國 立 成 功 大 學 醫 學 院
臨 床 醫 學 研 究 所

博 士 論 文

第一型血小板活化素在子宮頸癌化過程中扮演抑制腫瘤血管新生及基質反應角色之探討

The roles of thrombospondin-1 in tumor angiogenesis and stroma reaction during cervical carcinogenesis

研究生：吳銘斌

指導教授：周振陽教授、吳梨華副教授

2008 年 05 月 05 日

國立成功大學

博士論文

第一型血小板活化素在子宮頸癌化過程中扮演抑制腫瘤血管新生及基質反應角色之探討

The roles of thrombospondin-1 in tumor angiogenesis and stroma reaction during cervical carcinogenesis

研究生：吳銘斌

本論文業經審查及口試合格特此證明

論文考試委員：

指導教授：

系(所)主管：

中 華 民 國 97 年 5 月 5 日

中文摘要

血管新生表現型的出現(血管新生作用開關)，是子宮頸癌化過程是必須的步驟。而血管新生作用開關是由血管新生活化因子與抑制因子兩者相互抗衡的所調控。第一型血小板活化素(thrombospondin, TSP-1)是一種內生性血管新生抑制因子，具有多處大分子特殊結構(domains)及多個受體(receptor)。首先，我們探討在子宮頸鱗狀細胞癌化過程中，第一型血小板活化素的表現與人體子宮頸鱗狀細胞癌及其癌前病變之子宮頸檢體中，在時間與空間上的關聯性；以及其與腫瘤血管新生表現型的關聯性。我們的結果顯示：第一型血小板活化素主要表現於正常子宮頸基底上皮細胞層，如柵欄(我們命名為"第一型血小板活化素柵欄")一般阻止血管新生的出現。當子宮頸上皮細胞由低階鱗狀上皮內病變(LSIL)進入至高階鱗狀上皮內病變(HSIL)時，此柵欄發生崩解，與血管新生表現型的出現是同時發生的。由這樣的結果我們推測，第一型血小板活化素扮演抑制血管新生作用的調控角色。血管新生作用開始於子宮頸癌化過程中的早期，與增殖異常的上皮組織中發生第一型血小板活化素的下調節有相關。

基質作用(stroma reaction)是指腫瘤癌化過程中，基質由本來抑制轉為幫助上皮細胞成長，形成適合腫瘤成長環境的反應。因為，第一型血小板活化素除了已知抑制血管新生的角色外，同時也扮演著影響其他基質細胞(如纖維母細胞等)的生理作用的角色。所以，我們假設第一型血小板活化素可以經由抑制基質反應，進而達到抑制腫瘤成長。可能的作用機轉是經由抑制纖維母細胞的活性(移動性、侵襲性等生物特性)、以及抑制基質標記表現量。在本研究中我們探討：一：在臨床子宮頸癌及其癌前病變檢體中，檢視第一型血小板活化素的表現量與基質標記表現量之相關性；二：藉由免疫不全鼠(SCID mice)的異體腫瘤移植實驗，探討第一型血小板活化素經由抑制血管新生，以及基質反應而達到抗腫瘤成長的能力；三：探討第一型血小板活化素經由抑制肌纖維母細胞(活化型纖維母細胞)的移動力、侵襲力，達到抑制基質反應的能力。實驗結果顯示：一、在臨床病人檢體的免疫組織化學染色中發現，第一型血小板活化素表現量消失，會同時合併兩種基質標記 α-SMA 與 desmin 過度表現。顯示在子宮頸癌化過程中，第一型血小板活化素與腫瘤基質反應有關連性。二、經由轉染

第一型血小板活化素進入子宮頸癌細胞株(SiHa)，建立第一型血小板活化素過度表現的子宮頸癌細胞株(SiHa-TSP-1)。在體外模式中，具有抑制血管新生作用的生理功能。同時在免疫不全鼠的異體腫瘤移植實驗中，第一型血小板活化素過度表現可以經由降低腫瘤血管生長以及減少基質標記 α-SMA 與 desmin 的表現，達到抑制腫瘤生長的作用。三、經由轉染建立第一型血小板活化素過度表現的纖維母細胞株(NIH3T3-TSP-1)或是外加純化的第一型血小板活化素至纖維母細胞株(NIH3T3)中，發現第一型血小板活化素並不會直接影響纖維母細胞株(NIH3T3)之 α-SMA 與 desmin 蛋白質的表現量。在乙型變型性成長因子(transforming growth factor β, TGF-β)處理的情況下(乙型變型性成長因子，會增加纖維母細胞的活化作用及其細胞分化及 α-SMA 與 desmin 過度表現)，第一型血小板活化素也沒有抑制作用。相對地，第一型血小板活化素會明顯抑制乙型變型性成長因子處理纖維母細胞株(NIH3T3)後所增加纖維細胞移動力，並抑制金屬蛋白酶-2 (MMP-2)的活性及侵襲癌細胞群的能力。至於作用的細胞種類，不論是經由轉染至子宮頸癌細胞株中(SiHa-TSP-1)或是直接外加純化的第一型血小板活化素至子宮頸癌細胞株中時，對 NIH3T3 細胞株的侵襲癌細胞群的能力沒有影響。相對地，經由轉染至纖維母細胞株(NIH3T3-TSP-1)或是外加純化的第一型血小板活化素至纖維母細胞株(NIH3T3)中，對 NIH3T3 細胞株的侵襲癌細胞群的能力皆有影響。

綜合這些結果，我們發現第一型血小板活化素具有抑制基質反應功能的新角色。第一型血小板活化素會抑制免疫不全鼠異體移植腫瘤形成模式中基質標記的表現，與臨床病人子宮頸病變檢體基質標記的表現相符合。第一型血小板活化素經由抑制肌纖維母細胞(非宮頸癌細胞)移動力及侵襲能力，達到抑制基質反應並可能回復腫瘤基質反應到正常狀態。在未來，藉由控制腫瘤基質微環境，可能可以提高治療癌症的有效能力。

關鍵字：血管新生作用、第一型血小板活化素、α-平滑肌肌動蛋白、子宮頸癌、惡性腫瘤、纖維母細胞、肌纖維母細胞、活化型纖維母細胞、基底膜、基質反應

Abstract

The acquisition of an angiogenic phenotype (angiogenic switch) is essential for cervical carcinogenesis. The angiogenic switch is balanced by the angiogenic activators and inhibitors. Thrombospondin-1 (TSP-1) is an endogenous angiogenic inhibitor with multiple functional domains and interacting receptors. Our study was firstly aimed to examine the spatial and temporal relationship of TSP-1 expression in patients with squamous cell carcinoma of uterine cervix and its precursor lesions, and to correlate its expression with tumor angiogenesis. Our results indicate the disruption of TSP-1 fence (the expression of TSP-1 in basal epithelia) and the switch to angiogenic phenotype occurred concordantly during the transition from low grade squamous intraepithelial lesion (LSIL) into high grade squamous intraepithelial lesion (HSIL). This concordance suggests that TSP-1 play a role in the regulation of angiogenic switch during cervical carcinogenesis. We conclude that the onset of angiogenesis is an early event in cervical carcinogenesis due, in part, to the down-regulation of TSP-1 by the dysplastic epithelium.

Stroma reaction (also called stromagenesis) is a host reaction of stroma cells that, when induced in cancer, produces a progressive and permissive mesenchymal microenvironment, thereby supporting tumor progress. In addition to the well-known angiogenesis inhibitor, TSP-1 has been shown to exert different biological functions on various stromal cell types, e.g. fibroblasts. Therefore, we hypothesized that TSP-1 may play a role in stroma reaction, characterized by fibroblast activation. We firstly tried to elucidate the correlation between the TSP-1 expression and the overexpression of stroma markers in human cervical lesions; secondly, we tried to elucidate whether TSP-1 can exhibit its anti-tumor effects through the angio-inhibitory effects and the ability to inhibit tumor stroma reaction in SCID mice xenotransplant model; thirdly, we tried to elucidate whether TSP-1

can change the stroma reaction by inhibiting the migration and invasive ability of myofibroblast (activated fibroblasts) from invading tumor cell cluster. Our results revealed: First, immunohistochemistry staining of human clinical specimens showed the disappearance of TSP-1 coincided with the emergence of the overexpression of two stromal markers, α-SMA and desmin, in a stepwise pattern. Second, transfection of SiHa cervical cancer cells with a plasmid expressing the TSP-1 protein exhibited anti-angiogenic activity in vitro, and resulted in reduced tumor growth in SCID mice, which was accompanied by a decrease in tumor vascularization and lower expressions of α-SMA and desmin than those in the vector controls. Third, transfection with TSP-1 and purified TSP-1 added to NIH3T3 cells did not alter the protein levels of α-SMA and desmin which was increased by transforming growth factor β (TGF-β), a potent fibroblast activation and transdifferentiation factor, but significantly inhibited matrix metalloprotease-2 (MMP-2) activity. The increased migration ability and the invasive ability into tumor cluster of TGF-β-treated-NIH3T3 cells were dose-dependently inhibited by TSP-1. In contrast, ectopic TSP-1 expression in SiHa cells has little effect on the invasive ability of the NIH3T3 cells.

Together, these data demonstrate that TSP-1 possesses a novel role to reduce the expression of stromal markers in both human clinical specimens, and an *in vivo* tumor model. The inhibitory ability of TSP-1 to reverse stroma reaction could be partly attributed to the blockage of myofibroblasts from invading cancer. By targeting the tumor-stroma microenvironment, treatment effectiveness could be increased.

Keywords: activated fibroblasts, angiogenesis, α-smooth muscle actin, cervical neoplasms, desmin, fibroblasts, myofibroblasts, stromal reaction, thrombospondin-1

致謝 Acknowledgement

大約在 1999 年夏天，基於對自己所學深感不足及對追求科學知識渴望的情境下，一個必須再進修的念頭浮現腦海。在短暫思考後，就決定準備報考成大研究所博士班。經過近一年的準備及旁聽，於 2000 年 9 月正式進入臨床醫學所，開始了在腫瘤細胞生物科學領域的鑽研。經過漫長的二千八百個日子的奮鬥與等待，終於在與我同年入學的兒子小學畢業的一年後，完成渴望已久的博士學位。

首先，感謝對我影響最深，也認識我最久的老師，也就是我的指導教授─周振陽醫師(周醫師有婦癌醫學會理事長、成大癌症中心主任、卓越教授等等，許多偉大的頭銜，但是還是以最初認識時的稱呼最親切)。周醫師治學態度的嚴謹是眾所周知的，卻能容許我在研究領域中獨立自主，自由自在地讓思緒遨翔。然而，在我研究及實驗方向有所偏離時的重要時刻，卻總能給我即時且強而正確的指正。周醫師邏輯推理的能力，總是那麼令人佩服，許多片段的數據在他手中總能變成前後連慣合乎邏輯的故事。而且，在他非常忙碌的行政、臨床、研究工作之下，對於我請求一對一談話時間，卻永遠很願意熱心地撥出寶貴的時間來。回想許許多多的深夜裡，知道周醫師在電話的另一端仍埋頭修改我的論文，而且，在投稿審稿往返過程中一次又一次的修改，真是讓我非常感動。多少假日裡（包括農曆過年期間），找我一起討論分析時驗數據，並指導研究方向。自 1991 年認識周醫師以來，不僅是我在婦科學的啟蒙老師，更是博士班的指導教授，轉眼已經 18 年的師生情誼。師恩浩盪，銘感五內。

同時，感謝另一位指導教授─吳梨華老師，帶我進入了血管新生學的研究領域。在我入學前，在吳老師課堂旁聽的時候，就被吳老師的教學風格及研究熱忱所深深吸引。決定選擇腫瘤血管新生學為博士進修題目。感謝吳老師一直對我的包容與永不放棄的鼓勵。吳老師在實驗技術困難的排除、研究方向與實驗設計的指導更是不遺餘力。在吳老師的實驗室會議裡提出的問題，永遠不會被認為是愚蠢的問題，只有對科學真理的追求熱忱。吳老師是一位能與學生一起揮淚，一起耕耘，然後一起歡笑的老師。

感謝論文指導委員會的老師們劉校生老師(召集人)、吳華林老師、張文粲老師、曾慶誠醫師，長久以來的傾聽、陪伴。並且，在每次的進度報告中，給予重要的建議及指正。病理部曾醫師在病理切片免疫組織染色的相關研究，也給予大力的幫助與指導。同時，也感謝在最後學位考口試中，台大毒理所郭明良老師（兼台大醫學院副院長）及婦產部鄭文芳醫師，兩位老師同意撥冗前來擔任共同口試委員。在兩位卓越的研究前輩認可下畢業，更是與有榮焉。

感謝在臨醫所所有指導過我的教授老師們，或在教室課堂上，或在實驗室，或在走廊一隅，都有您們的指導及對科學思考的激發。感謝起初，臨醫所創所所長蘇益仁教授的鼓勵與賞識，允許我進入臨醫所就讀。同時，要特別感謝賴明德老師，在科學領域給我一位哲學家、科學家、及為人師表的風範。賴老師總是對我保持樂觀、鼓勵、積極態度。我幾次在實驗瓶頸情緒最低潮時，找賴老師墾談。每每與賴老師談完話，前面道路好像就突然出現一絲曙光。人生彷彿就像賴老師研究室窗外的天空一般明亮。

感謝我前後二位研究助理給我在實驗操作執行方面的幫忙。李秋琳小姐在我初進入成大實驗室時，陪我度過一段非常困難且辛苦的時日。秋琳總用柔和的心面對困境。在 2005 年，我出國進修回國後，因為研究方向有些轉變，在實驗技術執行面發生瓶頸時，楊明哲碩士適時地加入研究的行列，給我在技術操作上非常有效率的協助。終於能完成實驗，讓論文得以投稿成功發表。明哲也在老師，同儕一致看好下，準備開始進入博士生的生涯。明哲與我是研究及討論的夥伴，和我是具有革命情感的同袍兄弟。我們一起享受在科學領域內思緒自由飛翔的樂趣，也互相挑戰批判在假說與推論上不夠精確的地方。許多的下午，我們約在校園的樹下，或交談或閒聊或科學或人生，頗有華山煮酒論劍的豪情氣概。

感謝在研究所期間遇到許多年輕科學家、學弟妹們。陳雅惠、蔡婉琪兩位優秀的學妹都比我這老學長較早完成博士學位。連文慧現在西雅圖華盛頓大學攻讀博士學位，已有在著名期刊 Science 的文章發表。黃議賢思維嚴緊、實驗技術純熟，想必優秀論文發表也是指日可待。你們在實驗技術及研究經驗的分享，總是不曾笑話

這位老學長所知有限。並且給予毫不保留的指教及鼓勵。

感謝國科會研究計劃經費的補助及"千里馬博士生出國進修計劃"的贊助(2004.05- 2005.05)。讓我有機會在博士生階段出國進修，進入全世界最頂尖研究團隊的實驗室去觀摩學習。我感謝奇美醫院在院內及院際研究計劃經費的大力支持。院長邱仲慶教授(前醫教會主席)及副院長王志中教授(兼任醫研部主任)兩位長官及老師對我的栽培及賞識。感謝婦產部黃國峯主任及部內同仁，在這段時間分擔我部份臨床工作並給予許多的協助。

感謝Professor William A Frazier (Washington University, St. Louis, MO, U.S.A)接受並指導我在他的實驗室工作一年(國科會千里馬計劃)的時間。能在最先進的學術研究環境及不同研究模式題目中了解血小板活化素(thrombospondin)的各式不同的角色。Professor Frazier 審慎治學及開放的學術胸襟，充份散發世界級大師風範。感謝 Dr. Charles Tsai (WCP Pathology Lab)提供自己的病理實驗室，讓我在下班後及假日時，從事乳癌的基質反應及血管新生學的觀察與研究。同時，給我許多科學與人生價值的啟示。另外，Dr. Benjamin PH Tu (Washington University)也在 St. Louis 那段期間提攻自己的家讓我分住，同時給予生活上的照顧。並且分享很多的研究經驗，並提供許多科學思維及分子生物學重要慨念。更重要的是，Dr. Tu 夫妻帶我進入基督信仰的屬靈之旅。讓我對基督信仰由疑惑而深信，並且決志信 主，受洗禮成為基督徒。

最後，我要誠心地感謝我的愛妻淑玲，在這期間給我百分之百的支持。她的順服、耐心、包容與愛心是我一輩子也償還不了的。除此之外，還要忍受我在研究進展不順利、心情鬱悶及憤怒時，不理智的情緒宣泄。我不禁要說 "有才德的女子，是丈夫的冠冕"(箴言 12:4)。我感謝我這一對可愛、善良的兒女沛軒、沛芯。雖然，身於父親的我在你們成長中的許多的時刻是缺席的。但是，你們仍深深地敬愛及靜靜等待你們的父親，並且用心地長大。我感謝我的父母親及岳父母的耐心、寬容及愛。在這段忙碌的日子中，對您們的陪伴問候及關懷總是太少。反而，常常需要倚賴您們幫忙照顧兩個小孫子。

二千八百個日子過去了，多少個汗水夾雜多少的淚水，其中辛酸如寒天飲冰，非身在其中不知當中的滋味。然而，這一切就在召集人劉老師口中告知博士學位考通過時，一切的付出都得到最好的回饋了。我感謝 神的恩典及給予的榮耀。我更感謝 神應許在困難中，永不遺棄。雖然我不過是卑微的器皿，卻享受是尊貴 神的兒子這種神異恩典。 因為，全能的 神曾應許：”祈求就得著，尋找就尋見，叩門就為你開門”(路加 11:9)。(Ask and it will be given to you; Find and it will be found; Knock and the door will be opened to you; Luke 11:9)。

願以此著作獻給在辛勞、苦難、磨練中，仍不放棄追求真理的研究者。

同時，也獻給所有關心我、愛我及我所愛的人。

Thesis Contents

Table list

Figure list

Abbreviations

α-SMA: alpha-smooth muscle actin

CIS: carcinoma in situ

CM: conditioned medium

dKO: double knock out

HSIL: high grade squamous intraepithelial lesion

HUVEC: human umbilical vein endothelial cell

IHC: immunohistochemical

LSIL: low grade squamous intraepithelial lesion

MMP: matrix metalloprotease

MF: myofibroblast

MVD: microvessel density

NF: normal fibroblast

SCC: squamous cell carcinoma

SCID: severe combined immunodeficiency

TGF-β: Transforming growth factor β

TSR: type-1 repeat of thrombospondin

TSP: thrombospondin

TSP-1: thrombospondin-1

VEGF: vascular endothelial growth factor

Chapter 1 Background

1.1 Tumor angiogenesis overview.

1.1.1 Tumor growth is angiogenesis dependent.

Angiogenesis is defined as the formation of new blood vessels by proliferation of new capillaries from pre-existing microvessels. This process is distinct from vasculogenesis, which is defined as the formation of blood vessel de novo from angioblasts (Hanahan D and Folkman J 1996; Risau W 1997). Angiogenesis involves degradation of the basement membrane surrounding an existing capillary or venule, migration of endothelial cells through the basement membrane to create a sprout, proliferation of endothelial cells, formation of a lumen within the new sprout and joining of two sprouts to from a functional capillary loop, and vessel maturation (Bussolino F et al. 1997; Jendraschak E and Sage EH 1996). The idea that tumor growth is angiogenesis dependent was first proposed in 1971, allowing anti-angiogenic therapy to be used to treat cancer (Folkman J 1971). The development of a solid tumor progresses from a prevascular phase to a vascular phase. The prevascular tumor does not induce angiogenesis, is limited in size, and rarely metastasizes. The vascularized tumor induces host microvessels to undergo angiogenesis. The best characterized example is the hypoxia-dependent angiogenic switch in which host cell-derived endothelial cells invade into the tumor stroma to form new blood vessels (Carmeliet P and Jain RK 2000). One of the genetic proofs that tumor growth is angiogenesis-dependent is the induction of tumor angiogenesis by ras oncogene (Folkman J 1992). Ras induces the sequential activation of Myc, via a mechanism enables it to repress the expression of endogenous angiogenic inhibitors, e.g. thrombospondins (TSPs) (Watnick RS et al. 2003), and to activate angiogenic activators, e.g. vascular endothelial growth factor (VEGF) (Udagawa T et al. 2002). Thus, blocking angiogenesis can result in

tumor dormancy, tumors that can not expand beyond a microscopic size (Folkman J 2003b). Within the dormant tumors, the proliferating tumor cells are balanced by apoptotic tumor cells and few if any microvessels (Achilles EG et al. 2001).

1.1.2 Angiogenic activators and inhibitors are produced by tumor and host cells, respectively

In the early 1980's, it became more clearly that tumor cells can produce specific angiogenesis activators that stimulate the proliferation of capillary endothelial cells (Shing Y et al. 1984). The growth of tumor vasculature is no longer regarded as an inflammatory reaction. Angiogenesis activators are stored in extracellular matrix (ECM) in a "stand-by mode" before they become activated by specific enzyme when angiogenesis is required, physiologically or pathologically (Folkman J 2003b). Angiogenesis inhibitors can be expressed by tumor cells or by normal cells of the host and are endogenous molecular defense barriers in pathological hotspots of angiogenesis (Folkman J 2003b). Angiogenesis inhibitors are endogenous and from cryptic fragments of matrix protein, e.g. angiostatin from plasminogen (O'Reilly MS et al. 1994), endostatin from fragment of collagen XVIII (O'Reilly MS et al. 1997), and tumstatin from collage IV (Maeshima Y et al. 2002). The existence of naturally occurring angiogenic inhibitors which a tumor would have to overcome to induce angiogenesis also formed the basis for the subsequent concept of the "angiogenic switch" (Bouck N 1990; Hanahan D and Folkman J 1996).

1.1.3 Angiogenic switch depends on the balance of angiogenic activators and inhibitors

Bouck et al. proposed that the onset of angiogenesis was the result of a shift in the balance of angiogenic activators and inhibitors, which were controlled by

oncogenes and tumor suppressor genes (Bouck N 1990). The balance of activators and inhibitors governs the angiogenic switch (Hanahan D and Folkman J 1996; Hawighorst T et al. 2001). The angiogenic switch is characterized by down-regulation of angiogenic inhibitors or up-regulation of angiogenic activators or both. When inhibitors are over activators, the switch is in the “off” position. When activators are over inhibitors, the switch is in the “on” position (Hanahan D and Folkman J 1996). Change in the relative balance of activators and inhibitors activate the angiogenic switch, before stabilizer molecules activate the maturation of nascent blood vessels (Bussolino F et al. 1997). This shift takes place between the angiogenic activators and inhibitors within the tumor cell itself; and between the tumor cell’s angiogenic proteins and the host’s anti-angiogenic proteins (Hanahan D and Folkman J 1996). For a tumor to switch to the angiogenic phenotype, it must overcome natural angiogenic inhibitors that might exist in the host’s circulation, extracellular matrix or within the tumor cells.

The discoveries of endogenous angiogenesis inhibitors are associated with neovascularized tumors, suggest a new paradigm of tumorigenesis (Folkman J 1995a). The extent to which the inhibitors are decreased during this switch dictates whether a primary tumor grows rapidly or slowly and whether metastases grow at all (Folkman J 1995a). Rastinejad et al. demonstrated that tumor cells did not become angiogenic until they had significantly reduced their own production of TSP (Rastinejad F et al. 1989). In certain tumors, the angiogenic switch also involves down-regulation of endogenous angiogenic inhibitors, in addition to increased expression of angiogenic activator. For example, ras transfection decreases expression of TSP and increases VEGF expression (Kerbel R and Folkman J 2002).

1.1.4 The angiogenic process relies on a complex tumor-host interaction

The mechanisms of angiogenesis involve angiogenic activity arising from at least two sources: tumor cells that mediate the release of angiogenic activators and host cells (e.g. macrophages) recruited by the tumor from surrounding host ECM (Folkman J 1992). During angiogenic processes, there exist a complex interaction among cell components such as tumor, stromal, endothelial and inflammatory cells, growth factors, and the ECM, which are regulated by angiogenesis activators and inhibitors (Rice A and Quinn CM 2002).

Tissue mass, whether it is neoplastic or normal, may be regulated by microvascular endothelial cells (Folkman J 2003b). Both tumors and organs may limit their own growth by increasing the production of angiogenic inhibitors with size, until a critical size is reached where further growth is actively self-inhibited (Folkman J et al. 2000). Tumor mass, as well as normal organ size, is under the tight control of microvascular endothelium (Franck-Lissbrant I et al. 1998). If normal cells are similarly dependent upon endothelial-derived paracrine factors, the ratio of endothelial cells to normal parenchymal cells is likely to be lower than for tumor cells. Nevertheless, the regulation of tissue mass or organ size by vascular endothelial cells may be based upon mechanisms which also operate in tumors (Folkman J 1998). Cancer growth requires the proliferation of endothelial cells, in addition to malignant cells (Folkman J 1996). Without the proliferation of endothelial cells, a tumor cannot grow beyond the size of a colony. Folkman proposed the “two compartment model” explain the interaction of endothelial cells and tumor cells, which constitute two important compartments of a tumor. Within a tumor, these two cell compartments can stimulate each other’s growth via a perfusion effect or paracrine effect (Folkman J 1996). Coordinated tumor/vascular growth exploits an ultimate limitation to tumor size under angiogenic control, where

opposing angiogenic stimuli come into dynamic balance (Hahnfeldt P et al. 1999).

During tumor angiogenesis, there is continued recruitment of endothelia and continued expansion of the tumor mass. Ultimately, each newly recruited endothelial cell can support a large population of tumor cells. This leverage may be exploited to cancer therapeutic advantage (Folkman J et al. 2000). Administration of an angiogenic inhibitor, which is not directly cytotoxic to tumor cells, can increase tumor cell apoptosis and inhibit tumor growth by inhibiting endothelial proliferation and migration, or by inducing apoptosis of the endothelial cells (Folkman J 2003a). By administering angiogenic inhibitors to swift the stimulatory climate in the tumor back to inhibition, recruited endothelial cells are removed, followed by the subsequent loss of the relatively abundant amount of supported tumor cells (Folkman J et al. 2000; Hahnfeldt P et al. 1999).

1.2 Angiogenesis in cervical carcinogenesis

1.2.1 Angiogenesis plays an important role in cervical carcinogenesis.

Cervical cancer is the second most common malignancies among women worldwide, with nearly 80% of cases arising in less developed countries. There are approximately 500,000 diagnoses of cervical cancer per year, leading to 200,000 deaths each year (Ellenson LH and Wu TC 2004). It develops usually by a sequence of gradual, stepwise events starting at low-grade squamous intra-epithelial lesion (LSIL) and progressing through high-grade squamous intra-epithelial lesion (HSIL), until invasive cancer (Pinto AP and Crum CP 2000). Tumor development and metastasis is a complex process that includes transformation, proliferation, neovascularization, and metastatic spread. Among these processes, angiogenesis is now regarded as one of the most important events occurring in neoplastic processes, and its role in cervical neoplasm is

evident (Bremer GL et al. 1996; Sotiropoulou M et al. 2004). Hockel et al. reported an association between tumor hypoxia and progression of cervical cancer, and this would seem to correlate with the angiogenic potential of the tumor (Hockel M et al. 1996). However, trying to connect this finding with actual vessel counts or angiogenic factors has proven to be difficult. Two commonly used tumor vascularity measurements are microvessel density (MVD), the hotspot method that provides a histological assessment of tumor angiogenesis, and intercapillary distance (ICD), which is thought to reflect tumor oxygenation (West CM et al. 2001). Accumulating evidence has shown that angiogenesis is related to cervical neoplasm in histologic parameters, prognosis, survival (Cantu De Leon D et al. 2003) and therapeutic efficacy (Kohno Y et al. 1993; Siracha E et al. 1994).

1.2.2 Timing of angiogenic switch in cervical carcinogenesis

The timing of angiogenic switch during cervical carcinogenesis remains controversial. A debate exists regarding the ability of cervical intra-epithelial neoplasm (CIN) to induce angiogenesis (Abulafia O et al. 1996; Abulafia O et al. 1999; Smith-McCune KK and Weidner N 1994). Smith-McCune and Weidner found a significant increase of MVD in the CIN III lesions compared with those underlying low grade lesions such as condyloma and CIN I (Smith-McCune KK and Weidner N 1994). Sotiropoulou et al. reported that there was also a significant difference in the number of vessels between carcinoma in situ (CIS) and controls. Additionally, no significant correlation was found in relation to depth of invasion and histological grade of the microinvasive carcinomas (Sotiropoulou M et al. 2004). On the contrary, results from Abulafia el al. showing that microinvasive squamous cell carcinoma, but not CIS is angiogenic (Abulafia O et al. 1996). In order to eliminate the heterogeneity in the inborn characters of angiogenesis, Wu et al. examined the

surgical specimens that have cervical lesions with different severities within the same histologic slide, so that every lesion can be used as an internal control for each other (Wu MP et al. 2004). Their data showed that the angiogenic switch in cervical carcinogenesis occurred during the transition from low-grade CIN to high-grade CIN, and the neovascularization was largely confined to a narrow zone immediately underneath the dysplastic epithelium (Wu MP et al. 2004). This is in concordance with the results from Smith-McCune and Weidner (Smith-McCune KK and Weidner N 1994) and Sotiropoulou (Sotiropoulou M et al. 2004). Altogether, it seems that the onset of angiogenesis in cervical cancer is an early event, usually in a preinvasive stage, and further suggests that cervical carcinogenesis is angiogenesis-dependent (Folkman J 2003b; Sotiropoulou M et al. 2004).

1.3 Thrombospodin (TSP)

1.3.1 Gene function of thrombospondin 1 (TSP-1)

The TSPs are a family of extracellular proteins that participate in cell-to-cell and cell-to-matrix communication. Five family members have been identified (Lawler J 2000). TSP is a homotrimeric glycoprotein with disulfide-linked subunits of MW 180,000. TSP was first described by Baenziger and Majerus in 1970 as a large glycoprotein released from platelet α-granules upon activation on platelet aggregation (NCBI OMIM)(Baenziger NL et al. 1971). TSP is not limited to platelet and is synthesized and secreted for incorporation into the extracellular matrix (ECM) by a variety of cells including endothelial cells, fibroblasts, smooth muscle cells, and type II pneumocytes (OMIM). TSP binds heparin, sulfatides, fibrinogen, fibronectin, plasminogen, and type V collagen. Dixit et al. reported characterization of a cDNA encoding the amino-terminal 376 amino acid residues of human TSP (Dixit VM et al. 1986). Five family members, each representing a separate gene product, have been

identified (Lawler J 2000). TSP-1 and TSP-2 contain NH2-terminal, COOH-terminal, and procollagen homology domains, and type I (TSR or properdin), type II (EGF-like), and type III (Ca^{2+}-binding) repeats (Bornstein P 1992). The function of the TSP-1 in epithelial tumor development remained controversial.

TSP-1 is a matricellular glycoprotein that inhibits proliferation and migration of vascular endothelial cells in vitro and inhibits neovascularization in vivo, contributing to the normal quiescence of the vasculature (Bornstein P 2001; Jimenez B et al. 2000). Volpert et al. demonstrated that TSP-I, derive specificity for remodeling vessels from their dependence on Fas/Fas ligand-mediated apoptosis to block angiogenesis. TSP-1 upregulated FasL on endothelial cells, thereby specifically sensitizing the stimulated cells to apoptosis (Volpert OV et al. 2002). Isenberg et al. found that endogenous TSP-1 limited the angiogenic response to nitric oxide (NO) in mouse muscle explant assays. In human umbilical vein endothelial cells, TSP-1 was a potent antagonist of NO-induced chemotaxis, adhesion, and proliferation. TSP-1 antagonized these cGMP-dependent endothelial responses to NO both upstream and downstream of cGMP signaling (Isenberg JS et al. 2005b). Ridnour et al. found that slow and prolonged release of NO at various concentrations produced a triphasic response in TSP-1 protein expression in human umbilical vein endothelial cells (Ridnour LA et al. 2005).

Mammary tumor-prone mice that either lacked, or specifically overexpressed TSP-1 in the mammary gland were generated to ascertain the participation of the TSP-I in tumor progression (Rodriguez-Manzaneque JC et al. 2001). Tumor burden and vasculature were significantly increased in TSP1-deficient animals, and capillaries within the tumor appeared distended and sinusoidal. In contrast, TSP-1 overexpressors showed delayed tumor growth or lacked frank tumor development. Absence of TSP-1 resulted in increased association of VEGF with its

receptor VEGFR2 and higher levels of active matrix metalloproteinase 9 (MMP-9), a molecule previously shown to facilitate both angiogenesis and tumor invasion (Rodriguez-Manzaneque JC et al. 2001). In vitro, enzymatic activation of pro-MMP9 was suppressed by TSP-1. Together these results argued for a protective role of endogenous inhibitors of angiogenesis in tumor growth and implicated TSP-1 in the in vivo regulation of MMP-9 activation and VEGF signaling.

