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Cleavage of Focal Adhesion Kinase in Vascular Smooth Muscle Cells Overexpressing Membrane-Type Matrix Metalloproteinases

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Background—Membrane-type matrix metalloproteinases (MT-MMPs) were initially identified as cell surface activators of MMP-2 (gelatinase A). We have reported that MT1-MMPs and MT3-MMPs are expressed by activated vascular smooth muscle cells (SMCs) and play a role in the regulation of cell function. Overexpression of MT-MMPs results in cell rounding, decreased adherence, and increased migration. Because integrin-mediated cell adhesion regulates these events, we have investigated the functional relationship between MT-MMPs and focal adhesion assembly.

Methods and Results—Using adenoviral vectors we show that overexpression of MT-MMPs reduces the number of focal contacts, whereas the cell surface expression of integrin subunits remains unchanged. The 125-kDa focal adhesion kinase (FAK) is cleaved resulting in a 90-kDa fragment under these conditions, and paxillin is partially dissociated from FAK after its cleavage. Pretreatment of cells with BB94, a synthetic MMP inhibitor, rescues cell adhesion and prevents changes in focal adhesions, supporting a potential role for MT-MMP enzymatic activities.

Conclusions—This study provides the first evidence that MT-MMPs are not only important in matrix degradation but also may affect the function of focal adhesions through FAK cleavage. (*Arterioscler Thromb Vasc Biol.* 2004;24:839-844.)

Key Words: MT1-MMP ■ integrin ■ focal adhesion kinase ■ cell adhesion

Abnormal growth and migration of vascular smooth muscle cells (SMCs) contribute to vascular diseases, such as atherosclerosis and injury-induced intimal hyperplasia.^{1,2} These processes require remodeling of the extracellular matrix (ECM) surrounding the SMC, which alters their interaction with the ECM. SMCs express a series of matrix metalloproteinases (MMPs),³⁻⁵ zinc-dependent neutral proteinases, which degrade surrounding ECM, such as collagens, fibronectin, laminins, and proteoglycans.⁶⁻⁸ Membrane-type MMPs (MT-MMPs), a subgroup of MMPs, have a transmembrane sequence and act at the cell surface. They were initially identified as cell surface activators of MMP-2 (gelatinase A),^{9,10} and we have reported that MT1-MMP and its closely related molecule, MT3-MMP, are preferentially expressed by activated SMCs both in vitro and in vivo.^{11,12} Recently, we found that the overexpression of MT-MMPs in SMCs induces changes in cell shape, adhesion, and migration, although the precise molecular mechanism is still unclear.¹³

kinase (FAK) is a nonreceptor cytoplasmic protein tyrosine kinase associated with the cytoplasmic domain of integrin β subunit.¹⁵⁻¹⁷ FAK is responsible for integrin-mediated signaling to downstream molecules and connecting the adhesion complex to cytoskeletal actin. FAK associates with focal adhesion components, such as paxillin and talin, through its carboxyl-terminal focal adhesion targeting (FAT) sequence. The kinase activity of FAK is activated by the ligation of integrin subunits to the ECM or by growth factors. Activated FAK autophosphorylates tyrosine³⁹⁷ just upstream of the kinase domain and transmits cell migration and growth signals.¹⁵⁻¹⁷ The phosphotyrosine residue becomes a docking site for the SH2-domain-containing Src family protein tyrosine kinases, which can, in turn, phosphorylate additional tyrosine residues of FAK.

The function of FAK is controlled not only by phosphorylation but also by cleavage. For example, degraded collagen can induce the cleavage of FAK by activating calpain-like intracellular proteinases in human SMCs.¹⁸ A similar pattern of FAK cleavage by caspases has been observed in cells undergoing apoptosis¹⁹⁻²¹ or fibroblasts transformed by the *v-src* or *myc* oncogene.^{22,23} Under these conditions, FAK is cleaved into an amino-terminal 85- to 100-kDa fragment

See cover

Cell adhesion is mediated through integrin-cytoskeletal connections localized to the focal adhesion complex.¹⁴ Among numerous focal adhesion components, focal adhesion

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containing integrin-binding and kinase domains and a carboxyl-terminal 25- to 35-kDa fragment containing the FAT sequence. Cleavage can perturb the functions of FAK and alter its association with other focal adhesion proteins.¹⁸ The regulation of focal contacts is important not only for cell adhesion but also for cell growth, migration, and survival. In this study, we report that FAK is cleaved in MT-MMP-overexpressing cells and that integrin-mediated cell adhesion is reduced, even though cell surface expression of integrin proteins is not changed. Possible roles of MT-MMPs in regulating the function of focal adhesion in SMCs will be discussed.

Methods

Materials

A hydroxamate inhibitor of MMPs, BB94 (Batimastat), was provided by British Biotechnology Ltd (Oxford, UK). Cytochalasin D and monensin sodium salt were from ICN Pharmaceuticals (Costa Mesa, Calif). Phalloidin conjugated with fluorescein isothiocyanate (FITC) and ionomycin were from Sigma (Saint Louis, Mo). Pepsin-digested type I collagen (Vitrogen) was purchased from Celtrix Co (Richmond, Va). Antihuman integrin αv (LM609) and $\alpha 5\beta 1$ (JBS5) monoclonal antibodies and an antihuman MT3-MMP polyclonal antibody were purchased from Chemicon (Temecula, Calif). Antihuman integrin $\alpha 3$ (P1B5), $\beta 1$ (P4C10), and $\alpha v\beta 5$ (P1F6) monoclonal antibody ascites were from LifeTechnologies (Rockville, Md). Anti-MT1-MMP antiserum raised against a synthetic MT1-MMP sequence in the hemopexin-like domain is a kind gift from Amgen Inc (Seattle, Wash). An antipaxillin monoclonal antibody was from Transduction Laboratories (Lexington, KY). Two different antibodies against FAK were used, a polyclonal antibody recognizing the amino-terminus (Santa Cruz Biotechnology, Santa Cruz, Calif) and a monoclonal antibody against the kinase domain (Transduction Laboratories, Lexington, Ky). Antirabbit IgG conjugated with FITC and anti-mouse IgG conjugated with tetramethylrhodamine B isothiocyanate (TRITC) were from Zymed Laboratories (San Francisco, Calif). Other chemicals were purchased from Sigma (Saint Louis, Mo). Baboon aortic smooth muscle cells were cultured as described.¹³

