nuclei) cells by means of light microscopy. The TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted. Osteoclast bone resorption activity was assessed using a bone resorption assay kit (Cosmo Bio, Tokyo, Japan), in the same culture conditions as described above.

Gene knockdown by siRNA

Cells were transfected with MISSION® siRNA Universal Negative Controls, WHSC1, or resistin siRNA (Sigma-Aldrich). Immunoblot analyses showed that expression of WHSC1 remained low but detectable, whereas expression of GAPDH was unaffected by siRNA treatment.

Results

Upregulation of resistin were formed in the process of DC differentiation from CD14$^+$ monocytes

This study was identified the solutions factor enhancing cancer cell migration and released from DCs which were stimulated by cancer cells, the microarray was performed as the results showed that TADCs express high levels of resistin by 10.56-fold when compared to those DCs (Figure 1A). qRT-PCR analysis also showed that A549-CM increases the expression of resistin in A549-TADCs (Figure 1B). In addition, the protein levels of resistin are enhanced in A549-TADCs, as determined by ELISA analysis (Figure 1C). Furthermore, co-culture of A549 with DCs also increases the expression of resistin in DCs at mRNA and protein levels (Figure 1D and Figure 1E).

High levels of resistin in CD11c$^+$ DCs are found in the tumor sections of human lung cancer patients

The effects of resistin on TADC-mediated cancer progression in human were studied by using clinical lung cancer tissue samples. Immunofluorescence staining of the marginal regions of a human lung cancer specimen revealed the presence of many CD11c$^+$ DCs which had infiltrated the area around the tumor. These areas express higher levels of resistin in comparison with tissue sections of non-tumorous regions (Figure 2A). Furthermore, a marked statistical difference has been found between lung cancer patients and healthy donor serum (Figure 2B).

Resistin increases cancer migration, invasion, EMT and osteoclastogenesis

The effects of resistin on the proliferation, migration and EMT in A549 cells were invesgated to reveal of resistin on cancer progression. 72 h of treatment of lung cancer cells with resistin did not increase cell proliferation in the A549 cells (Figure 3A).

However, it did enhance the migration and invasion ability of the A549 cells (Figure 3B to 3D). In addition, resistin also causes the A549 cells to undergo EMT, including the downregulation of epithelial markers (E-cadherin and claudin-3), and upregulation of fibroblast markers (vimentin, fibronectin and N-cadherin) after 24 h treatment (Figure 3E). Levels of Twist, an important regulator of EMT, is increased in resistin-treated A549 cells after 6 h treatment (Figure 3E). Furthermore, resistin also increases the differentiation and activity of osteoclasts, which contribute to osteolytic metastases (Figure 3F and 3G).

Neutralization of Resistin by antibodies blocks A549-mdDCs-mediated cancer migration, invasion, EMT and osteoclastogenesis

To better understand the role of resistin, the activity of resistin was blocked by using specific neutralization antibodies. As shown in Figure 4A and B, TADC-CM increases the migration and invasion of A549 cells, whereas these effects are prevented by treating with anti-resistin antibodies, but not by an isotype control (Figure 4A and 4B). Similarly, the induction of A549-TADC-CM on EMT and Twist expression is also inhibited by anti-resistin antibodies (Figure 4C). The stimulation of A549-TADC-CM on osteoclast differentiation is also expressed by anti-resistin antibodies (Figure 4D). These data clearly demonstrate that TADC-derived resistin promotes lung cancer progression.

Resistin increases the expression of WHSC1, and changes the methylation of histone protein

To understand how resistin promotes lung cancer progression, the gene-wide expression profile of resistin-treated A549 cells was determined by using microarray. The results show that resistin up regulates 36 gene but down regulates 275 gene

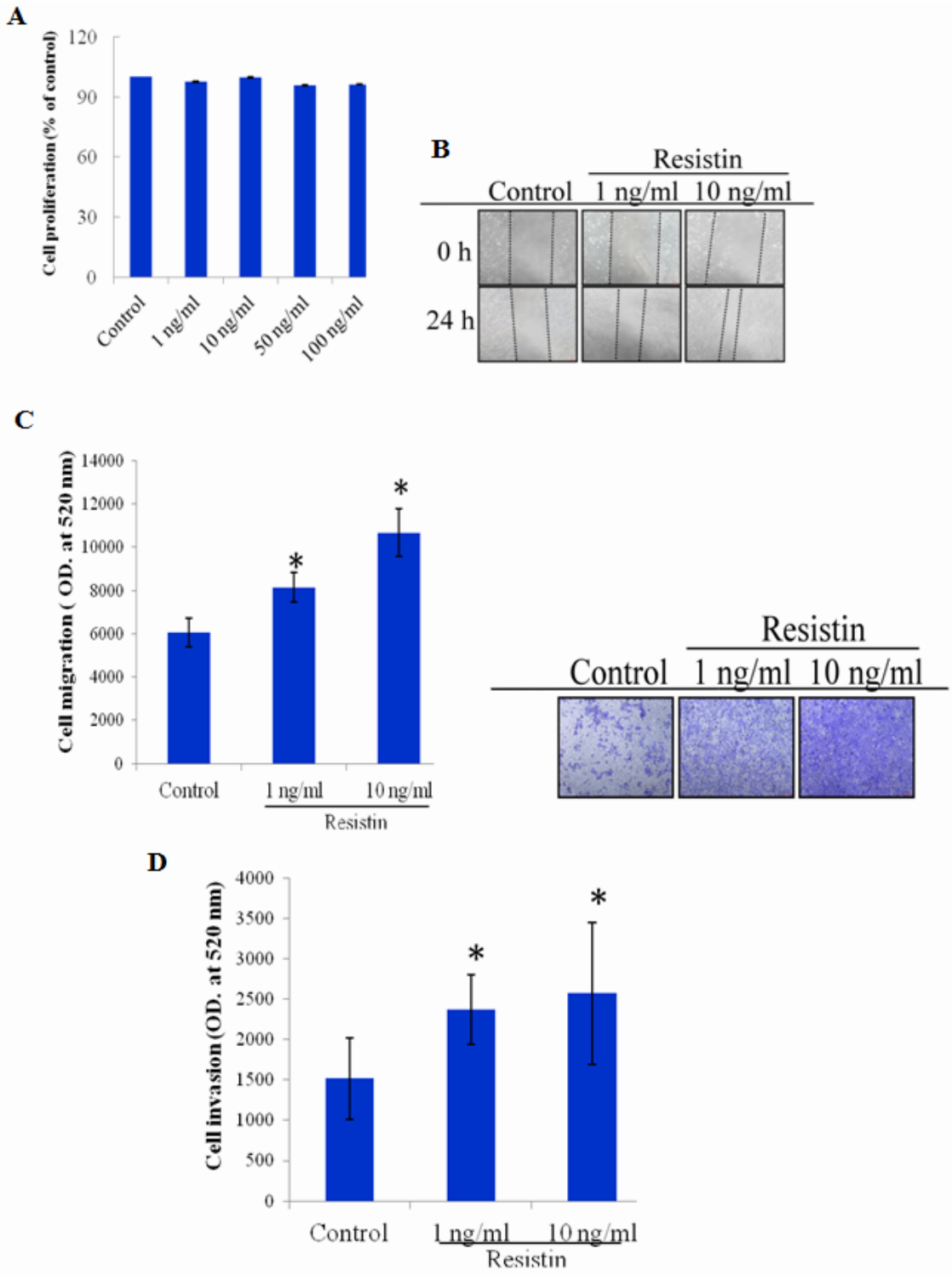
expressions. Among these upregulated genes, levels of WHSC1, an oncogenic histone lysine methyltransferase, increases 3.12-fold in resistin-treated A549 cells (Figure 5A). qRT-PCR analysis also showed that resistin increases the expression of WHSC1 mRNA in A549 cells (Figure 5B). The protein levels of WHSC1 were also enhanced in resistin-treated A549 cells after 6 h treatment, as determined by immunoblot analysis (Figure 5C). Furthermore, resistin treatment increases the dimethylation of H3K36 and decreases trimethylation of H3K27 in A549 cells (Figure 5C).

