Focal adhesion and actin organization by a cross-talk of TM4SF5 with integrin α2 are regulated by serum treatment

Sung-Yul Lee[1], Tai Young Kim[2,3], Mi-Sook Lee[1], Yong-Bae Kim[3], Eunji Chung[4], Semi Kim[5], and Jung Weon Lee[1,3,*], Cancer Research Institute, Departments of [1]Molecular and Clinical Oncology, and [3]Tumor Biology, College of Medicine, Seoul National University, 28, Yeongeon-dong, Jongno-gu, Seoul 110-799, [5]Korea Research Institute of Bioscience and Biotechnology, Taejon 305-333, Korea.

[2,4]Current Addresses: [2]Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, [4]Department of Biology, Scripps College, 1030, Columbia Ave., Claremont, CA 91711, USA.

[*]To whom proofs are to be addressed;

Jung Weon Lee, Ph.D. Cancer Research Institute, Departments of Tumor Biology, and Molecular & Clinical Oncology, College of Medicine, Seoul National University, 28, Yeongeon-dong, Jongno-gu, Seoul 110-799, Korea, Tel. 82-2-3668-7030; Fax. 82-2-766-4487; E-mail: jwl@snu.ac.kr

Running title: Cooperation of TM4SF5 with integrin- and serum-mediated signals

ABSTRACTS

The biological functions of transmembrane 4 L6 family member 5 (TM4SF5) homologues to a tumor-associated antigen L6 are unknown, although it is over-expressed in certain forms of cancer. In the present study, the ectopic expression of TM4SF5 in Cos7 cells reduced integrin signaling under serum-containing conditions, but increased integrin signaling upon serum-free replating on substrates. TM4SF5 regulated actin organization and focal contact dynamics via the serum treatment-dependent differential regulation of FAK Tyr925 and paxillin Tyr118 phosphorylations and their localizations on peripheral cell boundaries. Y925F FAK mutation abolished the TM4SF5 effects. TM4SF5 associated with integrin α2 subunit, and this association was abolished by serum treatment. Furthermore, functional blocking anti-integrin α2 antibody abolished TM4SF5-enhanced signaling activity and caused membrane blebbing with abnormal actin organization. TM4SF5 increased chemotactic but decreased haptotactic migration. Altogether, this study reveals the functions of TM4SF5 collaborative with integrin signaling to alter focal contact dynamics, actin reorganization, and migration. Furthermore, this study suggests a mechanism of cross-talk between TM4SF5 and integrin which is further regulated by growth factor signaling.

Key words: TM4SF5, actin organization, focal adhesion kinase, integrin, and growth factor

INTRODUCTION

Adhesion and spreading of normal epithelial and fibroblast cells regulates diverse cellular behaviors such as proliferation, migration, invasion, and gene transcription. The regulations generally involve bi-directional signal transduction between extracellular and intracellular spaces, which is generally through integrins alone and/or collaboration with other membrane receptors. Integrins, a family of cell adhesion receptors composed of an α and a β subunit, function not only in direct signaling via integrin-mediated interaction of cells to extracellular matrix (ECM) proteins, but also in indirect signaling collaborative with other membrane receptors (e.g., growth factor receptors, G-protein coupled receptors, cytokine receptors, syndecans, or TM4SF), leading to activation of diverse intracellular signaling molecules and reorganization of actin filaments [1-7].

Transmembrane 4 superfamily (TM4SF) proteins (also referred to as tetraspanins or tetraspans) are a group of hydrophobic proteins with four transmembrane domains, two extracellular loops and two short cytoplasmic tails [8]. They are abundantly expressed transmembrane proteins of 25–50 kDa, with at least 28 distinct family members in mammals. They display numerous physiological properties important for cell adhesion, motility, and proliferation [9, 10]. TM4SFs are well-known to form massive cell-surface complexes (i.e., tetraspanin-web structures) with cell adhesion molecules to collaboratively perform their biological functions [7, 11-17]. Transmembrane 4 L6 family member 5 (tetraspan transmembrane protein L6H or TM4SF5) was originally cloned as a protein of 190 amino acids as a member of TM4SF proteins and assigned to human chromosome 17p13.3 [18]. It is homologous to a tumor-associated antigen L6 (TM4SF1) and thus it forms a four-transmembranes L6 superfamily with L6, IL-TMP, and L6D that share just the membrane

topology but not other features found in other genuine TM4SF proteins [19]. TM4SF5 is highly expressed in pancreatic carcinoma [18], gastric carcinoma (Semi Kim, unpublished data), colon and hepatocellular carcinoma cell lines (S-Y Lee and JW Lee, unpublished data), and ACTH(corticotropin)-negative bronchial carcinoid tumors [20], and its mouse homologue is highly expressed in brain [21, 22]. However, its function in cancer development, brain maintenance, or any cell biological aspects has not been explored yet.

It is well-known that integrins regulate cellular migration and invasion [23]. Focal adhesion kinase (FAK) is activated by integrin-mediated adhesion and important for integrin-mediated cell migration and invasion [24, 25]. Upon cell adhesion, autophosphorylation of Tyr397 of FAK occurs and thereafter Tyr925 phosphorylation by c-Src bound to pY397 leads to recruitment of adaptor proteins (Grb2 and/or Shc) for activation of the Erk1/2 cascade [26]. The roles of integrins in cell migration are also influenced by growth factor signaling [2, 27], and/or by their interactions with TM4SF [14]. However, integrin cooperation with any of the four-transmembrane L6 family members is not known. Cell migration involves reorganization of cytoskeletal network including actin filaments, as cells migrate by protrusions in the forward end and retraction in the rear end [28-30].

In this study, we explored the function of TM4SF5 with regards to regulation of integrin-mediated signaling activities, actin organization, and migration by virtues of cooperation with integrins that is further regulated by growth factor signaling.

MATERIALS AND METHODS

Cells: Cos7 cells (ATCC) were cultured in DMEM-H with 10% (v/v) FBS at 37℃ and 5% CO_2.

Stable Cos7 cells were selected by G418 (400 μg/ml) after infection with retroviruses encoding for pLNCX or TM4SF5 cDNA (pLNCX-TM4SF5), which were prepared by separate transfection of PT67 packaging cells with the retroviral vector pLNCX with or without TM4SF5 cDNA insert. *TM4SF5 Cloning*: Human TM4SF5 (NM-003963) from a cDNA pool of hepatocyte Huh7 cells was amplified and cloned into diverse mammalian expression vectors by using PCR methods. Forward primer was 5'-ATG TGT ACG GGA AAA TGT GCC CGC T-3' and reward primer was 5'-AGT GAG GTG TGT CCT GTT TTT TC-3'. The cDNA was inserted into *XhoI*/*EcoRI* cloning sites of pEGFP-N1 (pEGFP-TM4SF5), *XhoI/EcoRI* sites of myc-$(His)_6$-pcDNA3.1 [myc-$(His)_6$-TM4SF5], or *HindIII/EcoRI* sites of pcDNA3 (TM4SF5). The plasmid-based siRNA construct against TM4SF5 (shTM4SF5) targets its sequence of 5'-^{123}CCA TCT CAG CTT GCA AGT C^{141}-3' and a control shRNA targets its scrambled sequence.

Y925F FAK mutation: pRC/CMV-$(HA)_3$-FAK WT (a kind gift of Dr. Jun-Lin Guan, Cornell University, Ithaca, NY, USA) was point-mutated at Tyr925 by using Quick-change site-directed mutagenesis kit (Stratagene). The mutated sequence was confirmed by direct sequence analysis.

Cell replating: Plasmids were transfected into cells using Lipofectamine 2000™ reagent (Invitrogen), according to the manufacture's protocols. Two days after the transfections, cells were harvested or processed for replating. Cells were kept in suspension (with rocking-over at 80 rpm) for 1 h and then were either kept in suspension or replated on ECM-precoated dishes [10 μg/ml collagen I (Chemicon), or 0.56 mg/ml matrigel for a thin coating (BD Biosciences)] or tissue culture dishes for 1 h at 37°C, as described previously [6]. In certain cases, cells were pretreated with 25 μM ML9 (Calbiochem) or premixed with 7.5 μg/ml anti-integrins α2 (P1E6),

α3 (P1B5), or α5 (P1D6) antibody (Chemicon), 30 min before the replating. Treatment of 10% FBS or 50 ng/ml PDGF-AB was done by adding them directly to the replating media (DMEM-H plus 1% BSA), 5 min before ending of the 1 h replating period.

Cell lysate preparation and Western blots: Whole cell lysates from control Cos7 cells or TM4SF5-expressing cells (at 70~80% confluence) in the normal culture media or cells kept in suspension or replated on ECM substrates were prepared by using a modified RIPA lysis buffer, as described previously [6]. The lysates were quantitated by BCA (Pierce) methods, normalized, and used in standard Western blots using antibodies against phospho-Y^{397}FAK, phospho-Y^{416}Src, RhoA, myc (9E10) (Santa Cruz Biotech.), integrin α2, α3, α4 (Chemicon), integrin α5, FAK, paxillin, α-tubulin, (BD Transduction Lab.), phospho-Y^{118}paxillin, phospho-Y^{925}FAK, phospho-$T^{508/505}$LIMK1/2, LIMK1, phospho-S^{3}cofilin, cofilin, phospho-S^{19}MLC, phospho-Erk1/2, and Erk1/2 (Cell Signaling Tech.).

Immunofluorescence microscopy: Parental or stable Cos7 cells were (co)transfected with diverse plasmids as indicated. Two days later, the transfected cells were replated on 10 μg/ml collagen I-precoated coverslips with or without ML9 pretreatment or 10% FBS treatment, as described above. After incubation for 1 or 2 h, cells were fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature, and washed three times with PBS. Washed cells were then incubated with the primary antibody for 1 h in a humidified chamber at 37°C and washed with PBS (3 times x 10 min). Cells were then incubated with secondary antibody-conjugated with FITC or TRITC (Chemicon) in a dark and humidified chamber for 1 h at 37°C. In some cases, cells on coverslips were stained with rhodamine-conjugated phalloidin (Molecular Probes) for 1 h at 37°C. Then the coverslips were processed to PBS washings (3 times x 10 min) and mounted with a mounting solution

(DakoCytomation, Germany). Mounted samples were visualized by a fluorescent microscopy (Bio-Rad MRC-500). Quantitative analyses were performed from three independent experiments where at least 10 separate immunofluorescent images with a few transfected and untransfected cells for each condition were recorded. Microscopic fields were scored to compare numbers and intensities of focal contacts/adhesions (FAs), levels of actin structures (i.e., lamellipodia, stress fiber, and membrane ruffle), or localization of pY^{925}FAK or pY^{118}paxillin in the TM4SF5-postive versus –negative cells before graphic presentations.

Immunoprecipitation: After the replating and FBS treatment, cells were twice washed with cold PBS and immediately lysed in an immunoprecipitation buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF, 1% Triton X-100 and 0.5% NP-40) on ice. The lysates of 500 μg proteins per condition were mixed with anti-Myc (Clone 9E10), anti-integrin α2 (P1E6), or anti-HA antibody (0.5 μg/condition) before rotation for 2 h at 4°C. Protein A/G sepharose beads (30 μl of 50% PBS slurry/condition, Upstate) were added to the mixtures before an additional rotation for 2 h at 4°C. Immunoprecipitates were collected, washed, and eluted prior to standard Western blots for the indicated molecules.

RhoA activity assay: RhoA activity was measured, as described previously [31]. Lysates of 1.0 mg total protein per condition and 30 μg of GST-RBD [GST-fused Rhotekin (amino acids 7–89), a kind gift from Dr. Keith Burridge, University of North Carolina, Chapel Hill, NC] bound to glutathione-sepharose beads (Amersham Pharmacia Biotech, Sweden) were used in the assay.

Wound healing assay: Stable Cos7 cells in 60 mm culture dishes were wounded by scraping through the cell monolayer with a yellow pipette tip. Cells were then washed twice with DMEM-H and incubated at 37°C and 5% CO_2 under DMEM-H with or without 10% FBS. After 12 h incubation, several images around wounds in each condition were taken.

Transwell migration assay: Transwell chambers (pore size of 8 μm) were precoated with 10 μg/ml collagen I. Stable Cos7 cells were replated into the transwell chamber in duplicates. The lower chamber was filled with DMEM-H with or without 10% FBS. After incubation for 12 h, cells inside of the upper chamber were removed. Cells beneath the membrane filter were fixed with 3.7% formaldehyde in PBS and stained with Diff-Quik® (Sysmex Cooperation, Japan) following the manufacture's protocols, and five images for each condition were taken and cells in each image were counted visually and mean ± standard deviation values were calculated for the graphic presentation.

RESULTS

While studying the effects of cell adhesion on gene expression, our oligonucleotide array experiments showed increased mRNA and protein levels of TM4SF5 as gastric carcinoma cells adhered on extracellular matrix (data not shown), indicating that it might be involved in cell adhesion processes. We cloned TM4SF5 cDNA from a cDNA pool of Huh7 hepatocarcinoma cells using a PCR approach, and its sequence was confirmed directly by sequence analysis (data not shown). We performed experiments to study its signaling pathways and biological functions, since functions of TM4SF5 are unknown.