Adenovirus Vector Development and Infection of the Cells

Replication-defective adenovirus expressing rat MT1-MMP cDNA and human MT3-MMP cDNA (AdMT1S and AdMT3, respectively) were generated as reported.¹³ As a negative control, AdMT1AS, which expresses rat MT1-MMP cDNA in an antisense orientation or Ad β -gal, which expresses β -galactosidase cDNA, was used. All adenoviruses were prepared at the University of Washington adenovirus core laboratory.²⁴

SMCs (1×10^6) were plated on a 10-cm dish 24 hours before infection. Adenovirus was incubated with the cells at 3×10^2 multiplicity of infection for 18 to 24 hours in 10% serum containing DME medium. Cells were then switched to serum-free culture and media conditioned by the cells or total cell lysates were recovered 12 to 72 hours after the incubation.

Indirect Immunofluorescent Microscopy

SMCs were plated onto plastic chamber slides (Nalge Nunc International, Rochester NY), infected with adenoviruses, and cultured as described. Cells were fixed with ice-cold acetone or 2% paraformaldehyde for 10 minutes and incubated with PBS containing 1% BSA, 1% goat serum and 0.1% Triton X-100 for 30 minutes. Incubation with primary antibodies was overnight at 4°C, followed by secondary antibodies conjugated with FITC or TRITC, and in some cases phalloidin conjugated with FITC. Fluorescence was analyzed by laser microscopy.

Cell Adhesion Assay

Cell adhesion was evaluated as reported previously with slight modifications.¹³ Briefly, cells infected with adenoviral vectors were cultured for 72 hours in the absence of serum with or without BB94 (10 μ mol/L). Cells were suspended with trypsin, incubated in serum to neutralize the trypsin, washed twice with DMEM, and resuspended in DMEM containing 1 mg/mL BSA. Cells (1.5×10^4 cells/well) were plated in 96-well plates coated with anti-integrin antibodies or with 3 μ g/mL of monomeric type I collagen. Ascites fluids were diluted 1:500 and purified IgG was diluted to 4 μ g/mL before coating. In some experiments, cell adhesion in the presence of BB94 was also examined. After a 45-minute incubation at 37°C, cells were washed with PBS to remove unattached cells, fixed with formalin, stained with toluidine blue dye, and photometrically quantified. Results are shown as the mean \pm SD of 4 independent experiments.

Flow Cytometry

SMCs were harvested with trypsin-EDTA (GIBCO/BRL), fixed with 2% paraformaldehyde, and incubated with the primary antibody in PBS containing 0.2% BSA on ice for 2 hours. Samples were washed 3 times with PBS containing BSA and incubated with phycoerythrin-conjugated goat anti-mouse IgG for 1 hour. After 3 washes, a minimum of 5000 cells were analyzed using a FACScan flow cytometer (Becton Dickinson).

Immunoprecipitation, Immunoblotting, and Gelatin Zymography

Cells in 10-cm culture dishes were washed with PBS containing 1 mmol/L MgCl₂ and 0.2 mmol/L CaCl₂ and lysed in buffer containing 50 mmol/L Tris HCl (pH 7.4), 150 mmol/L NaCl, 15 mmol/L CHAPS, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, and proteinase inhibitors (1.5 μ mol/L pepstatin, 50 μ mol/L leupeptin, and 5 μ g/mL aprotinin). Immunoprecipitation was performed by adding primary antibody (1 to 2 μ g) and 10 μ L of a 50% slurry of agarose conjugated with protein A or protein G (Roche Diagnostics, Indianapolis, Ind) overnight at 4°C with constant rotation. After 2 washes with lysis buffer, immunoprecipitates were solubilized in 30 μ L of SDS sample buffer, separated on 10% polyacrylamide gels, and electrophoretically transferred onto nitrocellulose membranes. After incubation with primary antibodies at 4°C overnight, bands were visualized by ECL (Amersham Biosciences Corp, Piscataway, NJ).

Zymography was performed on 10% polyacrylamide gels containing 1 mg/mL of gelatin, as described previously.¹³

Statistics

Sources of significant variation were determined by using ANOVA and the SNK test. Statistical significance was set at $P < 0.01$.

Results

Reduced Organization of Actin Filaments and Focal Adhesions in MT-MMP-Overexpressing Cells

As we previously reported, overexpression of MT-MMPs induces SMC rounding, decreases adherence, and stimulates migration.¹³ More than 90% of SMCs are transduced by this adenoviral system.¹³ We tested the possibility that MT-MMP may perturb the assembly of focal adhesion complexes, thereby affecting cytoskeletal arrangement in these cells. Baboon SMCs infected with AdMT3 were cultured with or without the MMP inhibitor, BB94, for 48 hours in the absence of FCS. Cells were then fixed and stained with phalloidin conjugated with FITC. In SMCs overexpressing MT3-MMP, actin stress fibers are poorly organized and accumulate in the periphery of the cells (Figure 1A). As reported previously, the morphological changes in MT3-

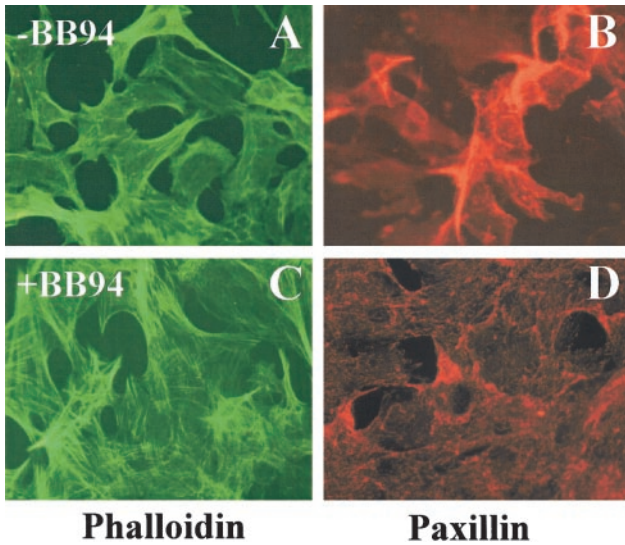


Figure 1. Fluorescent staining demonstrates altered actin filament and paxillin localization in SMCs overexpressing MT3-MMP. MT3-MMP-overexpressing SMCs were cultured without (A and B) or with (C and D) 10 μM BB94. Fixed cells were stained with phalloidin conjugated with FITC (A and C) and with anti-paxillin monoclonal antibody, followed by a secondary antibody conjugated with TRITC (B and D). Fluorescence was analyzed by laser microscopy.