Resistin increases Twist expression through WHSC1-mediated epigenetic regulation

An elevated level of WHSC1 has been reported to increase the expression of cancer invasion-related genes by changing the modification of histone 3. Next step assessed the binding of WHSC1 on Twist promoter. Therefore, chromatin immunoprecipitation assay was preformed that WHSC1 bound to the Twist promoter after resistin treatment in A549 cells (Figure 6A and 6D). Dimethylation of histone H3 at lysine 36 on the regions of the Twist promoter was thereby increased, whereas the trimethylation of histone H3 on the Twist promoter was decreased (Figure 6B to 6D).

WHSC1 is involved in resistin-mediated Twist regulation and cancer progression

The role of WHSC1 on the resistin-mediated upregulation of Twist and on cell migration and invasion was determined by using siRNA. Transfection of A549 cells with WHSC1 siRNA reduces the levels of WHSC1 (Figure 7A). Selective genetic inhibition of WHSC1 abrogated the resistin-mediated upregulation of Twist and H3K36me2 in cancer cells (Figure 7B). In addition, specific knockdown WHSC1 expression by WHSC1 siRNA decreases cell migration and invasion in resistin-treated cells (Figure 7C and 7D). These results suggest that WHSC1 is an upstream event of Twist, and involved in resistin-mediated cancer progression.


