รายงานวิจัยฉบับสมบูรณ์

โครงการ : บทบาทของโปรตีนเบต้า-1 อินทีกริน ต่อ กลไกการกระตุ้นเอนไซม์เอ็ม-เอ็ม-พี-2 ในการตอบสนองต่อการกระตุ้นของคอลลาเจน-1 ในเซลล์มะเร็งเต้านม

: The involvement of β1 Integrin on MMP-2 activation mechanism in response to Type I Collagen stimulation in Breast cancer cells.

โดย

ดร.กุลรัตน์ บริรักษ์วาณิชย์

พ.ศ. 2554

สัญญาเลขที่ TRG5180002

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
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Dr. Kulrut Borrirukwanit

Abstract

Project Code: TRG5180002
Project Title: The involvement of β1 Integrin on MMP-2 activation mechanism in response to Type I Collagen stimulation in Breast cancer cells.
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Integrins are a family of transmembrane adhesion receptors that mediate cell-matrix and cell-cell interactions, affecting tumour proliferation, migration and metastasis. MMP-2 is endopeptidases enzyme that also closely correlated with tumor metastasis. Type I collagen is the most abundant ECM protein in the body, which capable to activate MMP-2 through β1 integrin, a partner of the collagen-binding integrin receptors. However, the involvement of β1 integrin in collagen-stimulated MMP-2 activation is still unclear. Therefore, the aim of this study was to elucidate the involvement of β1 integrins in the Col I-induced MMP-2 activation mechanism. The siRNA technology was used and six different β1 intgerin siRNAs were designed. Three candidate of these siRNAs included β1/4, β1/5 and β1/6 were efficient knockdown β1integrin both at mRNA and protein levels. However, the best effective was β1/6 siRNA which completely knocked down β1integrin and most affect on biological function. This specific of β1 integrin siRNA showed a particular target site for the β1 integrin gene which exist good efficient knockdown of β1 integrin at appropriate time. At completely depleting of β1 integrin, reduction of Col I-induced MMP-2 activation was observed which depends on dose response threshold effect. Abrogation of this β1 integrin siRNA reduced MMP-2 activation via up-regulation of MT1-MMP which specific to type I collagen response. Collectively this reveal provides insight into certain mechanism involved in the reciprocal regulation of β1 integrin and MMPs of tumor cells.

Keywords: Integrin/ Matrix Metalloproteinase/ MMP-2 activation / Type I Collagen/ Breast Cancer/ Invasion and Metastasis

บทคัดย่อ

รหัสโครงการ: TRG5180002

ชื่อโครงการ: บทบาทของโปรตีนเบต้า-1 อินทีกริน ต่อ กลไกการกระตุ้นเอนไซม์เอ็ม-เอ็ม-พี-2 ในการตอบสนองต่อการกระตุ้นของคอลลาเจน-1 ในเซลล์มะเร็งเต้านม

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อินทีกรินเป็นโปรตีนที่ผนังเยื่อหุ้มเซลล์ ทำหน้าที่ในการเป็นตัวรับและยึดเกาะระหว่างเซลล์และเซลล์กับเมทริก โปรตีนอินทีกรินมีบทบาทสำคัญต่อการเจริญเติบโต การเดินทาง และ การแพร่กระจายของเซลล์มะเร็ง และเอ็ม-เอ็ม-พี-สองเป็นเอ็นไซม์ในกลุ่มเอนโดเปปติเดส ที่มีบทบาทสัมพันธ์กับการแพร่กระจายของเซลล์มะเร็งเช่นกัน โดยพบว่าคอลลาเจน-หนึ่งซึ่งเป็นโปรตีนที่พบมากในเมทริกของร่างกายนั้นสามารถกระตุ้นการทำงานของเอ็นไซม์เอ็ม-เอ็ม-พี-สอง ผ่านโปรตีนอินทีกรินเบต้า-หนึ่ง ซึ่งเป็นตัวรับของคอลลาเจน-หนึ่งบนผนังเซลล์ได้ แต่บทบาทของโปรตีนอินทีกรินเบต้า-หนึ่งต่อการกระตุ้นเอ็นไซม์เอ็ม-เอ็ม-พี-สองเมื่อถูกกระตุ้นด้วยคอลลาเจน-หนึ่งนั้นยังไม่ทราบแน่ชัด การวิจัยครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาบทบาทของโปรตีนอินทีกรินเบต้า-หนึ่ง ต่อการกระตุ้นของเอนไซม์เอ็ม-เอ็ม-พี-สองที่ตอบสนองต่อการกระตุ้นด้วยคอลลาเจน-หนึ่ง โดยใช้วิธี เอสไอ-อาร์เอนเอ เพื่อทำให้มีการยับยั้งการแสดงออกของโปรตีนอินทีกรินเบต้า-หนึ่ง ซึ่งได้ออกแบบเอสไอ-อาร์เอนเอจำนวน 6 ชนิด และพบว่า 3 ชนิด ได้แก่ เบต้า-หนึ่ง/สี่ เบต้า-หนึ่ง/ห้า และ เบต้า-หนึ่ง/หก มีประสิทธิภาพสูงในการยับยั้งการแสดงออกของอินทีกรินเบต้า-หนึ่งทั้งในระดับอาร์เอนเอและโปรตีน แต่ชนิดที่มีประสิทธิภาพสูงมากที่สุด คือ เบต้า-หนึ่ง/หก เอสไอ-อาร์เอนเอ เนื่องจากสามารถยับยั้งการแสดงออกของโปรตีนอินทีกรินเบต้า-หนึ่งได้สมบูรณ์และมีผลต่อหน้าที่ของโปรตีนอินทีกรินเบต้า-หนึ่งมากที่สุด ซึ่งเบต้า-หนึ่ง/หก เอสไอ-อาร์เอนเอนี้มีความจำเพาะต่อตำแหน่งของการยับยั้งการแสดงออกของอินทีกรินเบต้า-หนึ่งโดยตรง ที่พบว่าสามารถลดการการกระตุ้นของเอนไซม์เอ็ม-เอ็ม-พี-สองได้ โดยผลนี้ขึ้นกับระดับของการตอบสนอง ที่มีต่อระดับการยับยั้งการแสดงออกของยีนอินทีกรินเบต้า-หนึ่งโดยเอสไอ-อาร์เอนเอ ผลของการยับยั้งการแสดงออกของโปรตีนอินทีกรินเบต้า-หนึ่งด้วยเอสไอ-อาร์เอนเอนั้นเนื่องจากมีผลต่อกลไกการควบคุมการทำงานของเอนไซม์เอ็มทีวัน-เอ็ม เอ็ม พี และส่งผลต่อการลดการกระตุ้นเอ็นไซม์เอ็ม-เอ็ม-พี-สอง จากผลการศึกษาครั้งนี้ทำให้เข้าใจในกลไกของการควบคุมและตอบสนองร่วมกันของโปรตีนอินทีกรินเบต้า-หนึ่ง และเอ็นไซม์เมทริกเมทัลโลโปรติเนสของเซลล์มะเร็ง

คำหลัก : อินทีกริน, เมทริก เมทัลโลโปรติเนส, การกระตุ้น เอ็ม-เอ็ม-พี-2, คอลลาเจน-1, มะเร็งเต้านม, การลุกลามและการแพร่กระจาย

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CHAPTER 1
INTRODUCTION

Introduction

Cancer is the leading cause of death in the World**.** Although advances have been made in reducing mortality rates and improving survival, most deaths from cancer are due to metastatic disease [1]. Metastasis is a multi-step process which includes tumor cell migration, intravasation, adhesion to the vessel wall, extravasation, infiltration and proliferation in the target tissue. However, these basic steps occur in the context of different organs, emerge at different rates and are clinically managed in different ways depending on the type of cancer. Breast cancer is the second leading cause of death from cancer among women that displays an invasive phenotype [2]. The principles sites of metastasis in breast cancer are bone, lung, liver and brain [1]. The basic steps of metastasis include the progression of the primary tumour towards invasive carcinoma and dispersion of cancer cells through the lymphatic or blood vessels. Circulating cancer cells that survive could infiltrate distant organs. Infiltrated cells in the new microenvironment might proceed towards overt metastasis with or without an intervening period of latency. These steps are supported by functions of the cancer cells themselves or of the tumour stroma [3]. The metastatic process is often depicted as a multistage process in which malignant cells spread from the tumour of origin to colonize distant organs [4, 5]. Thus, in cancer, the extracellular matrix (ECM) within and surrounding a tumor undergoes continuous remodeling. The importance of proteolytic enzymes in facilitating invasive tumor growth had been recognized, with a hypothetical secretion by fibroblasts [6], and subsequently identified as hyaluronidases, serine proteinases, and matrix metalloproteinases. Recently, matrix metalloproteinases (MMPs) have been regarded as major critical molecules assisting tumor cells during metastasis [7, 8]. It was postulated from the earliest work on MMPs in

cancer, there has been a clear connection between MMPs, ECM degradation and cancer cell invasion [9, 10].

MMPs family consists of more than 26 endopeptidases that share homologous protein sequences, with conserved domain structures and specific domains related to substrate specificity and recognition of other proteins [11]. Most members the MMP family are organized into three basic, distinctive and well-conserved subsets based on specific domains and structural considerations: an amino-terminal propeptide, a catalytic domain and a hemopexin-like domain at the carboxyl-terminal. Based on their domain structure and substrate specificities, the MMPs have been categorized into the subgroups collagenases, stromelysins, gelatinases, membrane type metalloproteinases (MT-MMPs), and others [8]. The strong correlations between altered expression of MMPs at the protein and mRNA levels in different human cancers with poor disease prognosis have been already presented in a number of recent summaries [8, 13]. Comparative studies of MMP expression frequently demonstrate significant elevation of MMP levels from tumor cells in culture to primary tumors and to metastases in animal models. Recently, the role of MMPs in malignancy has been reviewed [12-15]. Stromelysin 3 expression is associated with human breast cancer progression, while matrilysin, but not stromelysin 1 or stromelysin 2, is prominent in human gastric and colonic carcinomas [16, 17]. Furthermore, stromelysin-2 mRNA was localized in tumor cells arranged along disrupted basement membranes [17]. Interstitial collagenase (MMP-1) and type IV collagenases (MMP-2, MMP-9) were first isolated from tumors, where it was generally considered that the enzymes were produced by the tumor cells to degrade interstitial collagen and basal laminae, respectively, to create space for invasive growth [18-20]. Overall, retrospective analysis of MMP expression in cancer patients, indicate that the presence or elevated expression of many MMPs, including MMP-1, -2, -3, -7, -9, -13, -14, in both primary tumors and /or metastases, are positively associated with tumor progression [12]. In contrast, human breast tumor cells that acquired the ability to

metastasize had dramatically reduced expression of MMP-8 compared to their non-metastatic counterpart [21]. Indeed, expression of both individual MMPs and their endogenous inhibitors (TIMPs) has been correlated with tumor progression. Therefore, MMP-9 and TIMP-1 have been found both elevated in the serum of patients with lung carcinomas [22]. Moreover, simultaneously elevated expression of TIMP-1 and TIMP-2 correlated with cancer progression and poor prognostic factors in cancer patients [23]. Although several MMPs have been involved in tumor progression, MMP-2 is one of the key enzyme that had been focused on due to its ability to digest type IV collagen, a major component of basement membranes and also type I collagen, the most abundant ECM protein in the body [24, 25].

MMP-2, also known as gelatinase A or type IV collagenase, is the most widely expressed of all the MMPs and is found in most tissues and cells. The previous studies demonstrated that, MMP-2 is associated with tumor tissues and the appearance of active MMP-2 is closely correlated with tumor metastasis [7, 26]. Almost all cancers expressed MMP-2 mRNA throughout the tumor in stromal cells close to noninvasive tumor clusters and well-differentiated invasive cancer cells [12]. Knock-out mice for MMP-2 show reduced tumor angiogenesis resulting in reduced tumor growth [27]. Moreover, MMP-2 has been shown to be a prognostic factor in different malignancies such as ovarian cancer, gastric carcinoma and stage I non-small cell lung carcinoma [28, 29]. It is clearly evidence that, all MMPs are secreted as inactive zymogens and are activated by cleavage of the N-terminal pro-domain [30]. Most MMPs are activated by tissue or plasma serine proteases, like plasmin. The propeptide sequence is cleaved at basic amino acid motifs and subsequently autocatalytical activation of the MMPs is induced. Thus, many cell types constitutively synthesize MMP-2 as a latent proenzyme, which requires proteolytic removal of the propeptide for activation. Three mechanisms for proMMP-2 activation have been described at this moment; autocatalytical activation, TIMP-2 dependent activation and cell-surface associated urokinase-type plasminogen activator (uPA)/

plasmin system dependent activation [31, 32]. The mechanisms differ substantially, but have a characteristic phenomenon. That is, all three mechanisms of activation involve processing of pro-MMP-2 to an intermediate form and the intermediate form is then further processed to the active MMP-2. Indeed, pro-MMP-2 is not readily activated by general proteinases. The main activation of pro-MMP-2 proceeds on the cell surface and is mediated by a tri-molecular complex of MT1-MMP, TIMP-2 and MMP-2. Pro-MMP-2 forms a tight complex with TIMP-2 through its C-terminal domain, permitting the N-terminal inhibitory domain of TIMP-2 to bind MT1-MMP on the cell surface [33, 34]. This MT1-MMP-TIMP-2-MMP-2 complex is then presented to an adjacent free MT1-MMP for initial cleavage of MMP-2 generating the activation intermediate, which further converts to the mature MMP-2 form via autocatalytic processing [35]. Moreover, the activation of pro-MMP-2 by MT1-MMP is considered to be a critical step during cancer cells traverse the basement membrane to achieve invasion and metastasis [36].

Membrane-type MMPs (MT-MMPs) is one of the subgroup of MMPs that differ from other MMPs since they are membrane anchored either by a transmembrane domain or glycosylphosphatidylinositol (GPI) linkage at the carboxyl terminus [37, 38]. Membrane type 1- MMP (MT1-MMP) is the first discovered member of the MT-MMP, encoding a 63-kDa enzyme that also known as MMP-14 [39]. MT1-MMP with transmembrane and cytoplasmic domains is a unique cell surface activator of pro-MMP-2 through the formation of trimolecular complex with TIMP-2 (tissue inhibitor of metalloproteinases) [39]. In addition, MT1-MMP has a large number of substrates including ECM and non-ECM molecules including collagens I, II, and III, gelatin, laminins 1 and 5, fibronectin, vitronectin, aggrecan, fibrin, and lumican [14]. Recently, MT1-MMP is regarded critical roles during tumor malignancy and is one of the best-validated proteolytic enzyme targets on cancer cells. MT1-MMP also plays a unique role in promotion of cell invasion by pericellular collagen degradation, since overexpression or downregulation of other

soluble MMPs such as MMP-1, -2, -8, -9, and -13 did not affect either invasive growth of tumor cells in 3-dimentional collagen gels *in vitro* or invasion activity *in vivo* in the CAM assay [40, 41]. Silencing of endogenous MT1-MMP markedly decreases invasive and migratory properties of breast cancer cells [42]. Similarly, my previous work indicated that introduction of MT1-MMP into poorly invasive MCF-7 breast cancer cells renders those cells more invasive in vitro and more tumorigenic in vivo [43]. Thus, cell surface MT1-MMP is a potent modifier of the tissue microenvironment, expression of which is closely associated with invasion and tumor growth advantage in animal models and cancer progression in patients [37-39].

Although MT1-MMP mediates the activation of pro-MMP-2, certain ECM molecules such as collagen type I can stimulate MMP-2-activation via MT1-MMP activity in several cell types [25, 44-46]. Collagen is a major ECM component that plays an important role in maintaining tissue architecture and in forming a stable scaffold for cells [14]. Type I collagen (Col I) is cleaved into characteristic 3/4 and 1/4 fragments by collagenolytic MMPs, including collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), MMP-2 (gelatinase A) and membrane-type 1-MMP (MMP-14). Among the collagen remodeling enzymes, MT1-MMP-mediated cleavage of Col I, thus one of the important functions of MT1-MMP is its collagen degrading activity. For MT1-MMP to cleave collagen on the cell surface, the enzyme is also required to be in a dimeric state, again disruption of dimerization of MT1-MMP effectively inhibited its collagenolytic activity [47]. Homodimerization of MT1-MMP has emerged as an important mechanism to regulate two major activities of MT1-MMP on the cell surface, pro-MMP-2 activation and collagen degradation. Col I can activate MMP-2 through 2 distinct mechanisms, increase MT1-MMP mRNA and protein levels, thereby activating MMP-2 (transcriptional response). Moreover, an alternate pathway has been observed more recently in which Col I increases the activity of pre-existing MT1-MMP molecules in order to activate MMP-2 [48]. Recent study also demonstrated that Col I induced MMP-2 activation via MT1-MMP by

blocks MT1-MMP internalization through a clathrin-coated pit-mediated mechanism involving the HPX domain of MT1-MMP, thereby stimulating MMP-2 activation [49]. In addition, another molecule is adhesion molecules that act as cell surface receptors that also involve in MMP-2 activation. The α2β1 integrin, a collagen binding receptor, thought to be implicated in MMP-2 activation that response to type I collagen stimulation and promote tumor invasion and metastasis.

Integrins are a family of transmembrane adhesion receptors that mediate cell-matrix and cell-cell interactions. These molecules are α/β heterodimers, composed of one of 19 α and 8 β subunits that interact noncovalently to form 25 different heterodimeric receptors [50]. Both the α- and β- subunits are integral membrane glycoproteins containing long extracellular domains that constitute the ligand binding regions. Thus, ligand binding specificity depends mostly on the specific α- and β- subunit chains present in the heterodimer [51]. Thus integrins play an important role in ECM and cell-cell interactions and function as bidirectional transducers of extracellelar and intracellular signals. In all cancer cell types, an altered integrin expression pattern allows cancer cells to recognize various matrices and may also lead to altered cell signaling and gene expression. Although the expression of integrin receptors is altered in malignant compared to normal cell, most tumors maintain normal expression of at least some of their integrins. The changes in integrin expression and the cell surface distribution are specific to the tumor cell type. Carcinomas of the breast, prostate and colon exhibit a loss of α3β1, α2β1, α5β1 and α6β4 integrin expressions, however, the highly malignant sarcomas overexpress the α2β1 integrin receptor, where it is believed to play a role in metastasis [52, 53]. Furthermore, the α2β1 integrin collagen and laminin receptor is highly expressed on the epithelium of ductules of normal breast tissue and benign fibroadenomas. In contrast, a marked decrease in α2β1 integrin expression, together with loss of expression of the estrogen receptor, was found on poorly differentiated breast adenocarcinoma cells. Also, in mammary tumor progression, the

expression of α2β1 integrin and α3β1 integrin were present in non-neoplastic and fibroadenomas but were low or absent in invasive mammary carcinomas [52, 53].

The α2β1 integrin has a positive effect on collagen gene expression [54, 55]. Culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of MMP-2 [44-46]. It has been demonstrated in ovarian cancer cells cultured in collagen gels that a strong pro-MMP-2 activation response can be mimicked by clustering of β1 integrin receptor, using β1 antibodies immobilized on beads and thus implicating β1 intergin in this process [54, 55]. However, in contrast, cultivation in three-dimensional (3-D) collagen suppresses the proliferation of numerous cell types. This growth-suppressive signal may be mediated by integrins, as integrin α2β1 mediates fibril collagen-induced inhibition of cell growth [58], and 3-D collagen induces down-regulation of cell-matrix adhesion proteins such as FAK, talin, and paxillin, which is blocked by integrin α2β1-neutralizing antibody [57]. The molecular details of the affinity modulation of α2β1integrin is well understood and involve the linkage of the α1 subunit to the cytoskeleton via talin, which facilitates an interaction between the α and β subunit polypeptides. This then leads to the unfolding of the α2 polypeptide and exposure of the α2I domain, the specific region of the complex that lead α2β1 integrin interacts with collagen [58]. However, previous studies also indicated that Collagen-induced MMP-2 activation occur either directly or indirectly through integrin signaling [25, 59]. Recent study indicated that inhibition of integrin β1 function and expression did not affect 3-D collagen-induced cell surface localization of MT1-MMP and MMP-2 activation, and strongly suggest that 3-D collagen scaffolding provide the direct and multivalent interaction with MT1-MMP, by which MMP-2 activation occur in abundant cell surface MT1-MMP-dependent manner, rather than a manner regulated by matrix stiffness and integrin β1 function [60]. In contrast, previous studies have indicated that cellular interaction with type I collagen is mediated largely through integrin α1β1, α2β1 and α3β1 receptors,

cross linking of integrin β1 could activate MMP-2 in ovarian carcinoma cells, suggesting direct involvement of integrin signaling in MMP-2 activation [25, 59]. The results of the present study, it seems apparent that collagen might directly interact with MT1-MMP, leading to increased surface expression of MT1-MMP and MMP-2 activation. Considering these reports, the involvement of β1 integrin in MMP-2 activation in response to type I collagen stimulation is still not precisely understood. Moreover, MMP-2 is not only important for degradation of denatured, then cleaved type I collagen but also has direct activity against type I collagen [61]. Because type I collagen is a major component of stromal tissue, and both normal and tumor cells digest collagen fibrils to grow or to invade connective tissues. Thus, type I collagen is recommended for the preferred substrate rather than Matrigel in studies investigating cancer invasive behaviors. This study focus on investigation of β1 integrin involve in MMP-2 activation in response to collagen type I stimulation in breast cancer cells model, to better approximate critical interactions and events associated with metastasis mechanism.

Aims and Objectives.

Aim: To investigate the involvement of β1 Integrin on MMP-2 activation mechanism in response to type I Collagen stimulation in breast cancer cells.

Hypothesis: That β1 integrin is necessary for MMP-2 activation in response to type I collagen stimulation in breast cancer cells.

Specific Objectives:

Objective 1: To investigate that knockdown of β1 intergin expression involves in MMP-2 activational responses to type I collagen stimulation in breast cancer cells.

Hypothesis 1: That knockdown of β1 integrin expression reduces MMP-2 activational responses to type I collagen stimulation in breast cancer cells.

Objective 2: To investigate the mechanism of abolishment of β1 integrin expression specifically reduces Type I collagen-induced MMP-2 activation in breast cancer cells.

Hypothesis 2: That abolishment of β1 integrin expression specifically reduces Type I collagen-induced MMP-2 activation in breast cancer cells.

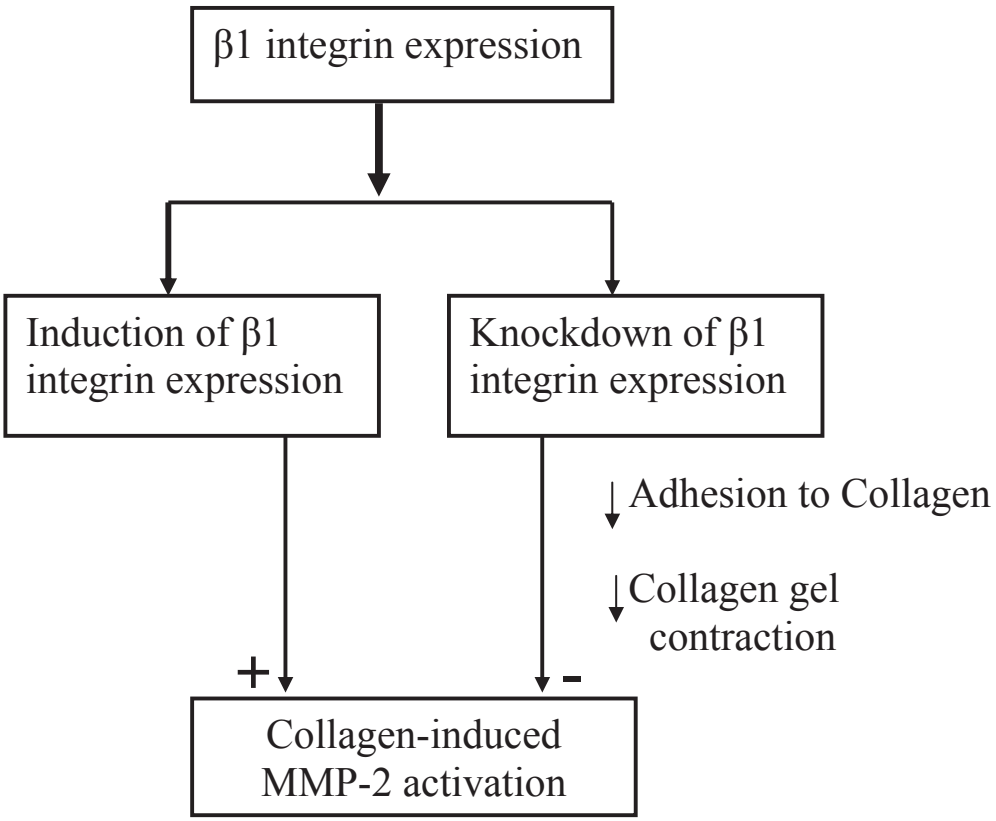


Figure 1: Summary diagram of Research objectives and hypotheses

CHAPTER 2
LITERATURE REVIEW

Breast Cancer Biology

Human cancer is a global health burden responsible for one in eight deaths worldwide [62]. Breast cancer is the most common malignancy with the highest incidence among women in the world. Metastasis is the major reason for breast cancer-related deaths [63]. Indeed, the term cancer was used to describe the crab-like gross appearance of malignant tumors with their tendrils of tumor tissue extending into the surrounding host tissue. Since the time of Hippocrates, it has been recognized that malignant tumors invade and destroy surrounding tissues and metastasize. It is know that defects in the source of molecules that regulate the cellular proliferation machinery are common in tumor cells. Cancer develops through a multi-step process in which normal, healthy cells in the body go through stages that eventually change them to abnormal cells that multiply out of control.

Breast tissue is particularly sensitive to developing cancer for several reasons. The female hormone estrogen stimulates breast cell division. This division can increase the risk of making damage to DNA permanent. Furthermore, breast cells are not fully matured in girls and young women who have not had their first full-term pregnancy. Breast cells that are not fully mature bind carcinogens more strongly and are not as efficient at repairing DNA damage as mature breast cells [64]. Most cancers of the breast initiate in the ductal or lobular tissue and as such would be classified as carcinoma or adenocarcinoma. Approximately 80% of all diagnosed breast cancers in the United States are of the ductal subtype [65]. More rarely, tumors can form in the stromal tissue which tumors deriving from the stromal (connective tissue) are called sarcomas. The tumors that form can be contained entirely within the duct or lobule and are then referred to as being term of in situ. A tumor originating in the ductal epithelia would therefore be a ductal carcinoma in situ (DCIS). These tumors are not invading

into surrounding tissue, however, that is certainly behavior that they can evolve. More aggressive tumors can invade into local tissues and eventually metastases to distant organ sites. Thus, There are several types of breast cancer, the most common is ductal carcinoma, which begins in the lining of the milk ducts of the breast. Another type, lobular carcinoma, begins in the lobules where breast milk is produced. Finally, if malignant tumor invades nearby tissue, it is known as infiltrating or invasive cancer. Since, the majority of breast cancer deaths are due to metastasis. Breast cancer that is detected prior to metastatic spread has considerably better prognosis than cancer that has already spread. Thus, if significant impact is to be made on the mortality figures for breast cancer, a better understanding of tumor progression and the process of metastasis in breast cancer are important.

Tumor Development and Progression

Normal cells in the body communicate with each other and regulate each other's proliferation. Cancer occurs when, for unknown reasons, cells become abnormal and divide without control or order [66]. All parts of the body are made up of cells that normally divide to produce more cells only when the body needs them. When cancer occurs, cells escape the normal controls on their growth and keep dividing even when new cells are not needed. This escape from control can happen through a variety of pathways.

Tumors can develop in different stages, these stages may or may not eventually lead to invasive and metastatic cancer. In most cases it takes many years for cancer to develop. Early detection of any tumor is important because it increases the chances of removing the cancer before it becomes life-threatening. Tumor development can be viewed as an evolutionary process where cells are constantly subjected to mutations. Eventually, altered genes and uncontrolled growth may produce a tumor that can be benign or malignant. Malignant tumor, further damage can lead to cells that break away from the primary tumor and form cancers at other sites in the body, known as the term of

metastasis. The three stages in cancer formation are initiation, promotion, and latency; they are as follow (Figure 2):

1. Initiation stage

The current predominant view of cancer is that of a disease involving irreversible genomic change. This genomic changes encompassing single mutations in specific genes, or alteration, amplification, or loss of large regions of the genome [67]. Genetically altered cells or Mutations in DNA, is defined as a change in the genetic code, which can happen by subtracting from, adding to, or rearranging the original code [63]. Mutations can happen randomly within the cell's DNA, but they can also be induced. Mutations in a gene may interfere with its ability to make a functional signal, or cause it to code for a protein that sends an incorrect signal to the cell. Most mutations are repaired by the cell, but in rare cases mutations do not get repaired. If a mutation is not repaired before a cell copies its DNA and divides into two cells, then the mutation is passed on to the two new daughter cells and becomes permanent. Mutations in most of a cell's DNA have no effect on whether the cell will become cancerous. However, the protein signals coded by a very small proportion of the total genes in each cell regulate cell growth and division. These regulatory genes include the two groups of genes called proto-oncogenes and tumor suppressor genes. In the past 25 years, a majority of cancer studies have focused on examining functional consequences of activating and/or inactivating mutations in critical genes implicated in cell cycle control. These studies have taught us a great deal about the functions of oncogenes and tumor suppressor genes and the signaling pathways regulating cell proliferation and/or cell death [68]. A series of mutations in the DNA of either and/or both groups of these growth controlling genes can eventually lead to cancer. This leads to more cells with the same mistake, and begin tumor development that may be take in several years. However, such studies have largely ignored the fact that cancers are heterogeneous cellular entities whose growth is dependent upon

reciprocal interactions between genetically altered initiated cells and the dynamic microenvironment in which they live [68].

2. Promotion stage

The initiation stage is overcome of the genomic alterations , which emerge of the initial cancer cells. This genomic alterations are irreversible and persists in otherwise normal tissue indefinitely until nonspecific stimulation occurs, typically following exposure of initiated cells to chemical irritants, such as phorbol esters, or from exposure to factors released at sites of chronic inflammation [70, 71]. Thus, during the promotion stage, the preneoplastic cell is stimulated to divide or is given preferential selection. A promoter is an agent that by itself does not cause cancer, but once the initiating carcinogenic event has occurred, it promotes or stimulates the cell containing the original damage [68]. Environmental and host factors have roles in cancer promotion, such as viral infection and chemical exposure. The National Toxicology Program lists 148 chemical agents and groups known to be carcinogenic in humans, including asbestos, benzene, vinyl chloride, nickel, soots, tars, formaldehyde, DDT, saccharin, and urethane [72]. In fact, Virchow hypothesized that cancer originates at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants (promoters) enhance cell proliferation largely because of the tissue injury and ensuing inflammation that they cause. When tissues are wounded or exposed to a chemical irritant, damaged cells are removed by induction of cell death pathways, while cell proliferation is enhanced to facilitate tissue regeneration or wound healing, thus maintaining homeostasis [73]. Proliferation and inflammation subside after the insulting agent is removed or the repair completed. By contrast, sustained proliferation of initiated cells in environments rich in inflammatory cells, growth/survival factors, activated stroma, and DNA damage–promoting agents potentiates and/or promotes neoplastic risk [71].

3. Latency stage

Cancer is developed, in which initial damage leads to a preneoplastic stage, followed by selection and proliferation of the neoplastic cell [68]. During latency, the transformed cell produces a number of different phenotypic clones through continued genetic diversity, although not all clones will be neoplastic [67-69]. Hyperplasia is called when cells look normal but grow too much, and further damage can lead to dysplasia. Dysplasia is namely when cells proliferate too much and look abnormal in shape and orientation. Cells are less responsive to surrounding cells and the body's signals to stop proliferating. Further damage and/or cell changes can lead to "in situ" cancer. Atypia is a general term describing cells look abnormal and how cells look, for example, one cell can appear atypical, but a group of cells display dysplasia. Several types of pre-malignant lesions, such as dysplasia and hyperplasia, can be detected in diverse organs prior to the appearance of fully malignant invasive tumors.

Tumor progression continues as additional mutations occur within cells of the tumor population. Some of these mutations confer a selective advantage to the cell, such as more rapid growth, and the descendants of a cell bearing such a mutation will consequently become dominant within the tumor population. The process is called clonal selection since a new clone of tumor cells has evolved on the basis of its increased growth rate or other properties. These properties are survival, invasion, or metastasis that confer a selective advantage. However, the pre-malignant lesions are caused either by genetic alterations which induce monoclonal expansion of the cells, or by environment factors, such as viral infection, which induce polyclonal expansion of the cells. Then, clonal selection continues throughout tumor development, so tumors continuously become more rapid-growing and increasingly malignant. Thus, it is defined tumor in three types as follow:

3.1 Benign tumor

Although cells are not normal, they do not have the ability to travel to other parts of the body. Cells in benign tumors are typically more differentiated (mature) and organized than cells in cancerous tumors. Chromosomal and enzymatic analyses indicate that all of the cancer cells of a tumor and its metastases are derivatives or clones of a single cell. The development of tumors from single cells that begin to proliferate abnormally. The original progenitor cell that gives rise to a tumor has initially acquired all of the characteristics of a cancer cell. In some cases a benign tumor may eventually become an invasive or metastatic tumor.

3.2 In situ carcinoma (cancer)

Cells become even more abnormal in growth and appearance but the tumor cells have not broken through the boundary around the tumor that separates it from surrounding tissues. This boundary is like a capsule that contains the tumor. Subsequently, accumulation of genetic alteration occur in one of the pre-malignant cells, and the cells convert into malignant ones of clonal origin and produce a primary tumor. However, at the early stage of primary tumor expansion, the cells are not invasive and metastatic [71]. Cells may acquire additional damage and/or changes which can lead to invasive cancer. Invasive cancer, the uncontrolled growth of cells in the tumor allow some cells to break through the capsule-like boundary and invade nearby tissues. Generally, invasive tumors are life-threatening if the cancer cells are present within a vital organ like the kidneys, lungs, or liver. Invasive tumors in non-vital organs like the breasts are not necessarily life-threatening unless they become malignant and migrate to a vital organ. Therefore, early detection of any tumor is important because it increases the chances of removing the cancer before it becomes life-threatening.

3.3 Metastatic cancer

Cells from the malignant primary tumor gain the ability to reestablish somewhere else in the body where they form new cancerous tumors. Malignant, cells from the invasive (primary) tumor

gain the ability to enter the blood stream or lymphatic system and to travel to distant areas in the body; form new secondary tumors are called metastases. Then, new clones with invasiveness and metastatic ability appear as a result of further accumulation of genetic alteration in the cells. Thus, fully malignant cells are invasive and metastatic; however, only a restricted fraction of the cells in primary tumor are considered to be highly metastatic. Namely, cells in a primary tumor are phenotypically and biologically heterogenous, and such a heterogeneity is caused but the difference in the genes altered in each cancer cell. Therefore, highly metastatic cells often acquire alterations in more genes than non-metastatic cells, and various genes are differentially expressed between metastatic and non-metastatic cells. Such cells selectively produce a metastatic tumor in a distant organ; thus, cells in the metastatic tumor are considered it carry all the genetic alterations necessary to maintain malignant phenotypes of cancer cells, including invasiveness and metastatic ability [71, 72].