1.3.2 TSP-1 is a matricellular protein with multiple receptors and diverse functions

TSPs are belong to the category of 'matricellular proteins', which has the has been applied to a group of extracellular proteins that (Bornstein P and Sage EH 2002). Is characterized by that (i). they do not subserve structural roles, but function contextually as modulators of cell-matrix interaction, (ii) bind to many cell-surface receptors, the ECM, growth factors, cytokines and proteases (Chen H et al. 2000), (iii) generally induce de-adhesion, in contrast to the adhesivity of most matrix protein (Bornstein P and Sage EH 2002). TSP-1 is a matricellular protein that acts as a molecular facilitator by bringing together cytokines, growth factors, matrix components, membrane receptors and extracellular proteases (Chen H et al. 2000). In addition to a well known endogenous angiogenesis inhibitor, TSP-1 has multiple diverse functions in in vitro and in vivo models (Rice A and Quinn CM 2002; Schneider BP and Miller KD 2005). TSP-1 interacts with multiple extracellular macromolecules and cell surface receptors, thus exerting a wide range of functions, among them CD36, CD47 and integrins are most well known to transmit the downstream signaling (Asch AS et al. 1991; Frazier WA 1991). The binding sites for these receptors on TSP-1 are dispersed throughout the molecule, with most domains binding multiple receptors. In some cases, TSP-1 binds to

multiple receptors concurrently (Chen H et al. 2000). TSP-1 may function to direct the clustering of receptors to specialized domains for adhesion and signal transduction (Chen H et al. 2000). As for breast cancer cell per se, TSP-1 has multiple functions on, linked with both a stimulatory (Wang TN et al. 1996) and inhibitory role (Zabrenetzky V et al. 1994) in tumor invasiveness and progression. Correlative expression data in breast cancer indicate that increased TSP-1 expression is associated with decreased malignant tumor growth, invasion and organ metastasis (Volpert OV et al. 1995). Conversely, antisense inhibition of TSP-1 expression in squamous cell carcinoma was reported to suppress tumor growth in vivo (Castle V et al. 1991). Thus, TSP-1 has been described a tumor suppressor and as a tumor promoter (Hawighorst T et al. 2002). The definite role of TSP-1 in breast carcinoma is still unclear. In vitro model showed TSP-1 promotes attachment, migration, and invasion of human MDA-MB-231 breast carcinoma cells (Tuszynski GP and Nicosia RF 1996; Wang TN et al. 1996). However, TSP-1 inhibits tumor growth in vivo through a reduction of angiogenesis (Dameron KM et al. 1994; Weinstat-Saslow DL et al. 1994). In addition, overexpression of TSP-1 in MDA-435 breast carcinoma cells shows that TSP-1 is pro-invasive and anti-angiogenesis in a controversial way. TSP-1 may also exert its bi-phasic effects by different concentrations (Tolsma SS et al. 1993) or even opposite activities via different domains (Taraboletti G et al. 2000). Moreover, TSP-1 may inhibit or stimulate endothelial cell proliferation and angiogenesis when binding with its different receptor CD36 (Dawson DW et al. 1997) or integrin, α6β1, α4β1, α3β1 (Calzada MJ et al. 2004).

Streit et al. found overexpression of TSP-1 inhibited tumor growth of A431 xenotransplants, and completely abolished tumor formation by SCC-13 cells (A431 and SCC-13 are human cutaneous squamous cell carcinoma cell lines). TSP-1

overexpressing A431 tumors were characterized by extensive areas of necrosis and by decreased tumor vessel number and size (Streit M et al. 1999). Hawighorst et al. subjected TSP-2-deficient and wild type mice to a two-step chemical skin carcinogenesis protocol. Loss of TSP-2 expression results in an early growth advantage of initiated/promoted epidermal cells, but not in enhanced malignant conversion rate. However, TSP-2 did not act as a promoter or an initiator. In TSP-2-deficient mice, tumor cell apoptosis was significantly reduced, without the change of tumor differentiation or proliferation. These results reveal upregulation of an endogenous angiogenesis inhibitor during multistep tumorigenesis. Enhanced stromal TSP-2 expression is a host anti-tumor defense mechanism (Hawighorst T et al. 2001). TSP-1 has been found to be more effective than TSP-2 as an inhibitor of endothelial cell migration and angiogenesis in a cornea assay (Volpert OV et al. 1995). By contrast, transfection of a full-length TSP-2 suppresses squamous cell carcinoma growth and angiogenesis more effectively than TSP-1 (Streit M et al. 1999).

The effects of TSP-1 on tumor cell growth were indirect since there is no change in tumor cell proliferation rates, anchorage-dependent and –independent growth, and susceptibility to apoptosis (Streit M et al. 1999)TSP-2 does not appear to contribute directly to the structural integrity of connective tissue elements. Instead, a model of action of TSP-2 acts ‘at a distance’, i.e. by modulating the activity and bioavailability of protease and growth factors in the pericellular environment, and very likely, by interaction with cell-surface receptors (Bornstein P 2000). The definite roles of TSP-1 in tumor progression remain controversial. The results by Dawson et al. indicated the protective effect of TSP-1 in angiogenesis (Dawson DW et al. 1997) while other studies showed the activatory roles of stromal TSP-1 in oral SCC by enhancing the motility and proteolytic activity (Hayashido Y et al. 2003). The contradictory findings

may be due to the interaction of TSP-1's multi-domains with their receptors (Chen H et al. 2000), such as CD36 (Dawson DW et al. 1997), CD47/integrin-associated protein (Manna PP and Frazier WA 2004) and integrin (Reynolds LE et al. 2002). For example, mice lacking β3 integrins not only support tumorigenesis, but have enhanced tumor growth as well (Reynolds LE et al. 2002).

1.3.3 TSP-1 and clinical cancer prognosis

In human adrenocortical carcinomas, non-small cell lung carcinomas, gliomas and thyroid carcinomas, TSP levels were lower in malignant tumors than in adenomas (Bunone G et al. 1999; de Fraipont F et al. 2000; Hsu SC et al. 1996; Kazuno M et al. 1999; Oshika Y et al. 1998; Tenan M et al. 2000). Several studies showed that TSP-1 expression is inversely correlated with tumor grade and survival rate in thyroid, colon and bladder carcinomas (Bunone G et al. 1999; Grossfeld GD et al. 1997; Tokunaga T et al. 1999). TSP-1 expression in the stromal area was much stronger in tumors than in normal tissue in breast tumors and cholangiocarcinomas (Bertin N et al. 1997; Clezardin P et al. 1993; Kawahara N et al. 1998; Pratt DA et al. 1989). Similarly, in colorectal carcinomas and pleural mesothelioma, the level of TSP-1 expression was higher in tumors than in normal tissue (Ohta Y et al. 1999; Yoshida Y et al. 1999). In all of these tumors, TSP-1 levels were of little prognostic value. While, in esophageal and gallbladder carcinomas, TSP-1 expression appeared to be directly correlated with the occurrence of metastases (Ohtani Y et al. 1999; Oshiba G et al. 1999).

1.4 Matrix metalloproteases (MMPs) and tumor progression

1.4.1 MMPs in angiogenesis and tumor progression

Invasion and metastasis of cancer cells are known to be initiated by an interaction between cancer cells and host cells, meanwhile, extracellular matrix (ECM) plays a major role in recognition and migration of cancer cells. Angiogenesis is an invasive process that required proteolysis of ECM, proliferation and migration of endothelial cells (EC), and synthesis of new matrix components (Stetler-Stevenson WG et al. 1996). MMPs are a family of extracellular endopeptidases that selectively degrade components of the ECM (Stetler-Stevenson WG et al. 1996). MMPs have been implicated in the mechanism involved in cellular invasion and migration. The mechanism includes cell-cell or cell-ECM attachment, proteolytic modification of ECM, and then migration through the modified matrix by a cancer cells (Brooks PC et al. 1996). The acquisition of an angiogneic phenotype may be associated with increased activation of MMPs (Bergers G et al. 2000; Fang J et al. 2000). Angiogenic tumor nodules were characterized by the presence of capillary vessels, and both angiogenic and proteolytic activities in vitro. MMP-2 and MMP-9, both belonging to the gelatinase subgroup, have been shown to play a major role in cancer invasion (Park CC et al. 2000). Bergers et al. showed that the switch from vascular quiescence to angiogenesis involves a MMP-9, which is upregulated in angiogenic islets and tumors, rendering VEGF more available to its receptors. Remarkably, MMP-9 is not expressed in tumor cells, but rather in a small number of cells that are proximal to the vasculature (Bergers G et al. 2000).

The presence of a prominent MMP-2 was detected in the angiogenic nodules, when compared with the preangiogenic ones. Suppression of MMP-2 activity by antisense oligonucleotides in the vascular nodules resulted in the loss of angiogenic potential both in vitro an in vivo in the chick chorioallantoic membrane assay (Fang J et al. 2000). TSP2-null mice display a variety of connective tissue abnormalities, including fragile skin and the presence of abnormally large collagen fibrils with

irregular contours in skin and tendon. TSP2-null skin fibroblasts show a defect in attachment to a number of matrix proteins, and a reduction in cell spreading (Yang Z et al. 2000).TSP-2 null fibroblasts produce a twofold increase in MMP-2 activity and protein, in comparison to wild-type cells. The adhesive defect of TSP-2-null fibroblasts was corrected by treatment with MMP inhibitors, or with a neutralizing antibody to MMP-2 (Bornstein P et al. 2000a; Yang Z et al. 2000). TSP-2 interacts directly with MMP-2. TSP-2 deficient fibroblast show enhanced MMP-2 activity in vitro suggest modulation of MMP activity as an additional mechanism of action by which TSP-2 may inhibit tumor angiogenesis (Bein K and Simons M 2000; Yang Z et al. 2000). TSP-2 binds MMP-2 and that the adhesive defect in TSP-2 null fibroblasts results from increased MMP-2 activity (Bornstein P et al. 2000a). As comparing TSP-1 and TSP-2 in modulating MMPs, TSP-1 modulates angiogenesis in vitro by up-regulation of matrix metalloproteinases-9 in endothelial cells (Qian X et al. 1997). TSP-2 binds MMP-2 and that the adhesive defect in TSP-null fibroblasts results from increased MMP-2 activity (Bornstein P et al. 2000a).

1.4.2 TSP-1 can modulate the matrix degradation by changing the MMPs

The interactions between tumor cells and the surrounding stroma may play an important role in the cancer progression, which is characterized by a strong desmoplastic reaction (Qian X et al. 2001). The different components of the ECM are capable of modulating tumor cell growth, motility, differentiation, and gene expression. MMPs represent a family of zinc containing enzyme, which degrade extracellular matrix components, and are believed to play a central role in tumor invasion and angiogenesis (Stetler-Stevenson WG 1999). MMP-9, one of the two type IV collagenases of the MMP family, is capable of cleaving a wide range of ECM components, including denatured collagens (gelatins). Alves F et al. used a

MMP inhibitor specifically to target certain MMPs and inhibits on tumor progression. This model shows that specifically targeting certain MMPs may have potency of novel anti-metastatic strategies (Alves F et al. 2001). Accumulating evidence suggests that TSP-1 functions as a modulator in tumor invasion and angiogenesis. TSP-1 was shown to promote breast tumor cell invasion both in vitro and in vivo (Wang TN et al. 1996). Also, TSP-1 has been noted to regulate the production of MMP-9 (or gelatinase B) in endothelial cells (Qian X et al. 1997). These observations suggest that TSP-1 may play a crucial role in proteolytic degradation of ECM, an essential process during tumor invasion and metastasis. TSP-1 augmented the production of MMP-9 in pancreatic cancer cells in vitro. The TSP-1-mediated stimulation of MMP-9 activity was specific and dose- and time-dependent (Qian X et al. 2001). The stromal TSP-1 from fibroblast also promote breast cancer cell invasion by upregulating tumor matrix metalloproteinase-9 production (Wang TN et al. 2002).

1.5 Stroma reaction and fibroblast activation during carcinogenesis

1.5.1 The invasion process of cancer cells is associated with stroma reaction

The cooperation between epithelial and mesenchymal cells is essential for embryonic development and also plays an important role in pathological phenomena such as wound healing and tumor progression. It is well known that many epithelial tumors are characterized by the local accumulation of connective tissue cells and extracellular material; this phenomenon has been called the stroma reaction. Stroma reaction, also known as stromagenesis, is a host reaction of connective tissue that, when induced in cancer, produces a progressive and permissive mesenchymal microenvironment, thereby supporting tumor progression (Amatangelo MD et al. 2005). The stromal microenvironment is

complex and comprises several cell types, including fibroblasts, the primary producers of the noncellular scaffolds known as ECM. The locally activated host microenvironment (both cellular and extracellular elements) in turn modifies the proliferative and invasive behavior of the tumor cells (Liotta LA and Kohn EC 2001).

Paget first propose ‘seed and soil hypothesis’ to highlight the influence of tumor growth by interactions between malignant cells and the tumor stroma in 1889 (Paget S 1889). In ‘seed and soil hypothesis’ (Paget S 1889) of cancer biology, cancer cells are the ‘seeds’, the microenvironment is the ‘soil’; in which the ‘seeds’ must find a receptive environment (Fidler IJ 2003). The normal host microenvironment is nonpermissive for neoplastic progression, tumor-reactive stroma promotes neoplastic growth and metastasis (Chlenski A et al. 2007b). Within the same microenvironment, vascular sprouts migrate and invade towards the tumor mass while tumor cells migrate outwards in the opposite direction (Carmeliet P and Jain RK 2000). Activation of the local invasive environment seems to create a permissive field for the malignant cells (Liotta LA and Kohn EC 2001). The presence of host cells is original considered as a reaction against the aberrant behavior of the cancer cells as a mechanical and immunological defense against the cancer cells. It was described by the pathologist as desmoplasia, inflammation and angiogenesis with accumulation of stromal fibroblasts, leukocytes and endothelial cells respectively. Recently, there is growing evidence that tumor-infiltrated host cells are recruited and diverted by the cancer cells to contribute to their malignant progression rather than to protect the host (Mueller MM and Fusenig NE 2004).

The complexity of multicellular organisms necessitates a high degree of coordination among a diverse range of specialized cell types. Maintaining this

organization requires a constant and dynamic stream of intercellular communication (Radisky DC and Bissell MJ 2004). Increasing evidence suggests that this organized exchange of information is essential for maintaining the differentiated state of cells, and that sustained disruption of key intercellular signaling pathways can predispose to malignancy (Bissell MJ and Radisky D 2001). It is well established that cellular tumorigenic potential is profoundly influenced by the microenvironment and that malignant cells can be induced to maintain a differentiated state by growth in an appropriate tissue microenvironment (Kenny PA and Bissell MJ 2003). Throughout the entire process of cancer etiology, progression and metastasis, the microenvironment of the local host tissue can be an active participant (Liotta LA and Kohn EC 2001). The invasion process of cancer cells is associated with the generation of specific stroma, called 'cancer-induced stroma' (Ishii G et al. 2005). The main constituents of cancer-induced stroma are inflammatory cells, (including lymphocytes, granulocytes, macrophages, and pericytes), fibroblasts and the endothelial cells of blood and lymph vessels. Inflammatory cells and endothelial cells are recruited into cancer stroma and involved in tumor immunity (Coussens LM and Werb Z 2002) and angiogenesis, respectively (Folkman J et al. 2000). New blood and lymph vessels (Carmeliet P and Jain RK 2000), immunocytes and inflammatory cells (Opdenakker G and Van Damme J 2004) as well as stromal fibroblasts (De Wever O and Mareel M 2003) don't inhibit but rather stimulate cancer invasion and metastasis, in line with Paget's "seed" and "soil" hypothesis in 1889 (Paget S 1889).

1.5.2 Stroma reaction is characterized by the fibroblast activation

The stromal cells that surround and sustain epithelia have been viewed primarily

as a source of oxygen, nutrients, and additional growth stimuli for tumors. Bhowmick et al. report that defective stromal cells stimulate the development of epithelial tumors, which suggests that normal stromal cells may prevent epithelia from becoming tumorigenic (Bhowmick NA et al. 2004). Fibroblasts, as the major component of stroma, are recruited and can convert into smooth muscle actin-positive fibroblasts, i.e., myofibroblasts or activated fibroblasts, during stroma reaction (Kunz-Schughart LA and Knuechel R 2002a; Kunz-Schughart LA and Knuechel R 2002b). The term “myofibroblast’ was firstly used to describe, in experimental granulation tissue of Wistar rats, fibroblastic cells with a smooth muscle cell-like morphology, a strongly developed microfilamentous apparatus and a contractile phenotype (Desmouliere A et al. 2004; Majno G et al. 1971). Myofibroblasts appear at the invasion front during stromal changes cells sharing characteristics with fibroblasts and smooth muscle cells (De Wever O and Mareel M 2003). Myofibroblast can also produce collagens and extracellular matrix proteins in response to several extracellular stimuli.

There assumably exists a continuous cross-signaling between the epithelial and the stromal compartment in normal and in pathological situations alterations that lead to invasion. The paracrine signaling interactions between cancer cells and associated fibroblasts play important roles in tumor formation and progression (Kunz-Schughart LA and Knuechel R 2002a; Kunz-Schughart LA and Knuechel R 2002b). Stromal fibroblasts create a context that promotes tumor progression (Barcellos-Hoff MH and Ravani SA 2000). Furthermore, investigators have found evidence that the proliferative activity of stromal fibroblasts in cancer-induced stroma is closely linked to lymph node and distant organ metastasis (Hasebe T et al. 2000) and that soluble factor secretion by stromal fibroblasts influences tumor progression.

Fibroblasts within cancer stroma have a migratory and invasive capacity similar to that of cancer cells and associate with cancer cells by transmitting reciprocal signals (Ishii G et al. 2005). It is unclear who is invading whom between epithelial and mesenchymal cells. The transition from normal to invasive carcinoma is preceded by, or is concomitant with, activation of local host stroma (Liotta LA and Kohn EC 2001).

Chapter 2 Materials and Methods

2.1 Materials and Methods in Chapter 3

2.1.1 Sample selections

Surgical specimen collections, obtained from cervical punch biopsy, conization, or hysterectomy, were retrieved from the Department of Pathology, Chi Mei Foundation Hospital. Thirty-six patients with a mean age of 49.2 years (range from 31 to 65 years) who had pathological diagnoses of cervical intra-epithelial neoplasm (CIN) 1, carcinoma in situ, and FIGO stage Ib squamous cell carcinoma (SCC) (n=12 from each group) were recruited in this study. They received cervical punch biopsy, conization, or radical abdominal hysterectomy, respectively. Two representative blocks that contained serial changes of cervical lesions from each specimen were analyzed. In each block, the cervical lesions of various severity as well as adjacent normal cervical epithelia were categorized into normal, LSIL, HSIL, and SCC epithelium. In addition, two tissue sections that contained normal cervical epithelium from 12 patients with benign uterine disease who underwent hysterectomy for uterine leiomyoma or adenomyosis were used as normal controls. The MVD counts and TSP-1 expression were assessed in these samples by immunohistochemistry.

2.1.2 Immunohistochemical (IHC) staining

Formalin-fixed, paraffin-embedded tissue blocks were cut with serial sections of 4 μm thickness. One section from each sample was stained with hematoxylin-eosin for the confirmation of histologic diagnoses. Adjacent sections were stained for von Willebrand factor antigen and TSP-1 by the use of standard immunoperoxidase staining methods. The paraffin was removed in xylene, and the tissue sections were rehydrated in descending dilutions of ethanol. After sections were heated and

boiled for 13 minutes in a microwave oven in 10 mM citrate (pH 6.0) buffer, they were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Specimens were then incubated with mouse anti-human von-Willebrand factor monoclonal antibody (clone F8/86; Dako Corp., Glostrup, Denmark) diluted to a 1:50 ratio, or mouse anti-human TSP-1 monoclonal antibody (Calbiochem Co. La Jolla CA, U.S.A.) with a 1:250 dilution. Slides were then incubated with supersensitive immuno-detection system of BioGenex (San Ramon, CA, U.S.A). The 3,3'-diaminobenzidin tetra-hydrochloride was used as chromogen. Human placenta, which was known to exhibit TSP-1 expression, was used as the positive control. A negative control, for which the primary antibody was substituted with the same concentration of the appropriate IgG, was used in each staining run. Sections were counter-stained lightly with hematoxylin.

2.1.3 Microvessel density (MVD) in different cervical lesions

Sections were scanned at low magnification (100x) to identify areas of capillaries. The vessels that were defined as any positively stained single cell or cluster of cells were counted to determine the MVD count per high-power microscopic field (200x; 20x objective lens and 10x ocular lens, 0.785 mm^2 per field). MVD were scored from three fields for each lesion by use of the microvessel counting protocol and criteria developed by Weidner et al, with minor modifications.(Weidner N et al. 1993) The quantization was simultaneously performed (with use of a multi-headed microscope) by three investigators who had to concur on the areas to be analyzed and on vessel identification and count.

2.1.4 TSP-1 expression in different cervical lesions

Preliminary results showed that TSP-1 expression was largely localized in the

basal layer of uterine cervical epithelium. Thus we measured TSP-1 intensity, and classified into 4 arbitrary units according to the percentage of TSP-1-expressing basal epithelial cells. Scores of 4, 3, 2, or 1 were given under the conditions that TSP-1 was expressed in 75 to 100%, 50 to 75%, 25 to 50%, or less than 25% of the basal epithelial cells, respectively.

2.1.5 Statistical analysis

All values were reported as mean ± S.D. (standard deviation). Due to small sample size and ordinal property of TSP-1 expression measurement, TSP-1 and MVD were subjected to non-parametrical data analysis method. Kruskal-Wallis test was performed to detect any difference of MVD and TSP-1 expression intensities among normal, LSIL, HSIL and SCC epithelium, and to detect any difference of MVD among the four levels of TSP-1 expression intensity. A value of $p < 0.05$ was considered as statistically significant. Dunn test was adapted as the post hoc comparing method to test the differences between groups pairwisely. Based on Bonfferroni correction method, the alpha level of each post hoc comparison was corrected into 0.008. In addition, statistical comparisons between different lesions on the same slide were made with Wilcoxon signed-rank test.

2.2 Material and Methods in Chapter 4

2.2.1 Sample selections

Surgical specimen collections, obtained from cervical conization, or hysterectomy, were retrieved from the Department of Pathology, Chi Mei Foundation Hospital, Tainan, Taiwan. Patients who had pathological diagnoses of carcinoma in situ (CIS), and International Federation of Gynecology and Obstetrics (FIGO) stage Ib squamous cell carcinoma (SCC), in which tumor is confined to cervix but beyond

microscopic lesion, (n= 15 from each group) were recruited in this study. They received conization for CIS, or radical hysterectomy for SCC, respectively. Two representative blocks from each specimen were analyzed. Additionally, two tissue sections containing normal cervical epithelium from 15 patients with benign uterine disease who underwent hysterectomy for uterine leiomyoma or adenomyosis were used as normal controls.

2.2.2 Immunohistochemical staining and scoring system

Formalin-fixed, paraffin-embedded tissue blocks were cut with serial sections of 4 μm thickness. One section from each sample was stained with hematoxylin-eosin for the confirmation of histological diagnoses by one of the authors (Tzeng C-C). Adjacent sections were stained for TSP-1, α-SMA, and desmin in human surgical specimens, and TSP-1, α-SMA, desmin, and CD34 in mice xenograft by the use of standard immunoperoxidase staining methods. The paraffin was removed in xylene, and the tissue sections were rehydrated in descending dilutions of ethanol. After sections were boiled for 13 minutes in a microwave oven in 10 mM citrate (pH 6.0) buffer, they were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. Specimens were then incubated with mouse anti-human TSP-1 (Calbiochem Co. La Jolla, CA; 1:250), α-SMA 1A4 (Dako, Glostrup, Denmark; 1:50), desmin D33 (Dako, 1:250), or CD34 monoclonal antibodies (Serotec, Oxford, UK; 1:250). Slides were then incubated with supersensitive immuno-detection system of BioGenex (San Ramon, CA). The 3,3'-diaminobenzidin tetra-hydrochloride was used as a chromogen. Human placenta was used as the positive control for TSP-1, whereas smooth muscle cells were used as the positive control for α-SMA and desmin. A negative control, for which the primary antibody was substituted with the same concentration of the appropriate IgG, was used in each staining run. Sections

were counter-stained with hematoxylin. All stained slides were examined by the authors (Tzeng C-C and Wu M-P) who were blinded to the patients' clinical information. The expression of α-SMA and desmin was categorized into 4 grades. They were arbitrarily scored as 0: no to little staining; 1: weak staining; 2: moderate staining; 3: strong staining. Microvessel density was defined as the number of blood vessels characterized as CD34-positive tube-like structures, and scored as: 0: no to scanty, 1: scanty, 2: moderate; 3. abundant.

2.2.3 Cell cultures and transfection

The cervical cancer SiHa cell line and mouse fibroblast NIH 3T3 cells were used to study TSP-1 function. Epithelial cells and fibroblasts derived from normal uterine cervix were used as representatives of normal, quiescent cells. Normal cervical epithelial cells were prepared as previously described (Chou CY et al. 1995). Normal cervical fibroblast cells were derived in a similar manner except that Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Biological Industries Co., Haemek, Israel) was used instead of serum-free keratinocyte medium for epithelial cells.

Full-length human TSP-1 cDNA construct, a kind gift from Professor Frazier, W. A. at Washington University, St. Louis, MO (Sheibani N and Frazier WA 1995), was subcloned into the eukaryotic expression vector pCDNA3.1 (Invitrogen, Rockville, MD), transfected into SiHa or NIH 3T3 cells by using lipofectamine 2000 (Invitrogen). G418 at a final concentration of 1 mg/ml was added 24 hours after transfection. The G418-resistant colonies were expanded and examined for the expression of TSP-1. To assess proliferation, cells were plated at the density of 10^5 per dish on 60-mm dishes and the medium was changed every two days. Viable cells were counted with a hemocytometer using trypan blue (0.4%) exclusion.

2.2.4 Immunoblotting

Equal amounts of protein lysates were fractionated by SDS-PAGE at 80-100V for 2.5-3 hrs. The proteins were transferred to PVDF membrane (Amershan Corp., Arlington Heights, IL). The blots were incubated with anti-human TSP-1 antibody (Neomarker, Fremont, CA; 1:1000), anti-human α-SMA 1A4 Ab, anti-desmin Ab (Abcam, Cambridge, MA) or anti-matrix metalloproteases 2 and 9 (MMP-2, -9) (Calbiochem, Darmstadt, Germany; 1: 500). Blots were then processed by chemiluminescent substrate detection system (Amersham).

2.2.5 Matrigel angiogenesis assay

Matrigel (BD Biosciences, Bedford, MA) was thawed overnight at 4^{o}C, then was added (80 μl) to each well of a 96-well plate at 37^{o}C and allowed to polymerize (Colorado PC et al. 2000). Before angiogenesis assay, HUVECs were cultured at low serum condition (M199 with 2% FBS) overnight. A suspension of 20,000 HUVECs in M199 medium was seeded into each well coated with Matrigel. The cells were treated with 40 ug/ml bovine serum albumin (BSA), indicated culture medium from different cell lines, or purified TSP-1 (Sigma, St. Louis, MO) in various concentrations. Cells were incubated for 6-8 hours at 37°C and viewed using an Olympus Optical (Tokyo, Japan) CK30 microscope. The formation of tubular network structures represents angiogenesis ability. All assays were performed in triplicate.

2.2.6 Animal models

Subcutaneous tumors were generated by inoculating 10^{7} SiHa-vector or SiHa-TSP-1 cells into six aged 9-week-old SCID mice in each group. All

manipulations were conducted under aseptic conditions in a laminar flow hood. The smallest and largest tumor diameters were measured weekly, using a digital caliper, and the tumor volumes were calculated using the following formula: volume= 4/3π × (1/2 smaller diameter)^2× 1/2 larger diameter. The mice were sacrificed for histological examination 12 weeks after the tumor cell inoculation. The animal experiments were performed according to the ethical guidelines and approved by the institutional ethical committee.

2.2.7 Fibroblast activation

Transforming growth factor β (TGF-β) (Sigma, St. Louis, MO) at 2-20 ng/ml was used to activate NIH3T3 fibroblasts for the indicated time (Meyer-Ter-Vehn T et al. 2006). TGF-β at 10 ng/ml was later used to activate fibroblasts for cell migration assay and 20 ng/ml for Matrigel multicellular co-culture invasion assays.

2.2.8 Cell migration assay and MMPs zymography

Cell migration was assayed in the 48-well Boyden chamber (Neuro Probe, Gaithersburg, MD) as described (Albini A et al. 1987). A 50 µl aliquot containing 5,000 cells of NIH3T3 or NIH3T3-TSP-1 were placed on the upper compartment, and fibronectin was used as the chemoattractant in the lower compartment of the chamber. The assays were run for 6 h in Dulbecco's modified Eagle's medium containing 0.1% BSA at 37°C. After incubation, cells were fixed with methanol, stained with Giemsa solution (Merck, Darmstadt, Germany), and counted immediately after staining. Conditioned medium from the culture was cleared of cells and debris by centrifugation at 3000 x g for 10 min and concentrated by 10-fold using Amicon Microcon (Ym-10). MMP-2 and MMP-9 activities in the conditioned medium were measured by gelatin zymography as previously

described (Shen MR et al. 2003).

2.2.9 Matrigel multi-cellular co-culture invasion assay of activated fibroblasts

To detect the invasive ability of fibroblasts into tumor cells cluster, we applied the multicellular Matrigel multi-cellular co-culture system as described by Walter-Yohrling et al. with a slight modification (Walter-Yohrling J et al. 2003). A layer of Matrigel (80 μl) was added to each well of a 96-well plate and allowed to polymerize. In the central area of each well, a Matrigel plug of approximately 2 μl was removed, and then filled with 10^5 cancer cells (SiHa or SiHa-TSP-1) in 5μl of Matrigel. The Matrigel was allowed to polymerize for 30 min to form a tumor cell cluster. A total of 10^5 fluorescent dye PKH26-labeled (Red) or PKH67-labeled (Green) (Sigma, St. Louis, MO) fibroblasts (normal fibroblasts, NIH3T3 or NIH3T3-TSP-1) in 150 μL aliquot were dispersed diffusely in the periphery of each well. Following 16 to 24-h incubation, red fluorescence for PKH26 dye or green fluorescence for PKH67 and bright field images were captured with an inverted fluorescent light microscope (Olympus IX70). Positive invasion of activated fibroblasts was defined as the presence of fibroblasts accumulation in the central tumor cluster following activated fibroblasts migration and invasion toward tumor cluster. In contrast, lack of invasion is defined as diffuse distribution of fibroblasts in the periphery of tumor clusters.

2.2.10 Statistical analysis

All values were reported as mean ± S.D. (standard deviation). Kruskal-Wallis test with Dunn post hoc comparison test was used for statistical analysis in α-SMA and desmin expression in normal cervix, CIS and SCC among the non-parametric measurement multi-groups comparison. Mann-Whitney U test was used for two

group comparison in SiHa-induced or SiHa-TSP-1-induced tumors. Repeated ANOVA measurement was used for tumor growth curves. Student t test was used for NIH3T3 cell migration assay in which TSP-1 was added in lower, upper or both, or comparing NIH3T3 with or without TGF-β treatment.

Chapter 3 TSP-1 acts as a fence to inhibit angiogenesis that occurs during cervical carcinogenesis

3.1 Summary

Purpose. The acquisition of an angiogenic phenotype (angiogenic switch) is essential for cervical carcinogenesis. This study was aimed to examine the spatial and temporal relationship of TSP-1 expression in patients with precursor lesions and squamous cell carcinoma of uterine cervix, and to correlate its expression with tumor angiogenesis.

Patients and Methods. TSP-1 expression and microvessel density (MVD) were assessed by immunohistochemistry in samples obtained from patients with pathological diagnoses of cervical intra-epithelial neoplasm I, carcinoma in situ, invasive squamous cell carcinoma (SCC), and benign disease (n=12 from each group). Two representative blocks that contained serial changes of cervical lesions from these 48 subjects were examined, and the pathological findings were categorized into the four groups of normal cervical epithelia, low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), and SCC.

Results. A total of 120 foci with various cervical lesions from 98 slides were examined and classified into normal (48), LSIL (36), HSIL (24), and SCC epithelium (12). Immunohistochemical studies showed that TSP-1 was mainly localized at the basal epithelial cells, and we named it as the 'TSP-1 fence'. The mean MVD counts and TSP-1 scores for normal, LSIL, HSIL, and SCC epithelium were 7.3 ± 2.9, 9.9 ± 3.4, 17.7 ± 5.1 and 22.8 ± 8.6, and 3.8 ± 0.4, 3.8 ± 0.4, 1.8 ± 0.4 and 1.5 ± 0.5, respectively. The TSP-1 intensities were significantly higher and the MVD counts lower in the groups of normal and LSIL epithelium, compared with those with HSIL and SCC epithelium ($p < .001$, Kruskal-Wallis test and Dunn post hoc comparison). In addition, MVD counts was negatively associated with the intensity of TSP-1 (p

<.001).

Discussion. Our results indicate the disruption of TSP-1 fence and the switch to angiogenic phenotype occurred during the transition from LSIL into HSIL. This concordance suggests that TSP-1 play a role in the regulation of angiogenic switch. We conclude that the onset of angiogenesis is an early event in cervical carcinogenesis due, in part, to the down-regulation of TSP-1 by the dysplastic epithelium.

Keywords: thrombospondin-1 (TSP-1), angiogenesis, microvessel density (MVD), cervical neoplasms

Published in: Cancer J 10(1):27-32;2004. (SCI, IF: 2.028, Oncol 78/127)

3.2 Introduction

Cervical cancer is the second most frequent causes of death from malignant neoplasms among women worldwide (Chou CY et al. 1994). It develops usually by a sequence of gradual, stepwise events starting at low-grade squamous intra-epithelial lesion (LSIL) and progressing through high-grade SIL (HSIL), until invasive cancer (Pinto AP and Crum CP 2000). Tumor development and metastasis is a complex process that includes transformation, proliferation, neovascularization, and metastatic spread. The angiogenic switch- acquisition of an angiogenic phenotype that is induced by a change in the balance of angiogenesis activators and inhibitors, is essential for tumor growth and metastasis (Hanahan D and Folkman J 1996; Hawighorst T et al. 2001). It has been reported that tumor microvasculature, accompanied by the overexpression of VEGF, was progressively up-regulated during the process of cervical carcinogenesis (Guidi AJ et al. 1995). However, the timing of angiogenic switch during cervical carcinogenesis remains to be debated (Smith-McCune KK and Weidner N 1994).