A
Cell proliferation (% of control)
120
90
60
30
0
Control
1 ng/ml
10 ng/ml
50 ng/ml
100 ng/ml
B
Resistin
Control
1 ng/ml
10 ng/ml
0 h
24 h
C
Cell migration (OD. at 520 nm)
14000
12000
10000
8000
6000
4000
2000
0
Control
1 ng/ml
10 ng/ml
Resistin
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Resistin
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1 ng/ml
10 ng/ml
D
Cell invasion (OD. at 520 nm)
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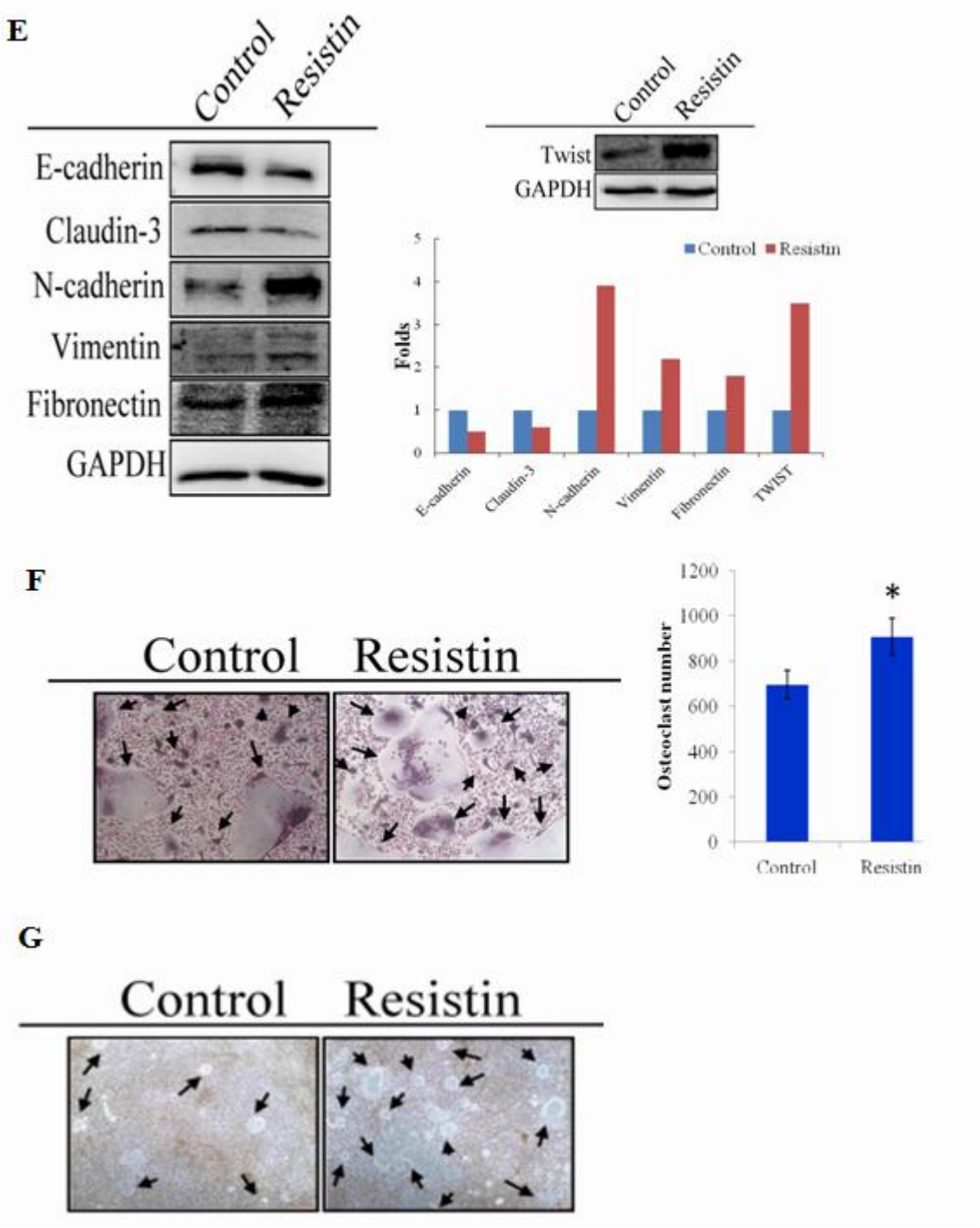
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Control
Resistin
E-cadherin
Claudin-3
N-cadherin
Vimentin
Fibronectin
GAPDH
Control
Resistin
Twist
GAPDH
Control
Resistin
Folds
E-cadherin
Claudin-3
N-cadherin
Vimentin
Fibronectin
TWIST
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Osteoclast number
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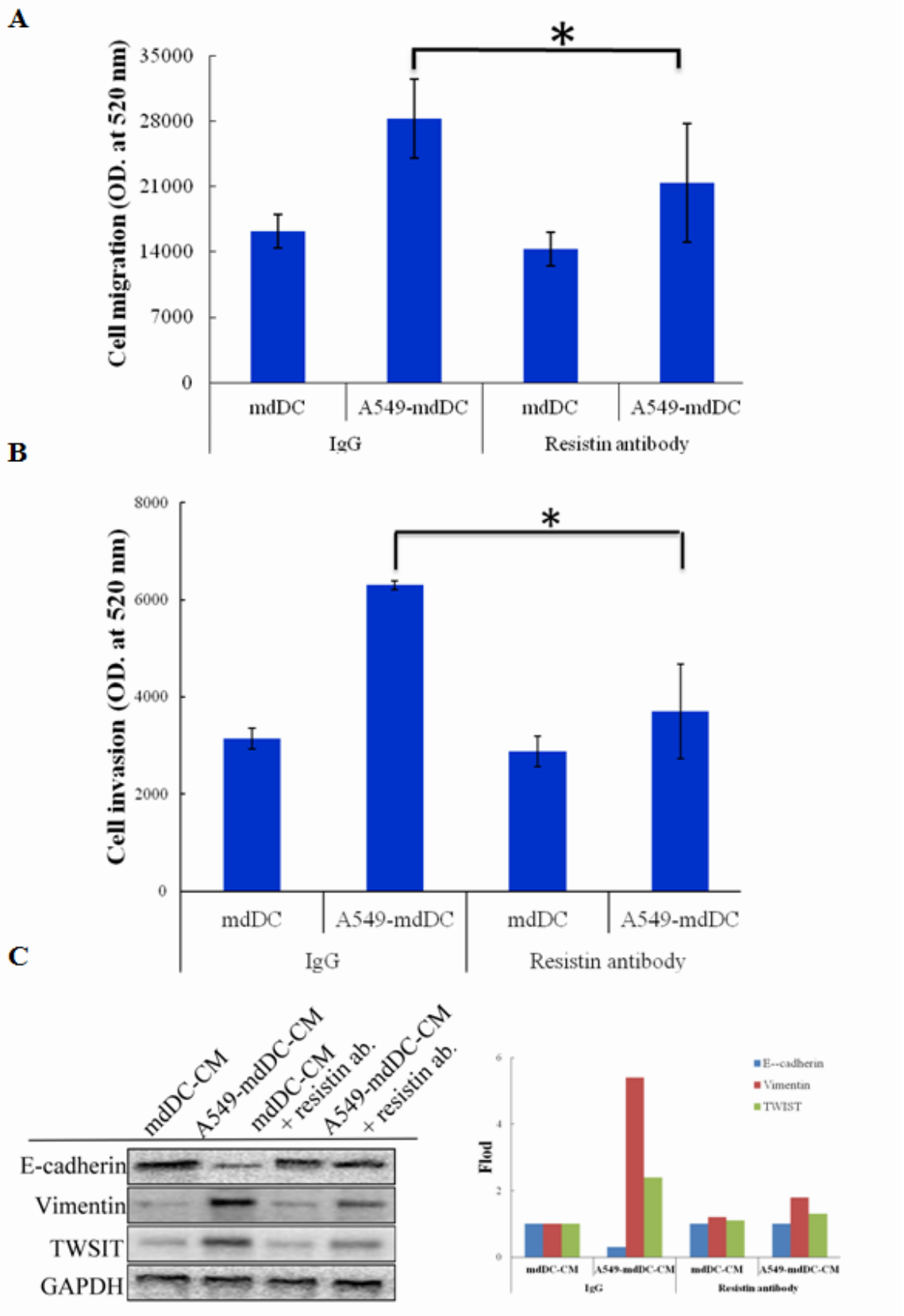
G
Control
Resistin