Tumor invasion and metastasis mechanism

Invasion into neighboring tissue and ectopic survival are required for cancer progression and are a requirement to form metastasis [69, 74]. It is known that the movement of neoplastic cells is not a random process. However, the mechanisms controlling the neoplastic cells movement, survival in foreign tissue environments, and choice of residence at a final destination are not clear. Tumor invasion can be defined as the active migration of neoplastic cells out of their tissue of origin and into adjacent tissues of different types. The invading tumor cells, therefore, end up in a tissue where they do not belong. Metastasis is the process whereby cancer cells spread throughout the body, establishing new colonies in organs at a distance from the one where the primary tumor originated [74]. There are three major routes for tumor dissemination: lymphatic vessels, blood vessels, and serosal surfaces. Tumor metastases occurring via these three routes are referred to as lymphatic, hematogenous, and transcoelomic, respectively. A tumor cell that initiates a metastatic colony has ability

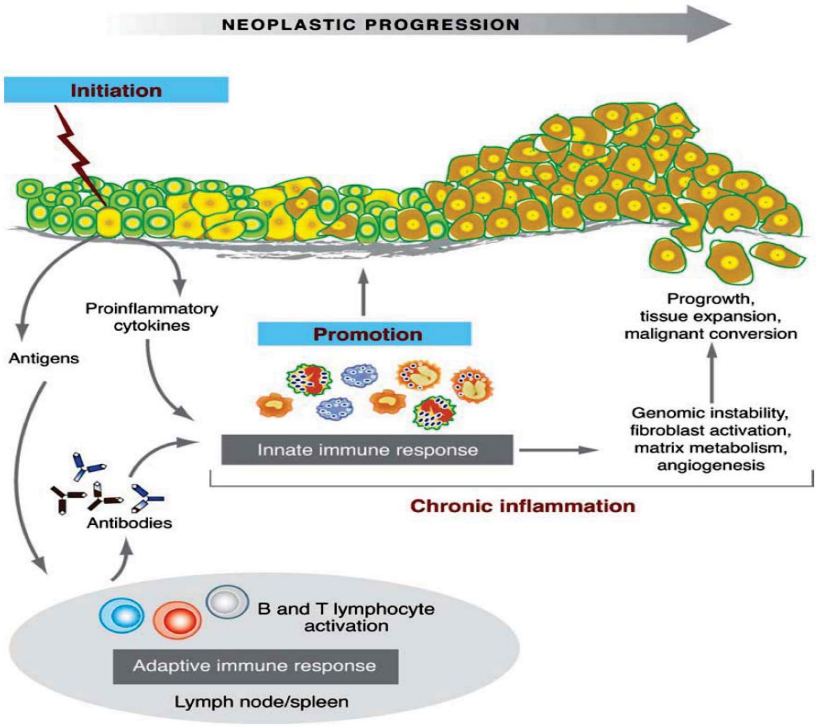


Figure 2: Model of tumor development and progression [69]

including, detach from the primary mass, invade the local host tissue stroma, penetrate local lymphatic and blood vessels, survive within the circulation, become arrested in capillaries or venules of other organs penetrate the corresponding parenchyma, adapt to the newly colonized milieu or subvert the local microenvironment to suit its own needs, and divide to form the new tumor (Figure 3) [74]. The mammalian organism is composed of a number of tissue compartments separated from each other by two types of ECM: basement membrane and interstitial stoma [75]. During the process of invasion, tumor cells must traverse these matrix barriers as they cross tissue boundaries. During the transition to invasive carcinoma, tumor cells penetrate the epithelial basement membrane and enter the underlying interstitial stoma [76]. After traversing the stoma, tumor cells gain access to lymphatics and blood vessels. For future dissemination through the wall of a capillary or venule the tumor cells must penetrate the sub-endothelial basement membrane. In the target organ where metastases are initiated, tumor cells that have extravasated must migrate through the perivascular

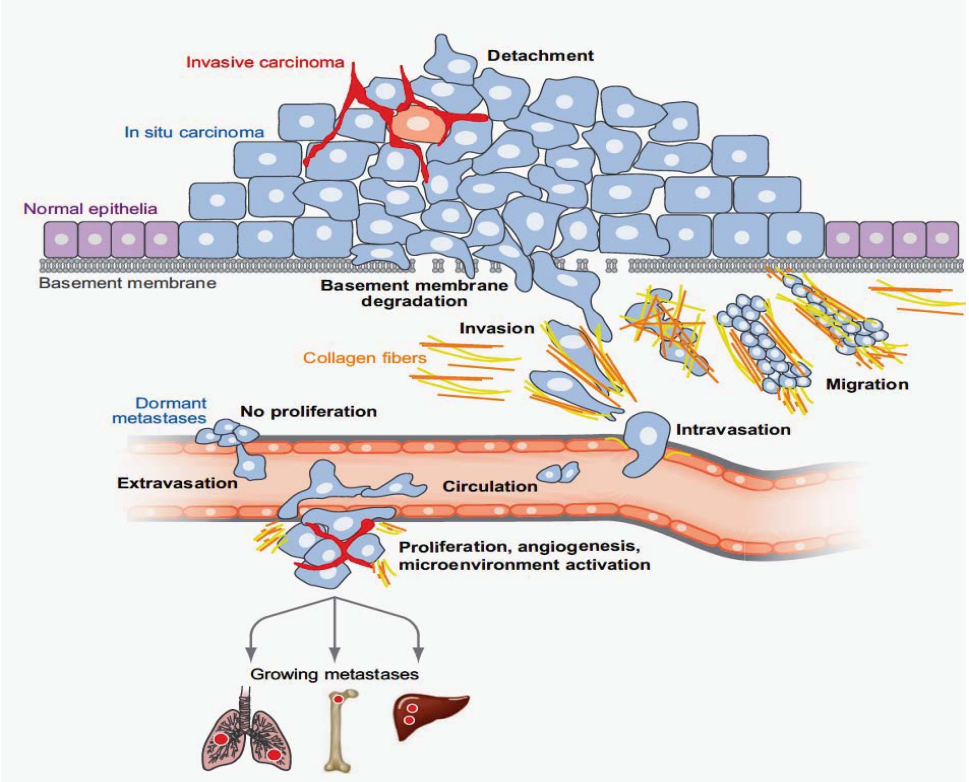


Figure 3: Principal steps in metastasis [74]

interstitial stoma before tumor colony growth occurs in the organ parenchyma. By penetrating this second basement membrane barrier and the layer of endothelial cells that form the vessel's inner lining, the cancer cell gains access to the blood stream and is carried elsewhere in the body. Thus, the metastatic cascade commences with the dislodgment of cells from the primary tumor mass and terminates in the establishment of tumors at secondary sites. As this suggests, metastasis is referred to as the spread of cancer to distant sites in the body. Therefore the process of cancer metastasis consists of a series of sequential interrelated steps. The outcome of the process is dependent on both the intrinsic properties of the tumor cells and the responses of the host. However, in principle, the steps or events in the pathogenesis of a metastasis are similar in all tumors. Because tumor cell penetration of extracellular matrix occurs at multiple stages in the metastatic cascade, many investigators are studying the interaction of

tumor cells with matrix in order to gain some insight into the mechanism of tumor invasion and metastasis.

Extracellular Matrix: ECM

The extracellular matrix (ECM) is a complex meshwork of glycoproteins and proteoglycans that determines architecture and conditions many biological activities. Components of the extracellular matrix provide a large variety of specific signals which directly influence cell growth, migration, morphology, proliferation, differentiation, and biosynthetic activities [77]. It has become the extracellular matrix is a complex meshwork which consists of collagen, glycoproteins, proteoglycans and glycosaminoglycans [77, 78]. It is a highly organized fibrillar meshwork, which serves as substratum for cell adhesion and migration. ECM also exists as a three-dimensional supporting scaffold that isolates tissue compartments, mediates cell attachment, and determines tissue architecture. All types of ECM such as basement membranes, interstitial stoma, bone and cartilage differ from each other in the amount and type of matrix components. Each type of matrix is composed of characteristic types of generally distinct collagen. For example, type IV collagen is found only in basement membrane, type II collagen is present mainly in cartilage, and type I collagen is present in both stroma and bone. Two structural and functionally distinct ECM categories are specialized ECMs of interstitial connective tissues and basement membranes.

Extracellular Matrix and Cancer Progression

The function of normal organs is determined by reciprocal communication between cells in an epithelial layer and their surrounding stroma, and the same organizational principles apply to cancer [79]. The progression from a hyperproliferative state to malignancy can be characterized by increasingly abnormal communications between all cell types comprising the tumor mass**.** In

addition, the tumor can modify the extracellular matrix in the following three ways [10, 80].

1. Degradation of matrix components associated with invasion: Matrix degradation in a malignant tumor is probably restricted to localized regions where active invasion is taking place. Frank loss or marked disorganization of basement membrane is a general finding during the transition from benign to malignant epithelial neoplasm.

2. Increased production of matrix components by host cells in response to the presence of the tumor. " Desmoplasia " is the term applied to the phenomenon of excessive accumulation of connective tissue associated especially with breast, bowel, and prostate cancers.

3. Tumor cell synthesis of matrix components. Matrix components synthesized by a tumor cell are generally of the same type produced by the normal cell counterpart. The actual amount of matrix produced by the tumor cells is frequently much less than the normal counterpart.

The three-dimensional ECM surrounding cells contains a mixture of fibrillar proteins, glycoproteins, proteoglycans, cytokines, and growth factors [81]. Composition of ECM varies considerably both between and within different tissues, and ECM changes temporally as an adaptation to varying signals during normal developmental processes and during pathological processes such as those that occur in cancer. The ECM of most tissues contains various types of fibrillar and nonfibrillar collagens, noncollagenous glycoproteins, and proteoglycans. However, collagens are the most important components of ECM and play an important role in cell adhesion, movement, differentiation, proliferation, and metastasis of tumor cells.

Collagens

Collagen is a structural protein found in abundance in extra-cellular matrices of connective tissues [77, 82]. Collagen molecular structure was first demonstrated by Ramchandran and Kartha and is still under investigation. Collagens are a heterogenous family of

proteins that form a major component of interstitial ECM in all tissues [83]. Twenty-eight different types of collagen composed of at least 46 distinct polypeptide chains have been identified in vertebrates, and many other proteins contain collagenous [84]. Collagen is a structural protein that gives tensile strength to bone, tendons, and various other tissues. It has a unique amino acid composition containing one-third glycine and rest is primarily praline and/or hydroxyproline or any other amino acid residue. This structure makes the collagen a molecule with high mechanical strength. Hydrogen bonds between glycine of one chain and proline of the other chain and water mediated bonds stabilize the triple helix. The molecular unit of collagen is made up of three polypeptide strands, each of which is a left-handed helix. These three left-handed helices are twisted together into a right-handed coiled coil, called as a triple helix [83]. It is noteworthy that, although the three polypeptide chains in the triple helix of each collagen type can be identical, heterotrimeric triple helices are more prevalent than are homotrimeric triple helices.

Collagen is synthesized intra-cellularly as a precursor procollagen molecule. The procollagen molecule by virtue of its globular N-terminal and C-terminal propeptide extensions confers solubility under physiological conditions and prevents aggregation and fibril formation inside the cell. Therefore there has to be controlled cleavage of the propeptides to initiate fibril formation in extracellular matrix. After secretion into the extracellular space these propeptides are then systematically cleaved to trigger fibril formation. [84] Collagen are composed of three α-chain polypeptides, each containing considerable stretches of repeating Gly-X-Y tripeptide sequence necessary for triple-helix formation. The X- and Y residues are, in most cases, proline and hydroxyproline, respectively [84-86]. Collagen α-chain synthesis follows the same general pathway as other secretory proteins. Common for all collagens are stretches of triple-helical domains where the α- chains are wound around each other in a coiled-coil conformation.[86] However, the peptide chains are extensively modified prior to secretion by the hydroxylation of proline and lysine

residues, glycosylation, chain association and addition of complex carbohydrate or glycosaminoglycan chains.

Fibrillar collagens

Fibrillar collagens, the fibril forming collagens, form a rather homogenous group consisting of collagens type I, II, III, V and XI [77, 78], structurally characterized by the feature that almost the entire molecule consists of one single collagenous domain. By intermolecular interactions, these collagens form fibers and fiber bundles that are the main providers of mechanical support and resistance in the ECM. Thus, fibrillar collagens are the most important molecules in conferring mechanical strength. The fibrillar collagens contain a single long, unbroken triple-helix domain and assemble into ordered polymers called fibrils. They are synthesized and secreted as procolllagens which are released into folds and furrows of the plasmalemma, which acts as molds that arrange the forming fibrils in the proper direction. The alignment of the collagen fibrils and fiber bundles is determined by the cells that synthesized them. Fibril diameters can range from 20 to 500 nm and the fibrils themselves can organize into diverse patterns including bundles, weaves, and layers. Procollagens have N- and C- terminal pro-peptides that are cleaved off after secretion into the extracellular space, resulting in helical rod-like molecules ending with short non-collagenous telopeptides [86]. Finally, they are processed to mature collagen by collagen monomers assembled into fibrils with a longitudinal head-to-tail association, followed by a quarter-staggered lateral aggregation. Collagens are classified into several subfamilies according to their molecular and supramolecular shapes [87]. The triple-helical molecules corresponding to collagen types I, II, III, V and XI self-associate to form cross-striated fibrils with a typical axial periodicity of 67 nm. The network-forming collagens contain types IV, VIII and X. Among these, collagen type IV forms basement membranes with other non-collagenous components, such as laminin, entactin and perlecan. Other collagen types are classified as fibril-associated collagens with interrupted triple helices,

transmembrane collagens and others. There are two major tissue-specific collagen fibrils: the collagen type II-containing fibrils, typical of cartilage, and collagen type I-containing fibrils present in most other interstitial connective tissues [82, 83]. Among the collagen types, even among whole mammalian proteins, collagen type I is the most ubiquitous and abundant [88].

Type I Collagen

Type I collagen is the major component of collagen fibrils in a variety of tissues including bone, skin, tendon and other fibrous tissues. Collagen type I consists of two α1 (I) chains and one α2 (I) chain, encoded by two different genes. In concert with other collagens and proteoglycans, collagen type I gives a variety of tissues their optimal functional structure. Apart from providing tissues tensile strength and integrity, collagen type I also induces specific signals upon cell attachment, vital for cellular functions including survival, migration and proliferation. Although, it is a major component of most connective tissues, collagen type I is synthesized only by a subset of cell types such as fibroblasts in the loose connective tissues, and in bone and dentin matrices by osteoblasts and odontoblasts respectively [89]. Although type I collagen is the most abundant collagen in skin, bone, and other tissues and organs, no type I collagen molecule and/or fragments have been demonstrated to have suppressive efficacy on malignant phenotypes of a tumor. However, variations in the production of type I collagen by tumors have been reported in the relationship with tumor progression, and transformed cells generally synthesize less collagen than their counterparts [85, 86]. The differential expression between hepatocellular carcinomas and normal liver was revealed, and type I collagen was down-regulated in hepatocellular carcinomas [90]. Type I collagen was observed to increase the proliferation and long-term survival of pancreatic cancer cells [91]. In a neuroblastoma, type I collagen biosynthesis is a helpful marker for studying specific patterns of transdifferentiation associated

with the loss of malignant potential [92]. In addition, type I collagen was able to inhibit growth and malignant transformation in human glioma cells [93], and also suppressed the motility and invasion of the glioma cell growth in vitro and in vivo [94].

Collagen triple helices show many important and specific biological functions, and many of these are initiated by specific interaction of collagen-binding molecules with epitopes displayed on the collagen triple helices [88]. Adhesion to type I collagen can affect cell morphology, differentiation, and cell cycle progression, depending on its structure. When polymerized into fibrils, as it is mostly found in vivo, type I collagen inhibits growth of a number of cell types including smooth muscle cells, melanoma cells [89], and hepatocytes [90], whereas adhesion to monomeric collagen stimulates cell cycle progression under similar culture conditions [88]. Unlike loss of adhesion, however, which blocks cell cycle progression and also induces apoptosis, adhesion to collagen gels promotes survival and increased differentiated function [95]. In addition, the triple helix of human collagen type I with a length of 300 nm contains binding sites for over 50 other molecules [86, 87]. The collagen-binding molecules include membrane-anchored receptors, secreted soluble factors and other ECM components, including themselves. Collagen triple helices can directly function as signalling ligands through specific activation of collagen receptors, generate vital cellular functions including migration and proliferation. Moreover, collagen is continually synthesized and degraded in the extracellular matrix. Collagen degradation is a normal part of collagen homeostasis; however excessive collagenolysis has been implicated in a number of human diseases such as arthritis, cancer, and atherosclerosis.

Collagen Degradation

Collagen degradation is a normal part of collagen homeostasis, proteolytic degradation of collagen is important in physiological processes like tissue remodeling, morphogenesis and angiogenesis. It also plays a vital role in induction and perpetuation of

pathological states like disuse atrophy, metastasis and cartilage degeneration. The fundamental importance of type I collagen can be inferred by its presence in almost all human tissues and by the nonviability of embryos deficient in this extracellular matrix (ECM) protein [96]. Type I collagen exerts its roles, as a load-bearing structure and regulator of cell function, only after it has polymerized into fibrils. However, this assembly process proceeds after proteolytic removal of the globular termini of the secreted procollagen molecules. Because type I collagen is a major component of stromal tissue, and both normal and tumor cells digest collagen fibrils to grow or to invade connective tissues.

Collagen is generally resistant to most proteinases, however, it is susceptible to cleavage via bacterial collagenase (Clostridium histolyticum) and members of Matrix Metalloproteinase (MMP) family [97]. Matrix metalloproteinases (MMP) are implicated in the tissue remodeling processes associated to the growth and development and in several pathological conditions such as osteoarthritis and tumor invasion and metastasis [7, 8]. MMPs are zinc-dependent proteinases which degrade most of the extra-cellular proteins in humans. The latter group includes collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), MMP-2 (gelatinase A) [97, 98] and membrane-type 1-MMP (MMP-14) [99]. These MMPs cleave the three a chains of native triplehelical type I, II and III collagens after Gly in a particular sequence, at a specific peptide bond between Gly775 and the residue in position 776, leading to the formation of 1⁄4 and 3⁄4 fragments [100]. Thus two fragments, a larger N-Terminal three-quarters fragment and a smaller C-terminal one-quarter fragment, are formed. They then get unfolded and are degraded further by gelatinases [100]. In recent study supports this indication, their observations suggest that the C-terminal telopeptide must be proteolyzed before collagenase can gain access to the cleavage site. Collagenase then binds to the substrates at interaction domain, which facilitates the triplehelix unwinding/dissociation function of the enzyme before collagenolysis [101. Moreover, MMP action on collagen proceeds by a

ratchet mechanism whereby the enzyme moves along collagen fibrils in steps, alternately binding to and cleaving the available monomers (Figure 4)[102]. A present study explains the mechanism of breakdown of collagen by collagenase, typically taken as MMP-1. According to this study, MMP-1 is proposed to first bind to, and then locally unwind, the triple helix so that each peptide may fit into the active site binding groove, before hydrolyzing the peptide bonds of each chain in succession (Figure 5) [103].

MMP2 is one type of MMPs that important for degradation of denatured, cleaved, type I collagen but also has direct activity against type I collagen [61, 97]. In parallel, type I collagen is also activates MMP-2, it was postulated that culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of MMP-2 [44-46]. Previous studies also indicated that Collagen-induced MMP-2 activation occur either directly or indirectly through integrin signaling [25, 59]. Interestingly, MMP2 had higher activity against mutant type I collagen than normal type I collagen at physiological temperatures, whereas the opposite was true for the classic collagenolytic MMPs. MMP2 unfolds type I collagen by a mechanism distinct from the classic collagenases [61], and might, therefore, be more efficient at degrading mutant type I collagen with slight conformational changes. Recently, it is suggesting that MMP2 activity is absolutely required for degradation of mutant type I collagen in vivo. Moreover, MMP2 was also able to degrade both wildtype and mutant type I collagen in vitro, that may be due to direct biochemical interactions between the MMP2 enzyme and one of its substrates, type I collagen [104]. Therefore, recently, MMPs represent the most prominent family of proteinases associated with ECM proteolysis mediates tissue homeostasis and tumorigenesis.

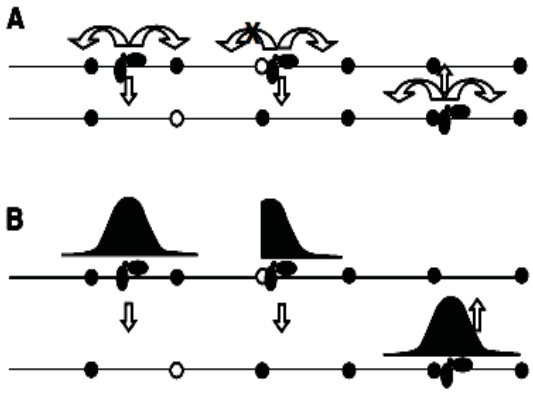


Figure 4: Burnt bridges mechanism of MMP-1 transport on a collagen fibril[102]

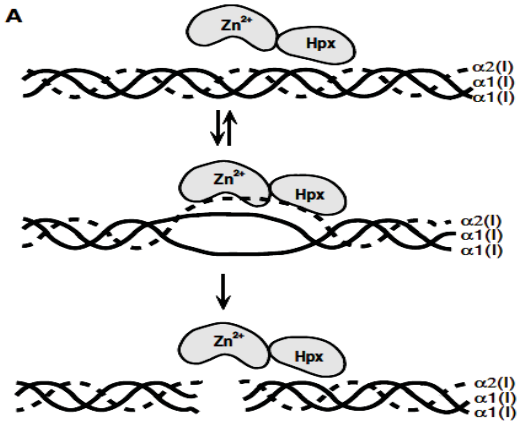


Figure 5: Steps involved in Collagenolysis [103]

Collagen gel contraction

Remodeling of the ECM plays a critical role in the reorganization of connective tissue under both normal and pathophysiological condition such as tissue morphogenesis and growth, angiogenesis, wound healing arthritis and tumor invasion [105, 106]. Collagen gel contraction is a process of reorganizing the collagen fibrils and contraction of the gel. It is used as an in vitro model of cell-mediated tissue remodeling, including wound contraction and maintenance of tissue homeostasis [107]. Two major experimental setups are used when studying collagen gel contraction, namely contraction of a relaxed or free-floating gel which has been released from the culture dish, and contraction of a stressed gel that is still attached. When cells are cultured in the attached gel, they generate a mechanical load and develop an isometric tension, resulting in flattened cell morphology and prominent actin stress fibres [108]. In contrast, cells grown in relaxed matrices become quiescent and take on a dendritic morphology [109, 110]. Several studies have demonstrated a role for β1 integrin, in particular α1β1 and α2β1 integrin, in mediating collagen gel contraction by fibroblast and osteoblastic cells [111-113].

There are some indications that MMPs involve in collagen gel contraction process. Culturing human fibroblast cells embedded into three-dimensional collagen gels, induced MMP-2 activation and eventually to contracted collagen lattices [111]. Previously study demonstrated the functional role of MT1-MMP in collagen gel contraction. Tumor cells that lacked MT1-MMP expression were unable to activated MMP-2 activation and contracted collagen gel lattices. In contrast, only tumor cells which were capable of both sufficient synthesis and activation of MMP-2 successfully reorganized and contract collagen lattices. These data indicated the significance of MMP-2 activation in cell induced collagen gel contraction which up-regulated MT1-MMP activity [114].

Matrix Metalloproteinase enzymes (MMPs)

Matrix metalloprotienases (MMPs) are a major group of enzymes that regulate cell-matrix composition. MMPs are a family of zinc-dependent endopeptidases first described by Gross and Lapiere, in 1962 [7, 8]. Nowadays, over 25 enzymes of the MMPs family were recognized that cleave the various components of the ECM [8, 13, 37]. The general structural blueprint of MMPs shows three domains that are common to almost all MMPs, the pro-peptide, the catalytic domain, and the hemopexin-like C-terminal domain that is linked to the catalytic domain via a flexible hinge region [37, 115]. All MMPs have the “minimal domain” in common, which contains three principal regions: an amino-terminal signal sequence (Pre) to be cleaved by the signal peptidase during entry into the endoplasmic reticulum, a pro-domain (Pro) containing a thiol-group (-SH) and a furin cleavage site, and the catalytic domain with a zinc-binding site (Zn2+). Interaction of the -SH group of the pro-domain with the zinc ion of the catalytic domain keeps the enzyme as an inactive zymogen. Activation of the zymogen is often mediated by intracellular furin-like proteinases that target the furin recognition motif (Fu) between the pro-domain and the catalytic domain. In addition to the minimal domain, most MMPs

possess a hemopexin-like region, a domain composed of four repeats that resemble hemopexin and contain a disulfide bond (S-S) between the first and the last subdomain, which is linked to the catalytic domain via a flexible hinge region (Figure 6) [115].

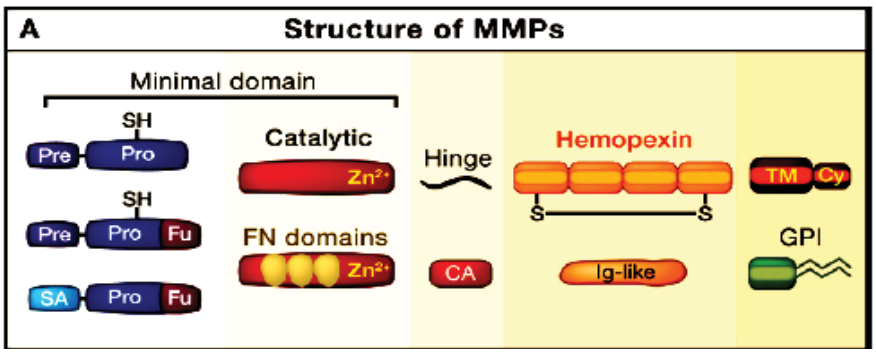


Figure 6: MMP Composition: Minimal domain structure [115].

Structural diversity of MMPs

The MMPs have been classified in to six subgroups based on their putative substrate specificity and internal homologies. However, analysis of the structural design of these enzymes has led to a new classification system based on MMP structures rather than on their substrate specificities (Figure 7) [116]. Most of them are organized around a conserved catalytic domain which incorporates a propeptide necessary to maintain enzyme latency, a signal peptide which directs their secretion from the cell, and a C-terminal hemopexin domain which contributes to substrate specificity and to interactions with endogenous inhibitors [117]. This archetypal MMP design is present in the subgroup of secreted proteases composed of the three human collagenases (MMP-1, MMP-8, and MMP-13), the two stromelysins (MMP-3 and MMP-10), and four additional MMPs with unique structural characteristics (MMP-12, MMP-19, MMP-20, and MMP-27). Besides the archetypal conformation, the two matrilysins

(MMP-7 and MMP-26) lack the hemopexin domain [118]. Two gelatinases, MMP-2 and MMP-9 are named for their selective ability to cleave gelatin and also type IV collagen. The special feature of the gelatinases is the three repeats homologous to the type II module of fibronectin in the catalytic domain, which has been show to be involved in binding to denatured collagen or gelatin and type III collagen [120, 121]. It is important to mention the tissue inhibitors of metalloproteinases (TIMP) family, which presently includes four proteins, TIMP-1, -2, -3, and –4 [160, 161]. Binding of all TIMPs to the catalytic domain result in efficient inhibition of enzymatic activity of almost all MMPs [122, 123]. In addition to these secreted MMPs, there are six membrane-type (MT)-MMPs localized at the cell surface through a C-terminal transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) or by a glycosylphosphatidylinositol anchor (MT4- and MT6-MMP) [119]. The MT-MMPs also have an additional insertion of basic residues between the propeptide and the catalytic domain.

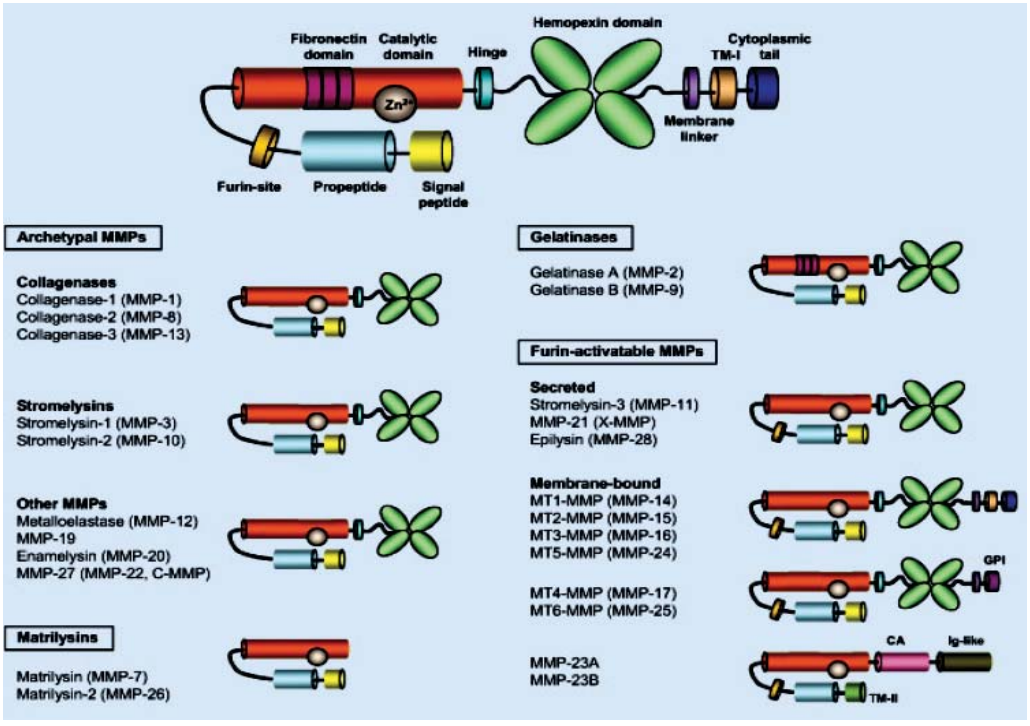


Figure 7: Structural classification of human MMPs based on their domain organization[116]

Regulation of MMP activity

The complexity of the tumor microenvironment allows for a variety of regulatory cascades that determine the functions of the diverse MMPs expressed. MMPs are regulated in many levels although it is not fully understood how MMPs act in the processes of development, homeostasis and diseases. They are tightly regulated transcriptionally and on the posttranslational level. MMPs are secreted as inactive proMMPs. Their activity is controlled at the protein level by their activators, inhibitors and through their cell surface localization. Recently, it is well recognized that proteolytic activity of MMPs can be regulated at different levels as follow [115, 116].

1. Transcriptional regulation

MMPs are highly regulated proteins, and the first level of regulation is transcriptional regulation. The basal gene-expression level and mRNA stability can be rapidly changed when remodeling of the extracellular matrix is required. This change can be achieved by change of extracellular matrix components or by growth factors or cytokines [7, 8, 12, 13]. Not all MMPs contain well-defined transcriptional elements; one of these MMPs is MMP-2. Formerly, it was thought that MMP-2 could only be post-transcriptionally regulated, nowadays a number of well-known transcriptional elements have been identified in the MMP-2 promoter [124, 126]. Structural and functional analysis of the promoter regions from a number of MMP genes has provided a better understanding of the mechanisms that regulate their expression. These studies have revealed the existence of an AP-1 binding site in the promoter of most MMP genes. Likewise, the PEA3 site which binds the ETS family of oncoproteins, is also present in many MMP gene promoters. It has been demonstrated that the ETS and AP-1 binding sites cooperate to enhance transcription, although the presence of other upstream elements such as NF-κB or Cbfa1 binding sites is also necessary to precisely regulate MMP gene expression and tissue specificity [115, 124].

2. Proenzyme activation

MMPs, like most proteolytic enzymes, are synthesized as inactive zymogens. The second essential form of regulation of MMPs is activation of latent MMPs. All MMPs are secreted as inactive zymogens and are activated by cleavage of the N-terminal pro-domain [37, 124]. Most MMPs are activated by tissue or plasma serine proteases, like plasmin. The propeptide sequence is cleaved at basic amino acid motifs and subsequently autocatalytical activation of the MMPs is induced. Once activated, MMP-2, -3, -7, -9, and -12 may launch a negative feedback signal, for example, by degrading plasminogen and thus interfering with plasminogen conversion to active plasmin [115]. Exceptions for this activation cascades are the membrane-type MMPs (MT-MMPs) and proMMP-2. MT-MMPs are activated intracellular before transport to the cell surface, resulting in an active membrane protein. ProMMP-2 however lacks a basic amino acid motif and can therefore not be activated by serine proteases [8, 12, 13, 15]. Three mechanisms for proMMP-2 activation have been described at this moment; autocatalytical activation, TIMP-2 dependent activation and cell-surface associated urokinase-type plasminogen activator (uPA)/plasmin system dependent activation [31–36]. The mechanisms differ substantially, but have a characteristic phenomenon. That is, all three mechanisms of activation involve processing of proMMP-2 to an intermediate form and the intermediate form is then further processed to the active MMP-2. However, mechanisms by which proMMP-2 can be activated are still under investigation, resulting in the elucidation of other mechanisms for proMMP-2 activation. Examples hereof are proMMP-2 activation by thrombin in a MT1-MMP dependent manner [127] and activation of proMMP-2 by MT2-MMP in a TIMP-2 independent manner [128]. The complexity of the different activation mechanisms indicates however, that there is a complicated network regulating extracellular matrix degradation.

3. Inhibition of MMPs

A third form of regulation of MMPs described so far occurs via natural endogenous inhibitors of MMPs. Several natural inhibitors of MMPs exist, α2-macroglobulin, which mainly blocks MMP activity in plasma and tissue fluids, whereas the most common inhibitors TIMPs (tissue inhibitors of metalloproteinases) are more specific. TIMPs are a group of endogenous proteins capable of inhibiting MMPs. Four TIMPs have been identified in vertebrates include TIMP-1, TIMP-2 and TIMP-4, that share a conserved structure divided into an N- and a C-terminal domain and containing three conserved disulfide bonds [30, 31]. In general, binding of TIMPs to the active site of the MMPs results in an efficient reversible inhibition of enzymatic activity, but TIMPs do differ in their ability to inhibit the various MMPs. Also the tissue distribution of TIMPs is very diverse [106, 107]. The inhibitory activities of TIMPs suggest that the net balance between MMPs and TIMPs is a major determinant of the proteolytic potential of tumours. However, several studies have shown that TIMP levels are also increased during tumour progression and may exhibit growth promoting activities on a number of cell types, indicating that their role in cancer progression is much more complex than that derived from MMP inhibitory function [8, 30, 39].

4. Compartmentalization of MMPs

The localization or compartmentalization of MMPs under physiological conditions often dictates their biological function. MMPs are secreted and anchored to the cell membrane, thereby targeting their catalytic activity to specific substrates within the pericellular space. Specific cell-MMP interactions have been reported. Several MMPs interact with surface receptors such as integrins or localize to specific areas of the ECM, which potentiates MMP activity by increasing their local concentration and also may interfere with accessibility to endogenous inhibitors [12-15]. The binding of MMP-2 to integrin αvβ3 via its hemopexin domain is crucial for mesenchymal

cell invasive activity [35]. Moreover, several studies indicate binding of gelatinase B to CD44, and binding of matrilysin to surface proteoglycans [124]. Progelatinase A also interacts with tissue inhibitor of metalloproteinases TIMP-2 and MT1-MMP on the cell surface, and this trimeric complex is essential for activation of this gelatinase [14, 15]. It is likely that other MMPs are also attached to cells via specific interaction to membrane proteins, and determining these anchors will lead to identifying activation mechanisms and pericellular substrates.

However, the second of third controls seem to be important primarily for fine tuning the rate of MMP activity since the major level of regulation is at synthesis. Thus, MMPs are as a rule synthesize only upon demand. With the exception of the MT-MMPs, all of these enzymes are soluble, synthesized as latent proenzymes and are secreted into the extracellular space as zymogens that are kept inactive by their propeptides. To facilitate regulation of MMPs production, the MMPs are not stored in most cell types except neutrophils and macrophages [8]. Usually, they are not synthesized and secreted into the matrix until a clear signal arrives that they are needed. When the signal ceases or negative signals arrive, synthesis is stopped or falls to a low level.

The role of MMPs in cancer invasion and metastasis.