Down-regulation of integrin-related signaling by TM4SF5 in cells under a serum-containing condition

Since Cos7 fibroblasts showed no endogenous TM4SF5 (data not shown), we transiently transfected GFP alone or GFP-tagged TM4SF5 cDNA into Cos7 cells prior to analysis of TM4SF5-mediated effects on cell adhesion-related intracellular signaling pathways. The GFP-TM4SF5 expression level was comparable to GFP alone and showed intermolecular multimerization by immunoblottings under non-reducing conditions (Figure 1A, right), as shown for other TM4SF members [32]. Among intracellular signaling molecules, integrin-related signaling molecules such as FAK and paxillin were affected by TM4SF5 expression. FAK Tyr925, interestingly but not Tyr397, and paxillin Tyr118 phosphorylations were reduced by TM4SF5 expression (Figure 1A). Although FAK Tyr925 phosphorylation (pY^{925}FAK) was reduced, the Erk1/2 phosphorylation (i.e., activity) was unchanged, indicating that TM4SF5-mediated pY^{925}FAK has an unusual significance in this system, rather than leading to Erk1/2 activation (Figure 1A, left). Since integrin signaling regulates focal adhesion (FA) formation and actin filament reorganization, we next examined the effects of TM4SF5 expression on FA formation. FAs visualized by anti-FAK immunostaining were also reduced in the TM4SF5-

ectopically expressing cells, as compared with neighboring untransfected cells (Figure 1B). We compared cells positive versus negative for TM4SF5 transfection in terms of FA number and intensity. On average, we observed that 79% (±18.9, standard deviation) of GFP-TM4SF5-transfected cells showed fewer FAs than neighboring untransfected cells. In addition, the effect of TM4SF5 expression on actin filament organization was also examined. Typically cells expressing (GFP-tagged) TM4SF5 showed actin structures with fewer stress fibers and actin-enriched lamellipodia and membrane ruffles (Figure 1C). We quantitatively compared levels of actin structures (i.e., lamellipodia, stress fibers, and/or membrane ruffles) in cells positive and negative for TM4SF5 transfection. On average, we observed that 75% (±8.3, standard deviation) of GFP-TM4SF5-transfected cells showed fewer actin structures, compared with neighboring untransfected cells. However, the cells transfected with pEGFP alone did not show such significant changes in FA formation and actin structure, and cotransfection of TM4SF5 expression plasmid (pcDNA3-TM4SF5) with pcDNA3-GFP vector resulted also in less significant actin structures only in TM4SF5-expressing cells (data not shown). Furthermore, this less significant actin structure (especially stress fiber formation) in pEGFP-TM4SF5-transfected cells was recovered by cotransfecting plasmid-based shRNA against TM4SF5 (Figure 1D). Plasmid-based shRNA against TM4SF5 successfully suppressed the expression of GFP_B-TM4SF5 at the monomer and multimer forms and reversed TM4SF5-mediated decreases in pY^{925}FAK and pY^{118}paxillin levels under normal serum-containing condition (Figure 1E). These observations indicate that TM4SF5 expression in Cos7 cells under serum-containing conditions down-regulates integrin-related signaling activity (especially pY^{925}FAK), FA formation, and actin filament organization.

Positive regulation of integrin-mediated signaling by TM4SF5 in cells replated on collagen I

in serum-free condition

In general, integrin signaling activity is analyzed in cells replated on ECM substrates in the absence of serum components [33], since such conditions allow cells to trigger only the integrin/ECM-mediated signaling activity. Therefore, we next performed experiments to determine the effects of TM4SF5 expression in cells kept in suspension or replated on ECMs without serum. Cos7 cells were transfected with either pEGFP-TM4SF5, myc-$(His)_6$-TM4SF5, or pcDNA3-TM4SF5 and kept in suspension or replated on tissue culture dishes for 1 h, and examined for integrin-related signaling molecules. TM4SF5-expressing cells suspended or replated onto tissue culture dishes did not significantly change the activities of FAK and paxillin, compared to control 'vector only'-transfected cells (Figure 2A, left). However, replating cells on matrigel-precoated dishes showed increased pY^{925}FAK (but not pY^{397}FAK), pY^{118}paxillin, and pS^{3}cofilin levels in cells transfected with different TM4SF5 constructs, as compared with cells transfected with a control vector only (Figure 2A, right). Next, we examined the effects of replating onto other ECMs, such as, fibronectin, collagen I, and laminin I. TM4SF5 expression caused more obvious increases in signaling activity in cells replated on collagen I, among these ECMs (Figure 2B, and data not shown). Replating of TM4SF5-expressing cells on collagen I under serum-free conditions increased pY^{925}FAK (but not pY^{397}), pY^{118}Paxillin, pY^{416}c-Src, $pT^{508/505}$LIMK1/2, and pS^{3}Cofilin (Figure 2B). These were opposite to the effects of TM4SF5 under serum-containing conditions. In addition, TM4SF5 expression resulted in activation of RhoA and increased phosphorylation of myosin light chain (MLC) (Figure 2B, right), which are known to be involved in actin reorganization. When FA formation and actin structure were analyzed in cells replated on collagen I, TM4SF5-expressing cells showed more significant FAs visualized by integrin β1 immunostaining (Figure 2C, upper panels) and actin structures by virtue

of actin-enriched lamellipodia and ruffles and stress fibers (Figure 2D, upper panels). These observations indicate that under serum-free conditions TM4SF5 positively regulates integrin-mediated signaling activities leading to actin filament organization, unlike under serum-containing conditions. Next, we examined if the pharmacological inhibition of actin polymerization-related intracellular signaling molecules affects TM4SF5-mediated signaling activity, FA formation, and actin organization. We found that treatment with ML9 [a specific and potent inhibitor of myosin light chain kinase (MLCK)] abolished TM4SF5-mediated signaling activities (Figure 2E). ML9 treatment might cause a reduced cofilin phosphorylation presumably through influencing ROCK and/or cofilin phosphatases activities without significantly affecting LIMK1/2 phosphorylation [34-36]. Furthermore, ML9 treatment blocked TM4SF5-enhanced FA formation (Figure 2C, lower panels) and actin structure (Figure 2D, lower panels). These observations indicate that cross-talk between TM4SF5- and integrin-signaling pathways is linked to actin reorganization.

The TM4SF5 effects in the replated cells depend on serum components treatment

We observed that TM4SF5-expression in cells under serum-containing conditions reduced integrin-related signaling (Figure 1). Therefore, we recapitulated the serum effects even in cells newly replated on collagen I in the absence of serum, via a direct addition of serum components 5 min before ending the 1 h replating incubation. Interestingly, treatment of PDGF-AB or serum (i.e., FBS at a 10% final concentration) abolished TM4SF5-mediated increases in pY^{925}FAK and pY^{118}paxillin (Figure 3A). As expected, their treatments increased the phosphorylation of Erk1/2 (Figure 3A), indicating that mitogenic signal stimulation by serum components had occurred. However, EGF treatment (50 ng/ml) did not alter TM4SF5-increased phosphorylations, indicating a specificity of serum components to regulate the effects of TM4SF5 (data not shown). Consistent with the effects of FBS on pY^{925}FAK and pY^{118}paxillin levels,

serum treatment abolished TM4SF5-enhanced FA formation (Figure 3B, upper panels) and actin reorganization (Figure 3B, lower panels), resulting in no significant difference between TM4SF5-null and TM4SF5-expressing cells. Although it was observed that cells in normal serum (10% FBS)-containing medium showed TM4SF5-mediated decreases in integrin signal activities (Figure 1), serum treatment (i.e., treatment with FBS at a final concentration of 10% for 5 min) to cells replated on collagen I resulted in no increases in TM4SF5-mediated activities upon serum-free replating, indicating that serum treatment might not completely mimic the normal serum containing culture condition. Quantitatively, about 67% (±5.92, standard deviation) of GFP and TM4SF5 positively cotransfected cells up-regulated actin structures and 33% (±3.05) showed no significant changes when they were replated on collagen I (Figure 3C, left bars). However, 50% (±6.95, standard deviation) cells of GFP and TM4SF5 positively cotransfected cells showed a down-regulation, 42% (±4.23) showed no significant change, and about 8% (±3.76) showed a slight up-regulation in actin structures when cells replated on collagen I were serum-treated (Figure 3C, right bars). Even after a longer incubation after replating (e.g., 2 h), TM4SF5-mediated effects were similar (data not shown), indicating that the effects appeared not to be dependent on different cell spreading stages after replating. Therefore, TM4SF5-enhanced actin structures and FAs upon serum-free replating were almost abolished by serum treatment.

TM4SF5 affects also localization of pY^{925}FAK and pY^{118}paxillin

Next we analyzed phosphorylation of the integrin signaling molecules that were regulated by TM4SF5 expression, by indirect immunofluorescent microscopy. Interestingly, we found that TM4SF5-transfected cells showed more peripheral localization of pY^{925}FAK than neighboring untransfected cells (Figure 4A). Therefore, we analyzed if the cellular localizations

of pY^{925}FAK and pY^{118}paxillin were altered by TM4SF5 expression in a serum treatment-dependent manner using pLNCX or pLNCX-TM4SF5 stable cells. Control TM4SF5-null pLNCX Cos7 cells showed pY^{925}FAK located internally from the cell boundaries after being replated on collagen I, but TM4SF5-expressing pLNCX-TM4SF5 stable cells under the same condition clearly showed pY^{925}FAK at peripheral cell boundaries (Figure 4B, upper). This change in localization accompanied changes in the sizes and numbers of pY^{925}FAK-postive spots; the spots positive for pY^{925}FAK in pLNCX cells were longer, fewer in number, and radially and outwardly directed, whereas they were shorter, more numerous, and more located at boundaries, presumably indicating the localization of more pY^{925}FAK at focal contacts around the cell boundaries of pLNCX-TM4SF5 cells. Changes in the localizations of pY^{925}FAK were blocked not only by ML9 but also by serum treatment (Figure 4B). To avoid artifacts due to the cell-spreading stage-dependent effects on their localizations, we tried to confirm TM4SF5-mediated effects at different incubation periods after replating (e.g., 2 h). Even after 2 h incubation, TM4SF5-mediated effects were similar (data not shown). Quantitatively, 79.2% (±5.03, standard deviation) of GFP and TM4SF5 positively-cotransfected cells located pY^{925}FAK on peripheral plasma membranes, whereas 16.6% (±7.37) and 4.2% (±4.89) of cells located pY^{925}FAK at both periphery/internal areas and mostly at internal areas, respectively, upon serum-free replating on collagen I (Figure 4D, upper, left bars). However, an increased trend toward internal localization of pY^{925}FAK was observed by serum treatment; more cells showed pY^{925}FAK located internally (35.1%±4.97) or both peripherally and internally (51.4%±5.10) rather than on peripheral boundaries (13.5%±3.02) (Figure 4D, upper, right bars). Therefore, the TM4SF5-mediated peripheral localization of pY^{925}FAK after serum-free replating decreased and simultaneously its internal localization increased by serum treatment.

The localization of pY^{118}paxillin was also regulated by TM4SF5, but in the manner opposite to that of pY^{925}FAK. pY^{118}paxillin in control pLNCX cells was located usually at the peripheries, whereas in TM4SF5-expressing cells it was located internally from boundaries after serum-free replating on collagen I (Figure 4C, upper panels). However, serum treatment appeared to abolish the TM4SF5-mediated translocation of pY^{118}paxillin, since a certain population of pY^{118}paxillin in TM4SF5-expressing cells remained at cell peripheries after serum treatment (Figure 4C, lower panels). Quantitatively, 73.9% (±4.96, standard deviation) of GFP and TM4SF5 positively-cotransfected cells showed pY^{118}paxillin localized internally, whereas 8.7% (±6.73) and 17.4% (±6.92) of cells located pY^{118}paxillin either to both peripheral and internal areas or to peripheral boundaries, respectively, after being serum-freely replated on collagen I (Figure 4D, lower, left bars). However, TM4SF5-transfected cells showed still significant pY^{118}paxillin localization on peripheral boundaries even after serum treatment; more cells showed pY^{118}paxillin at peripheral boundaries and at both peripheral and internal areas (34.8%±3.89 and 47.8%±6.94, respectively) rather than at internal areas (17.4%±3.82) (Figure 4D, lower, right bars). Therefore, TM4SF5 expression was found to affect the cellular localizations of pY^{925}FAK and pY^{118}paxillin, depending on serum treatment.

pY^{925}FAK appears to be important for the effects of TM4SF5

Since TM4SF5 appeared to regulate pY^{925}FAK and its localization, FA formation and actin organization, we next examined the significance of pY^{925}FAK on the TM4SF5 effects. We mutated the Tyr925 of FAK to prevent its phosphorylation by changing it to Phe925. Cell lysates were prepared 2 days after transiently transfecting Cos7 cells with either $(HA)_3$-FAK WT or $(HA)_3$-Y925F FAK, and immunoprecipitated with anti-HA antibody prior to immunoblotting. As shown in Figure 5A, Y925F FAK transfection caused no Tyr925 phosphorylation, as was

expected. To determine the significance of pY^{925}FAK in TM4SF5-mediated actin organization, pLNCX-TM4SF5 Cos7 cells were transfected with WT or Y925F mutant FAK plasmid 2 days before replating on collagen I-precoated dishes or cover glasses. Y925F FAK-transfected cells showed less significant TM4SF5-enhanced actin structures in terms of stress fibers and lamellipodia, compared to neighboring cells with endogenous FAK in which Tyr925 would be phosphorylated on serum-free replating (Figure 5B). Furthermore, the TM4SF5-enhanced phosphorylation of MLC was abolished in Y925F FAK-expressing cells, compared with in neighboring cells presumably with endogenous pY^{925}FAK (Figures 5C and D). However, Y925F FAK did not cause any changes in actin structure or in the p-MLC level of TM4SF5-null pLNCX cells (data not shown). Furthermore, pY^{925}FAK located randomly and irrelevantly with respect to GFP-β-actin in pLNCX cells transfected with GFP-tagged β-actin, whereas it located on lamellipodia around cell boundaries and mostly overlapped with GFP-β-actin in pLNCX-TM4SF5 cells (Figure 5E), suggesting a functional relevance between pY^{925}FAK and actin at the peripheries of TM4SF5-expressing cells after serum-free replating. These observations indicate that pY^{925}FAK appears to be important for TM4SF5-mediated actin organization.

TM4SF5 collaborates with integrin α2

Generally TM4SF proteins are cooperative with integrins for cellular functions via direct associations [7]. Therefore, we next investigated which integrin subunits are involved in the effects of TM4SF5 by using a co-immunoprecipitation approach. Cell lysates from Cos7 cells transfected separately with either myc-$(His)_6$-TM4SF5 or its control myc-$(His)_6$-pcDNA3 plasmid were immunoprecipitated with an anti-myc or anti-integrin α2 antibody. When anti-myc immunoprecipitates were immunoblotted with antibodies against integrin subunits, integrin α2 was shown to be co-precipitated with myc-$(His)_6$-TM4SF5 (Figure 6A, left and upper).