Figure 3


A
Cell migration (OD. at 520 nm)
35000
28000
21000
14000
7000
0
*
mdDC
A549-mdDC
mdDC
A549-mdDC
IgG
Resistin antibody
B
Cell invasion (OD. at 520 nm)
8000
6000
4000
2000
0
*
mdDC
A549-mdDC
mdDC
A549-mdDC
IgG
Resistin antibody
C
mdDC-CM
A549-mdDC-CM
mdDC-CM + resistin ab.
A549-mdDC-CM + resistin ab.
E-cadherin
Vimentin
TWSIT
GAPDH
Flod
E--cadherin
Vimentin
TWIST
mdDC-CM
A549-mdDC-CM
mdDC-CM
A549-mdDC-CM
IgG
Resistin antibody

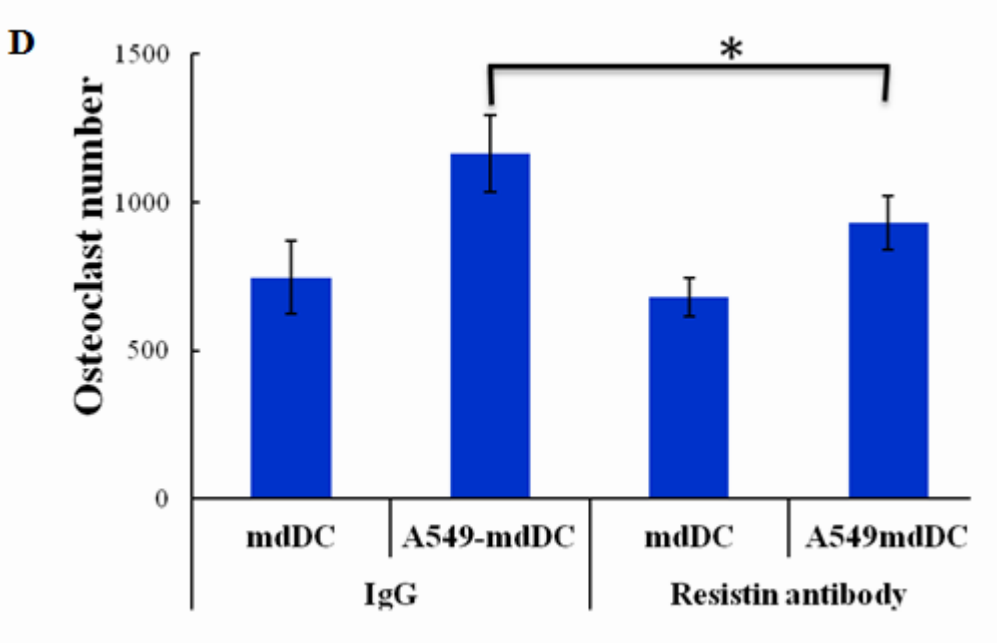

D
Osteoclast number
1500
1000
500
0
*
mdDC
A549-mdDC
mdDC
A549mdDC
IgG
Resistin antibody