MMP-overexpressing cells are blocked in the presence of BB94.¹³ In fact, when the cells are cultured with BB94 (10 μmol/L), they show highly organized actin filaments (Figure 1C), comparable to those in both normal uninfected cells and control cells infected with Adβ-gal (data not shown). When MT3-MMP-overexpressing cells are stained with anti-paxillin antibody, the staining tends to cluster (Figure 1B). In the presence of BB94, however, paxillin shows punctuate staining throughout the cell (Figure 1D). These dots represent focal adhesion sites and the pattern of uninfected and Adβ-gal-infected cells is indistinguishable (data not shown). Similar localization of actin and paxillin is observed in MT1-MMP-overexpressing SMCs (data not shown). These results suggest that the organization of focal adhesions and actin filaments are reduced in cells overexpressing MT1-MMPs and MT3-MMPs and that enzymatic activity is necessary for these changes. We are unable to localize the MT1-MMP or MT3-MMP in the MT-MMP-overexpressing cells because of the high background staining with the antibodies.

Reduced Integrin-Mediated Cell Adhesion of the MT-MMP-Overexpressing Cells

We examined the contribution of specific integrins to SMC adhesion using a modified cell adhesion assay with specific integrin antibodies on culture plates (Figure 2). Control cells infected with Adβgal or AdMT1AS adhere to the anti-α3, anti-β1, and anti-α5β1 antibody-coated plates to the same extent as to type I collagen-coated plates, but not to normal IgG coated plates. The poor adhesion of the cells to anti-αvβ3 and anti-αvβ5 antibodies is likely caused by the low expression of these integrins by these SMC (manuscript in preparation). Both MT1-MMP-overexpressing and MT3-MMP-

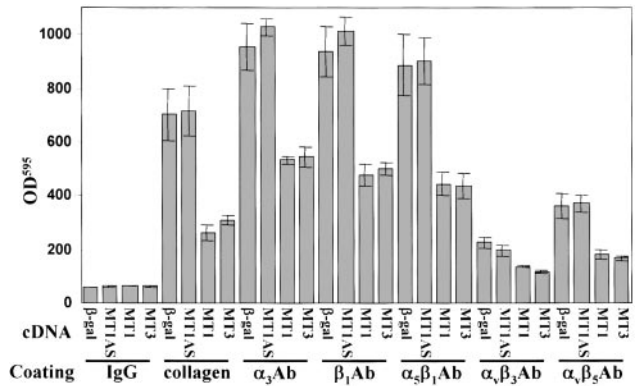


Figure 2. Integrin-mediated cell adhesion is reduced in SMCs transduced with MT-MMP cDNAs. Baboon SMCs transduced with MT1-MMP (MT1) or MT3-MMP (MT3) cDNA were plated onto the 96-well ELISA plate coated with a series of integrin function-blocking antibodies, collagen, or normal IgG. Control cells were infected with AdMT1AS (MT1AS) or Adβ-gal (β-gal). Cells were cultured with 10 μM BB94 for 3 days before but not during the assay. Adherent cells were fixed, stained, and quantified photometrically. Results are expressed as the mean±SD of 4 wells. The results are representative of 3 independent experiments.

overexpressing cells show significant reductions (40% to 60%) in adhesion to all of the antibodies tested ($P < 0.001$ versus control AdMT1AS cells), and there are no differences between MT1-MMP-overexpressing and MT3-MMP-overexpressing cells.

To investigate whether the reduced adhesion requires proteolytic activity of MT-MMPs, BB94 was used. As shown in Figure 3, when cells are incubated for 3 days with BB94 before the adhesion assay, both α3- and β1-dependent adhesion of MT-MMP-overexpressing cells is restored ($P > 0.01$ versus control). In contrast, BB94 has little effect on cell adhesion when it is added during the assay (Figure 1, available online at <http://atvb.ahajournals.org>). These results show that an extended exposure to the proteolytic activity of MT-MMPs triggers the reduction of integrin-dependent cell adhesion.

Because the observed reduction in cell adhesion may be mediated by a reduction of integrin expression on the cell surface, we performed flow cytometric analysis of integrin expression. Baboon SMCs express substantial levels of β1, α2, α3, and α5 integrin subunits (median fluorescent inten-

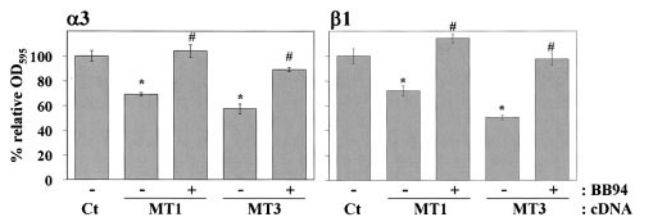


Figure 3. BB94 rescues the adhesion defect of MT-MMP-overexpressing cells. Cells infected with AdMT1 (MT1), AdMT3 (MT3), or control AdMT1AS (Ct) were cultured in the presence or absence of 10 μM BB94 for 3 days as indicated. ELISA plates (96-well) were coated with anti-α3 (α3) or anti-β1 (β1) integrin function-blocking antibodies. Cells were washed, trypsinized, and plated onto the wells to evaluate the cell adhesion. * $P < 0.001$, # $P > 0.01$.