Moreover, this association between TM4SF5 and integrin α2 was abolished by serum treatment (Figure 6A left and upper, lanes of 3 and 4). However, integrins α3, α4, and α5 were not co-precipitated with TM4SF5 (Figure 6A, left and lower, and data not shown). In a reverse approach, integrin α2 co-precipitated myc-$(His)_6$-TM4SF5 and their association was lost after serum treatment (Figure 6A, right). Next, we performed experiments to determine how the preincubation of TM4SF5-expressing cells with functional blocking anti-integrin α2 antibody affected the TM4SF5 effects. TM4SF5-enhanced phosphorylations of FAK Tyr925 and paxillin Tyr118 upon serum-free replating were abolished by preincubating cells with integrin α2 antibody, but not with α3 or α5 antibody (Figure 6B and data not shown). However, Erk1/2 activity, which appeared unrelated to TM4SF5 expression, was not affected by integrin α2 blocking (Figure 6B, left). Furthermore, when TM4SF5 cells premixed with functional blocking antibody against integrin α2 were replated, they showed severe membrane blebbing through abnormal actin reorganization, whereas anti-α3 or α5 antibodies did not cause significant changes in actin organization or membrane integrity (Figure 6C). These observations suggest that TM4SF5-mediated phosphorylation and actin organization may involve cooperation between TM4SF5 and integrin α2.

TM4SF5-mediated regulation of cell migration also depends on serum treatment

Next we examined whether TM4SF5 plays a function in the regulation of cell migration, since other TM4SF proteins are involved in cell migration [7, 10, 37]. First, we performed wound healing analysis using pLNCX or pLNCX-TM4SF5 Cos7 cells under normal serum-containing or serum-free conditions. Interestingly, wound healing was increased by TM4SF5 expression under normal serum-containing conditions, whereas no significant wound healing occurred under serum-free conditions (Figure 7A). However, this enhanced wound healing did

not result from serum-induced changes in cell proliferation, since cell numbers and Erk1/2 activities were not significantly different between pLNCX and pLNCX-TM4SF5 cells under serum-containing conditions (data not shown and Figure 1A). Next, we confirmed our observations by Transwell migration assays, where the bottom side of membrane filter of a transwell chamber was precoated with collagen I. When 10% FBS-containing culture media was in the bottom chamber, migration toward FBS was increased by TM4SF5 expression (Figure 7B, left graph bars), which was consistent with wound healing analysis in the presence of serum. Meanwhile, haptotactic migration toward collagen I was lower in TM4SF5-expressing cells than in control cells not-expressing TM4SF5 (Figure 7B, right bars). These observations indicated that TM4SF5 may differently regulate cell migration mediated by chemotactic or haptotactic stimuli.

DISCUSSION

Cross-talks between TM4SF5-, integrins- and growth factor receptor-mediated signal transduction

Based on the observations made during this study, we suggest that TM4SF5 expression affect integrin signaling activities including the phosphorylations of FAK (pY^{925}FAK), paxillin, LIMK1/2, and cofilin, which are correlated with the modulation of actin reorganization, focal contact (or adhesion) formation, and eventually cell migration. To this end, TM4SF5 appears to cooperate with integrin α2. Evidences for this include; (a) an association between TM4SF5 and integrin α2 was observed by co-immunoprecipitation, (b) TM4SF5-expressing cells on collagen I or matrigel affected integrin signaling activities more significantly than on fibronectin (Figure 2 and data not shown), and (c) the TM4SF5-enhanced phosphorylations of FAK and paxillin (but not of Erk1/2 irrelevant to TM4SF5 as shown in figures 1A, 3A, and 6B) were abolished after serum-free replating of cells premixed with anti-integrin α2 antibody. However, an interesting aspect in TM4SF5 roles was observed with respect to growth factor signaling dependence. That is, treatment of TM4SF5-expressing cells with serum or growth factors reduced TM4SF5-mediated intracellular signaling activity and actin organization, abolished the association between TM4SF5 and integrin α2, and consequently enhanced TM4SF5-mediated migration or wound healing. These findings imply that TM4SF5 participates in FA disassembly, presumably at the rear of a migratory cell in serum-available environments (see below), through cross-talk between integrins-, TM4SF5-, and growth factor receptor-mediated signal transduction.

However, it cannot be ruled out at this time that unknown cytoplasmic mediator(s) such as kinases or phosphatases might be involved in the cross-talk. TM4SF members of CD53 and CD63 have been shown to be co-immunoprecipitated with tyrosine phosphatase activities that

targeted tyrosine kinase Lck [38]. Integrin α3β1-CD151 complex was also found to contain a transmembrane protein tyrosine phosphatase PTPμ and to regulate cell-cell adhesion [39]. Meanwhile, it is also likely that cross-talk might occur at the level of the receptors. TM4SF proteins are well-known to form homophilic or heterophilic multimers (as shown in Figure 1A, right), with creating tetraspanin-web structures or tetraspanin-enriched microdomains that can function in architectural maintenance of diverse cell membrane proteins including TM4SF themselves, integrins, immunoglobulin super family (IgSF), and/or other membrane receptors [7, 11-17, 32, 40]. The interaction of a specific TM4SF protein appears to be diverse, depending on TM4SF types and/or the availability of their binding partners on the surfaces of specific cell types [40]. For example, CD81 tetraspanin associates with the extracellular domain of CD19 IgSF member on B cells [41], and with intracellular domain of another IgSF partner of EWI-2 on embryonic kidney cells [42]. It has thus been suggested that changes in the compositions of the tetraspanin-webs on the membrane or ligands occupancy of the integrin-TM4SF5 complexes may control its functional properties or outcomes [7]. Therefore, it may be possible that activated growth factor receptors (except for EGFR, since EGF did not cause any changes, data not shown) may replace integrin α2 from the TM4SF5-web complex on the cell membrane, and thereby that TM4SF5-enhanced integrin signaling activity be abolished by serum treatment following serum-free replating.

TM4SF5-mediated Tyr925 FAK phosphorylation, actin organization, and FA dynamics.

In this study, we observed that TM4SF5 reduced pY^{925}FAK in the presence of serum, but increased it in the absence of serum. MLC phosphorylation and actin reorganization were also differentially regulated by TM4SF5, depending on serum treatment. The expression of Y925F FAK mutant leading to a loss of pY^{925}FAK in TM4SF5-expressing cells abolished TM4SF5-

enhanced actin reorganization and phosphorylation of MLC upon serum-free replating. These observations indicate that TM4SF5-mediated pY^{925}FAK might mediate actin reorganization in this system, depending on serum components-mediated signaling.

It was interesting to find that TM4SF5 did not affect pY^{397}FAK and TM4SF5-mediated alteration of pY^{925}FAK did not accompany Erk1/2 activity changes (Figures 1, 3, and 6). In addition to the well-established effect of pY^{925}FAK on the activation of the Erk1/2 cascade [43, 44], one previous study showed that pY^{925}, but not pY^{397}, of FAK is altered in postmigratory oligodendrocytes and appears to be involved in the remodeling of cell morphology through the regulation of actin-linkage to integrin αvβ3/ECM at inset of myelination [45]. Moreover, the association of dynamin to pY^{925}FAK via Grb2 was also shown to be important for the microtubule-induced disassembly of focal adhesions during cell migration [46, 47], as an evidence of the significance of pY^{925}FAK in focal adhesion turnover rather than of Erk1/2 activation. In addition to the Y925F FAK mutant study where pY^{925}FAK was found to be important for TM4SF5-mediated p-MLC and actin organization, we also observed that pY^{925}FAK in TM4SF5-expressing cells, but not in TM4SF5-null cells, colocalized with exogenously expressed GFP-β-actin in lamellipodia around peripheral cell boundaries, thus supporting the functional relevance of pY^{925}FAK in actin organization.

In addition to their phosphorylation statuses, TM4SF5 also affected the cellular localizations of pY^{925}FAK and pY^{118}paxillin. pY^{925}FAK located prominently in internal areas of TM4SF-null Cos7 cells, but mainly on the peripheral boundary of TM4SF5-expressing cells, indicating the TM4SF5-mediated involvement of pY^{925}FAK in FA formation around peripheries. In the case of paxillin, pY^{118}paxillin located oppositely. That is, it located mainly at the peripheries of TM4SF5-null cells but in internal areas of TM4SF5-expressing cells. This observation indicates

that TM4SF5 may affect FA dynamics via having paxillin recruited to focal adhesions located centrally from peripheral boundaries. These effects on the localizations of pY^{925}FAK and pY^{118}paxillin were blocked by a short serum treatment or by inhibition of MLCK, a regulator of actin polymerization and thus of cellular contractility. It was previously shown that Src-mediated pY^{925}FAK is associated with FAK exclusion from FAs [48], FA turnover [49], and Src-induced epithelial-mesenchymal transition (EMT) through the MEK-Erk1/2-MLCK pathway [50, 51]. However, our study shows that TM4SF5-mediated FA dynamics do not correlate with Erk1/2 activity (Figures 1A, 3A, and 6B). It has also been reported that the FAK associations with GRAF (a RhoA-specific GTPase activating protein), ASAP1 (Arf1 and Arf6 GTPase-activating protein), and p190RhoGEP (a guanine nucleotide exchange factor for Rho) provide important linkages between integrin-mediated adhesion processes and actin reorganization, which also involve Src-mediated phosphorylation of tyrosine residues in FAK [52, 53]. Consistent with these, TM4SF5 expression increased RhoA activity in addition to pY^{925}FAK and pY^{416}Src levels, after serum-free replating. Therefore, our study and previous reports suggest significance of pY^{925}FAK on FA dynamics and actin reorganization. In addition, this current study presents evidence that TM4SF5 might influence pY^{925}FAK-involved FA dynamics and actin reorganization at the peripheral edges. During a follow-up study, we observed that the modulation of exogenous or endogenous TM4SF5 protein levels via siRNA introduction into hepatocarcinoma cell lines caused actin reorganization via the RhoA pathway (data not shown, manuscript in preparation).

Differential effects of TM4SF5 on chemotactic and haptotactic migration

In this study, the effects of TM4SF5 on serum-induced chemotactic or ECM-induced haptotactic migration were stimulatory or inhibitory, respectively. We also observed that the effects of TM4SF5 on actin reorganization, focal contact/adhesion formation, and intracellular

signaling activity were negative or positive in serum-treated or serum-free conditions, respectively. With regard to differential effects on cell migration, therefore, we speculate that TM4SF5-mediated signaling activity including p-MLC along peripheral boundaries (Figure 5D) and FA formation after serum-free replating might result in firm FAs that may lead to no wound healing and less efficient haptotactic migration. However, when serum-mediated signals are triggered and the TM4SF5-integrin α2 association is disrupted, actin organization and FA dynamics are inversely regulated for FA disassembly, presumably at the rear of a migrating cell, thus leading to a TM4SF5-enhanced-chemotactic migration.

Cell migration occurs through a complex and integrated process that involves the continuous, coordinated assembly and disassembly of cell adhesion structures including peripheral FAs and central 'fibrillar adhesions' around both cell front and rear [54]. It was previously shown that cell migration is differentially regulated by peripheral or central FAs and p-MLC, after the inhibitions of either myosin light chain kinase (MLCK) or Rho-associated kinase (ROCK) [55]. Moreover, TM4SF5-enhanced FA formation and actin organization under serum-free conditions might favor cell migration if they are dynamically prominent at the front edges of a migratory cell, but not always favor if they are strongly functional at rear edges. Cell migration might not be favored, if TM4SF5-enhanced actin reorganization is radial rather than being directional (i.e., polarized). Thus, it would be interesting to investigate how TM4SF5 regulates FA/fibrillar adhesion dynamics and actin reorganizations, especially at the sub-cellular level in migrating cells.

Altogether, this present study suggests that TM4SF5 participate in the regulation of integrin signaling (e.g., pY^{925}FAK) for actin reorganization and for FA dynamics at cell peripheries and thereby in migration, depending on growth factor signal transduction.

ACKNOWLEDGEMENTS

We authors deeply thank Drs. Sarah M. Short and Eun-Kyung Suh (Harvard Medical School, Boston, MA, USA) for valuable discussion and review for the manuscript, Haruhiko Bito (Department of Neurochemistry, University of Tokyo, Tokyo, Japan), Jun-Lin Guan (Cornell University, College of Veterinary Medicine, Ithaca, NY, USA), and Keith Burridge (University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) for expression vectors such as pEGFP-β-actin, pRC/CMV-$(HA)_3$-FAK, and GST-RBD, respectively. This work was supported by a Korea Research Foundation Grant (KRF-2004-015-C00445 to J W Lee) and by Cancer Research Institute, College of Medicine, Seoul National University (CRI-05-06 to J W Lee).

REFERENCES

[1] Short, S. M., Boyer, J. L., and Juliano, R. L., Integrins regulate the linkage between upstream and downstream events in G protein-coupled receptor signaling to mitogen-activated protein kinase, J. Biol. Chem. 275 (2000) 12970-12977.

[2] Eliceiri, B. P., Integrin and growth factor receptor crosstalk, Circ. Res. 89 (2001) 1104-1110.

[3] Yamada, K. M., and Even-Ram, S., Integrin regulation of growth factor receptors, Nat. Cell Biol. 4 (2002) E75-76.

[4] Juliano, R. L., Signal transduction by cell adhesion receptors and the cytoskeleton: Functions of Integrins, Cadherins, Selectins, and Immunoglobulin-Superfamily Members, Annu. Rev. Pharmacol. Toxicol. 42 (2002) 283-323.

[5] Bhowmick, N. A., Zent, R., Ghiassi, M., McDonnell, M., and Moses, H. L., Integrin β1 signaling is necessary for transforming growth factor-β activation of p38MAPK and epithelial plasticity, J. Biol. Chem. 276 (2001) 46707-46713.

[6] Lee, M. S., Ko, S. G., Kim, H. P., Kim, Y. B., Lee, S. Y., Kim, S. G., Jong, H. S., Kim, T. Y., Lee, J. W., and Bang, Y. J., Smad2 mediates Erk1/2 activation by TGF-β1 in suspended, but not in adherent, gastric carcinoma cells, Int. J. Oncol. 24 (2004) 1229-1234.

[7] Berditchevski, F., Complexes of tetraspanins with integrins: more than meets the eye, J. Cell Sci. 114 (2001) 4143-4151.

[8] Stipp, C. S., Kolesnikova, T. V., and Hemler, M. E., Functional domains in tetraspanin proteins, Trends Biochem. Sci. 28 (2003) 106-112.

[9] Berditchevski, F., and Odintsova, E., Characterization of integrin-tetraspanin adhesion complexes: role of tetraspanins in integrin signaling, J. Cell Biol. 146 (1999) 477-492.