MMPs have been implicated in cancer for more than 40 years, and the notion that MMP-mediated ECM degradation leads to cancer cell invasion and metastasis has been a guiding principle in MMP research [9]. First evidence for the role of MMPs in cancer came from knock-out mice. Knock-out mice for MMP-2 show reduced tumor angiogenesis resulting in reduced tumor growth [27]. Significant evidence has accumulated to directly implicate members of the gene family of matrix metalloproteinases in tumor invasion and metastasis formation. Because most components of the ECM and basement membrane can be degraded by MMPs, MMPs are thought to play an important role in tumor invasion and metastasis. Recently, the role of MMPs in malignancy has been reviewed [7, 8, 12, 13, 115]. The

discovery that inhibition of MMPs suppresses the invasive potential of tumors in animal studies was swiftly implemented into clinical trials. Yet, these failed to increase survival rate of the patients [129]. It is now evident that MMP function is more complex than initially thought, given that these enzymes do more than degrade physical barriers. Rather, they also affect multiple signaling pathways that modulate the biology of the cell in normal physiological processes and in disease. The recent insights gained on cancer invasion and metastasis processes modulated by MMPs, including, MMPs affect on growth signals, apoptosis regulation, tumor vasculature, initiate of neoplastic progression and tumor invasion and metastasis, which are likely to have significant consequences on the tumor microenvironment [115].

Unregulated proliferation is a common feature of cancer cells. MMP-9 is compartmentalized to the cell surface by docking to the surface receptor CD44 and then proteolytically activates TGF-β [105]. Similarly, MMP-14 and MMP-2 proteolytically activate TGF-β1 [130]. Given that tumor cells often acquire nonresponsiveness to TGF-β, this suggests that proteolytic activation of TGF-β by MMPs has tumor-promoting effects by selectively driving stroma-mediated invasion and metastasis of the tumor [115]. MMPs are also regulate apoptosis mechanism. Evading programmed cell death, or apoptosis, is another strategy that increases the cell number and size of tumors. MMP-7 is reported in this process, it cleaves Fas ligand from the surface of doxorubicin-treated cancer cells, lowering the impact of chemotherapy on the tumor by abrogating apoptosis [131]. The interaction between MMP-7 and Fas ligand also may play a role in pancreatic ductal adenocarcinoma, as MMP-7 is expressed in human pancreatic cancer specimens and mice deficient in MMP-7 or carrying a nonfunctional Fas ligand mutation show greatly reduced metaplasia during pancreatic duct ligation [132].

An important part in the process of cancer is tumor angiogenesis. It is known that angiogenesis is a process in which new vessels are formed from existing vessels. Several lines of evidence support an important function of MMPs in angiogenic or

lymphangiogenic processes. The major MMPs involved in tumor angiogenesis are MMP-2, -9, and -14, and to a lesser extent MMP-1 and -7. Given that several MMPs are expressed in all tumors, it is now evident that each MMP can contribute to distinct vascular events in the same tumor [133]. MMP-9 has a distinct role in tumor angiogenesis, mainly regulating the bioavailability of vascular endothelial growth factor (VEGF), inflammatory cells convey MMP-9, enables an angiogenic switch by making sequestered VEGF bioavailable for its receptor VEGFR2 in pancreatic islet tumors [134]. In contrast, TIMPs can interfere with tumor angiogenesis in different ways include, inhibiting the degradation of ECM, TIMP-2 can also interfere with tumor angiogenesis through their role in activating MMPs and their several effects on tumor cell proliferation and apoptosis, independent of their capacity to inhibit MMP-activity [125, 126].

Finally, MMPs involved in initiation of neoplastic progression and invasion and metastasis mechanism. The initial process of tumor invasion shares many characteristics with the epithelial-mesenchymal transition (EMT) program during developmental processes including loss of cell-cell adhesion and increased cellular mobility [7, 11]. Overexpression of several MMPs, including MMP-3, -7, and -14 results in carcinoma formation [8, 12, 13]. MMP-2 and -9 are the most studied MMPs in the processes of cancer, including the process of metastasis. A number of studies have linked elevated MMP-2 and -9 expression to increased metastasis and tumor in breast tumors and acute leukemia [8, 126]. Accumulated lines of evidence have demonstrated that MMP-2 is always activated in tumor tissues, in which MT1-MMP is expressed [34-39]. Furthermore, MT1-MMP expression and MMP-2 activation levels are well co-related and closely associated with invasiveness and malignancy of tumors, suggesting that MT1-MMP is as critical a factor for tumor invasion and metastasis as MMP-2 activator. [15, 36, 47].

MMP-2

MMP-2 (Gelatinase A, 72kDa type IV collagenase) is a widely studied matrix metalloproteinase. It was first described and purified from highly metastatic murine tumors [9]. MMP-2 is abundantly expressed in fibroblasts, endothelial and epithelial cells [135, 136]. MMP-2 and -9 are gelatinase subgroup, which differ from the other MMPs by containing three head-to-tail repeats homologous to the type II repeat of the collagen-binding domain of fibronectin. These domains are required to bind and cleave type IV collagen, gelatin and elastin [137]. The hemopexin domain does not affect MMP-2 binding to collagen , but it is critical for the initial cleavage of the triple helical type I collagen [120]. Although, both MMP-2 and MMP-9 share common structure features, however, the specific substrates for MMP-2 and MMP-9 are different. Collagenous substrates including collagen types IV, V, VII, X, XIV are both substrates for MMP-2 and MMP-9, but collagen type I and gelatin are only specific substrates for MMP-2. In addition, MMP-2 is capable of binding various types of non-structural ECM component substrates including active MMP-9, active MMP-13, FGF R I, IGF-BP-3, IGF-BP-5, IL-1β, recombinant TNF-α peptide and TGF-β. In contrast, MMP-9 is able to bind CXCL5, IL-1β, IL-2R, plasminogen, pro-TNF-α, SDF-I and TGF-β [12-15].

Evidence for the enhanced expression of gelatinase A in human tumors come from many experimental studies correlating enzyme expression and tumor grade. Immunocytochemical and in situ hybridization studies have shown increased expression of MMP-2 in many human tumors, including carcinoma of the pancreas, prostate, bladder, skin, breast and ovary [8, 11]. Studies in breast cancer found increased levels of MMP-2 in invasive regions rather than the benign regions, and the same was found in gastric carcinomas, colorectal carcinoma and non small cell lung carcinoma [7, 8]. However, MMP-2 has a specific cell surface mode of activation that differs from others members of the family, and it has become well-accepted that MMP-2 activation requires MT1-MMP and TIMP-2 and is tightly regulated

[32-35]. Many recent studies have turned to measure the ratio of activated to latent MMP-2 rather than detect the level of MMP-2 mRNA or protein alone. Thus, the measurement of active enzyme levels may be more informative with respect to the stage and aggressive behavior of the tumor.

MT1-MMP

MT1-MMP is the first discovered member of the MT-MMP, encoding a 63-kDa enzyme that has a similar domain structure with most MMPs [39]. To date, six MT-MMPs have been identified by cDNA cloning, and the proteolytic activation of pro-MMP-2 was shown experimentally for all MT-MMPs. Their protein products have been named membrane type (MT)-MMPs including MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP, MT6-MMP respectively [14-15, 138-139]. MT1-, MT2-, MT3- and MT5-MMP possess a transmembrane domain (type I transmembrane) and a cytoplasmic tail at the C-terminal region. MT4-MMP and MT6-MMP are localised to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor. However, these enzyme are closely related to each other, sharing sequence homology and a common multidomain structure [12-15]. Thus, MT-MMPs are unique in that they contain a furin recognition motif between the pro- and catalytic domains, and are processed by proprotein convertases such as furin in the secretory pathway [36-38]. In contrast to secretory form of MMPs, MT-MMPs are presented to the cell surface in active form. As a result they can act only in the pericellular space. The restricted distribution of the MT-MMPs provides these enzymes with a unique functional property in that they can alter the immediate microenvironment surrounding the cells [36, 38. Recently particular attention has been given to MT1-MMP, because this enzyme affects cellular functions in a variety of ways.

MT1-MMP has a basic amino acid motif of RRKR at the end of the propeptide which is cleaved by furin or related proprotein convertases [39]. Thus, activation of MT1-MMP takes place during

secretion in the Golgi , and the enzyme is expressed on the cell surface as the active form [15]. However, due to rapid turnover rate of active MT1-MMP, its concentration on cell surface is very low, and the active form of MT1-MMP predominantly localizes on the cell surface by forming a complex with the inhibitor [39]. MT1-MMP degrades various extracellular matrix (ECM) macromolecules including collagen I, II and III, gelatin, fibronectin, tenascin, nidogen, aggrecan, prelecan and laminin-1, and 5, fibrin, aggrecan, as well as pro-TNF-α fusion protein [12-15]. On the cell surface, MT1-MMP also activates other members of the MMP family, for example, proMMP-2 and proMMP-13 (collagenase 3), creating a wider proteolytic repertoire on the cell surface [119]. Because MT1-MMP acts on the cell surface, it modifies the immediate environment of the cell resulting in alteration of signaling from the ECM or adhesion molecules, thereby influencing cellular functions. MT1-MMP cleaves CD44 and laminin-5 which promotes cell migration. MT1-MMP also promotes angiogenesis by means other than stimulation of endothelial cell invasion. Expression of MT1-MMP in tumors stimulates angiogenesis in vivo by stimulating VEGF synthesis from the tumor cells [12, 13]. Taken together, MT1-MMP is an important pericellular microenvironment modifier which affects cell functions and promotes invasion in tissue. Thus, MT1-MMP is expressed on the surface of various tumor cells including gastric carcinoma, and by stromal cells of human colon, breast cancer and head and neck carcinomas [140, 141]. In breast cancer cell lines, MT1-MMP expression is restricted to vimentin-positive and invasive phenotype. MT1-MMP was initially considered mainly in the light of pro-MMP-2 activation. MMP-2 activation is impaired in tissues of MT1-MMP and TIMP-2 deficient mice, confirming that the function of MT1-MMP and TIMP-2 is important for MMP-2 activation [7, 8, 27]. Thus, one of the important functions of MT1-MMP is its collagen degrading activity. MT1-MMP expression is induced in fibroblast and endothelial cells by culture in three dimensional collagen matrixes [46, 55, 114]. It has been shown that only MT1-MMP can promote cellular invasiveness into collagen I matrix in epithelial cells and fibroblasts

[38, 39]. Interestingly, pericellular collagen degradation is essential not only for invasion, but also for tumor cells to grow within a collagen-based 3-D matrix [38, 39]. Again, only MT1-MMP as a collagen degrading enzyme is found to play a critical role. The effect of MT1-MMP is specific to 3-D cultures, suggesting that it is acting through pericellular degradation of collagen matrix [14].

Mechanism of MMP-2 activation

As proMMP-2 activation is an important step for cancer cells to invade into basal lamina, the mechanism of activation has been extensively studied. Like many members of MMPs family, MMP-2 is secreted as latent proenzyme and must be activated extracellularly. According to the current model of pro-MMP-2 activation, MT1-MMP on the cell surface acts as a receptor for TIMP-2. This inhibitor is a bifunctional molecule capable of binding via its N-terminal domain to the MMP-2 active site and via its COOH-terminal domain to the junction of the outer-rim of the β- blades III and IV of the MMP-2 hemopexin-like COOH-terminal domain [31-34]. Evidence from studies involving truncated enzymes and cross-linking method suggests the following model. The N-terminal domain of TIMP is inhibitory, while the C-terminal domain confers binding specificity. So, while the N-terminal domain of TIMP-1 and TIMP-2 bind to the N-terminal domain of active MMP-2, only the C-terminal domain of TIMP-2 will bind specifically to the C-terminal of pro-MMP-2 [32]. As the other two partners of the ternary complex, TIMP-2 mediates the cell surface activation of pro-MMP-2 by binding to a MT1-MMP containing complex on the cell surface, such that the activated MT1-MMP acts as a TIMP-2 receptor [15]. Interaction of TIMP-2 with pro-MMP-2 may facilitate cell surface-mediated activation, whereas interaction with active-MMP-2 results in inhibition [32-34]. Formation of MT1-MMP/TIMP-2 complex appears to be the initial event preceding further binding of pro-MMP-2, and its activation by, an adjacent TIMP-2-free active MT1-MMP molecule [37-39]. Activation of pro-MMP-2 in this

model is only possible if the TIMP-2 concentration is low, with sufficient TIMP-2 to generate the trimolecular complex but not enough to saturate all the MT1-MMPs required for proteolysis of the propeptide. Whereas, high levels of TIMP-2 will inhibit all MT1-MMP activity, and reduce the concentration of free MT1-MMP available to activate pro-MMP-2, low levels of TIMP-2 fail to localize pro-MMP-2 to the cell surface for activation [14, 15]. Also, if no TIMP-2 is present, MT1-MMP undergoes autocatalytic processing to a 45 kDa inactive form.

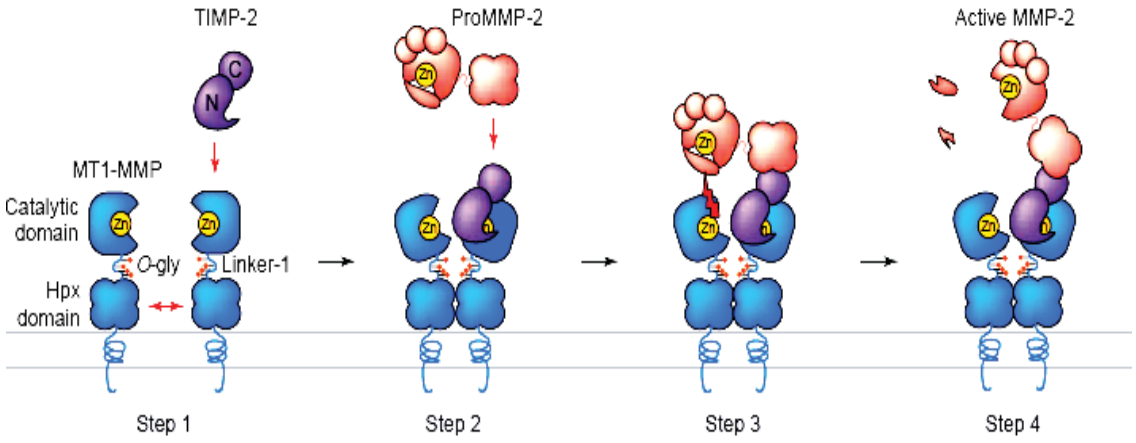


Figure 8: Current model of pro-MMP-2 activation by MT1-MMP [38]

In conclusion, pro-MMP-2 activation is a two step process. The first activation pathway for pro-MMP-2 is initiated by MT1-MMP. This initial cleavage of the 68 kDa pro-MMP-2 generates the 64- kDa activation intermediate. A free MT1-MMP molecule in close proximity to the trimolecular complex of MT1-MMP/TIMP-2/MMP-2 will cleave the propeptide of pro-MMP-2 at Asn37-Leu38 [121]. This is followed by the second step of activation in which further proteolysis of the propeptide results in cleavage at Asn80-Phe81 through an autocatalytic mechanism, generating the 62-kDa fully active enzyme [125]. However, MT1-MMP turns over very rapidly due to autolysis if not inactivated / stabilized by TIMP-2. The time course of

MT1-MMP production and its binding to TIMP-2 on the cell surface is a critical factor; however, this still remains unsolved [15].

Although MT1-MMP mediates the activation of pro-MMP-2, certain ECM molecules such as collagen type I can stimulate MMP-2-activation via MT1-MMP activity in several cell types [25, 44-46]. Collagen is a major ECM component that plays an important role in maintaining tissue architecture and in forming a stable scaffold for cells [14]. As mentioned above, MMP-2 is one type of MMPs that important for degradation of denatured, cleaved, type I collagen but also has direct activity against type I collagen. However, it is well accepted that MT1-MMP plays a pivotal role in invasive growth in collagen gel, but the role of MMP-2 still remains unclear. A possible explanation for intensive digestion of collagen gel is that MT1-MMP as a collagenase cleaves type I collagen to initiate denaturation into gelatin, which is subsequently digested to pieces by MMP-2 gelatinase. Furthermore, MT1-MMP stimulates integrin-mediated extracellular signal-regulated kinase (ERK) activation by enhancing the turnover rate of focal adhesions on fibronectin or collagen. In cellular regulation of MT1-MMP, MT1-MMP is regulated by internalization, that β1 integrin can colocalize with MT1-MMP at the migration, where MTCBP-1 can bind to the cytoplasmic domain of MT1-MMP, suppressing its pro-migratory effects [14]. Even though, MT1-MMP is a critical molecule in regarding of type I collagen induced MMP-2 activation. Another molecule is adhesion molecules that act as cell surface receptors that also involve in MMP-2 activation. Especially, the α2β1 integrin, a collagen binding receptor, thought to be implicated in MMP-2 activation that response to type I collagen stimulation and promote tumor invasion and metastasis.

Integrins

Integrins are a family of transmembrane glycoprotein receptors that mediate matrix and cell-cell interaction. The term integrin is derived from the ability of these proteins to link the ECM

proteins with the intracellular cytoskeleton, consequently, play an important role as a bi-directional transducer of extra- and intracellular signals [144]. The integrin receptors always contain one α chain and one β chain, and are thus called heterodimeric. Heterodimer integrins consist of two transmembrane glycoproteins, α and β subunit that are non-covalently bound, with a large extracellular domain, a single transmembrane span and short cytoplasmic tails. The α subunits all have some homology to each other, as do the β subunits. To date 18 α and 8 β subunits combined into 24 different integrins are identified in mammalians [50]. However, most α subunits combine with only one or two and β subunits except αV which is capable of forming heterodimers with 5 different β subunits including β1, β3, β5, β6 and β8 [50, 51]. Both subunits fold into an N-terminal globular head, that combines to create the ligand-binding surface, a long stalk region connecting the ligand-binding head with a transmembrane type I stretch and the C-terminal cytoplasmic tail [145, 146]. The cytoplasmic tails are short and of variable length. They provide a link between the receptors and the cytoskeleton of the cell but do not contain any catalytic activity or consensus protein binding-motifs. Several α- and β- subunits exist in different splice variants, and all known alternative splicing of integrins occurs in the cytoplasmic tail.

Integrin Structure

Integrins are composed of α and β subunits, each containing a large extracellular domain (700–900 amino acids), a short transmembrane domain, and a short cytoplasmic domain (20–60 amino acids) [51]. The structures of the α subunits are very similar, but they vary in size from 150 to 200 kDa. Structural analysis of the α subunits have revealed seven homologous repeats in the N-terminal part that fold into a seven-bladed β propeller [147, 148]. The three or four repeats that are most extracellular, contain sequences with cation-binding properties. These sequence are thought to be involved in the

binding of ligands. All the α subunits share the 5 amino acid motif GFFKR, which is located directly under the transmembrane region. In addition, α subunits may be composed of a heavy and a light chain which are bridged by a disulfide bond. Half of the α subunits such as α1, α2, α10 contain an I-domain which inserted between the second and third propeller repeat [149]. The I-domain is exposed on the upper surface of the propeller and is involved in direct recognition and binding of the ligand [150].

Integrin β subunits are also highly homologous to each other. Most are 90-110 kDa in size, with the exception of β4 which is considerably larger (210 kDa). The β subunit has an N-terminal cysteine-rich region called a PSI domain. The PSI domain cooperates with a more C-terminal cysteine-rich region in restraining the integrin in an active state via a long-range disulfide bond [50, 51]. The β subunits also contain an I-domain-like domain that can be associated with an α subunit lacking an I-domain. This I-domain-like structure takes a direct part in ligand binding. As in the α subunits there is a stalk region in the C-terminal part of the extracellular domain but, unlike the α subunits, this region contain four EGF-like cysteine-rich repeats important in signal transduction and activation of the integrin (Figure 9) [151].

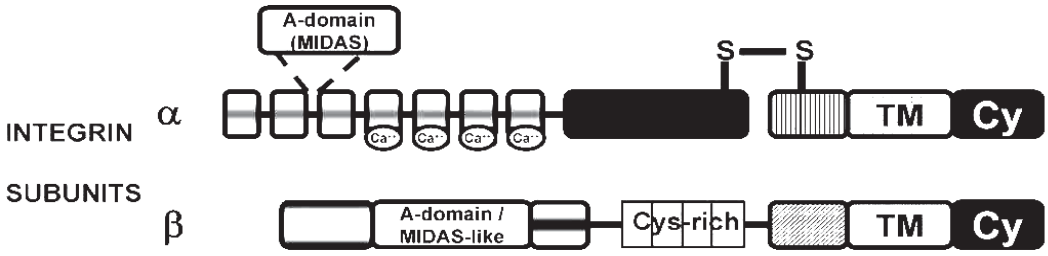


Figure 9: Domain structure of integrins [151]

The specificity of integrin binding to ECM components including laminins, collagens, and fibronectin depends on the extracellular domains of the α and β integrin subunits. Integrins α1β1,

α2β1, α10β1 and α11β1 represent the primary collagen receptors [145, 150]; integrins α3β1, α6β1, α6β4 and α7β1 are the major laminin receptors [147]; and integrins α5β1, α8β1, αIIbβ3 are the major fibronectin receptors and the αvβ3 is specific for vitronectin receptor that bind in an RGD-dependent manner [145]. Most integrin heterodimers are widely expressed in many tissues; however, some are more restricted in their expression. For example, αIIbβ3 is only found on platelets, α6β4 on keratinocytes, and αEβ7, α4β7, α4β1, and the β2 integrin families are restricted to leukocytes (Figure 10) [152].

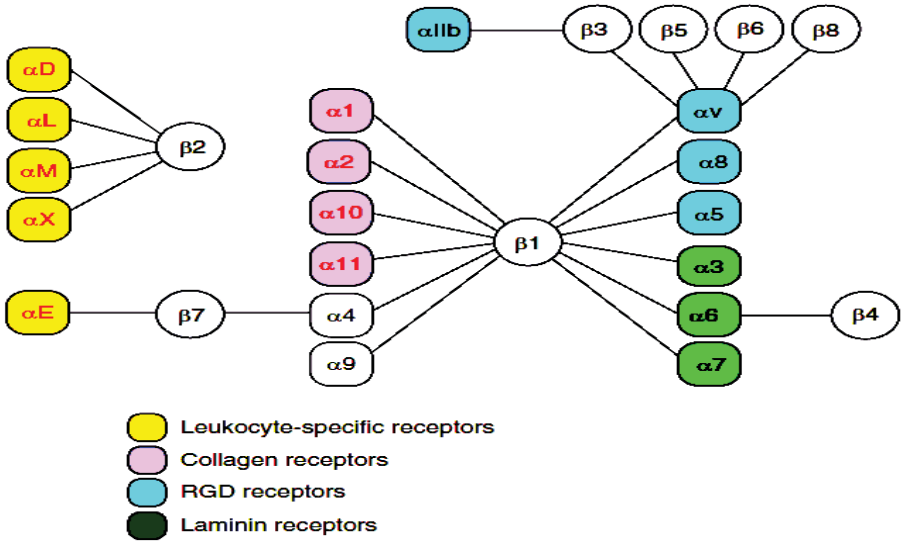


Figure 10: Classification of The integrin family Heterodimers [152]

The β1 integrin

The β1 integrin is one of β subunits which is highly homologous to each other. Five β1 variant subunits, β1A, β1B, β1C, β1C-2 and β1D, generated by alternative splicing, have been described [153]. However, most integrin β tails, include β1 integrin contain one or two NPxY/F motifs that are part of a canonical recognition sequence for phosphotyrosine-binding (PTB) domains, which are protein

modules present in a wide variety of signaling and cytoskeletal proteins [154]. There is also striking homology among the β-subunit cytoplasmic tails, but the α-subunit tails are highly divergent except for a conserved GFFKR motif next to the transmembrane region, which is important for association with the β tail. The β1 integrin subunit is expressed in most cells from the earliest stages of development until adulthood, and it combines with 12 distinct α subunits to form receptors that bind to fibronectin, laminins, collagens, and a variety of other matrix components. Many of the insights gained from these studies extrapolate to the β1 integrins because several of the β1 and both β1 integrins share RGD recognition specificity [2]. Four subunits containing an a-I-domain, include α1, α2, α10 and α11, combine with β1 and form a distinct laminin/collagen-binding subfamily.

Integrin β1 is ubiquitously expressed and can bind multiple a partners, and thus it is not surprising that knockout of β1 results in embryonic lethality due to a complete block in preimplantation development. In contrast, knockouts of α1, α2, α10, and α11 integrin subunits, which each exclusively heterodimerize with β1 to function as primary collagen receptors, are all viable and fertile but possess distinct characteristic abnormalities. The α1 integrin knockout mice develop increased collagen synthesis and display reduced tumor vascularization, while the α2 integrin knockout mouse has a more subtle phenotype with mild platelet function abnormalities and increased vascularization following wounding [155, 156]. These findings support the idea that there is significant redundancy and compensation amongst the collagen receptor integrins. In present, from genetic studies using mouse models have demonstrated that β1 integrins have unique roles in embryonic development, hematopoiesis, wound healing, and cancer [2, 144].

The role of β1 integrin in Cancer

Integrins are intimately involved in tumor invasion into healthy tissues in a complex manner. Several in vivo and in vitro studies have demonstrated the association between deregulation of integrin expression and cancer. Changes in the integrin pattern during malignant transformation are highly dependent on the type of cancer. An altered integrin pattern allows the cancer cells to recognize various matrices. The β1 integrin signaling has been shown to mediate diverse roles in cancer progression including invasion, migration and metastasis [157]. Indeed, expression of β1 integrin was shown recently to be necessary for formation of mammary tumors in engineered murine models. Despite compelling evidence that β1 integrin plays multipotent roles in breast cancer progression, its role as a potential prognostic marker is not well defined. The highly organized predominantly basal distribution of β1 integrin in normal ductal architecture. However, primary myoepithelial tumors are rare, and the role of myoepithelial cells in malignant progression is not well understood [158]. In prostate cancer, primary tumor growth has been shown to be affected by expression of the β1 cytoplasmic variant, β1C, which is downregulated completely prevents tumor growth by inhibiting IGF-IR signaling [159]. Recently, increased levels of β1 and its ligand, fibronectin, have been shown to be associated with decreased survival of breast cancer patients, but this finding has not been reported in prostate cancer [160]. The study in non-small cell lung cancer also demonstrated a clinical significance of β1 integrin expression as a prediction marker for radiotherapy and β1 integrin as novel prognostic marker, respectively [161].

Some tumor cells lose their ability to attach to fibronectin after transformation, loss of α5β1 integrin expression led to enhanced tumorigenicity, while increase α5β1 expression showed a loss of tumorigenicity and reduced proliferation of tumor cells [162]. Loss expression of the integrin α2β1 in breast epithelial cells is correlated with the transformed phenotype [152]. Further, by re-expression of

integrin α2β1, the ability of poorly differentiated breast cancer cells to differentiate into gland-like structures has been restored, and the in vivo tumorigenicity has been clearly reduced. Moreover, the integrin α2β1 has been shown to impart metastatic abilities to some tumor cells. Several early studies, both *in vivo* and *in vitro,* demonstrated decreased expression of α3β1 integrin by tumors, particularly those of epithelial origin [163]. Interaction of α3β1 integrin with the adjacent basement membrane might transduce signals that inhibit the invasive behavior of the epithelial cells. Overall, these results are promising and indicate that β1 integrin facilitate activation of selective signaling pathways that support cancer progression. It is possible that different heterodimers of β1 integrin are acting in cancer cell, and indeed, several are aberrantly expressed in cancer. Moreover, several studies illustrate that β1 integrin plays a central role in mediating resistance regardless of the mechanism of cytotoxic injury and is a promising therapeutic target for several cancers [160].

The involvement of β1 integrin in MMP-2 activation

Physical associations between β1-integrin and several classes of proteases, endogenous inhibitors of those proteases or protease binding partners have been identified, yet a mechanism for β1-integrin involvement in the proteolytic remodeling of ECM has not been established [164]. There are numerous studies that have linked β1-integrin expression to alterations in proteases. In the current study, down regulation of β1-integrin expression and/or function revealed differential effects on protease expression and secretion depending on the tumor cell type. β1 integrin is also linked to matrix metalloproteinases (MMPs) via interactions with tissue inhibitors of metalloproteinases (TIMPs) and with TIMPs through tetraspanins. These interactions play roles in cell survival, apoptosis, angiogenesis, but are independent of MMP activity [165]. The study in both breast and prostate carcinoma cell, it also revealed that a reduction of β1-integrin expression and activity decreased the cell migration and

adhesion to type I and IV collagen. Down-regulation of β1-integrin reduces expression of MMP-14, reduces secretion of MMP-13, TIMP-1 and -2 and cysteine protease cathepsin B, and increases secretion of TIMP-3. Moreover, decreases in the expression and activity of β1-integrin reduced digestion of collagen IV which correspondingly their invasion through and migration on reconstituted basement membrane [164].

MMP-2 activation is important for degradation of denatured and cleaved type I collagen, in parallel type I collagen is also activates MMP-2. It was postulated that culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of MMP-2 [44-46]. Collagen type I can activate MMP-2 in certain cell types. MMP-2 activation is induced when cultured on type I collagen in human skin fibroblasts [45] and endothelial cells [166]. Several studies have shown that collagen-binding integrins interact with collagen involve in this mechanism. To date there are four integrins know to bind triple-helical interstitial collagen, and all of these integrins are closely related structurally. They share the common β1 integrin subunit and have four unique α subunits, resulting in the four heterodemers: α1β1, α2β1, α10β1 and α11β1 [85, 86]. Integrin α1β1 and α2β1 are known to be primarily collagen receptors. The α1β1 integrin seems to bind principally to type IV collagen, but it can also recognize type I-VI and type XIII collagen. While integrin α2β1 is a receptor for types I-VIII collagen, integrin α3β1 can interact with some collagen types, but it may not be primarily a collagen receptor. Instead, laminins-1 and –5 seem to be the main ligands for α3β1 [85]. Recently two novel collagen-binding integrin α10β1 and α11β1, have been discovered. Integrin α10β1 was originally purified due to its binding to type II collagen, and integrin α11β1 has been shown to bind to at least type I collagen [150].

Although, both α1β1 and α2β1 integrins are mainly collagen-binding integrins, α2β1 integrin is a functional cellular receptor which can bind to type I collagen fibrils, whereas α1β1 integrin may only effectively bind type I collagen monomers. In

addition, α2β1 is preferentially for type I collagen binding by endothelial cells [167]. Binding of α2β1 integrins to type I collagen in three dimensional, up regulate expression of MMP-1, which in turn is able to degrade fibrillar collagens [168]. Collagen degradation by MMP-1 is followed by denaturation, uncovering RGD sequences. The RGD motif acts as ligand for αVβ3 integrin, which in turn induce the production of MMP-2, and this enzyme completes the collagen degradation. Gilles, et al have shown that type I collagen induced MMP-2 activation and concomitantly increased the MT1-MMP mRNA levels in invasive human breast carcinoma cell lines [48]. In addition, MMP-2 was activated when cultured ovarian carcinoma cells inside a three-dimensional collagen [59], and this has also been seen in breast cancer cells which have been cultured in collagen gel [44]. Furthermore, the induction of pro-MMP-2 and TIMP-2 occurs after treatment of cells with soluble anti-β1 integrin antibody. MT1-MMP-mediated pro-MMP-2 activation accumulated after integrin aggregation, suggesting that integrin aggregation can regulate the MMP-2 activation process [59].

The involvement of β1 integrin in MMP-2 activation in regarding to collagen stimulation is still investigation. Treatment of human endothelial cells with function blocking α2β1 integrin antibody can inhibit MMP-2 activation, whereas blocking ligand interaction to α3β1 integrin had no effect. These studies demonstrated that binding of α2β1 integrin to collagen is necessary for MMP-2 activation process [166]. Previous studies have indicated that cellular interaction with type I collagen is mediated largely through integrin α1β1, α2β1 and α3β1 receptors, cross linking of integrin β1 could activate MMP-2 in ovarian carcinoma cells, suggesting direct involvement of integrin signaling in MMP-2 activation [25, 59]. However, recent study indicated that inhibition of integrin β1 function and expression did not affect 3-D collagen-induced cell surface localization of MT1-MMP and MMP-2 activation, and strongly suggest that 3-D collagen scaffolding provide the direct and multivalent interaction with MT1-MMP, by which MMP-2 activation occur in abundant cell surface MT1-MMP-

dependent manner, rather than a manner regulated by matrix stiffness and integrin β1 function [60]. In this result, it seems apparent that collagen might directly interact with MT1-MMP, leading to increased surface expression of MT1-MMP and MMP-2 activation. In contrast, in endothelial cells, MT1-MMP participates in cooperation with β1-integrin during migration of these cells on various ECMs [34]. MT1-MMP was also found colocalize with β1-integrin in actin-rich, collagenolysis-free leading edges of migrating fibrosarcoma and breast carcinoma cells grown on a 3D collagen matrix [169]. Moreover, It was postulated that the association of β1-integrin with MT1-MMP involves cell migration. Furthermore, the study using functional imaging of live cells demonstrates an involvement for β1-integrin expression in the degradation of collagen IV via MT1-MMP expression and MMP-13, TIMP-1, -2 and -3 secretions [160]. Considering these reports, the involvement of β1 integrin in MMP-2 activation in response to type I collagen stimulation is still not precisely understood. Moreover, MMP-2 is not only important for degradation of denatured, then cleaved type I collagen but also has direct activity against type I collagen [61]. Because type I collagen is a major component of stromal tissue, and both normal and tumor cells digest collagen fibrils to grow or to invade connective tissues. Thus, type I collagen is recommended for the preferred substrate rather than Matrigel in studies investigating cancer invasive behaviors. This study focus on investigation of β1 integrin involve in MMP-2 activation in response to collagen type I stimulation in breast cancer cells model, to better approximate critical interactions with respect to their roles in β1-integrin mediated ECM degradation and invasion of tumor cells.

CHAPTER 3
MATERIALS AND METHODS

1. Materials

1.1 Antibodies and Reagents

Monoclonal antibodies toward integrins β1 (AB2510), αV (L320), α1 (MAB19732), and α3 (MAB19522), were purchased from Chemicon Australia (Boronia, Australia). Polyclonal antibody anti MT1-MMP (AB815) was purchased from Chemicon Australia (Boronia, Australia). Monoclonal antibody toward α2 integrin (Ak7) was a kind gift from Dr. Michael Berndt (Monash University, Melbourne) and antibody toward pan-actin was obtained from Biosource International (Camarillo, CA). Con A, TPA, Vitronectin (VN), fibronectin (FN) and laminin-1 (LN) were obtained from Sigma (Castle Hill, Australia). Recombinant full-length pro-MMP-2 expressed in a vaccinia virus system (rMMP-2) was a kind gift from Dr. Rafael Fridman (Wayne State University, Detroit, MI). Vitrogen 100 Collagen type I was obtained from Cohesion (Palo Alto CA). Alexa Fluor 568 conjugated goat anti-mouse secondary antibody and Alexa Fluor 488 conjugated donkey anti-rabbit secondary antibody were obtained from Molecular Probes (Eugene, OR).

1.2 β1 integrin siRNA

In this study, 6 different β1-integrin siRNAs were designed as indicated in the Table 1 below and synthesized by Proligo (Australia).

Table 1: β1 integrin siRNA sequences

β1-integrin siRNA type (number)	first base of β1-integrin siRNAs	last base of β1-integrin siRNAs	β1 integrin siRNAs target sequence
1	527	547	AATTCAAGAGAGCTGAAG ACT
2	760	780	AACTGCACCAGCCCATTT AGC
3	1042	1062	AATATGTACACAATGAGC CAT
4	1407	1427	AATTAGCATAACTTCAAA TAA
5	1816	1836	AATGGCTTAATTTGTGGA GGA
6	2380	2400	AAGCTTTTAATGATAATTC AT

1.3 DNA constructions

The pcDNA3.1 vector was purchased from Invitrogen, Melbourne, Australia and used for subclone target human gene in this study. The orientation of pcDNA3.1 vector was showed in Figure 11. Qiagen RNeasy mini-column kit was obtained from Qiagen, GmbH, Germany.