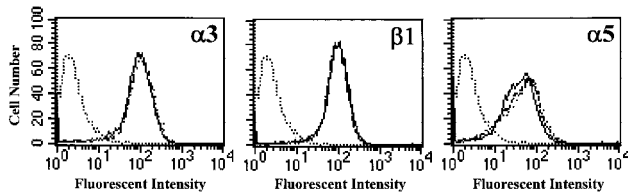


Figure 4. Cell surface expression of integrin subunits is not altered in MT-MMP-overexpressing cells. SMCs infected with AdMT1S (bold dotted lines) or AdMT3 (sparse dotted lines) and uninfected control cells (solid lines) were trypsinized, fixed, and incubated with anti- $\alpha 3$ (left), $\alpha 5$ (right), and $\beta 1$ (middle) antibodies. To analyze the cell surface expression of integrin subunits, cells were incubated with PE-conjugated goat anti-mouse antibody and analyzed by flow cytometry. In these experiments, negative control staining was performed without first antibodies (dotted lines). The results shown are representative of 3 experiments.

sity $>10^1$), but only low levels of αv and $\beta 3$ (median fluorescent intensity $<10^1$) (manuscript in preparation). As shown in Figure 4, overexpression of MT1-MMP or MT3-MMP does not affect the cell surface expression of integrin $\alpha 3$ and $\beta 1$ subunits. There is a subtle but reproducible induction of $\alpha 5$ subunit expression, which seems to be independent of the dramatic reduction in cell adhesion. We also see the stable expression of integrin subunits on the cell surface of MT-MMP-overexpressing cells by surface biotinylation and immunoblot analysis (Figure II, available online at <http://atvb.ahajournals.org>). Therefore, the observed reduction in cell adhesion in MT-MMP-overexpressing cells cannot be explained by changes in levels of integrin subunits on the cell surface.

Cleavage of FAK and Reduced Association of Paxillin with FAK in MT-MMP-Overexpressing Cells

In SMCs infected with AdMT1AS or Ad β -gal, FAK is detected as a single 125-kDa band in the presence or absence of BB94 (10 μ M; Figure 5A, upper). However, in both AdMT1S and AdMT3-infected cells, the intensity of the 125-kDa band is decreased and a 90-kDa band appears. The ratio of cleaved (90kDa) to total FAK is increased dramatically (Figure 5B; $P<0.01$ versus control AdMT1AS). BB94 reduces the level of FAK cleavage below that of control cells (Figure 5B), suggesting that the enzymatic activity of MT-MMPs is required for FAK cleavage. At least 1 μ M BB94 is required to inhibit FAK cleavage effectively (Figure III, available online at <http://atvb.ahajournals.org>). When SMCs overexpressing MT1-MMP are preincubated 3 days with BB94 and then the inhibitor is removed, the cleaved form emerges 1 to 3 hours later. The pattern is similar to that of MMP-2 activation, suggesting MT1-MMP may mediate both events (Figure IV, available online at <http://atvb.ahajournal-s.org>). Because FAK is immunoprecipitated with an antibody recognizing the amino-terminus and detected by an antibody against the kinase domain, cleavage of FAK must occur at the carboxyl terminus. For the same reasons, the smaller 35-kDa fragment of FAK cannot be detected by this method. Reprobing these blots with antibody against paxillin shows that co-precipitation of paxillin with FAK decreases in MT1-

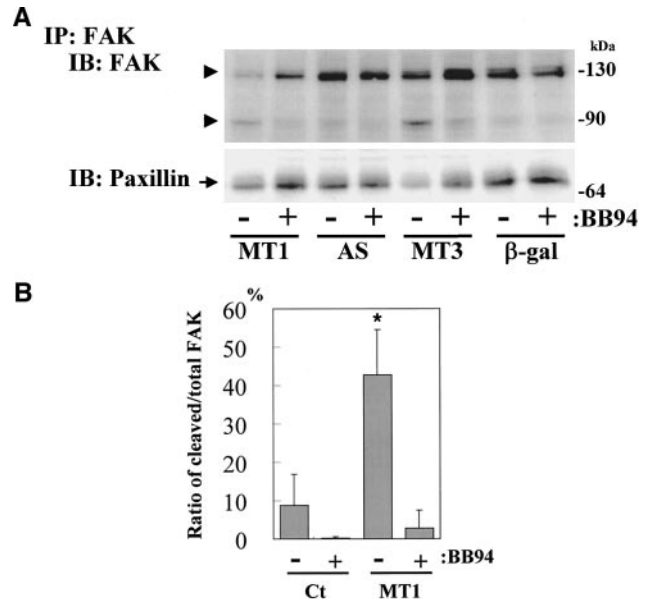


Figure 5. FAK cleavage is induced and the association of FAK with paxillin is reduced in SMCs overexpressing MT-MMPs. A, Cell lysates prepared from SMCs expressing MT1-MMP (MT1) or MT3-MMP (MT3) cultured with (\pm) or without ($-$) 10 μ M BB94 were compared with control cells infected with AdMT1AS (AS) or Ad β -gal (β -gal). FAK was immunoprecipitated from the lysates with an anti-FAK polyclonal antibody and detected by immunoblotting with the monoclonal antibody (upper panel). The same blot was reprobed with an antipaxillin monoclonal antibody (lower panel). Arrowheads, Immunoreactive bands obtained by screening for FAK (upper panel) and paxillin (lower panel), respectively. B, The ratio of cleaved (90 kDa) FAK to total FAK was determined by densitometric analysis of 6 individual experiments ($*P<0.01$).

MMP-overexpressing and MT3-MMP-overexpressing cells (Figure 5A, lower). In addition, both intact and cleaved forms of FAK are detected in an immunocomplex with anti- $\beta 1$ -integrin antibody, suggesting that the cleaved form of FAK associates with $\beta 1$ integrin (Figure V, available online at <http://atvb.ahajournals.org>). Thus, FAK cleavage might be responsible for the reduction in focal adhesions and actin filament organization.

FAK Cleavage by Endogenous MT-MMP Activity

To rule out the possibility that FAK cleavage was promoted by nonphysiological levels of MT-MMP, normal SMCs were stimulated with factors known to increase the expression or activity of MMPs. Concanavalin A²⁵ and cytochalasin D²⁶ induce MMP-2 activation, whereas ionomycin prevents MMP expression.²⁷ Phorbol ester and monensin stimulate MT-MMP expression in several cell lines.^{25,28} Both concanavalin A and cytochalasin D activate MMP-2 (Figure 6) and MT1-MMP protein expression (data not shown) in baboon SMCs. Under these conditions, FAK cleavage is simultaneously observed, and both MMP-2 activation and FAK cleavage are inhibited by BB94. In phorbol ester-stimulated cells, we observe low levels of the FAK fragment, which are not affected by BB94. Thus, induction of endogenous MT1-MMP is associated with FAK cleavage and MMP-2 activation.