[10] Boucheix, C., Duc, G. H., Jasmin, C., and Rubinstein, E., Tetraspanins and malignancy, Expert Rev. Mol. Med. 2001 (2001) 1-17.

[11] Hemler, M. E., Mannion, B. A., and Berditchevski, F., Association of TM4SF proteins with integrins: relevance to cancer, Biochim. Biophy. Acta 1287 (1996) 67-71.

[12] Yang, X., Claas, C., Kraeft, S. K., Chen, L. B., Wang, Z., Kreidberg, J. A., and Hemler, M. E., Palmitoylation of tetraspanin proteins: modulation of CD151 lateral interactions, subcellular distribution, and integrin-dependent cell morphology, Mol. Biol. Cell 13 (2002) 767-781.

[13] Kazarov, A. R., Yang, X., Stipp, C. S., Sehgal, B., and Hemler, M. E., An extracellular site on tetraspanin CD151 determines α3 and α6 integrin-dependent cellular morphology, J. Cell Biol. 158 (2002) 1299-1309.

[14] Charrin, S., Manie, S., Billard, M., Ashman, L., Gerlier, D., Boucheix, C., and Rubinstein, E., Multiple levels of interactions within the tetraspanin web, Biochem. Biophys. Res. Commun. 304 (2003) 107-112.

[15] Tarrant, J. M., Robb, L., van Spriel, A. B., and Wright, M. D., Tetraspanins: molecular organisers of the leukocyte surface, Trends Immunol. 24 (2003) 610-617.

[16] Yang, X., Kovalenko, O. V., Tang, W., Claas, C., Stipp, C. S., and Hemler, M. E., Palmitoylation supports assembly and function of integrin-tetraspanin complexes, J. Cell Biol. 167 (2004) 1231-1240.

[17] Gesierich, S., Paret, C., Hildebrand, D., Weitz, J., Zgraggen, K., Schmitz-Winnenthal, F. H., Horejsi, V., Yoshie, O., Herlyn, D., Ashman, L. K., and Zoller, M., Colocalization of the tetraspanins, CO-029 and CD151, with integrins in human pancreatic adenocarcinoma: impact on cell motility, Clin. Cancer Res. 11 (2005) 2840-2852.

[18] Muller-Pillasch, F., Wallrapp, C., Lacher, U., Friess, H., Buchler, M., Adler, G., and Gress,

T. M., Identification of a new tumour-associated antigen TM4SF5 and its expression in human cancer, Gene 208 (1998) 25-30.
[19] Wright, M. D., Ni, J., and Rudy, G. B., The L6 membrane proteins--a new four-transmembrane superfamily, Protein Sci. 9 (2000) 1594-1600.
[20] Pascual-Le Tallec, L., Dulmet, E., Bertagna, X., and de Keyzer, Y., Identification of genes associated with the corticotroph phenotype in bronchial carcinoid tumors, J. Clin. Endocrinol. Metab 87 (2002) 5015-5022.
[21] Garcia-Frigola, C., Burgaya, F., Calbet, M., de Lecea, L., and Soriano, E., Mouse Tspan-5, a member of the tetraspanin superfamily, is highly expressed in brain cortical structures, Neuroreport 11 (2000) 3181-3185.
[22] Garcia-Frigola, C., Burgaya, F., de Lecea, L., and Soriano, E., Pattern of expression of the tetraspanin Tspan-5 during brain development in the mouse, Mech. Dev. 106 (2001) 207-212.
[23] Friedl, P., and Wolf, K., Tumour-cell invasion and migration: diversity and escape mechanisms, Nat. Rev. Cancer 3 (2003) 362-374.
[24] Schlaepfer, D. D., and Mitra, S. K., Multiple connections link FAK to cell motility and invasion, Curr. Opin. Genet. Dev. 14 (2004) 92-101.
[25] Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D., Focal adhesion kinase: in command and control of cell motility, Nat. Rev. Mol. Cell Biol. 6 (2005) 56-68.
[26] Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P., Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase, Nature 372 (1994) 786-791.
[27] Guo, W., and Giancotti, F. G., Integrin signalling during tumour progression, Nat. Rev. Mol. Cell Biol. 5 (2004) 816-826.
[28] Brakebusch, C., and Fassler, R., The integrin-actin connection, an eternal love affair, EMBO J. 22 (2003) 2324-2333.
[29] Rafelski, S. M., and Theriot, J. A., Crawling toward a unified model of cell mobility: spatial and temporal regulation of actin dynamics, Annu. Rev. Biochem. 73 (2004) 209-239.
[30] Ponti, A., Machacek, M., Gupton, S. L., Waterman-Storer, C. M., and Danuser, G., Two distinct actin networks drive the protrusion of migrating cells, Science 305 (2004) 1782-1786.
[31] Arthur, W. T., and Burridge, K., RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity, Mol. Biol. Cell 12 (2001) 2711-2720.
[32] Yunta, M., and Lazo, P. A., Tetraspanin proteins as organisers of membrane microdomains and signalling complexes, Cell Signal. 15 (2003) 559-564.
[33] Juliano, R. L., Aplin, A. E., Howe, A. K., Short, S., Lee, J. W., and Alahari, S., Integrin regulation of receptor tyrosine kinase and G protein-coupled receptor signaling to mitogen-activated protein kinases, Methods Enzymol. 333 (2001) 151-163.
[34] Bain, J., McLauchlan, H., Elliott, M., and Cohen, P., The specificities of protein kinase inhibitors: an update, Biochem. J. 371 (2003) 199-204.
[35] Goeckeler, Z. M., and Wysolmerski, R. B., Myosin Phosphatase and Cofilin Mediate cAMP/cAMP-dependent Protein Kinase-induced Decline in Endothelial Cell Isometric Tension and Myosin II Regulatory Light Chain Phosphorylation, J. Biol. Chem. 280 (2005) 33083-33095.
[36] Gohla, A., Birkenfeld, J., and Bokoch, G. M., Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics, Nat. Cell Biol. 7 (2005) 21-29.
[37] Boucheix, C., and Rubinstein, E., Tetraspanins, Cell Mol. Life Sci. 58 (2001) 1189-1205.
[38] Carmo, A. M., and Wright, M. D., Association of the transmembrane 4 superfamily molecule CD53 with a tyrosine phosphatase activity, Eur. J. Immunol. 25 (1995) 2090-2095.

[39] Chattopadhyay, N., Wang, Z., Ashman, L. K., Brady-Kalnay, S. M., and Kreidberg, J. A., α3β1 integrin-CD151, a component of the cadherin-catenin complex, regulates PTPμ expression and cell-cell adhesion, J. Cell Biol. 163 (2003) 1351-1362.
[40] Levy, S., and Shoham, T., Protein-protein interactions in the tetraspanin web, Physiology (Bethesda) 20 (2005) 218-224.
[41] Bradbury, L. E., Goldmacher, V. S., and Tedder, T. F., The CD19 signal transduction complex of B lymphocytes. Deletion of the CD19 cytoplasmic domain alters signal transduction but not complex formation with TAPA-1 and Leu 13, J. Immunol. 151 (1993) 2915-2927.
[42] Stipp, C. S., Kolesnikova, T. V., and Hemler, M. E., EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily, J. Biol. Chem. 276 (2001) 40545-40554.
[43] Schlaepfer, D., and Hunter, T., Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases., Mol. Cell. Biol. 16 (1996) 5623-5633.
[44] Schlaepfer, D. D., and Hunter, T., Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src, J. Biol. Chem. 272 (1997) 13189-13195.
[45] Fox, M. A., Alexander, J. K., Afshari, F. S., Colello, R. J., and Fuss, B., Phosphodiesterase-Iα/autotaxin controls cytoskeletal organization and FAK phosphorylation during myelination, Mol. Cell Neurosci. 27 (2004) 140-150.
[46] Ezratty, E. J., Partridge, M. A., and Gundersen, G. G., Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase, Nat. Cell Biol. 7 (2005) 581-590.
[47] Burridge, K., Foot in mouth: do focal adhesions disassemble by endocytosis?, Nat. Cell Biol. 7 (2005) 545-547.
[48] Katz, B. Z., Romer, L., Miyamoto, S., Volberg, T., Matsumoto, K., Cukierman, E., Geiger, B., and Yamada, K. M., Targeting membrane-localized focal adhesion kinase to focal adhesions: roles of tyrosine phosphorylation and SRC family kinases, J. Biol. Chem. 278 (2003) 29115-29120.
[49] Brunton, V. G., Avizienyte, E., Fincham, V. J., Serrels, B., Metcalf, C. A., 3rd, Sawyer, T. K., and Frame, M. C., Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior, Cancer Res. 65 (2005) 1335-1342.
[50] Avizienyte, E., Fincham, V. J., Brunton, V. G., and Frame, M. C., Src SH3/2 domain-mediated peripheral accumulation of Src and phospho-myosin is linked to deregulation of E-cadherin and the epithelial-mesenchymal transition, Mol. Biol. Cell 15 (2004) 2794-2803.
[51] McLean, G. W., Carragher, N. O., Avizienyte, E., Evans, J., Brunton, V. G., and Frame, M. C., The role of focal-adhesion kinase in cancer - a new therapeutic opportunity, Nat. Rev. Cancer 5 (2005) 505-515.
[52] Zhai, J., Lin, H., Nie, Z., Wu, J., Canete-Soler, R., Schlaepfer, W. W., and Schlaepfer, D. D., Direct interaction of focal adhesion kinase with p190RhoGEF, J. Biol. Chem. 278 (2003) 24865-24873.
[53] Westhoff, M. A., Serrels, B., Fincham, V. J., Frame, M. C., and Carragher, N. O., SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling, Mol. Cell Biol. 24 (2004) 8113-8133.
[54] Webb, D. J., Parsons, J. T., and Horwitz, A. F., Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again, Nat. Cell Biol. 4 (2002) E97-100.
[55] Totsukawa, G., Wu, Y., Sasaki, Y., Hartshorne, D. J., Yamakita, Y., Yamashiro, S., and

Matsumura, F., Distinct roles of MLCK and ROCK in the regulation of membrane protrusions and focal adhesion dynamics during cell migration of fibroblasts, J. Cell Biol. 164 (2004) 427-439.

FIGURE LEGENDS

Figure 1. Ectopic expression of TM4SF5 in Cos7 cells decreases integrin-related signaling, focal adhesion formation, and actin reorganization. (A) TM4SF5-null Cos7 cells were transiently transfected with pEGFP or pEGFP-TM4SF5. Two days later, whole cell lysates were prepared from the cells in normal serum-containing culture media and immunoblotted against the indicated molecules under non-reducing conditions. (B and C) Two days after transfection with pEGFP-TM4SF5, cells were replated on serum-coated coverslips in the presence of 10% FBS prior to FAK immunostaining to visualize focal adhesions (B) or actin-staining using rhodamine-conjugated phalloidin (C). (D) Cos7 cells were cotransfected with pEGFP-TM4SF5 and either a scrambled control shRNA (upper panel) or TM4SF5 shRNA (shTM4SF5, lower panel), and 24 h later cells were replated on serum-coated coverslips in 10% FBS-containing culture media. One day after the replating, cells were stained for actin by using rhodamine-conjugated phalloidin. (E) Cos7 cells were co-transfected with pEGFP$_B$-TM4SF5 with either shRNA against scrambled (Cont) or TM4SF5 sequence (shTM4SF5), as explained in the Materials and Methods. Two days later, cell lysates were prepared and equal amounts of proteins were subjected to standard Western blots against the indicated molecules. Please notice that GFP-TM4SF5 at monomer and multimer forms decreased by shTM4SF5 cotransfection in the anti-GFP blots under non-reducing conditions. Data shown are representative from three independent experiments.

Figure 2. Replating of TM4SF5-expressing cells on extracellular matrix proteins in the absence of serum increased the TM4SF5-mediated effects. Cos7 cells were transiently transfected with pcDNA3 (as a control vector, Vec), pEGFP-TM4SF5 (GFP-TM4SF5), myc-(His)$_6$-TM4SF5 (MH-TM4SF5), or pcDNA3-TM4SF5 (TM4SF5) (A), or stably infected with either retrovirus with pLNCX (C) or pLNCX-TM4SF5 (T) (B). Two days later, the cells were

kept in suspension or replated on non-precoated tissue culture dishes, matrigel- (0.56 mg/ml for a thin coating, A), or collagen I- (10.0 μg/ml, B and E) coated dishes for 1 h with vehicle DMSO or ML9 (25 μM) pretreatment (E), as explained in the Materials and Methods. Whole cell lysates were then prepared prior to standard Western blots for the indicated molecules (A, B, and E). (C and D) Cells were transiently cotransfected with pcDNA3-GFP and pcDNA3-TM4SF5 (TM4SF5). Two days later, the cells were pretreated with DMSO or ML9 (25 μM), 30 min before replating on collagen I-precoated coverslips for 1 h. Cells were then immunostained for integrin β1 (C) or stained for actin by using rhodamine-conjugated phalloidin (D). (E) Cells transfected either with pcDNA3 (Vec) or pcDNA3-TM4SF5 (TM4SF5) were pretreated with DMSO or ML9 (25 μM) 30 min before replating on collagen I-coated dishes. Cell lysates were prepared 2 days after the transfection, and equal amounts of protein were subjected to standard Western blots against the indicated molecules. Data shown are representative from three independent experiments.