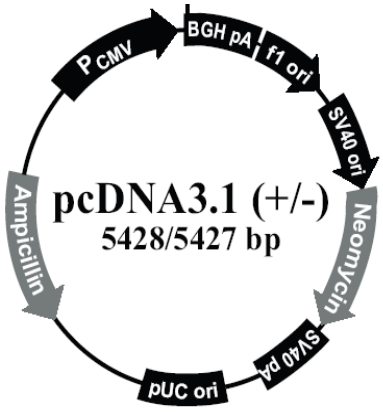


Figure 11: pcDNA3.1 plasmid vector

1.4 Quantitative Reverse Transcription-PCR Primers

Gene expression analysis by Quantitative Reverse Transcription-PCR including α1, α2, α10, α11, and β1 integrin. The L32 expression was utilized as an internal control. Primer sequences of all of human gene are described as Table 2.

Table 2: Human gene primer sequences

Human gene	Forward primer:	Reverse primer:
L32	CAGGGTTCGTAGAAGATTCAAGGG	CTTGGAGGAAACATTGTGAGCGATC
α1 integrin	GCTGACCAGTCAGCAGCTTCATTT	CTCCAGAAGAAGCAGTAGCAGAGTTT
α2 integrin	GACCTATCCACTGCCACATGTGAAAAA	CCACAGAGGACCACATGTGAGAAAA
α3 integrin	CGCAGGTGGGCGCCTATTTT	GGCACCCCCTACTTCCTCTTT
αV integrin	GGCAGTGCCATAGCTCCTTT	CCCACTGCCCTTCAAGGGATTT
β1 integrin	GACTGATCAGTTCAGTTTGCTGTGTGTTT	CCCTGCTTGTATACATTCTCCACATGATTT

2. Methods

2.1 Cell culture

The MCF-7 breast cancer cell line was engineered to generate a model for stable human MT1-MMP expression. MCF-7-MT1 cells were generated by stably transfecting a vector encoding the MT1-MMP cDNA under the transcriptional control of the CMV promoter pCNC (MT1-MMP) into a MCF-7 breast adenocarcinoma

clone already transfected with bacterial β-galactocidase gene (ML20). MCF-7-MT1 and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM. Life Technologies, Auckland, New Zealand), supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS). Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere.

2.2 Constructed β1 integrin plasmid DNA for over expression of β1 integrin in MCF7-MT1 cells

Human β1 integrin cDNA was amplified by RT-PCR and subsequently subcloned into a pcDNA3.1 vector (Fig 11) after ECOR I restriction. The wild type pcDNA-β1 integrin plasmid was transformed into the XL-1 Blue supercompetent cells where the repair of the nicks in plasmid occurred. Subsequently, the successful of insertion was determined by restriction enzyme method. Transient transfection of β1 integrin plasmid DNA in MCF7-MT1 cells was performed. The day before transfection, MCF-7-MT1 cells were trypsinized, diluted with fresh medium without antibiotics and seeded ($4x10^5$ cells/well) in 2 ml of culture medium into 6-well culture plates. Fugene6 reagent (Roche) was used for transfection as per the manufacturer's instruction. For each transfection, 2 ug of either pcDNA-VC or pcDNA-β1 integrin were diluted in 50 μl Serum-free DMEM (SFM/DMEM). Fugene6 (6 μl) was mixed in 50 μl SFM/DMEM, then mixed with each diluted pcDNA-VC or pcDNA-β1 integrin and subsequently incubated for 30 minutes at room temperature to allowed the pcDNA-VC or pcDNA-β1 integrin: Fugene6 complexes to form. Then 100 μl of each of the pcDNA-VC or pcDNA-β1 integrin: Fugene6 complexes were added to each well and incubated with the cells at 37°C in a CO_2 incubator for 24-72 hours. Over-expression of β1 integrin was determined by quantitative RT-PCR and immunoblotting.

2.3 Gene expression analysis by Quantitative Reverse Transcription-PCR

Total RNA was isolated using Qiagen RNeasy mini-column kit according to manufacturer's recommendations (Qiagen, GmbH, Germany). 100 ng of total RNA was used to generate cDNA using Superscript II reverse transcriptase (Invitrogen) and random primers (50 ng/µl). The samples were heated at 65° C for 5 minutes and cooled to 4° C for 5 minutes. The reaction mixture containing First strand buffer (Gibco), 10 mM DTT (Gibco) and 1.25 mM dNTPs (New England Biolabs, Inc, MA, USA) and 200 Unit Superscript II reverse transcriptase were added for a final volume of 10 µl, and subsequently incubated at 42° C for 2 hours. Quantitative RT-PCR was performed on an ABI Prism 5700 Sequence Detection System (PE Applied Biosystems, Sydney, NSW, Australia) on an equivalent of 1:10 dilution of cDNA from RT-PCR products generated from an equivalent of 100 ng of total RNA. The PCR reaction was performed in 25 µl of 10 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 50 mM KCl, 200µM dNTPs, 1/40,000 dilution SYBR Green I (Molecular Probes), 1 µg/ml 6-carboxy-X-rhodamine (6-ROX) (Molecular Probes), 8% DMSO, 200 nM primers and 0.625 U AmpliTaq Gold polymerase (Applied Biosystems). The PCR conditions were 50° C for 2 minutes, followed by 10 minutes at 95° C and 50 cycles of 95° C for 15 seconds and 60° C for 1 minute. Melt curve analysis was performed at the end of each run from 60° C to 95° C. The difference in average cycle threshold (dCT) between L32 (housekeeping) and the gene of interest was determined by quadruplicate readings for each sample and the mean and standard deviation determined. The L32 expression was utilized as an internal control. Primer sequences of all of human gene are described as Table 2 including α1, α2, α10, α11, and β1 integrin.

2.4 β1-integrin siRNA transfection to knockdown β1 integrin expression

Short interference RNA (siRNA) technology was used to knockdown β1 integrin expression levels. All 6 putative β1-integrin siRNAs were designed to target all known β1 integrin transcript variants. β1-integrin siRNAs targets were selected from regions lacking strong local mRNA secondary structure. The specificity of each putative β1-integrin siRNAs target was confirmed using blasta (www.ncbi.nlm.nih.gov). The day before transfection, MCF-7-MT1 cells were trypsinized, diluted with fresh medium without antibiotics and seeded ($1x10^5$ cells/well) in 0.5 ml of culture medium into 24-well culture plates. Lipofectamine ^{TM} 2000 (Invitrogen, Life Technologies) was used for transfection as per the manufacturer's instruction. For each transfection, 20 pMol of the β1-integrin siRNA was diluted in 50 µl Serum-free DMEM (SFM/DMEM). Lipofectamine ^{TM} 2000 (1 µl) was mixed in 50 µl SFM/DMEM, then mixed with each diluted β1-integrin siRNA, and subsequently incubated for 20 minutes at room temperature to allowed the β1-integrin siRNA:Lipofectamine ^{TM} 2000 complexes to form. Then 100 µl of each of the β1-integrin siRNA:Lipofectamine ^{TM} 2000 complexes was added to each well and incubated with the cells at 37^{o}C in a CO_2 incubator for 24-72 hours. β1 integrin knockdown efficiency was determined by quantitative RT-PCR and immunoblotting.

2.5 Flow cytometry

Cells were trypsinized and resuspended in FACS buffer (2% FCS in PBS, 0.02% sodium azide) at $1x10^6$ cells/ml. Cells were incubated with 10 µg/ml antibodies specific to integrins α1, α2, α3, αV, and β1 for 60 minutes at room temperature. Following several washes with FACS buffer, cells were incubated with 1:100 dilution of goat anti-mouse or swine anti-rabbit IgG conjugated with FITC (Dako) for 45 minutes at room temperature. Thereafter, cells were analyzed on a

FACScan flow cytometer Becton-Dickinson, Mountain View, CA). Population gates were set by using control cells incubated only with the secondary antibody.

2.6 Immunofluorescence

Cells were plated on Teflon printed glass slides (Electron Microscopy Sciences, Ft. Washington PA) at 37°C, 5% CO_2. The following day, cells were fixed with 3% paraformaldehyde/PBS for 20 minutes at room temperature and blocked with 5% BSA/0.25% Triton X-100 for 30 min. Cells were then washed in PBS and incubated with appropriate primary antibodies toward β1 integrin or MT1-MMP at 4°C overnight. Primary antibodies were detected with a donkey anti-rabbit secondary Alexa Fluor 488 conjugated antibody (1:500 dilution in 1% BSA/PBS) for 1 hour at room temperature. After several PBS washes, nuclei were stained with propidium iodide (0.25mg/ml in Triton-X100/RNase) for 30 minutes at room temperature. Cells were then washed in PBS and mounted using fluorescent mounting medium (Dako) and viewed by confocal microscopy (BioRad MRC 1024, Hemel Hempstead, UK).

2.7 Cell lysates and Western Blot

Cells were grown on 6-well plates and washed with ice-cold Phosphate Buffered Saline (PBS). The cells were then resuspended in Lysis Buffer (10 mM Tris-HCL pH 7.6, 10 mM NaCL, 3 mM $MgCl_2$ and 1% Nonidet P-40) containing 1:10 Protease Inhibitor Cocktail (Roche, Mannheim, Germany). The cells were scraped with a rubber cell scraper and left on ice for 1 hour with periodic mixing, followed by centrifugation at 6000 rpm for 10 minutes. The protein concentrations were determined with a BCA protein quantification assay (Pierce, Rockford IL). Equal amounts of total protein lysates were electrophoresed on 10% SDS-PAGE under reducing condition (100 mM DTT). After electroproresis, the proteins were transferred to

a PVDF membrane, blocked with 5% skim milk powder in Tris Buffered Saline (TBS) containing 0.1% (v/v) Tween-20 (TBST). Membranes were incubated with primary antibodies overnight at 4^0 C, then probed with an HRP-conjugated secondary antibody (Pierce). Detection of the secondary antibody was performed using the ECL+Plus system (Pierce) and the membranes expose to ECL hyperfilm to visualize protein bands (Amersham Pharmacia Biotech).

2.8 Conditioned medium isolation

Cells were plated for near confluence at 37^o C overnight. After each treatment, cells were washed twice with serum-free medium (SFM: unsupplemented DMEM), then replaced with fresh SFM. Recombinant full-length pro-MMP-2 (kindly, provided by Dr. Rafael Fridman, Wayne State University, Detroit, MI, USA) was added at 100 ng/ml concentration. At the appropriate time point, the conditioned medium was collected, transferred to a microcentrifuge tube and centrifuged at 6000 rpm for 10 minutes at 4^o C in order to remove any cells in suspension. For the MMP-2 activation assay, equal amounts of conditioned media were mixed with non-reducing sample buffer and analyzed by gelatin zymography as described below.

2.9 Gelatin Zymography for MMP-2 activation analysis

Cells were plated and grown to confluence and then washed twice with SFM, incubated with fresh SFM containing 25% conditioned-medium from MCF-7 cells stably expressing MMP-2. At the appropriate time point, the conditioned medium was collected and transferred to an Eppendorf tube and centrifuge at 6000 rpm for 10 minutes at 4^o C in order to remove any cells in suspension. Equal amounts of conditioned media were analyzed by gelatin zymography using 5% polyacrylamide stacking gel and a 10% polyacrylamide resolving gel (Bio-Rad Co., Richmond, VA) containing 1 mg/ml gelatin (BDH Laboratory Supplies, Poole, UK). Conditioned-medium

or cellular proteins were mixed with SDS sample buffer under non-reducing conditions. After electroproresis, gels were washed twice in 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$ and 2.5% (v/v) Triton X-100 for 30 minutes each wash, and then incubated in 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$ at 37^o C overnight. Gels were stained with 0.25% (w/v) Coomassie Brilliant Blue (R-250) dye in 10% (v/v) acetic acid and 10% (v/v) isopropanol, then destained in 10% (v/v) acetic acid and 10% (v/v) isopropanol until clear bands of MMP-2 were visualized**.**

2.10 Collagen gel contraction assay for functional activity of β1 integrin on ECM remodeling Determination

Collagen gels were made from collagen type I (vitrogen 100, 3 mg/ml), neutralized with 1M sodium hydroxide, pH 8.0 and then dilution in PBS to a final concentration of 2 mg/ml of Col I in 10 mM of sodium hydroxide. Cells were resuspended such that one part of the cell suspension was mixed with nine parts of the collagen solution, to give a final concentration of $4x10^5$ cell/ml. A total of 500 µl of cell-collagen mixture was added to 24 well plates and gels were allowed to polymerize for 60 min at 37^0 C. In order to release the gels, a yellow tip was inserted around and underneath the gels. The relaxed, floating gels were immersed with 1 ml medium and incubated at 37^0 C. The gel diameters were measured daily for 5 days using an inverted microscope. Collagen gel contraction is indicated as the decrease in gel area expressed as a percentage. Each experiment was performed in triplicate and with 3 independent experiments. Statistical analysis was performed by student t-test.

2.11 Adhesion assay for functional activity of β1 integrin

Matrix molecules were collagen type I, fibronectin, and vitronectin. ECM molecules were serially diluted in serum-free DMEM containing 0.1% BSA (DMEM/BSA), and 50 µl of each dilution was added in triplicate to 96-well plates. The coated-plates

were incubated at 37^{o} C for 60 minutes. Wells were then washed with 100 µl PBS containing 3% (w/v) BSA for 30 minutes at 37^{o} C. Cells were detached from culture by VERSENE (PBS containing 0.2 mg/ml EDTA), then washed twice with DMEM/BSA, and resuspended at 2.5x 10 ^{5} cells/ml in DMEM/BSA. Cells were incubated at 37^{o} C for 60 minutes to allow recovery of cell surface proteins, after which 100 µl (2.5x 10^{4} cells) were added to each of the triplicate wells, which were then incubated at 37^{o} C for 60 minutes. The adherent cells were stained with 0.5% (w/v) Crystal Violet in 25% (v/v) methanol for 5 minutes at room temperature. Wells were then gently rinsed 5 times and allowed to air-dry overnight at room temperature. The determination of cell adhesion was achieved by solubilizing the remaining crystal violet with 0.1 M sodium citrate containing 50%(v/v) ethanol for 15 minutes and measuring absorbance at 540 nm by microplate reader (Power Wave X, Bio-Tek Instruments, Inc., Winooski, VT, USA). Each experiment was performed in triplicate and 3 independent experiments were performed. A student t-test was used for statistical analysis.

2.12 Point mutations of human β1 integrin DNA

Human β1 integrin cDNA was amplified by RT-PCR and subsequently subcloned into a pcDNA3.1 vector (Fig. 7) (Invitrogen, Melbourne, Australia) after ECOR I restriction. Human β1 integrin point mutation was generated by QuickChange Site-directed Mutagensis kit (Invitrogen, Melbourne, Australia). Two synthetic oligonucleotide primers were designed to generate the desired point mutation of β1/6 integrin siRNA recognition site. These primer sequences are as follows: human β1 integrin mutant forward primer TGC TGA TAT GGA AAC TAC TTA TGA TTA TAC ACG ACA GAA GGGAGT, human β1 integrin mutant reward primer ACT CCC TTC TGT CGT GTA TAA TCA TAA GTA GTT TCC ATA TCA GCA. To generate the human β1 integrin mutation, 50 ng of pcDNA-β1 was used and amplified by PCR reaction in 50 µl of 1X reaction

buffer, 125 ng of oligonucleotide forward and reward primers, 2.5 units of *PfuTurbo* DNA polymerase and dNTPs. The PCR conditions were 95 ^{o} C for 30 seconds, followed by 12 cycles of 95^{o} C for 30 seconds, 55^{o} C for 1 minute and 68 ^{o} C for 18 minutes. Following temperature cycling, the samples were cooled down 2 minutes at 37^{o} C. 10 units of Dpn I restriction enzyme was added to digest the methylated, non-mutated parental DNA, then incubated at 37^{o} C for 60 minutes. The mutated-pcDNA-β1 plasmid was transformed into the XL-1 Blue supercompetent cells where the repair of the nicks in mutated-plasmid occurred. Subsequently, the successful of mutagenesis was determined by restriction enzyme method.

2.13 Co-transfection of human β1 integrin and β1 integrin siRNA

MCF-7-MT1 and MDA-MB-231 were tested, cells were trypsinized, diluted with fresh medium without antibiotics and seeded ($4x10^5$ cells/well) in 2 ml of culture medium into 6-well culture plates. Following day, cells were transfected with β1/6-integrin siRNA, Lipofectamine ^{TM} 2000 (Invitrogen, Life Technologies) was used for transfection as described above (3.2). The transfected cells were then incubated at 37^{o}C in a CO_2 incubator for 24 hours. Medium was removed and added with 2 ml of fresh supplement medium. The siRNA-transfectd cells were subsequently transfected with either wild-type or mutant human β1 integrin as indicated. FuGENE 6 (Roche) was used for transfection as per the manufacturer's instruction using 2 μg plasmid DNA with 6 μl FuGENE 6 per treatment. After 24 hours transfection, cells were washed twice with serum-free medium (SFM: unsupplemented DMEM), then replaced with fresh SFM. Recombinant full-length pro-MMP-2 (kindly, provided by Dr. Rafael Fridman, Wayne State University, Detroit, MI, USA) was added at 100 ng/ml concentration. At the appropriate time point, the conditioned

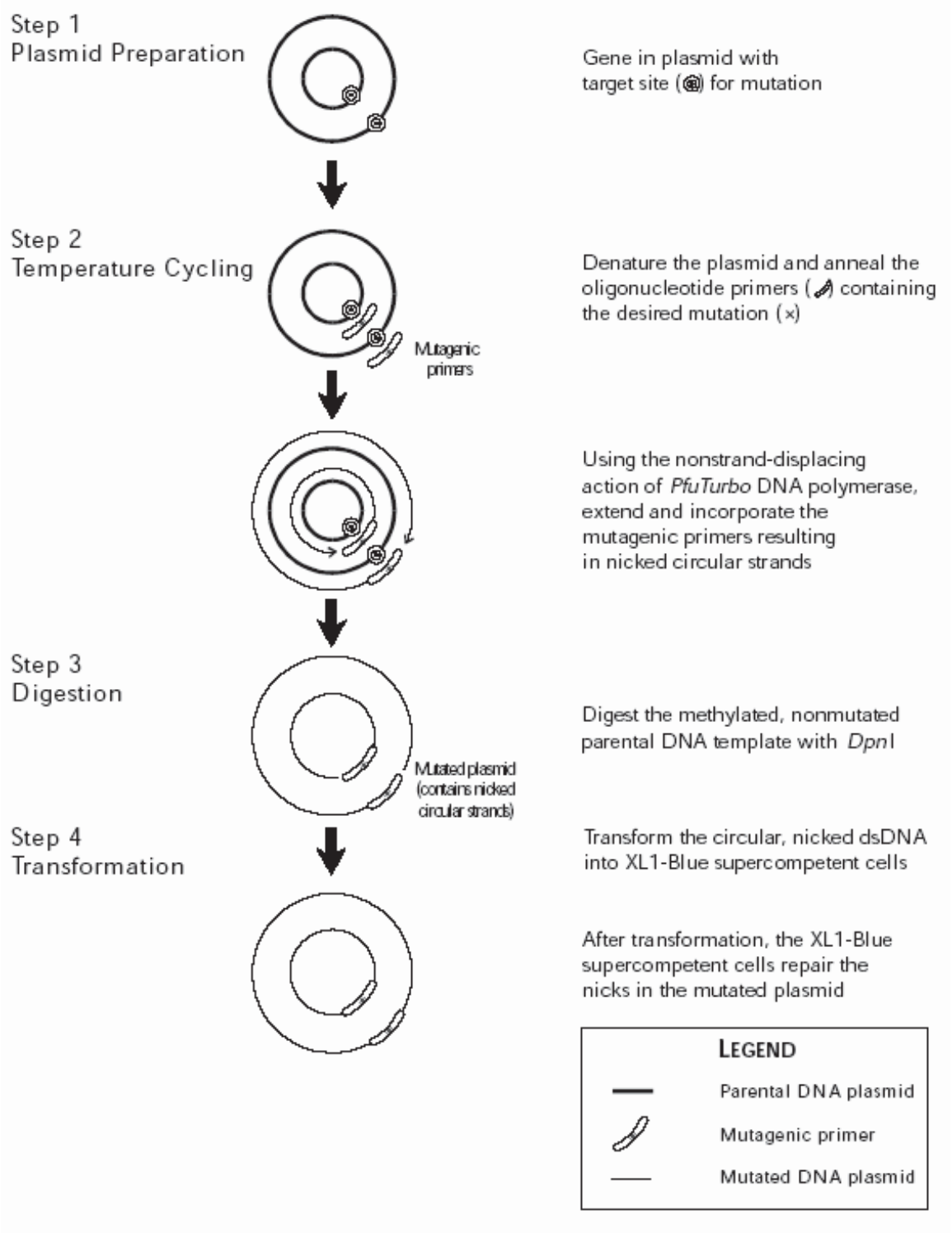
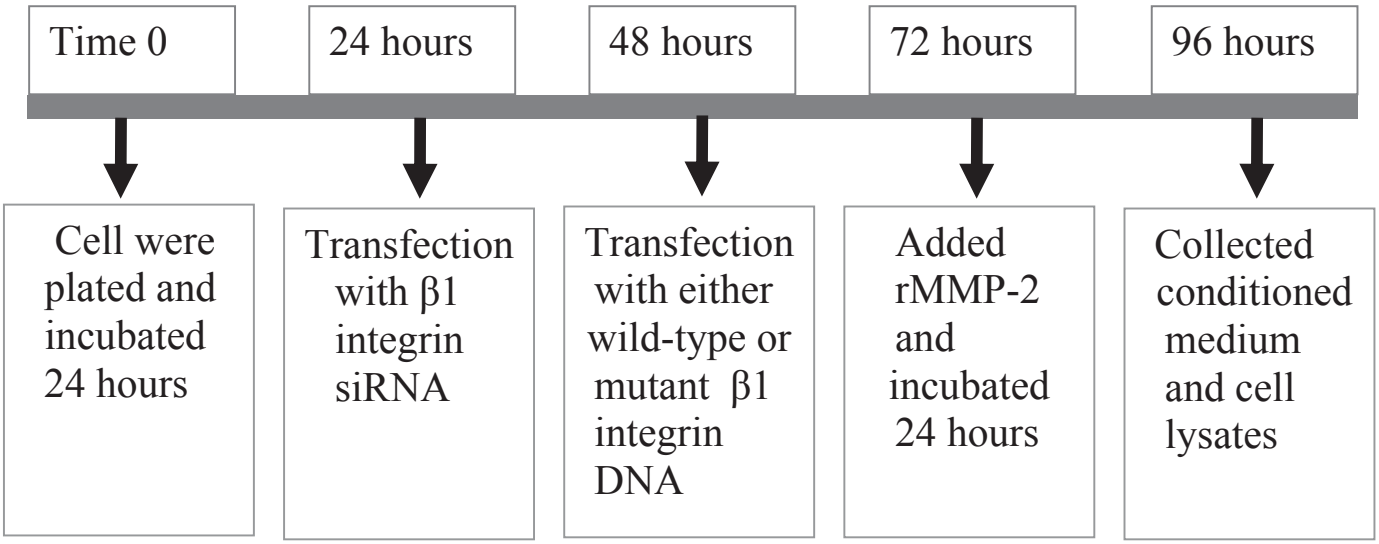


Figure 12: Overview of site-direct mutagenesis method

medium and cell lysates were collected for MMP-2 activation by Zymograpgy and β1 inetgrin expression by Western blot respectively.



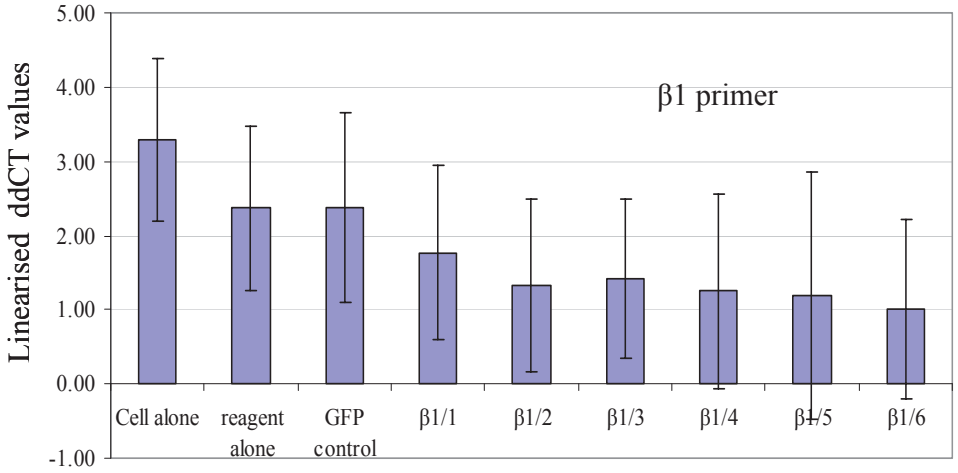
CHAPTER 4
RESULTS

1. Three candidates of β1 integrin siRNAs, namely β1/4, β1/5 and β1/6 were efficiently knockdown β1 integrin expressin at 72, 96 and 120 hours.

The β1 integrin is an important partner of the collagen-binding integrin receptors. However, the involvement of β1 integrin in collagen-stimulated MMP-2 activation is still unclear. β1 integrin siRNA technology was used to determine the influence of β1 integrin on MMP-2 activational responses to type I collagen stimulation. Six different β1 siRNAs were designed, namely β1/1, β1/2, β1/3, β1/4, β1/5 and β1/6 siRNA, and transfected in MCF-7-MT1 breast cancer cells which had previously been stably transfected MT1-MMP. Although, all 6 siRNAs showed decreased β1 integrin expression at both the RNA and protein levels, only 3 of them (β1/4, β1/5 and β1/6 siRNAs) showed substantial knockdown of the β1 integrin expression, with the most efficient knockdown being from β1/6 siRNA (Fig. 13 A and 13 B).

To determine the interval time for suppression of β1 integrin expression by siRNA technology, only β1/4, β1/5 and β1/6 siRNAs were selected. Both MCF-7-MT1 and MDA-MB-231 breast cancer cells were used. MCF-7-MT1 which lack of endogenous MT1-MMP and also showed low level of β1 integrin expression, while MDA-MB-231 present high level of endogenous MT1-MMP and β1 integrin expression. All of the β1/4, β1/5 and β1/6 siRNAs initiated knockdown of β1 integrin expression at 24, 48, 72, 96 and 120 hours after transfection. However, β1 integrin expression was completely knocked down at 72, 96 and 120 hours in both MCF-7-MT1 (Fig. 14 A) and MDA-MB-231cells (Fig. 14 B).

A. qRT-PCR



B. Western Blot

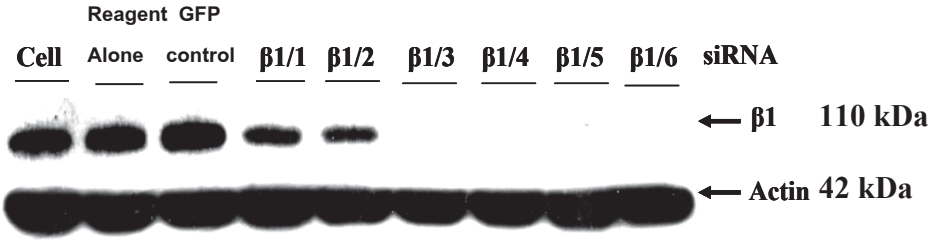


Figure 13: Knockdown of β1 integrin expression by β1 siRNA technology in MCF-7-MT1 cells. All 6 different β1 siRNAs were transfected into MCF-7-MT1 cell. RNA was collected after 24 hours transfection, and 100 ng was analyzed for β1 integrin expression by qRT-PCR **(A)**. Proteins were harvested at 48 hours after transfection and analyzed for β1 integrin expression by Western Blot **(B).**

A. Western Blot: MCF-7-MT1 **B. Western Blot: MDA-MB-231**

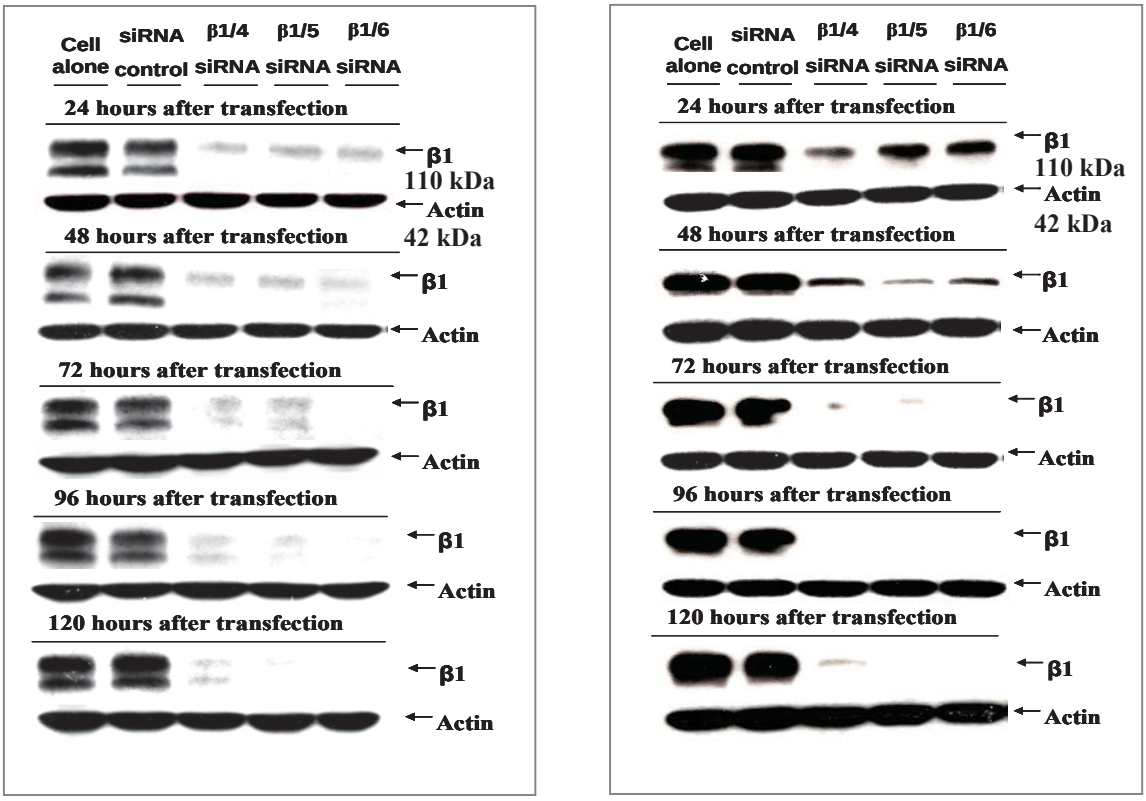


Figure 14: Time course of β1 integrin knockdown after β1siRNA transfection. MCF-7-MT1 and MDA-MB-231 cells were plated in 6 well plates and transfected on the following day with β1/4, β1/5, and β1/6 siRNAs. Controls included cell alone and control siRNA. At appropriate time points, cell lysates were collected for analysis of β1 integrin levels by Western blot in MCF-7-MT1 (A) and MDA-MB-231 (B).

2. Abrogation of β1 integrin expression reduced cells adhesion to Type I collagen, Fibonectin, but not Vitronectin.

The specificity of integrin binding to ECM components including laminins, collagens, and fibronectin depends on the extracellular domains of the α and β integrin subunits. Integrins α1β1, α2β1, α10β1 and α11β1 represent the primary collagen receptors [145, 150]; integrins α3β1, α6β1, α6β4 and α7β1 are the major laminin receptors [147]; and integrins α5β1, α8β1, αIIbβ3 are the major fibronectin receptors and the αvβ3 is specific for vitronectin receptor that bind in an RGD-dependent manner [145]. Since, β1 integrin is ubiquitously expressed and can bind multiple a partners. To examined the functional activity of abrogated-β1 expressing, adhesion analysis was performed with ECM substrates including VN, FN, and Col I after β1 integrin siRNAs transfection, where β1 integrin was completely knockdown. Cells expressing β1 integrin (control and siRNA control) showed significantly stronger adhesion to type I Col and FN compared to abrogated-β1 expressing cells. However, β1/6 integrin siRNA showed the lowest adhesion to both type I Col and FN. Specifically in type I Col adhesion, only β1/6 integrin siRNA showed the lowest adhesion significantly ($p < 0.05$), while β1/4 and β1/5 siRNAs were not clearly decrease adhesion ($p > 0.05$) (Fig. 15 A). Decreasing adhesion to FN was also observed, all of β1/4, β1/5 and β1/6 siRNAs adhered less than β1-integrin expressing cells statistically significance, $p < 0.05$, 0.05 and 0.01 respectively. Similarly, β1/6 integrin siRNA showed the lowest adhesion to FN compared to β1/4 and β1/5 knockdown cells (Fig. 15 B). Vitronectin is specific for the αvβ3 cell receptor, thus it was used for control in this experiment. All of abrogated-β1 expressing cells were not different adhesion to VN, confirm that abrogation of β1 expressions were not effect to unspecific ECM molecule of β1 integrin (Fig. 15 C).

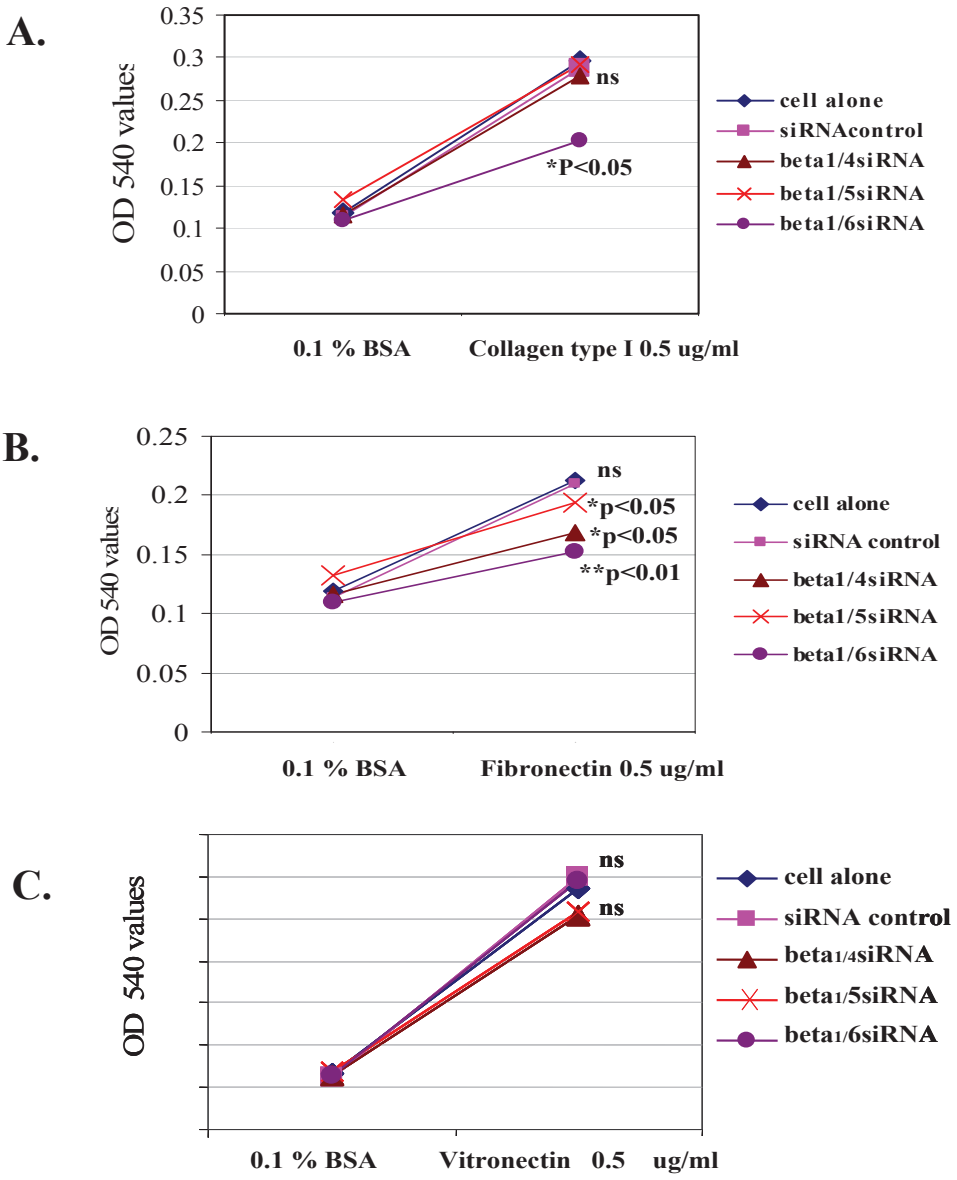


Figure 15: Functional activity reduction of abrogated-β1 integrin expressing cells: Adhesion assay of β1 expression in MCF-7-MT1. Adhesion analysis was performed to ECM substrates including Col I, FN, and VN which were used at o.5 ug/ml as shown. Each experiment was performed in triple and student t-test was used for statistical analysis.