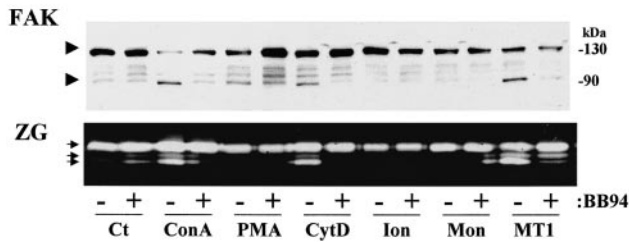


Figure 6. Induction of endogenous MT-MMPs is associated with FAK cleavage and MMP-2 activation. Normal baboon SMCs were starved for 24 hours and stimulated with concanavalin A (ConA, 100 $\mu\text{g}/\text{mL}$), phorbol ester (PMA, 100 ng/mL), cytochalasin D (CytD, 1 $\mu\text{g}/\text{mL}$), ionomycin (Ion, 0.5 mM), monensin (Mon, 10 mM), or no stimulation (Ct) in the presence (\pm) or absence ($-$) of BB94 for 24 hours. SMCs infected with AdMT1S were also analyzed (MT1). Cells lysates were prepared and FAK was immunoprecipitated and detected by immunoblotting (upper panel). MMP-2 in the lysates was analyzed by gelatin zymography (lower panel). Arrowheads, Immunoreactive FAK (upper panel). Arrows indicate location of pro-, intermediate, and active forms of MMP-2 (lower panel).

Discussion

Focal adhesion complexes regulate multiple cellular functions including adhesion, survival, apoptosis, and migration.¹⁵⁻¹⁷ Among numerous proteins composing the focal contact, FAK is especially important for connecting matrix-integrin complexes with downstream signaling molecules and actin stress fibers. In this study, we provide the first evidence of the possible regulation of focal adhesions by cell surface matrix metalloproteinase activity in SMCs. The overexpression of MT1-MMPs and MT3-MMPs reduces integrin-mediated cell adhesion without changing the cell surface expression and assembly of integrin subunits. Overexpression of MT-MMPs is associated with FAK cleavage and partial dissociation of paxillin from the integrin-FAK complex, all of which are blocked by BB94. Loss of the FAK/paxillin interaction may be a major contributor to the reduced organization of focal adhesions and actin filaments in cells overexpressing MT-MMPs (Figure 1). As a result integrin-mediated cell adhesion may be decreased.

The mechanism by which MT-MMPs mediate cleavage of FAK is not clear including whether MT-MMPs cleave FAK directly or indirectly. Some reports suggest an indirect mechanism. Carragher et al reported that degraded collagen can induce FAK cleavage and cell rounding,¹⁸ which is relevant given the ability of MT1-MMP to degrade native collagens.²⁹ In this study, we demonstrated that FAK cleavage is directly catalyzed by intracellular calpain-like enzymes and is inhibited by the membrane permeable calpain inhibitor, ALLN.¹⁸ However, in MT1-MMP-overexpressing cells, 50 $\mu\text{mol}/\text{L}$ ALLN does not affect FAK cleavage (Figure VI, available online at <http://atvb.ahajournals.org>). We were unable to test a higher concentrations because of its toxic effect on baboon SMCs. Carragher et al also suggested that α 2-integrin mediates the effect of degraded collagen, because among the integrin antibodies tested, only the function blocking antibody against α 2-integrin reduces FAK cleavage. In contrast, none of the antibodies against integrin α subunits affects FAK cleavage or the morphological changes in MT-MMP-overexpressing cells (data not shown). Caspases

are also involved in FAK cleavage when cells undergo apoptosis;^{19,21} however, ZVAD, a caspase inhibitor, does not prevent cell rounding of MT1-MMP-overexpressing cells and only partially inhibits FAK cleavage (Figures VI and VII, available online at <http://atvb.ahajournals.org>). Under our experimental conditions, the addition of 1 $\mu\text{mol}/\text{L}$ BB94 suppresses changes in focal adhesion (Figure 1) and the majority of FAK cleavage in MT1-MMP-overexpressing cells (Figure III). Importantly, this concentration is needed to inhibit the activation of MMP-2 by MT1-MMP-expressing cells,¹¹ whereas BB94 inhibits soluble MMPs, such as MMP-1, MMP-2, MMP-3, and MMP-9 at much lower concentrations.³⁰ Our results are consistent with MT-MMP-mediated cleavage of FAK.

However, MT-MMPs are transmembrane proteases with the reactive site in the extracellular space, whereas FAK is located in the cytoplasm. Therefore, it is unlikely that MT-MMPs could directly cleave FAK. However, Lee et al reported that an MT-MMP-like activity can activate MMP-2 intracellularly when fibroblasts are cultured on a type I collagen lattice.³¹ In addition, MT1-MMP is stored in an intracellular compartment of tumor cells.³² We detect substantial levels of MT1-MMP and MT3-MMP in the 1% Triton X-100 insoluble cytoskeletal fraction (data not shown). It is known that a portion of FAK is also localized in the same fraction, probably because of its tight connection to the cytoskeleton.³³ Still another possibility is that MT-MMPs might cleave and induce the activation of some other cell surface or extracellular proteins, which in turn directly induce FAK cleavage. Our current model of these possibilities is illustrated in Figure VIII (available online at <http://atvb.ahajournals.org>). MT-MMPs could activate a FAK-cleaving activity through their pericellular proteolytic activity or directly cleave FAK intracellularly. Either way, FAK cleavage would promote the dissociation of actin filaments from the focal adhesion complex where integrins mediate cell/matrix contact. Therefore, this might be the primary mechanism for changes in cell shape and adhesion when MT-MMPs are overexpressed.