Figure 3. Treatment of serum components of cells replated on collagen I abolished TM4SF5-mediated signaling activity, FA formation, and actin organization on replating. (A) Stable Cos7 cells with pLNCX (C) or pLNCX-TM4SF5 (T) were replated on collagen I (10 μg/ml)-coated dishes with or without growth factor(s) (50 ng/ml PDGF-AB or FBS at a final concentration of 10%). The growth factors were added for the last 5 min of the 1 h replating period. Whole cell lysates were prepared and equal amounts of protein were subjected to the standard Western blots for the indicated molecules. Data shown are representative from three isolated experiments. (B) Cos7 cells were cotransfected with pcDNA3-GFP and pcDNA3-TM4SF5. Replating and treatment with serum were performed as in (A), except for replating

cells on collagen I-coated coverslips. Then immunostaining processes for integrin β1 (upper and right panel) or staining for actin by using rhodamine-conjugated phalloidin (lower and right panel) were followed, as explained in Materials and Methods. The GFP and the immunostained images at the same fields are shown in parallel. Data shown are representative from three independent experiments. (C) Quantitative analysis of TM4SF5-mediated effects on actin structure organization was performed through three independent experiments, where cells were replated on collagen I-precoated coverslips for 1 h without (Collagen I) or with FBS treatment at a final concentration of 10% for the last 5 min (Collagen I + FBS) prior to staining for actin. More than 10 separate images with a few transfected and untransfected cells were analyzed to see if (GFP-positive) TM4SF5-expressing cells increased ($q<1$), not-changed ($q=1$), or decreased ($q>1$) actin structures including lamellipodia, stress fiber, and/or ruffling. The q equals to $[\text{GFP-negative}]_{actin}/[\text{GFP-positive}]_{actin}$. Each graph bar represents percentages (average ± standard deviation) of the numbers of cells within each q value group out of the total transfected cells. * indicates $p < 0.05$ for quantitative significance.

Figure 4. TM4SF5-mediated localization of pY^{925}FAK or pY^{118}paxillin on replating is regulated by serum treatment. (A) Cos7 cells were cotransfected with pcDNA3-GFP and pcDNA3-TM4SF5 expression plasmids, and two days later cells were replated on collagen I-coated coverslips for 1 h prior to immunostaining for anti-pY^{925}FAK, as explained in the Materials and Methods. Note that a GFP-positive (and thus TM4SF5-postive) cell showed more pY^{925}FAK on peripheral plasma membranes, compared to neighboring untransfected (GFP-negative) cells. (B to D) Stable Cos7 cells with pLNCX or pLNCX-TM4SF5 were replated on collagen I-precoated coverslips with or without serum treatment, as in figure 3. In some cases,

cells were pretreated with ML9 (25 μM) 30 min prior to replating. Cells were then immunostained for pY^{925}FAK (B) or pY^{118}paxillin (C). Data shown are representative from three independent experiments. (D) Quantitative analysis for figure 4C. Quantitative analysis for localization of p^{Y925}FAK or pY^{118}paxillin in pLNCX-TM4SF5 cells replated on collagen I for 1 h without or with FBS (at final concentration of 10% for 5 min in the end of the 1 h replating period) were performed as explained in the Materials and Methods. Each graph bar represents percentages of cell numbers at average ± standard deviation (SD), and * indicates $p < 0.05$ for quantitative significance. P; peripheral plasma membranes, I; internal areas of a cell distal from plasma membranes, B; both peripheral membranes and internal areas.

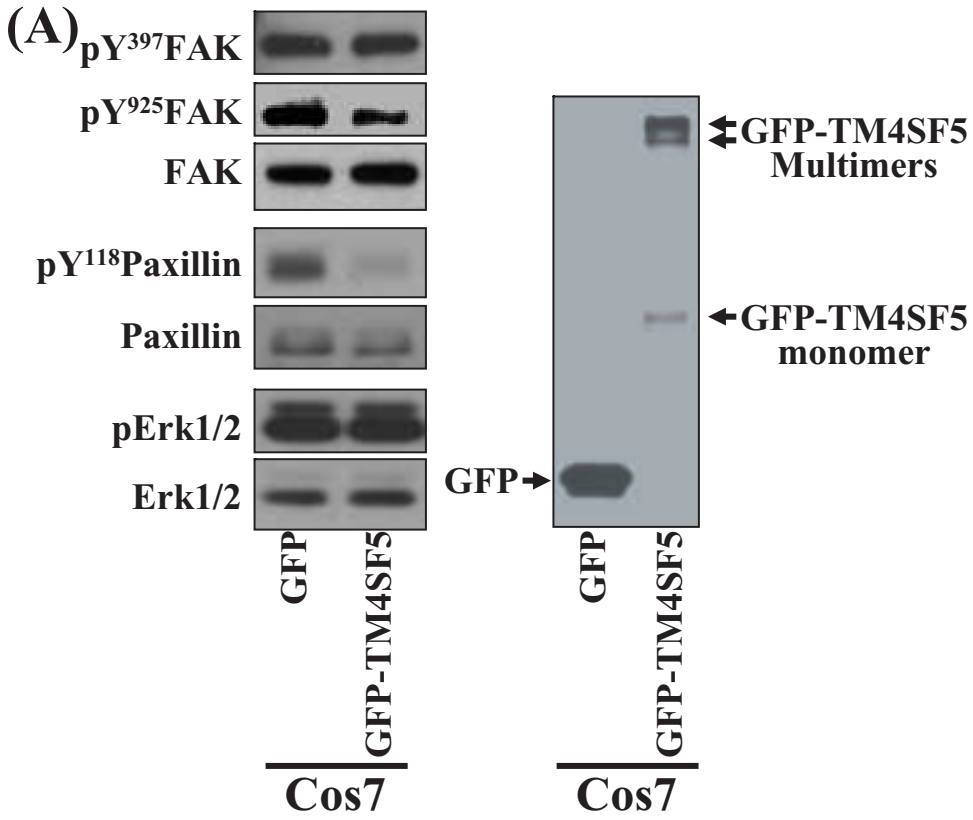
Figure 5. Y925F mutation of FAK abolished TM4SF5-mediated integrin-signaling, actin reorganization, and MLC phosphorylation. (A) Cos7 cells were transiently transfected with either pRC/CMV-$(HA)_3$-WT FAK (WT) or pRC/CMV-$(HA)_3$-Y925F FAK (Y925F) plasmid, as explained in the Materials and Methods. Two days later, cells were harvested for whole cell lysates prior to immunoprecipitation using anti-HA antibody and immunoblotting against the indicated molecules. (B to E) pLNCX-TM4SF5 Cos7 cells were cotransfected with pEGFP and Y925F FAK plasmids (B and D), transfected with WT or Y925F FAK plasmid (C), or transfected with pEGFP-β-actin (E). Two days later, the cells were harvested for the whole cell lysates for immunoblotting against the indicated molecules (C), or replated on collagen I (10 μg/ml) -precoated coverslips prior to staining with either rhodamine-conjugated phalloidin (B), anti-phospho-MLC (D), or anti-pY^{925}FAK (E), as explained in the Materials and Methods. (B, D, and E) The GFP (left) and the immunostained images (right) at the same fields are shown in parallel. Data shown are representative from three independent experiments.

Figure 6. TM4SF5 association with integrin α2 is involved in TM4SF5-mediated signaling activity and actin organization depending on serum treatment. (A) Cos7 cells were transiently transfected with myc-$(His)_6$ control (C) or myc-$(His)_6$-TM4SF5 (T) plasmid. Two days later, cells were replated on collagen I-coated dishes with or without serum treatment, as explained above. Whole cell lysates were then prepared and equal amounts of protein were subjected to an immunoprecipitation by using anti-myc (9E10 clone) or anti-integrin α2 (P1E6) antibody. Immunoblottings of the immunoprecipitates were performed by using anti-integrin subunits (left and right) or -myc tag antibody (right). Lysates themselves showed expression of myc-$(His)_6$-TM4SF5, as expected, and equal expression levels of integrins α2, α3, or α5 in the cells transfected with myc-$(His)_6$ control (C) or myc-$(His)_6$-TM4SF5 (T) plasmid (data not shown). The marked (*) bands indicate a non-specific band. (B and C) Stable Cos7 cells with pLNCX (C) or pLNCX-TM4SF5 (T) were replated on collagen I-coated dishes. When indicated, the cells were preincubated with functional blocking anti-integrin α2 (P1E6), α3 (P1B5), or α5 (P1D6) antibody at 7.5 μg/ml before replating. After incubation on collagen I for 1 h, cell lysates were prepared and subjected to standard Western blots for the indicated molecules. Functional blocking experiments by using integrin α2 (P1E6) antibody were shown twice in separate experiments (B). Alternatively, antibody premixed cells were replated on collagen I-coated coverslips in the absence of serum for 1 h prior to taking phase-contrast images and staining for actin (C). Arrows indicate the blebby membranes. Data shown represent three independent experiments.

Figure 7. TM4SF5-expressing Cos7 cells migrate differentially depending on chemotactic or

haptotactic stimuli. (A) Stable Cos7 cells with pLNCX or pLNCX-TM4SF5 were subjected to wound healing in 10% FBS-containing or serum-free conditions. The degree of wound healing was measured 12 h after initiating the wounds with a pipette tip. The dotted lines indicate the start lines of wound healing. Data shown represent three different experiments. (B) In the transwell (with membrane filters precoated with 10 μg/ml collagen I) migration assay, stable Cos7 cells with pLNCX (C) or pLNCX-TM4SF5 (T) were replated at 5 x 10^4 cells per chamber into the upper chamber. Bottom chambers were filled with DMEM-H without (Coll) or with 10% FBS (FBS). Twelve hours later, migrated cells were stained after removing the non-migrated cells, images were taken, and average (± standard deviation) values from 5 different images for each condition were calculated and graphed. Data shown were representative from three independent experiments.

Figure 1:.ECR-06-138



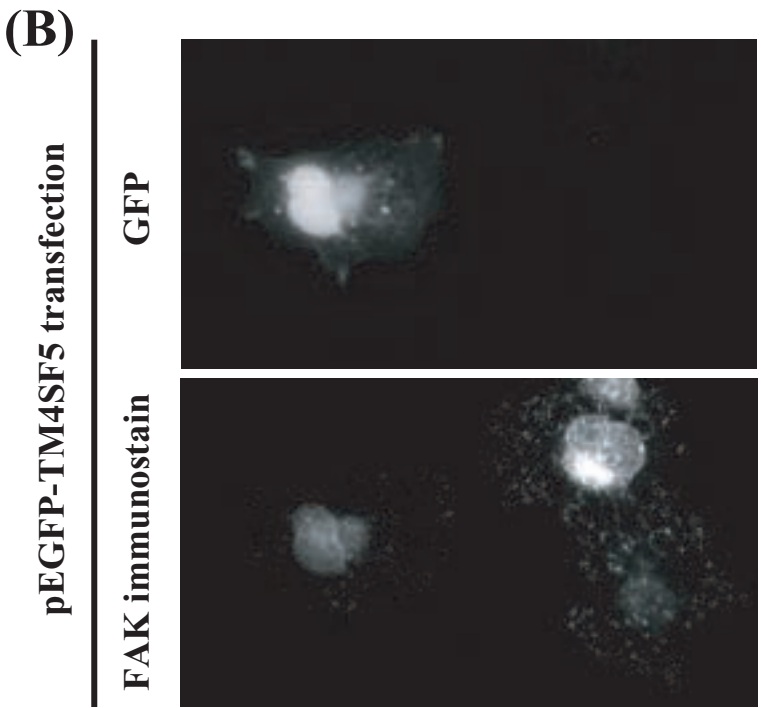


Figure 1:.ECR-06-138

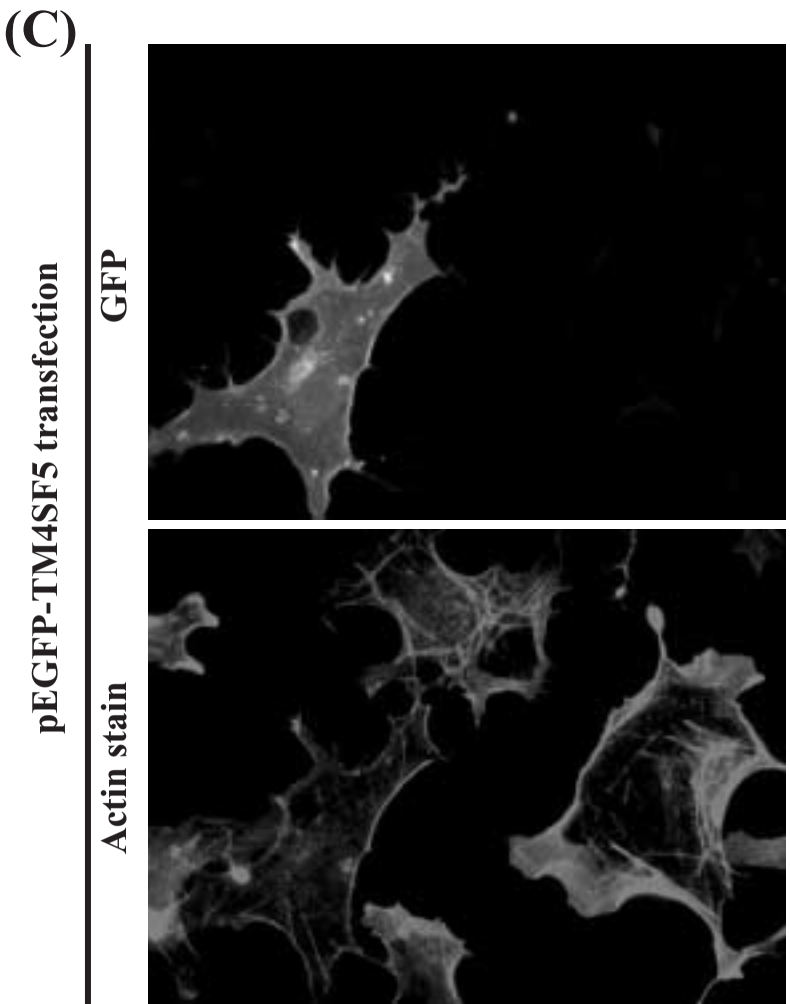
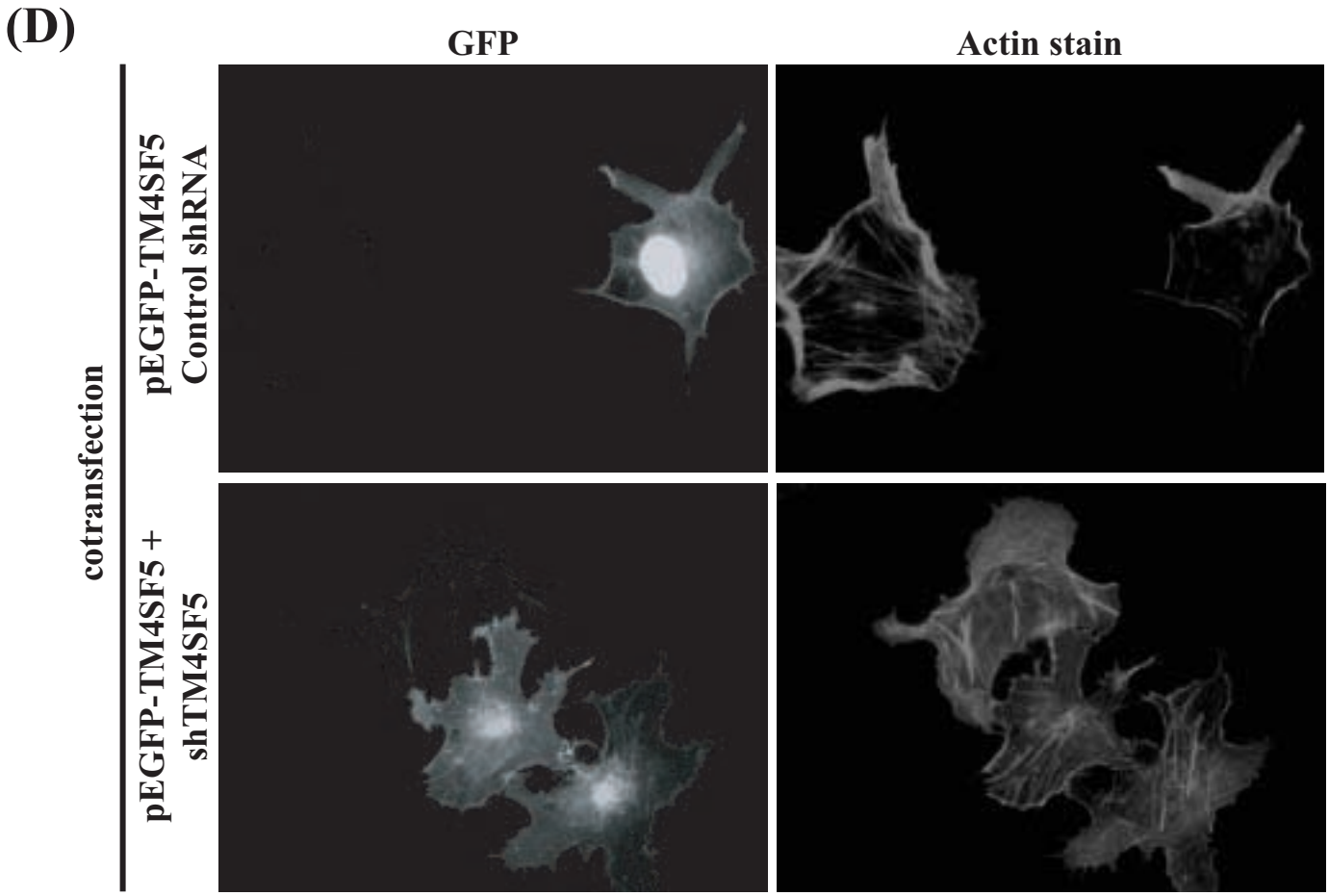