Figure 4

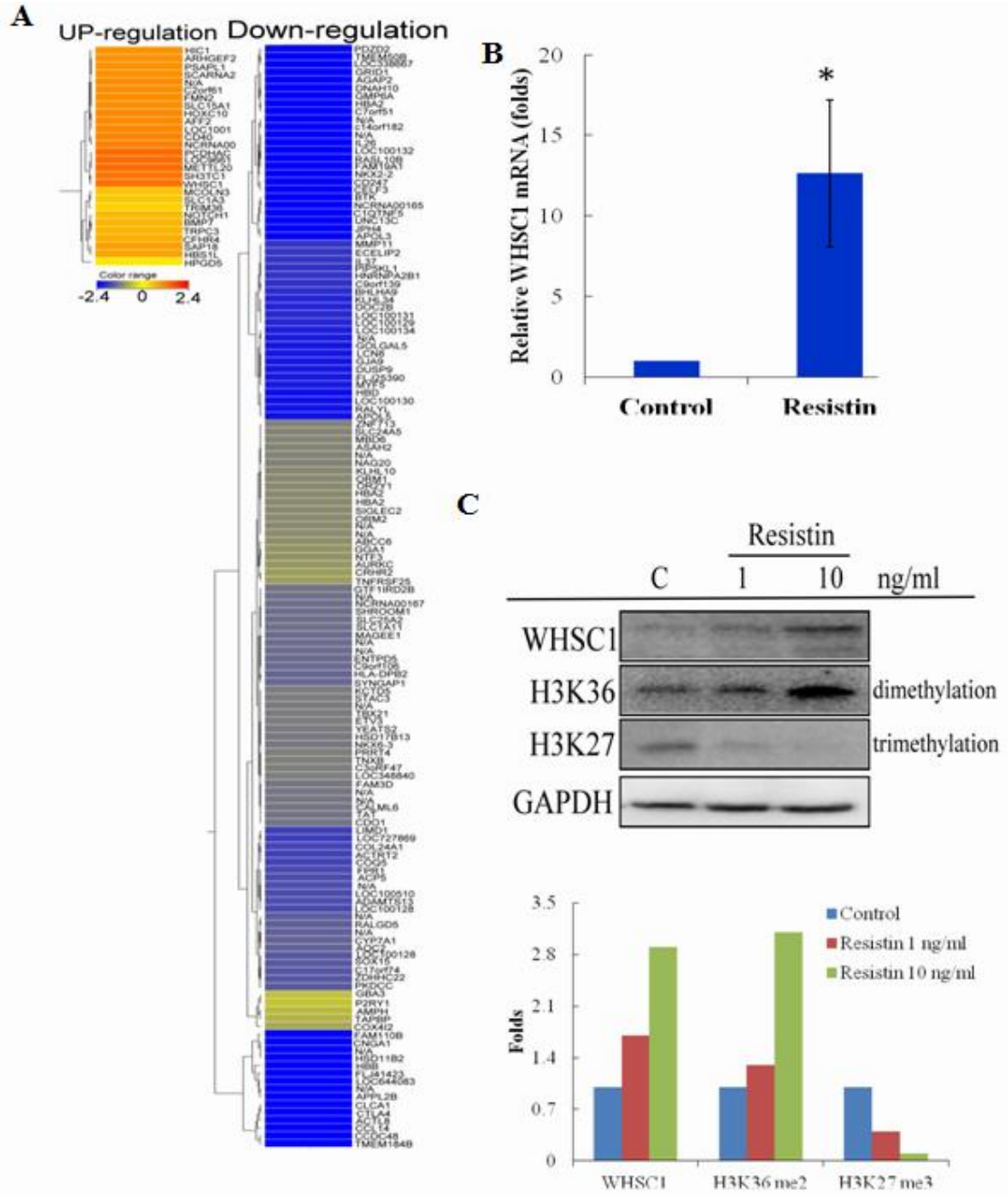
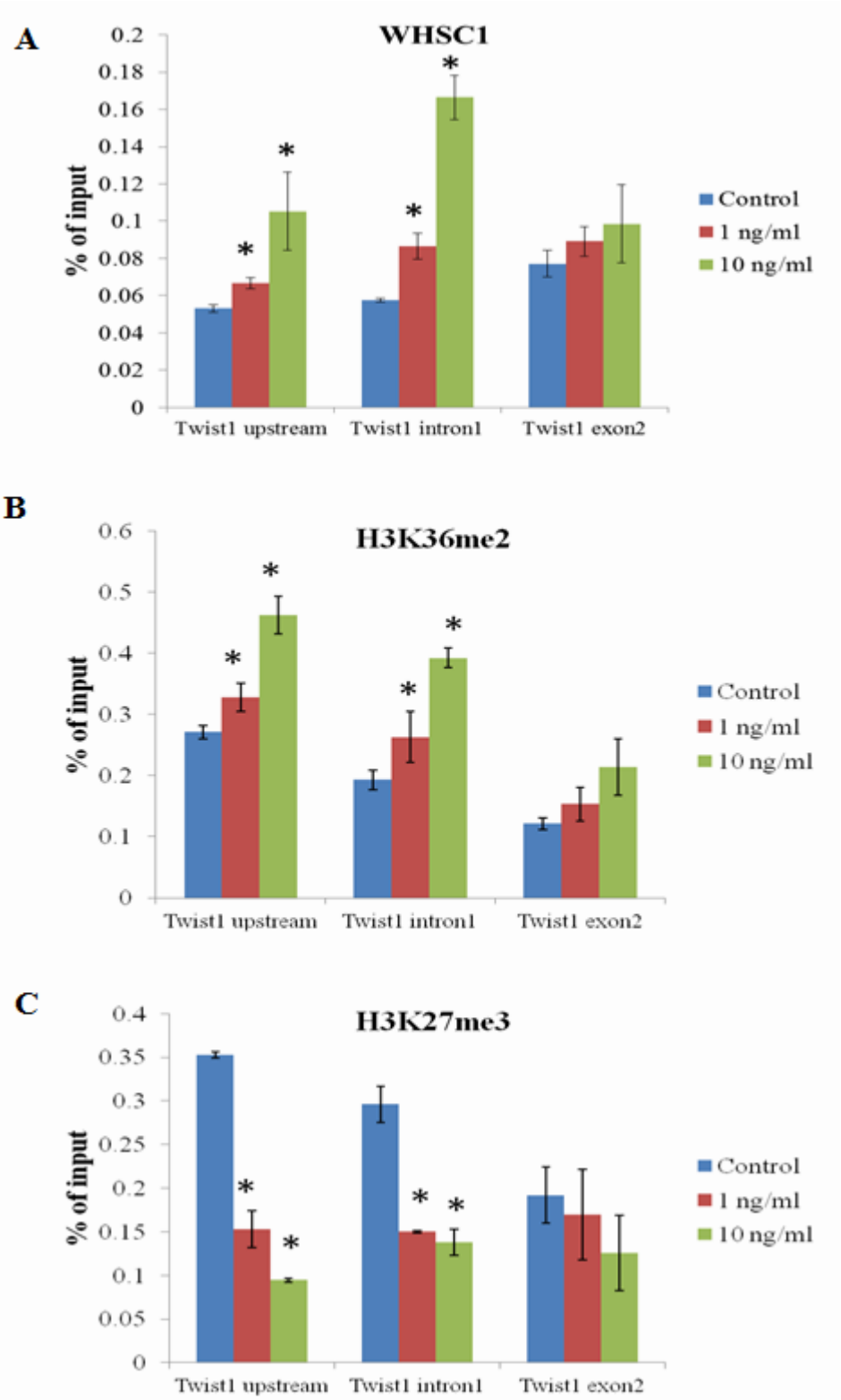

A
UP-regulation
Down-regulation
Color range
-2.4
0
2.4
B
Relative WHSC1 mRNA (folds)
20
15
10
5
0
*
Control
Resistin
C
Resistin
C
1
10
ng/ml
WHSC1
H3K36
dimethylation
H3K27
trimethylation
GAPDH
Folds
3.5
2.8
2.1
1.4
0.7
0
Control
Resistin 1 ng/ml
Resistin 10 ng/ml
WHSC1
H3K36 me2
H3K27 me3

Figure 5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WHSC1
% of input
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Twist1 upstream
Twist1 intron1
Twist1 exon2
Control
1 ng/ml
10 ng/ml
B
H3K36me2
% of input
0.6
0.5
0.4
0.3
0.2
0.1
0
Twist1 upstream
Twist1 intron1
Twist1 exon2
Control
1 ng/ml
10 ng/ml
C
H3K27me3
% of input
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Twist1 upstream
Twist1 intron1
Twist1 exon2
Control
1 ng/ml
10 ng/ml