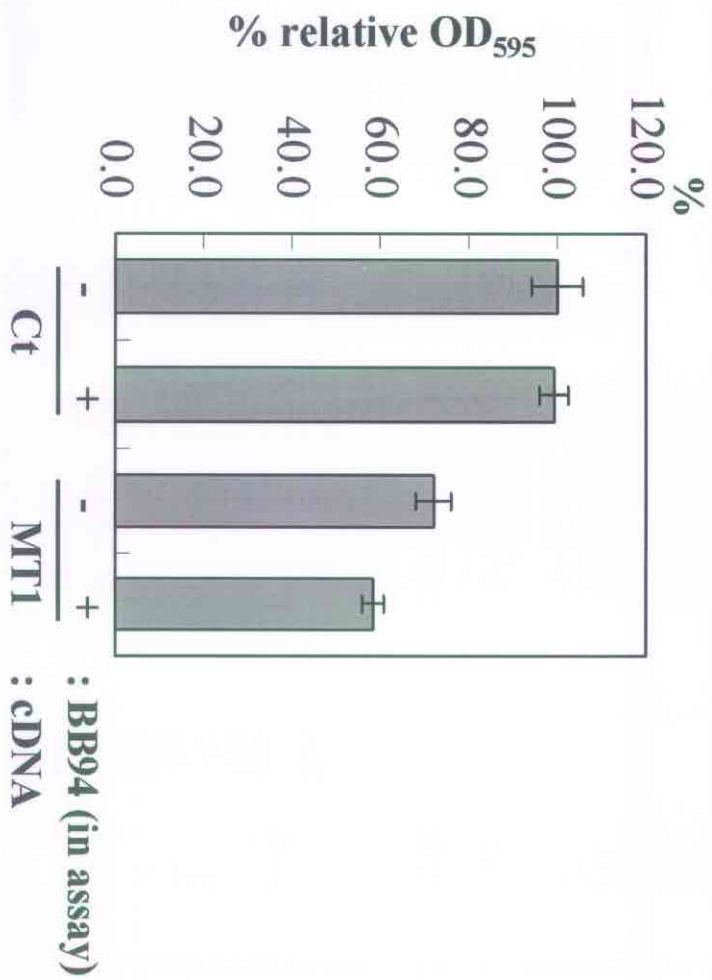
In conclusion, our results suggest that MT-MMPs play a role in modulating the function of focal adhesions. This is the first report of the contribution of MT-MMP activity to the function of focal adhesion complexes by a direct or indirect effect on FAK. Recently, novel substrates and binding proteins for MT-MMPs have been described including CD44,³⁴ transglutaminase,³⁵ claudin5,³⁶ and β 1 and β 3 integrins.³⁷ Future work will investigate whether one or more of these proteins may mediate the modulation of focal adhesions reported here.

Acknowledgments

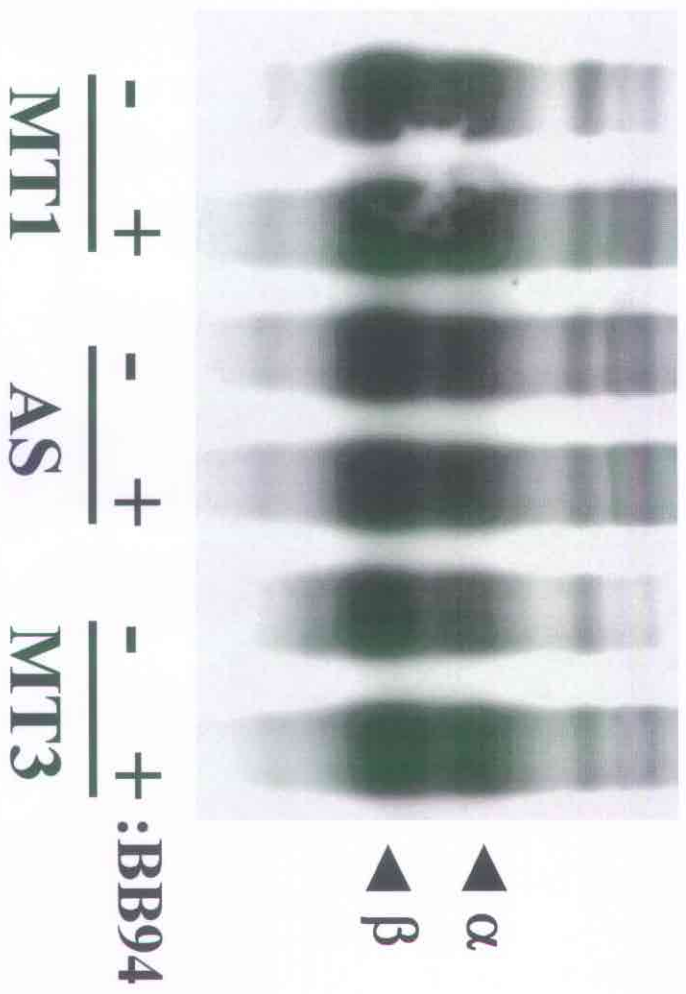
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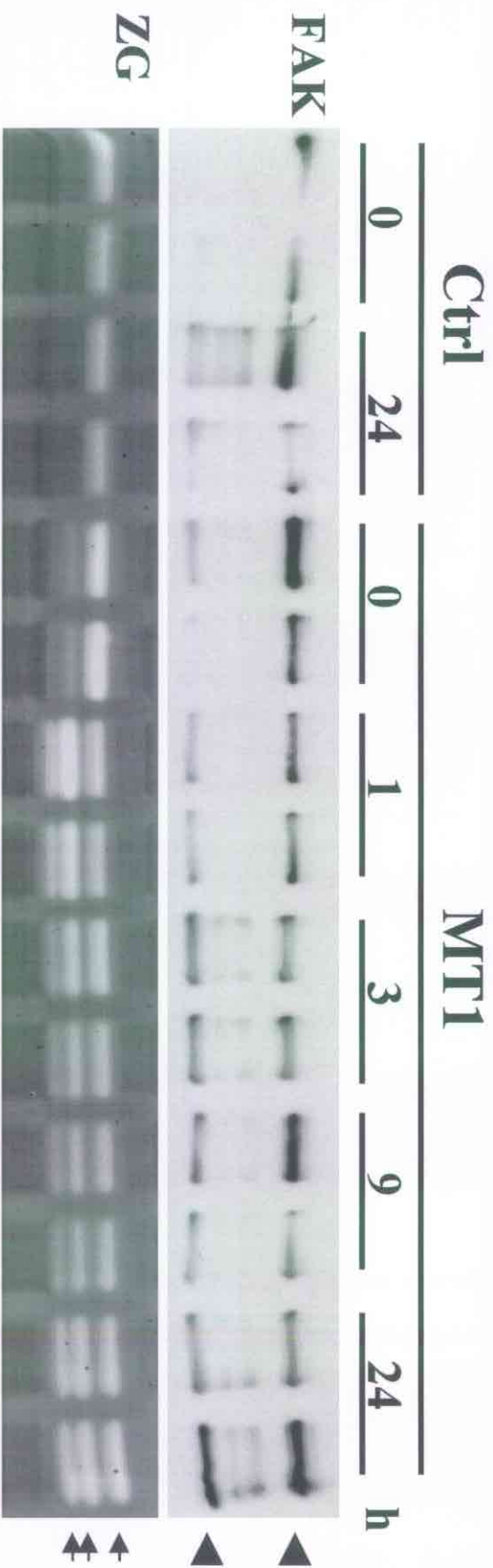
Shofuda *et al.* Suppl. Fig. 1