Figure 1C

Figure 1:.ECR-06-138



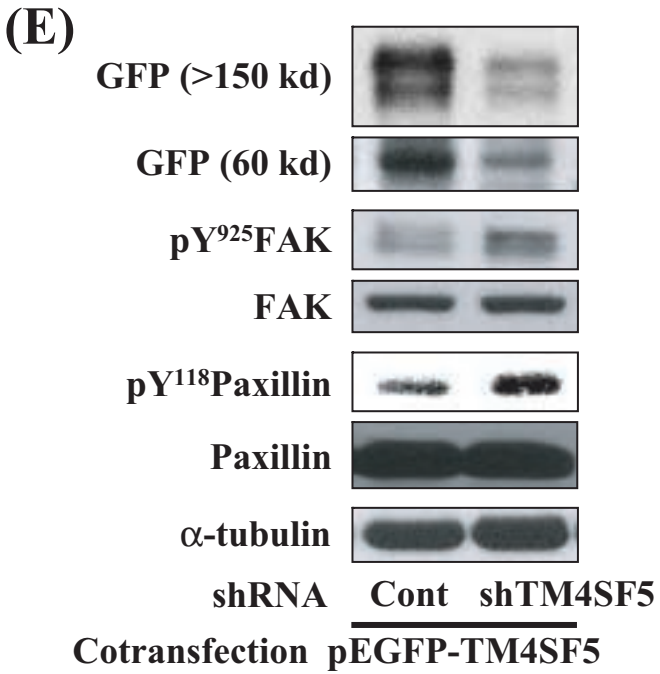
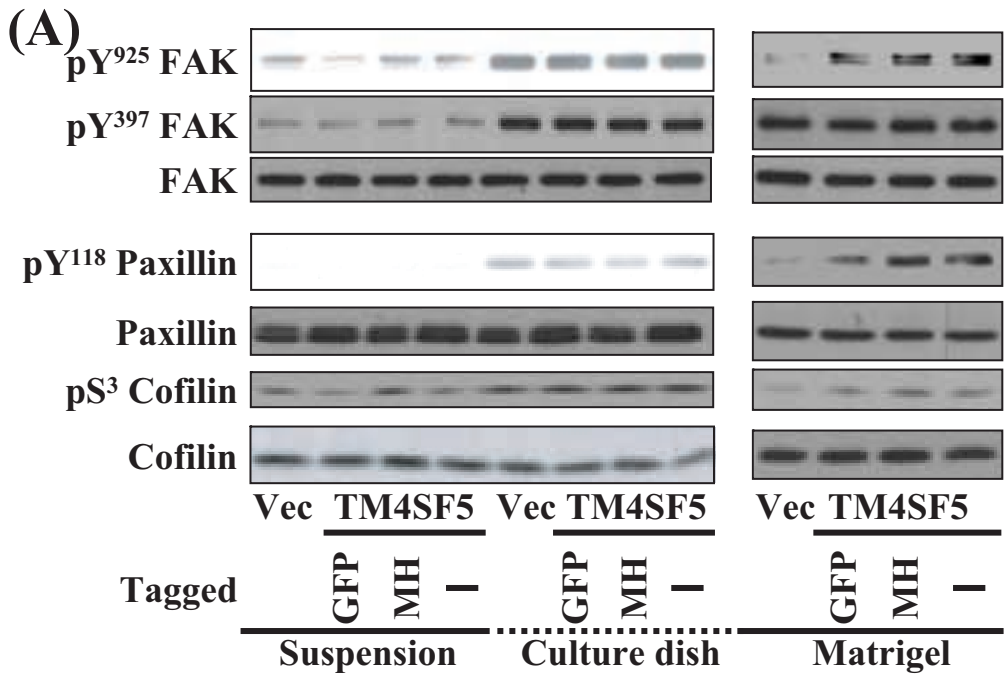