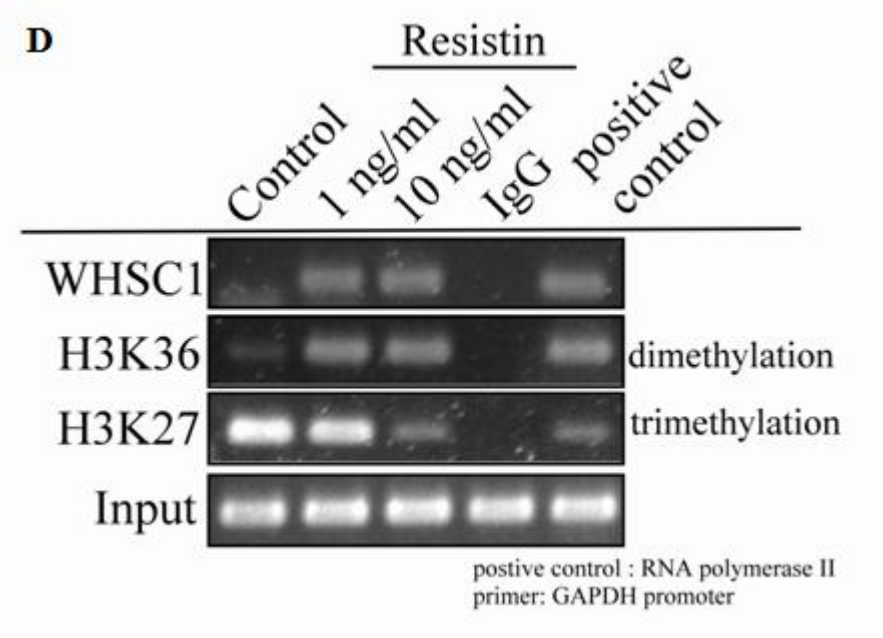
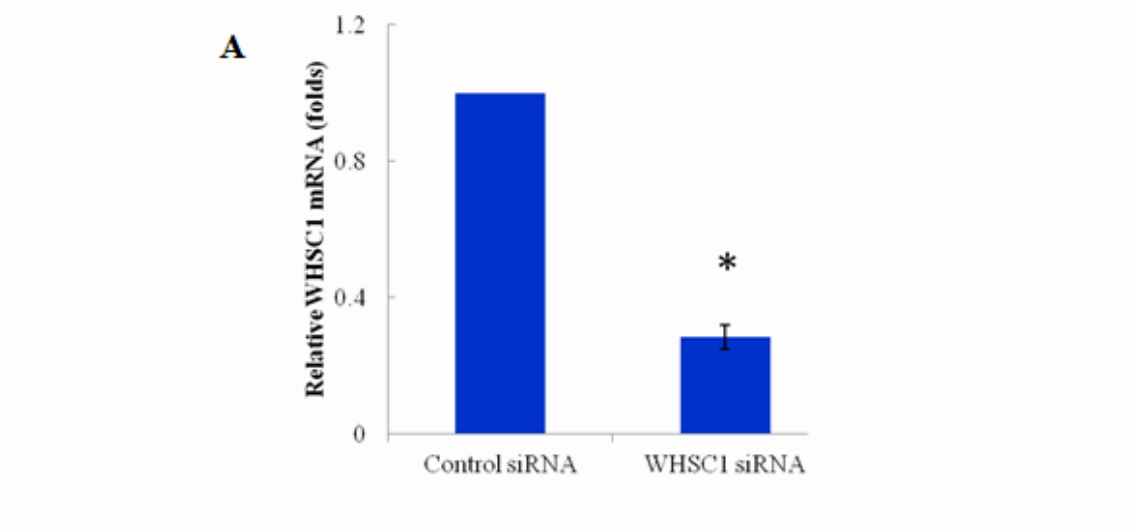

D
Resistin
Control
1 ng/ml
10 ng/ml
IgG
positive control
WHSC1
H3K36
dimethylation
H3K27
trimethylation
Input
postive control : RNA polymerase II
primer: GAPDH promoter