3. The β1/6 siRNA was the most efficient reduction of collagen gel contraction, while β1/4 and β1/5 siRNAs were only partial reduction of collagen gel contraction.

Remodeling of the ECM plays a critical role in the reorganization of connective tissue in tumor invasion [105, 106]. Collagen gel contraction is a process of reorganizing the collagen fibrils and contraction of the gel. It is used as an in vitro model of cell-mediated tissue remodeling, including wound contraction and maintenance of tissue homeostasis [107]. Several studies have demonstrated a role for β1 integrin, in particular α1β1 and α2β1 integrin, in mediating collagen gel contraction by fibroblast and osteoblastic cells [111-113]. To determine the functional effects of β1 integrin knockdown by siRNA technology. Contraction of a relaxed or free-floating gel experiment was performed. Abrogation of β1 integrin expressions were induced by β1/4, β1/5 and β1/6 siRNAs transfection. After 72 hours trasfection, both MCF-7-MT1 and MDA-MB-231 cells were grown in 3 dimensional (3D) collagen gels, and free-floating gel contractions were monitored for 5 days. Collagen gel contraction was clearly observed with MDA-MB-231 cells. While, MCF-7-MT1 cells invaded through the collagen gel and some cells stayed inside collagen gel, and only partial contraction of collagen gel was seen. All of abrogated- β1 integrin expressing cells show less contractile than β1 integrin expressing cells (cell and siRNA control) (Fig. 16 A). However, only β1/6 siRNA initiated failing of contraction at the first day and keep failing until the last day, while β1/4 and β1/5 siRNAs initiated failing of contraction only at the first day, then started to contract at the second day until the last day (Fig. 16 B). Interestingly, only β1/6 siRNA indicated the most efficient reduction of collagen gel contraction significantly (16.49 %, $p < 0.001$), while β1/4 and β1/5 siRNAs were not significant difference from β1 integrin expressing cells (cell and siRNA control) as shown in Figure 16 C.

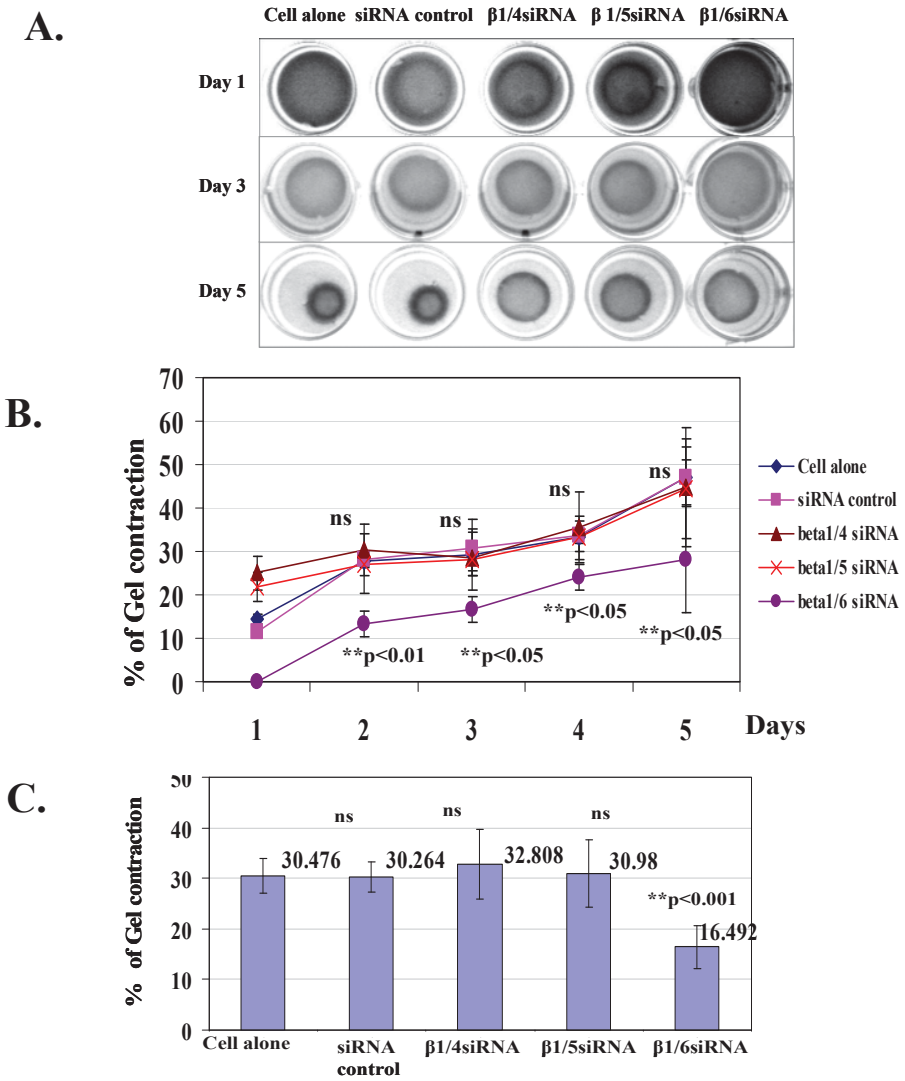


Figure 16: Knockdown of β1 expression by β1/6 siRNA reduced collagen gel contraction in MDA-MB-231 cells. Abrogation of β1 expression was induced by β1/4, β1/5 and β1/6 siRNAs trasfection. After 72 hours transfection, MDA-MB-231 cells were plated within 3D type I collagen gels. On the following day, gels were released as free-floating gels (A). Measurements of gel diameters were performed as indicated daily for 5 days (B). Collagen gel contractions were calculated as mean values in relation to the gel diameters before releasing. Three independent experiments were performed, and student's t-test was used for statistical analysis (C).

4. β1/4, β1/5 and β1/6 siRNA each efficiently knocked down β1 integrin, however, only β1/6 effected a reduction in Col I-induced MMP-2 activation.

The β1 integrin is an important partner of the collagen-binding integrin receptors. However, the involvement of β1 integrin in collagen-stimulated MMP-2 activation is still unclear. Since, all of β1/4, β1/5 and β1/6 siRNAs initiated knockdown of β1 integrin expression at 24, 48, 72, 96 and 120 hours after transfection (Fig. 14 A, B). Thus, to determine the interval time for suppression of β1 integrin and subsequent effects on MMP-2 activation by β1 siRNA, only β1/6 siRNA was transfected in to MCF-7-MT1 and MDA-MB-231 cells. The β1/6 siRNA showed a reduction of MMP-2 activation at 72 and 96 hours after transfection in parallel with β1 integrin expression in both MCF-7-MT1 and MDA-MB-231 cells (Fig. 17 B), where β1 integrin expression was completely knocked down at 72 hours (Fig. 17 A) . No effect on MMP-2 activation was seen at 24 or 48 hours after transfection, at which times β1 integrin expression was not completely knocked down. This indicated that, substantially knock down of β1 integrin expression showed a reduction of Type I Col-induced MMP-2 activation in both MCF-7-MT1 and MDA-MB-231 cells.

To determined further the effects of β1/4, β1/5 and β1/6 siRNAs on MMP-2 activation in MCF-7-MT1 cells. As described above, MCF-7-MT1 is a breast cancer cell line which lack of endogenous MT1-MMP expression and induced MT1-MMP expression by stably transfected with MT1-MMP. In this cell model, Col I causes activation of MMP-2 despite MT1-MMP being under the control of a heterologous promoter. Furthermore, when these cells were treated with cycloheximide to block new protein synthesis, some reduction in MMP-2 activation was seen, but the Col I response was still observed. Thus, MCF-7-MT1cells provide a model for non- VV Vtranscriptional regulation of Col I-induced MMP-2 activation. As the result found that β1 integrin expression was completely knocked down at 72 hours after transfection (Fig. 18 A). An MMP-2 activation time

course analysis was conducted at 72, 96 and 120 hours after transfection. All 3 types of β1 integrin siRNA; β1/4, β1/5 and β1/6 were knockdown β1 inetgrin at 72, 96 and 120 hours as shown in Figure 15 A. Although, β1/4, β1/5 and β1/6 siRNA each were efficient in the knockdown of β1 integrin, however, only β1/6 had a convincing effect on MMP-2 activation reduction at all time points (Fig. 18 B).

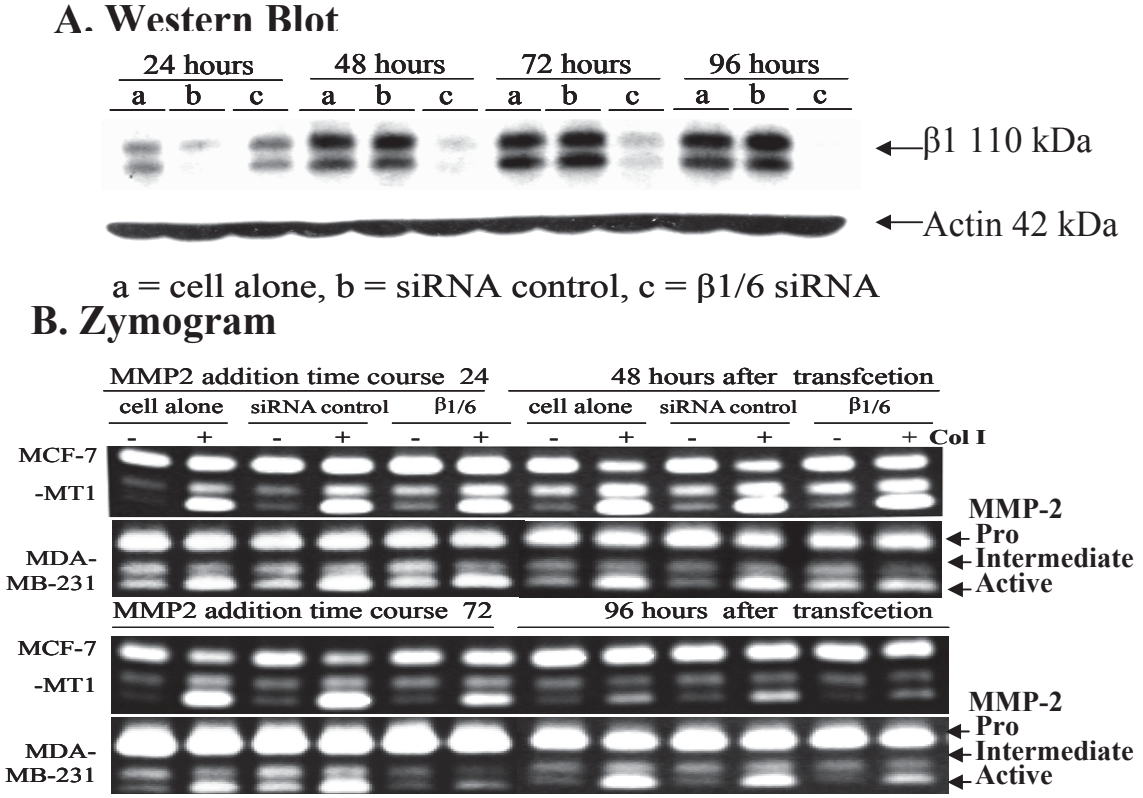


Figure 17: Time course of β1 integrin knockdown after β1/6 siRNA transfection in MCF-7-MT1 and MDA-MB-231 cell. MCF-7-MT1 and MDA-MB-231cells were plated in 6 well plates and transfected on the following day with β1/6 siRNA. Controls included cell alone and control siRNA. Cells were washed and 100 ng/ml of rMMP-2 was added where indicated, then cells were untreated and treated with 100 μg/ml of Col I. At appropriate time points, cell lysates were collected for analysis of β1 integrin levels by Western blot (A). Conditioned media were also harvested for MMP-2 activation status determination by gelatin zymogram (B). Pro, Intermediate, and Active MMP-2 are indicated by arrow.

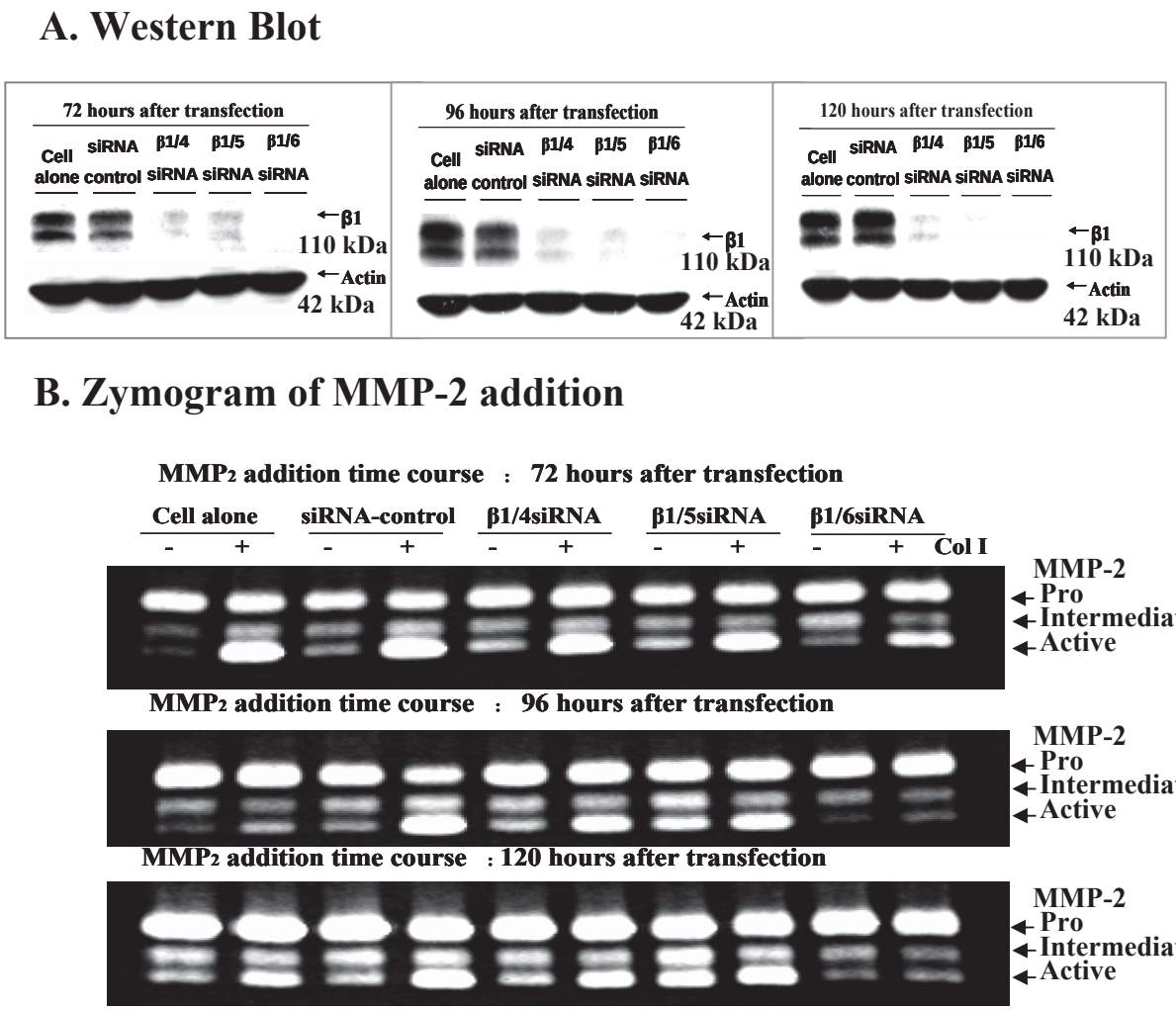


Figure 18: Time course of β1 integrin knockdown and MMP-2 activation in MCF-7-MT1 cell. (A). Western Blot. MCF-7 cells were transfected with β1/4, β1/5 and β1/6 siRNAs. Cell lysates were collected at 72, 96 and 120 hours after transfection. (B). Zymogram. 100 ng of rMMP-2 was added at 72, 96 and 120 hours after β1 integrin siRNA transfection, then cells were untreated or treated 100 μg/ml of Col I. Conditioned-medium was collected at 24 hours incubation and MMP-2 activation status in response to type I collagen stimulation assessed by zymogram. Pro, Intermediate, and Active MMP-2 are indicated by arrow.

5. β1/4, β1/5 and β1/6 siRNAs were not only efficient in the knockdown of β1 integrin at the cell surface, but also knocked down α2 and α3 integrin subunits.

Integrin α1β1, α2β1, α10β1 and α11β1 are the major collagen-binding integrin receptors. Because β1/4, β1/5 and β1/6 siRNAs were all efficient in the knockdown of β1 integrin, but only β1/6 reduced MMP-2 activation in response to type I collagen. We hypothesized that these 3 different β1 siRNAs have different effects on the β1 integrin knockdown and also on the α subunits which comprise collagen-binding integrin heterodimers. FACS analysis was performed to assess the α2, α3, and αV integrins, since MCF-7-MT1 cells were found to have very low levels of α1 integrin, and no detectable levels of α10 and α11 integrin subunits when examined by qRT-PCR (data not shown). Interestingly, β1/4, β1/5 and β1/6 siRNAs each not only knocked down β1 integrin, but also the α2 and α3 integrin subunits. However, no effect on the levels of the αV integrin subunit was observed (Fig. 19). This data indicated that each of the β1 integrin siRNAs (β1/4, β1/5 and β1/6) had closely similar effects on collagen-binding integrin receptors, α2β1 and α3β1 integrin heterodimers.

6. Dose response effect of β1 integrin siRNA on knockdown of β1 integrin expression.

There are several factors that may influence the efficiency of RNAi in mammalian system including the choice of the target site of degradation, the transfection method and the turn over rate of the protein [170]. In addition, the mRNA degradation rate is an important factor influencing target gene knockdown by siRNA technology [170-172]. This effect is dependent on the dose response to siRNA concentration. To determined β1/4, β1/5 and β1/6 siRNAs had dose-dependent effects on the abrogation of β1 integrin mRNA expression, and whether this was relative to the reduction seen in MMP-2 activation. We hypothesized that the β1/6 siRNA showed a quicker

mRNA degradation rate than either β1/4 or β1/5 siRNA. Serial concentrations of β1/6 siRNA (2.5, 5, 10, 15 and 20 μM) were tested. The data showed that at the lowest concentration (2.5 μM), β1/6 siRNA was able to knockdown β1 integrin expression to an extent comparable to the highest concentration (20 μM) of β1/4 or β1/5 siRNA (Fig. 20 A). The β1 integrin expression was below the threshold of detection 72 hours after transfection with 2.5 and 5 μM of β1/6 siRNA, and this was also seen with β1/4 and β1/5 siRNA at 20 μM. In contrast to β1 integrin expression, MMP-2 activation reductions were observed only at 20 μM of β1/6 integrin siRNA (Fig. 20 B). These results indicated that the reduction of type I collagen-induced MMP-2 activation seen only with β1/6 may require the more rapid and complete knock down of β1 integrin seen with this siRNA. A threshold of β1 integrin which cannot be surpassed by β1/4 or β1/5 siRNAs may exist for the MMP-2 activation effect.

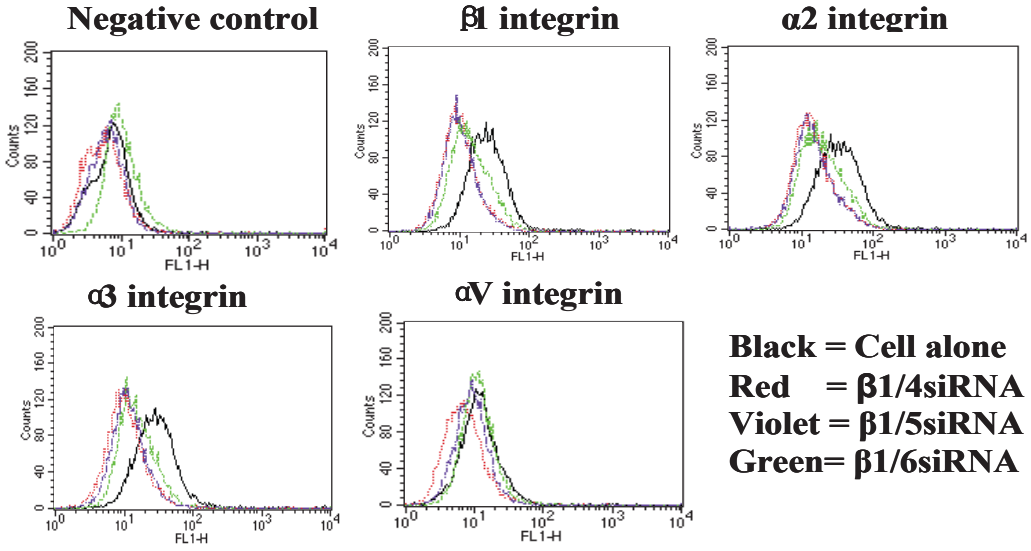
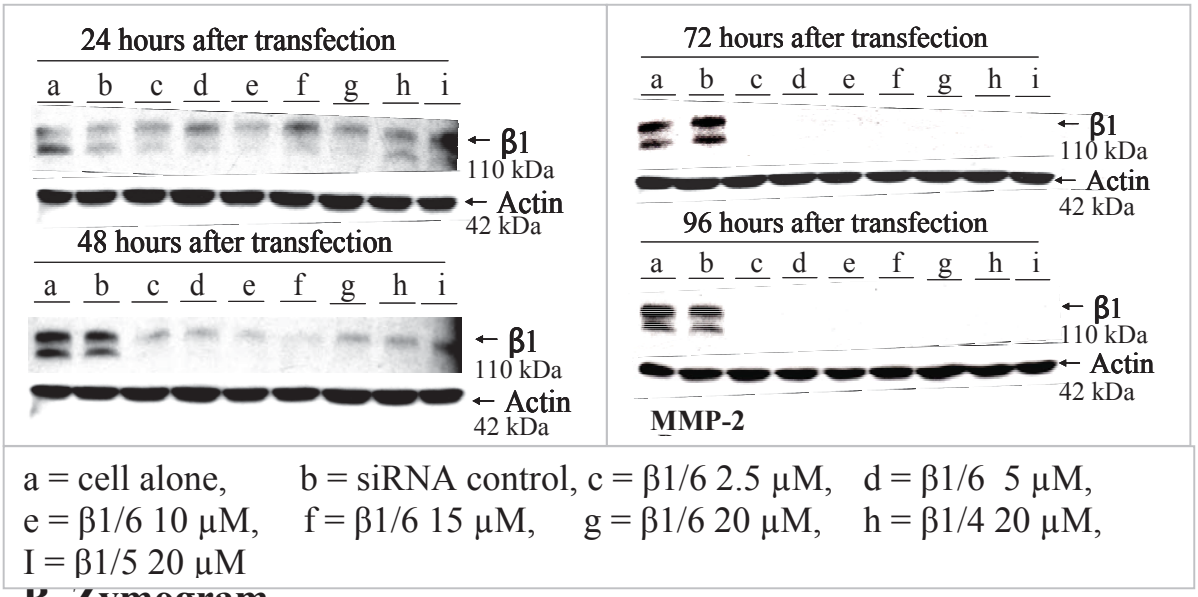


Figure 19: FACS analysis of β1 integrin expression at cell surfaces. MCF-7-MT1 cells were transfected with β1/4, β1/5 and β1/6 siRNAs. Cells were washed and incubated with specific antibodies toward α2, α3, αV and β1 integrin, and analyzed by FACScan flow cytometer. The β1 as well as α2 and α3 integrin subunits were knockdown, but not αV integrin.

A. Western Blot



a = cell alone, b = siRNA control, c = β1/6 2.5 μM, d = β1/6 5 μM, e = β1/6 10 μM, f = β1/6 15 μM, g = β1/6 20 μM, h = β1/4 20 μM, I = β1/5 20 μM

B. Zymogram

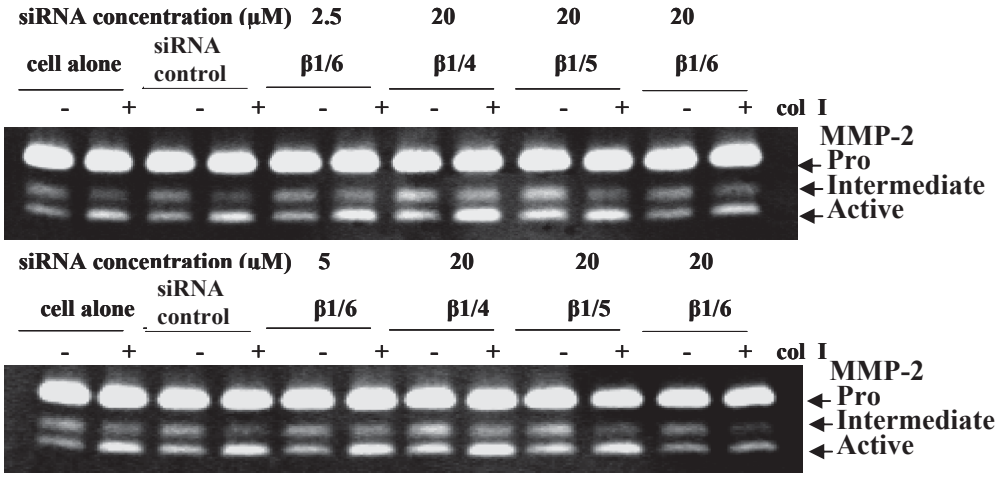
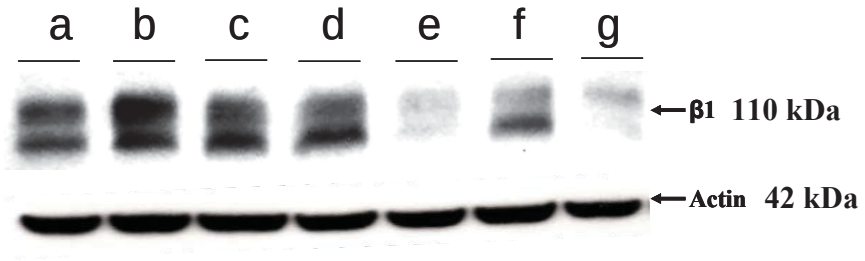


Figure 20: Dose response effect of β1 integrin siRNA on MMP-2 activation in MCF-7-MT1 cells. Cells were plated and transfected with β1 integrin siRNA. Serial dilutions of β1/6 siRNA (2.5, 5, 10, 15 and 20 μM) were used in comparison with the 20 μM concentrations of β1/4 and β1/5 siRNAs. After 72 hours transfection, β1 integrin expressions were determined by Western Blot (A). MMP-2 activation was analyzed by Zymography method. Pro, Intermediate, and Active MMP-2 are indicated by arrow (B).

7. The target cleavage site of β1/6 siRNA was specific to β1 integrin gene.

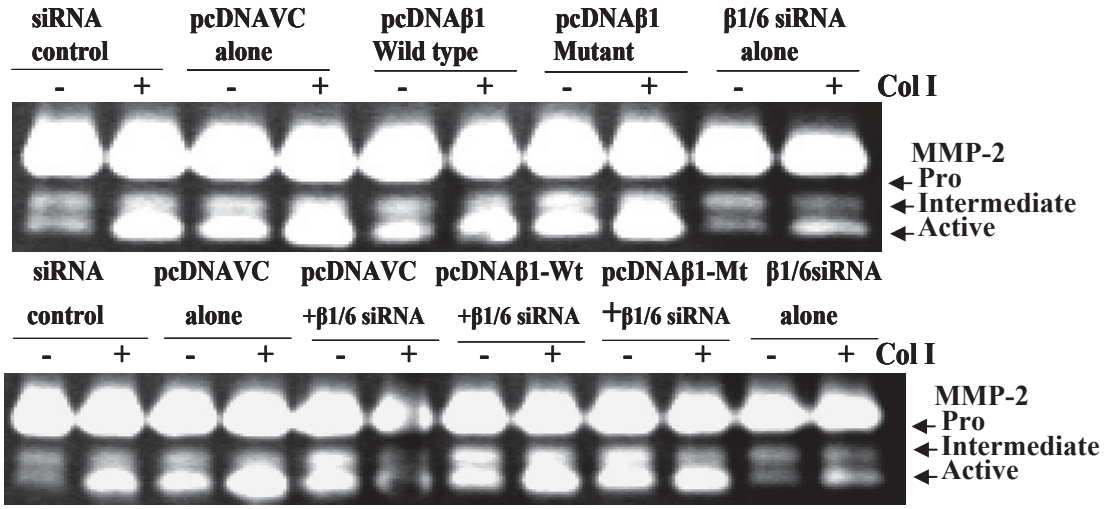
Although β1/4, β1/5 and β1/6 siRNAs each provided good knockdown of β1 integrin expression, only the β1/6 integrin siRNA reduced MMP-2 activation in response to type I collagen stimulation. In addition, the results demonstrated that the reduction in MMP-2 activation by β1/6 siRNA was dose-dependent on siRNA concentration. Because of the lack of effect of β1/4 and β1/5 siRNAs on MMP-2-activation, we concerned that the β1/6 integrin siRNA may elicit a nonspecific gene knockdown which caused the reduction in MMP-2 activation. To assess this, a point mutation technique was used to codon-swap the DNA sequence at the siRNA target site, with no effect on the protein sequence. Co-transfection of the β1 integrin-mutant with β1 integrin siRNA should avoid the mRNA degradation by β1 integrin siRNA, and rescue the effect on MMP-2 activation reduction. In contrast, co-transfection of wild type β1 integrin with β1 integrin siRNA should also lead to mRNA degradation by β1/6 integrin siRNA. MCF-7-MT1 cells were transfected with either wild-type β1 integrin (Wt) or β1 integrin mutant (Mt), and also either wild-type β1 integrin or β1 integrin mutant were co-transfected with the β1/6 integrin siRNA. Then, the effect on MMP-2 activation in response to type I collagen stimulation was assessed. Co-transfection of β1 integrin mutant and β1/6 siRNA failed to knockdown β1 integrin, whereas co transfection of wild-type β1 integrin and β1/6 siRNA, or transfection with β1/6 integrin siRNA alone, successfully knocked down β1 integrin (Fig. 21 A). Expectedly, Co-transfection of β1 integrin mutant and β1/6 siRNA could rescue MMP-2 activation reduction (Fig. 21 B). Even though, a reduction of MMP-2 activation rescue effect by β1 integrin mutant was not convincing. This may be due to complications of the effects of the co-transfection conditions on MMP-2-activation, which was elevated. However, the same result was also observed in MDA-MB-231 cells. (Fig. 21 C). These results indicated that the target cleavage site of β1/6 integrin siRNA was specific to β1 integrin gene.

A. Western Blot



a = siRNA control, b = pcDNA-VC, c = pcDNA-β1-wild type, d = pcDNA-β1-mutant, e = co-transfection pcDNA-β1-wild type with β1/6 siRNA, f = co-transfection pcDNA-β1-mutant with β1/6 siRNA g = β1/6 siRNA alone.

B. Zymogram: MCF-7-MT1



C. Zymogram: MDA-MB-231

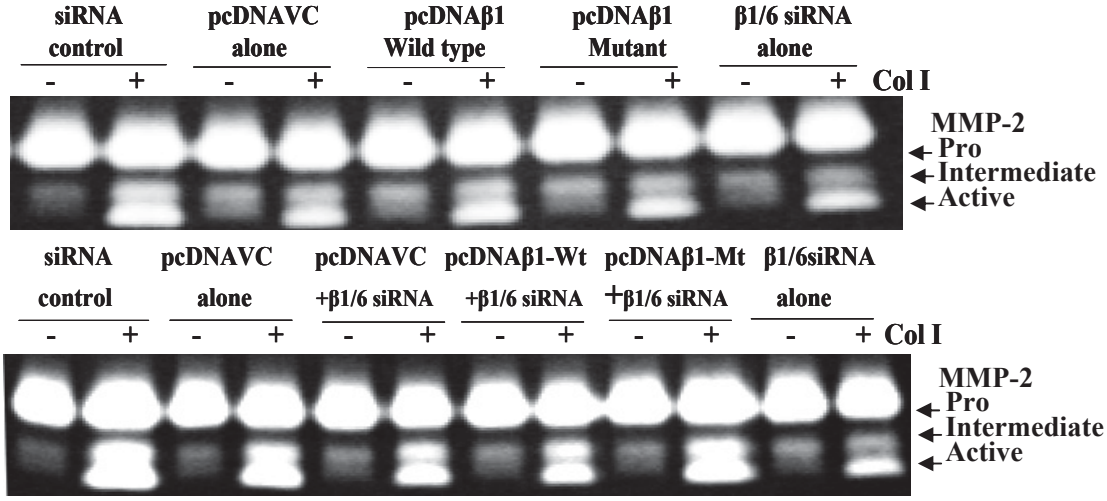


Figure 21: The target site of β1/6 integrin siRNA was specific to β1 integrin gene. Human β1 integrin cDNA was subcloned into the pCDNA3.1 vector. Site-specific mutagenesis primers were designed around the target site of the β1/6 siRNA. Co-transfections of β1/6 siRNA and either wild type β1 integrin or the β1 integrin mutant were performed. **(A)** Western blot analysis of β1 integrin expression, and the effects on MMP-2 activation were examined by gelatin zymography in both MCF-7-MT1 cells (B) and MDA-MB-231 cells (C). Pro, Intermediate, and Active MMP-2 are indicated by arrow.

8. A reduction of MMP-2 activation by β1/6 siRNA was specific to Type I Col stimulation.

Although Collagen type I was reported induction of MMP-2 activation [45], artificial activation of MMP-2 can be effected by a variety of agents. Cellular activation of MMP-2 has been documented recently upon treatment of different cell types with TPA, TGF-0, Con A and APMA [173, 174]. It was suggested that the response to interstitial collagen and Con A may be mediated by integrins, since a number of integrin family members are their cell surface receptors. Thus, to investigated that a reduction of MMP-2 activation by abrogation of β1 integrin expression was specific to Type I Col stimulation. Non-specific substrates include Con A and TPA were used to generate induction of MMP-2 activation. MCF-7-MT1 cells were treated with Type I Col, Con A or TPA after 72 hours after β1/6 siRNA transfcetion. Interesting, knockdown of β1 integrin expression by β1/6 siRNA abolished substantially only Type I Col-induced MMP-2 activation. Importantly, MMP-2 activation in regarding of either Con A or TPA induction, abrogation was not seen by β1/6 siRNA transfection (Fig. 22 A). Nevertheless, to investigate unspecific effect of gene knockdown by siRNA technology that may be manipulate on Type I Col-induced MMP-2 activation. Other unspecific siRNAs including Vimentin-1, Vimentin-2, Slug-1, Gal3-1 and Gal3-2 were further tested effect on Type I Col-induced MMP-2 activation. It was found that, only β1/6 siRNA transfection convinced abrogation of Type I Col-induced MMP-2 activation. While all of other unspecific siRNAs were not seen, except Slug-1 showed a little reduction (Fig. 22 B). Theses data were support that, knockdown of β1 integrin expression by β1/6 siRNA had specific effect on MMP-2 activation reduction in response to Type I collagen stimulation.