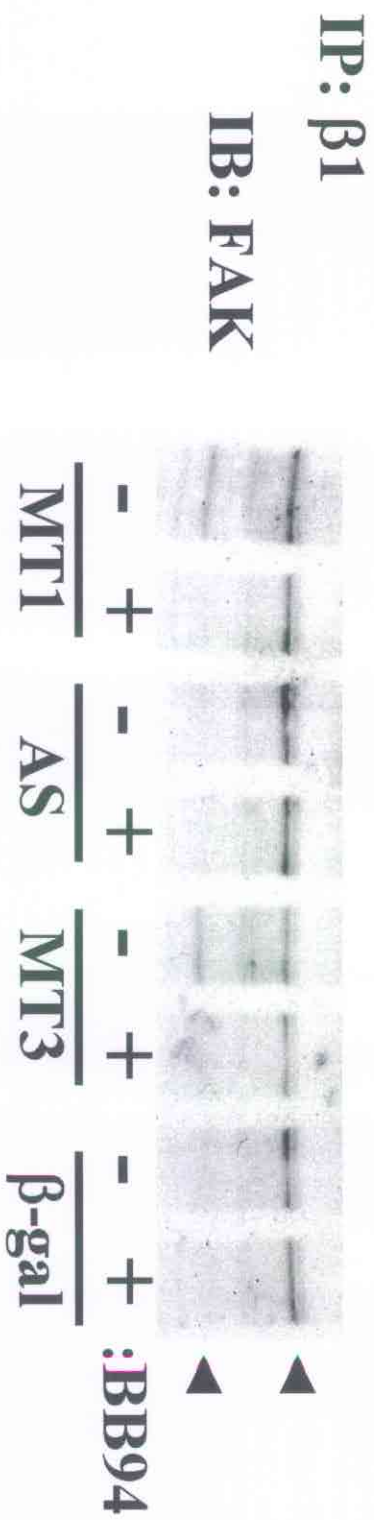
Shofuda *et al.* Suppl. Fig. 2



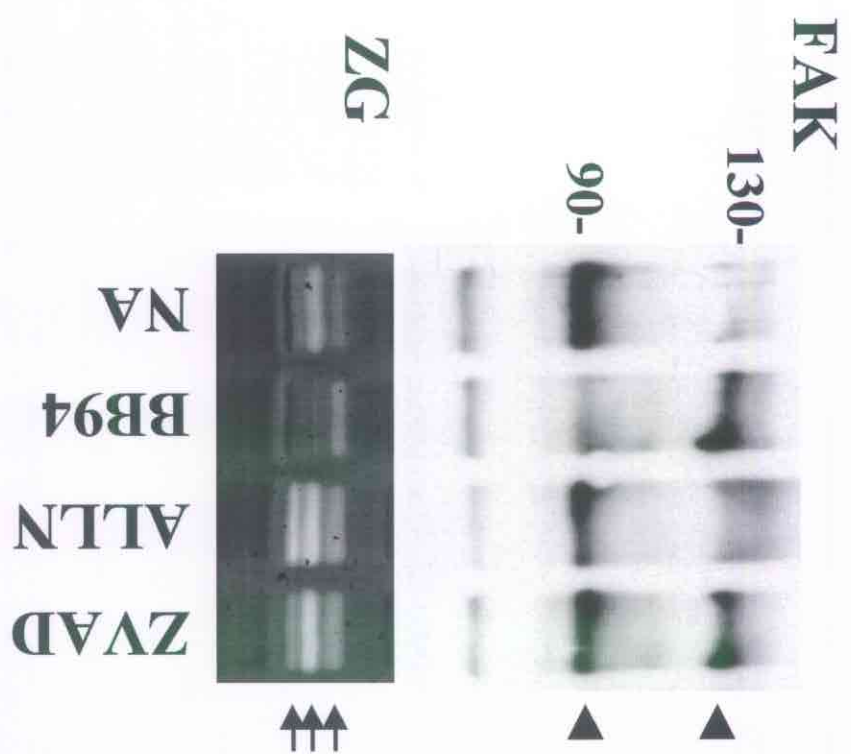
Shofuda *et al.* Suppl. Fig. 3



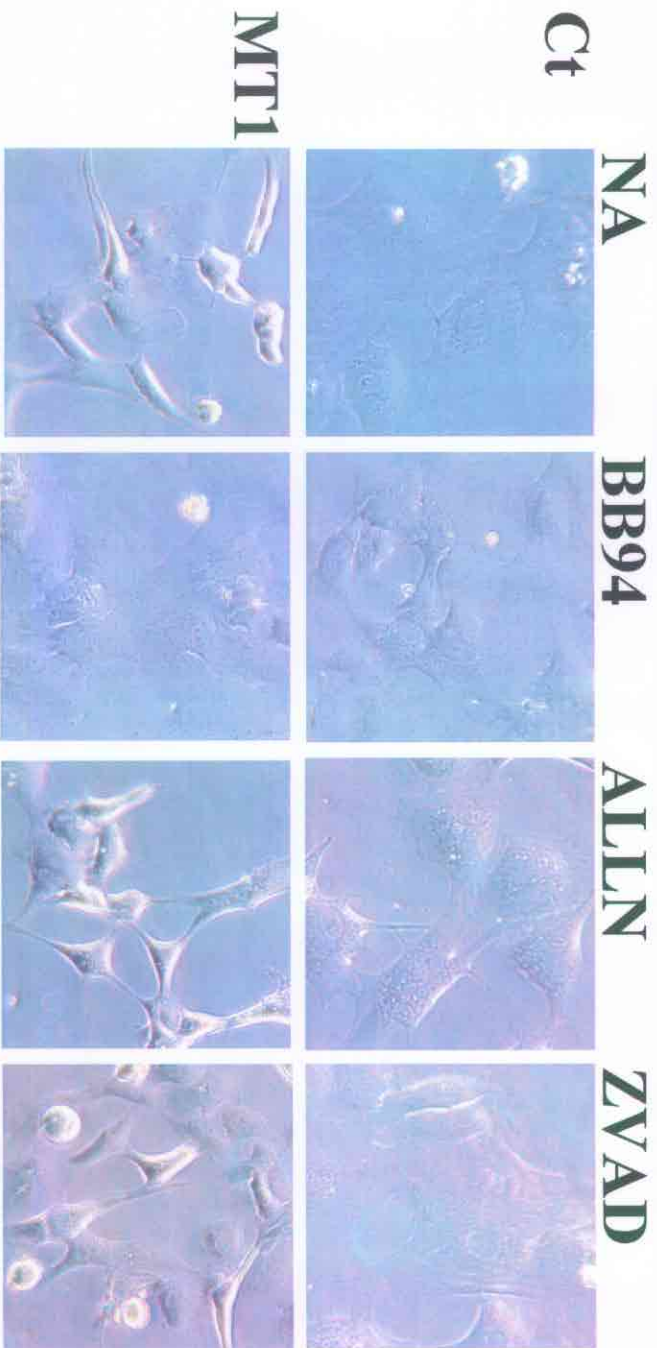