The TSPs are a family of extracellular proteins that participate in cell-to-cell and cell-to-matrix communication. Five family members have been identified (Lawler J 2000). Recent work has identified the roles of TSP-1 as an endogenous angiogenic inhibitor that plays an important role in the angiogenic switch in skin, prostate and bladder cancers (Campbell SC et al. 1998; Hawighorst T et al. 2001; Jin RJ et al. 2000). Evidence revealing the role of TSP-1 in cervical carcinogenesis is currently lacking. The purpose of this study was aimed to examine the spatial and temporal expression of TSP-1 in patients with preinvasive and invasive squamous cell carcinoma of the uterine cervix, and correlated TSP-1 expression with microvessel density (MVD).

3.3 Results

3.3.1 MVD during cervical carcinogenesis

A total of 96 tissue sections were recruited for this study. Among them, different extent of cervical lesions i.e. LSIL, HSIL and invasive cancer that appeared in the same slide were scored individually and regarded as different lesions. Accordingly, normal cervical epithelia in 48 slides, LSIL in 36 slides, HSIL in 24 slides, and SCC in 12 slides were eligible for analysis. The MVD count and TSP-1 expression in the four groups are summarized in Table I.

A representative immunohistochemical staining of von-Willebrand factor is shown in Fig. 1. Microvessels were only scarcely visible in the groups of normal cervical epithelium and LSIL, whereas in HSIL and SCC groups, microvessels were abundant (Fig. 1). It is noteworthy that the neovascularization in HSIL is confined to a narrow zone immediately underneath the dysplastic epithelium and along the basement membrane. The mean MVD counts for normal, LSIL, HSIL, and SCC epithelium were 7.3 ± 2.9, 9.9 ± 3.4, 17.7 ± 5.1 and 22.8 ± 8.6, respectively. Statistical analyses with Kruskal-Wallis test revealed that the MVD counts were significantly different among these four groups ($p < .001$). Furthermore, the MVD counts were significantly lower in the groups of low vascularity i.e. normal and LSIL epithelium, compared with those with high vascularity i.e. HSIL and SCC ($p <.001$, Dunn post hoc comparison). Together, these results clearly indicate that MVD counts increase during the transition from LSIL into HSIL.

Since more than one category of cervical epithelium may apply to a block and two blocks were taken from each patient, we also analyzed the data as dependent samples. Pairwise comparisons between different lesions on the same slide with Wilcoxon signed-rank test showed that the mean MVD counts for normal/ LSIL epithelium, LSIL/ HSIL epithelium, and HSIL/ SCC epithelium were 7.1 ± 3.0/$9.9 \pm$

3.4, 9.8 ± 3.5/17.7 ± 5.1, and 18.4 ± 4.7/22.8 ± 8.6 respectively, a significant difference (Table 2).

3.3.2 TSP-1 expression during cervical carcinogenesis

A representative immunostaining of TSP-1 in various cervical epithelial lesions is shown in Fig. 2. In the groups of normal and LSIL epithelium, intense TSP-1 expression was found and localized mainly in the basal epithelial cell layer. It arrayed like a barrier therefore we named it as 'TSP-1 fence' (Fig. 2). In contrast, this "TSP-1 fence" disappeared partially in HSIL, and became nearly invisible in SCC epithelium. The mean TSP-1 scores for normal, LSIL, HSIL, and SCC epithelium were 3.8 ± 0.4, 3.8 ± 0.4, 1.8 ± 0.4 and 1.5 ± 0.5, respectively. The TSP-1 scores were significantly different among these four groups ($p < .001$, Kruskal-Wallis test), and Dunn post hoc comparison further confirmed that the TSP-1 intensities were significantly higher in the groups of low vascularity, compared with those with high vascularity ($p < .001$). These results indicate the disruption of TSP-1 fence occurred during the transition from LSIL into HSIL.

When two blocks from the same patient were considered as dependent samples, TSP-1 score was significantly different only between LSIL/HSIL epithelium (Table 2). The mean TSP-1 scores for normal/LSIL, LSIL/ HSIL, and HSIL/SCC epithelium were 3.8 ± 0.4/ 3.8 ± 0.4, 3.8 ± 0.4/ 1.8 ± 0.4 and 1.8 ± 0.4/1.5 ± 0.5, respectively.

3.3.3 Association of MVD and TSP-1

Among the 120 cervical lesions examined, 66 cases were grade 4 positive for TSP-1 staining, 18 cases were grade 3 positive, 24 cases were grade 2 positive, and 12 were grade 1 positive. The mean MVD counts for TSP-1 grades 1, 2, 3 and

4 cervical lesions were 21.0 ± 9.2, 18.6 ± 5.3, 8.0 ± 3.1 and 8.6 ± 3.4, respectively. These results indicate that the down-regulation of TSP-1 grade 3 into TSP-1 grade 2 was accompanied by a significant elevation of MVD counts. A Kruskal-Wallis analysis revealed that MVD counts was negatively associated with the intensity of TSP-1 ($p < .001$). A p-value less than 0.001 from Dunn post hoc comparison test further pointed out the difference of MVD counts between low TSP-1 expression group (score 1 or 2) and high TSP-1 expression group (grade 3 or 4).

3.4 Discussion

The timing of angiogenic switch during cervical carcinogenesis remains controversial. A debate exists regarding the ability of CIN to induce angiogenesis (Abulafia O et al. 1996; Abulafia O et al. 1999; Smith-McCune KK and Weidner N 1994). Smith-McCune and Weidner found a significant increase of MVD in the CIN III lesions compared with those underlying low grade lesions such as condyloma and CIN I (Smith-McCune KK and Weidner N 1994). On the contrary, reports from Abulafia el al. showing that microinvasive squamous cell carcinoma is angiogenic, but not carcinoma in situ (Abulafia O et al. 1996). In the present study, we intend to examine the slides that contain different severities of cervical lesions in the same slide, so that every lesion can be used as an internal control for each other. Thus, the heterogeneity in the inborn characters of angiogenesis can be eliminated. Our data showed that the angiogenic switch in cervical carcinogenesis occurred during the transition from LSIL to HSIL, and the neovascularization was largely confined to a narrow zone immediately underneath the dysplastic epithelium. This is in concordance with the results from Smith-McCune and Weidner (Smith-McCune KK and Weidner N 1994). and further suggests that cervical carcinogenesis is angiogenesis-dependent.

Our results also showed that TSP-1 was mainly localized on basal cervical epithelial cells, and arrayed like a barrier. We therefore name it as the ‘TSP-1 fence’. TSP-1 decreases significantly during the transition from LSIL to HSIL, which is concomitant with the increase of MVD counts. The temporal and spatial concordance of TSP-1 down-regulation and the emergence of angiogenic imply that the “TSP-1 fence” may act as an angiogenic barrier to inhibit angiogenesis which occurred in early phase of cervical carcinogenesis. The disappearance of the angiogenic barrier may induce a vigorous angiogenic response for tumor growth

and perhaps tumor metastasis (Sheibani N and Frazier WA 1999). Evidence from Kodama et al. showing that TSP-1 mRNA expression was significantly lower in advanced-stage cervical cancer, and its expression is of value as a prognostic factor in cervical cancer (Kodama J et al. 2001). The origin of TSP-1 is currently unknown, and two possible origins including tumor cells themselves and host cells (endothelial cells) may be responsible for the production (Folkman J 1992). Our data suggest that the basal epithelial cells contribute to the production of TSP-1 under physiological condition, and lose the ability to secrete TSP-1 during the transformation from LSIL to HSIL.

Argument has been raised that our statistical comparisons were made between different lesions, irrespective of the patients. Since more than one category of cervical epithelium may apply to a block and two blocks were taken from each patient, we also analyzed the data as dependent samples. Pairwise comparisons with Wilcoxon signed-rank test further confirm the significant decrease of TSP-1 and elevation of MVD during the transition from LSIL to HSIL.

Accumulating evidence indicates that for most tumors, the switch to the angiogenic phenotype depends upon the outcome of a balance between angiogenic stimulators and angiogenic inhibitors (Folkman J 1992). Up-regulation of angiogenesis activators alone may not be enough for the emergence of angiogenic switch; it accompanies with down-regulation of some angiogenesis inhibitors in the same time (Folkman J 1995b). TSP-1 and VEGF appear to be the constituents of a "switch" that regulates in concert many components of the angiogenic and differentiated phenotypes of endothelial cells (Sheibani N and Frazier WA 1999). In skin cancer model, down-regulation of TSP-1 and up-regulation of VEGF happened coincidently and had spatial correlation throughout the consecutive stages of tumorigenesis (Hawighorst T et al. 2001). In

contrast, down-regulation of TSP-1 secretion is a key event in the switch from anti-angiogenic to an angiogenic phenotype, while VEGF seems to play little role in bladder cancer (Campbell SC et al. 1998). VEGF has been found to increase significantly in high-grade cervical intraepithelial lesions as compared with low-grade intraepithelial lesions and benign epithelium (Guidi AJ et al. 1995). We here suggest that TSP-1, as one of the endogenous angiogenic inhibitors, may play the comparable role to other angiogenic activators such as VEGF in the angiogenesis balance during cervical carcinogenesis.

The angiogenic response, which is induced by the disappearance of “TSP-1 fence”, modulates the peri-cellular environment, and can potentially change the cell-matrix interactions associated with cell movement and further progression. TSP-1 does not appear to contribute directly to the structural integrity of connective tissue elements. Instead, TSP-1 acts by modulating the activity and bioavailability of protease and growth factors and by interaction with cell-surface receptors (Bornstein P et al. 2000b; Stetler-Stevenson WG 1999). MMPs have been shown to play an active role in the neovascularization of tumors through their ability to degrade the extracellular matrix (Liotta LA et al. 1982; Zetter BR 1990). Bergers et al. showed that the switch from vascular quiescence to angiogenesis involves MMP-9, which is upregulated in angiogenic islets and tumors, rendering VEGF more available to its receptors (Bergers G et al. 2000). Notably, MMP-9 is negatively modulated by TSP-1. Thus, TSP-1 acts as a multifunctional modulator of angiogenesis by modulating through the activity and bioavailability of MMP-9. How does TSP-1 modulate MMPs deserves for further studies.

Acknowledgement:

This work was supported by a grant from Chi Mei Foundation Hospital (CMFHR No.

9107). We thank Miss Chin-Li Lu, Section of Statistic Consultation, Department of Medical Research, Chi Mei Foundation Hospital, for statistic analytic assistance.

Chapter 4 A novel role of TSP-1 in cervical carcinogenesis: Inhibit stroma reaction by inhibiting activated fibroblasts from invading cancer

4.1 Summary

TSP-1, a potent angiogenesis inhibitor, has been shown to exert different biological functions on various cell types. Here we investigate the role of TSP-1 in tumor stroma reaction, which is mainly characterized by fibroblast activation to create a permissive microenvironment for tumor progression. Immunohistochemistry examinations in the human surgical specimens showed the downregulation of TSP-1 during the progression of cervical carcinogenesis was accompanied by the emergence of the upregulation of stroma markers, α-SMA and desmin. Transfection of SiHa cervical cancer cells with a plasmid expressing the TSP-1 protein exhibited anti-angiogenic activity in vitro, and resulted in reduced tumor growth in SCID mice, which was accompanied by a decrease in tumor vascularization and lower expressions of α-SMA and desmin than those in the vector controls. Transfection with TSP-1 and purified TSP-1 added to NIH3T3 cells did not alter the protein levels of α-SMA and desmin, but significantly inhibited matrix metalloprotease-2 (MMP-2) activity. Transforming growth factor β (TGF-β), a major factor to activate fibroblasts, increased α-SMA and desmin expression, and the ability of cell migration and invasion in NIH3T3 cells. The increased migration ability and the invasive ability into tumor cluster of TGF-β-treated-NIH3T3 cells were dose-dependently inhibited by TSP-1. In contrast, ectopic TSP-1 expression in SiHa cells has little effect on the invasive ability of the NIH3T3 cells. Together, our findings demonstrate a novel role of TSP-1 to inhibit tumor stroma reaction that could be attributed to the blockage of activated fibroblasts from invading cancer cells.

Keywords: thrombospondin-1, stromal reaction, cervical neoplasms, angiogenesis, activated fibroblasts

Published in: Carcinogenesis 2008 In Press, (SCI, IF: 5.366, Oncol 18/127).

4.2 Introduction

Our understanding of cancer has largely come from the analysis of aberrations within the tumor cell population. Recent evidence has begun to emerge of the important roles of tumor microenvironment in tumorigenesis (Mueller MM and Fusenig NE 2004). Stroma reaction, also known as stromagenesis, is a host response of mesenchymal alteration induced in cancer that produces a progressive and permissive mesenchymal microenvironment, thereby supporting tumor progression (Amatangelo MD et al. 2005). Stroma reaction is characterized by the activation of quiescent fibroblasts, the predominant cell type within a normal stroma, into activated fibroblast. Activated fibroblasts are defined by the expression of α-smooth muscle actin (α-SMA), desmin, vimentin, etc in the fibroblasts (Lazard D et al. 1993). Activated fibroblasts can produce noncellular scaffolds in response to extracellular stimuli, and create a context to promote tumor progression (Barcellos-Hoff MH and Ravani SA 2000). Additionally, activated fibroblasts within tumor stroma have a propensity to migrate and invade like cancer cells (Ishii G et al. 2005). The proliferative activity of activated fibroblasts in cancer-induced stroma is closely linked to tumor progression, lymph node and distant organ metastasis of breast cancer (Hasebe T et al. 2000).

TSPs consist of a family of five extracellular proteins that participate in cell-to-cell and cell-to-matrix communications (Lawler J 2000). Among them, TSP-1 is a 450-kDa homo-trimeric matricellular glycoprotein with potent anti-angiogenic effects. In many tumor types, TSP-1 can block in vivo neovascularization and decrease malignant tumor growth (Kodama J et al. 2001; Streit M et al. 1999); whereas in others, it promotes cancer cell adhesion, migration and invasion (Bertin N et al. 1997). The differential effects of TSP-1 on tumorigenesis indicate that TSP-1 exerts different biological functions in different

cell types.

We previously demonstrated that the downregulation of TSP-1 in cervical epithelium temporally and spatially coincided with the emergence of angiogenic switch during cervical carcinogenesis (Wu MP et al. 2004). However, the exact biologic roles of TSP-1 in tumor stroma reaction and progression remain to be further explored. This study was initiated to test the hypothesis that TSP-1 may inhibit stroma reaction which supports tumor progression through inhibiting activated fibroblasts from invading cancer cells. By studies in cell lines, SCID mice xenograft and clinical human specimens, we demonstrated a novel role of TSP-1 by which it inhibited tumor progression, at least in part, through blocking the migration and invasion of activated fibroblasts and leading to stroma normalization.

4.3 Specific aims and strategies:

Aim I. To correlate the TSP-1 expression and stroma marker expression in clinical human specimens during cervical carcinogenesis

This is to offer the clinical correlation that TSP-1 downregulation associates with stroma marker overexpression (i.e. stroma reaction) of different cervical lesions during cervical carcinogenesis. The correlative data was followed by the further related study for the causal relationship.

Strategies (**Human cervical lesion specimens)** Immunohistochemistry (IHC) stains were used to evaluate the TSP-1 expression and two stroma markers (α-smooth muscle actin and desmin). Patients with different stages of cervical lesion were recruited for IHC study, in which 15 cases with diagnosis of benign gynecologic lesion, 15 cases with carcinoma in situ (CIS), and 15 cases of FIGO stage Ib squamous cell carcinoma (SCC). The expression of two stroma markers, α-SMA and desmin, were assessed in these samples. The expression of different markers was categorizes into 4 grades. They were arbitrarily scored as 0: no to little; 1: weak; 2: moderate; 3: strong in α-SMA and desmin.

To elucidate the anti-tumor effects of TSP-1 through its angio-inhibitory effects and its ability to modulate the stroma reaction.

IIa. To elucidate that TSP-1 inhibits tumor formation (TSP-1-overexpressing tumor is smaller, as compared with vector control); and the anti-tumor effect is through the angio-inhibitory effects of TSP-1 (angiogenesis is measured by microvessel density, MVD).

Strategies (SCID xenograft model) To elucidate that TSP-1-overexpressing tumor (as compared with vector control) may have smaller and less vascularized tumor formation. The established TSP-1-overexpressing clones with SCID

xenograft model were used to elucidate the causal relationship between TSP-1 and tumor angiogenesis and stroma reaction. Human TSP-1 cDNA is a kindly gift from Professor Frazier WA (Washington University, School of Medicine, St. Louis, MO, U.S.A). The expression plasmid pcDNA/TSP-1 contains the human TSP-1 cDNA under the control of the CMV promoter and a neomycin selection cassette. The plasmid pcDNA was used as the empty vector control. Human cervical squamous cell carcinoma cell line (SiHa) was transfected with pcDNA /TSP-1 or pcDNA with lipofectamine 2000. We inoculated SCID mice with SiHa-TSP-1 or SiHa-pcDNA, to demonstrate that TSP-1 can suppress tumor growth and angiogenesis in xenografts. The tumor sizes of the inoculation sites can be seen subsequently from TSP-1 expressing tumor, as compared with the vector controls. The SCID mice were sacrificed for the evaluation for tumor size, and tumor angiogenesis (by immuno-histochemistry stains for microvessel densities).

IIb. To elucidate that TSP-1 inhibits stroma reaction measured by marker expressions (TSP-1-overexpressing tumor may have less stroma marker expression as compared with those of vector control-induced tumors).

Strategies. After sacrificing experimental animals, the harvested tumor specimens will be sent for IHC, which are counterstained with hematoxylin, α-SMA, and desmin in TSP-1-overexpressing tumor, and Siha-induced tumor. α-SMA, and desmin were uses as stroma markers for the evaluation of stroma reaction. Tumor-associated stroma from SiHa-TSP-1-induced tumor tumors will be compared with SiHa-induced tumor. The morphologic pattern, as well as the levels of expression of α-SMA and desmin within the tumor-associated stroma will be evaluated.

Aim III. To elucidate that TSP-1 can change the invasive ability of activated

fibroblasts during stroma reaction

IIIa. To elucidate that endogenous TSP-1 can weaken the invasive ability of myofibroblasts from invading tumor cell cluster, either via transfection into tumor cells or myofibroblasts.

Strategies (multicellular matrigel coculture model) To elucidate that TSP-1 can change the Matrigel invasive ability of normal fibroblasts or myofibroblasts during stroma reaction. In addition to the angiogenesis tube formation assay, Matrigel was used as a multi-cellular co-culture invasion assay. We established Matrigel multicellular co-culture system, which was modified from the report of Walter-Yohrling J et al. (Walter-Yohrling J et al. 2003), for the evaluation of the tumor stromal interaction, which comprises cancer cells (SiHa vs SiHa-TSP-1), and (myo)fibroblast (293T, NIH3T3, 7-4) with or without TSP-1-overexpression. The details of multicellular matrigel co-culture system in vitro model were in the Methodology section. Fluorescent labels were used to visualize the localization of the HUVECs and myofibroblasts in the presence of the cancer cells cluster. The interaction between the tumor cells and mesenchymal compartments (fibroblasts or myofibroblasts) creates a local heterotypic ‘invasion field’. It is unclear who is invading whom, i.e. cancer cells invading fibroblasts or fibroblasts invading cancer cells. 293T, NIH-3T3, and 7-4 were used for (myo)fibroblasts. Among them, 7-4 and NIH3T3 are either with or without ras overexpression. SiHa, as well as SiHa-TSP-1, was placed in the central well, while NIH3T3 with or without TSP-1 overexpression were placed in the peripheral to see the effect of endogenous TSP-1 on the invasion ability.

IIIb. To elucidate that exogenous TSP-1 can weaken the invasive ability of myofibroblasts from invading tumor cell cluster, either via the addition into tumor cells or myofibroblasts.

Strategies. To elucidate that exogenous TSP-1 can weaken the chemo-invasive ability of myofibroblasts from invading tumor cell cluster, either in tumor cells or myofibroblasts. NIH3T3 could invade SiHa cell cluster in the absence of TSP-1. Different concentrations of purified TSP-1, either low concentration, 10, 20 ug/ml, or high concentration 40 ug/ml, were added into SiHa cell clusters or NIH3T3 in the peripheral to see the inhibitory effects of TSP-1 in the myofibroblast invasion.

4.4 Results

4.4.1 The downregulation of TSP-1 coincides with the upregulation of stroma markers during cervical carcinogenesis

A representative immunohistocheminsty staining of TSP-1, α-SMA and desmin in various human cervical epithelial lesions was shown in Figure 1. In the group of normal cervical epithelium, TSP-1 expression was localized mainly in basal epithelial cell layer, but became invisible in CIS and SCC epithelia (upper panel, Figure 3). In contrast, the stains for α-SMA (middle panel, Figure 3) and desmin (lower panel, Figure 3) were hardly visible in the stromal area of normal cervix. The staining intensities of α-SMA and desmin increased overtly in stromal area of CIS and SCC. The mean α-SMA and desmin scores in the three groups are summarized in Table 3. Statistical analyses with Kruskal-Wallis test revealed that α-SMA and desmin scores were significantly different among normal, CIS and SCC groups. In addition, α-SMA scores were significantly different when compared normal with CIS, CIS with SCC, normal with SCC, with p values of 0.002, 0.006, and <0.001, respectively. Similarly, desmin scores were also significantly different among pair-wise comparisons with p values of 0.019, <0.001, <0.001, respectively (Dunn post hoc comparison test).

4.4.2 TSP-1 inhibits angiogenesis, tumor development and SCC-induced stromal reaction in vivo

To test whether manipulation of TSP-1 activity would alter tumor formation in vivo and whether TSP-I is involved in tumor stroma reaction, we developed SiHa-TSP-1 cell lines which were stably transfected with the full-length TSP-1 cDNA. Immunoblots confirmed the overexpression of TSP-1 in C44 and C57 clones, as compared with parental cells (SiHa) or vector control (C5, C9 and C12) (Figure 4,

A). The C44 clone (SiHa-TSP-1) was used for further study. As shown in Figure 4, B, increased expression of TSP-1 did not alter the growth rate of cancer cells. We next examined the anti-angiogenic activity of TSP-1 by Matrigel angiogenesis assay. HUVECs seeded on Matrigel rapidly formed tubules networks within 6 hours (left upper panel, Figure 4, C), which was attenuated by either the purified TSP-1 at 10 μg/ml or by the conditioned medium (CM) from normal cervical epithelium (upper panel, Figure 4, C). In the same line of observations, only TSP-1 containing CM from SiHa-TSP-1 (SiHa-TSP-1 CM) but neither parental SiHa (SiHa CM) nor vector control cells (SiHa-vector CM) had potent inhibitory effect on the tube forming ability of HUVECs (lower panel, Figure 4, C).

The SiHa-TSP-1 and its vector control were inoculated into SCID mice subcutaneously. Rapid tumor growth was evident in SCID mice inoculated with vector control cells. In contrast, SiHa-TSP-1 cells exhibited significantly reduced rate of tumor growth and tumor size ($p= 0.013$, ANOVA test; Figure 5, A and B). Moreover, the SiHa-TSP-1-innoculated tumor was less vascularized, as compared with SiHa-vector control (CD34, Figure 5, C) (0.58 ± 0.18 vs 1.67 ± 0.19 ($n=6$), $p= 0.002$. In SiHa-vector inoculated tumor, the expression level of TSP-1 was negligible, while TSP-1 was much highly expressed in tumor site (T) and stroma area (S) of SiHa-TSP-1 inoculated tumor. In contrast, the expression levels of α-SMA and desmin were high in SiHa-vector inoculated tumor, but significantly reduced in SiHa-TSP-1 inoculated tumor (Figure 5, C; 0.95 ± 0.42 vs 2.17 ± 0.43 for α-SMA, $p= 0.002$; 0.48 ± 0.46 vs 1.52 ± 0.63 for desmin, $p= 0.015$, Mann-Whitney U test).

4.4.3 TSP-1 does not downregulate α-SMA and desmin expression but inhibits MMP-2 activity

Phenotypic switching of quiescent fibroblasts into activated fibroblasts is the most prominent stromal reaction during carcinogenesis. Activated fibroblasts are often characterized by acquisition of high α-SMA expression. Due to difficulties in long-term culturing primary cells, NIH3T3 fibroblasts were used instead. Western blot analysis showed that NIH3T3 cells had higher level of α-SMA expression than those in normal primary cervical fibroblasts (NF) and HUVECs (Figure 6, A). To examine if TSP-1 could directly modulate the α-SMA expression, we established clones stably expressing TSP-1 protein in NIH3T3 cells (NIH3T3-TSP-1). Western blot confirmed the expression of TSP-1 protein in NIH3T3-TSP-1 but not NIH3T3 or vector control cells (Figure 6, A and B). TSP-1 overexpression by transfection or by exogenous addition of TSP-1 (1-20 μg/ml) did not have any detectable effect on the expression of the α-SMA and desmin in these cells (Figure 6, B). We then tested the effect of TSP-1 on the MMP-2 and MMP-9 activities which are key regulators for matrix degradation. Elevated levels of TSP-1 either by plasmid transfection or by direct addition of TSP-1 at concentrations ranging from 1 to 20 μg/ml did not downregulate the expression of MMP-2 protein level (Figure 6, B; the MMP-9 level was negligible, and therefore the data were not shown). However, MMP-2 activity was inhibited by both ectopic expression and the addition of TSP-1 in a dose-dependent manner using gelatin zymography (Figure 6, C).

4.4.4 TSP-1 inhibits the migration of untreated and TGF-β treated NIH3T3 cells

TGF-β, a potent fibroblast activation and trandifferentiation factor, was used to activate NIH3T3 fibroblasts (Bierie B and Moses HL 2006). To elucidate the biological functions associated with TGF-β-mediated fibroblast activation, we examined the effects of TGF-β on α-SMA, desmin expression and MMP activity in

NIH3T3 cells. TGF-β at as low as 2 ng/ml potently enhanced the expression of α-SMA and desmin TSP-1 was added to TGF-β-treated NIH3T3 to see whether or not it can change α-SMA expression after TGF-β treatment. The TGF-β-increased α-SMA expression can not blocked with TSP-1 (Figure 6, D). No significant change of MMP-2 protein level and its gelatinyltic activity was observed in TGF-β-treated cells (Figure 6, D and E). We then compared the effects of TSP-1 on the migration ability of untreated and TGF-β-treated NIH3T3 cells using cell migration assay. TGF-β at 10ng/ml increased NIH3T3 cell migration (p=0.038). The stimulatory effect of TGF-β on cell migration was inhibited by purified TSP-1, p= 0.010, 0.007, 0.006. The migration ability of untreated NIH3T3 was also markedly inhibited by TSP-1 at 10 μg/ml in the lower, upper or both chambers (with p=0.021, 0.009, and 0.009, Student t test) (Figure 4, F). The inhibitory effect was in a dose-dependent manner at TSP-1 concentrations of 0.1-10 μg/ml (data not shown). Reduced cell migration was also observed in NIH3T3-TSP-1 cells compared with NIH3T3-vector cells, p=0.044 (Figure 6, F).

4.4.5 TSP-1 inhibits activated fibroblasts from invading tumor cell cluster

Activated fibroblasts, like other invasive cancer cells, have the ability to invade tumor cell clusters (Ishii G et al. 2005). Since TSP-1 directly reduced MMP-2 activity and TGF-β-induced migration ability, we examined the effect of TSP-1 on invasive ability of activated fibroblasts into tumor cell cluster. NIH3T3 cells but not human normal fibroblasts (NF), expressed α-SMA and had the ability to invade SiHa tumor cluster, regardless of the expression status of TSP-1 in tumor cells (Figure 7, A). Exogenous TSP-1 (10, 20 or 40 μg/ml) in tumor cluster also failed to inhibit NIH3T3 from invading SiHa tumor cluster (Figure 7, B). By contrast, the invasive ability of NIH3T3-TSP-1 cells to SiHa (or SiHa-TSP-1) tumor cluster was

significantly reduced when compared with NIH3T3, indicating a direct inhibitory effect of TSP-1 on the invasive ability of NIH3T3 (Figure 7, A). We next examined the effect of exogenous TSP-1 on the invasive ability of NIH3T3 cells. Consistent with stable expression of TSP-1 in NIH3T3 cells, we found a dosage-dependent inhibition of NIH3T3 cell invasion. A complete inhibition was observed at 20 μg/ml or higher (Figure 7, C). We further used TGF-β to activate NIH3T3 and investigated the effect of TGF-β on TSP-1-mediated inhibition of NIH3T3 invasion. TSP-1 potently inhibited fibroblast invasion regardless of the presence of TGF-β although a higher dose of TSP-1 was required for a complete inhibition of TGF-beta-treated NIH3T3 cells (Figure 7, C and D). Taken together, our results indicate that the TSP-1-mediated inhibition of the invasive ability could only be demonstrated when the manipulation of TSP-1 expression was in fibroblasts, but not tumor cells.

4.5 Discussion

Our current understanding on the role of TSP-1 in tumor progression and clinical prognosis has extended far beyond anti-angiogenesis. The present study provides evidence that TSP-1 has the potential to inhibit stroma reaction during cervical carcinogenesis. This conclusion is supported by the following findings: (i) The concordance of the downregulation of TSP-1 and the upregulation of stroma markers in surgical specimens of cervical carcinoma suggests that TSP-1 plays an inhibitory role in stroma reaction. (ii) Transfection of SiHa cells with a plasmid expressing the TSP-1 protein resulted in reduced tumor growth in SCID mice that was accompanied by a decrease in tumor vascularization and a lower level of stroma markers, α-SMA and desmin, than the vector transfection. (iii) Ectopic expression of TSP-1 or by addition of purified TSP-1 manifested an inhibition of MMP-2 activity, TGF-β-enhanced cell migration, and the invasive ability of activated fibroblasts from tumor cell clusters.

We previously pointed out that the switch of angiogenic phenotype, partly due to the down-regulation of TSP-1, occurred during the transition from low-grade to high-grade squamous intra-epithelial lesion (Wu MP et al. 2004). In this study, we demonstrated a temporal inverse correlation of TSP-1 and stromal marker expression during cervical carcinogenesis using human clinical specimens. The inhibitory effect of TSP-1 on stromal marker expression was further confirmed in SCID mouse xenografts using transfection of TSP-1 cDNA expression vectors. Genetic manipulation of TSP-1 expression level in the cells demonstrated that TSP-1-mediated inhibition of stroma reaction was primarily due to the inhibition of activated fibroblast migration and invasion, rather than a direct effect on the stromal maker expression. These results indicate that TSP-1 participates not only in the negative regulation of angiogenesis but also stroma reaction during cervical

carcinogenesis.

Cancer progression is a complex process involving transformation, invasion, angiogenesis and metastasis (Hanahan D and Weinberg RA 2000). Although TSP-1 is commonly believed to have anti-tumor effects due to its anti-angiogenic ability, however, results from various studies have demonstrated different correlations among the levels of TSP-1 and tumor progression and clinical prognosis in different tumor types, indicating different biological functions of TSP-1 in different cancer cell types. The lower expression levels of TSP-1 in cervical cancer than those in normal tissue is similar to some human tumors in which decreased TSP-1 expression is associated with malignancy (Bunone G et al. 1999; de Fraipont F et al. 2000). Among these cancers, TSP-1 expression is inversely correlated with tumor grade and survival rate in thyroid, colon and bladder carcinomas (Bunone G et al. 1999; de Fraipont F et al. 2001). The prognostic value of TSP-1 in cervical cancer requires further investigation. In contrast, TSP-1 expression was higher in tumors or tumor-associated stroma than in normal epithelial or stroma tissue in other cancer types (Bertin N et al. 1997). Thus, TSP-1 seems to have activatory as well as inhibitory properties in tumor progression. There are several likely explanations to account for its dualistic effects. Firstly, TSP-1 interacts with multiple extracellular macromolecules and cell surface receptors, thus exerting a wide range of responses (Brown EJ and Frazier WA 2001; de Fraipont F et al. 2001). Secondly, the exposure to high stromal TSP-1 may induce expression of angiogenesis activators in tumor cells, which override the effects of TSP-1 (Fontana A et al. 2005). Thirdly, TSP-1 exerts its effects on multiple types of stromal cells such as inhibiting fibroblasts migration (Streit M et al. 2000), decreasing the recruitment of inflammatory cells (Doyen V et al. 2003), inducing apoptosis of endothelial cells (Reiher FK et al. 2002; Streit M et al. 1999),

or activating smooth muscle cells proliferation (Isenberg JS et al. 2005a). Fourthly, some limitations still remain among the various models (Hlatky L et al. 2002).