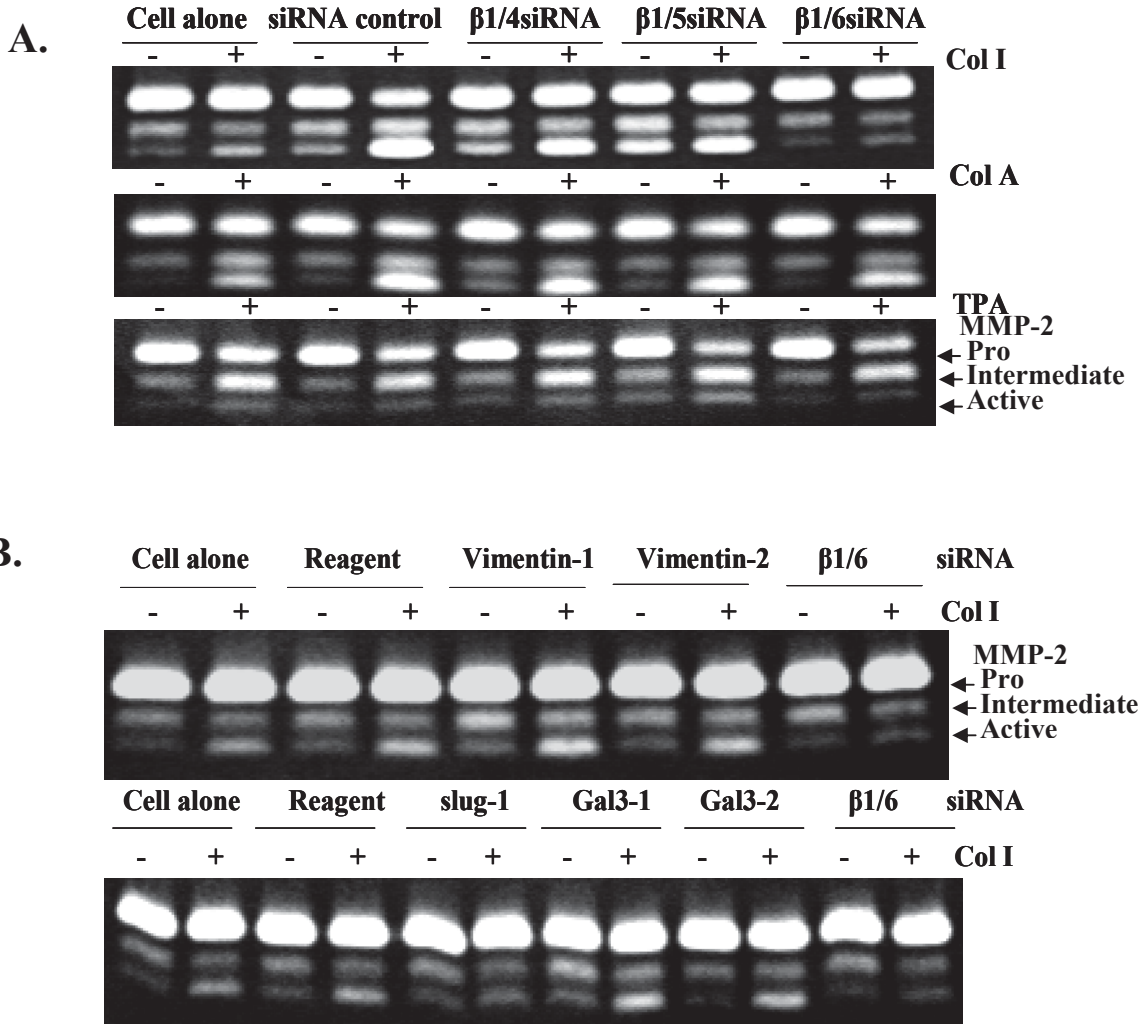


Figure 22: Abrogation of β1 integrin was specific effect to Type I Col-induced MMP-2 activation reduction. MCF-7-MT1 cells were treated with Type I Col, Con A or TPA after 72 hours of β1/6 siRNA transfection at 100 ng/ml, 10 μM/ml and 10 $^{-7}$ M/ml respectively. Conditioned medium was collected at 24 hours incubation after 100 ng of rMMP-2 was added and cells were untreated or treated with Col I, Con A or TPA. The effects on MMP-2 activation were examined by gelatin zymography (A). Other unspecific siRNAs were transfected in MCF-7-MT1 cells at 20 μM. MMP-2 activation effect was performed in the same pattern and examined by gelatin zymography. Pro, Intermediate, and Active MMP-2 are indicated by arrow (B).

(B). **Abrogation of β1 integrin expression suppressed Type I Col-induced MMP-2 activation via MT1-MMP up-regulation.**

Collagen type I can stimulate MMP-2 activation via MT1-MMP activity in several cell types [25, 44-46], involves both a transcriptional increase in MT1-MMP expression, and a non-transcriptional response mediated by pre-existing MT1-MMP. Type I collagen stimulated MMP-2 activation functions principally via MT1-MMP in fibroblasts, as no MMP-2 activation occurs in MT1-MMP-deficient mouse fibroblasts in response to Col I and reduced levels of active MMP-2 are seen in MT1-MMP-deficient mice [46, 55, 114]. In this study, it found apparently abrogation of β1 integrin expression was able to suppress Type I Col-stimulated MMP-2 activation. Thus, the influence of β1 integrin on MMP-2 activation in regarding of MT1-MMP activity was examined. Again, MCF-7-MT1 which stably transfected MT1-MMP was used as the non-transcriptional response of MT1-MMP regulation model. MCF-7-MT1 cells were transfected with β1/4, β1/5 and β1/6 siRNAs, protein levels of both β1 integrin and MT1-MMP were examined by Western blot at 72 hours after transfection. Interestingly, all of β1/4, β1/5 and β1/6 siRNAs showed decrease MT1-MMP levels, particularly, the β1/6 siRNA was the strongest weakness of MT1-MMP which correspond to β1 integrin level compared to controls (Cell alone and siRNA control) (Fig 23 A). Subsequently, β1/6 siRNA was obvious suppression of Col I-induced MMP-2 activation (Fig. 23 B). However, due to rapid turnover rate of active MT1-MMP, its concentration on cell surface is very low, and the active form of MT1-MMP predominantly localizes on the cell surface. Even through, western blotting may be a more suitable alternative method, however, the preferred method for analyzing a protein knockdown is immunofluorescence detection using a specific antibody that recognizes the targeted gene. Thus, detection of cell surfaces β1 integrin and MT1-MMP were also investigated using Immunofluorescence method at 6 hours and 24 hours incubation after cells were untreated or treated with Col I. All abrogated-β1 integrin

expressing cells displayed different morphology comparison with cells expressing β1 integrin (Cell alone and siRNA control). Cells expressing β1 integrin demonstrated better spread, whereas, all abrogated-β1 integrin expressing cells displayed rounder and less spread morphology, somehow the β1/6 siRNA was predominant (Fig. 24 A). In addition, the cell surface expression of β1 integrin localized to the cell periphery was strongly observed, while MT1-MMP was more diffusely distributed on the cell surface, somehow concentration of MT1-MMP was associate with β1 integrin. Althrough, abrogated-β1 integrin expressing cells knocked down β1 integrin, but the cell surface expression of β1 integrin was still detected. However, cell surfaces of β1 integrin and MT1-MMP were similar between untreated and treated with Col I. Interestingly, only β1/6 siRNA indicated less of β1 integrin and MT1-MMP at the cell surface compared to β1/4 and β1/5 siRNAs in both untreated and treated with Col I conditions (Fig. 24 B).

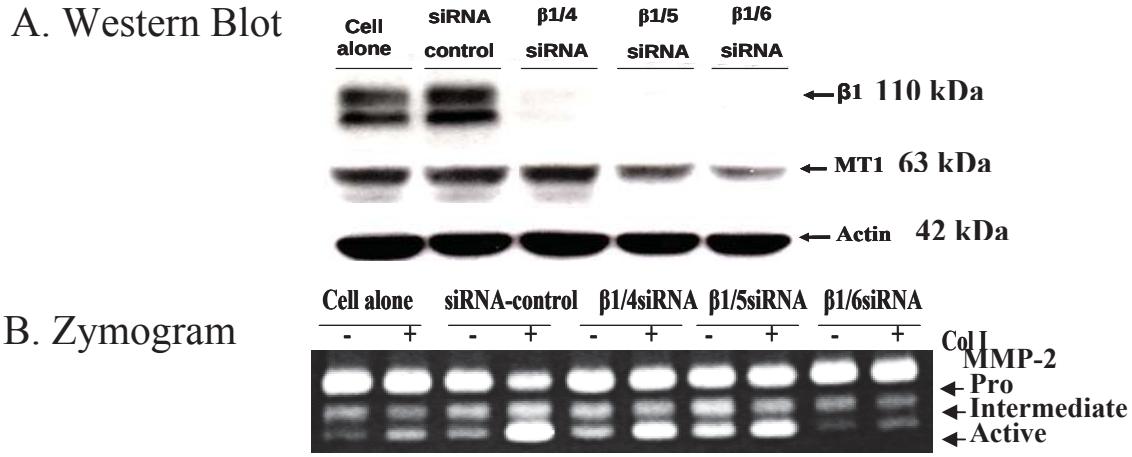
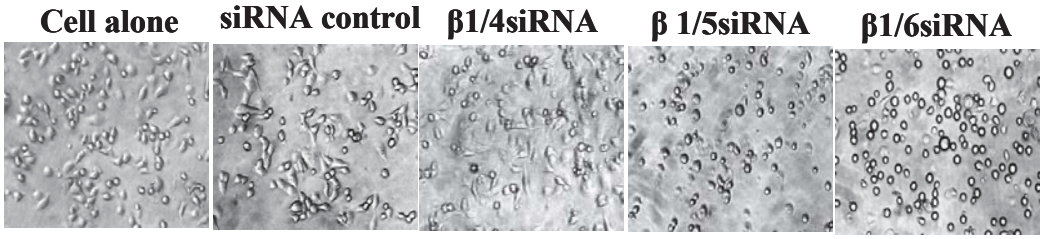
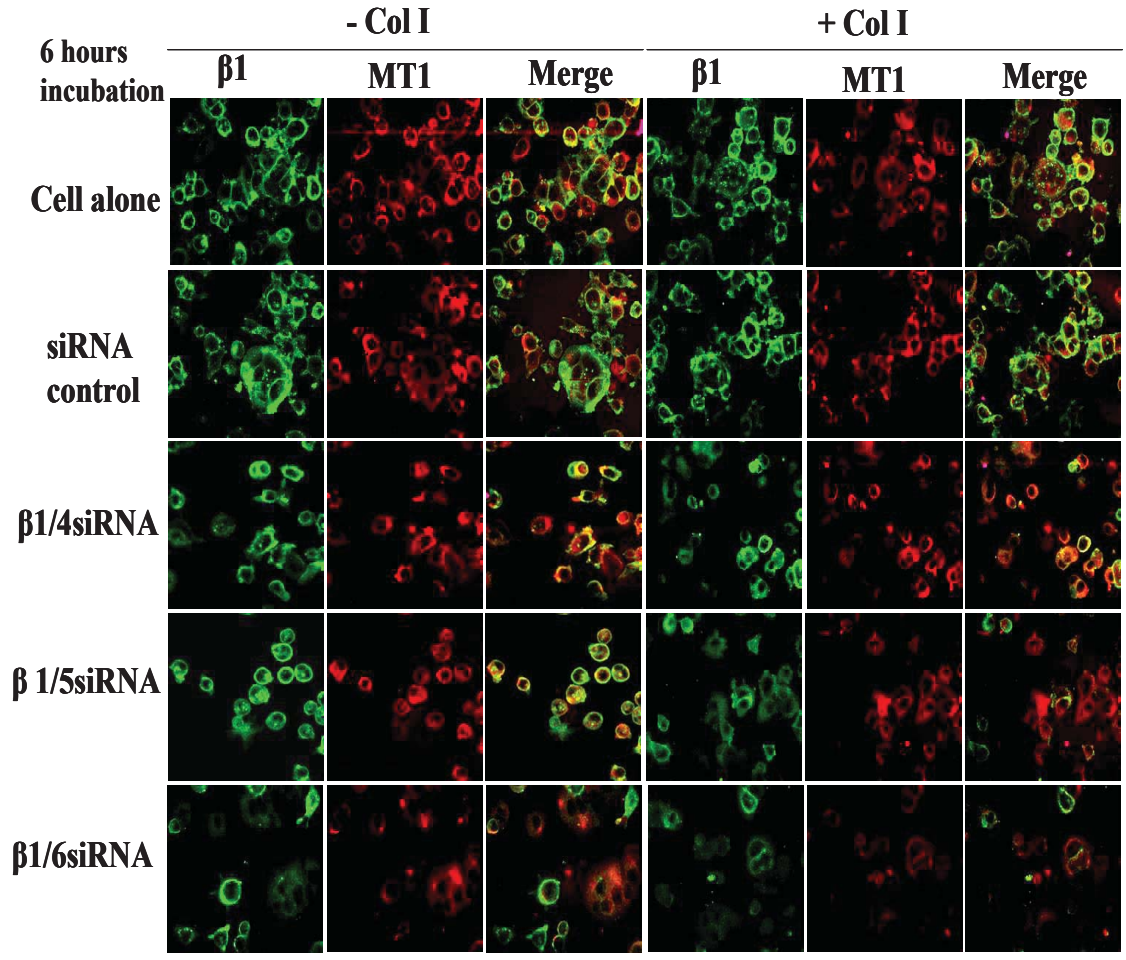


Figure 23. MT1-MMP levels were reduced in abrogated-β1 integrin expressing cells. MCF-7-MT1 cells were transfected with β1/4, β1/5 and β1/6 siRNAs, protein levels of both β1 integrin and MT1-MMP were examined by Western blot at 72 hours after transfection (A). Conditioned media were collected at 24 hours after cells were untreated and treated with Col I and gelatin zymography was performed for MMP-2 activation. Pro, Intermediate, and Active MMP-2 are indicated by arrow (B).

A.


Cell alone
siRNA control
β1/4siRNA
β 1/5siRNA
β1/6siRNA

B.


6 hours incubation
- Col I
+ Col I
β1
MT1
Merge
β1
MT1
Merge
Cell alone
siRNA control
β1/4siRNA
β 1/5siRNA
β1/6siRNA

C.

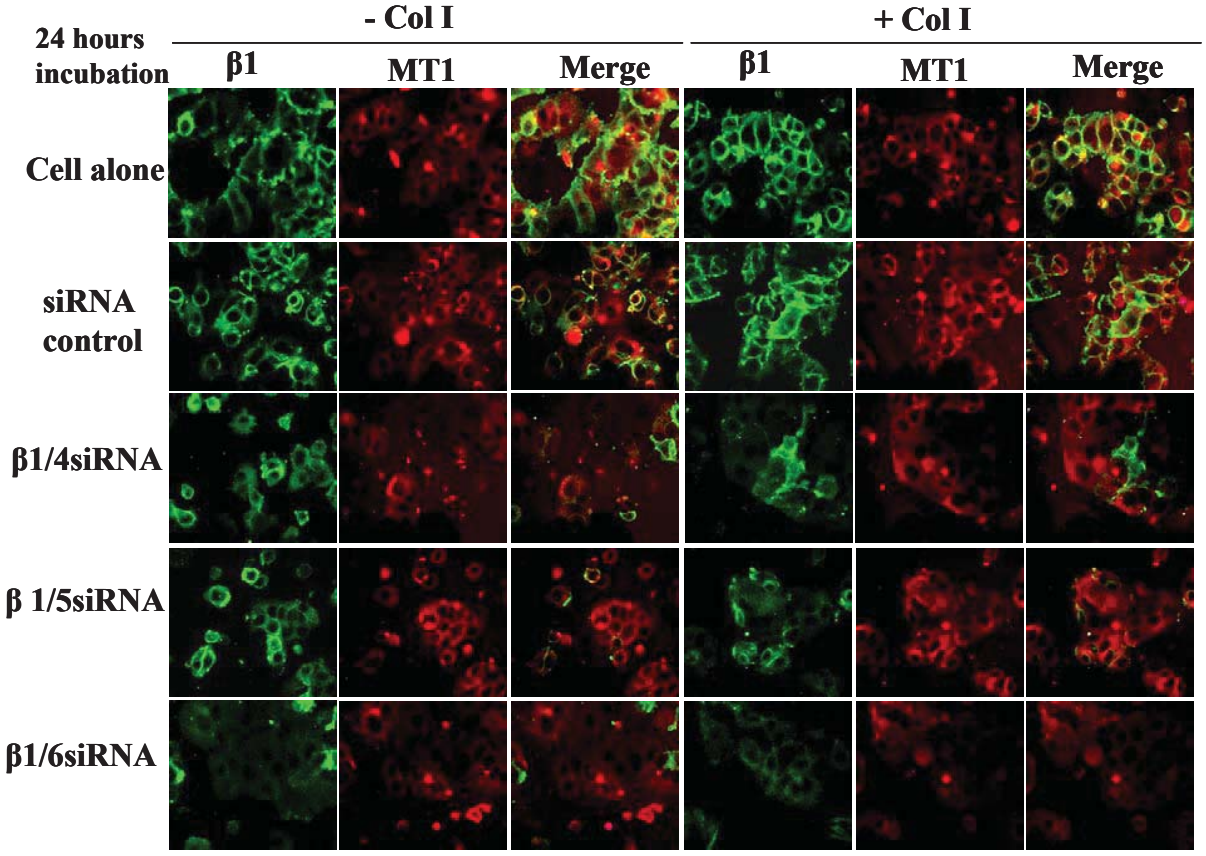


Figure 24. Immunofluorescence analysis for β1 integrin and MT1-MMP at the MCF-7-MT1 cell surface. All different types of β1/4, β1/5 and β1/6 siRNAs were transfected into MCF-7-MT1 cells. After 72 hours, cells were plated on Teflon printed glass slides. The following day, cells were untreated and treated with Col I. At appropriate time points, cells were incubated with primary antibodies toward MT1-MMP (AB815) or β1 integrin (AB2511). Primary antibody was detected with a donkey anti-rabbit secondary Alexa Fluor 488 (green for β1 integrin) or Alexa Fluor 568 (red for MT1-MMP) conjugated antibody for 1 hour at room temperature. Cells were then mounted and viewed by confocal microscopy. Cell morphologies were captured at 6 hours incubation (A). Immunofluorescence analysis were

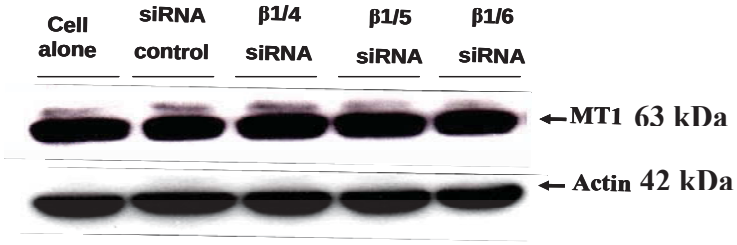
performed after 6 hours (B) and 24 hours (C) stimulation with and without Col I.

Since usage of siRNA technology, the transient reduction in mRNA levels can be detected in less than a day and lasts for several days depending on the turnover rate of the RNA and/or the protein. Immunofluorescence detection was further performed at 24 hours incubation of β1 integrin and MT1-MMP at the cell surface (Fig. 24 C). At this time, β1 integrin localized to the cell periphery was strongly observed in control include cell alone and siRNA control. In the other hand, all abrogated-β1 integrin expressing cells showed little β1 integrin at the cell periphery. Obviously, β1 integrin was more diffusely distributed on the cell surface and disappear from cell periphery in β1/6 siRNA knockdown cells. When cells were stimulated with Col I, only β1/6 siRNA knockdown cells distinctly displayed MT1-MMP less than untreated with Col I condition which related to the most efficiently knockdown of β1 integrin. Importantly, both β1/4 and β1/5 siRNAs expressing cells were able to knock down β1 integrin, however, β1 integrin localized at cell periphery was still detected. Additionally, β1 integrin and MT1-MMP levels examined by immunofluorescence were corresponding to Western blot determination (Fig 23 A). Thus, it was conceivable that β1/6 siRNA was not only the most efficient knockdown of β1 integrin but also the highest significantly effected down regulation of MT1-MMP level. Taken together, only knockdown β1 integrin by β1/6 siRNA showed significantly MMP-2 activation reduction, while β1/4 and β1/5 siRNAs were not.

However, to confirm that this knockdown of β1 integrin was up-regulation of MT1-MMP. The reciprocal experiment was further investigated. Human MT1-MMP cDNA was subcloned into a pCEP-4 plasmid under neomycin selection. MCF-7-MT1 cells were transfected with β1/4 and β1/5 β1/6 siRNAs, subsequently, cell were transiently transfceted with pCEP-MT1-MMP upon indication, controls were also including. Untreated and treated with Col I conditions were parallel set up. Ectopic expression of MT1-MMP were

determined by Western blot, the reduction of MT1-MMP in all different β1 integrin siRNAs knockdown cells were increasing back (Fig 25A). Expectedly, suppression of MMP-2 activation by β1/6 siRNA was rescued by MT1-MMP induction in both non- and stimulation with Col I. (Fig. 25 B). More over, to confirm that β1 integrin was up regulation of MT1-MMP activity, the reciprocal induction of β1 integrin was further investigated. Human β1 integrin cDNA was subcloned into a pcDNA3.1 vector. Transient expression of β1 integrin in MCF7-MT1 cells, full length β1 integrin plasmid DNA (pcDNAβ1) and empty vector (pcDNA-VC) transfected counterparts, were generated. MCF-7-MT1 cells were transiently transfected with either pcDNA-VC or pcDNA-β1, subsequently, rMMP-2 was added and MMP-2 activation was performed at serial times include 6, 12, 24, 48 and 72 hours incubation. The β1 integrin protein levels were also determined at serial time points in parallel with MMP-2 activation. The β1 integrin strongly increased at 12 and 24 hours, and then start to go down at 48 hours and reach to baseline at 72 hours after transfection (Fig. 26 A). Interestingly, increasing of β1 integrin induced MT1-MMP parallel together at 12 hours until 72 hours. This effect, subsequently, enhance Col I-induced MMP-2 activation by MT1-MMP activity. Col I-induced MMP-2 activations were convincingly observed at 12 and 48 hours, then MMP-2 activation levels were similar between non- and induction of β1 integrin (Fig. 26 B). These results indicated that β1 integrin was up regulation of MT1-MMP, subsequently, effect on MMP-2 activation in response to Col I stimulation. Thus, it strongly recommends that the β1 integrin involve in Col I-induced MMP-2 activation via up regulation of MT1-MMP activity.

A. Western Blot



B. Zymogram

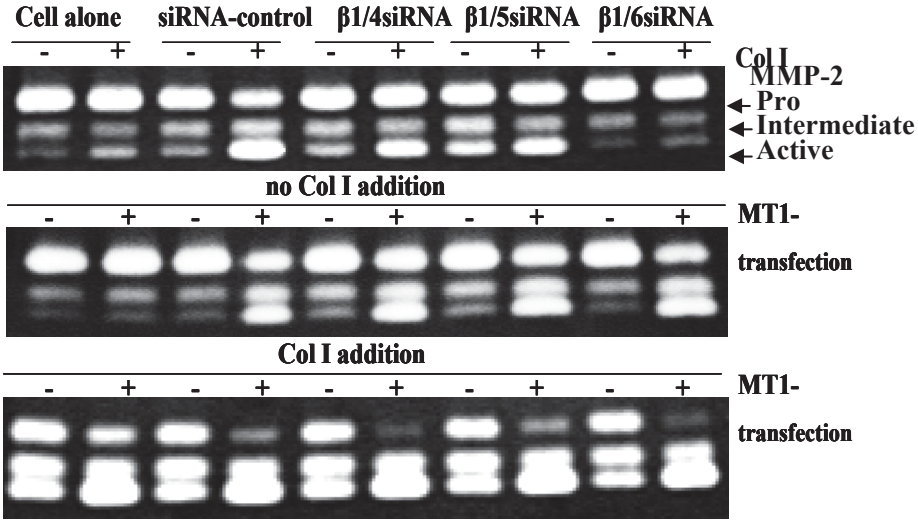


Figure 25. Ectopic MT1-MMP expression rescued suppression of Col I-induced MMP-2 activation by β1/6 siRNA. MCF-7-MT1 cells were transfected with all different β1/4, β1/5 and β1/6 siRNAs. Following day, cells were subsequently transient transfected with pCEP-MT1-MMP as indication. Then, cells were untreated or treated with Col I. Efficiency of MT1-MMP inductions were determined by Western Blot (A.) and MMP-2 activation was analyzed by gelatin zymography. Pro, Intermediate, and Active MMP-2 are indicated by arrow (B.)

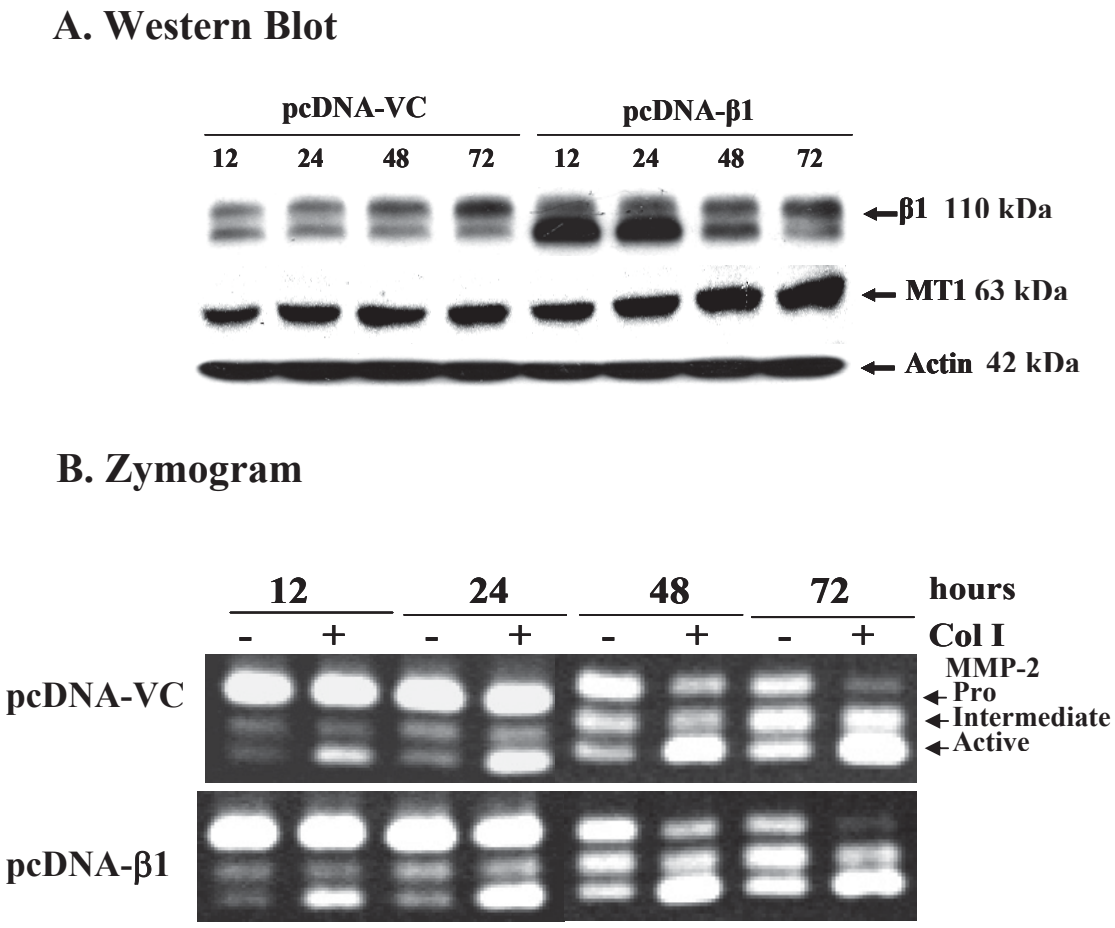


Figure 26. The β1 integrin was up regulation of MT1-MMP activity. MCF-7-MT1 cells were transient transfected with either pcDNA-VC or pcDNA-β1 integrin. Cells were untreated or treated with Col I and Conditioned media were collected at appropriate time points. Inductions of β1 integrin were determined by Western Blot at serial times including 12, 24, 48 and 72 hours (A). MMP-2 activation was also investigated at similar time points by Zymogram method. Pro, Intermediate, and Active MMP-2 are indicated by arrow (B.)

CHAPTER 5
DISCUSSION

1. Three candidates of β1/4, β1/5 and β1/6 integrin siRNAs were efficiently knockdown β1 integrin expression at 72, 96 and 120 hours.

Integrin β1 is ubiquitously expressed and can bind multiple a partners. One important function of β1 integrin is partner of the collagen-binding receptors, to date they are four integrin receptor including α1β1, α2β1, α10β1 and α11β1 are the major collagen-binding receptors [145, 150]. Even through, physical associations between β1-integrin and several classes of proteases, endogenous inhibitors of those proteases or protease binding partners have been identified, yet a mechanism for β1-integrin involvement in the proteolytic remodeling of ECM has not been established [164]. It was postulated that culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of MMP-2 [44-46]. Several studies have shown that collagen-binding integrins interact with collagen involve in this mechanism [55-60]. However, the involvement of β1 integrin in type I collagen-stimulated MMP-2 activation is still unclear. Thus, this study aimed to elucidate β1 integrin involve in Col I-induced MMP-2 activation. siRNA technology was used to determine β1 integrin function in this study. Since this method is a powerful technique used to investigate gene function by degrading a specific mRNA target in a cell or organism and thus knocking out or knocking down the level of the encoded protein [175]. Despite the high success rate of this technique, still controls are important. It was recommended that it is essential to find an optimal siRNA sequence, and several different siRNAs should be used in the experimental system [176].

Thus, six different β1 siRNAs were designed, namely β1/1, β1/2, β1/3, β1/4, β1/5 and β1/6 siRNA, and transfected in MCF-7-MT1 breast cancer cells which had previously been stably transfected MT1-MMP. Although, all 6 siRNAs showed decreased β1 integrin expression at both the RNA and protein levels, only 3 of them (β1/4, β1/5 and β1/6 siRNA) showed substantial knockdown of the β1 integrin expression, with the most efficient knockdown being from β1/6 siRNA (Fig. 10 A and 10 B). This is due to the efficiency of target RNA cleavage, even for single-nucleotide displacements of the siRNA relative to the target, is quite variable [175]. Apparently, siRNAs can target genes as effectively as long dsRNAs [175] and are widely used today for assessing gene function in cultured mammalian cells. Several biological roles for this short RNA triggered gene silencing mechanism have been elucidated and/or postulated. Mechanistically, RNAi involves a multistep process, briefly; the dsRNA is recognized by an RNase III family member and is cleaved into siRNAs of 21–23 nucleotides. In the nextstep, the siRNAs are incorporated into an RNAi targeting complex known as RISC (RNA-induced silencing complex), which destroys mRNAs that are homologous to the integral siRNA [177, 178]. The target mRNA is cleaved in the center of the region complementary to the siRNA [176] with the net result being a rapid degradation of the target mRNA and a decrease in protein expression. The most potent siRNA duplexes are 21 nucleotides long, comprising a 19 bp sequence with a 2-uridine 3′ overhang at each end [176]. After target RNA cleavage, the mRNA cleavage products are released and RISC may be reactivated for another round of catalytic target RNA cleavage. Nevertheless, in this study, all 6 putative β1-integrin siRNAs were designed to target all known β1 integrin transcript variants. β1-integrin siRNAs targets were selected from regions lacking strong local mRNA secondary structure. The 21 nucleotides of siRNA size were identical in all 6 β1-integrin siRNAs. Apparently, the estimates of siRNA size vary in the literature between 21 and 25 nt [176-179]. SiRNA duplexes composed of 21-nucleotide sense and 21-nucleotide antisense strands, paired in a

manner to have a two-nucleotide 3′ overhang, are the most efficient triggers of sequence-specific mRNA degradation in tissue culture systems [180]. The target RNA cleavage reaction guided by siRNAs is highly sequence-specific [177]. However, not all positions of an siRNA contribute equally to target recognition. Until now, duplexes of 21 nt siRNAs with 2 nt 3' overhangs were shown to be the most efficient triggers of RNAi-based mRNA degradation [178]. Somehow, only three candidates of β1 integrin siRNAs including β1/4, β1/5 and β1/6 siRNAs were efficiently knockdown. Thus, β1/4, β1/5 and β1/6 siRNAs were reasonably selected for further examined. Since, the reduction in target mRNA and protein should be followed over time to define the window of effective knockout of siRNA [175, 176]. Determination of the interval time for suppression of β1 integrin expression by siRNA technology was performed. Both MCF-7-MT1 and MDA-MB-231 breast cancer cells were used. MCF-7-MT1 which lack of endogenous MT1-MMP and stably transfected with MT1-MMP, thus, it was used as the non-transcriptional response of MT1-MMP regulation model. Moreover, this cell also showed low level of endogenous β1 integrin expression, while MDA-MB-231 present high level of endogenous MT1-MMP and β1 integrin expression (data not show). The results showed that, all of β1/4, β1/5 and β1/6 siRNAs initiated knockdown of β1 integrin expression at 24, 48, 72, 96 and 120 hours after transfection. However, β1 integrin expression was completely knocked down at 72, 96 and 120 hours in both MCF-7-MT1 (Fig. 14 A) and MDA-MB-231cells (Fig. 14 B). This indicated that all of β1/4, β1/5 and β1/6 were highly efficient siRNAs. The presence of this knockdown behavior and, therefore, these time points were conducted in next experiments. Since, protein knockdowns mediated by exogenous siRNAs are transient because the targeted protein levels of siRNA-treated cells recover, typically between 5 and 7 days after siRNA transfection [179]. Reasonably, β1/1, β1/2 and β1/3 siRNAs were excluded, due to, they displayed low efficiency. One mechanistic explanation for the existence of inactive siRNAs is that this asymmetry in the ends of the duplex leads to differential loading

into RISC of one RNA strand over the other, in Drosophila embryo lysates [181].

2. The β1/6 siRNA was the most efficient effect to β1 integrin functional activity reduction, while β1/4 and β1/5 siRNAs were less effectively.