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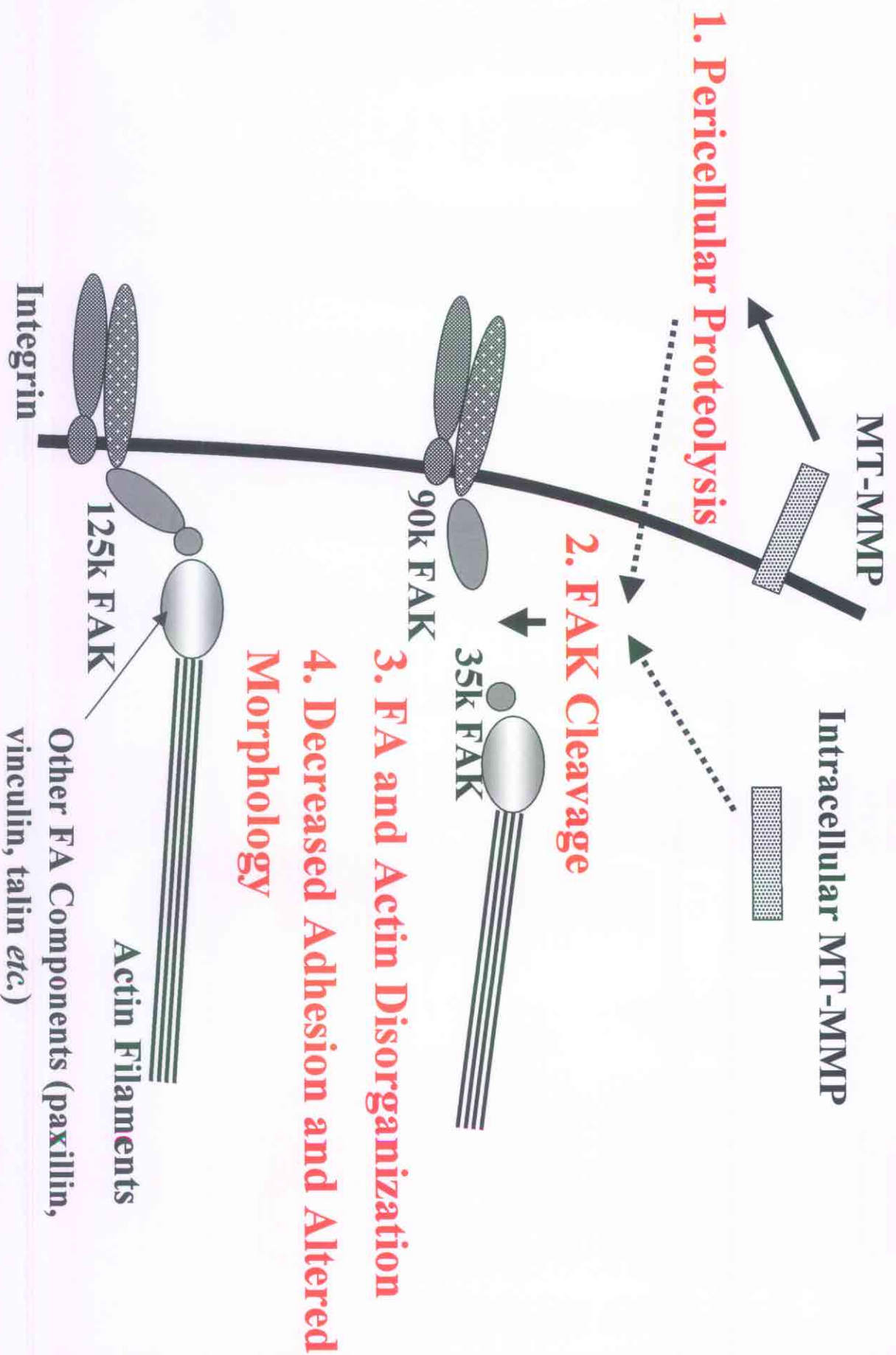
Shofuda *et al.* Suppl. Fig. 5



Shofuda *et al.* Suppl. Fig. 6



Shofuda *et al.* Suppl. Fig. 7



Shofuda *et al.* Suppl. Fig. 8