Figure 2:.ECR-06-138



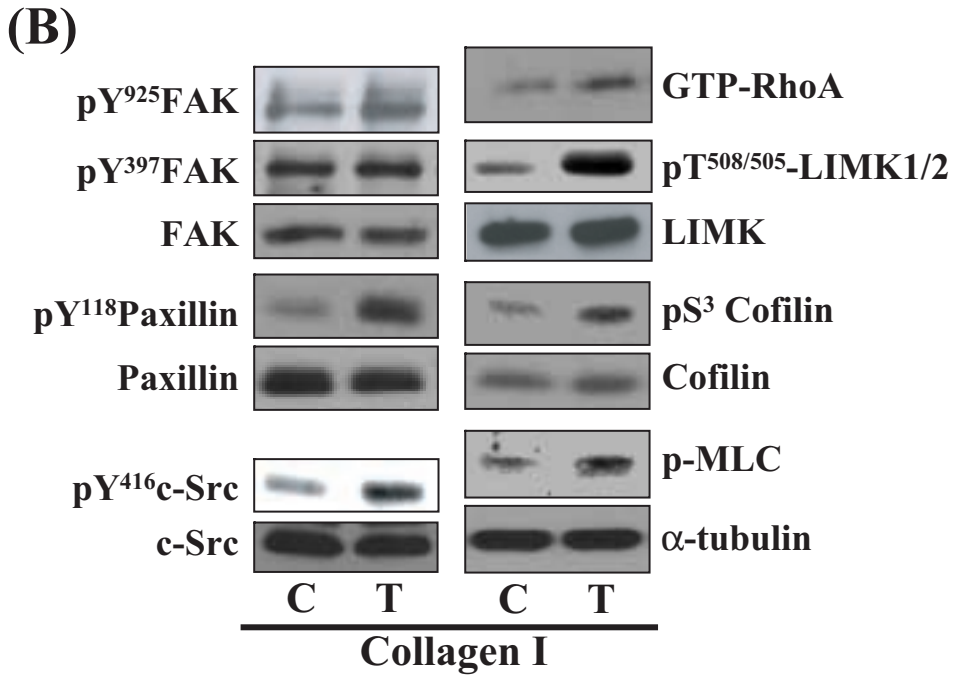


Figure 2:.ECR-06-138

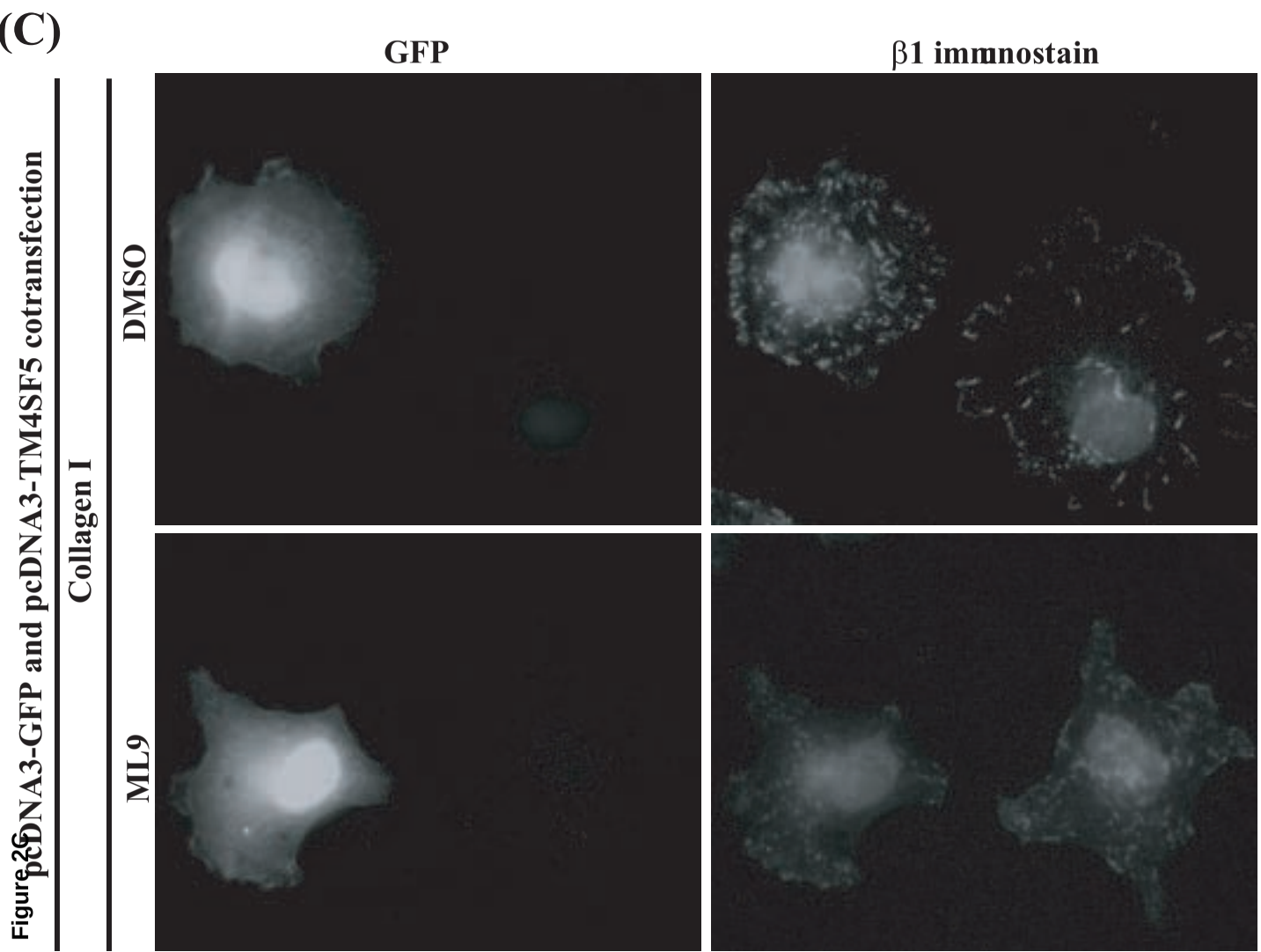


Figure 2C

Figure 2:.ECR-06-138

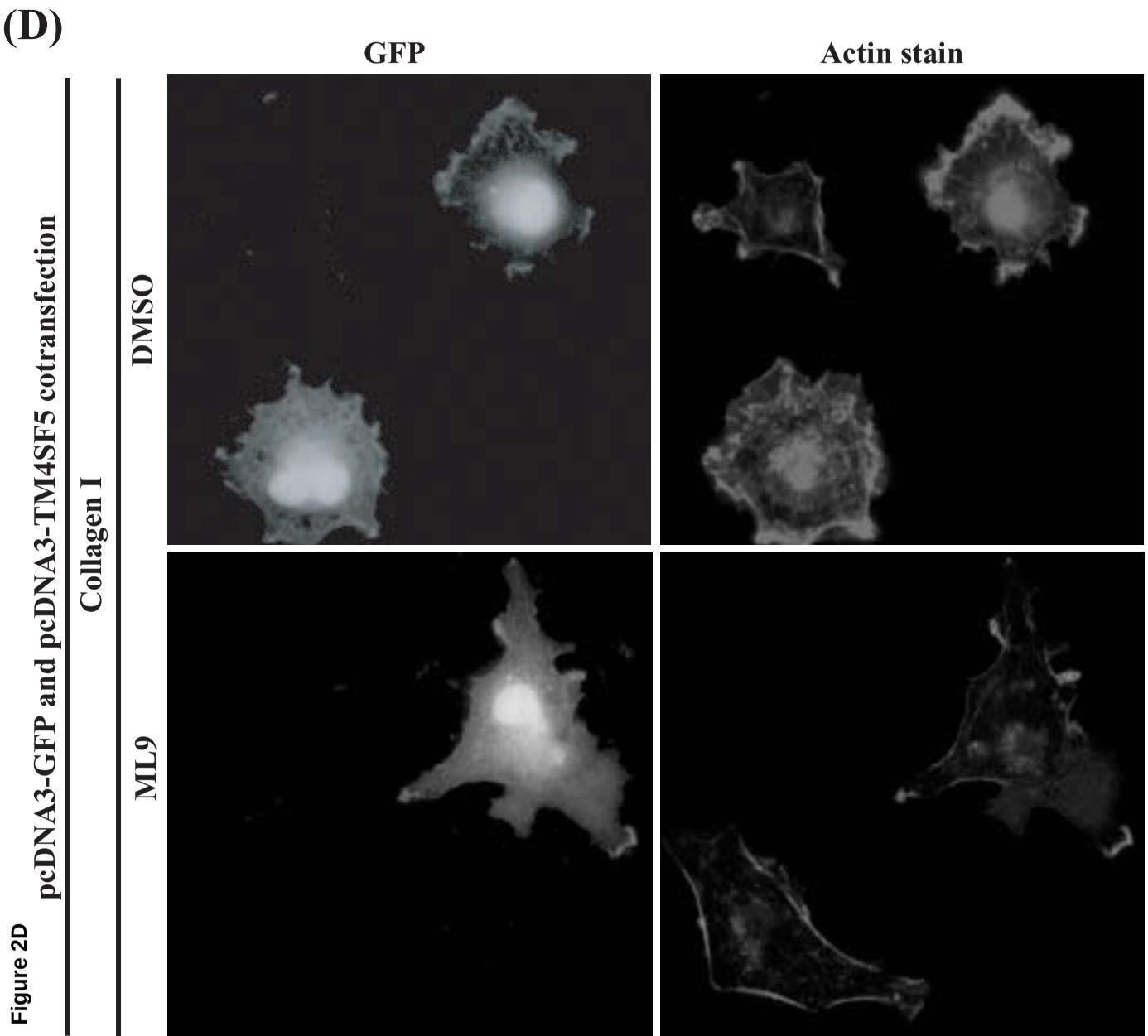


Figure 2D

Figure 2E

Figure 2:.ECR-06-138

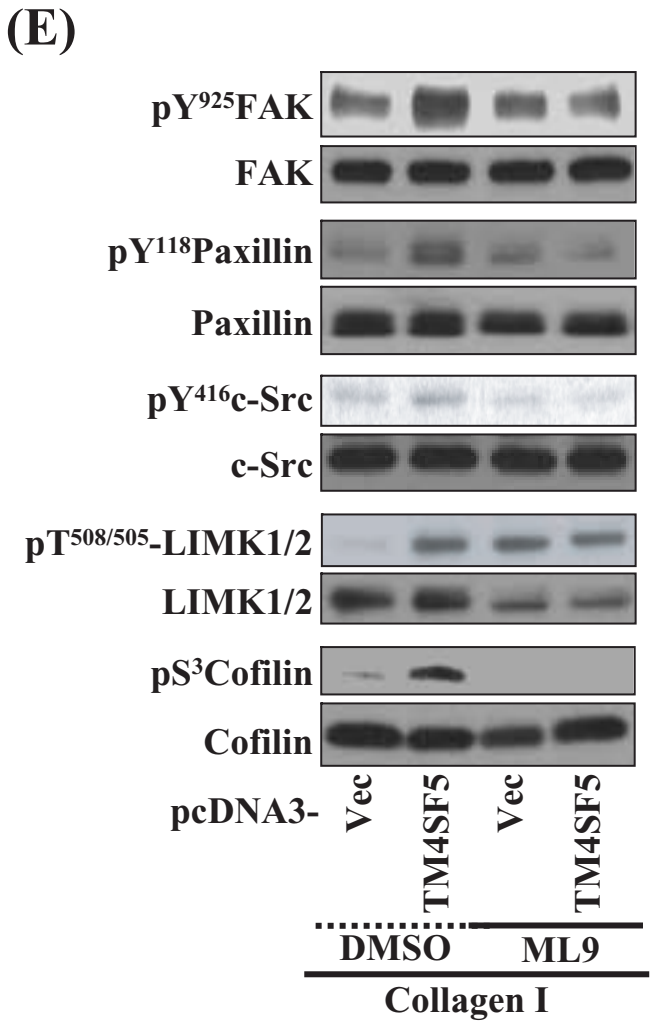


Figure 3A

Figure 3:.ECR-06-138

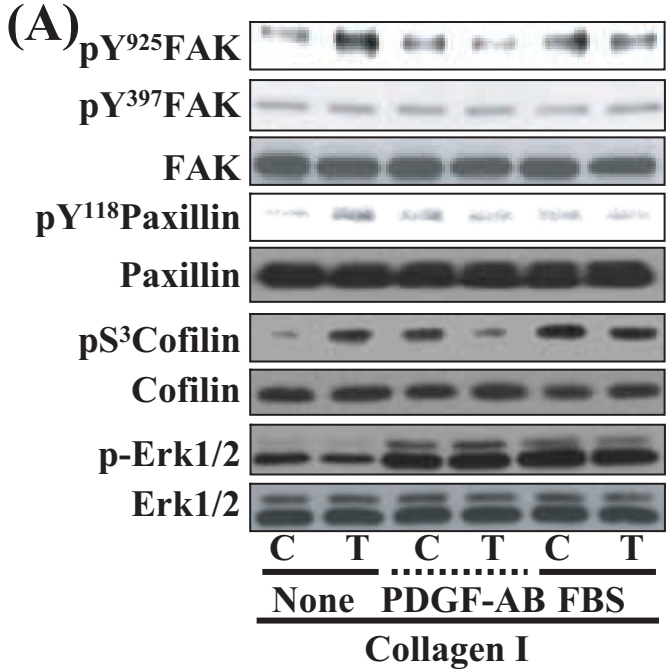


Figure 3:.ECR-06-138

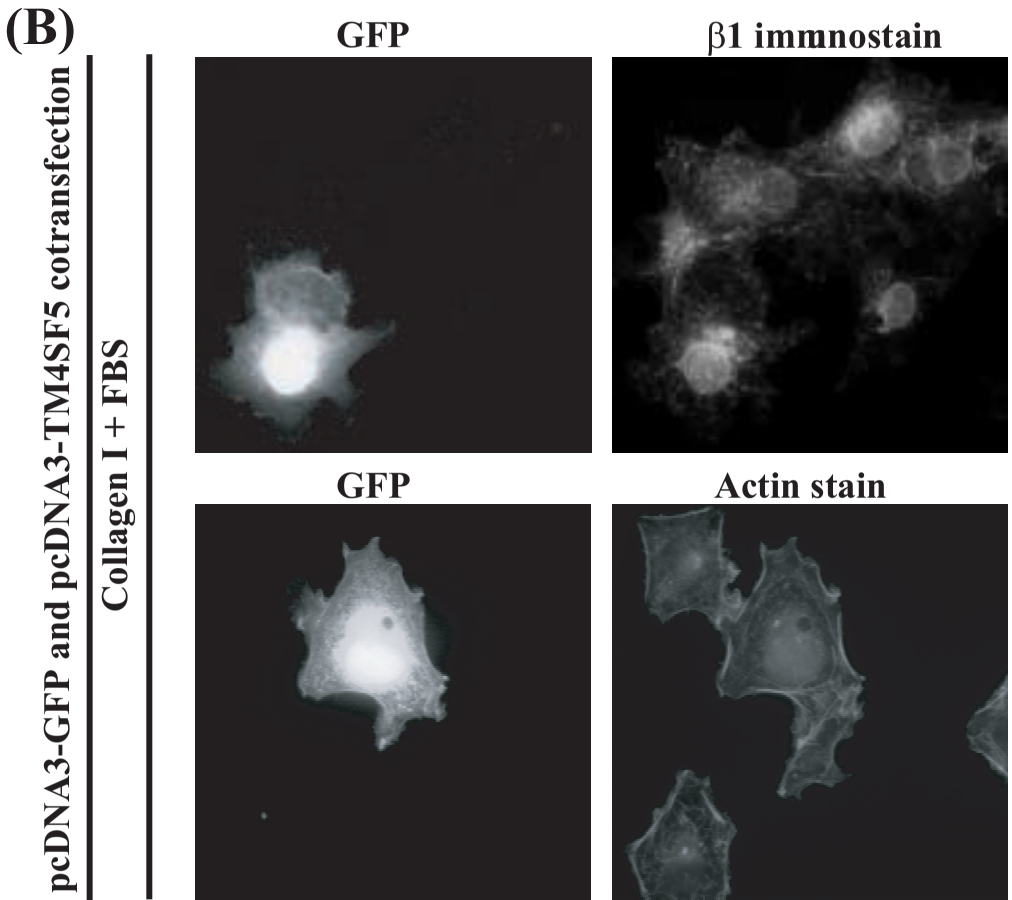


Figure 3B

Figure 3C

Figure 3:.ECR-06-138