It has become increasing clear that, from the context of tumor-stroma interactions, stroma plays an active role in tumor progression (Mueller MM and Fusenig NE 2004). Stromal cells can acquire oncogenic transformation following the exposure to carcinogen (Kalas W et al. 2005), manipulation of MMPs (Rodriguez-Manzaneque JC et al. 2001), and the recruitment of inflammatory cells to the stroma (Doyen V et al. 2003; Vallejo AN et al. 2000). Stroma reaction is often accompanied with stromal marker expression and functional changes into an invasive phenotype. Inhibition of stroma reaction by TSP-1 might be through reduced expression of stromal markers and invasiveness or inhibition of activated fibroblast migration and recruitment to tumor stroma. Using Western blot analysis, migration and Matrigel co-culture invasion assays, we demonstrated that TSP-1 potently exerted inhibitory effect on the migration and invasion of fibroblasts with or without TGF-β treatment, however, it has little effect on the expression level of α-SMA expression, a typical marker for activated fibroblasts. Unlike TSP-1, secreted protein, acidic and rich in cysteine (SPARC) was shown to inhibit fibroblast activation by blocking α-SMA overexpression (Chlenski A et al. 2007b). Although SPARC and TSP-1 are both matricellular proteins that inhibit angiogenesis and interfere with the organization of the extracelluar matrix; however, TSP-1 inhibits stroma reaction through a mechanism distinct from SPARC.

It is worth mentioning that the stroma reaction occurred mainly at the tumor-stroma junction as shown in both clinical and animal specimens, this phenomenon was attenuated by the upregulation of TSP-1. Furthermore, this highlights the importance of the interaction between tumor and stroma, and

suggests a likely possibility that modulation of tumor microenvironment can potentially change the cell-matrix interactions associated with cell movement and further progression. Consistent with this notion, it has been shown that TSP-1 inhibits the activity of MMP-2 and MMP-9 via a direct interaction with these proteases in specific regions (Bein K and Simons M 2000; Rodriguez-Manzaneque JC et al. 2001). In this study, no MMP-9 activity could be detected in NIH3T3 cells. Instead, we observed that TSP-1 inhibited MMP-2 activity, which was not altered by TGF-β treatment, followed by a decrease in the migration and invasive ability of activated fibroblasts. More studies are needed to address the exact mechanism whereby TSP-1 decreased MMP-2 activity in the context of activated fibroblasts.

Our present results show that TSP-1 inhibited TGF-β-enhanced migration and the invasive ability of NIH3T3. TSP-1 is known to function as an activator of TGF-β and this activity is mapped to the type-1 repeats of TSP-1 (TSRs) (Crawford SE et al. 1998; Kawataki T et al. 2000). Moreover, the activation of TGF-β by TSP-1 can either inhibit (Miyanaga K et al. 2002) or increase (Kawataki T et al. 2000) malignancy depending on cell types. The complexity and duality in the functions of TSP-1 and TGF-β may be due to their ability to suppress tumor cell proliferation at early stages, but to enhance tumor invasion and metastasis by enhancing the host stroma reaction at later stages (Radisky DC and Bissell MJ 2004). Further investigation is needed to dissect the dynamic interaction between TSP-1 and TGF-β in the regulation of cervical cancer growth.

Furthermore, TSP-1 expression in both the stroma and tumor areas of SCID mouse xenografts were different from that mainly expressed in basal epithelia of normal human cervix. This discrepancy may stem from species differences, differential sensitivities of antibodies to murine and human samples, host compensatory response or different study model (heterotopic versus orthotopic

animal model). In addition, clinical observations and genetic manipulations in our study have pointed to TSP-1 as a central mediator to inhibit the invasive ability of activated fibroblasts and their subsequent recruitment to stroma. However, we cannot completely rule out the possibility of other factors, e.g. the interaction with TGF-β, involved in stroma reaction (Hugo C 2003).

To summarize, our current study demonstrates that TSP-1 plays a broader role in tumor cell biology including the inhibition of activated fibroblast migration and invasion, in addition to its well-known anti-angiogenic effects. In viewing the fact that stromal therapy has recently emerged as a strategy for cancer treatment (Meyerhardt JA and Mayer RJ 2005), this study carries significant implications with regard to the application of targeted interventions on the TSP-1-mediated stroma reaction may represent a potentially new strategy for inhibiting the progression of cancer.

Acknowledgements:

This work was supported by grants, 93-CM-TMU-02, 94CM-KMU-03 and 95CM-TMU-03 from Chi Mei Foundation Hospital, and Center of Excellence for Clinical Trial and Research in Oncology Specialty, Ministry of Health, Taiwan. We thank Professor Tien Kuo, Department of Molecular Pathology, MD Anderson Cancer Center for critical reading of this manuscript, and Miss Chin-Li Lu, Chi Mei Foundation Hospital, for statistic analysis assistance.

Chapter 5 Discussion

5.1 How does TSP-1 regulate angiogenesis in cervical neoplasm

5.1.1 The roles of angiogenic activators in cervical neoplasm

VEGF is disulfide-bonded glycoprotein whose transcription and expression is upregulated with hypoxia and appears to play an important role in the development and growth of tumors (Senger DR et al. 1994). The tumor microvasculature, accompanied by the overexpression of VEGF, was progressively upregulated during the process of cervical carcinogenesis. VEGF has been found to increase significantly in HSIL as compared with LSIL and benign epithelium (Guidi AJ et al. 1995). Meanwhile, Dobb et al. found the progressive increases in MVD and VEGF expression starting at CIN I through CIN III to invasive SCC. There was a strong correlation between MVD and VEGF expression, and both were associated with histological grade of CIN. The onset of angiogenesis is an early event in premalignant changes of the cervix due to, in part, enhanced expression of VEGF by the abnormal epithelium (Dobbs SP et al. 1997). Cheng et al. found that cytosol VEGF might be a biomarker for the status of pelvic lymph nodes in early-stage cervical carcinoma and an independent prognostic indicator of its overall survival (Cheng WF et al. 2000). On the contrary, Raleigh et al. found no relationship between hypoxia and VEGF expression in cervical cancer (Raleigh JA et al. 1998). In summary, it appears that hypoxia is important in the response of cervical tumors to treatment, but the actual angiogenic factors that play a role in the development and progression of cervical tumors are not yet clear (Wolf JK and Ramirez PT 2001).

5.1.2 TSP-1 may play a comparative role to VEGF

TSP-1 and -2 can inhibit angiogenesis in response to many angiogenic cytokines

and growth factors such as basic fibroblast growth factor (bFGF), VEGF and others (Tolsma SS et al. 1993). Evidence suggests that TSP-1 is involved in angiogenesis. However, the mechanisms by which TSP-1 regulates angiogenesis are not well known, and the exact role of TSP-1 in angiogenesis has been controversial; both stimulatory (Qian X et al. 1997) and inhibitory effects (Streit M et al. 1999) of TSP-1 on angiogenesis have been reported.

Up-regulation of angiogenesis activators alone may not be enough for the emergence of angiogenic switch; it accompanies with down-regulation of some angiogenesis inhibitors in the same time (Folkman J 1995b). Rastinejad et al. demonstrated that tumor cells did not become angiogenic until they had significantly reduced their own production of TSP (Rastinejad F et al. 1989). VEGF has been found to increase significantly in HSIL as compared with low-grade intraepithelial lesions and benign epithelium (Guidi AJ et al. 1995). TSP-1, as one of the endogenous angiogenic inhibitors, may play the comparable role to other angiogenic activators such as VEGF in the angiogenesis balance during cervical carcinogenesis. In skin cancer model, down-regulation of TSP-1 and up-regulation of VEGF happened coincidently and had spatial correlation throughout the consecutive stages of tumorigenesis (Hawighorst T et al. 2001). Additionally, Kwak et al. showed higher VEGF and lower TSP-1 expression in prostate cancer than in non-cancerous tissues (Kwak C et al. 2002). Genetic evidences also imply that TSP-1 and VEGF play a comparative role to each other; the overexpression of VEGF and down-regulation of TSP-1 can be trigger by the same oncogene, ras; and the loss function of tumor suppressor genes, p53. Activating mutations in K-ras and H-ras up-regulate VEGF expression and down-regulate expression of TSP-1 (Udagawa T et al. 2002),(Rak J et al. 2000). Wild-type p53 normally suppresses tumor angiogenesis by up-regulating TSP-1 (Dameron KM et al. 1994), and

suppressing transcription of VEGF (Zhang L et al. 2000). TSP-1 and VEGF appear to be the constituents of a "switch" that regulates in concert many components of the angiogenic and differentiated phenotypes of endothelial cells.

5.1.3 TSP-1 specifically induces endothelial cell apoptosis

TSP-1 inhibits angiogenesis and modulates endothelial cell adhesion, motility, and growth. The type I repeats (TSR) of TSP-1 induced programmed cell death (PCD) in bovine aortic endothelial cells based on morphological changes, assessment of DNA fragmentation, and internucleosomal DNA cleavage. Intact TSP1 also induced DNA fragmentation (Guo N et al. 1997). The endothelial cell response was specific and not dependent on the activation of TGF-β (Guo N et al. 1997; Miao WM et al. 2001). TSP-1 induces endothelial cell apoptosis by involving interaction with the microvascular endothelial cell receptor CD36, which is modulated by hypoxia and by oncogenes (de Fraipont F et al. 2001). The in vivo and the induction of apoptosis by TSP-1 all required the sequential activation of CD36, p59fyn, caspase-3 like proteases and p38 mitogen-activated protein kinases (Jimenez B et al. 2000; Nor JE et al. 2000). Moreover, TSP-1 was significantly reduced in tumor-derived endothelial cells which display increased survival and angiogenic properties in respect to normal endothelial cells. Tumor-derived endothelial cells were shown to display a basal upregulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. TSP-1 production by tumor-derived endothelial cells was enhanced by the treatment with LY294002 and wortmannin (the PI3K inhibitors) and with rapamycin (the mammalian target of rapamycin (mTOR) inhibitor). The blockade of TSP-1 with an anti-TSP-1 antibody in negative dominant Akt tumor-derived endothelial cells restored their proangiogenic phenotype. The upregulation of the PI3K/Akt/mTOR

pathway is responsible for the inhibition of TSP-1 synthesis which is critical in determining the proangiogenic phenotype of TEC (Bussolati B et al. 2006). A recent report by Yamauchi et al. revealed a a novel antiangiogenic pathway of TSP-1 mediated by suppression of the cell cycle, different to the known mechanism of CD36-dependent endothelial cell apoptosis (Yamauchi M et al. 2007). The HUVEC, , TSP-1 induces little apoptosis of endothelial cells, which did not express CD36 at detectable levels; but causes cell-cycle arrest, increasing the amounts of p21(CIP/WAF-1) and unphosphorylated retinoblastoma (Rb) (Yamauchi M et al. 2007).

5.1.4 TSP-1 acts as "an angiogenic fence" during cervical carcinogenesis

In bladder cancer, the down-regulation of TSP-1 secretion is a key event in the switch from anti-angiogenic to an angiogenic phenotype, while VEGF seems to play little role (Campbell SC et al. 1998). Recent work has identified TSP-1 as an endogenous angiogenic inhibitor that plays an important role in the angiogenic switch in skin, prostate and bladder cancers (Campbell SC et al. 1998; Hawighorst T et al. 2001; Jin RJ et al. 2000). Wu et al. examined the spatial and temporal expression of TSP-1 in patients with preinvasive and invasive squamous cell carcinoma of the uterine cervix (Wu MP et al. 2004). Temporal relationship is that TSP-1 decreases significantly during the transition from LSIL to HSIL, which is concomitant with the increase of MVD counts. In spatial relationship, TSP-1 was mainly localized on basal cervical epithelial cells, and arrayed like a barrier, and was therefore proposed as the "TSP-1 fence". The temporal and spatial concordance of TSP-1 down-regulation and the emergence of angiogenesis which occurred in early phase of cervical carcinogenesis imply that the "TSP-1 fence" may act as an angiogenic barrier to inhibit angiogenesis. The disappearance of the

angiogenic barrier may induce a vigorous angiogenic response for tumor growth and perhaps tumor metastasis (Sheibani N and Frazier WA 1999).

The origin from where TSP-1 is produced is currently unknown, and two possible origins including tumor cells themselves and host cells (e.g. stromal, endothelial cells, etc) may be responsible for the production (Folkman J 1992). Previous data suggested that the basal epithelial cells contribute to the production of TSP-1 under physiological condition, and lose the ability to secrete TSP-1 during the transition from LSIL to HSIL (Wu MP et al. 2004). As for the TSP-1 expression and prognosis, Kodama et al. showed that TSP-1 mRNA expression was significantly lower in advanced-stage cervical cancer, and its expression is of value as a prognostic factor in cervical cancer (Kodama J et al. 2001). The inverse correlation between TSP and MVD expressions also indicates that decreased TSP expression may be associated with an angiogenic phenotype in this class of neoplasm.

5.1.5 TSP-1 may influence angiogenesis by changing the ECM

The disappearance of “TSP-1 fence” modulates the peri-cellular environment, and can potentially change the cell-matrix interactions associated with cell movement and further progression. The basement membrane (BM), a specialized form of ECM, has been recognized to be involved in the regulation of tumor angiogenesis (Kalluri R 2003). TSP-1 does not appear to contribute directly to the structural integrity of connective tissue elements. Instead, TSP-1 acts by modulating the activity and bioavailability of protease and growth factors and by interaction with cell-surface receptors (Bornstein P et al. 2000b; Stetler-Stevenson WG 1999). MMPs have been shown to play an active role in the neovascularization of tumors through their ability to degrade the ECM (Liotta LA et al. 1982; Zetter BR 1990).

Bergers et al. showed that the switch from vascular quiescence to angiogenesis involves MMP-9, which is up-regulated in angiogenic islets and tumors, rendering VEGF more available to its receptors (Bergers G et al. 2000). Notably, MMP-9 is negatively modulated by TSP-1. Thus, TSP-1 acts as a multifunctional modulator of angiogenesis by modulating through the activity and bioavailability of MMP-9 (Albo D et al. 2002; Qian X et al. 1997). How does TSP-1 modulate MMPs in cervical carcinogenesis deserves further studies.

5.1.6 Do TSP-1 or other angiogenic inhibitors play a physiologic gatekeeper role in cancer prevention

TSP-1 has been proposed to act as “an angiogenic fence” to inhibit angiogenesis that occurs during cervical carcinogenesis (Wu MP et al. 2004). The loss of the TSP-1 barrier in early cervical cancer lesions leads to more aggressive and more vascular cancer phenotype. In a related situation, elevated angiogenesis inhibitor endostatin level in conjunction with elevated VEGF are associated with either more aggressive or with metastasis or shortened survival in renal cell cancer, colon cancer, and soft tissue sarcoma (Feldman AL et al. 2001a; Feldman AL et al. 2002; Feldman AL et al. 2001b). These observations showed that the angiogenic inhibitors can be modulated as a result of changes in the tumor environment or in tumor disease burden (Feldman AL et al. 2000; Ozatli D et al. 1999). Although circulating angiogenic activators such as bFGF, VEGF and angiogenin have been evaluated not only as diagnostic and/or prognostic factors in cancer patients, little is known about the clinical significance of angiogenic inhibitors. Neither the source nor the mechanism of TSP-1 protein externalization has been clarified in detail (Kuroi K and Toi M 2001).

The cause-effect relationship of TSP-1 as a gatekeeper during cervical

carcinoma has not been clearly established. Although the levels of TSP-1 decreased with increasing malignancy potential of lesions, these determinations are only semi-quantitative and may be influenced by the degree of antibody reactivity and the samples variability (Libutti SK 2004). Meanwhile, the inverse relationship between TSP-1 staining and severity of tumor lesions may be influenced directly or indirectly by other processes, e.g angiogenic factors. Similarly, a cause-effect relationship has never been established between increasing MVD and decreasing TSP-1 levels (Libutti SK 2004). The prevention of blood vessel development appears to be the mechanism of action of many successful chemopreventive drugs of natural or synthetic origin, which is termed "angioprevention". The hypothesis that anti-angiogenesis is at the basis of tumor prevention also suggests that many anti-angiogenic drugs could be used for chemoprevention in higher risk populations or in early intervention. There is a growing body of experimental evidence that anti-angiogenic strategies will contribute to the future therapy of cancer (Bisacchi D et al. 2003). Further work for angiogenic inhibitors as therapeutic implications is to elucidate the causal-effect relationship between the change of these angiogenic inhibitors and tumor progression. Proof of such a relationship would provide a rationale for the use of angiogenic inhibitors as preventive agents in patients at high risk for developing cancer.

5.2 MVD and other modalities in evaluation of angiogenesis status

5.2.1 The usefulness of MVD in assessing angiogenesis

One often-quantified aspect of tumor vasculature is MVD. However, does MVD measurement reflect every aspect of angiogenesis remains controversial? The use of MVD measurement within isolated regions of high vessel concentration (i.e.,

hotspots) was reported by Weidner et al. in the early 1990s, as a significant and independent prognostic indicator in early-stage breast carcinoma (Weidner N et al. 1992). MVD is a measure of the number of vessels per high-power (microscope) field which means inter-capillary distance. Inter-capillary distances are determined at the local level by the net balance between angiogenic activators and inhibitors in each micro-environment; it does not reflect the angiogenic activity or angiogenic dependence of a tumor (Hlatky L et al. 2002). From this point, MVD may be a useful prognostic indicator; however, it may not be a good indicator to monitor anti-angiogenic treatment efficacy such as inclusion criteria or follow-up parameters (Hlatky L et al. 2002). Though, both vascularity measurements, ICD and MVD, provided independent prognostic information in multivariate analysis in cervical neoplasm. There was a significant correlation between tumor hypoxia and ICD but not MVD (Weber WA et al. 2001).

5.2.2 MVD used as a prognostic indicator in cervical neoplasm

MVD has been shown to serve as a prognostic indicator in cervical squamous cell carcinomas (Abulafia O et al. 1999; Bremer GL et al. 1996; Di Leo S et al. 1998). MVD together with depth of invasion, regional lymph node status, and vascular invasion, is a strong independent prognostic indicator for overall survival in patients with clinical stage IB cervical carcinoma (Dellas A et al. 1997). MVD may play a role in predicting recurrence and survival in patients with stage II squamous cell cancer (Cantu De Leon D et al. 2003). Nevertheless, two reports independently showed that high MVD predicts improved survival in cervical cancer patients treated with radiotherapy (Siracha E et al. 1994) or intra-arterial chemotherapy (Kohno Y et al. 1993). The vascular densities were significantly higher in the effective group than the non-effective group. Rutgers et al. reported

that increased angiogenesis in squamous cell carcinoma of the cervix is not associated with a worsened prognosis (Rutgers JL et al. 1995). Another report further addressed that MVD was not a significant prognostic factor, although it might associate with the presence of lymph node metastases and vascular space invasion of tumor cells (Graflund M et al. 2002).

It is possible that the discrepancies of results from MVD assay are due to the methodology being used. For example, different types of antibody, as well as whether MVD is assessed at the periphery or center of the tumor can influence its value as a prognostic indicator. Vieira et al. compared the performance of three different monoclonal antibodies, i.e. anti-CD31, anti-CD34 and BNH9 in the quantification of angiogenesis in cervical cancer. The results showed that MVD value estimated by anti-CD34 and BNH9 was significantly higher than that estimated by anti-CD31. Furthermore, the agreement between anti-CD34 and BNH9 to quantify microvessel density is higher than anti-CD31 with others (Vieira SC et al. 2004).

5.2.3 MVD may not be an indicator of anti-angiogenic treatment efficacy

It is a common misconception that anti-angiogenic treatment can be applied only to cancers with high MVD. However, MVD does not reflect the angiogenic activity or angiogenic dependence of a tumor (Hlatky L et al. 2002). Since virtually all tumors are angiogenesis-dependent, low MVDs within tumors are not sufficient criteria to exclude patients from treatment with angiogenesis inhibitors (Folkman J et al. 2000). Also, MVD is not predictive of tumor response under anti-angiogenic treatment and therefore is not useful for stratifying patients for clinical trials (Hlatky L et al. 2002). There was a considerable degree of heterogeneity in the intensity of angiogenesis within each tumor. In many situations, the MVD count of a tumor is

often lower than that of its corresponding normal tissue (Eberhard A et al. 2000). Also, rapid tumor growth does not imply high MVD, because tumor cells can remain viable at lower oxygen concentrations. In other words, they can exist at greater distances from the vasculature than can their normal-cell counterparts.

Individual tumors can make a wide variety of angiogenic activators, and the relative expressions of these factors can change over time. The net angiogenic influence of the tumor microenvironment should be thought of as the sum of the angiogenic activators and inhibitors that arise from both the tumor cells (Hahnfeldt P et al. 1999) and host tissues (Hlatky L et al. 1994). Tumor MVD count may not vary in accordance with the tissue or blood levels of any single angiogenic activator (Hlatky L et al. 2002). Anti-angiogenic therapy has an ultimate limitation to tumor size under angiogenic control, where opposing angiogenic stimuli come into dynamic balance (Hahnfeldt P et al. 1999). All tumor vessels are not equal in their ability to provide oxygen and nutrients to the tumor cells they support. Some can be ineffective. Thus, inhibition of ineffective vessels has little consequence in the reduction of tumor growth (Hlatky L et al. 2002). The lack of a parallel relationship between tumor size and tumor MVD points to the error in interpreting the lack of a decrease in MVD during therapy as a failure to inhibit vascularization. Thus, the efficacy of anti-angiogenic agents cannot be simply visualized by alterations in MVD during treatment period (Hlatky L et al. 2002).

5.2.4 Other models for angiogenesis study

Though MVD offers a convenient way for the evaluation of angiogenesis, it still has its limitations. Hypoxia my not be morphologically detectable and is more likely to be detected by functional imaging than by histological tissue assessment (Mayr NA et al. 1999). Cheng et al. developed a novel method to evaluate in vivo

angiogenesis in patients with cervical carcinoma. Using color Doppler ultrasound and a quantitative image processing, they found that intratumoral vascularity index (VI), but not resistant index (RI), showed better correlation with tumor stage, tumor size, depth of stromal invasion, lymphovascular emboli, and pelvic lymph node metastases. In addition, the in vivo indicator of angiogenic activity (VI) is well correlated with the conventional indicator of tumor angiogenic activity, MVD (Cheng WF et al. 1999). Recently, the combined use of three-dimensional (3D) imaging and power Doppler provides the possibility to assess the cervical cancer volume and quantify the power Doppler signal in the whole target organ, which is in contrast to two-dimensional (2D) ultrasound where information on vascularization and blood flow is restricted to one subjectively chosen 2D plane. We have demonstrated that 3D power Doppler ultrasound system is accurate in the measurement of cervical cancer volume and provides a global assessment of the vascular heterogeneity in cervical cancer (Hsu KF et al. 2004). Therefore, it can be used in the monitoring of response and vascular changes of tumors after anti-angiogenesis therapy. Tumor vascularity and oxygenation have long been implicated as important determinants of radiation therapy (Mayr NA et al. 1999), and tumor oxygenation levels can be measured by using a sterile polarographic needle electrode (Cooper RA et al. 2000). Alternatively, dynamic contrast-enhanced pattern of magnetic resonance imaging (MRI) are used to asses tissue microcirculation. Tumor perfusion imaging is non-invasive and has the potential to be easy to implement clinically, and reproducible for therapy monitoring (Hawighorst H 1999; Ueda T et al. 1999). The dynamic contrast MRI is based on the combination of rapid dynamic MRI techniques and bolus injection of gadolinium (Gd) contrast agent. In the first-pass (bolus injection) method, contrast enhancement patterns are used to assess tissue microcirculation (Ueda T et al.

1999). The equilibrium method is based on tracer-kinetic model to measure tissue perfusion (Hawighorst H 1999). There is reportedly a significant correlation between tumor oxygenation measured on pO_2 histograph and maximal enhancement of tumor in dynamic contrast-enhanced MRI. However, intra-tumor MVD and MRI parameters are not significantly correlated (Cooper RA et al. 2000). Results from Mayr et al. further suggested that MR microcirculation parameters do not always correlate with histo-morphometric parameters, while there is evidence that MR parameters predict patients' treatment outcome. It can be interpreted as that MR microcirculation assessment may reflect the "dynamic", instead of “static” single time point by histo-morphologic parameters, angiogenic and metabolic status of a tumor (Mayr NA et al. 1999).

Various in vivo and in vitro models have been developed to test the effects of angiogenic agonists or antagonists. In developing an animal model for angiogenesis study, Guedez et al. described the directed in vivo angiogenesis assay (DIVAA) to overcome the difficulties for quantifying the angiogenic response. The assay consists of subcutaneous implantation of semi-closed silicone cylinders (angio-reactors) into nude mice, and the results seemed reproducible and quantitative in dose-response analysis and was capable of identifying the effective doses of angiogenesis-modulating factors in vivo (Guedez L et al. 2003). Alternatively Matrigel-perfusion assay is widely used to measure angiogenesis. Matrigel, an extracellular matrix gel-like plug, contains angiogenic factors and/or experimental inhibitor is implanted into the skin of mice. The new blood vessels that grow into the plug can be quantified by measuring perfusion of hemoglobin or large fluorescently tagged molecules (such as intravenously administered dextran) into the plugs (Kerbel RS and Kamen BA 2004). Subsequently, Guidolin et al. described an automatic image analysis method to evaluate the angiostatic activity

by using the in vitro Matrigel assay. This method allows to establish several parameters, such as dimensional (area % covered by endothelial cells and the total length of the cellular network per field), topological (the number of meshes and the number of branching points per field), and fractal (fractal dimension, lacunarity) of the capillary-like network (Guidolin D et al. 2004). Their results indicate that both topological and fractal parameters allowed a characterization of the spatial texture generated by endothelial cells during in vitro angiogenesis, and it can be implemented in a computer program to facilitate calculation, and exhibit a wider dynamic range than the fractal ones (Guidolin D et al. 2004).

5.3 Anti-angiogenic therapy offers a paradigm shift for anti-cancer therapy

5.3.1 Tumor vasculature as a therapeutic target

There exist some limitations in conventional chemotherapy, e.g. tumor cells are easy to develop resistance to cytotoxic agents that cause DNA damage or disrupt DNA replication, a phenomenon related to their genomic instability after varying periods of sensitivity (Kerbel RS et al. 2001). Conventional chemotherapy scheduling are normally given on one or a few consecutive days, followed by 3 to 4-week periods of rest, to allow recovery of normal proliferating cells, mainly bone marrow progenitors. The applied clinical strategy involves multidrug regimens designed to kill as many tumor cells as possible by administering combined cytotoxic agents at the maximum tolerated dose (MTD). The goal is to obtain total eradication of the cancer cells (Gasparini G 2001). However, most solid neoplasms are the result of multiple genetic abnormalities and may contain heterogeneous subpopulations of cells with different cell kinetics, invasive, and metastatic properties (Fidler IJ and Ellis LM 2000).

There are more and more researches highlight the importance of tumor-host

interactions and of the surrounding microenvironment in tumor development, invasiveness, metastasis, and responsiveness to therapy. There are some advantages to regard tumor vasculature as the therapeutic targets. Tumor endothelia, as compared with tumor cells, are composed of more genetically stable cells. It is less likelihood of emergence of acquired chemo-resistance. There are fewer systemic side-effects and less toxicity. They offer a more feasibility of long-term administration and possibility of combination with other cytostatic and/or molecularly-targeted therapy (Gasparini G 2001). Furthermore, tumor endothelia are qualitatively distinct from normal endothelia at the molecular level, so they offer a specific and selective target (St Croix B et al. 2000). Systemic administration of inhibitors can easily target with no difficulty in reaching the concentration of drug needed (Gasparini G 2001). Tumor endothelia may proliferate under the stimulus of known growth factors, so therapeutic neutralization of endothelial cell growth factors can also be used as a therapeutic modality (Klement G et al. 2000). The proliferation and migration of tumor endothelia can be inhibited by naturally occurring angiogenesis inhibitors, e.g. endostatin, angiostatin, TSP-1, etc. (Browder T et al. 2000; O'Reilly MS et al. 1997). Recently, promising results have been reported in an adult phase I study of ABT-510, a peptide derivative of TSP-1. Total cessation of tumor growth was achieved by the combination therapy with a histone deacetylase inhibitor (valproic acid, VPA), while the microvascular density was significantly reduced as compared with controls or tumors treated with single agents in neuroblastoma xenografts. In addition, the number of structurally abnormal vessels was reduced, suggesting that these agents may "normalize" the tumor vasculature (Yang Q et al. 2007). Endothelial cells do not appear to acquire resistance to some antiangiogenic agents. It offer the possibility of re-inducing a response after interruption of therapy (Boehm T et al. 1997)..

5.3.2 Low-dose ‘metronic’ chemotherapy is anti-angiogenic

Surprisingly, cytotoxic chemotherapy is found to have anti-angiogenic effects, particularly when administered at low and frequent doses. This scheduling is more effective in targeting tumor endothelia than large single bolus doses followed by long rest period (Browder T et al. 2000). Conventional cytotoxic chemotherapeutic drugs were designed to treat cancer by directly killing or inhibiting the proliferation of rapidly dividing tumor cells. However, recent studies have highlighted the possibility that cytotoxic agents might reasonably be considered to have meaningful anti-angiogenic activity as a secondary mechanism (Miller KD et al. 2001). Browder et al. developed an alternative anti-angiogenic schedule for administration of cyclophosphamide that provided more sustained apoptosis of endothelial cells within the vascular bed of a tumor (Browder T et al. 2000). The use of chronically administering chemotherapeutic agents in a frequent, even daily, significantly low doses below the MTD, with no prolonged drug-free breaks is called “anti-angiogenic” or “metronomic” chemotherapy (Hanahan D et al. 2000). Evidences of this antiangiogenic activity of metronomic chemotherapy have been conducted in vivo mouse corneal model (O'Leary JJ et al. 1999), disc model (Presta M et al. 1999), chorio-allantoic membrane (CAM) (Presta M et al. 1999). The sustained and potent anti-angiogenic effects are based on targeting the endothelial cells of newly growing tumor blood vessels (Browder T et al. 2000; Klement G et al. 2000). The activated, differentiated endothelial cells, as well as circulating endothelial progenitor cells, are sensitive to low-dose chemotherapy (Bertolini F et al. 2003; Wang J et al. 2003).

The potential advantages of metronomic chemotherapeutic include: (i) it may significantly delay the onset of mutation-dependent mechanisms of acquired drug

resistance, because the target of the therapy is presumed to be the genetically stable, activated endothelial cells rather than the genetically unstable highly mutable cancer cells (Bocci G et al. 2003); (ii) it would facilitate the efficacy and durability of long-term integration of chemotherapy drugs with targeted antiangiogenic agents (Klement G et al. 2000); (iii) it can reduce or lose traditional toxic side effects due to the high sensitivity and selectivity characters (Bocci G et al. 2002; Wang J et al. 2003); (iv) it could induce an antiangiogenic effect by decreasing the mobilization and/or viability of circulating bone marrow-derived endothelial precursor cells (CEPs) (Lyden D et al. 2001).

5.3.3 TSP-1 plays the angio-inhibitory roles via a mediator of the low-dose metronomic chemotherapy, in addition to direct endothelial targeting

TSP-1 has been shown to possess potent angio-inhibitory effects in epithelial tumor development. Experiment showed that human squamous cell carcinoma cell lines, stably transfected to overexpress human TSP-1 exhibit inhibited tumor growth or completely abolished tumor formation in xenotransplants (Streit M et al. 1999). In addition to the direct targeting effects, Bocci et al. reported that protracted exposure of endothelial cells in vitro to low concentrations of cytotoxic chemotherapeutic drugs caused marked induction of gene and protein expression of TSP-1. Increases in circulating TSP-1 were also detected in the plasma of human tumor-bearing severe combined immunodeficient mice treated with metronomic low-dose cyclophosphamide (Bocci G et al. 2003). The induced angiogenic inhibitor can cause further growth arrest or apoptosis of endothelial cells. The induction of TSP-1, as a secondary mediator of the anti-angiogenic effects, in low-dose metronomic chemotherapy regimens can explain the ‘indirect’ pathway induce growth arrest or apoptosis of endothelial cells (Kerbel RS and

Kamen BA 2004). In summary, TSP-1 may exhibit its anti-angiogenic effects in two ways: one is its direct targeting on endothelia; the other one is that it acts as a mediator of metronomic chemotherapy.

5.4 Host stroma is an active participant during tumor progression

5.4.1 Myofibroblasts in host stroma stimulate cancer invasion

Interactions between cancer cells and the tissue microenvironment play an essential role in controlling tumor development and progression (Willhauck MJ et al. 2007). Maintenance of epithelial tissues needs the stroma. When the epithelium changes, the stroma inevitably follows. The changes in the stroma drive invasion and metastasis, the hallmarks of malignancy (De Wever O and Mareel M 2003). However, Kinzler and Volgestein proposed landscape defect to describe that changes in the epithelial compartment might be secondary to alterations of the stroma stroma reaction occurs before epithelial changes (Kinzler KW and Vogelstein B 1998). A subcutaneous syngeneic colon cancer (PROb) model revealed the invasive cancers were rich in myofibroblasts localized mainly at the front of invasion (Dimanche-Boitrel MT et al. 1994). However, the original PROb cells harvested from routine cell culture failed to invade when confronted in vitro with collagen, Matrigel or embryonic chick heart tissue. By contrast, freshly dissociated tumor cell suspensions, containing PROb cells and tumor-associated stromal cells, were invasive in all three in vitro assays (Dimanche-Boitrel MT et al. 1994). Therefore, epithelial compartment and stroma compartment might invade each other.

Fibroblasts are regarded as precursors of tumor-associated myofibroblasts, however, they are not the only ones. Other precursors to be considered comprise preexisting myofibroblasts, CD34-positive stem cells, smooth muscle cells and

pericytes. In cocultures of epithelial organoids, vascular smooth muscle cells and pericytes participated at the formation of myofibroblasts to a lesser extent than fibroblasts (Ronnov-Jessen L et al. 1995).