Since, it was found that three candidate siRNAs showed effectively knock down of β1 integrin both at the level of mRNA and protein. The reduction in target mRNA and protein were followed over time, 72 hours after transfection was accomplished knockout. Even through, siRNA induce RNA degradation through a natural gene-silencing pathway and has rapidly become the most widely used approach for gene knockdown because of its potency. However, several recent reports have suggested that non-specific effects can be induced by siRNAs, both at the level of mRNA and protein [184-186]. The functional effects of β1 integrin depleting were further investigated. Apparently, integrins are highly regulated receptors that can function in both cell–substrate and cell– cell adhesion. Since, β1 integrin is ubiquitously expressed and can bind multiple a partners. Heterodimer of β1 integrins include α1β1, α2β1, α10β1 and α11β1 represent the primary collagen receptors [145, 150]; integrins α3β1, α6β1, and α7β1 are the major laminin receptors [147]; and integrins α5β1, α8β1, are the major fibronectin receptors and the αvβ3 is specific for vitronectin receptor that bind in an RGD-dependent manner [145]. Moreover, the collagen gel contraction was widely used an in vitro model to investigate of cell-mediated tissue remodeling, including wound contraction and maintenance of tissue homeostasis [107]. Several studies have demonstrated a role for β1 integrin, in particular α1β1 and α2β1 integrin, in mediating collagen gel contraction by fibroblast and osteoblastic cells [111-113]. Thus, in order to investigate the biological consequences of integrin depleting by β1 integrin siRNAs, analysis of cell adhesion and collagen gel contraction were

performed. It was clearly that, abrogation of β1 integrin expression reduced cells adhesion to specific substrate of β1 integrin including, type I collagen and fibonectin, but not vitronectin, a nonspecific of β1 integrin substrate. Even though, all of β1/4, β1/5 and β1/6 siRNAs were efficient knockdown, reduction of functional adhesion to FN and Col I were statistically in only β1/6 siRNA (Fig. 15). Moreover, collagen gel contraction effects were also clearly observed. All of abrogated-β1 integrin expressing cells showed less contractile than β1 integrin expressing cells (cell and siRNA control) (Fig. 16 A). Similarly, only β1/6 siRNA displayed the most efficient reduction of collagen gel contraction significantly (16.49 %, $p < 0.001$), while β1/4 and β1/5 siRNAs were not significant difference from β1 integrin expressing cells (Fig. 16 C). These data indicated that, knockdown of β1 integrin by β1/4, β1/5 and β1/6 siRNAs were specific target to β1 integrin gene, consequently, abrogated functional β1 integrin activities. Since, one of biological function of integrins is cell adhesion which plays a fundamental role in regulating cell survival, migration, proliferation, and differentiation as well as during disease states such as tumor progression, atherosclerosis, asthma, and rheumatoid arthritis [187]. It was postulated that, integrins mediate many adhesive interactions via recognition both cell–surface and extracellular matrix ligands [188, 189]. In fact, a number of anti-β1 mAbs have been identified that can activate integrins directly, resulting in increased cell adhesion, presumably through increased integrin-binding affinity for ligand [190]. Integrin activation can be defined as the enhanced ability of integrins to mediate ligand binding and/or cell adhesion. However, not all β1 integrin activating antibodies can stimulate cell– cell adhesion, may be several variable factors that involve [183]. Conversely, this study successfully abrogated cell and ligand adhesions by knock down of β1 integrin, therefore, presume efficiency of these β1 integrin siRNAs, especially β1/6 siRNA which the most efficient. Furthermore, it found that only β1/6 siRNA initiated failing of contraction at the first day and keep failing until the last day, while β1/4 and β1/5 siRNAs initiated failing of contraction only at the first

day, then started to contract at the second day until the last day (Fig. 13 B). Similarly, only β1/6 siRNA indicated the most efficient reduction of collagen gel contraction significantly (16.49 %, $p < 0.001$), while β1/4 and β1/5 siRNAs were not significant difference from β1 integrin expressing cells (Fig. 16 C). Collagen gel contraction is a process of reorganizing the collagen fibrils and contraction of the gel. It is used as an in vitro model of cell-mediated tissue remodeling, including wound contraction and maintenance of tissue homeostasis [107]. In cancer, remodeling of the ECM plays a critical role in the reorganization of connective tissue in tumor invasion [105, 106]. Several studies have demonstrated a role for β1 integrin, in particular α1β1 and α2β1 integrin, in mediating collagen gel contraction by fibroblast and osteoblastic cells [111-113]. Reorganization of collagen fibrils, seen as a contraction of a floating collagen gel, reflects the ability of cells to interact with the collagen matrix. In accordance to several studies have been demonstrated a role for β1 integrin, in particular α1β1 and α2β1 integrin, in mediating collagen gel contraction by fibroblast and osteoblastic cells [111-113]. However, it seems that the function of integrins may vary in different cell types. In some cell types α1β1 integrin or αVβ3 integrin, a principal receptor for fibronectin and vitronectin, can also mediate collagen gel contraction [191]. Nonetheless, MCF-7-MT1 cell lines that used in this study had none endogenous β3 integrin expression, therefore, only β1 integrin plays a critical role in this process. Previous studies have been suggested the importance of high affinity binding of the α2β1 integrin to fibrillar collagen is essential for biological integrin function, a collagen gel contraction process [112, 113]. Independently, only β1/6 siRNA demonstrated strongly abrogation of functional β1 integrin both of Col I adhesion and collagen gel contraction. These revealing data indicate that, successful abrogation of β1 integrin among three different β1/4, β1/5, and β1/6 siRNAs, but they had different efficiency rates on biological of β1 integrin activities.

3. β1/4, β1/5 and β1/6 siRNA each efficiently knocked down β1 integrin, however, only β1/6 effected a reduction in Col I-induced MMP-2 activation.

Indeed, MMPs in cancer were first associated with tumor cell invasion and metastasis by enabling tumor cells to invade through the ECM and vascular basement membrane [9]. The recent insights gained on cancer invasion and metastasis processes modulated by MMPs, including, MMPs affect on growth signals, apoptosis regulation, tumor vasculature, initiate of neoplastic progression and tumor invasion and metastasis, which are likely to have significant consequences on the tumor microenvironment [115]. MMP-2 and MMP-9, which have been associated predominantly with the ability of tumors to invade and become neovascularized [192]. Thus, this study focuses on MMP-2 activity in regarding of Col I stimulation. Since type I collagen is a prevalent matrix encountered by invasive carcinomas. Previous works have shown that a three-dimensional collagen matrix can stimulate pro-MMP-2 activation in a variety of cell lines [44-46]. In addition, β1 integrin is an important partner of the collagen-binding integrin receptors. However, the involvement of β1 integrin in collagen-stimulated MMP-2 activation is still unclear and yet investigating. Determinations the interval time for suppression of β1 integrin and subsequent effects on MMP-2 activation by β1 siRNA were performed. As the result found that β1 integrin expression was completely knocked down at 72 hours after transfection (Fig. 18 A). An MMP-2 activation time course analysis was conducted at 72, 96 and 120 hours after transfection. All 3 types of β1 integrin siRNA; β1/4, β1/5 and β1/6 were knockdown β1 inetgrin at 72, 96 and 120 hours as shown in Figure 18 A. Although, β1/4, β1/5 and β1/6 siRNA each were efficient in the knockdown of β1 integrin, however, only β1/6 had a convincing effect on MMP-2 activation reduction at all time points (Fig. 18 B). As described above, MCF-7-MT1 is a breast cancer cell line which lack of endogenous MT1-MMP expression and induced MT1-MMP expression by stably transfected with MT1-MMP. In this

cell model, Col I causes activation of MMP-2 despite MT1-MMP being under the control of a heterologous promoter. Furthermore, when these cells were treated with cycloheximide to block new protein synthesis, some reduction in MMP-2 activation was seen, but the Col I response was still observed. Thus, MCF-7-MT1-MMP cells provide a model for non-transcriptional regulation of Col I-induced MMP-2 activation. However, MDA-MB-231 cells which different from MCF-7-MT1 cells since they had endogenous MT1-MMP, therefore it was used for transcriptional regulation of Col I-induced MMP-2 activation. Similar result was found, only β1/6 siRNA showed a reduction of MMP-2 activation at 72 and 96 hours after transfection in parallel with β1 integrin expression in both MCF-7-MT1 and MDA-MB-231 cells (Fig. 17 B), where β1 integrin expression was completely knocked down at 72 hours (Fig. 17 A) . No effect on MMP-2 activation was seen at 24 or 48 hours after transfection, at which times β1 integrin expression was not completely knocked down. Again, only β1/6 siRNA was the effective reduction of Col I-induced MMP-2 activation, whereas, β1/4 and β1/5 siRNAs had no effect in any time point. Therefore, we hypothesized that these 3 different β1 siRNAs have different effects on the β1 integrin knockdown and also on the α subunits which comprise collagen-binding integrin heterodimers. FACS analysis was performed to assess the α2, α3, and αV integrins, since MCF-7-MT1 cells were found to have very low levels of α1 integrin, and no detectable levels of α10 and α11 integrin subunits when examined by qRT-PCR (data not shown). Interestingly, β1/4, β1/5 and β1/6 siRNAs each not only knocked down β1 integrin, but also the α2 and α3 integrin subunits. Expectedly, no effect on the levels of a non partner of collagen-binding integrin, the αV integrin subunit was observed (Fig. 19). This data indicated that each of the β1 integrin siRNA (β1/4, β1/5 and β1/6) had closely similar effects on collagen-binding integrin receptors, α2β1 and α3β1 integrin heterodimers. Generally, integrin-mediated adhesion is thought to be initiated on the cell membrane by the engagement of individual integrin dimers with their respective ligand. The number of receptor–ligand pairs may then

grow by increasing the cell–substrate contact area and by receptor diffusion within that zone. Subsequent adhesion strengthening occurs through integrin clustering and linkage to the cytoskeleton. In addition, the ligand affinity of the integrins may increase as a result of intracellular regulatory pathways [194]. In this study, abrogation of β1 integrin by β1/4, β1/5 and β1/6 siRNAs were also depleting α2 and α3 subunit integrin partners. Thus, all these siRNAs were accomplished abrogation of heterodimers at the cell surface, subsequently; fail to engage of individual integrin dimers with their respective substrates. Col I is the specific substrate of α2β1 and α3β1 integrin heterodimers and fibronectin, is different specific substrate for αVβ1 integrin. Although, αV integrin subunit was not altered, however, abrogated- β1 integrin expressing cell still indicated less adherent to FN comparison to β1 integrin expressing cell. Similarity data was observed, β1/6 siRNA demonstrated strongly depletion of both Col I and FN adhesion. Moreover, only β1/6 siRNA was strongly rescindable of collagen gel contraction. Even though, β1/4, β1/5 and β1/6 siRNAs each had closely similar effects on α2β1 and α3β1 integrin heterodimers, but only β1/6 siRNA reach threshold effect on inetgrin heterodimers engagement failure. Recently, using the high-force resolution of the AFM was able to observe adhesion events mediated by single α2β1 integrins. This study show that cells adhere to the collagen type I in a two-step process: α2β1-mediated adhesion as weak initial during first contact times 60 s, cell adhesion increase slowly. Afterward, single-integrin–mediated binding events are superseded by strong adhesive interactions involving receptor cooperativity. When substrate contact is sustained for 60 s, overall cell adhesion strongly increases as cells switch to an “activated” adhesion state. In activated cells the smallest discrete rupture events rise above the single-integrin level, suggesting the establishment of cooperative integrin receptor binding [195]. From this report, knockdown threshold of integrin heterodimers by β1 integrin siRNAs are obvious importance. That may be only β1/6 siRNA is completely knockdown β1 integrin heterodimers which reach

threshold effect depleting cell adhesion to Col I and subsequently, fail to contacts collagen gel. In contrast, β1/4 and β1/5 siRNAs were able to knockdown β1 integrin heterodimers, somehow they still leave some β1 integrin heterodimers at cell surface, therefore, turn on activated cell adhesion state and allow the establishment of cooperative integrin receptor binding. However, non-specific gene suppressions by these siRNAs that may be involve in this phenomenon still be concern.

4. Dose response effect of β1 integrin siRNA on knockdown of β1 integrin expression.

This study used siRNA technology to knockdown β1 integin and explored its roles in MMP-2 activation in response to type I collagen to increase an understanding. 6 types of β1 integrin siRNA were designed, somehow, only 3 types of them were good efficient knockdown β1 integrin expression in both RNA and protein levels. Interestingly, all 3 types of β1 integrin siRNA not only knockdown β1 integrin but also α2 and α3, no effect on αV integrin were observed (Fig. 19). Although, 3 types of β1 integrin siRNA were good knockdown β1 integrin, only one type was able to reduce MMP-2 activation in response to type I collagen stimulation in MCF-7-MT1 breast cancer cell line. The important thing should be considered is off-target effect of siRNAs. Several reports have been indicated that siRNAs can affect the expression of unintended targets. An early glimpse into the possible existence of off-target gene regulation by siRNAs came from gene expression profiling of siRNA studies [184]. The non-specific effects of siRNAs on gene expression depend on siRNA concentration and specific sequence. It is highly recommended to identify the most potent sequences and to ensure that the sequence is specific to the target gene by performing a BLAST search [198]. Thus, the specificity of each putative β1-integrin siRNAs target was already confirmed using blasta (www.ncbi.nlm.nih.gov). So, dose response effect of siRNAs must be concerned to avoid ineffective siRNAs that

can lead to false negative conclusions, whereas non-specific siRNAs can produce false-positive conclusions regarding the role of the target gene in functional assays [186]. To explaine this effect, determination of the dose response of β1 integrin siRNA was investigated. Serial concentrations of MMP-2-reduced-β1 integrin siRNA (β1/6) were performed; usual concentration of β1/4, β1/5 and β1/6 were also included for comparative relation. This result found that lower concentration include 2.5 and 5 μM showed similar levels of β1 integrin expression compare to usual concentration (20 μM) of non-reduced-MMP-2- β1 integrin siRNA (β1/4 and β1/5). As expectation, at lower concentration of MMP-2-reduced-β1 integrin siRNA failed to decrease MMP-2 activation, whereas, at 20 μM still able to so doing this effect, even though β1 integrin expression similarly disappear. Two studies have investigated siRNAs concentration on non-specific gene effect [196, 197]. Five different siRNAs that were designed against each of three endogenous genes, this revealed that many non-specific transcripts were significantly regulated only at a high concentration of siRNA (100 nM). Some of these genes were regulated by multiple siRNAs, and this was interpreted to reflect a cellular response to the toxic effects of siRNA. At lower concentrations of siRNA, target transcripts were silenced efficiently without generating the siRNA response signature, and all five siRNAs for the same gene produced similar gene expression patterns [196]. It also demonstrated concentration-dependent regulation of numerous genes in HeLa cells that were transfected with an siRNA for luciferase, which should not contain significant homology to any gene in the human genome [197]. In this study, siRNAs concentration was used at 20 μM. Even though, the siRNAs concentration seems slightly high, however, toxic effects were not appearing. All abrogated-expressing cells (β1/4, β1/5 and β1/6) displayed only rounder morphology compared with control and siRNA control that used as the same concentration. This effect should be form β1 integrin knockdown effect which decrease cell surface adhesion molecules, subsequently influence spreadable cell function. Since, at lower concentration of β1/6 siRNA include 2.5 and 5 μM

failed to decrease MMP-2 activation, whereas, at 20 μM able to so doing this effect, even though β1 integrin expression similarly disappear. This data correlate to β1/4 and β1/5 siRNA effect, at 20 μM failed to reduce MMP-2 activation even though β1 integrin expressions similarly disappear. Apparently, an effective siRNA should be titrated; siRNAs are functional at surprisingly low concentrations and they should be used at the lowest effective level in order to minimize potential side effects since there is experimental evidence that the RISC complex is saturable [186]. Considering β1/4 and β1/5 siRNAs potency, they were efficiency knockdown β1 integrin expression, but not reach threshold to reduce Col I-induced MMP-2 activation. However, higher concentration should not be used, due to the toxic effects of siRNA that may be inducing off-target effect. This finding supports that β1 integrin knockdown threshold was depend on dose of siRNA which significantly effect on mRNA degradation efficiency. Somehow, β1 inetrgin expression knockdown threshold was necessary for MMP-2 activation reduction.

5. The target cleavage site of β1/6 siRNA was specific to β1 integrin gene.

The current challenge facing the use of siRNA need to combine high specificity with high efficacy. Ineffective siRNAs can lead to false negative conclusions, whereas non-specific siRNAs can produce false-positive conclusions regarding the role of the target gene in functional assays [186]. Ideally, siRNAs would be absolutely specific, regulating only the target gene of interest. SiRNAs were first reported to affect unintended mRNA transcripts that are partially complementary in sequence [184] . More recently, siRNAs have been reported to affect translation of unintended transcripts containing partial complementarily [185], and to induce the non-specific interferon response [199]. Taken together, these various reports indicate that siRNAs can affect the expression of unintended targets. Mindful attention to these liabilities is very important to prevent

mistake and permit enhanced experimental design and interpretation of gene silencing data. Considering all data, it seems to be β1/6 siRNA is the most effective compare to β1/4 and β1/5 siRNAs in regarding to depletion of β1 integrin expression and also biological function. Experimental design to affirmative specific target site of β1 integrin siRNA was further examined. Site-direct mutation was used to mutagenized at desire site. Importantly, the mutant β1 inetrgin was successful to protect β1 integrin knockdown when co-transfection with β1 integrin siRNA, while the wild type β1 integrin still able to knockdown β1 integrin expression. Expectedly, Co-transfection of β1 integrin mutant and β1/6 siRNA could rescue MMP-2 activation reduction (Fig. 21 B). Even though, a reduction of MMP-2 activation rescue effect by β1 integrin mutant was not convincing. This may be due to complications of the effects of the co-transfection conditions on MMP-2-activation, which was elevated. Moreover, because of MCF-7-MT1 not only has low level of endogenous β1 integrin expression, but also low level of glycosylated form (130 kDa). Several roles for integrin glycosylation have been suggested, this was particularly well-illustrated for β1 integrin function. In addition, it had been reported that N-glycosylation of β1 integrin is essential for optimal function [200, 201]. It found that overexpression of either β1 integrin wild type or mutant, only precursor form (110 kDa) of β1 integrin was increased. When co-tranfection of β1 integrin mutant and β1 integrin siRNA, precursor form of β1 integrin was detected and limittation of glycosylation was also seen. In addition, MMP-2 becomes three forms after it was activated including pro, intermediate and active MMP-2. Strongly bands of active MMP-2 were observed; therefore, they were saturation and could not further stronger than their limit. However, when tried to avoid this effect by decrease volume loading, they showed very little amount of different (data not show). For this reason, finding of rescue effect on MMP-2 activation reduction in co-transfection of β1 integrin mutant and β1 integrin siRNA were not convincing. However, to ensure this result, MDA-MB-231 cells which have very high endogenous β1 integrin were repeated examined, the

same result was also observed (Fig. 21 C). These results indicated that the target cleavage site of β1/6 integrin siRNA was specific to β1 integrin mRNA degradation and subsequently specific to reduced Col I-induced MMP-2 activation.

Experimental design was further performed to insist whether a reduction of MMP-2 activation by β1/6 siRNA was specific to Type I Col stimulation. Although Collagen type I was reported induction of MMP-2 activation [45], artificial activation of MMP-2 can be effected by a variety of agents. Cellular activation of MMP-2 has been documented recently upon treatment of different cell types with TPA, TGF-0, Con A and APMA [173, 174]. It was suggested that the response to interstitial collagen and Con A may be mediated by integrins, since a number of integrin family members are their cell surface receptors. Non-specific substrates include Con A and TPA were used to generate induction of MMP-2 activation. Interestingly, knockdown of β1 integrin expression by β1/6 siRNA abolished substantially only Type I Col-induced MMP-2 activation. Importantly, MMP-2 activation in regarding of either Con A or TPA induction, abrogation was not seen by β1/6 siRNA transfection (Fig. 22 A). Nevertheless, to investigate unspecific effect of gene knockdown by siRNA technology that may be manipulate on Type I Col-induced MMP-2 activation. Other unspecific siRNAs including Vimentin-1, Vimentin-2, Slug-1, Gal3-1 and Gal3-2 were further tested effect on Type I Col-induced MMP-2 activation. It was found that, only β1/6 siRNA transfection convinced abrogation of Col I-induced MMP-2 activation. While all of other unspecific siRNAs were not seen in Col I-induced MMP-2 activation reduction, except Slug-1 showed a little reduction (Fig. 22 B). These data were support that, knockdown of β1 integrin expression by β1/6 siRNA had specific effect on MMP-2 activation reduction in response to Type I collagen stimulation.

6. Abrogation of β1 integrin was up-regulation of MT1-MMP, subsequently, effect on reduction of Col I-induced MMP-2 activation.

MMP-2 activation is important for degradation of denatured and cleaved type I collagen, in parallel type I collagen is also activates MMP-2. It was postulated that culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of MMP-2 [44-46]. Collagen type I can activate MMP-2 in certain cell types. MMP-2 activation is induced when cultured on type I collagen in human skin fibroblasts [45] and endothelial cells [166]. Several studies have shown that collagen-binding integrins interact with collagen involve in this mechanism. To date there are four integrins know to bind triple-helical interstitial collagen, and all of these integrins are closely related structurally. They share the common β1 integrin subunit and have four unique α subunits, resulting in the four heterodemers: α1β1, α2β1, α10β1 and α11β1 [85, 86]. In this study, it was found that three candidate β1 inetgrin siRNAs displayed unique knockdown, however, the β1/6 siRNA is the best effective. In addition, abrogation of β1 inetgrin by siRNAs reduced MMP-2 activation in response to type I collagen stimulation. This finding correlate with previous studies that indicated Collagen-induced MMP-2 activation occurs either directly or indirectly through integrin signaling [25, 59]. Cellular interaction with type I collagen is mediated largely through integrin α1β1, α2β1 and α3β1 receptors, cross linking of integrin β1 could activate MMP-2 in ovarian carcinoma cells, suggesting direct involvement of integrin signaling in MMP-2 activation [25, 59]. However, these argue recent study that indicated inhibition of integrin β1 function and expression by β1 siRNA did not affect 3-D collagen-induced cell surface localization of MT1-MMP and MMP-2 activation, by which MMP-2 activation occur in abundant cell surface MT1-MMP-dependent manner, rather than a manner regulated by integrin β1 function [60]. Because of this study investigate MMP-2 activation at

24 hours incubation and no longer time was further determined, therefore MMP-2 activation reduction could not observed. Moreover, interval time points of knockdown effect was only elucidate at short time including 5 and 24 hours after transient transfection. This revealed that successful knockdown both β1 integrin at cell surface and protein level, while MMP-2 activation was not effect at 24 hours. It seems apparent that collagen might directly interact with MT1-MMP, leading to increased surface expression of MT1-MMP and MMP-2 activation. Actually, this finding correlate with us, due to at this time point, even though, β1 integrin was knocked down, but was not complete. Thus, MMP-2 activation was still activated, while at longer times include 72, 96, and 120 hours, β1 integrin was completely knocked down whereas MMP-2 activation reduction strongly occur.

Mechanism of abrogated-β1 integrin expressing cells reduced Col I-induced MMP-2 activation was further elucidated. Collagen type I can stimulate MMP-2 activation via MT1-MMP activity in several cell types [25, 44-46], involves both a transcriptional increase in MT1-MMP expression, and a non-transcriptional response mediated by pre-existing MT1-MMP. Type I collagen stimulated MMP-2 activation functions principally via MT1-MMP in fibroblasts, as no MMP-2 activation occurs in MT1-MMP-deficient mouse fibroblasts in response to Col I and reduced levels of active MMP-2 are seen in MT1-MMP-deficient mice [46, 55, 114]. In this study, it found apparently abrogation of β1 integrin expression was able to suppress Type I Col-stimulated MMP-2 activation. Thus, the influence of β1 integrin on MMP-2 activation in regarding of MT1-MMP activity was examined. Interestingly, all of β1/4, β1/5 and β1/6 siRNAs showed decrease MT1-MMP levels, particularly, the β1/6 siRNA was the strongest weakness of MT1-MMP which correspond to β1 integrin level compared to controls (Cell alone and siRNA control) (Fig 23 A). Subsequently, β1/6 siRNA was obvious suppression of Col I-induced MMP-2 activation (Fig. 23 B). However, due to rapid turnover rate of active MT1-MMP, its concentration on cell surface is very low, and the active form of MT1-MMP predominantly localizes on the cell surface.

Even though, western blotting may be a more suitable alternative method, however, the preferred method for analyzing a protein knockdown is immunofluorescence detection using a specific antibody that recognizes the targeted gene. All abrogated-β1 integrin expressing cells displayed different morphology comparison with cells expressing β1 integrin (Cell alone and siRNA control). Cells expressing β1 integrin demonstrated better spread, whereas, all abrogated-β1 integrin expressing cells displayed rounder and less spread morphology, somehow the β1/6 siRNA was predominant (Fig. 24 A). In addition, the cell surface expression of β1 integrin localized to the cell periphery was strongly observed, while MT1-MMP was more diffusely distributed on the cell surface, somehow concentration of MT1-MMP was associate with β1 integrin. Although, abrogated-β1 integrin expressing cells knocked down β1 integrin, but the cell surface expression of β1 integrin was still detected. However, cell surfaces of β1 integrin and MT1-MMP were similar between untreated and treated with Col I. Interesting, only β1/6 siRNA indicated less of β1 integrin and MT1-MMP at the cell surface compared to β1/4 and β1/5 siRNAs in both untreated and treated with Col I conditions (Fig. 24 B). Since usage of siRNA technology, the transient reduction in mRNA levels can be detected in less than a day and lasts for several days depending on the turnover rate of the RNA and/or the protein. Immunofluorescence detection was further performed at 24 hours incubation of β1 integrin and MT1-MMP at the cell surface (Fig. 24 C). At this time, β1 integrin localized to the cell periphery was strongly observed in control include cell alone and siRNA control. In the other hand, all abrogated-β1 integrin expressing cells showed little β1 integrin at the cell periphery. Obviously, β1 integrin was more diffusely distributed on the cell surface and disappear from cell periphery in β1/6 siRNA knockdown cells. When cells were stimulated with Col I, only β1/6 siRNA knockdown cells distinctly displayed MT1-MMP less than untreated with Col I condition which related to the most efficiently knockdown of β1 integrin. Importantly, both β1/4 and β1/5 siRNAs expressing cells were able to knock down β1

integrin, however, β1 integrin localized at cell periphery was still detected. Additionally, β1 integrin and MT1-MMP levels examined by immunofluorescence were corresponding to Western blot determination (Fig 23 A). Thus, it was conceivable that β1/6 siRNA was not only the most efficient knockdown of β1 integrin but also the highest significantly effected down regulation of MT1-MMP level. Taken together, only knockdown β1 integrin by β1/6 siRNA showed significantly MMP-2 activation reduction, while β1/4 and β1/5 siRNAs were not. However, to confirm that MT1-MMP was down regulation of this knockdown β1 integrin. The reciprocal experiment was further investigated. Human MT1-MMP were transiently transfceted upon indication in MCF-7-MT1 cells, controls were also including. Untreated and treated with Col I conditions were parallel set up. Ectopic expression of MT1-MMP were determined by Western blot, the reduction of MT1-MMP in all different β1 integrin siRNAs knockdown cells were increasing back (Fig 25A). Expectedly, suppression of MMP-2 activation by β1/6 siRNA was rescued by MT1-MMP induction in both non- and stimulation with Col I. (Fig. 25 B). More over, to confirm that β1 integrin was up regulation of MT1-MMP activity, the reciprocal induction of β1 integrin was further investigated. Transient expression of β1 integrin in MCF7-MT1 cells, full length β1 integrin plasmid DNA (pcDNAβ1) and empty vector (pcDNA-VC) transfected counterparts, were generated. MCF-7-MT1 cells were transiently transfected with either pcDNA-VC or pcDNA-β1, subsequently, rMMP-2 was added and MMP-2 activation was performed at serial times include 6, 12, 24, 48 and 72 hours incubation. The β1 integrin protein levels were also determined at serial time points in parallel with MMP2-activation. The β1 integrin strongly increased at 12 and 24 hours, then start to go down at 48 hours and reach to baseline at 72 hours after transfection (Fig. 26 A). Interestingly, increasing of β1 integrin induced MT1-MMP parallel together at 12 hours until 72 hours. This effect, subsequently, enhance Col I-induced MMP-2 activation by MT1-MMP activity. Col I-induced

MMP-2 activations were convincingly observed at 12 and 48 hours, then MMP-2 activation levels were similar between non- and induction of β1 integrin (Fig. 26 B). These results indicated that β1 integrin was up regulation of MT1-MMP, subsequently, effect on MMP-2 activation in response to Col I stimulation. Thus, it strongly recommends that the β1 integrin involve in Col I-induced MMP-2 activation via up regulation of MT1-MMP activity.

CHAPTER 6
CONCLUSIONS

Breast cancer is the most common malignancy with the highest incidence among women in the world. Tumors can develop in different stages; these stages may or may not eventually lead to invasive and metastatic cancer. Metastasis is the major reason for breast cancer-related deaths [63]. Tumor invasion can be defined as the active migration of neoplastic cells out of their tissue of origin and into adjacent tissues of different types. The invading tumor cells, therefore, end up in a tissue where they do not belong. During the process of invasion, tumor cells must traverse the matrix barriers as they cross tissues boundaries. After traversing these barriers, tumor cells gain access to lymphatic and blood vessels for further dissemination, and finally colonies a distant organ – the so-called metastasis process. MMPs are the enzyme which play important roles in tumor invasion and metastasis, due to their ability to degrade several matrix and non-matrix proteins. To invade and penetrate the surrounding tissues, both cancer cells and stroma cell release theses enzymes to degrade and remodel the ECM. Therefore, ECM degradation and tissue remodeling are important phenomena in malignant progression.

MMP-2 is a widely studied matrix metalloproteinase. It was first described and purified from highly metastatic murine tumors [9]. MMP-2 is abundantly expressed in fibroblasts, endothelial and epithelial cells [135, 136]. Studies in breast cancer found increased levels of MMP-2 in invasive regions rather than the benign regions, and the same was found in gastric carcinomas, colorectal carcinoma and non small cell lung carcinoma [7, 8]. Many cell types constitutively synthesize MMP-2 as a latent proenzyme, requiring proteolytic removal of the propeptide for activation. It has become well-accepted that MMP-2 activation requires MT1-MMP and TIMP-2 and is tightly regulated [32-35]. Moreover, Type I collagen (Col I)-stimulated MMP-2 activation via

MT1-MMP involves both a transcriptional increase in MT1-MMP expression, and a non-transcriptional response mediated by pre-existing MT1-MMP.

The involvement of β1 integrin in MMP-2 activation in regarding to collagen stimulation is still investigation. Treatment of human endothelial cells with function blocking α2β1 integrin antibody can inhibit MMP-2 activation, whereas blocking ligand interaction to α3β1 integrin had no effect. Previous studies have indicated that cellular interaction with type I collagen is mediated largely through integrin α1β1, α2β1 and α3β1 receptors, cross linking of integrin β1 could activate MMP-2 in ovarian carcinoma cells, suggesting direct involvement of integrin signaling in MMP-2 activation [25, 59]. However, recent study indicated that inhibition of integrin β1 function and expression did not affect 3-D collagen-induced cell surface localization of MT1-MMP and MMP-2 activation, and strongly suggest that 3-D collagen scaffolding provide the direct and multivalent interaction with MT1-MMP, by which MMP-2 activation occur in abundant cell surface MT1-MMP-dependent manner, rather than a manner regulated by matrix stiffness and integrin β1 function [60]. Considering these reports, the involvement of β1 integrin in MMP-2 activation in response to type I collagen stimulation is still not precisely understood.

In this study demonstrated the involvement of β1 integrin on MMP-2 activation in response to type I collagen stimulation. β1 integrin siRNA technology was utilized and examined the β1 integrin knockdown effect on MMP-2 activation mechanism. The results can be summarized as follows.

1. Six different β1 integrin siRNAs were designed and tested, and each successfully knocked down β1 integrin at both RNA and protein levels. Three showed high efficiency knockdown of β1 integrin, and were chosen for further study.
2. The β1 integrin siRNA not only knocked down β1 integrin, but also resulted in the detectable reduction of α subunits including α2 and α3 integrins, but not αV integrin (detected by FACS analysis).

3. The 3 different β1 integrin siRNAs tested further showed knockdown of β1 integrin expression after 24 transfection, however, complete knockdown of β1 integrin was observed at 72 hours after transfection.

4. The 3 different β1 integrin siRNAs tested further showed good knockdown of β1 integrin, but only one caused a reduction in collagen-stimulated MMP-2 activation. The reduction in MMP-2 activation occurred at 72 hours of MMP-2 addition, commensurate with the complete abrogation of β1 integrin.

5. The β1 integrin siRNA which blocked collagen-induced MMP-2-activation was more potent in terms of both dose response and time course to inhibition than the other 2 tested, suggesting that an important threshold may exist for the level of β1 integrin required to mediate collagen-stimulated MMP-2-activation.

6. The one β1 integrin siRNA with MMP-2-activation inhibition could be reversed with transfection of a β1 integrin constructed in which the specific target site of the siRNA had been mutated, therefore, co-transfection of the β1 integrin mutant and the β1 integrin siRNA could rescue the reduction in MMP-2 activation.

7. The one β1 integrin siRNA with MMP-2-activation suppression was specific to type I collagen stimulation, but not Con A or TPA. Non-specific other siRNAs were also no effect on Col I-induced MMP-2 activation.

8. Abrogation of β1 integrin was up-regulation of MT1-MMP, subsequently, suppressed Col I-induced MMP-2 activation. Reciprocal test by transfection of MT1-MMP back, it returned MT1-MMP to base line could rescue Col I-induced MMP-2 activation reduction. Moreover, ectopic of β1 integrin induction also increase MT1-MMP, subsequently, increase MMP-2 activation in response to Col I stimulation.

In conclusion, . this study demonstrated in regarding the MMP-2 activation effects of β1 integrin, knockdown of β1 integrin expression was able to reduce MMP-2 activation. However, knockdown of β1 integrin that inhibited MMP-2 activation depended on a precise dose threshold effect of β1 integrin siRNA.

These findings suggest that the β1 integrin is an important molecule that involve in MMP-2 activation in response to collagen type I stimulation. Abrogation of β1 integrin reduced MMP-2 activation was specific to type I collagen stimulation. A reduction of Col I-induced MMP-2 activation is via MT1-MMP up regulation. This reveal provides insight into certain mechanism involved in the reciprocal regulation of integrin and MMPs of tumor cells

REFERENCES

1. Klein, A.C. Parallel progression of primary tumours and metastases. *Nature Reviews/ Cancer*. 2009; 9(6): 302-312.
2. White, E.D. and Muller, J.W. Multifaceted Roles of Integrins in Breast Cancer Metastasis. *J Mammary Gland Biol Neoplasia.* 2007; (12) :135–142.
3. Nguyen, X,D, Bos, D,P, and Massagué, J. Metastasis: from dissemination to organ-specific colonization. *Nature Reviews/ Cancer.* 2009; 9(6): 274-285.
4. Christofori, G. New signals from the invasive front. *Nature.* 2006; (441): 444–450.
5. Gupta, G. P. & Massague, J. Cancer metastasis: building a Framework. *Cell.* 2006; (127): 679–695.
6. Gersh, I., Catchpole, H.R. The organization of ground substance and basement membrane and its significance in tissue injury, disease and growth. *Am J Anat*. 1949; 85(3): 457–507.
7. Lynch CC, Matrisian LM: Matrix metalloproteinases in tumor host cell communication. *Differentiation*. 2002; (70): 561-573.
8. Fingleton, B. Matrix metalloproteinases: roles in cancer and metastasis. *Front Biosci*. 2006; (11): 479–491.
9. Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie. S: Metastatic potential correlates with enzymatic degradation of basement-membrane collagen.*Nature*.1980; (284): 67-68.
10. Liotta LA: Tumor invasion and metastases-role of the extracellular matrix: Rhoads memorial award lecture. *Cancer Res*. 1986; (46): 1–7.
11. Amaleini,C.,et al. Matrixmetalloproteinases involvement in pathologic conditions. *Romanian Journal of Morphology and Embryology.* 2010; 51(2): 215–228.
12. Deryugina, E.I. and Quigley,J.P. Matrix metalloproteinases and tumor metastasis.*Cancer Metastasis Rev*.2006; 25(1): 9–34.
13. Fridman, R. Metalloproteinases and cancer. *Cancer Metastasis Rev*.2006; 25(1): 7–8.