Figure 6


A
Relative WHSC1 mRNA (folds)
1.2
0.8
0.4
0
*
Control siRNA
WHSC1 siRNA

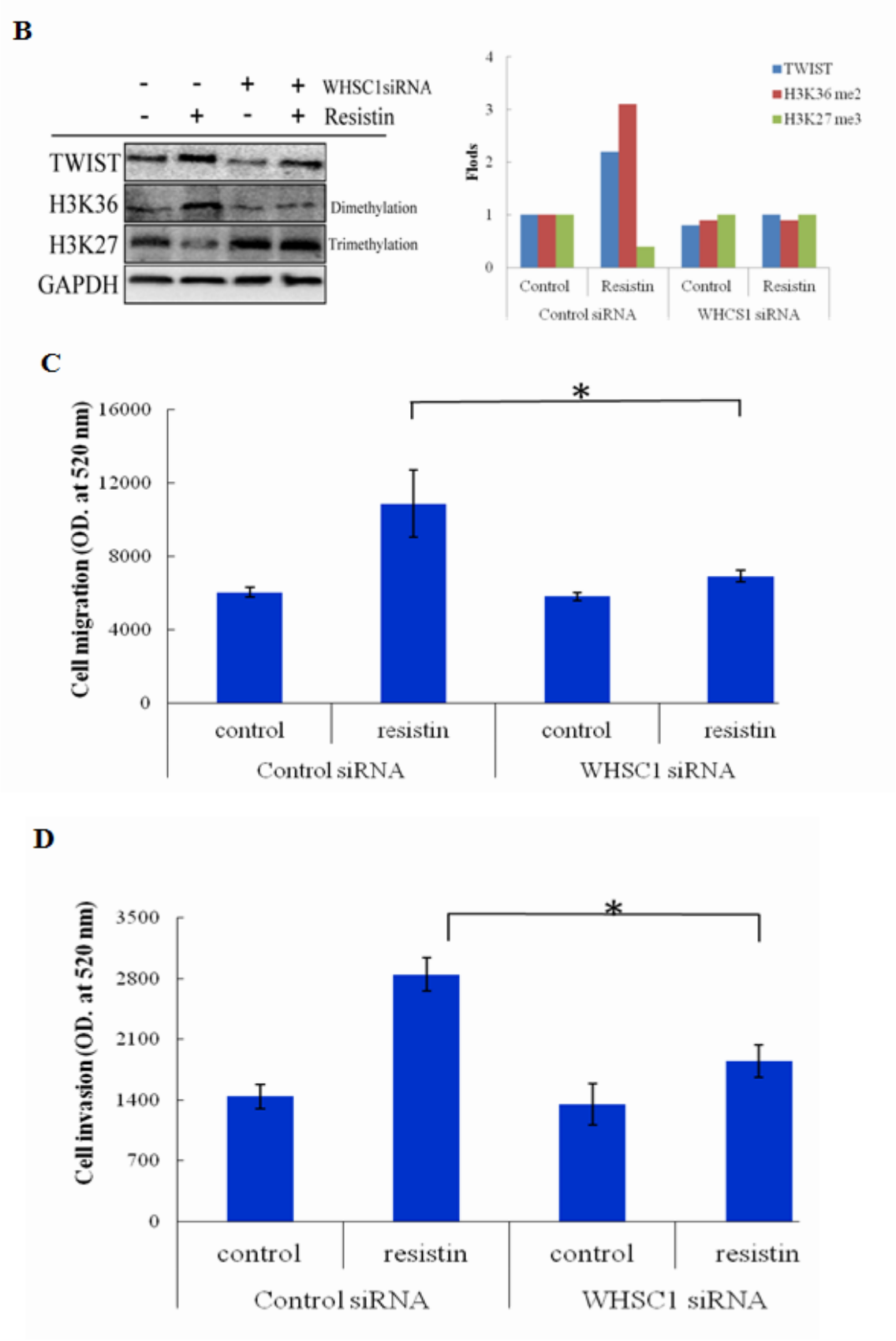

B
- - + + WHSC1siRNA
- + - + Resistin
TWIST
H3K36
Dimethylation
H3K27
Trimethylation
GAPDH
TWIST
H3K36 me2
H3K27 me3
Flods
Control
Resistin
Control
Resistin
Control siRNA
WHCS1 siRNA
C
Cell migration (OD. at 520 nm)
16000
12000
8000
4000
0
*
control
resistin
control
resistin
Control siRNA
WHSC1 siRNA
D
Cell invasion (OD. at 520 nm)
3500
2800
2100
1400
700
0
*
control
resistin
control
resistin
Control siRNA
WHSC1 siRNA

Figure 7

Discussion

Lung cancer is one of the most common cancers in developed and developing countries (1-3, 21). In the present study, it has demonstrated that resistin, produced by cancer-infiltrating DCs, was able to increase the migration and invasiveness of lung cancer. Furthermore, resistin also shows a direct stimulatory effect on the differentiation of osteoclasts, which is an important pathological factor for the development of bone metastasis in lung cancer. These findings suggest that TADC-derived resistin may be a novel candidate in conferring the ability for lung cancer to develop.

Resistin is a recently identified adipocyte-derived hormone that was originally thought to link obesity with diabetes (10, 22). Resistin is upregulated in several cancers, such as breast, colon and prostate cancer, and higher levels are associated with the highest histological grade (15-17, 23, 24). Resistin has been reported to increase the proliferation of prostate cancer by activating PI3K/AKT signaling (23). It has also been indicated that resistin increases the expression of MMPs, decreases the synthesis of tissue inhibitors of metalloproteinase production, and thereby enhances the invasiveness of trophoblast-like cells (24). In humans, adipose tissue is not the primary source of resistin and RELMs, which are instead secreted by PBMCs, macrophages, bone marrow and pancreatic cells (25). The results show that the source of resistin production is secretion from tumor-surrounding dendritic cells. TADCs produce high levels of resistin, which increases lung cancer migration and invasion and cause lung cancer cells to undergo EMT. Neutralization of resistin by antibodies, however, prevents this effect of TADCs on cancer progression. Moreover, TADC-derived resistin increases osteoclast differentiation and bone resorption activity, which suggests that TADC-derived resistin is responsible for the development of osteolytic bone metastasis. Investigation of the paradoxical roles of DCs in lung cancer development and malignant pathogenesis is

very important for gaining insight into the intact tumor microenvironment.

Interestingly, this study has found that resistin-producing $CD11c^+$ DCs infiltrate the lungs of cancer patients. In addition, levels of resistin in the serum of lung cancer patients are also higher than those of healthy donors. These results have been consistently observed in cell culture studies and, more importantly, in human specimens. These findings provide unique evidence that TADCs enhance the development of lung cancer by secreting the tumorigenic factor resistin.

Twist is a class II member of the basic helix-loop-helix (bHLH) transcription factor essential for regulating EMT, a process required for enhancement of cell mobility (26, 27). Twist is upregulated in various human solid cancers and is strongly associated with cancer invasion and metastasis (28, 29). Twist binds the E-box DNA elements of specific gene promoters, resulting in the repression of E-cadherin expression (30). In this study, we found that treatment of A549 cells with resistin resultes in an increase in Twist expression and a decrease in E-cadherin. Inhibition of Twist expression by specific shRNA transfection decreases the effects of resistin on cell migration and E-cadherin upregulation in A549 cells, suggesting that the activation of Twist signaling plays a crucial role in the development of lung cancer growth and metastasis-induced TADCs or resistin.