The interaction between the epithelial and mesenchymal compartments creates a local heterotypic 'invasion field' from which the metastatic cell emerges and disseminates. De Wever et al. showed the pro-invasive activity of myofibroblasts using human colon cancer cells from established cell lines with myofibroblasts compared with fibroblasts. Stromal cells isolated from surgical colon cancer fragments yielded myofibroblasts, while, stromal cells from normal mucosa at some distance from the tumor yield normal mucosa (De Wever O et al. 2004a). In 48-hour cultures, the colon cancer cells invaded into the collagen only when myofibroblasts but not fibroblasts were added to the collagen. The pro-invasive activity was found also with conditioned media from myofibroblast but not from fibroblast cultures instead of the cells themselves (De Wever O et al. 2004a). Ronnove-Jessen et al. yielded fibroblasts and myofibroblasts isolated primary cultures from normal breast tissue and from carcinomas, respectively (Ronnov-Jessen L et al. 1992). Breast cancer cells formed smooth-edged spheres when alone inside collagen, however they invaded into the collagen when cocultured with myofibroblasts (Ronnov-Jessen L et al. 1995). This illustrates that the source of the fibroblasts and the coculture conditions are crucial for the interaction with cancer cells (Kunz-Schughart LA et al. 2001).

5.4.2 Myofibroblasts themselves are invasive

Myofibroblasts are present in the stroma of many malignant tumors and they are frequently localized at the front of invasion (De Wever O and Mareel M 2003). That myofibroblasts may participate at the transition from the noninvasive toward

the invasive phenotype is compatible with their appearance in benign lesions that have a high risk of progression toward invasive cancer. In CIN (cervical intraepithelial neoplasia), α-SMA-positive stromal cells were considered as a sign of imminent invasion (Cintorino M et al. 1991). Moreover, the presence of myofibroblasts has been positively correlated with poor prognosis (Seemayer TA et al. 1979).

Experimental and clinical observations indicate that myofibroblasts produce pro-invasive signals. N-Cadherin and its soluble form act as invasion-promoters. N-Cadherin is expressed in invasive cancer cells and in host cells such as myofibroblasts, neurons, smooth muscle cells, and endothelial cells. N-Cadherin-dependent heterotypic contacts may promote matrix invasion, perineural invasion, muscular invasion, and transendothelial migration; the extracellular, the juxtamembrane and the beta-catenin binding domain of N-cadherin are implicated in positive invasion signalling pathways (De Wever O et al. 2004a).

Myofibroblasts invade the tumor site and this invasion may proceed angiogenic invasion. Fukumura et al. used solid tumor implantation in transgenic mice expressing GFP under the control of the VEGF promoter leads to induction of VEGF promoter activity in myofibroblast-like cells. Subsequently, GFP-positive myofibroblast-like cells invade the whole tumor site (Fukumura D et al. 1998). During avascular growth of developing hepatic metastases, myofibroblast-like cells are already present, before endothelial cell recruitment (Olaso E et al. 2003). De Wever et al. studied the invasion mechanism in collagen type I of myofibroblasts treated with colon cancer cell-derived TGF-β1. The experiments demonstrate that TGF-β stimulates migration on solid substrate and invasion into collagen of myofibroblasts and that N-cadherin is a necessary element in the pro-invasive loop

(De Wever O et al. 2004b). N-cadherin activity was implicated in TGF-β stimulated invasion.

5.5 Factors acting on fibroblast trans-differentiation and activation

5.5.1 Factors that enhance fibroblasts activation and transdifferentiation

The main precursors of myofibroblasts are fibroblasts. There are many factors which can enhance Factors that enhance fibroblasts activation and transdifferentiation, e.g. TGF-β, p311, Unc 119, etc.

TGF-β is a potent inhibitor of epithelial cell growth and migration (Annes JP et al. 2003). On the other side, it can stimulate migration, proliferation and contractility of mesenchymal cells, and increases the production of typical extracellular-matrix components. TGF-β is one of the candidate signals from the cancer cells converted the fibroblasts into myofibroblast which in their turn made the cancer cells invasive (Desmouliere A et al. 1993; Ronnov-Jessen L and Petersen OW 1993; Ronnov-Jessen L et al. 1992). Cancer cell-derived TGF-β is able to directly transdifferentiate fibroblasts to myofibroblasts, characterized by morphological changes and up-regulation of α-SMA in vitro and in vivo (Desmouliere A et al. 1993; Ronnov-Jessen L and Petersen OW 1993; Tuxhorn JA et al. 2002b). The in vitro findings that TGF-β induces myofibroblast transdifferentiation in cancer were confirmed in prostate- and colon-cancer mice models (Tuxhorn JA et al. 2002a; Tuxhorn JA et al. 2002b). Moreover, TGF-β causes accumulation of N-cadherin at the tip of myofibroblast filopodia which was implicated in myofibroblast invasion (De Wever O et al. 2004b). TGF-β causes cancer progression through paracrine and autocrine effects (De Wever O and Mareel M 2003). Paracrine effects of TGF-β implicate stimulation of angiogenesis, escape from immuno-surveillance and recruitment of myofibroblasts. Autocrine effects of TGF-β in cancer cells with a

functional TGF-β receptor complex may be caused by a convergence between TGF-β signalling and beta-catenin or activating Ras mutations (De Wever O and Mareel M 2003). Fibroblasts, migrating from vascular structures in vitro were converted to myofibroblasts by breast carcinoma cells MCF-7 or their conditioned medium (Ronnov-Jessen L et al. 1992). In resting fibroblast cultures deprived of serum, α-SMA was induced without concurrent cell proliferation by addition of TGF-β or conditioned medium of MCF-7 cell cultures. The activity of the conditioned medium was neutralized by antibody against TGF-β (Ronnov-Jessen L and Petersen OW 1993). In 3-D cultures cancer cells and TGF-β induced α-SMA but the response was more variable than in 2-D cultures on solid substrate (Kunz-Schughart LA et al. 2003). With human colon fibroblasts, the induction of α-SMA was dependent upon the time of treatment and the concentration of TGF-β the α-SMA-inducing activity of conditioned media from colon cancer cell cultures was neutralized by an antibody against TGF-β (De Wever O et al. 2004a).

TSP-1 is one of the key regulators of TGF-β (Crawford SE et al. 1998). TSP-1 has been reported to function as an activator of TGF-β and this activity has been mapped to the type-1 repeats of TSP-1 (TSRs) (Crawford SE et al. 1998; Kawataki T et al. 2000). The activation of TGF-β by TSP-1 can either inhibit (Miyanaga K et al. 2002) or increase (Kawataki T et al. 2000) malignancy in different cell types. The complexity and duality of function of TSP-1 and TGF-β may be due to the ability to suppress tumor cell proliferation at early stages, and to enhance tumor invasion and metastasis by affecting the host stroma in later stages (Radisky DC and Bissell MJ 2004). Therefore the mechanisms supporting TGF-β-mediated inhibition of tumor growth and tumor angiogenesis are complex and vary with the type of tumor.

TSP-1, in our model, inhibits the migration, and invasive ability of

myofibroblasts, but not α-SMA expression. TSP-1 may inhibit the myofibroblasts activity in a TGF-β-irrelevant pathway (Wu MP et al. 2008). The relationships between TSP-1 and TGF-β in tumor growth need to be further investigated in a variety of tumor in order to determine important common signaling pathways for the regulation of tumor growth (Ren B et al. 2006). Moreover, the interaction between TGF-β and TSP-1 and the final biologic effects on tumor progression is still unclear. The underlying mechanisms that lead to the activation of storma and, more precisely, to the conversion and/or recruitment of fibroblasts that exhibit myofibroblastic phenotype need further exploration (Micke P and Ostman A 2004).

P311, an intracellular protein involved in cell migration, is found in human wound myofibroblast precursors (proto-myofibroblasts) and myofibroblasts. By binding to the TGF-β1 latency associated protein (LAP), P311 induced NIH 3T3 cells to transform into non-fibrogenic myofibroblasts characterized by lack of TGF-β1 production (Shi J et al. 2006). Meanwhile, P311-induced myofibroblasts migrate in an ameboid, which is characterized by lack of focal adhesions and stress fibers, rather than a mesenchymal pattern. Ameboid migration is also characterized by absence of integrins and MMPs clustering/activation and changes in small GTPases activity, all leading to increased cell motility. TGF-beta1, the major stimulus of collagen production during wound repair, also reversed the ameboid phenotype to mesenchymal. P311 promotes a motile proto-myofibroblast and myofibroblast phenotype specifically adapted to rapidly populate the initial wound matrix (Shi J et al. 2006).

Unc119 is an adaptor protein that is involved in the development of the vertebrate nervous system. Unc119 was found to stimulate the induction of α-SMA and myofibroblast differentiation by TGF-β in human lung fibroblasts (Vepachedu R et al. 2007). Unc119 increases the kinase activity of Fyn and associates with it in

coprecipitation and colocalization studies. Phosphorylation and activation of Fyn in response to TGF-β and platelet-derived growth factor is delayed in Unc119-deficient cells. This delay translates into suppressed cell proliferation. In Src family kinase-deficient (SYF) cells, Unc119 knockdown does not affect cell proliferation. Unc119 overexpression does not stimulateα-SMA in SYF cells and this defect is restored upon reconstitution with Fyn indicating that Unc119 stimulation of α-SMA requires at least Fyn. Unc119 overexpression stimulated p38, but not JNK, phosphorylation. Blocking p38 MAPK resulted in reducedα-SMA expression by Unc119 suggesting that the p38 pathway regulates Unc119-induced myofibroblast differentiation. Unc119 stimulates the production of TGF-β and IL-6, known inducers of myofibroblast differentiation. The results suggest that Unc119 plays an important role in fibrotic processes through myofibroblast differentiation (Vepachedu R et al. 2007).

5.5.2 Factors that inhibit fibroblasts activation and transdifferentiation

Reversine, a 2,6-disubstituted purine, was discovered that induces lineage reversal of C2C12 myoblast cells to become multipotent progenitor cells which can redifferentiate into osteoblasts and adipocytes (Chen S et al. 2004). Moreover, reversine is active in multiple cell types, including 3T3E1 osteoblasts and human primary skeletal myoblasts. Biochemical and cellular experiments suggest that reversine functions as a dual inhibitor of nonmuscle myosin II heavy chain and MEK1, and that both activities are required for reversine's effect (Chen S et al. 2007). This and other such molecules are likely to provide new insights into the molecular mechanisms that control cellular dedifferentiation

Adenylyl cyclase agoinst was found to inhibit cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl

cyclase. A 24-h incubation of cardiac fibroblast with TGF-βbeta or angiotensin II increased α-SMA expression, which was inhibited by the adenylyl cyclase agoinst forskolin and a cAMP analog that activates protein kinase A. Treatment with forskolin blunted serum-, TGF-β, and angiotensin II-stimulated collagen synthesis. Thus, adenylyl cyclase stimulation blunts collagen synthesis and, in parallel, the transformation of adult rat cardiac fibroblast to myofibroblasts. (Swaney JS et al. 2005)

Scaffold. Willhauck *et al.* report that stromal modulation induced by a biodegradable meshwork (Hyalograft 3D) inhibited tumor vascularization and invasion of the locally invasive low-grade malignant human HaCaT-ras II-4 keratinocytes in a surface xenotransplantation assay (Willhauck MJ et al. 2007). The scaffold caused formation of an active granulation tissue that shifted to a fibrotic-type connective tissue with accumulation of myofibroblasts and collagen bundles (De Wever O et al. 2004a). Most importantly, in transplants with scaffolds, the epithelial-stromal border was normalized developing an ultrastructurally complete basement membrane (BM) including hemidesmosomes. The observed reversion of the tumor phenotype was not due to decreased tumor cell proliferation but correlated with normalization of epidermal differentiation, condensation of ECM and reduction of peritumoral protease activity Furthermore, inhibited invasion was paralleled by eliminated tumor vascularization (Willhauck MJ et al. 2007). This was substantiated by a diminished endothelial VEGF-receptor (VEGFR) expression and, in turn, by a concomitant increase in TSP-1 and endostatin, known to impair angiogenesis. This clearly demonstrates that the scaffold-modulated connective tissue not only blocks tumor invasion but reverts the tumor phenotype (Willhauck MJ et al. 2007).

SPARC is a matricellular glycoprotein that is capable of inhibiting the growth of

several different types of cancer by enhancing tumor stroma formation and prevents fibroblast activation (Chlenski A et al. 2007b). SPARC was noted to impair the growth of xenografts comprised of 293 cells. In addition to enhancing stroma formation, SPARC prevents fibroblast activation in 293 xenografts, suggesting that the anti-cancer effects of SPARC may be due, at least in part, to the formation of tumor stroma that is not supportive of tumor growth. In vitro, 3T3 fibroblasts cocultured with SPARC-transfected 293 cells remain negative for α-SMA, whereas wild-type 293 cells induce fibroblast activation. Moreover, activation of 3T3 cells and primary fibroblasts by TGF-β is blocked by SPARC treatment. Therefore, in addition to blocking angiogenesis, SPARC may inhibit tumor growth by promoting the assembly of stroma that is non-permissive for tumor progression (Chlenski A et al. 2007b). SPARC has deadhesive effects on cultured cells and have been characterized as antiproliferative factors in some cellular contexts (Framson PE and Sage EH 2004).

Although, TSP-1 has significant effects on stroma marker expression, myofibroblast migration and Matrigel invasion, however, it has little effect on the transdifferentiation of quiescent fibroblasts into myofibroblasts, characterized by the overexpression of α-SMA, either by transfection or direct addition in vitro. It is different from other study by Chlenski A, et al. that SPARC prevent fibroblast activation by downregulating of α-SMA in myofibroblasts, as well as enhancing normal fibroblast migration ability and eventually increasing unresponsive tumor stroma formation that is not supportive of tumor growth (Chlenski A et al. 2007a). In contrast, TSP-1 inhibits myofibroblast migration, but limited effect on α-SMA expression. Thus, we speculate that the alteration of α-SMA expression may not be necessarily in TSP-1-induced decrease stroma reaction in all kinds of cancer.

Chapter 6 Conclusion and implications

6.1 Tumor angiogenesis can be used as a therapeutic target

Angiogenesis plays a critical role in the growth and metastasis of tumors. TSP-1 is a potent angiogenesis inhibitor, and the down-regulation of TSP-1 has been suggested to alter tumor growth by modulating angiogenesis in a variety of tumor types. Expression of TSP-1 is up-regulated by the tumor suppressor gene, p53, and down-regulated by oncogenes such as Myc and Ras. TSP-1 inhibits angiogenesis by inhibiting endothelial cell migration and proliferation and by inducing apoptosis. An understanding of the molecular basis of TSP-1-mediated inhibition of angiogenesis and tumor progression will aid in the development of novel therapeutics for the treatment of cancer (Ren B et al. 2006).

Anti-angiogenesis effect of metronomic scheduling has cause the paradigm shift from conventional dose-density chemotherapy to metronomic scheduling. Significant anti-angiogenic and anti-tumor effects are unlikely to be achieved in the clinical setting with a single chemotherapeutic agent at metronomic doses. Pioneering studies by Kakeji and Teicher showed the potentiality or synergism when angiogenic inhibitors were combined with standard schedules of certain cytotoxic agents (Kakeji Y and Teicher BA 1997). The efficacy of metronomic chemotherapy can be significantly increased when administered in combination with anti-angiogenic drugs, such as antibodies against VEGF or VEGFR2 (Klement G et al. 2000). Browder et al. have used cyclophospamide and TNP-470 to reveal the antiangiogenic capability of cancer chemotherapy to eradicate chemo-resistant tumors (Browder T et al. 2000). The proposed rationale for the beneficial effect of such combinations was based on their ability to target both the parenchymal and stromal components of neoplasia (Gasparini G and Harris AL 1994). Tumor endothelial targeting and tumor cell targeting should not be thought

of as mutually exclusive. Antiangiogenic therapy can be added to chemotherapy, radiotherapy, immunotherapy, gene therapy or any other traditionally cancer cell-directed modality (Folkman J et al. 2000).

A “new” paradigm for dosing of chemotherapy is theoretically based on targeting the vascular system of the tumor rather than the tumor itself by using low-dose continuous chemotherapy (Kamen BA et al. 2000). As angiogenic inhibitors become more widely used in anti-cancer therapy. It will be important to reduce the harsh side-effects and risk of drug resistance of conventional chemotherapy (Folkman J 2003b). The paradigm of anti-cancer treatment may shift from cancer-centered to epigenic, endothelia-centered therapy (Folkman J et al. 2000). The focus is not on the gene alterations within tumor cells, but on physiological constraints imposed on the overall tumor system (Kamen BA et al. 2000). Conventional anticancer therapy use cytotoxic drugs to kill tumor cells and to achieve the goal of cancer eradication. Homogeneous groups of patients are treated with the same chemotherapeutic schedule (Gasparini G 2001). While, individual tailored therapy use metronomic therapy and/or molecular target treatments (e.g. angiogenic inhibitors) to target the molecular abnormalities involved in tumor and endothelia to achieve the goal of cancer control in each patient (Gasparini G 2001). The final goals of anti-angiogenesis therapy are not to cure cancer, instead, it is to make cancer more survivable and controllable, and eventually to be converted to a chronic manageable disease, like heart disease or diabetes, especially in conjunction with radiation, chemotherapy and other treatment (Ezzell C 1998).

6.2 Stroma reaction can be used as a therapeutic target

In viewing the fact that stromal therapy has recently emerged as a strategy for

cancer treatment. The clinical studies have shown the effective treatment for many types of cancer (Meyerhardt JA and Mayer RJ 2005). TSP-1 is potentially an example of a molecule that is capable of altering the composition of the tumor stroma, in addition to inhibiting angiogenesis. By inhibiting vascular cells, inhibiting the recruitment and preventing fibroblast activation, TSP-1 may play a role in 'normalizing' the tumor stroma and creating a microenvironment that is on-permissive for tumor growth (Wu MP et al. 2008). Myofibroblasts, the major component of stroma, plays a major role in tumor progression (Desmouliere A et al. 2004). However, the definite role of TSP-1 in myofibroblasts still remains to be determined. Both the upregulation (Castle VP et al. 1993) and repression (Sheibani N and Frazier WA 1996) of TSP-1 have been reported to cause NIH3T3 into the malignant phenotype. Our data revealed the inhibitory effect of TSP-1 in myofibroblast migration and invasion, like those in the wound healing processes (Streit M et al. 2000). Integrin-ERK1/2 pathway is related to migratory ability and spindle-like morphological alterations, as evidenced by actin polyperization and focal adhesion-complex aggregation (Chen PS et al. 2007). It has been reported that TSP-1, cooperatively with integrin, affects focal adhesion kinase-dependent signaling to induces focal adhesion disassembly and spreading (Orr AW et al. 2004; Sipes JM et al. 1999). Further work on the biologic mechanisms of myofibroblast recruitment is needed, before myofibroblasts could be used as a therapeutic target or biologic tracer of cancer cells.

6.3 Conclusion

After the clinical observation that TSP-1 plays an important role in the regulation of angiogenic switch during cervical carcinogenesis (Wu MP et al. 2004), we further elucidate the cause-effect relationship between TSP-1 and tumor-stroma

interaction (Wu MP et al. 2008). The in vivo xenotransplant animal model elucidates the anti-angiogenic effect of TSP-1 and the ability to modulate tumor-stroma interaction morphologically and functionally. Furthermore, TSP-1 reduces the stroma reaction by changing the behaviors of stromal cells, i.e. myofibroblasts in multicellular Matrigel co-culture model. Much work on the biologic mechanisms of fibroblast recruitment is needed, but the present study may offer the evidence that TSP-1 can change the tumor-stroma reaction during tumor progression by acting on the activity of myofibroblasts (Wu MP et al. 2008). It suggests that fibroblasts could be used as a biologic tracer of cancer cells and could act as an efficient drug delivery system to prevent or slow the local growth of cancer cells. Elucidation of the mechanism by which fibroblasts are recruited into cancer stroma could lead to new insights into not only the mechanisms of cancer progression but also strategies for cancer treatment. A better understanding of stromal contributions to cancer progression will likely increase our awareness of the importance of the combinatorial signals that support and promote growth, dedifferentiation, invasion, and ectopic survival and eventually result in the identification of new therapeutics targeting the stroma (De Wever O and Mareel M 2003).

References

1. Abulafia O, Triest WE, Sherer DM. Angiogenesis in squamous cell carcinoma in situ and microinvasive carcinoma of the uterine cervix. Obstet Gynecol 88(6):927-932;1996.
2. Abulafia O, Triest WE, Sherer DM. Angiogenesis in malignancies of the female genital tract. Gynecol Oncol 72(2):220-231;1999.
3. Achilles EG, Fernandez A, Allred EN, Kisker O, Udagawa T, Beecken WD*, et al.* Heterogeneity of angiogenic activity in a human liposarcoma: a proposed mechanism for "no take" of human tumors in mice. J Natl Cancer Inst 93(14):1075-1081;2001.
4. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM*, et al.* A rapid in vitro assay for quantitating the invasive potential of tumor cells. Cancer Res 47(12):3239-3245;1987.
5. Albo D, Shinohara T, Tuszynski GP. Up-regulation of matrix metalloproteinase 9 by thrombospondin 1 in gastric cancer. J Surg Res 108(1):51-60;2002.
6. Alves F, Borchers U, Padge B, Augustin H, Nebendahl K, Kloppel G*, et al.* Inhibitory effect of a matrix metalloproteinase inhibitor on growth and spread of human pancreatic ductal adenocarcinoma evaluated in an orthotopic severe combined immunodeficient (SCID) mouse model. Cancer Lett 165(2):161-170;2001.
7. Amatangelo MD, Bassi DE, Klein-Szanto AJ, Cukierman E. Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts. Am J Pathol 167(2):475-488;2005.
8. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. J Cell Sci 116(Pt 2):217-224;2003.
9. Asch AS, Tepler J, Silbiger S, Nachman RL. Cellular attachment to thrombospondin. Cooperative interactions between receptor systems. J Biol

Chem 266(3):1740-1745;1991.

10. Baenziger NL, Brodie GN, Majerus PW. A thrombin-sensitive protein of human platelet membranes. Proc Natl Acad Sci U S A 68(1):240-243;1971.
11. Barcellos-Hoff MH and Ravani SA. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. Cancer Res 60(5):1254-1260;2000.
12. Bein K and Simons M. Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. J Biol Chem 275(41):32167-32173;2000.
13. Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K*, et al.* Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat Cell Biol 2(10):737-744;2000.
14. Bertin N, Clezardin P, Kubiak R, Frappart L. Thrombospondin-1 and -2 messenger RNA expression in normal, benign, and neoplastic human breast tissues: correlation with prognostic factors, tumor angiogenesis, and fibroblastic desmoplasia. Cancer Res 57(3):396-399;1997.
15. Bertolini F, Paul S, Mancuso P, Monestiroli S, Gobbi A, Shaked Y*, et al.* Maximum tolerable dose and low-dose metronomic chemotherapy have opposite effects on the mobilization and viability of circulating endothelial progenitor cells. Cancer Res 63(15):4342-4346;2003.
16. Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S*, et al.* TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 303(5659):848-851;2004.
17. Bierie B and Moses HL. TGF-beta and cancer. Cytokine Growth Factor Rev 17(1-2):29-40;2006.
18. Bisacchi D, Benelli R, Vanzetto C, Ferrari N, Tosetti F, Albini A. Anti-angiogenesis and angioprevention: mechanisms, problems and perspectives. Cancer Detect Prev 27(3):229-238;2003.

19. Bissell MJ and Radisky D. Putting tumours in context. Nat Rev Cancer 1(1):46-54;2001.
20. Bocci G, Francia G, Man S, Lawler J, Kerbel RS. Thrombospondin 1, a mediator of the antiangiogenic effects of low-dose metronomic chemotherapy. Proc Natl Acad Sci U S A 100(22):12917-12922;2003.
21. Bocci G, Nicolaou KC, Kerbel RS. Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. Cancer Res 62(23):6938-6943;2002.
22. Boehm T, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. Nature 390(6658):404-407;1997.
23. Bornstein P. Thrombospondins: structure and regulation of expression. FASEB J 6(14):3290-3299;1992.
24. Bornstein P. Matricellular proteins: an overview. Matrix Biol 19(7):555-556;2000.
25. Bornstein P. Thrombospondins as matricellular modulators of cell function. J Clin Invest 107(8):929-934;2001.
26. Bornstein P, Armstrong LC, Hankenson KD, Kyriakides TR, Yang Z. Thrombospondin 2, a matricellular protein with diverse functions. Matrix Biol 19(7):557-568;2000a.
27. Bornstein P, Kyriakides TR, Yang Z, Armstrong LC, Birk DE. Thrombospondin 2 modulates collagen fibrillogenesis and angiogenesis. J Investig Dermatol Symp Proc 5(1):61-66;2000b.
28. Bornstein P and Sage EH. Matricellular proteins: extracellular modulators of cell function. Curr Opin Cell Biol 14(5):608-616;2002.
29. Bouck N. Tumor angiogenesis: the role of oncogenes and tumor suppressor genes. Cancer Cells 2(6):179-185;1990.

30. Bremer GL, Tiebosch AT, van der Putten HW, Schouten HJ, de Haan J, Arends JW. Tumor angiogenesis: an independent prognostic parameter in cervical cancer. Am J Obstet Gynecol 174(1 Pt 1):126-131;1996.
31. Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG*, et al.* Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. Cell 85(5):683-693;1996.
32. Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS*, et al.* Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res 60(7):1878-1886;2000.
33. Brown EJ and Frazier WA. Integrin-associated protein (CD47) and its ligands. Trends Cell Biol 11(3):130-135;2001.
34. Bunone G, Vigneri P, Mariani L, Buto S, Collini P, Pilotti S*, et al.* Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. Am J Pathol 155(6):1967-1976;1999.
35. Bussolati B, Assenzio B, Deregibus MC, Camussi G. The proangiogenic phenotype of human tumor-derived endothelial cells depends on thrombospondin-1 downregulation via phosphatidylinositol 3-kinase/Akt pathway. J Mol Med 84(10):852-863;2006.
36. Bussolino F, Mantovani A, Persico G. Molecular mechanisms of blood vessel formation. Trends Biochem Sci 22(7):251-256;1997.
37. Calzada MJ, Zhou L, Sipes JM, Zhang J, Krutzsch HC, Iruela-Arispe ML*, et al.* Alpha4beta1 integrin mediates selective endothelial cell responses to thrombospondins 1 and 2 in vitro and modulates angiogenesis in vivo. Circ Res 94(4):462-470;2004.
38. Campbell SC, Volpert OV, Ivanovich M, Bouck NP. Molecular mediators of angiogenesis in bladder cancer. Cancer Res 58(6):1298-1304;1998.

39. Cantu De Leon D, Lopez-Graniel C, Frias Mendivil M, Chanona Vilchis G, Gomez C, De La Garza Salazar J. Significance of microvascular density (MVD) in cervical cancer recurrence. Int J Gynecol Cancer 13(6):856-862;2003.
40. Carmeliet P and Jain RK. Angiogenesis in cancer and other diseases. Nature 407(6801):249-257;2000.
41. Castle V, Varani J, Fligiel S, Prochownik EV, Dixit V. Antisense-mediated reduction in thrombospondin reverses the malignant phenotype of a human squamous carcinoma. J Clin Invest 87(6):1883-1888;1991.
42. Castle VP, Ou X, O'Rourke K, Dixit VM. High level thrombospondin 1 expression in two NIH 3T3 cloned lines confers serum- and anchorage-independent growth. J Biol Chem 268(4):2899-2903;1993.
43. Chen H, Herndon ME, Lawler J. The cell biology of thrombospondin-1. Matrix Biol 19(7):597-614;2000.
44. Chen PS, Wang MY, Wu SN, Su JL, Hong CC, Chuang SE*, et al.* CTGF enhances the motility of breast cancer cells via an integrin-alphavbeta3-ERK1/2-dependent S100A4-upregulated pathway. J Cell Sci 120(Pt 12):2053-2065;2007.
45. Chen S, Takanashi S, Zhang Q, Xiong W, Zhu S, Peters EC*, et al.* Reversine increases the plasticity of lineage-committed mammalian cells. Proc Natl Acad Sci U S A 104(25):10482-10487;2007.
46. Chen S, Zhang Q, Wu X, Schultz PG, Ding S. Dedifferentiation of lineage-committed cells by a small molecule. J Am Chem Soc 126(2):410-411;2004.
47. Cheng WF, Chen CA, Lee CN, Wei LH, Hsieh FJ, Hsieh CY. Vascular endothelial growth factor and prognosis of cervical carcinoma. Obstet Gynecol 96(5 Pt 1):721-726;2000.
48. Cheng WF, Lee CN, Chu JS, Chen CA, Chen TM, Shau WY*, et al.* Vascularity index as a novel parameter for the in vivo assessment of angiogenesis in

patients with cervical carcinoma. Cancer 85(3):651-657;1999.

49. Chlenski A, Guerrero LJ, Yang Q, Tian Y, Peddinti R, Salwen HR*, et al.* SPARC enhances tumor stroma formation and prevents fibroblast activation. Oncogene;2007a.
50. Chlenski A, Guerrero LJ, Yang Q, Tian Y, Peddinti R, Salwen HR*, et al.* SPARC enhances tumor stroma formation and prevents fibroblast activation. Oncogene 26(31):4513-4522;2007b.
51. Chou CY, Shen MR, Wu SN. Volume-sensitive chloride channels associated with human cervical carcinogenesis. Cancer Res 55(24):6077-6083;1995.
52. Chou CY, Wang ST, Kuo HC, Tzeng CC, Yao BL. Serum level of squamous cell carcinoma antigen and tumor size are useful to identify preoperatively patients at high risk of cervical cancer. Cancer 74(9):2497-2501;1994.
53. Cintorino M, Bellizzi de Marco E, Leoncini P, Tripodi SA, Xu LJ, Sappino AP*, et al.* Expression of alpha-smooth-muscle actin in stromal cells of the uterine cervix during epithelial neoplastic changes. Int J Cancer 47(6):843-846;1991.
54. Clezardin P, Frappart L, Clerget M, Pechoux C, Delmas PD. Expression of thrombospondin (TSP1) and its receptors (CD36 and CD51) in normal, hyperplastic, and neoplastic human breast. Cancer Res 53(6):1421-1430;1993.
55. Colorado PC, Torre A, Kamphaus G, Maeshima Y, Hopfer H, Takahashi K*, et al.* Anti-angiogenic cues from vascular basement membrane collagen. Cancer Res 60(9):2520-2526;2000.
56. Cooper RA, Carrington BM, Loncaster JA, Todd SM, Davidson SE, Logue JP*, et al.* Tumour oxygenation levels correlate with dynamic contrast-enhanced magnetic resonance imaging parameters in carcinoma of the cervix. Radiother Oncol 57(1):53-59;2000.
57. Coussens LM and Werb Z. Inflammation and cancer. Nature 420(6917):860-867;2002.

58. Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO*, et al.* Thrombospondin-1 is a major activator of TGF-beta1 in vivo. Cell 93(7):1159-1170;1998.
59. Dameron KM, Volpert OV, Tainsky MA, Bouck N. Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 265(5178):1582-1584;1994.
60. Dawson DW, Pearce SF, Zhong R, Silverstein RL, Frazier WA, Bouck NP. CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells. J Cell Biol 138(3):707-717;1997.
61. de Fraipont F, El Atifi M, Gicquel C, Bertagna X, Chambaz EM, Feige JJ. Expression of the angiogenesis markers vascular endothelial growth factor-A, thrombospondin-1, and platelet-derived endothelial cell growth factor in human sporadic adrenocortical tumors: correlation with genotypic alterations. J Clin Endocrinol Metab 85(12):4734-4741;2000.
62. de Fraipont F, Nicholson AC, Feige JJ, Van Meir EG. Thrombospondins and tumor angiogenesis. Trends Mol Med 7(9):401-407;2001.
63. De Wever O and Mareel M. Role of tissue stroma in cancer cell invasion. J Pathol 200(4):429-447;2003.
64. De Wever O, Nguyen QD, Van Hoorde L, Bracke M, Bruyneel E, Gespach C*, et al.* Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. Faseb J 18(9):1016-1018;2004a.
65. De Wever O, Westbroek W, Verloes A, Bloemen N, Bracke M, Gespach C*, et al.* Critical role of N-cadherin in myofibroblast invasion and migration in vitro stimulated by colon-cancer-cell-derived TGF-beta or wounding. J Cell Sci 117(Pt 20):4691-4703;2004b.
66. Dellas A, Moch H, Schultheiss E, Feichter G, Almendral AC, Gudat F*, et al.* Angiogenesis in cervical neoplasia: microvessel quantitation in precancerous

lesions and invasive carcinomas with clinicopathological correlations. Gynecol Oncol 67(1):27-33;1997.