14. Itoh, Y. and Seiki, M. MT1-MMP: A Potent Modifier of Pericellular Microenvironment. *Journal of Cellular Physiology*. 2006; 206: 1–8.
15. Sato, H., Takinol, T. Coordinate action of membrane-type matrix metalloproteinase-1 (MT1-MMP) and MMP-2 enhances pericellular proteolysis and invasion. *Cancer Science*. 2010; 101(4): 843–847.
16. Elnemr, A., Y. Yonemura, et al. Expression of collagenase-3 (matrix metalloproteinase-13) in human gastric cancer. *Gastric Cancer* 6. 2003; (1): 30-8
17. Gallegos, N.C., C. Smales, et al. The distribution of matrix metalloproteinases and tissue inhibitor of metallo-proteinase in colorectal cancer. *Surgical Oncol.* 1995; 4: 21-29.
18. Taylor, A.C., B.M. Levy and J.W. Simpson. Collagenolytic activity of sarcoma tissues in culture. *Nature*. 1970; 228: 366-7.
19. Dresden, M.H., S.A. Helman and J.D. Schmidt. Collagenolytic enzymes in human neoplasms. *Cancer Res.*1972; 32: 993-6.
20. Liotta, L.A., K. Tryggvason, et al. Partial purification and characterization of a neutral protease which cleaves type IV collagen. *Biochemistry*. 1981; 20: 100-104.
21. Agarwal D, Goodison S, Nicholson B, Tarin D, Urquidi V: Expression of matrix metalloproteinase 8 (MMP-8) and tyrosinaserelated protein-1 (TYRP-1) correlates with the absence of metastasis in an isogenic human breast cancer model. *Differentiation*. 2003; 71: 114–125.
22. Jumper C, Cobos E, Lox C: Determination of the serum matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) in patients with either advanced small-cell lung cancer or non-small-cell lung cancer prior to treatment. *Respir Med*. 2004; 98: 173–177.
23. Kallakury BV, Karikehalli S, Haholu A, Sheehan CE, Azumi N, Ross JS: Increased expression of matrix metalloproteinases 2 and 9 and tissue inhibitors of metalloproteinases 1 and 2 correlate with poor prognostic variables in renal cell carcinoma. *Clin Cancer Res*. 2001; 7: 3113–3119.

24. Murphy, G., M. I. Cockett, et al. Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). *Biochem J.* 1991; 277 (1): 277-9.
25. Ellerbroek, S. M., Y. I. Wu, et al. Functional interplay between type I collagen and cell surface matrix metalloproteinase activity. *J Biol Chem*. 2001; 276(27): 24833-42.
26. Polette, M., N. Gilbert, et al. Gelatinase A expression and localization in human breast cancers. An in situ hybridization study and immunohistochemical detection using confocal microscopy. *Virchows Arch.* 1994; 424(6): 641-5.
27. Itoh T, Tanioka M, Yoshida H, et al. Reduced angiogenesis and tumor progression in gelatinase A deficient mice. *Cancer Res.* 1998; 58: 1048–51.
28. Monig SP, Baldus SE, Hennecken JK, et al. Expression of MMP-2 is associated with progression and lymph node metastasis of gastric carcinoma. *Histopathology*. 2001; 39: 597–602.
29. Herbst RS, Yano S, Kuniyasu H, et al. Differential expression of E-cadherin and type IV collagenase genes predicts outcome in patients with stage I non-small cell lung carcinoma. *Clin Cancer Res*. 2000; 6: 790–7.
30. Curran S, Murray GI. Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis. *Eur J Cancer*. 2000; 36: 1621–30.
31. Kinoshita T, Sato H, Okada A, et al. TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. *J Biol Chem*. 1998; 273: 16098–103.
32. Overall CM, Tam E, McQuibban GA, et al. Domain interactions in the gelatinase A TIMP-2 MT1-MMP activation complex. *J Biol Chem*. 2000; 275: 39497–506.

33. Sato, H., T. Takino, et al. Cell surface binding and activation of gelatinase A induced by expression of membrane-type-1-matrix metalloproteinase (MT1-MMP). *FEBS Lett.* 1996; 385(3): 238-40.
34. Strongin, A. Y., I. Collier, et al. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. *J Biol Chem.* 1995; 270(10): 5331-8.
35. Deryugina, E. I., B. Ratnikov, et al. MT1-MMP initiates activation of pro-MMP-2 and integrin alphavbeta3 promotes maturation of MMP-2 in breast carcinoma cells. *Exp Cell Res.* 2001; 263(2): 209-23.
36. Seiki M. Membrane-type 1 matrix metalloproteinase: A key enzyme for tumor invasion. *Cancer Lett.* 2003; 194: 1–11.
37. Visse, R. and H. Nagase. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res.* 2003; 92(8): 827-39.
38. Itoh, Y. and M. Seiki. MT1-MMP: an enzyme with multidimensional regulation. *Trends. Biochem Sci.* 2004; 29(6): 285-9.
39. Sato, H., T. Takino, et al. A matrix metalloproteinase expressed on the surface of invasive tumour cells. *Nature.* 1994; 370 (6484): 61-5.
40. Brooks, P. C., R. A. Clark, et al. Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science.* 1994; 264(5158): 569-71.
41. Hodivala-Dilke, K.M., et al. Beta3 integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Cli Invest.* 1999; 103(2): 229-38.
42. Davies, G., Martin, T. A., Parr, C., Watkins, G., Mason, M. D., and Mansel, R. E. Expression of membrane type-1 matrix metalloproteinase, MT1-MMP in human breast cancer and its impact on invasiveness of breast cancer cells. Int. *J. Mol. Med*. 2006; 17: 583–590.

43. Borrirukwanit, K., Lafleur, M. A., Mercuri, F. A., Blick, T., Price, J. T., Fridman, R., Pereira, J. J., Leardkamonkarn, V., and Thompson, E. W. The type I collagen induction of MT1-MMP-mediated MMP-2 activation is repressed by alphaVbeta3 integrin in human breast cancer cells. *Matrix Biol.* 2007; 26: 291–305.
44. Thompson, E. W., M. Yu, et al. Collagen induced MMP-2 activation in human breast cancer. *Breast Cancer Res Treat.* 1994; 31(2-3): 357-70.
45. Azzam, H. S. and E. W. Thompson. Collagen-induced activation of the M(r) 72,000 type IV collagenase in normal and malignant human fibroblastoid cells. *Cancer Res.* 1992; 52(16): 4540-4.
46. Kim, I.Y., Jeong, S.J., Kim, S.R., Kim, S.H. and Moon, A. Type I Collagen-induced Pro-MMP-2 Activation is differentially Regulated by H-Ras and N-Ras in Human Breast Epithelial Cells. *Journal of Biochemistry and Molecular Biology*. 2007; 40(5): 825-831.
47. Itoh, Y., A. Takamura, et al. Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. *Embo J.* 2001; 20(17): 4782-93.
48. Gilles, C., M. Polette, et al. Implication of collagen type I-induced membrane-type 1-matrix metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma. *Lab Invest.* 1997; 76(5): 651-60.
49. Lafleur, M. A., Mercuri, F. A., Ruangpanit, N., Seiki, M., Sato, H., and Thompson, E. W. Type I collagen abrogates the clathrin-mediated internalization of membrane type 1 matrix metalloproteinase (MT1-MMP) via the MT1-MMP hemopexin domain. J. Biol. Chem. 2006; 281: 6826–6840.
50. Humphries, M. J. Integrin structure. *Biochem Soc Trans.* 2000; 28(4): 311-39.
51. Travis, M. A., J. D. Humphries, et al. An unraveling tale of how integrins are activated from within. *Trends Pharmacol Sci.*

2003; 24(4): 192-7.
52. Zutter MM., et al. Re-expression of the α2β1 integrin abrogates the malignant phenotype of breast carcinomas cells. *Proc Natl Acad Sci USA.* 1995; 92: 7411- 7415.
53. Keely, P.J., et al. Alteration of collagen-dependent adhesion, motility and morphogenesis by the expression of antisense ha2 integrin in mRNA in mammary cells. *J Cell Sci.* 1995; 108: 595-607.
54. Ivaska, J., H. Reunanen, et al. Integrin alpha2beta1 mediates isoform-specific activation of p38 and upregulation of collagen gene transcription by a mechanism involving the alpha2 cytoplasmic tail. *J Cell Biol.* 1999; 147(2): 401-16.
55. Haas, T. L., S. J. Davis, et al. Three-dimensional type I collagen lattices induce coordinate expression of matrix metallo-proteinases MT1-MMP and MMP-2 in microvascular endothelial cells. *J Biol Chem.* 1998; 273(6): 3604-10.
56. Koyama, H. Raines, E.W., Bornfeldt,K.E., et al., Fibrillar collagen inhibits arterial smooth muscle proliferation through regulation of Cdk2 inhibitors, *Cell* , 1996; 87: 1069-1078.
57. Wang, K.Y., Wang, H.Y., Wang, Z.C., et al., Rigidity of collagen fibrils controls collagen gel-induced down-regulation of focal adhesion complex proteins mediated by alpha2beta1 integrin, *J. Biol. Chem*. 2003; 278: 21886-21892.
58. Handin, R, Escaping the niche. *Blood.* 2009; 113(25): 6271-73.
59. Ellerbroek, S. M., D. A. Fishman, et al. Ovarian carcinoma regulation of matrix metalloproteinase-2 and membrane type 1 matrix metalloproteinase through beta1 integrin. *Cancer Res.* 1999; 59(7): 1635-41.
60. Sakai,K., Nakamura, T. Suzuki, Y., et al. 3-D collagen-dependent cell surface expression of MT1-MMP and MMP-2 activation regardless of integrin b1 function and matrix stiffness. *Biochemical and Biophysical Research Communications*. 2011; 412: 98–103.

61. Tam EM, Moore TR, Butler GS, Overall CM. Characterization mechanisms of matrix metalloproteinase 2 and 14 (gelatinase A and MT1-MMP): the differential roles of the MMP hemopexin c domains and the MMP-2 fibronectin type II modules in collagen triple helicase activities. *J Biol Chem*.2004; 279: 43336–43344.
62. Ferlay J, Shin HR, Bray F, et al . GLOBOCAN 2008, Cancer incidence and mortality worldwide. *IARC Cancer Base*. 2010; No. 10: http://globan.iarc.fr/.
63. Shi, M., Liu, D. Duan, H., et al. Metastasis-related miRNAs, active players in breast cancer invasion,and metastasis. *Cancer Metastasis Rev*. 2010; 29: 785–799.
64. Dairkee, S.H., and H.S. Smith, The Genetic Analysis of Breast Cancer Progression. *Journal of Mammary Gland Biology and Neoplasia*. 1996; 1(2): 139-149.
65. Tavassoli FA, Devilee P. *World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Breast and Female Genital Organs*. Lyon, Fr.: IARC, 2003.
66. Chambers, A.F., Welch, D.R. and Bratist, G. Breast Cancer Progression and Metastasis:Experimental Studies and Clinical Translation. *Journal of Mammary Gland Biology and Neoplasia.* 2002; 6(4): 373-374.
67. Albertson DG, Collins C, McCormick F and Gray JW. Chromosome aberrations in solid tumors. *Nat. Genet.* 2003; 34: 369–76.
68. Weinstein, I. B.; Gattoni-Celli, S.; Kirschmeir, P.; Lambert, M.; Hsiao, W.; Backer, J.; and Jeffrey, A. Multistage carcino-genesis involves multiple genes and multiple mechanisms. *J. Cell. Physiol. (Suppl.)*1984; 3: 127-137.
69. Tlsty, T.D. and Coussens, L.M. Tumor Stroma and Regulation of Cancer Development. *Annu. Rev. Pathol. Mech. Dis*. 2006; 1: 119-150.
70. Fitzpatrick FA. Inflammation, carcinogenesis and cancer. *Int. Immuno.*2001; 1: 1651–6.

71. Coussens LM,Werb Z. Inflammation and cancer. *Natur.* 2002; 420:860–6.
72. National Toxicology Program. *Fourth annual report on carcinogens. Summary 1985 [NTP 85-002].* Washington, DC: Department of Health and Human Services, 1985.
73. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet.* 2002*;* 357:539–4.
74. Bacac, M. and Stamenkovic, I. Metastatic Cancer Cell. *Annu. Rev. Pathol. Mech. Dis*. 2008; 3: 241-248.
75. Timpl, R. Macromolecular organization of basement membranes. *Curr Opin Cell Biol.* 1996; 8: 618-624.
76. Terranova, V. P., E. S. Hujanen, et al. Basement membrane and the invasive activity of metastatic tumor cells. *J Natl Cancer Ins*t. 1986; 77(2): 311-6.
77. Aumailley, M. and B. Gayraud. Structure and biological activity of the extracellular matrix. *J Mol Med.* 1998; 76: 253-265.
78. Zagris, N. Extracellular matrix in development of the early embryo. *Micron.* 2001; 32: 427-438
79. Bissell MJ, Aggeler J. Dynamic reciprocity: How do extracellular matrix and hormones direct gene expression? *Prog*. *Clin. Biol. Res.* 1987; 249: 251–62.
80. De Wever, O. and M. Mareel. Role of tissue stroma in cancer cell invasion. *J Pathol.* 2003; 200(4): 429-47.
81. Duffy,MJ., McGowan, PM. and Gallagher, WM. Cancer invasion and metastasis: changing views. *J Pathol* . 2008; 214: 283–293.
82. Van der Rest, M. and R. Garrone. Collagen family of proteins. *Faseb J.* 1991; 5(13): 2814-23.
83. Ramachandran, G.N. and G. Kartha. Structure of collagen. *Nature*. 1955; 176(4482): 593-5.
84. Brinckmann J. Collagens at a glance. *Top. Curr. Chem*. 2005; 247: 1-6.
85. Prockop, D.J. and Kivirikko. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem.* 1995; 64: 403-4.

86. Shoulders, M.D. and Raines, R.T. Collagen Structure and Stability. *Annu Rev Biochem*. 2009 ;78 : 929–958.
87. Ricard-Blum, S., Ruggiero, F. & van der Rest, M. The collagen superfamily. *Top. Curr. Chem*. 2005; 247: 35–84.
88. Koide, D. Designed triple-helical peptides as tools for collagen biochemistry and matrix engineering. Phil. Trans. R. Soc. B. 2007; 362: 1281–1291.
89. Rossert, J., C. Terraz, and S. Dupont. Regulation of type I collagen genes expression. *Nephrol Dial Transplant.* 2000; 15 suppl 6: 66-8.
90. Tannapfel A, Anhalt K, Hausermann P, et al. Identification of novel proteins associated with hepatocellular carcinomas using protein microarrays. *J Pathol.* 2003, 201:238-249.
91. Armstrong, T., Packham, G., Murphy, L.B., et al. Type I Collagen Promotes the Malignant Phenotype of Pancreatic Ductal Adenocarcinoma. *Clinical Cancer Research*. 2004; 10(1): 7427–37.
92. DeClerck YA, Bomann ET, Spengler BA, Biedler JL: Differential collagen biosynthesis by human neuroblastoma cell variants. *Cancer Res.* 1987, 47:6505-6510.
93. Savaraj N, Wu C, Landy H, Wangpaijit M, Wei Y, Kuo MT, Robles C, Furst AJ, Lampidis T, Feun L: Procollagen alpha 1 type 1: a potential aide in histopathological grading of glioma. *Cancer Invest.* 2005; 23: 577-581.
94. Honma, K., Miyata, T. and Ochiya, T. Type I collagen gene suppresses tumor growth and invasion of malignant human glioma cells. *Cancer Cell International.* 2007; 7(12): 1-9.
95. Gomez-Lechon, M. J., Jover, R., Donato, T., et al. Long-term expression of differentiated functions in hepatocytes cultured in three-dimensional collagen matrix. *J. Cell. Physiol*. 1998; 177: 553–562.
96. Jaenisch R, Harbers K, Schnieke A, Lohler J, Chumakov I, Jahner D, Grotkopp D, Hoffmann E: Germline integration of Moloney murine leukemia virus at the Mov13 locus leads to recessive lethal mutation and early embryonic death. *Cell.* 1983; 32: 209–216.

97. Aimes RT, Quigley JP. Matrix metalloproteinase-2 is an Interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. *J Biol Chem.* 1995; 270: 5872–76.
98. Patterson ML, Atkinson SJ, Kna¨uper V, Murphy G. Specific collagenolysis by gelatinase A, MMP-2, is determined by the hemopexin domain and not the fibronectin-like domain. *FEBS Lett.* 2001; 503: 158–162.
99. Ohuchi E, Imai K, Fujii Y, Sato H, Seiki M, Okada Y. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J Biol Chem*. 1997; 272: 2446–51.
100. Hasty, K.A., et al., The collagen substrate specificity of human Neutrophil collagenase. *J Biol Chem*., 1987; 262(21): 10048-52.
101. Perumal, S., Antipova, O. and. Orgel., P. R. O. J. Collagen fibril architecture, domain organization, and triple-helical conformation govern its proteolysis. *PNAS*. 2008; 105(8): 2824–29.
102. Saffarian, S., et al., Interstitial collagenase is a Brownian ratchet driven by proteolysis of collagen. *Science*. 2004; 306(5693): 108-11.
103. Chung, L., et al., Collagenase unwinds triple-helical collagen prior to peptide bond hydrolysis. *Embo J*. 2004; **23**(15): 3020-30.
104. Egeblad, M., Shen, H.-C.J., Behonick1, J.D., et al. Type I Collagen Is a Genetic Modifier of Matrix Metalloproteinase-2 in Murine Skeletal Development. *Dev Dyn*. 2007; 236(6): 1683–93.
105. Vu, T. H. and Z. Werb. Matrix metalloproteinases: effectors of development and normal physiology. *Genes Dev.* 2000; 14(17): 2123-33.
106. Matrisian, L. M. The matrix-degrading metalloproteinases. *Bioessays.* 1992; 14(7): 455-63.

107. Finesmith, T. H., K. N. Broadley, et al. Fibroblasts from wounds of different stages of repair vary in their ability to contract a collagen gel in response to growth factors. *J Cell Physiol.* 1990; 144(1): 99-107.
108. Tamariz, E. and F. Grinnell. Modulation of fibroblast morphology and adhesion during collagen matrix remodeling. *Mol Biol Cell.* 2002; 13(11): 3915-29.
109. Nakagawa, S., P. Pawelek, et al. Long-term culture of fibroblasts in contracted collagen gels: effects on cell growth and biosynthetic activity. *J Invest Dermatol.* 1989; 93(6): 792-8.
110. Rosenfeldt, H. and F. Grinnell. Fibroblast quiescence and the disruption of ERK signaling in mechanically unloaded collagen matrices. *J Biol Chem.* 2000; 275(5): 3088-92.
111. Seltzer, J. L., A. Y. Lee, et al. Activation of 72-kDa type IV collagenase/gelatinase by normal fibroblasts in collagen lattices is mediated by integrin receptors but is not related to lattice contraction. *Exp Cell Res.* 1994; 213(2): 365-74.
112. Gullberg, D., A. Tingstrom, et al. β1 integrin-mediated collagen gel contraction is stimulated by PDGF. *Exp. Cell Res*. 1990; 186: 264-72.
113. Carver, W., I. Molano, et al. Role of the α1β1 integrin complex in collagen gel contraction in vivo by fibroblasts. *J. Cell Physiol.* 1995; 165: 425-37.
114. Deryugina, E. I., M. A. Bourdon, et al. Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2. *Cancer Res.* 1998; 58(16): 3743-50.
115. Kessenbrock, K., Plaks, V., and Werb, Z. Matrix Metallo-proteinases: Regulators of the Tumor Microenvironment. *Cell,* 2010; 141: 52-67.
116. Folgueras, A., Pendas, M.A., Sanchez, M.L. Lopez-Otin, C. Matrix metalloproteinases in cancer: from new functions to improved inhibition strategies. *Int. J. Dev. Biol.* 2004; 48: 411-424.
117. Overall, C. M. and C. Lopez-Otin. Strategies for MMP inhibition in cancer: innovations for the post-trial era. *Nat Rev Cancer.*

2002; 2(9): 657-72.
118. UriaBalbin, M., Freije, J. M. and Lopez-Otin, C. Differential effects of transforming growth factor-beta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. *J. Biol. Chem*. 1998; 273: 9769-77.
119. Zucker, S., Pei, D., Cao, J. and Lopez-Otin, C. Membrane type-Matrix metalloproteinases (MT-MMP). *Curr. Top Dev. Biol.* 2003; 54: 1-74.
120. Allan, J. A., A. J. Docherty, et al. Binding of gelatinases A and B to type-I collagen and other matrix components. *Biochem J.* 1995; 309 (Pt 1): 299-306.
121. Murphy, G., F. Willenbrock, et al. The C-terminal domain of 72 kDa gelatinase A is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases. *Biochem J.* 1992; 283 (Pt 3): 637-41.
122. Docherty, A.J., A. Lyons, et al. Sequence of human tissue inhibitor of metalloproteinase and its identity to erythoid-potentiating activity. *Nature.* 1985; 318: 66-9.
123. Greene, J., M. Wang, et al. Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J Biol Chem.* 1996; 271: 30375-80.
124. Chakraborti, S., M. Mandal, et al. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem.* 2003; 253(1-2): 269-85.
125. Birkedal HH, Moore WG, Bodden MK et al. Matrix metallo-proteinases: a review. *Crit Rev Oral Biol Med*. , 1993; (4): 197–250.
126. Klein, G., Vellenga, E., Fraaije, M.W., et al. The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. *Critical Reviews in Oncology /Hematology*. 2004; 50: 87–10.
127. Lafleur MA, Hollenberg MD, Atkinson SJ, et al. Activation of pro-(matrix metalloproteinase-2) (pro-MMP-2) by thrombin is membrane-type-MMP-dependent in human

umbilical vein endothelial cells and generates a distinct 63 kDa active species. *Biochem J.* 2001; 357: 107–15.

128. Morrison CJ, Butler GS, Bigg HF, et al. Cellular activation of MMP-2 (gelatinase A) by MT2-MMP occurs via a TIMP-2-independent pathway. *J Biol Chem.* 2001; 276: 47402–10.
129. Coussens, L.M., Fingleton, B., and Matrisian, L.M. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. *Science*. 2002; 295: 2387–92.
130. Mu, D., Cambier, S., Fjellbirkeland, L., et al. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. *J. Cell Biol*. 2002; 157: 493–507.
131. Mitsiades, N., Yu, W.H., Poulaki, V., Tsokos, M., and Stamenkovic, I. Matrix metalloproteinase-7-mediated cleavage of Fas ligand protects tumor cells from chemotherapeutic drug cytotoxicity. Cancer Res. 2001; 61: 577–581.
132. Crawford, H.C., Scoggins, C.R., Washington, M.K., Matrisian, L.M., and Leach, S.D. Matrix metalloproteinase-7 is expressed by pancreatic cancer precursors and regulates acinar-to-ductal metaplasia in exocrine pancreas.*J. Clin. Invest*. 2002; 109: 1437–1444.
133. Littlepage, L.E., Sternlicht, M.D., Rougier, N., et al. Matrix metalloproteinases contribute distinct roles in neuroendocrine prostate carcinogenesis, metastasis, and angiogenesis progression. *Cancer Res*. 2010; 70: 2224–2234.
134. Bergers, G., Brekken, R., McMahon, G., et al. Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat. Cell Biol*. 2000; 2: 737–744.
135. Salo T, Liotta LA & Tryggvason K. Purification and characterization of a murine basement membrane collagen-degrading enzyme secreted be metastatic tumor cells. *J Biol Chem.* 1983; 258: 3058–63.
136. Hipps DS, Hembry RM, Docherty AJ, Reynolds JJ & Murphy G. Purification and characterization of human 72-kDa gelatinase (type IV collagenase). Use of immunolocalisation to demonstrate the non-coordinate regulation of the 72-kDa

and 95-kDa gelatinases by human fibroblasts. *Biol Chem Hoppe-Seyler.* 1991; 372: 287–296.

137. Murphy, G., Q. Nguyen, et al. Assessment of the role of the fibronectin-like domain of gelatinase A by analysis of a deletion mutant. *J Biol Chem.* 1994; 269: 6632-6636.
138. Will, H., and B. Hinzmann. cDNA sequence and mRNA tissue distribution of a novel human matrix metalloproteinase with a potential transmembrane segment. *Eur J Biochem .* 1995; 231: 602-8.
139. Takino, T., H. Sato, A. Shinagawa, and M. Seiki. Identification of the second membrane-type matrix metalloproteinase (MT-MMP-2) gene from a human placenta cDNA library. MT-MMPs form a unique membrane-type subclass in the MMP family. *J Biol Chem*. 1995; 270: 26333-20.
140. Ishigaki, S., M. Toi, et al. Significance of membrane type 1 matrix metalloproteinase expression in breast cancer. *Jpn J Cancer Res.* 1999; 90(5): 516-22.
141. Okada, A., J. P. Bellocq, et al. Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. *Proc Natl Acad Sci U S A.* 1995; 92(7): 2730-4.
142. Xiong, J. P., T. Stehle, et al. New insights into the structural basis of integrin activation. *Blood.* 2003; 102(4): 1155-9.
143. O'Toole, T. E., J. Ylanne, et al. Regulation of integrin affinity states through an NPXY motif in the beta subunit cytoplasmic domain. *J Biol Chem.* 1995; 270(15): 8553-8.
144. Chen, K. and Chen, X. Integrin Targeted Delivery of Chemotherapeutics. *Theranosttiics.* 2011; 1: 189-200.
145. Hynes, R.O. Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002; 110(6): 673-87.
146. Shimaoka, M., J. Takagi., and T.A. Springer. Conformational regulation of integrin structure and function. *Annu Rev Biophys Biomol Struct.* 2002; 31: 485-516.
147. Green, L. J., A. P. Mould, et al. The integrin beta subunit. *Int J Biochem Cell Biol.* 1998; 30(2): 179-84.

148. Clark, E. A. and J. S. Brugge. Integrins and signal transduction pathways: the road taken. *Science.* 1995; 268(5208): 233-9.
149. Shimaoka, M., J. Takagi., and T.A. Springer. Conformational regulation of integrin structure and function. *Annu Rev Biophys Biomol Struct.* 2002; 31: 485-516.
150. Tulla, M., O. T. Pentikainen, et al. Selective binding of collagen subtypes by integrin alpha 1I, alpha 2I, and alpha 10I domains. *J Biol Chem.* 2001; 276(51): 48206-12.
151. Manso, M.A., Elsherif, L., Kang, S-M., and Ross, A.R. Integrins, membrane-type matrix metalloproteinases and ADAMs: Potential implications for cardiac remodeling. *Cardiovascular Research.* 2006; 69: 574 – 584.
152. Srichai, M,B.anf Zent, R. Integrin Structure and Function. *Cell-Extracellular Matrix interaction in Cancer*. 2010, 1-314. http://www.springer.com/978-1-4419-0813-1.
153. Goel, L.H., Li, J., Kogan, S. and Languino, L. R. Integrins in prostate cancer progression. *Endocrine-Related Cancer*. 2008; 15: 657–664.
154. Takada, Y., Ye, X. and Simon, S. The integrins. *Genome* 2007; 8 (5): 1-9.
155. Pozzi A, Zent R. Integrins: sensors of extracellular matrix and modulators of cell function. *Nephron Exp Nephro*. 2003; l94: 77–84.
156. Gardner H, Broberg A, Pozzi A, Laato M, Heino J. Absence of integrin alpha1beta1 in the mouse causes loss of feedback regulation of collagen synthesis in normal and wounded dermis. *J Cell Sci.* 1993; 112: 263–272.
157. Elliott, B. E. , Ekblom, P. , Pross, H. , Niemann, A. andRubin, K. Anti-beta 1 integrin IgG inhibits pulmonary macrometastasis and the size of micrometastases from a murine mammary carcinoma. *Cell Adhesion & Communication.* 1994; 1: 319-332.
158. Adriance, M. C. , Inman, J. L. , Petersen, O. W. and Bissell, M. J. Myoepithelial cells: Good fences make good neighbors. *Breast Cancer Research* . 2005; 7: 190-197.
159. Goel HL, Fornaro M, Moro L, et al. Selective modulation of type

1 insulin-like growth factor receptor signaling and functions by b1 integrins. *Journal of Cell Biology*. 2004; 166: 407–418.

160. Cordes, N. and Park, C.C. β1 integrin as a molecular therapeutic target. *International Journal of Radiation Biology*. 2007; 83(11-12): 753 – 760.
161. Okamura, M. , Yamaji, S. , Nagashima, Y. , Nishikawa, et al. Prognostic value of integrin beta1-ILK-pAkt signaling pathway in non-small cell lung cancer. *Human Pathology*. 2007; 38: 1081-1091.
162. Giancotti, F.G. and Ruoslati, E. Elevated levels of the α5β1 receptor suppress the transformed phenotype Chinese hamster ovary cells. *Cell.* 1990; 60: 849-859.
163. Pignatelli, M., A.M. Hanby, and G.W. Stamp. Low expression of β1,α2 and α3 subunits of VLA integrins in malignant mammary tumors. *J Pathol.* 1991; 165: 25-32.
164. Sameni, M., Dosescu, J., Yamada, M.K.,Sloane, F.B. and Cavallo-Medved, D. Functional Live-Cell Imaging Demonstrates that β1-Integrin Promotes Type IV Collagen Degradation by Breast and Prostate Cancer Cells. *Mol Imaging*. 2008; 7(5): 199–213.
165. Chirco R, Liu XW, Jung KK, Kim HR. Novel functions of TIMPs in cell signaling. *Cancer Metastasis Rev*. 2006; 25:99–113.
166. Nguyen, M., J. Arkell, et al. Three-dimensional collagen matrices induce delayed but sustained activation of gelatinase A in human endothelial cells via MT1-MMP. *Int J Biochem Cell Biol.* 2000; 32(6): 621-31.
167. Jokinen, J., E. Dadu, et al. Integrin-mediated cell adhesion to type I collagen fibrils. *J Biol Chem.* 2004; 279(30): 31956-63.
168. Riikonen, T., J. Westermarck, et al. Integrin alpha 2 beta 1 is a positive regulator of collagenase (MMP-1) and collagen alpha 1(I) gene expression. *J Biol Chem.* 1995; 270(22): 13548-52.
169. Wolf, K., Wu, YI, Liu, Y., et al. Multi-step pericellular proteolysis controls the transition from individual to

collective cancer cell invasion. Nat Cell Biol. 2007; 9:893–904.
170. Kim, N.V. RNA Interference in Functional Genomics and Medicine. *J Korean Med Sci.* 2003; 18: 309-18.
171. Lipardi, C., Q. Wei, and B.M. Paterson. RNAi as random degradative PCR. siRNA primers convert mRNA into dsRNA that are degraded to generate new siRNAs. *Cell.* 2001; 107: 297-307.
172. Tushl, T., P.D. Zamore, et al. Targetd mRNA degradation by double-stranded RNA in vitro. *Genes Dev*. 1999; 13(24): 3191-7.
173. Overall, C. M., and Sodek, J. Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. *J. Biol. Chem.,* 1990; 265: 21141- 51.
174. Ward, R. B., Atkinson, S. J., Slocombe, P., et al. Tissue inhibitor of metalloproteinases-2 inhibits the activation of 72 kDa progelatinase by fibroblast membranes. *Biochim. Bio phys. Acta,* 1991; 1079: 242-246.
175. Tuschl. T. Targeting genes expressed in mammalian cells using siRNAs. *Nature method*s. 2004; 6: 13-17.
176. Schütze, N. At the Cutting Edge siRNA technology. *Molecular and Cellular Endocrinology*. 2004; 213: 115–19.
177. Elbashir, S.M., Lendeckel, W. & Tuschl, T. RNA interference is mediated by 21 and 22 nt RNAs. *Genes Dev.* 2001; 15: 188–200.
178. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W. & Tuschl, T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J.* 2001; 20: 6877–88.
179. Holen, T. Mechanisms of RNAi: mRNA cleavage fragments may indicate stalled RISC. *Journal of RNAi and Gene Silencing.* 2005; 1(1): 21-25.
180. Elbashir S.M., Harborth J., Weber K., and Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods.* 2002; 26: 199–213.

181. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N and Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, 2003; 115: 199-208.
182. Symington, B. E., Takada, Y., and Carter, W. G. Interaction of integrins alpha-3-beta-1 and alpha-2-beta-1: Potential role in keratinocyte intercellular-adhesion. *J. Cell Biol.*1993; 120: 523–535.
183. Whittard, D.J. and Akiyama, K.S. Activation of b1 Integrins Induces Cell–Cell Adhesion. *Experimental Cell Research*. 2001; 263: 65–76.
184. Jackson, A.L. et al. Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol*. 2003; 21: 635–637.
185. Saxena, S. et al. Small RNAs with imperfect match to endogenous mRNA repress translation: implications for off-target activity of siRNA in mammalian cells. *J. Biol. Chem.* 2003; 278: 44312–44319.
186. Jackson, A.L. and Linsley, P.S. Noise amidst the silence: off-target effects of siRNAs? *TRENDS in Genetics*. 2004; 20(11): 521-524.
187. Steinberg, M. S., and Foty, R. A. Intercellular adhesions as determinants of tissue assembly and malignant invasion. *J. Cell. Physiol.* 1997; 173: 135–139.
188. Akiyama, S. K., Olden, K., and Yamada, K. M. Fibronectin and integrins in invasion and metastasis. *Cancer Metastasis Re.* 1995; 14: 173–189.
189. Sanders, R. J., Mainiero, F., and Giancotti, F. G. The role of integrins in tumorigenesis and metastasis. *Cancer Invest,* 1998; 16: 329–344.
190. Cruz, M. T., Dalgard, C. L., and Ignatius, M. J. Functional partitioning of b1 integrins revealed by activating and inhibitory mAbs. *J. Cell Sc,* 1997; 110: 2647–2659.
191. Grundstrom, G., D. F. Mosher, et al. Integrin alphavbeta3 mediates platelet-derived growth factor-BB-stimulated collagen gel contraction in cells expressing signaling deficient integrin alpha2beta1. *Exp Cell Res.* 2003; 291(2): 463-73.

192. Djonov V, Cresto N, Aebersold DM, et al. Tumor cell specific expression of MMP-2 correlates with tumor vascularisation in breast cancer. *Int J Oncol*. 2002; 21: 25–30.
193. Zhang A, Meng L, Wang Q, et al. Enhanced in vitro invasiveness of ovarian cancer cells through up-regulation of VEGF and induction of MMP-2. *Oncol Rep*. 2006; 15: 831–836.
194. Gallant, N. D., and Garcia, A. J. Model of integrin-mediated cell adhesion strengthening. J. Biomechanics. 2007; 40(6): 1301-1309.
195. Taubenberger, A., Cisneros, D.A., Friedrichs, J., et al . Revealing Early Steps of α2β1 Integrin-mediated Adhesion to Collagen Type I by Using Single-Cell Force Spectroscopy. *Molecular Biology of the Cell*. 2007; 18: 1634–1644.
196. Semizarov, D. et al. Specificity of short interfering RNA determined through gene expression signatures. *Proc. Natl. Acad. Sci. U. S. A.* 2003; 100: 6347–6352.
197. Persengiev, S.P. et al. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA*. 2004; 10: 12–18.
198. Dallas, A., and Vlassov A.V. RNAi: A novel antisense technology and its therapeutic potential. *Med Sci Monit,* 2006; 12(4): RA67-74.
199. Sledz, C.A. et al. Activation of the interferon system by shortinterfering RNAs. *Nat. Cell Biol*. 2003; 5: 834–839.
200. Pochec, E., A.Lytinska, et al Glycosylation profile of integrin α3β1 changes melanoma progression. *Biochimica Biophysica Acta.* 2003; 1643: 113-23.
201. Bellis, L. S. Variant glycoslation : an underappreciated regulatory mechanism for β1 integrin. *Biochimica Biophysica Acta.* 2004; 1663: 56-60.

RESEARCH OUTPUTS

The reveal data from this work recommend that β1 integrin is an important adhesion cell receptor involve in MMP-2 activation in response to type I collagen stimulation. Despite compelling evidence that β1 integrin plays multipotent roles in cancer progression, recently, β1 integrin was thought as a molecular therapeutic target in cancer. Thus, the advantage output of this study was as follow:

1. This study will be publishing in international journal for distribution of knowledge to better understanding of functional role of β1 integrin in cancer. The detail of this publishes paper as below.

Borrirukwanit, K., Pavasant, P., Sripairojthikoon, W., Lafleur, M. and Thompson, E. Abrogation of β1 integrin suppressed Col I-induced MMP-2 activation via up-regulation of MT1-MMP. Matrix Biology. (This paper is still approving from reviewers)

2. This work has been presented for knowledge sharing at Kanazava University, Japan in 2009. Since we received research fund from Kanazava University for oversea knowledge sharing. This research group focus on involvement of integrin and MMPs in cancer, therefore this work has been presented and discuss for advantage from this work at Kanazava University.