WHSC1 (also known as NSD2 or MMSET), located at chromosome 4p16.3, encodes a histone lysine methyltransferase (31). WHSC1 acts as a transcriptional regulator and interacts with developmental transcription factors whose defects overlap with the human disease Wolf-Hirschhorn syndrome (32). Methylation of histone at lysine residues promotes or prevents the binding of transcription factors and other proteins that access particular regions of the genome, resulting in the regulation of the

transcriptional potential of the underlying genes (33, 34). Dimethylation of histone H3 at lysine 36 (H3K36me2) is associated with actively transcribed regions, and has been implicated as the mechanism for continuing transcription (35). WHSC1 is ubiquitously expressed in early development embryonic tissues, whereas it is silent in most healthy adult tissues except for the thymus and testes (36). WHSC1 has been found to be overexpressed in various types of carcinomas, and elevation of WHSC1 has been shown to promote cellular growth, migration, invasion and tumorigenicity in multiple myeloma, glioblastoma and prostate cancers (18, 20, 37). WHSC1 triggers oncogenic Wnt and NF-κB signaling by increasing trimethylation of histone H3 at lysine 36, resulting in the promotion of the cancer development (20, 37). WHSC1 has also been reported to be an activator of Twist and to contribute to the EMT and invasive properties of prostate cancer (18, 20). This study demonstrated that A549 cells treated with resistin dramatically increase the expression of WHSC1, in turn enhancing the dimethylation of histone H3 at lysine 36, resulting in an increase of Twist expression. Furthermore, selective inhibition of WHSC1 by shRNA transfection also decrease the effects of resistin on cell migration, invasion and E-cadherin downregulation, suggesting that the phenotypic transition of lung cancer by TADCs involves resistin-mediated WHSC1 upregulation and subsequent WHSC1-mediated Twist gene expression by epigenetic modification.

In summary, present studies show a novel mechanism for lung cancer progression, in which TADC-derived resistin promotes phenotypic transition and reinforces cancer progression. Resistin enhances the expression of WHSC1 and subsequently increases the expression of Twist by epigenetic modification, resulting in enhanced lung cancer development. The findings of this study suggest a new molecular determinant to target the vicious cycle of DCs and cancer, and provide a new rationale for using anti-resistin

strategies to target invasive lung cancer.

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Figure legends

Figure 1. Upregulation of resistin is found in tumor-associated DCs. A549-CM increases the expression of resistin at the mRNA level, as determined by microarray (A) and qRT-PCR (B). A549-CM increases the expression of resistin in protein levels (C). Co-culture of A549 cells with DCs increases the expression of resistin at mRNA (D) and protein (E) in DCs. The asterisk indicates a significant difference between the two test groups, as analyzed by the Student's *t* test (*, $P < 0.05$).

Figure 2. High levels of resistin in $CD11c^+$ DCs are found in the tumor sections of human lung cancer patients. (A) $Resistin^{high}$ $CD11c^+$ DCs are found to have infiltrated cancer sections. Non-tumorous and tumorous regions (n=10) were cut and stained, then samples were analyzed by confocal microscopy. (B) High levels of resistin have also been found in the serum of lung cancer patients. Resistin levels in the sera of lung cancer patients (n=46) and healthy donors (n=24) were assessed by ELISA. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by the Student's *t* test (*, $P < 0.05$).

Figure 3. Resistin increases lung cancer progression and osteoclastogenesis. Resistin did not affect cell proliferation. (A) Resistin increases cell migration, as determined by wound healing (B) and the transwell system (C). Resistin increases cell invasion (D), EMT and Twist expression (E) in A549 cells. Resistin increases osteoclast differentiation (F) and activity (G). Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference compared with the controls, as analyzed by Student's *t* test (*, $P < 0.05$).

Figure 4. **Inhibition of resistin by its neutralizing antibody decreases TADC-CM -mediated cancer progression.** Resistin antibodies decreased the effect of

TADC-CM on cell migration (A), invasion (B), EMT (C) and osteoclastogenesis (D). A549 cells were treated **with** mdDC-CM or TADC-CM presenting in resistin antibodies or IgG. A549 cells were seeded in the upper insert and were treated with or without resistin antibody (2 μg/ml) or IgG (2 μg/ml), and TADC-CM (20%) was added into the lower well to act as a chemoattractive agent for 24 h. The migratory and invasive cells were quantified as described above. PBMCs were treated with mdDC or TADC-CMs presenting in RANKL (100 ng/mL) and M-CSF (200 ng/mL) for 21 days. Osteoclast cells were stained for TRAP activity. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by the Student's *t* test (*, $P < 0.05$).

Figure 5. Resistin induces histone modification of Twist promoter by increasing WHSC1 expression. (A) The profile of gene expression in resistin-treated A549 cells. Cells were treated with resistin (10 ng/ml) for 6 h, and gene expression assessed by microarray. Resistin changes WHSC1 expression at mRNA (B) and protein as well as the methylation modification of histone proteins (C). Cells were treated with resistin (10 ng/ml) for the indicated times, and the levels of various proteins were assessed by immunoblot assays. The asterisk indicates a significant difference between the two test groups, as analyzed by the Student's *t* test (*, $P < 0.05$).

Figure 6. Resistin increases Twist expression through WHSC1-mediated epigenetic regulation. (A) The binding of WHSC1 on promoter regions of Twist. The change of H3K27me3 (B) and H3K36me2 (C) on promoter regions of Twist in A549 cells.(D) The agarose electrophoresis data of ChIP. Dimethylation and trimethylation of histone H3 and H4 were assessed by ChIP assay. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Student's *t* test (*, $P < 0.05$)

Figure 7. WHSC1 is involved in resistin-mediated Twist upregulation and cancer progression. (A) The efficacy of WHSC1 siRNA transfection. Blockade of WHSC1 prevents resistin-mediated Twist upregulation (B) cell migration (C) and invasion (D). Cells were transfected with either **cont**

rol siRNA or WHSC1 siRNA, and the efficacy of siRNA was assessed by qRT-PCR. siRNA-transfected cells were treated with resistin (10 ng/ml) for the specified times (Twist and histone methylation, 6 h; cell migration, 48 h; invasion, 48 h). Protein levels were assessed by immunoblot assays. Cell migration and invasion were assessed by QCM™ 24-well Cell Migration and Invasion Assay kits. Dimethylation and trimethylation of histone H3 and H4 were assessed by Western blot assay. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by Student's *t* test (*, $P < 0.05$)