67. Desmouliere A, Geinoz A, Gabbiani F, Gabbiani G. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J Cell Biol 122(1):103-111;1993.
68. Desmouliere A, Guyot C, Gabbiani G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int J Dev Biol 48(5-6):509-517;2004.
69. Di Leo S, Caschetto S, Garozzo G, Nuciforo G, Cassaro N, Meli MT*, et al.* Angiogenesis as a prognostic factor in cervical carcinoma. Eur J Gynaecol Oncol 19(2):158-162;1998.
70. Dimanche-Boitrel MT, Vakaet L, Jr., Pujuguet P, Chauffert B, Martin MS, Hammann A*, et al.* In vivo and in vitro invasiveness of a rat colon-cancer cell line maintaining E-cadherin expression: an enhancing role of tumor-associated myofibroblasts. Int J Cancer 56(4):512-521;1994.
71. Dixit VM, Hennessy SW, Grant GA, Rotwein P, Frazier WA. Characterization of a cDNA encoding the heparin and collagen binding domains of human thrombospondin. Proc Natl Acad Sci U S A 83(15):5449-5453;1986.
72. Dobbs SP, Hewett PW, Johnson IR, Carmichael J, Murray JC. Angiogenesis is associated with vascular endothelial growth factor expression in cervical intraepithelial neoplasia. Br J Cancer 76(11):1410-1415;1997.
73. Doyen V, Rubio M, Braun D, Nakajima T, Abe J, Saito H*, et al.* Thrombospondin 1 is an autocrine negative regulator of human dendritic cell activation. J Exp Med 198(8):1277-1283;2003.
74. Eberhard A, Kahlert S, Goede V, Hemmerlein B, Plate KH, Augustin HG. Heterogeneity of angiogenesis and blood vessel maturation in human tumors: implications for antiangiogenic tumor therapies. Cancer Res

60(5):1388-1393;2000.

75. Ellenson LH and Wu TC. Focus on endometrial and cervical cancer. Cancer Cell 5(6):533-538;2004.
76. Ezzell C. Starving tumors of their lifeblood. Sci Am 279(4):33-34;1998.
77. Fang J, Shing Y, Wiederschain D, Yan L, Butterfield C, Jackson G*, et al.* Matrix metalloproteinase-2 is required for the switch to the angiogenic phenotype in a tumor model. Proc Natl Acad Sci U S A 97(8):3884-3889;2000.
78. Feldman AL, Alexander HR, Jr., Bartlett DL, Kranda KC, Miller MS, Costouros NG*, et al.* A prospective analysis of plasma endostatin levels in colorectal cancer patients with liver metastases. Ann Surg Oncol 8(9):741-745;2001a.
79. Feldman AL, Alexander HR, Jr., Yang JC, Linehan WM, Eyler RA, Miller MS*, et al.* Prospective analysis of circulating endostatin levels in patients with renal cell carcinoma. Cancer 95(8):1637-1643;2002.
80. Feldman AL, Pak H, Yang JC, Alexander HR, Jr., Libutti SK. Serum endostatin levels are elevated in patients with soft tissue sarcoma. Cancer 91(8):1525-1529;2001b.
81. Feldman AL, Tamarkin L, Paciotti GF, Simpson BW, Linehan WM, Yang JC*, et al.* Serum endostatin levels are elevated and correlate with serum vascular endothelial growth factor levels in patients with stage IV clear cell renal cancer. Clin Cancer Res 6(12):4628-4634;2000.
82. Fidler IJ. The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer 3(6):453-458;2003.
83. Fidler IJ and Ellis LM. Chemotherapeutic drugs--more really is not better. Nat Med 6(5):500-502;2000.
84. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 285(21):1182-1186;1971.
85. Folkman J. The role of angiogenesis in tumor growth. Semin Cancer Biol 3(2):65-71;1992.

86. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat Med 1(1):27-31;1995a.
87. Folkman J. Seminars in Medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med 333(26):1757-1763;1995b.
88. Folkman J. Tumor angiogenesis and tissue factor. Nat Med 2(2):167-168;1996.
89. Folkman J. Is tissue mass regulated by vascular endothelial cells? Prostate as the first evidence. Endocrinology 139(2):441-442;1998.
90. Folkman J. Angiogenesis and apoptosis. Semin Cancer Biol 13(2):159-167;2003a.
91. Folkman J. Fundamental concepts of the angiogenic process. Curr Mol Med 3(7):643-651;2003b.
92. Folkman J, Hahnfeldt P, Hlatky L. Cancer: looking outside the genome. Nat Rev Mol Cell Biol 1(1):76-79;2000.
93. Fontana A, Filleur S, Guglielmi J, Frappart L, Bruno-Bossio G, Boissier S*, et al.* Human breast tumors override the antiangiogenic effect of stromal thrombospondin-1 in vivo. Int J Cancer 116(5):686-691;2005.
94. Framson PE and Sage EH. SPARC and tumor growth: where the seed meets the soil? J Cell Biochem 92(4):679-690;2004.
95. Franck-Lissbrant I, Haggstrom S, Damber JE, Bergh A. Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. Endocrinology 139(2):451-456;1998.
96. Frazier WA. Thrombospondins. Curr Opin Cell Biol 3(5):792-799;1991.
97. Fukumura D, Xavier R, Sugiura T, Chen Y, Park EC, Lu N*, et al.* Tumor induction of VEGF promoter activity in stromal cells. Cell 94(6):715-725;1998.
98. Gasparini G. Metronomic scheduling: the future of chemotherapy? Lancet Oncol 2(12):733-740;2001.

99. Gasparini G and Harris AL. Does improved control of tumour growth require an anti-cancer therapy targeting both neoplastic and intratumoral endothelial cells? Eur J Cancer 30A(2):201-206;1994.
100. Graflund M, Sorbe B, Hussein A, Bryne M, Karlsson M. The prognostic value of histopathologic grading parameters and microvessel density in patients with early squamous cell carcinoma of the uterine cervix. Int J Gynecol Cancer 12(1):32-41;2002.
101. Grossfeld GD, Ginsberg DA, Stein JP, Bochner BH, Esrig D, Groshen S*, et al.* Thrombospondin-1 expression in bladder cancer: association with p53 alterations, tumor angiogenesis, and tumor progression. J Natl Cancer Inst 89(3):219-227;1997.
102. Guedez L, Rivera AM, Salloum R, Miller ML, Diegmueller JJ, Bungay PM*, et al.* Quantitative assessment of angiogenic responses by the directed in vivo angiogenesis assay. Am J Pathol 162(5):1431-1439;2003.
103. Guidi AJ, Abu-Jawdeh G, Berse B, Jackman RW, Tognazzi K, Dvorak HF*, et al.* Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. J Natl Cancer Inst 87(16):1237-1245;1995.
104. Guidolin D, Vacca A, Nussdorfer GG, Ribatti D. A new image analysis method based on topological and fractal parameters to evaluate the angiostatic activity of docetaxel by using the Matrigel assay in vitro. Microvasc Res 67(2):117-124;2004.
105. Guo N, Krutzsch HC, Inman JK, Roberts DD. Thrombospondin 1 and type I repeat peptides of thrombospondin 1 specifically induce apoptosis of endothelial cells. Cancer Res 57(9):1735-1742;1997.
106. Hahnfeldt P, Panigrahy D, Folkman J, Hlatky L. Tumor development under angiogenic signaling: a dynamical theory of tumor growth, treatment response, and postvascular dormancy. Cancer Res 59(19):4770-4775;1999.

107. Hanahan D, Bergers G, Bergsland E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J Clin Invest 105(8):1045-1047;2000.
108. Hanahan D and Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86(3):353-364;1996.
109. Hanahan D and Weinberg RA. The hallmarks of cancer. Cell 100(1):57-70;2000.
110. Hasebe T, Sasaki S, Imoto S, Ochiai A. Proliferative activity of intratumoral fibroblasts is closely correlated with lymph node and distant organ metastases of invasive ductal carcinoma of the breast. Am J Pathol 156(5):1701-1710;2000.
111. Hawighorst H. Dynamic MR imaging in cervical carcinoma. Radiology 213(2):617-618;1999.
112. Hawighorst T, Oura H, Streit M, Janes L, Nguyen L, Brown LF*, et al.* Thrombospondin-1 selectively inhibits early-stage carcinogenesis and angiogenesis but not tumor lymphangiogenesis and lymphatic metastasis in transgenic mice. Oncogene 21(52):7945-7956;2002.
113. Hawighorst T, Velasco P, Streit M, Hong YK, Kyriakides TR, Brown LF*, et al.* Thrombospondin-2 plays a protective role in multistep carcinogenesis: a novel host anti-tumor defense mechanism. EMBO J 20(11):2631-2640;2001.
114. Hayashido Y, Nakashima M, Urabe K, Yoshioka H, Yoshioka Y, Hamana T*, et al.* Role of stromal thrombospondin-1 in motility and proteolytic activity of oral squamous cell carcinoma cells. Int J Mol Med 12(4):447-452;2003.
115. Hlatky L, Hahnfeldt P, Folkman J. Clinical application of antiangiogenic therapy: microvessel density, what it does and doesn't tell us. J Natl Cancer Inst 94(12):883-893;2002.
116. Hlatky L, Tsionou C, Hahnfeldt P, Coleman CN. Mammary fibroblasts may influence breast tumor angiogenesis via hypoxia-induced vascular endothelial

growth factor up-regulation and protein expression. Cancer Res 54(23):6083-6086;1994.

117. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56(19):4509-4515;1996.

118. Hsu KF, Su JM, Huang SC, Cheng YM, Kang CY, Shen MR*, et al.* Three-dimensional power Doppler imaging of early-stage cervical cancer. Ultrasound Obstet Gynecol 24(6):664-671;2004.

119. Hsu SC, Volpert OV, Steck PA, Mikkelsen T, Polverini PJ, Rao S*, et al.* Inhibition of angiogenesis in human glioblastomas by chromosome 10 induction of thrombospondin-1. Cancer Res 56(24):5684-5691;1996.

120. Hugo C. The thrombospondin 1-TGF-beta axis in fibrotic renal disease. Nephrol Dial Transplant 18(7):1241-1245;2003.

121. Isenberg JS, Calzada MJ, Zhou L, Guo N, Lawler J, Wang XQ*, et al.* Endogenous thrombospondin-1 is not necessary for proliferation but is permissive for vascular smooth muscle cell responses to platelet-derived growth factor. Matrix Biol 24(2):110-123;2005a.

122. Isenberg JS, Ridnour LA, Perruccio EM, Espey MG, Wink DA, Roberts DD. Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner. Proc Natl Acad Sci U S A 102(37):13141-13146;2005b.

123. Ishii G, Sangai T, Ito T, Hasebe T, Endoh Y, Sasaki H*, et al.* In vivo and in vitro characterization of human fibroblasts recruited selectively into human cancer stroma. Int J Cancer 117(2):212-220;2005.

124. Jendraschak E and Sage EH. Regulation of angiogenesis by SPARC and angiostatin: implications for tumor cell biology. Semin Cancer Biol 7(3):139-146;1996.

125. Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N.

Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med 6(1):41-48;2000.

126. Jin RJ, Kwak C, Lee SG, Lee CH, Soo CG, Park MS*, et al.* The application of an anti-angiogenic gene (thrombospondin-1) in the treatment of human prostate cancer xenografts. Cancer Gene Ther 7(12):1537-1542;2000.

127. Kakeji Y and Teicher BA. Preclinical studies of the combination of angiogenic inhibitors with cytotoxic agents. Invest New Drugs 15(1):39-48;1997.

128. Kalas W, Yu JL, Milsom C, Rosenfeld J, Benezra R, Bornstein P*, et al.* Oncogenes and Angiogenesis: down-regulation of thrombospondin-1 in normal fibroblasts exposed to factors from cancer cells harboring mutant ras. Cancer Res 65(19):8878-8886;2005.

129. Kalluri R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer 3(6):422-433;2003.

130. Kamen BA, Rubin E, Aisner J, Glatstein E. High-time chemotherapy or high time for low dose. J Clin Oncol 18(16):2935-2937;2000.

131. Kawahara N, Ono M, Taguchi K, Okamoto M, Shimada M, Takenaka K*, et al.* Enhanced expression of thrombospondin-1 and hypovascularity in human cholangiocarcinoma. Hepatology 28(6):1512-1517;1998.

132. Kawataki T, Naganuma H, Sasaki A, Yoshikawa H, Tasaka K, Nukui H. Correlation of thrombospondin-1 and transforming growth factor-beta expression with malignancy of glioma. Neuropathology 20(3):161-169;2000.

133. Kazuno M, Tokunaga T, Oshika Y, Tanaka Y, Tsugane R, Kijima H*, et al.* Thrombospondin-2 (TSP2) expression is inversely correlated with vascularity in glioma. Eur J Cancer 35(3):502-506;1999.

134. Kenny PA and Bissell MJ. Tumor reversion: correction of malignant behavior by microenvironmental cues. Int J Cancer 107(5):688-695;2003.

135. Kerbel R and Folkman J. Clinical translation of angiogenesis inhibitors. Nat Rev Cancer 2(10):727-739;2002.

136. Kerbel RS and Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. Nat Rev Cancer 4(6):423-436;2004.
137. Kerbel RS, Yu J, Tran J, Man S, Viloria-Petit A, Klement G*, et al.* Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. Cancer Metastasis Rev 20(1-2):79-86;2001.
138. Kinzler KW and Vogelstein B. Landscaping the cancer terrain. Science 280(5366):1036-1037;1998.
139. Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ*, et al.* Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest 105(8):R15-24;2000.
140. Kodama J, Hashimoto I, Seki N, Hongo A, Yoshinouchi M, Okuda H*, et al.* Thrombospondin-1 and -2 messenger RNA expression in invasive cervical cancer: correlation with angiogenesis and prognosis. Clin Cancer Res 7(9):2826-2831;2001.
141. Kohno Y, Iwanari O, Kitao M. Prognostic importance of histologic vascular density in cervical cancer treated with hypertensive intraarterial chemotherapy. Cancer 72(8):2394-2400;1993.
142. Kunz-Schughart LA, Heyder P, Schroeder J, Knuechel R. A heterologous 3-D coculture model of breast tumor cells and fibroblasts to study tumor-associated fibroblast differentiation. Exp Cell Res 266(1):74-86;2001.
143. Kunz-Schughart LA and Knuechel R. Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? Histol Histopathol 17(2):599-621;2002a.
144. Kunz-Schughart LA and Knuechel R. Tumor-associated fibroblasts (part II): Functional impact on tumor tissue. Histol Histopathol 17(2):623-637;2002b.
145. Kunz-Schughart LA, Wenninger S, Neumeier T, Seidl P, Knuechel R.

Three-dimensional tissue structure affects sensitivity of fibroblasts to TGF-beta 1. Am J Physiol Cell Physiol 284(1):C209-219;2003.

146. Kuroi K and Toi M. Circulating angiogenesis regulators in cancer patients. Int J Biol Markers 16(1):5-26;2001.

147. Kwak C, Jin RJ, Lee C, Park MS, Lee SE. Thrombospondin-1, vascular endothelial growth factor expression and their relationship with p53 status in prostate cancer and benign prostatic hyperplasia. BJU Int 89(3):303-309;2002.

148. Lawler J. The functions of thrombospondin-1 and-2. Curr Opin Cell Biol 12(5):634-640;2000.

149. Lazard D, Sastre X, Frid MG, Glukhova MA, Thiery JP, Koteliansky VE. Expression of smooth muscle-specific proteins in myoepithelium and stromal myofibroblasts of normal and malignant human breast tissue. Proc Natl Acad Sci U S A 90(3):999-1003;1993.

150. Libutti SK. Do angiogenesis inhibitors perform a physiologic gatekeeper role in cancer prevention? Cancer J 10(1):12-14;2004.

151. Liotta LA and Kohn EC. The microenvironment of the tumour-host interface. Nature 411(6835):375-379;2001.

152. Liotta LA, Thorgeirsson UP, Garbisa S. Role of collagenases in tumor cell invasion. Cancer Metastasis Rev 1(4):277-288;1982.

153. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L*, et al.* Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 7(11):1194-1201;2001.

154. Maeshima Y, Sudhakar A, Lively JC, Ueki K, Kharbanda S, Kahn CR*, et al.* Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science 295(5552):140-143;2002.

155. Majno G, Gabbiani G, Hirschel BJ, Ryan GB, Statkov PR. Contraction of granulation tissue in vitro: similarity to smooth muscle. Science

173(996):548-550;1971.

156. Manna PP and Frazier WA. CD47 mediates killing of breast tumor cells via Gi-dependent inhibition of protein kinase A. Cancer Res 64(3):1026-1036;2004.

157. Mayr NA, Hawighorst H, Yuh WT, Essig M, Magnotta VA, Knopp MV. MR microcirculation assessment in cervical cancer: correlations with histomorphological tumor markers and clinical outcome. J Magn Reson Imaging 10(3):267-276;1999.

158. Meyerhardt JA and Mayer RJ. Systemic therapy for colorectal cancer. N Engl J Med 352(5):476-487;2005.

159. Meyer-Ter-Vehn T, Gebhardt S, Sebald W, Buttmann M, Grehn F, Schlunck G*, et al.* p38 inhibitors prevent TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. Invest Ophthalmol Vis Sci 47(4):1500-1509;2006.

160. Miao WM, Seng WL, Duquette M, Lawler P, Laus C, Lawler J. Thrombospondin-1 type 1 repeat recombinant proteins inhibit tumor growth through transforming growth factor-beta-dependent and -independent mechanisms. Cancer Res 61(21):7830-7839;2001.

161. Micke P and Ostman A. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? Lung Cancer 45 Suppl 2S163-175;2004.

162. Miller KD, Sweeney CJ, Sledge GW, Jr. Redefining the target: chemotherapeutics as antiangiogenics. J Clin Oncol 19(4):1195-1206;2001.

163. Miyanaga K, Kato Y, Nakamura T, Matsumura M, Amaya H, Horiuchi T*, et al.* Expression and role of thrombospondin-1 in colorectal cancer. Anticancer Res 22(6C):3941-3948;2002.

164. Mueller MM and Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 4(11):839-849;2004.

165. Nor JE, Mitra RS, Sutorik MM, Mooney DJ, Castle VP, Polverini PJ. Thrombospondin-1 induces endothelial cell apoptosis and inhibits angiogenesis by activating the caspase death pathway. J Vasc Res 37(3):209-218;2000.
166. Ohta Y, Shridhar V, Kalemkerian GP, Bright RK, Watanabe Y, Pass HI. Thrombospondin-1 expression and clinical implications in malignant pleural mesothelioma. Cancer 85(12):2570-2576;1999.
167. Ohtani Y, Kijima H, Dowaki S, Kashiwagi H, Tobita K, Tsukui M*, et al.* Stromal expression of thrombospondin-1 is correlated with growth and metastasis of human gallbladder carcinoma. Int J Oncol 15(3):453-457;1999.
168. Olaso E, Salado C, Egilegor E, Gutierrez V, Santisteban A, Sancho-Bru P*, et al.* Proangiogenic role of tumor-activated hepatic stellate cells in experimental melanoma metastasis. Hepatology 37(3):674-685;2003.
169. O'Leary JJ, Shapiro RL, Ren CJ, Chuang N, Cohen HW, Potmesil M. Antiangiogenic effects of camptothecin analogues 9-amino-20(S)-camptothecin, topotecan, and CPT-11 studied in the mouse cornea model. Clin Cancer Res 5(1):181-187;1999.
170. Opdenakker G and Van Damme J. The countercurrent principle in invasion and metastasis of cancer cells. Recent insights on the roles of chemokines. Int J Dev Biol 48(5-6):519-527;2004.
171. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS*, et al.* Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell 88(2):277-285;1997.
172. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Cao Y*, et al.* Angiostatin: a circulating endothelial cell inhibitor that suppresses angiogenesis and tumor growth. Cold Spring Harb Symp Quant Biol 59471-482;1994.
173. Orr AW, Pallero MA, Xiong WC, Murphy-Ullrich JE. Thrombospondin induces

RhoA inactivation through FAK-dependent signaling to stimulate focal adhesion disassembly. J Biol Chem 279(47):48983-48992;2004.

174. Oshiba G, Kijima H, Himeno S, Kenmochi T, Kise Y, Tanaka H*, et al.* Stromal thrombospondin-1 expression is correlated with progression of esophageal squamous cell carcinomas. Anticancer Res 19(5C):4375-4378;1999.

175. Oshika Y, Masuda K, Tokunaga T, Hatanaka H, Kamiya T, Abe Y*, et al.* Thrombospondin 2 gene expression is correlated with decreased vascularity in non-small cell lung cancer. Clin Cancer Res 4(7):1785-1788;1998.

176. Ozatli D, Kocoglu H, Haznedaroglu IC, Kosar A, Buyukasik Y, Ozcebe O*, et al.* Circulating thrombomodulin, thrombospondin, and fibronectin in acute myeloblastic leukemias. Haematologia (Budap) 29(4):277-283;1999.

177. Paget S. Distribution of secondary growths in cancer of the breast. Lancet 1571-573;1889.

178. Park CC, Bissell MJ, Barcellos-Hoff MH. The influence of the microenvironment on the malignant phenotype. Mol Med Today 6(8):324-329;2000.

179. Pinto AP and Crum CP. Natural history of cervical neoplasia: defining progression and its consequence. Clin Obstet Gynecol 43(2):352-362;2000.

180. Pratt DA, Miller WR, Dawes J. Thrombospondin in malignant and non-malignant breast tissue. Eur J Cancer Clin Oncol 25(2):343-350;1989.

181. Presta M, Rusnati M, Belleri M, Morbidelli L, Ziche M, Ribatti D. Purine analogue 6-methylmercaptopurine riboside inhibits early and late phases of the angiogenesis process. Cancer Res 59(10):2417-2424;1999.

182. Qian X, Rothman VL, Nicosia RF, Tuszynski GP. Expression of thrombospondin-1 in human pancreatic adenocarcinomas: role in matrix metalloproteinase-9 production. Pathol Oncol Res 7(4):251-259;2001.

183. Qian X, Wang TN, Rothman VL, Nicosia RF, Tuszynski GP. Thrombospondin-1 modulates angiogenesis in vitro by up-regulation of matrix

metalloproteinase-9 in endothelial cells. Exp Cell Res 235(2):403-412;1997.

184. Radisky DC and Bissell MJ. Cancer. Respect thy neighbor! Science 303(5659):775-777;2004.

185. Rak J, Yu JL, Klement G, Kerbel RS. Oncogenes and angiogenesis: signaling three-dimensional tumor growth. J Investig Dermatol Symp Proc 5(1):24-33;2000.

186. Raleigh JA, Calkins-Adams DP, Rinker LH, Ballenger CA, Weissler MC, Fowler WC, Jr., *et al.* Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. Cancer Res 58(17):3765-3768;1998.

187. Rastinejad F, Polverini PJ, Bouck NP. Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. Cell 56(3):345-355;1989.

188. Reiher FK, Volpert OV, Jimenez B, Crawford SE, Dinney CP, Henkin J, *et al.* Inhibition of tumor growth by systemic treatment with thrombospondin-1 peptide mimetics. Int J Cancer 98(5):682-689;2002.

189. Ren B, Yee KO, Lawler J, Khosravi-Far R. Regulation of tumor angiogenesis by thrombospondin-1. Biochim Biophys Acta 1765(2):178-188;2006.

190. Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, *et al.* Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 8(1):27-34;2002.

191. Rice A and Quinn CM. Angiogenesis, thrombospondin, and ductal carcinoma in situ of the breast. J Clin Pathol 55(8):569-574;2002.

192. Ridnour LA, Isenberg JS, Espey MG, Thomas DD, Roberts DD, Wink DA. Nitric oxide regulates angiogenesis through a functional switch involving thrombospondin-1. Proc Natl Acad Sci U S A 102(37):13147-13152;2005.

193. Risau W. Mechanisms of angiogenesis. Nature 386(6626):671-674;1997.

194. Rodriguez-Manzaneque JC, Lane TF, Ortega MA, Hynes RO, Lawler J,

Iruela-Arispe ML. Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. Proc Natl Acad Sci U S A 98(22):12485-12490;2001.

195. Ronnov-Jessen L and Petersen OW. Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia. Lab Invest 68(6):696-707;1993.

196. Ronnov-Jessen L, Petersen OW, Koteliansky VE, Bissell MJ. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J Clin Invest 95(2):859-873;1995.

197. Ronnov-Jessen L, Van Deurs B, Nielsen M, Petersen OW. Identification, paracrine generation, and possible function of human breast carcinoma myofibroblasts in culture. In Vitro Cell Dev Biol 28A(4):273-283;1992.

198. Rutgers JL, Mattox TF, Vargas MP. Angiogenesis in uterine cervical squamous cell carcinoma. Int J Gynecol Pathol 14(2):114-118;1995.

199. Schneider BP and Miller KD. Angiogenesis of breast cancer. J Clin Oncol 23(8):1782-1790;2005.

200. Seemayer TA, Lagace R, Schurch W, Tremblay G. Myofibroblasts in the stroma of invasive and metastatic carcinoma: a possible host response to neoplasia. Am J Surg Pathol 3(6):525-533;1979.

201. Senger DR, Brown LF, Claffey KP, Dvorak HF. Vascular permeability factor, tumor angiogenesis and stroma generation. Invasion Metastasis 14(1-6):385-394;1994.

202. Sheibani N and Frazier WA. Thrombospondin 1 expression in transformed endothelial cells restores a normal phenotype and suppresses their tumorigenesis. Proc Natl Acad Sci U S A 92(15):6788-6792;1995.

203. Sheibani N and Frazier WA. Repression of thrombospondin-1 expression, a natural inhibitor of angiogenesis, in polyoma middle T transformed NIH3T3 cells. Cancer Lett 107(1):45-52;1996.
204. Sheibani N and Frazier WA. Thrombospondin-1, PECAM-1, and regulation of angiogenesis. Histol Histopathol 14(1):285-294;1999.
205. Shen MR, Chou CY, Hsu KF, Hsu YM, Chiu WT, Tang MJ*, et al.* KCl cotransport is an important modulator of human cervical cancer growth and invasion. J Biol Chem 278(41):39941-39950;2003.
206. Shi J, Badri KR, Choudhury R, Schuger L. P311-induced myofibroblasts exhibit ameboid-like migration through RalA activation. Exp Cell Res 312(17):3432-3442;2006.
207. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 223(4642):1296-1299;1984.
208. Sipes JM, Krutzsch HC, Lawler J, Roberts DD. Cooperation between thrombospondin-1 type 1 repeat peptides and alpha(v)beta(3) integrin ligands to promote melanoma cell spreading and focal adhesion kinase phosphorylation. J Biol Chem 274(32):22755-22762;1999.
209. Siracha E, Sirachy J, Pappova N. Vascularization and radiocurability in cancer of the uterine cervix. Neoplasma 29183-188;1994.
210. Smith-McCune KK and Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. Cancer Res 54(3):800-804;1994.
211. Sotiropoulou M, Diakomanolis E, Elsheikh A, Loutradis D, Markaki S, Michalas S. Angiogenic properties of carcinoma in situ and microinvasive carcinoma of the uterine cervix. Eur J Gynaecol Oncol 25(2):219-221;2004.
212. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E*, et al.* Genes expressed in human tumor endothelium. Science 289(5482):1197-1202;2000.

213. Stetler-Stevenson WG. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. J Clin Invest 103(9):1237-1241;1999.
214. Stetler-Stevenson WG, Hewitt R, Corcoran M. Matrix metalloproteinases and tumor invasion: from correlation and causality to the clinic. Semin Cancer Biol 7(3):147-154;1996.
215. Streit M, Velasco P, Brown LF, Skobe M, Richard L, Riccardi L*, et al.* Overexpression of thrombospondin-1 decreases angiogenesis and inhibits the growth of human cutaneous squamous cell carcinomas. Am J Pathol 155(2):441-452;1999.
216. Streit M, Velasco P, Riccardi L, Spencer L, Brown LF, Janes L*, et al.* Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. Embo J 19(13):3272-3282;2000.
217. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA. Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. Proc Natl Acad Sci U S A 102(2):437-442;2005.
218. Taraboletti G, Morbidelli L, Donnini S, Parenti A, Granger HJ, Giavazzi R*, et al.* The heparin binding 25 kDa fragment of thrombospondin-1 promotes angiogenesis and modulates gelatinase and TIMP-2 production in endothelial cells. Faseb J 14(12):1674-1676;2000.
219. Tenan M, Fulci G, Albertoni M, Diserens AC, Hamou MF, El Atifi-Borel M*, et al.* Thrombospondin-1 is downregulated by anoxia and suppresses tumorigenicity of human glioblastoma cells. J Exp Med 191(10):1789-1798;2000.
220. Tokunaga T, Nakamura M, Oshika Y, Abe Y, Ozeki Y, Fukushima Y*, et al.* Thrombospondin 2 expression is correlated with inhibition of angiogenesis and metastasis of colon cancer. Br J Cancer 79(2):354-359;1999.
221. Tolsma SS, Volpert OV, Good DJ, Frazier WA, Polverini PJ, Bouck N. Peptides derived from two separate domains of the matrix protein thrombospondin-1

have anti-angiogenic activity. J Cell Biol 122(2):497-511;1993.

222. Tuszynski GP and Nicosia RF. The role of thrombospondin-1 in tumor progression and angiogenesis. Bioessays 18(1):71-76;1996.

223. Tuxhorn JA, McAlhany SJ, Dang TD, Ayala GE, Rowley DR. Stromal cells promote angiogenesis and growth of human prostate tumors in a differential reactive stroma (DRS) xenograft model. Cancer Res 62(11):3298-3307;2002a.

224. Tuxhorn JA, McAlhany SJ, Yang F, Dang TD, Rowley DR. Inhibition of transforming growth factor-beta activity decreases angiogenesis in a human prostate cancer-reactive stroma xenograft model. Cancer Res 62(21):6021-6025;2002b.

225. Udagawa T, Fernandez A, Achilles EG, Folkman J, D'Amato RJ. Persistence of microscopic human cancers in mice: alterations in the angiogenic balance accompanies loss of tumor dormancy. Faseb J 16(11):1361-1370;2002.

226. Ueda T, Yuh WT, Taoka T. Clinical application of perfusion and diffusion MR imaging in acute ischemic stroke. J Magn Reson Imaging 10(3):305-309;1999.

227. Vallejo AN, Mugge LO, Klimiuk PA, Weyand CM, Goronzy JJ. Central role of thrombospondin-1 in the activation and clonal expansion of inflammatory T cells. J Immunol 164(6):2947-2954;2000.

228. Vepachedu R, Gorska MM, Singhania N, Cosgrove GP, Brown KK, Alam R. Unc119 regulates myofibroblast differentiation through the activation of Fyn and the p38 MAPK pathway. J Immunol 179(1):682-690;2007.

229. Vieira SC, Zeferino LC, Da Silva BB, Aparecida Pinto G, Vassallo J, Carasan GA*, et al.* Quantification of angiogenesis in cervical cancer: a comparison among three endothelial cell markers. Gynecol Oncol 93(1):121-124;2004.

230. Volpert OV, Pili R, Sikder HA, Nelius T, Zaichuk T, Morris C*, et al.* Id1 regulates angiogenesis through transcriptional repression of thrombospondin-1. Cancer Cell 2(6):473-483;2002.

231. Volpert OV, Stellmach V, Bouck N. The modulation of thrombospondin and other naturally occurring inhibitors of angiogenesis during tumor progression. Breast Cancer Res Treat 36(2):119-126;1995.
232. Walter-Yohrling J, Pratt BM, Ledbetter S, Teicher BA. Myofibroblasts enable invasion of endothelial cells into three-dimensional tumor cell clusters: a novel in vitro tumor model. Cancer Chemother Pharmacol 52(4):263-269;2003.
233. Wang J, Lou P, Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. Anticancer Drugs 14(1):13-19;2003.
234. Wang TN, Albo D, Tuszynski GP. Fibroblasts promote breast cancer cell invasion by upregulating tumor matrix metalloproteinase-9 production. Surgery 132(2):220-225;2002.
235. Wang TN, Qian X, Granick MS, Solomon MP, Rothman VL, Berger DH*, et al.* Thrombospondin-1 (TSP-1) promotes the invasive properties of human breast cancer. J Surg Res 63(1):39-43;1996.
236. Watnick RS, Cheng YN, Rangarajan A, Ince TA, Weinberg RA. Ras modulates Myc activity to repress thrombospondin-1 expression and increase tumor angiogenesis. Cancer Cell 3(3):219-231;2003.
237. Weber WA, Haubner R, Vabuliene E, Kuhnast B, Wester HJ, Schwaiger M. Tumor angiogenesis targeting using imaging agents. Q J Nucl Med 45(2):179-182;2001.
238. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol 143(2):401-409;1993.
239. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH*, et al.* Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 84(24):1875-1887;1992.
240. Weinstat-Saslow DL, Zabrenetzky VS, VanHoutte K, Frazier WA, Roberts DD,

Steeg PS. Transfection of thrombospondin 1 complementary DNA into a human breast carcinoma cell line reduces primary tumor growth, metastatic potential, and angiogenesis. Cancer Res 54(24):6504-6511;1994.

241. West CM, Cooper RA, Loncaster JA, Wilks DP, Bromley M. Tumor vascularity: a histological measure of angiogenesis and hypoxia. Cancer Res 61(7):2907-2910;2001.

242. Willhauck MJ, Mirancea N, Vosseler S, Pavesio A, Boukamp P, Mueller MM*, et al.* Reversion of tumor phenotype in surface transplants of skin SCC cells by scaffold-induced stroma modulation. Carcinogenesis 28(3):595-610;2007.

243. Wolf JK and Ramirez PT. The molecular biology of cervical cancer. Cancer Invest 19(6):621-629;2001.

244. Wu MP, Tzeng CC, Wu LW, Huang KF, Chou CY. Thrombospondin-1 acts as a fence to inhibit angiogenesis that occurs during cervical carcinogenesis. Cancer J 10(1):27-32;2004.

245. Wu MP, Young MJ, Tzeng CC, Tzeng CR, Huang KF, Wu LW*, et al.* A novel role of TSP-1 in cervical carcinogenesis: Inhibit stroma reaction by inhibiting activated fibroblasts from invading cancer. Carcinogenesis In Press;2008.

246. Yamauchi M, Imajoh-Ohmi S, Shibuya M. Novel antiangiogenic pathway of thrombospondin-1 mediated by suppression of the cell cycle. Cancer Sci 98(9):1491-1497;2007.

247. Yang Q, Tian Y, Liu S, Zeine R, Chlenski A, Salwen HR*, et al.* Thrombospondin-1 peptide ABT-510 combined with valproic acid is an effective antiangiogenesis strategy in neuroblastoma. Cancer Res 67(4):1716-1724;2007.

248. Yang Z, Kyriakides TR, Bornstein P. Matricellular proteins as modulators of cell-matrix interactions: adhesive defect in thrombospondin 2-null fibroblasts is a consequence of increased levels of matrix metalloproteinase-2. Mol Biol Cell 11(10):3353-3364;2000.

249. Yoshida Y, Oshika Y, Fukushima Y, Tokunaga T, Hatanaka H, Kijima H*, et al.* Expression of angiostatic factors in colorectal cancer. Int J Oncol 15(6):1221-1225;1999.
250. Zabrenetzky V, Harris CC, Steeg PS, Roberts DD. Expression of the extracellular matrix molecule thrombospondin inversely correlates with malignant progression in melanoma, lung and breast carcinoma cell lines. Int J Cancer 59(2):191-195;1994.
251. Zetter BR. Cell motility in angiogenesis and tumor metastasis. Cancer Invest 8(6):669-671;1990.
252. Zhang L, Yu D, Hu M, Xiong S, Lang A, Ellis LM*, et al.* Wild-type p53 suppresses angiogenesis in human leiomyosarcoma and synovial sarcoma by transcriptional suppression of vascular endothelial growth factor expression. Cancer Res 60(13):3655-3661;2000.

Tables

Table 1. Distribution of mean microvessel density count and thrombospondin-1 score in the four groups of normal epithelia (Normal), low-grade squamous intraepithelial lesions, high grade SIL, or invasive squamous cell carcinoma (SCC).

Cervical		MVD		TSP-1 score	
lesions	No.	Mean ± SD	p-value	Mean ± SD	p-value
Normal	48	7.3 ± 2.9	<0.001	3.8 ± 0.4	<0.001
LSIL	36	9.9 ± 3.4		3.8 ± 0.4	
HSIL	24	17.7 ± 5.1		1.8 ± 0.4	
SCC	12	22.8 ± 8.6		1.5 ± 0.5	

Distribution of mean microvessel density (MVD) count and thrombospondins-1 (TSP-1) score in the four groups of normal cervical epithelia (normal), low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), or invasive squamous cell carcinoma (SCC). TSP-1 decreased significantly during the transition from LSIL to HSIL, which was concomitant with the increase of MVD counts. Statistical analyses with Kruskal-Wallis test revealed that MVD counts and TSP-1 scores significantly differed among the four groups ($p < .001$). In addition, MVD counts were significantly lower ($p < .001$) and TSP-1 scores higher ($p < .001$) in subjects with normal and LSIL epithelium as compared with those with HSIL and SCC epithelium (Dunn post hoc comparison test) (Wu MP et al. 2004).

Table 2. Paired comparisons of MVD and TSP-1 score between normal/LSIL, LSIL/HSIL, and HSIL/SCC epithelium groups

Cervical lesions		MVD		TSP-1 score	
	No.	Mean ± SD	p-value	Mean ± SD	p-value
Normal / LSIL	36	7.1±3.0/ 9.9±3.4	<0.001	3.8±0.4/ 3.8±0.4	n.s.
LSIL / HSIL	24	9.8±3.5/ 17.7±5.1	<0.001	3.8±0.4/ 1.8±0.4	<0.05
HSIL / SCC	12	18.4±4.7/ 22.8±8.6	<0.05	1.8±0.5/ 1.5±0.5	n.s.

Wilcoxon signed- rank test was applied for paired comparisons between different lesions on the same slide. MVD was significantly different between normal/ LSIL epithelium, LSIL/ HSIL epithelium, and HSIL/ SCC epithelium. In contrast, the TSP-1 score was significantly different only between LSIL/HSIL epithelium. note, n.s.: not significantly different (Wu MP et al. 2004).

Table 3. Distribution of stroma markers, α-SMA and desmin scores, in the three groups of normal, CIS or SCC

	α-SMA			desmin			
Cervical lesions	No.	Mean ± SD	p-value	Cervical lesions	No.	Mean ± SD	p-value
Normal[a,c]	15	0.67±0.42	<0.001	Normal[d,f]	15	0.21±0.18	<0.001
CIS[a,b]	15	2.13±0.61		CIS[d,e]	15	0.57±0.31	
SCC[b,c]	15	2.78±0.21		SCC[e,f]	15	1.78±0.44	

Statistical analyses with Kruskal-Wallis test revealed that α-SMA and desmin scores were significantly different among normal, CIS and SCC groups with both p-value < 0.001. In addition, α-SMA scores were significantly different when compared with normal and CIS[a], CIS and SCC[b], normal and CIS[c], with p-value of 0.002, 0.006, and <0.001, respectively (Dunn post hoc comparison test). Desmin scores were also significantly different when compared with normal and CIS[d], CIS and SCC[e], normal and CIS[f], with p-value 0.019, <0.001, <0.001, respectively (Dunn post hoc comparison test).

Table 4. Distribution of CD34-positive microvessel density, α-SMA and desmin scores in the SiHa-vector induced (SiHa-vector) or SiHa-TSP-1-induced (TSP-1) tumors

		CD34		α-SMA		desmin	
tumor	No.	Mean± SD	p-value	Mean± SD	p-value	Mean± SD	p-value
SiHa-vector	6	1.67± 0.19	0.002	2.17± 0.43	0.002	1.52± 0.63	0.015
SiHa-TSP-1	6	0.58± 0.18		0.95± 0.42		0.48± 0.46	

Statistical analyses with Mann-Whitney U test revealed that microvessel density, α-SMA and desmin scores significantly differed between SiHa-vector induced SiHa-TSP-1-induced.

Figures

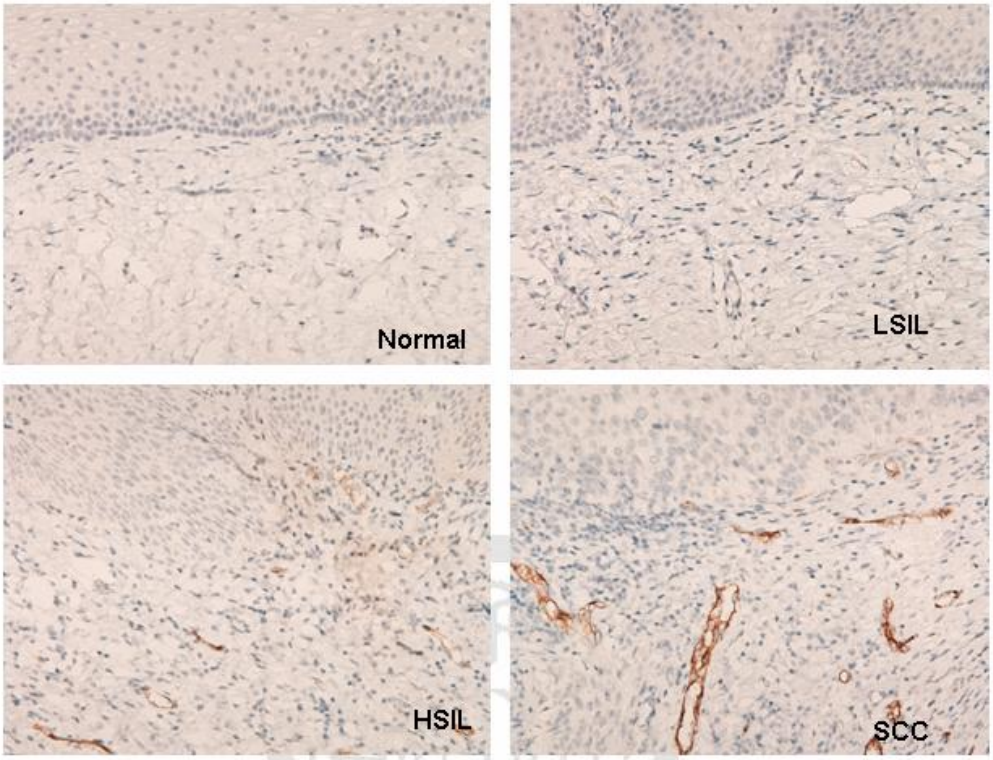

Fig. 1 Microvessels in different cervical lesions. Immunohistochemistry (X 200) of representative subjects with normal cervical epithelia (normal), low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), or invasive squamous cell carcinoma (SCC) revealed that microvessels were only scarcely visible in normal and LSIL epithelium. In contrast, microvessels were abundant in HSIL and SCC epithelium.

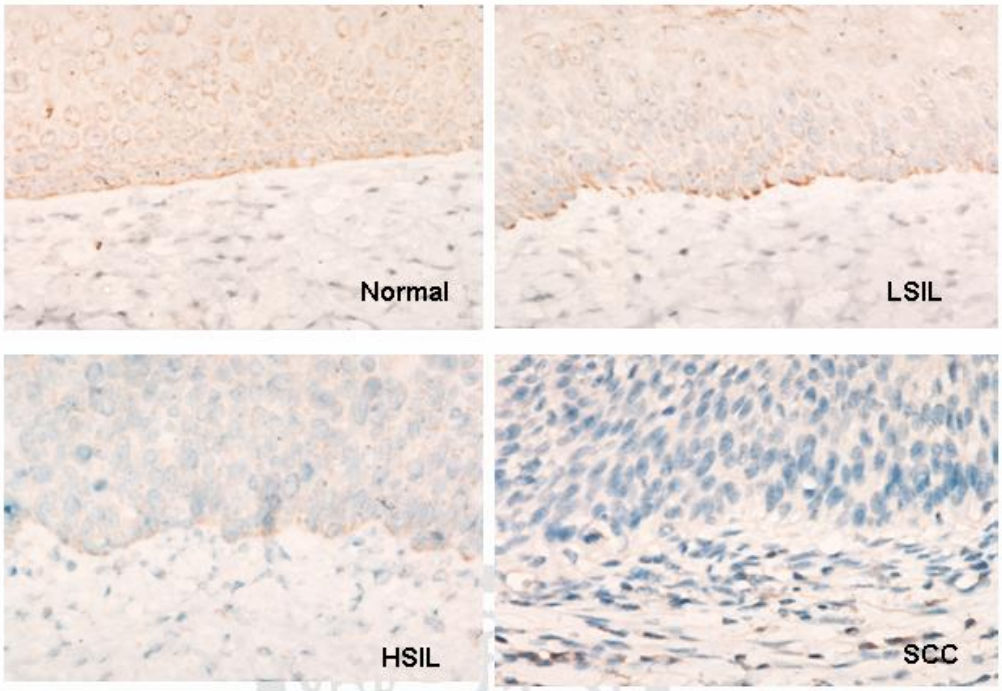

Fig. 2. TSP-1 expression in different cervical lesions. Immunohistochemistry (X 400) of representative subjects with normal cervical epithelia (normal), low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), or invasive squamous cell carcinoma (SCC) revealed that thrombospondins-1 (TSP-1) expression was localized mainly in the basal epithelial cell layer (upper panel). The staining intensity gradually decreased in cells of superficial layers; therefore, we named it 'TSP-1 fence'. This "TSP-1 fence" existed only in normal and LSIL epithelium, became much less obvious in HSIL epithelium, and was hardly visible in SCC epithelium.

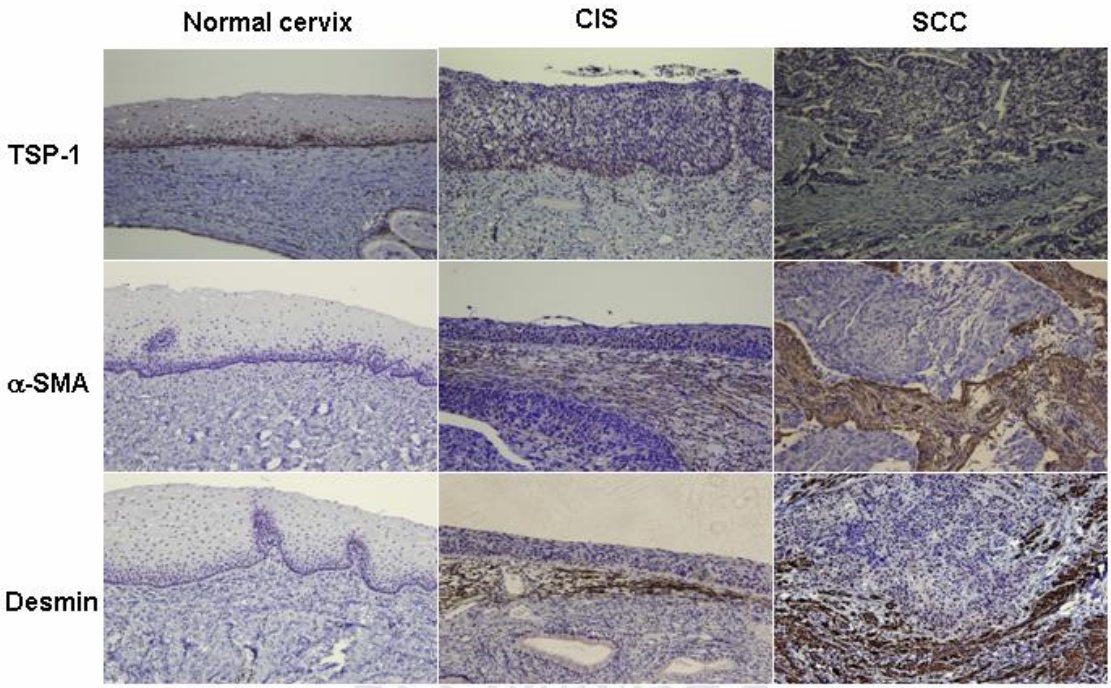

Fig. 3 Immunohistochemistry (X200) of representative subjects with normal cervical epithelium, carcinoma in situ (CIS), and squamous cell carcinoma (SCC) revealed that TSP-1 was localized in basal epithelial layer of normal cervix, and was downregulated in CIS and SCC. In direct contrast, the staining intensities of α-SMA and desmin were significantly upregulated in the stroma of CIS and SCC lesions compared with those in normal cervix.

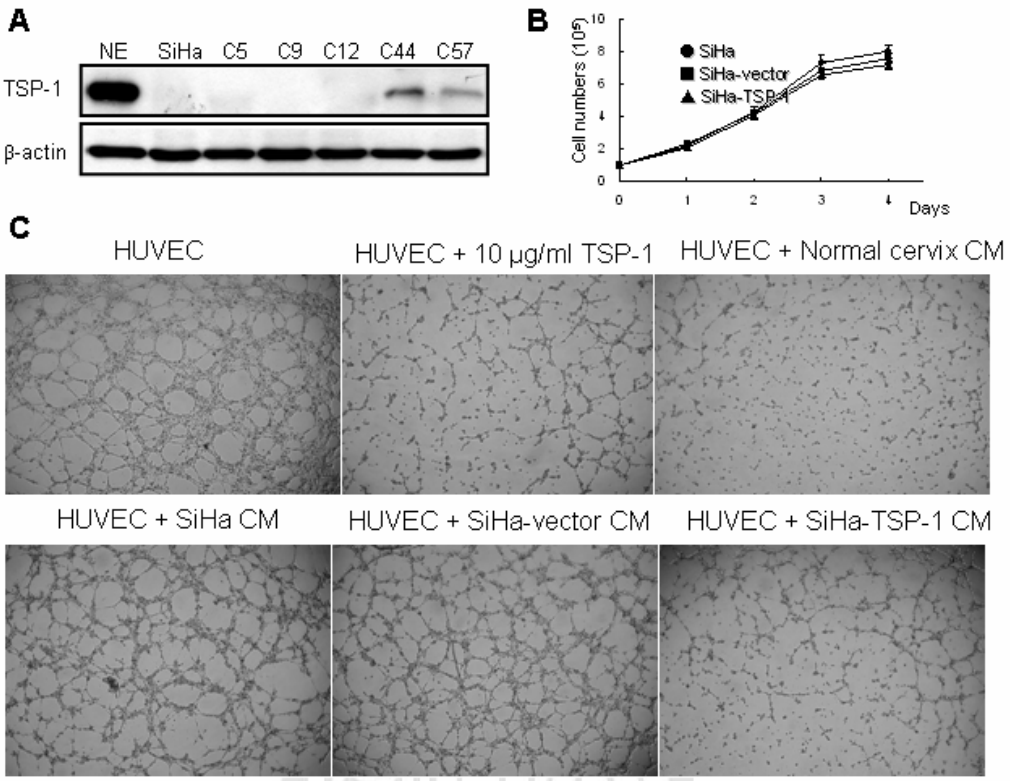

Fig. 4 **Molecular characterization and functional expression of TSP-1 in SiHa cells. A.** TSP-1 levels measured by Western blotting in parental cells (SiHa), cells transfected with empty vector (C5, C9, C12), and cells transfected with human TSP-1 cDNA (C44, C57). Positive control was total protein isolated from normal cervical epithelial cells (NE). **B.** Cell numbers were counted at the indicated time points with a hemocytometer by using trypan blue exclusion to monitor cell viability. **C.** The anti-angiogenic ability of TSP-1. Shown were the representative results of Matrigel angiogenesis assay to determine the formation of vascular network by human umbilical vein endothelial cells (HUVECs) under normal culture condition, treated with indicated CMs from different cell lines (SiHa, SiHa-vector, and SiHa-TSP-1), or purified TSP-1 at 10ug/ml. All assays were performed in triplicate.

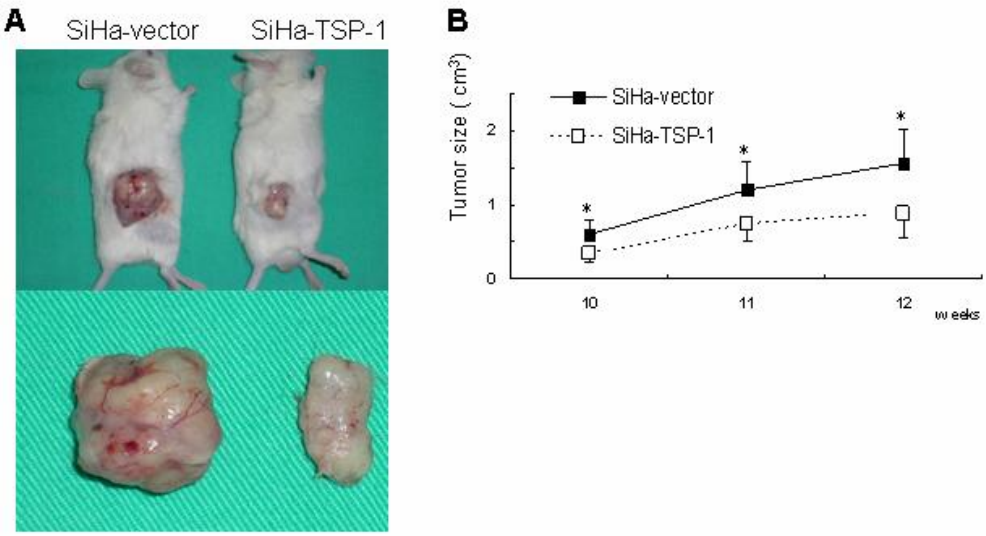

Fig. 5 A, B TSP-1 inhibits tumor development, tumor angiogenesis, and stroma marker expression in SCID mice. A. Representative gross tumor xenografts resulting from the transplantation of vector control (SiHa-vector) or cells transfected with human TSP-1 cDNA (SiHa-TSP-1) at 12 weeks after tumor transplantation. **B.** Tumor growth curves of SCID mice subcutaneously inoculated with SiHa-vector or SiHa-TSP-1 cell lines. Each point is mean ± S.D. (n= 6 in each group).

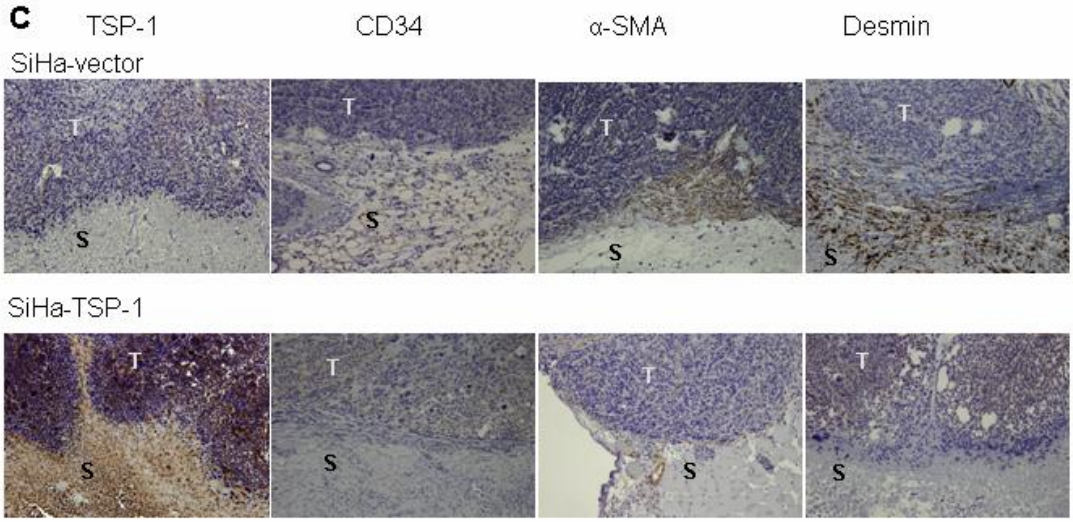

Fig. 5C TSP-1 inhibits tumor development, tumor angiogenesis, and stroma marker expression in SCID mice. C. Immunohistochemistry (X200) of representative sections from SCID mice subcutaneously inoculated with SiHa-vector or SiHa-TSP-1 cell lines revealed a negligible level of TSP-1 in tumor (T) and stroma (S) area, highly vascularized as shown by positive CD34 stain, and strong staining intensities for α-SMA and desmin in SiHa-vector-induced tumor stroma. In contrast, the SiHa-TSP-1-induced tumor shows less vascularization and overexpressed TSP-1 with downregulated α-SMA and desmin staining intensities compared with the SiHa-vector-induced tumor.

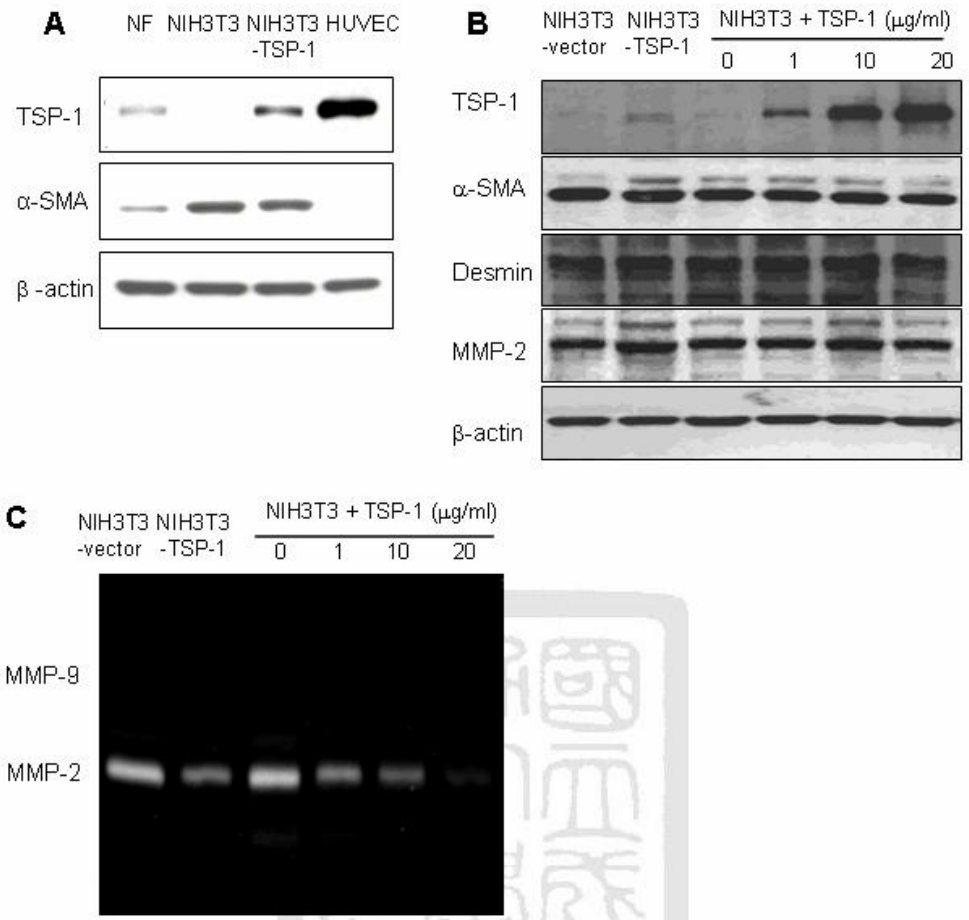

Fig. 6A, B, C Augmentation of TSP-1 activity is associated with decreased NIH3T3 cell migration and MMP-2 activity, but not with α-SMA and desmin expression. **A.** A representative Western immunoblot showing that stable transfection of TSP-1 in NIH3T3 cells upregulated the protein level of TSP-1, but not downregulated the protein level of α-SMA, as compared with normal fibroblast (NF) derived from normal uterine cervix with higher protein level of TSP-1, and lower α-SMA. **B.** A representative Western immunoblot showing that TSP-1 transfection (NIH3T3-TSP-1), as well as purified TSP-1 at various concentrations, did not change the protein levels of α-SMA, desmin and MMP-2. Experiments were repeated three times. **C.** Decreased MMP-2 activity was noted with the transfection of TSP-1 cDNA or the addition of TSP-1 to NIH3T3 cells. Shown is a gelatin zymography of conditioned media (a representative of three similar experiments).

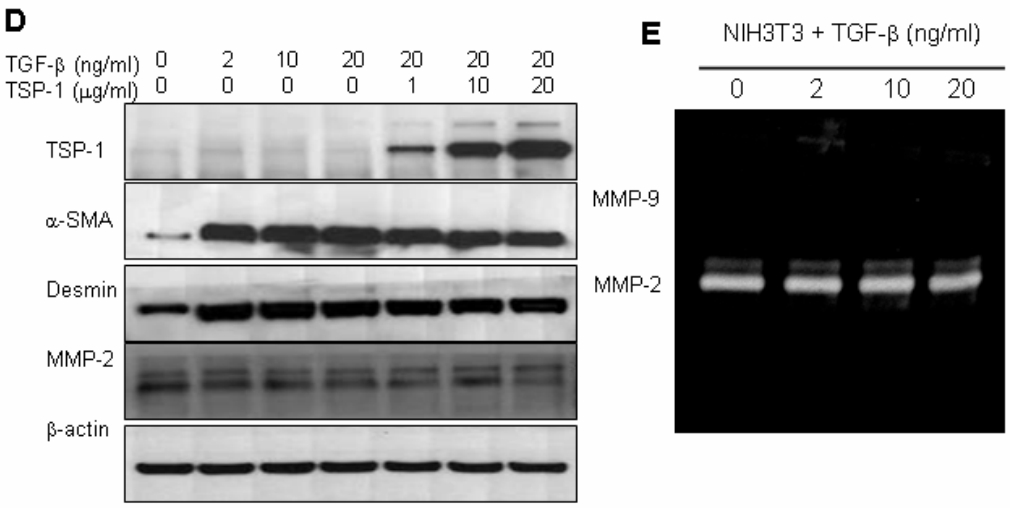

Fig. 6D, E TGF-β treatment for 24 hours increases NIH3T3 cell migration, and α-SMA and desmin expression. The TGF-β-increased α-SMA expression can not be blocked by TSP-1. **D.** A representative Western immunoblot showing that TGF-β-treatment (2, 10, 20 ng/ml) upregulated α-SMA and desmin, but did not change MMP-2 expression in NIH3T3 cells. Experiments were repeated three times. The addition of TSP-1 AT 1, 10, 20, μg/ml did not block TGF-β-increased α-SMA expression. **E.** There was no change on the MMP-2 activity after TGF-β treatment. Shown is a gelatin zymography of conditioned media (a representative of three similar experiments).

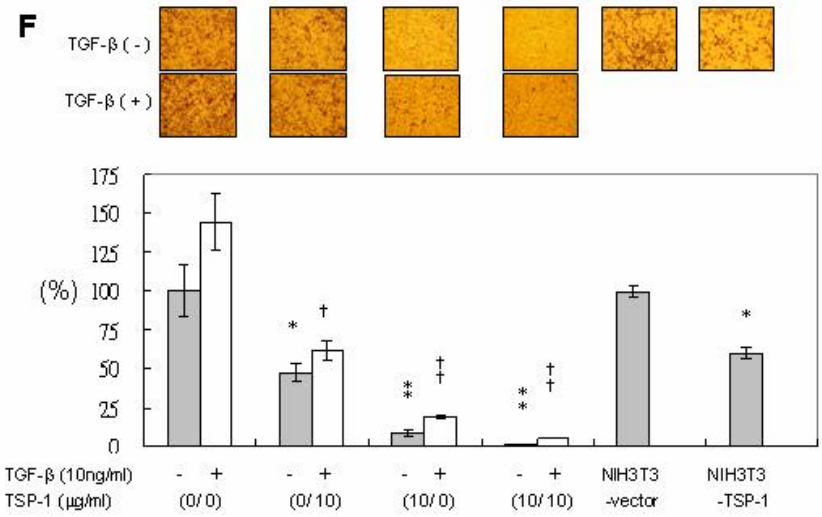

Fig. 6 F. Cell migration assay with modified Boyden chamber of NIH3T3-vector, NIH3T3-TSP-1, or NIH3T3 cells in the absence (0/ 0) or presence of purified TSP-1. Each column represents mean ± S.D. (n= 6). TSP-1 was added on the lower (0/ 10), upper (10/ 0) or both (10/ 10) compartments of the Boyden chamber. ∗ or †, $p < 0.05$; ∗∗ or ††, $p < 0.01$.

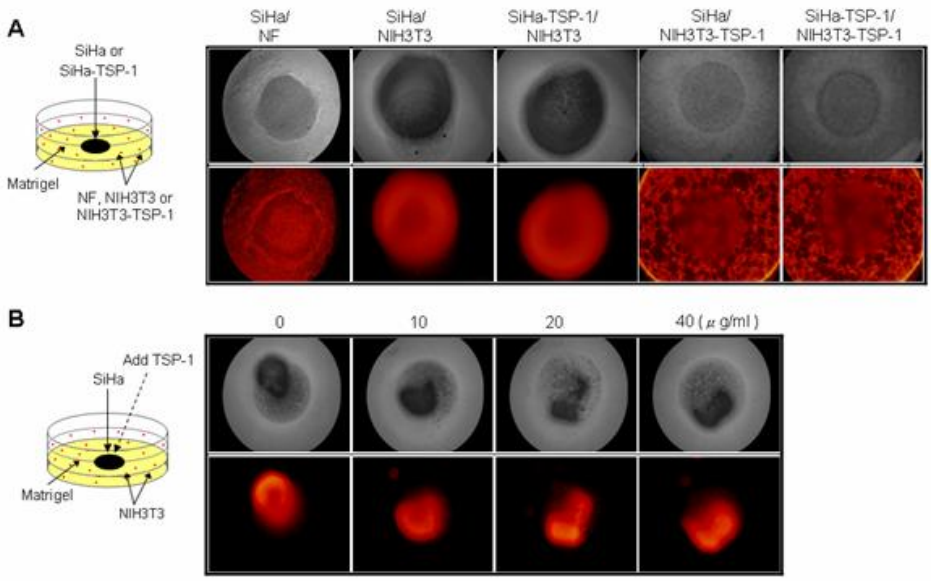

Fig. 7 A, B TSP-1 attenuates the invasive ability of activated fibroblasts from invading into tumor clusters. A Matrigel multi-cellular co-culture system was used to evaluate the invasive ability of fibroblasts into tumor cell cluster. Brightfield and fluorescent images were captured on an inverted phase microscope using a 4× objective for the same field. **A.** To test the effects of gain of TSP-1 function on tumor cells and fibroblasts, tumor cells with or without TSP-1 transfection (SiHa or SiHa-TSP-1) were placed into the central area of the Matrigel to form the tumor cell cluster; fluorescence (PKH26)-labeled fibroblasts (NIH3T3 or NIH3T3-TSP-1) (red) were evenly dispersed on the peripheral area. Primary cervical normal fibroblasts (NF) in the central area with SiHa tumor cell cluster in the peripheral (SiHa/ NF) was used as negative control. **B.** To test whether the addition of TSP-1 to tumor cell cluster or fibroblasts in the absence or presence of TGF-β can prevent the fibroblasts from invading into tumor cluster, SiHa cells were placed in the central area, while NIH3T3 cells (labeled with red fluorescence PKH26) were placed in the peripheral area. Purified TSP-1 was added into SiHa with different concentrations.

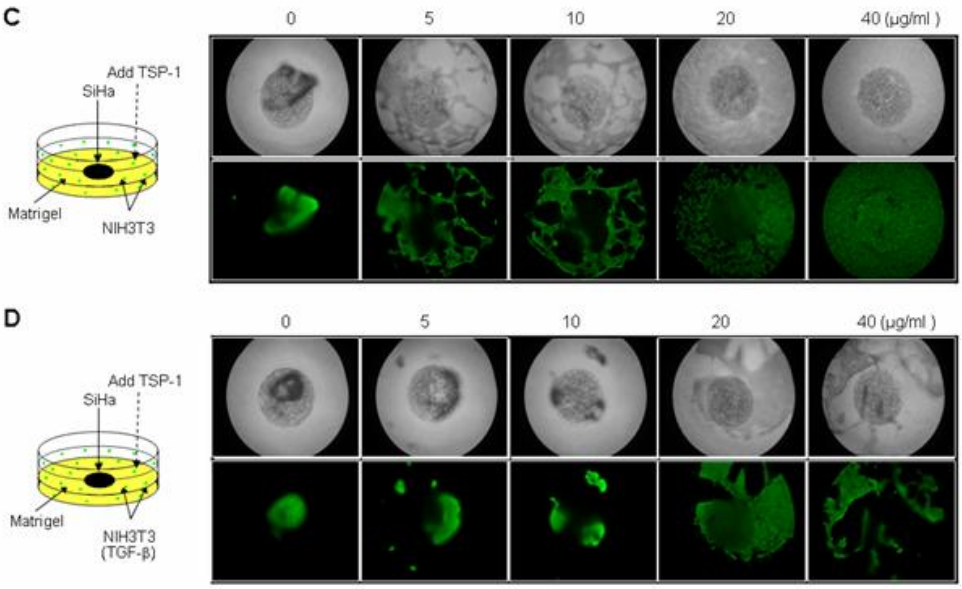

Fig. 7 C, D. To test whether the addition of TSP-1 to tumor cell cluster or fibroblasts in the absence or presence of TGF-β can prevent the fibroblasts from invading into tumor cluster, SiHa cells were placed in the central area, while NIH3T3 cells (labeled green fluorescence PKH67 in **C** and **D**) were placed in the peripheral area. Purified TSP-1 was added into untreated NIH3T3 cells (**C**) or TGF-β (10ng/ml)-treated NIH3T3 cells (**D**) with different concentrations.

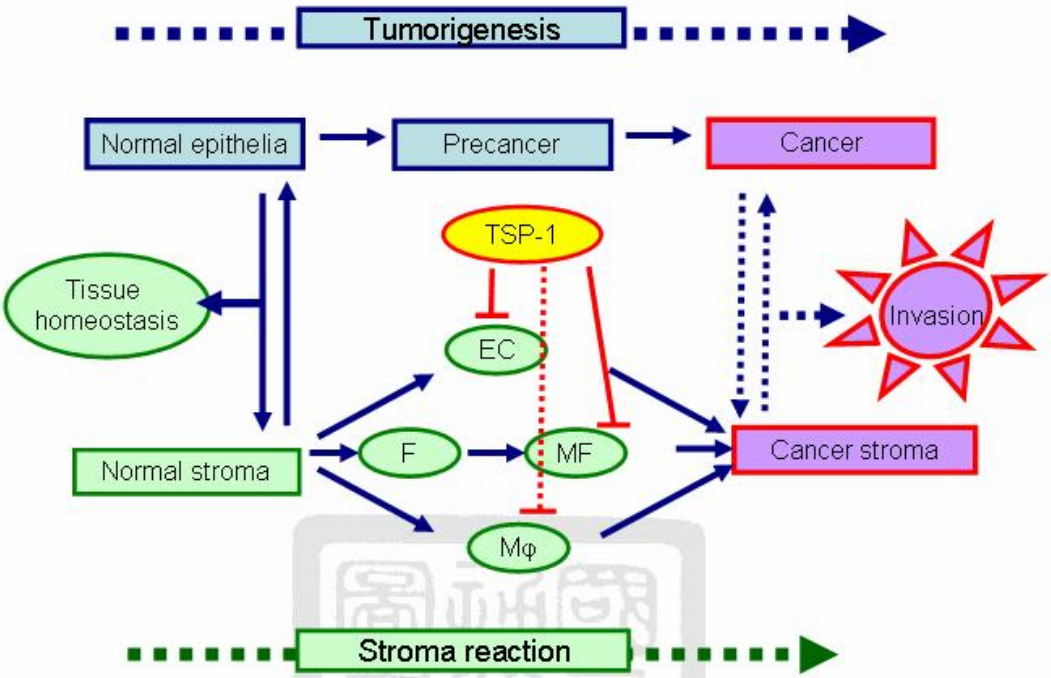

Fig. 8 TSP-1 inhibited cancer progression via inhibiting stroma reaction. Stroma reaction occurs parallel to tumorigenesis. In addition to the epithelial component, host stroma, including endothelial cells, (myo)fibroblasts, and inflammatory cells, e.g. macrophage, etc, plays an active participant. TSP-1 can inhibit angiogenesis status, the migration and invasive ability of myofibroblasts. (F: fibroblast; MF: myofibroblast; Mφ: macrophages).

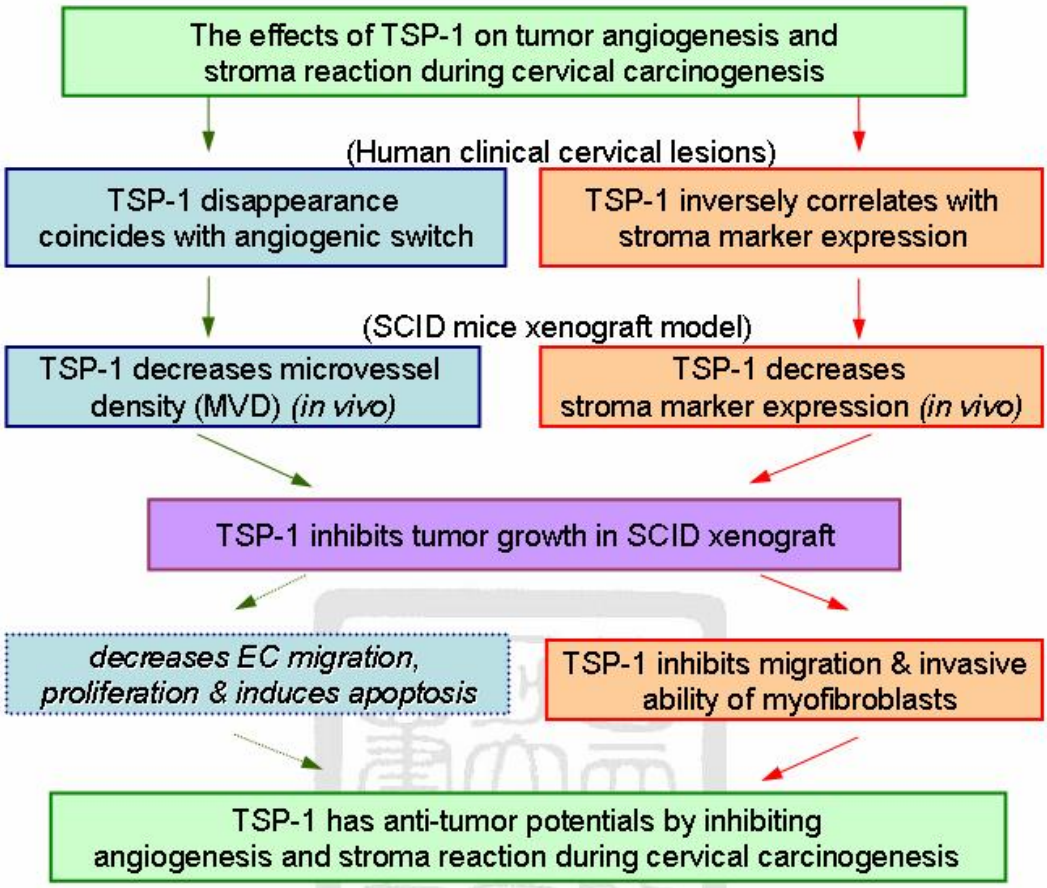

Fig. 9 The flow chart of this study. After obtained the correlative observations from clinical specimens of cervical cancer, we further elucidate the cause-effect relationship between TSP-1 and tumor-stroma interaction. The inhibitory ability to reverse stroma reaction could be partly attributed to the blockage of myofibroblasts from invading cancer. Dotted lines: not performed in this study.

Appendix- supplementary articles and manuscripts

S.1 (Review) Angiogenesis, thrombospondin-1, and cervical carcinogenesis

Authors: Ming-Ping Wu, Cheng-Yang Chou

Journal: Taiwanese J Obstet Gynecol (Taiwan) 44(2):128-138;2005.

Abstract:

Angiogenesis, the growth of new vessels from existing vasculature, plays an essential role in tumor development. The process involves interaction among cancer cells, endothelial cells, and components of the extracellular matrix, and is regulated by the balance of angiogenesis activators and angiogenesis inhibitors. This review profiles some fundamental concepts of angiogenesis, the importance of angiogenesis in cervical neoplasm, and the roles of thrombospondin-1 (TSP1), as an angiogenic inhibitor, in cervical carcinogenesis. The usefulness and limitations of microvessel density (MVD) in evaluation of angiogenic status are discussed. From the recent researches and evolving concepts, it comes up the paradigm shift for anticancer therapy, from conventional cancer-centered chemotherapy to angiogenic or “metronomic” chemotherapy and/or combined angiogenic inhibitors. The epigenetic strategy which views the tumor system as a whole, transcends the cancer gene-centered approach.

S.2 Tumor/normal counterpart microvessel density ratio has a better correlation with clinicopathologic parameters in endometrial carcinoma than tumor microvessel density alone

Authors: Ming-Ping Wu, Yu-Jen Cheng, Yue-Shan Lin, Chieh-Yi Kang, Kuo-Feng Huang, Ching-Cherng Tzeng

Journal: Taiwanese J Obstet Gynecol (Taiwan) 43(3):199-205;2004.

Abstract:

Objective: Angiogenesis is essential for tumor growth and metastasis. Angiogenesis regulators, which are controlled by ovarian hormones, play an important role during endometrial carcinogenesis. The aims of the study were to evaluate: (1) the relationship between angiogenesis and clinico-pathologic parameters; (2) the possible regulatory roles of estrogen and progesterone receptor status in the angiogenesis of endometrial carcinoma.

Materials and Methods: Twenty patients undergoing surgery for endometrial carcinoma were enrolled. The microvessel density (MVD) of the excised tumor was assessed immuno-histochemically. The relationships between the MVDs of the tumor or tumor/normal ratio and clinico-pathologic parameters including histologic grading, myometrium invasion, cervical involvement, lymph node involvement, lympho-vascular space involvement, menopausal status, histologic type, stage, and estrogen progesterone receptor status were analyzed.

Results: MVDs (x 200 fields) of the clinico-pathologic parameters including histologic grading, myometrium invasion, cervical involvement, lymph node involvement, lympho-vascular space involvement, menopausal status, histologic type, stage, and estrogen progesterone receptor status were not significantly different in these twenty patients when only tumor lesions were counted. However, several clinico-pathologic parameters were significantly different when MVDs were

measured by tumor/normal ratio. Significantly, higher MVD tumor-normal ratios (T/N ratios) were noted in tumors of high histologic grade (grade 2 and 3) (1.31 compared with 1.07, P =0.010), with lymphovascular space involvement (1.32 compared with 1.13, P =0.029), with serous type (1.48 compared with 1.13, P=0.021) compared with tumors of histologic grade 1, without lymphovascular space involvement, or with endometrioid type. Meanwhile, the MVD T/N ratios of tumors were higher in tumors of estrogen receptor (-), progesterone receptor (-) and both receptor (-) (1.33 compared with 1.02, p=0.002) as compared with receptor (+), progesterone receptor (+) and both receptor (+).

Conclusion: There was a better correlation between MVD T/N ratios and clinico-pathologic parameters, even when the existence of inborn heterogeneity characters of endometrial cancer or the case number is limited. Meanwhile, the (anti-)angiogenesis factors may have close relationship with the ovarian hormone receptor status in different pathogenesis of endometrial carcinoma.

S.3 TSP-1/ApoE and CD47/ApoE doubly null mice are protected from neointima formation in response to carotid injury but not from atherosclerosis

Authors: Ming-Ping Wu, Loretta Pappen, Dan Ye, Anna Oldenborg, Trey Coleman, Clay Semenkovich, Jack Lawler, Kathryn J Moore, William A Frazier

Journal: In preparing

Abstract:

Nitric oxide (NO) is a pervasive regulator of the vascular system whose functions are critical for vascular homeostasis. Further, a number of vascular pathologies, such as neointimal hyperplasia/restenosis, are ameliorated by increasing NO bioavailability and signaling. Recent data has revealed that TSP-1 and its non-integrin receptor CD47 function to restrain NO-cGMP signaling in vascular cells. Here we have used knockouts of TSP-1 and CD47 on the ApoE-null background (dKO mice fed low or high fat diets) to investigate the role of these proteins in the arterial response to injury after temporary carotid ligation. TSP-1 dKO but not CD47 dKO mice died due to massive lung inflammation on the high fat diet. The morphological response to injury was identical in TSP-1 dKOs on the low fat diet and CD47 dKOs on either diet: neointimal areas were reduced 50% relative to ApoE single null controls, while medial thickening in the dKO strains was not reduced and was the same as controls. However, the thickened media were depleted of cells relative to controls, while the recruitment of macrophages to the neointima was significantly reduced. We also tested CD47-null/C57Bl/6 mice in a hyperinflation/overstretch model and found a similar reduction of neointimal area relative to C57Bl/6 controls. All of these effects shared by both TSP-1- and CD47-null mice are consistent with a unique ligand-receptor relationship of TSP-1 and CD47 and are congruent with those predicted by a model in which TSP-1,

acting via CD47, chronically opposes NO-cGMP signaling in vascular cells.

S.4 Her2/neu overexpression is correlated with TSP-1-related angiogenesis and TSP-1-unrelated lymphangiogenesis in breast cancer

Authors: Ming-Ping Wu, Cheng C. Tsai, Yu-Feng Tian, Cindy Kilpack, Matthew J Morgan, Cheng-Yang Chou, Eing Mei Tsai, Ching-Cherng Tzeng

Journal: In preparing

Abstract:

Her-2/neu overexpression is linked to a poor prognosis and a significant predictor for the response to trastuzumab (herceptin) therapy in breast cancer patients. Recently, Her-2/neu was noted to promote angiogenesis with thrombospondin-1 (TSP-1) as a mediator during cancer development and progression in breast cancer. However, the clinical quantitative evaluation of Her-2/neu and specified localization of TSP-1 is currently lacking. With the assistance of Automated Cellular Imaging System (ACIS), we offered a quantitative evaluation and clinical correlation of Her-2/neu-induced angiogenesis and lymphangiogenesis; and specifically localized TSP-1 in either tumor or stromal area in breast cancer. Fifty-six with invasive ductal or lobular invasive breast cancer were recruited into this study for Her-2/neu (Fig. S1), TSP-1, CD31 (blood vessels) and D2-40 (lymphatic vessel) stains (Fig. S2). High-Her-2/neu group (≥2.0) had less ER, PR and more Ki-67 status, as compared with low-Her-2/neu group (<2.0) (p-value <0.001, 0.001 and <0.001, respectively). High-Her-2/neu group had higher angiogenesis status (microvessel density, MVD-CD31) (13.8±2.4 versus 7.9±3.6, p< 0.001), and higher lymphangiogenesis status (MVD-D2-40) (4.4±1.6 versus 3.0±1.5, p= 0.002). Meanwhile, high-Her-2/neu group had lower TSP-1 expression in tumor area (TSP-1/T) and higher TSP-1 in stromal area (TSP-1/S) (pattern 1, 17/28 cases), while low-Her-2/neu had high TSP-1/T and low TSP-1/S (pattern 2, 23/28 cases). There were two distinct TSP-1 expression patterns with pattern 1

prone to happen in high-Her-2/neu group, pattern 2 to low-Her-2/neu group, respectively. The expression of TSP-1 in tumor or stromal area correlated with different level of angiogenesis, but not lymphangiogenesis (Fig. S3). In conclusion, Her-2/neu affects biological manifestations of breast cancers by increasing both angiogenesis and lymphangiogenesis, which are TSP-1-related and TSP-1-unrelated, respectively. TSP-1 expressed reciprocally in tumor and stroma area, resulting in different angiogenesis status, but not lymphangiogenesis.

Publications during 2000-2008

I. TSP-1 in tumor angiogenesis and stroma reaction

1. Ming-Ping Wu, Ming-Jer Young, Ching-Cherng Tzeng, Chii-Ruey Tzeng, Kuo-Feng Huang, Li-Wha Wu, Cheng-Yang Chou: A novel role of thrombospondin-1 in cervical carcinogenesis: Inhibit stroma reaction by inhibiting activated fibroblasts from invading cancer. Carcinogenesis 2008 (In Press) (SCI, IF: 5.366, Oncology 18/127).
2. Ming-Ping Wu, Cheng-Yang Chou: Angiogenesis, thrombospondin-1, and cervical carcinogenesis. Taiwanese J Obstet Gynecol (Taiwan) 44(2):128-138;2005.
3. Loretta K. Pappan, Ming-Ping Wu, Dan Ye, Anna Oldenborg, Trey Coleman, Clay F. Semenkovich, Jack Lawler, Kathryn Moore, William A. Frazier. ApoE-null mice lacking thrombospondin-1 or its receptor CD47 are protected against neointimal thickening of injured arteries but not against diet-induced atherosclerosis. Cir Res 97:1201-1202;2005. (SCI, IF: 9.854, Cardiac and Cardiovascualr system 2/74)
4. Ming-Ping Wu, Ching-Cherng Tzeng, Li-Wha Wu, Kuo-Feng Huang, Cheng-Yang Chou: The thrombospondin-1 acts as a fence to inhibit angiogenesis that occurs during cervical carcinogenesis. Cancer J 10(1):27-32;2004. (SCI, IF: 2.028, Oncology 78/127)

II. Urogynecology and Minimally invasive Gynecology

1. Ming-Ping Wu: The use of prostheses for pelvic reconstructive surgery: joys or toys? Taiwanese J Obstet Gynecol 2008 (In press)
2. Tian-Ni Kuo, Ming-Ping Wu (Corresponding): Concomitant transvaginal pelvic floor reconstructive surgery with tension free vaginal mesh (TVM) technique

and tendion-free midurethral sling in pelvic organ prolapse (POP) and occult stress urinary incontinence. Taiwanese J Obstet Gynecol 2008 (In press)

3. Ming-Ping Wu, Kuan-Hui Huang: Tension-free midurethral sling surgeries in stress urinary incontinence. Incont Pelvic Floor Dysfun 2008 (In press).
4. Ming-Ping Wu, Hann-Chorng Kuo: Office-based Assessment and Physical Examination in Women with Urinary Incontinence and Pelvic Organ Prolapse. Incont Pelvic Floor Dysfun 1(4):131-136;2007.
5. Yu-Feng Tian, Yue-Shan Lin, Chun-Chieh Chia, Kuo-Feng Huang, Tune-Yie Shih, Kun-Hung Shen, Ming-Ting Chung, Yung- Chieh Tsai, Chien-Hsun Chao, Ming-Ping Wu* (Corresponding) Major Complications in Operative Gynecologic Laparoscopy in Southern Taiwan: a follow-up study. J Minim Invasive Gynecol 14:284-292;2007.
6. Chao-Hsiun Tang, Ming-Ping Wu, Jin-Tan Liu, Herng-Ching Lin, Chun-Chyang Hsu: Delayed Parenthood and the Risk of Cesarean Delivery — Is Paternal Age an Independent Risk Factor? Birth— Issues on Perinatal Care 33(1):18-26;2006. (SCI).
7. Ming-Ping Wu, Chin-Chuan Lin, Yu-Feng Tian, Kuo-Feng Huang , Allen W Chiu: The feasibility of internal bladder retractor in facilitating bladder dissection during laparoscopic-assisted vaginal hysterectomy (LAVH). J Am Assoc Gynecol Laparosc 11(2):283-284;2004. (SCI)
8. Chun-Chieh Chia, Ming-Ping Wu, Kuo-Feng Huang, Chang-Chuan Su: Primary stromal sarcoma of the ovary: a case report. Taiwanese J Obstet Gynecol (Taiwan) 43(2):110-112;2004.
9. Ming-Ping Wu, Chung-Chang Shen, Kuo-Feng Huang, Cheng-Yang Chou: Is laparoscopic high McCall colpopexy effective in treating uterine prolapse with uterine preservation? J Am Assoc Gynecol Laparosc 10(2):307;2003. (SCI)

10. Allen W Chiu, Vijayan Radhakrishnan, Chia-Hsing Lin, Steve K Huan, Ming-Ping Wu: Internal bladder retractor for laparoscopic cystectomy. J Urol 168:1479-1481;2002. (SCI)
11. Ming-Ting Chung, Yue-Shan Lin, Ming-Ping Wu, Kuo-Feng Huang: Laparoscopic surgery for omental pregnancy. J Am Assoc Gynecol Laparosc 9(1):84-86;2002. (SCI)
12. Ming-Ping Wu, Yue-Shan Lin, Cheng-Yang Chou: Major complications in operative gynecologic laparoscopy in southern Taiwan: 1507 cases review. J Am Assoc Gynecol Laparosc 8(1):61-67;2001. (SCI)
13. Ming-Ping Wu, Chau-Su Ou, Shwu-Ling Chen, Ernest YT Yen, Rowbotham R: Complications and recommended practices for electrosurgery in laparoscopy. Am J Surg 179:67-73;2000. (SCI)
14. Ming-Ping Wu, Wen-Yin Chen, Cheng-Yang Chou, Chi-Yu Cho, Ernest YT Yen, Lein-Ray Mo, Wen-Chun Lin, Mei-Hwang, Chao Mei-Yu Chao: Electosurgical injury in laparoscopy. Show Chwan Med J (Taiwan) 2(1):9-15;2000.
15. Wen-Chun Lin, Stephen Tang, Ming-Ping Wu (corresponding author), Man-Pun Yau, Shwu-Ling Chen, Daniel Liu, Jack CR Tsai: The feasibility of using laparoscopy in adnexectomy, appendectomy, cholecystectomy during pregnancy. Adv Obstet Perinatol (Taiwan) 11(2):91-98;2000.
16. Ming-Ping Wu, Shwu-Ling Chen, Chiung-Yu Tseng, Hsi-Yao Chen, Jean-Shi Chen, Tsai-Bei Lin, Mei-Yu Chao, Mei-Hwang Chao: Pregnancy carried to fetal viability in a chronic hemodialysis woman. Adv Obstet perinatol (Taiwan) 11(2):83-90;2000.

III. Educational articles

1. 人工網膜在骨盆底重建手術的角色：優缺點及疑慮 台灣婦產科內視鏡暨微創醫

學會月刊 11:12-14;2007.

2. 中段尿道吊帶術在婦女應力性尿失禁的應用 台灣婦產科內視鏡暨微創醫學會月刊 11:12-14;2007.
3. 抗血管新生療法在人類腫瘤的應用(一) 秀傳醫學雜誌 Show Chwan Med J (Taiwan). 5(3-4):125-136;2004.
4. 抗血管新生療法在人類腫瘤的應用(二) 秀傳醫學雜誌 Show Chwan Med J (Taiwan). 4(3-4):137-148;2004.
5. 骨盆鬆弛之解剖學異常 台灣醫學雜誌 Formosan J Med 8(6):1-9;2004.
6. 復發性應力性尿失禁 台灣醫學雜誌 Formosan J Med 7(1):115-122;2003.
7. 以基本門診評估項目建立婦女尿失禁的臨床診斷 台灣醫界 Taiwan Med J 46(3):25-30;2003.
8. 電刺激治療儀在婦女尿失禁之臨床應用 台灣醫界 Taiwan Med J 45(1):22-24;2002.
9. 遺傳與人類疾病 台灣醫學雜誌 Formosan J Med 5(5):569-575;2001.

簡歷

姓名：吳銘斌 (1964 年)

婚姻：已婚 配偶 陳淑玲 (小兒專科、小兒神經次專科醫師)

育有一子(沛軒 國中一年級) 一女(沛芯 國小四年級)

學歷：國立成功大學醫學院臨床醫學研究所博士班 (2000. 09—2008.05)

美國聖路易華盛頓大學醫學院生化及分子物理所進修 (2004.05—2005.05)

台北醫學院醫學系 (1982.09- 1989.06)

台南一中

台南市立後甲國中

台南市立忠義國小

現任：財團法人奇美醫院婦產部婦女泌尿暨骨盆重建醫學科主任

財團法人奇美醫院教師培育中心副主任

台北醫學大學醫學院婦產學科專任助理教授

台灣婦產科內視鏡暨微創醫學會監事

證照：教育部定助理教授(助理字第 005891 號) 2001. 02

婦產科專科醫師 (婦專醫字 2011 號) 1995.11

婦女泌尿暨骨盆重建專科醫師(婦泌專字第 036 號) 2008. 08

婦產科內視鏡暨微創醫學會骨盆重建醫學組專科醫師(台鏡專字 93 第 035 號)

2004. 08

榮譽：中華民國婦女泌尿暨骨盆重建醫學會暨台灣尿失禁防治協會 2007 年聯合學術

討論會學術論文比賽第二名 (2007. 12. 01)

國科會補助博士生赴國外研究(千里馬專案) (2004. 05-2005. 05)

台南縣醫師公會年度學術研究獎二次 (2003. 11; 2005. 11)

中華民國婦產科醫學會優秀論文獎第二名 (台灣 台中) (2000. 03)

美國婦科腹腔鏡醫學會科學海報展示第一名 (美國 芝加哥) (1996. 09)

聯絡方式：e-mail： mpwu@mail.chimei.org.tw

地址：台南縣 710 永康市中華路 901 號 財團法人奇美醫院 婦產部

電話：(06) 2812811； 傳真：(06)2828928

網頁：http://www.chimei.org.tw/ 永康院區介紹【科部室介紹】

婦女泌尿暨骨盆重建醫學

Curriculum Vitae

Name: Ming-Ping Wu

Marital status: married, spouse: Shwu-Ling Chen, M.D. (Pediatrician)

a son (Daniel), a daughter (Cindy)

Year of birth: 1964

Place of birth: Tainan, Taiwan

Present position:

1. Director, Division of Urogynecology and Pelvic Floor Reconstruction, Department of Obstetrics & Gynecology, Chi Mei Foundation Hospital, Tainan, Taiwan
2. Deputy Chairman, Center of Faculty Development (CFD), Chi Mei Foundation Hospital, Tainan, Taiwan
3. Assistant Professor, Department of Obstetrics & Gynecology, College of Medicine, Taipei Medical University, Taipei, Taiwan

Office address:

Division of Urogynecology and Pelvic Floor Reconstruction, Department of Obstetrics & Gynecology, Chi Mei Foundation Hospital,

No. 901, Chung Hwa Rd., Yung Kang, Tainan 710, Taiwan

Office telephone: (886)-6-2812811 ext 53270

Office fax: (886)-6-2828928

e-mail: mpwu@mail.chimei.org.tw or mpwu.stl@msa.hinet.net

Website:

http://www.chimei.org.tw/main/right/right01/cmh_department/57728/index.htm

Medical education:

2000, 08 – 2008. 05. Ph.D. Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan

1982, 09--1989, 06 M.D., Department of Medicine, Taipei Medical College, Taipei, Taiwan,

Postgraduate training

2007, 05- 2007, 08 Exchange Scholar, Division of Gynecology, Department of Obstetrics & Gynecology, Kaohsiung Chang Gung Memorial Hospital,, Kaohsiung, Taiwan

2004, 05—2005, 05 Research Associate in Department of Biochemistry and Molecular Biophysics (PI: Professor William A. Frazier), School of Medicine, Washington University, St. Louis, MO, U.S.A. (Supported by the “Ph.D. student study abroad program”, National Science Council, Taiwan No. 93-2917-I-006-002)

1999, 08-- 1999, 09 Fellowship in Urogynecology Section, Department of Obstetrics & Gynecology, Mackay Memorial Hospital, Taipei, Taiwan.

1996, 08-- 1996, 09 Fellowship in Laparoscopic Surgery Section, Department of Obstetrics & Gynecology, Northwest Hospital, Seattle, WA, U.S.A.

1991, 07-- 1995, 06 Resident, and Chief Resident, Department of Obstetrics & Gynecology, National Cheng Kung University Hospital, Tainan, Taiwan.

1988, 06-- 1989, 05 Internship, Mackay Memorial Hospital, Taipei, Taiwan.

Employment:

2007, 11 till now, Director, Division of Urogynecology and Pelvic Floor Reconstruction, Department of Obstetrics & Gynecology, Chi Mei Foundation Hospital, Tainan, Taiwan

2000, 06 till now, Attending Physician, Department of Obstetrics & Gynecology, Chi Mei Foundation Hospital, Tainan, Taiwan

1998, 11-- 2000, 05 Chairman, Gynecologic Laparoscopy Research and Development Unit, Department of Obstetrics & Gynecology, Tainan Municipal Hospital, Tainan, Taiwan

1995, 07-- 2000, 05 Attending Physician, Department of Obstetrics & Gynecology, Tainan Municipal Hospital, Tainan, Taiwan

1991, 07-- 1995, 06 Resident, and Chief Resident, Department of Obstetrics & Gynecology, National Cheng Kung University Hospital, Tainan, Taiwan

License and certification:

Subspecialist of Urogynecology and Pelvic Floor Reconstruction, Taiwan Urogynecology Association, 2007, 08

Subspecialist of Pelvic Floor Reconstructive Medicine, The Taiwan Association for Minimally Invasive Gynecology, 2004, 08

Specialist, The Taiwan Association of Obstetrics and Gynecology, 1995, 11

Instructor, The Taiwan Association of Gynecologic Endoscopists, 1998, 09

Instructor, The Society of Ultrasound in Medicine, R.O.C.

Academic appointments:

Assistant Professor, Department of Obstetrics & Gynecology, College of Medicine, Taipei Medical University, Taipei, Taiwan. 2004, 02.

Assistant Professor, Chung Hwa University of Medical Technology, Tainan, Taiwan. 2001, 01.

Lecturer, Department of Obstetrics & Gynecology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, 2000, 08

Lecturer, Chung Hwa College of Medical Technology, Tainan, Taiwan 1998, 07

Awards and honor:

1.Second prize, The Scientific Papers, 2007 Annual Conjoin Meeting of The Taiwan Urogynecology Association and Taiwan Continence Society, 2007 Dec 01-02. An *in vitro* extracellular matrix model to evaluate the biological behaviors of host stroma cell responses to different synthetic prostheses.

2.Third prize, the Excellent Scientific Writing, Chi Mei Foundation Hospital, Tainan, Taiwan, October, 2005. Main article entitled: “The thrombospondin-1 acts as a fence to inhibit angiogenesis that occurs during cervical carcinogenesis. Cancer J 10(1):27-32;2004.”

3.Annual Best Academic Research Achievement Award, Tainan County Medical Association, Tainan County, Taiwan, November, 2005.

4. Recipient of the grant for ‘Ph.D. Student Study Abroad Program 2004-2005’ sponsored by National Science Council, Taiwan. No. 93-2917-I-006-002.

5. International who’s who member 2005. Category: Professionals.

6. Organizing faculty and invited presentation. Asian American Multi-Specialty Congress of Laparoscopy and Minimally Invasive Surgery organized by Society of Laparoendoscopic Surgeons (SLS) Feb. 2004, Honolulu, Hawaii, U.S.A.

7.Annual Best Academic Research Achievement Award, Tainan County Medical Association, Tainan County, Taiwan, November, 2003.

8.Second prize, the Excellent Scientific Writing, Chi Mei Foundation Hospital, Tainan, Taiwan, December, 2002. Main article entitled: “Major complications in operative gynecologic laparoscopy in southern Taiwan: 1507 cases review. J Am Assoc Gynecol Laparosc 8(1):61-67;2001.”

Research Grants:

1. The roles of thrombospondin-1 in tumor angiogenesis and tumor-stroma microenvironement; and in vivo and in vitro study. PI. NSC 95-2314-B-384-006- National Science Council, R.O.C. 20060801—20070731.
2. The anti-tumor potential of thrombospondin-1 through its receptor CD47, integrins in breast and cervical cancer; and its downstream regulation of MMPs. PI 95CM-TMU-03, Chi Mei Foundation Hospital and Taipei Medical University 2006-02-01—2007-0131.
3. The in vivo and in vitro study of CD36 in associated with its ligands, TSP-1 and TSP-1-derived peptides, in angiogenesis inhibition; also studies on the CD36-SHP-1 signaling pathway and induced endothelial cell apoptosis. PI 93CM-KMU003 Chi Mei Foundation Hospital and Kaohsiung Medical University 20050301—20060228.
4. The molecular mechanisms of angio-inhibitory effects of TSP-1 in cervical carcinogenesis; studies on the upstream regulation signaling, the interaction with VEGF, and the TSP-1-integrin-IAP complex. PI. NSC 93-2314-B-384-006- National Science Council, R.O.C. 20040801—20050731.
5. The application of RAN interference (RNAi) in anti-angiogenesis therapy during cervical carcinogenesis. PI. 93CM-TMU-02 Chi Mei Foundation Hospital and Taipei Medical University. 20040101-20041231.
6. The angio-inhibitory effects of TSP-1 in cervical carcinogenesis; and its molecular mechanisms with CD36, TGF-b, and MMP-9. PI. NSC 92-2314-B-3B4-012- National Science Council, R.O.C. 20030801-20040731.