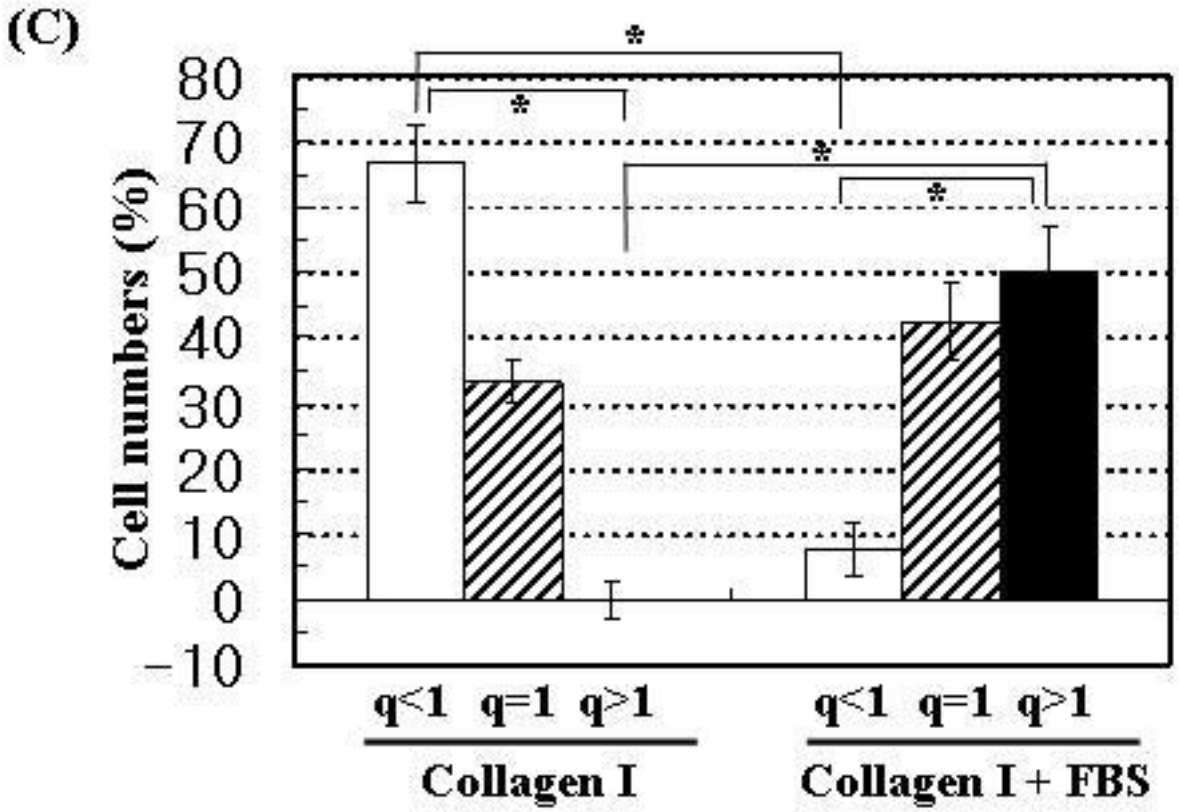


Figure 4:.ECR-06-138

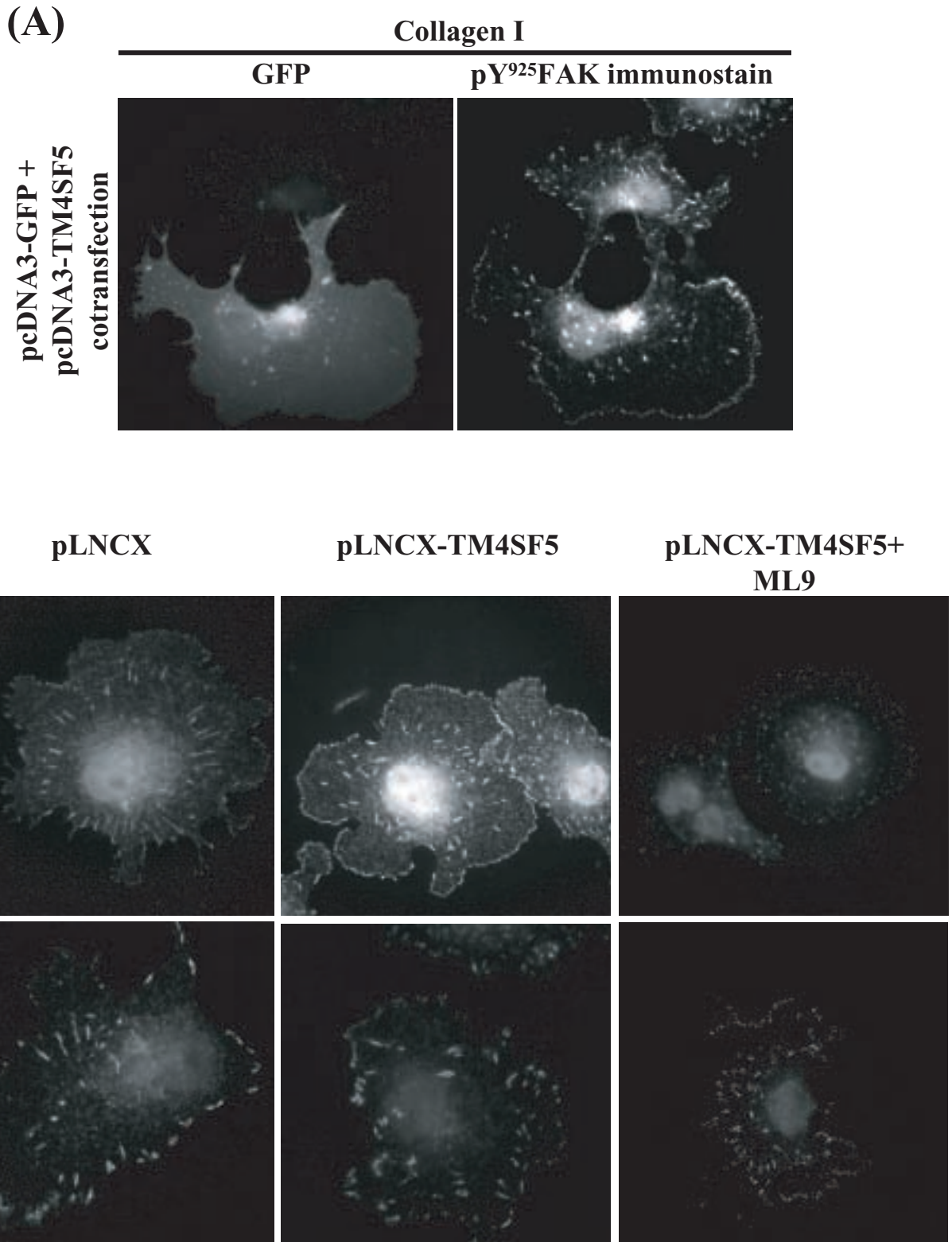


Figure 4C

Figure 4:.ECR-06-138

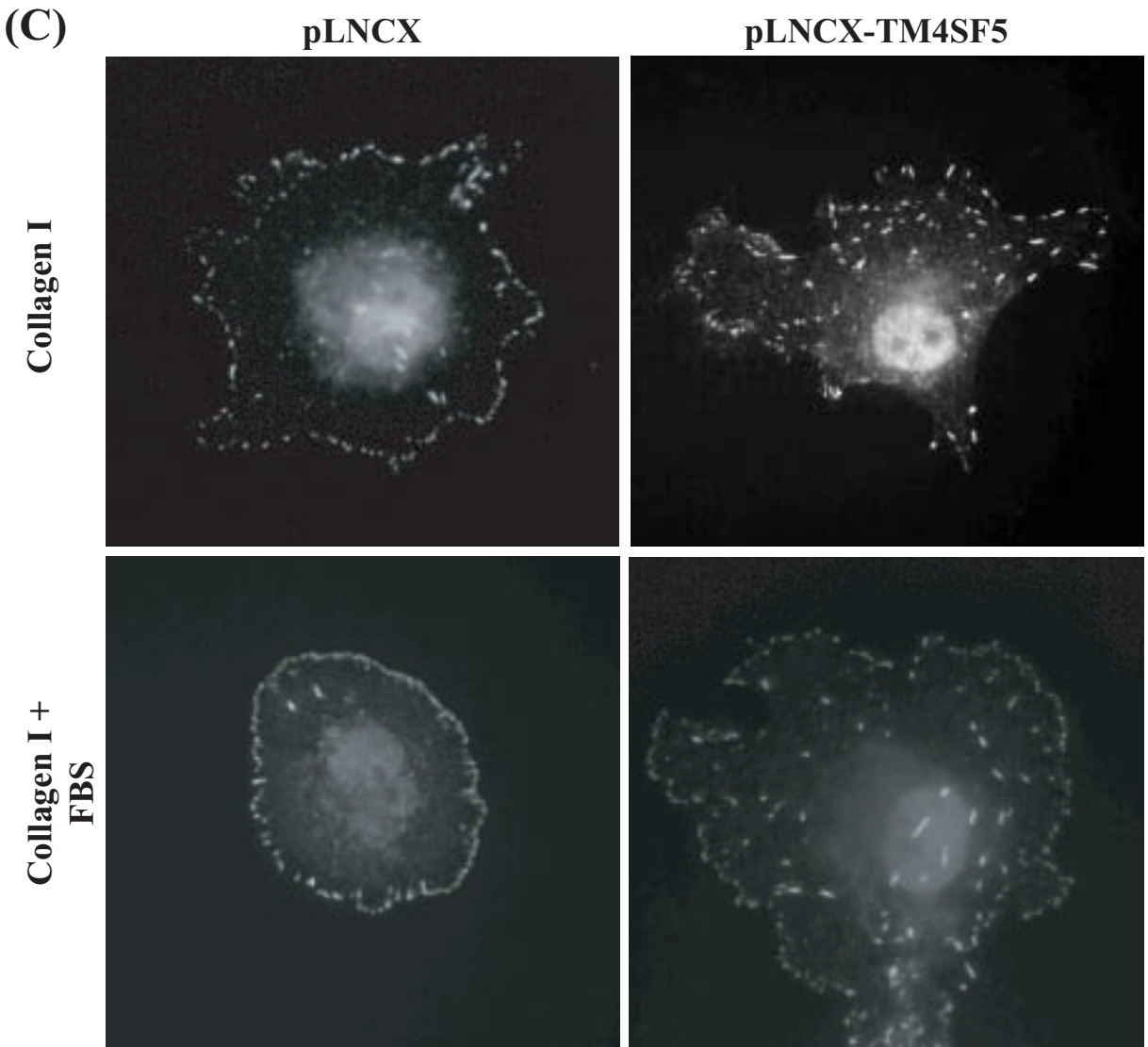


Figure 4D

Figure 4:.ECR-06-138

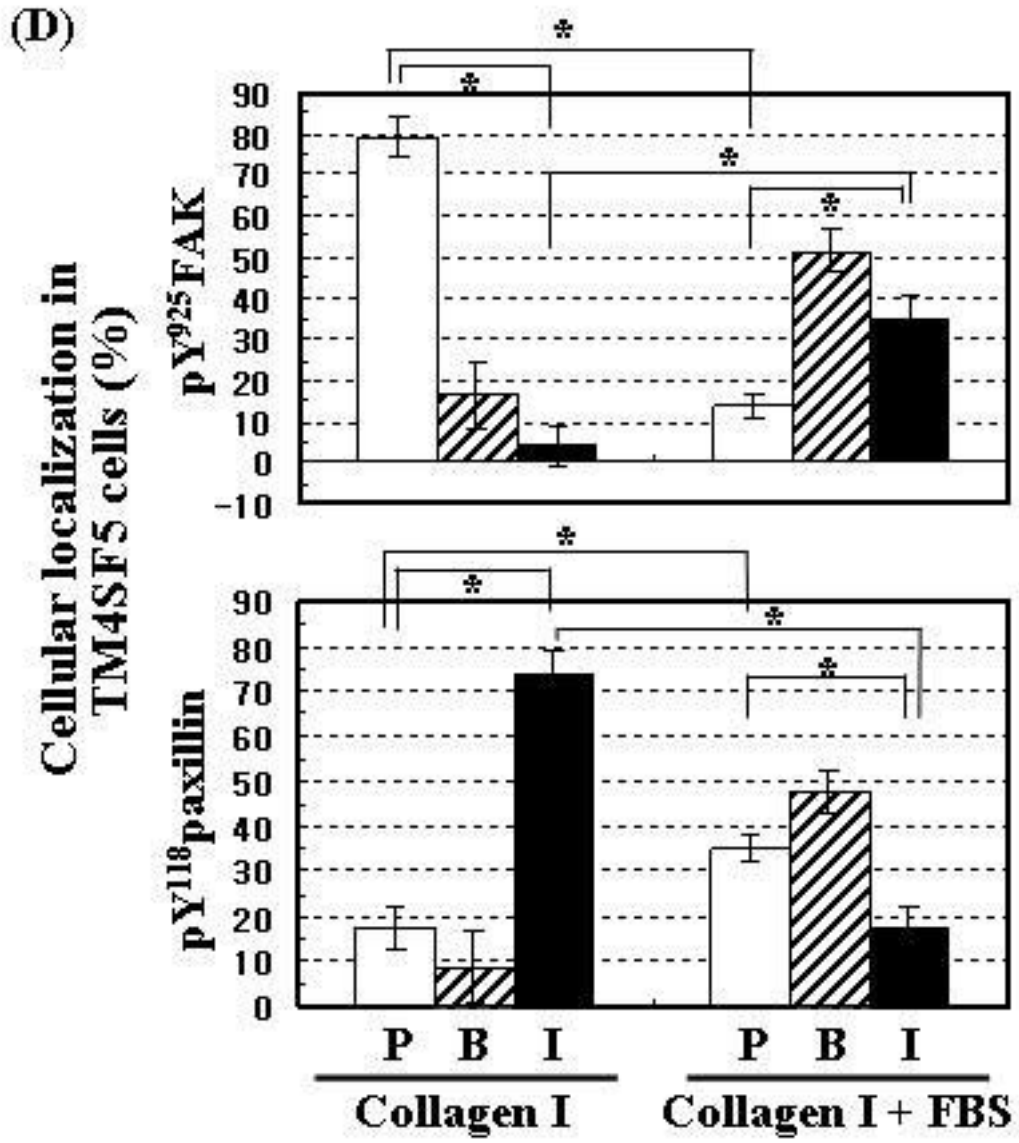
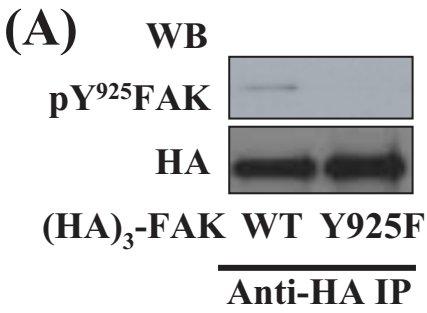
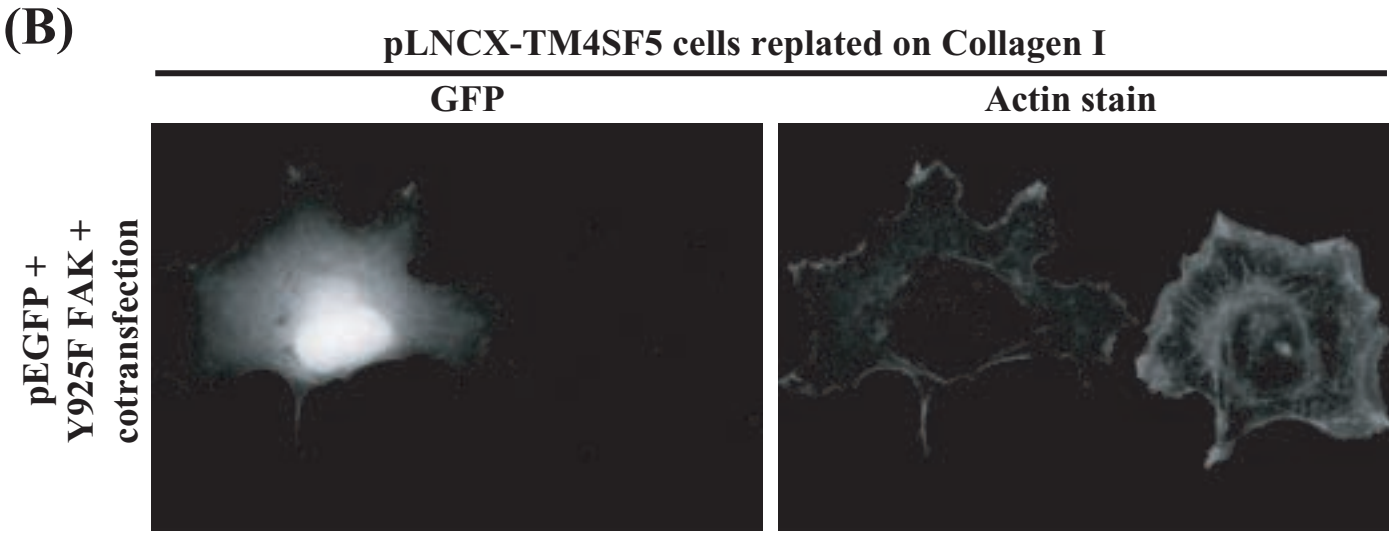


Figure 5:.ECR-06-138





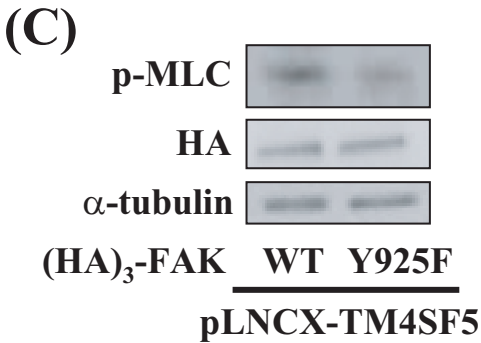
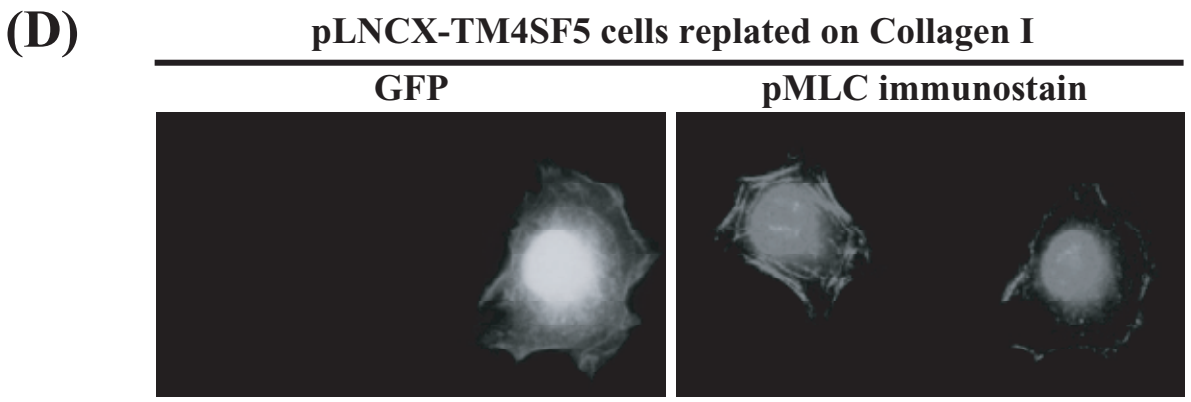


Figure 5:.ECR-06-138



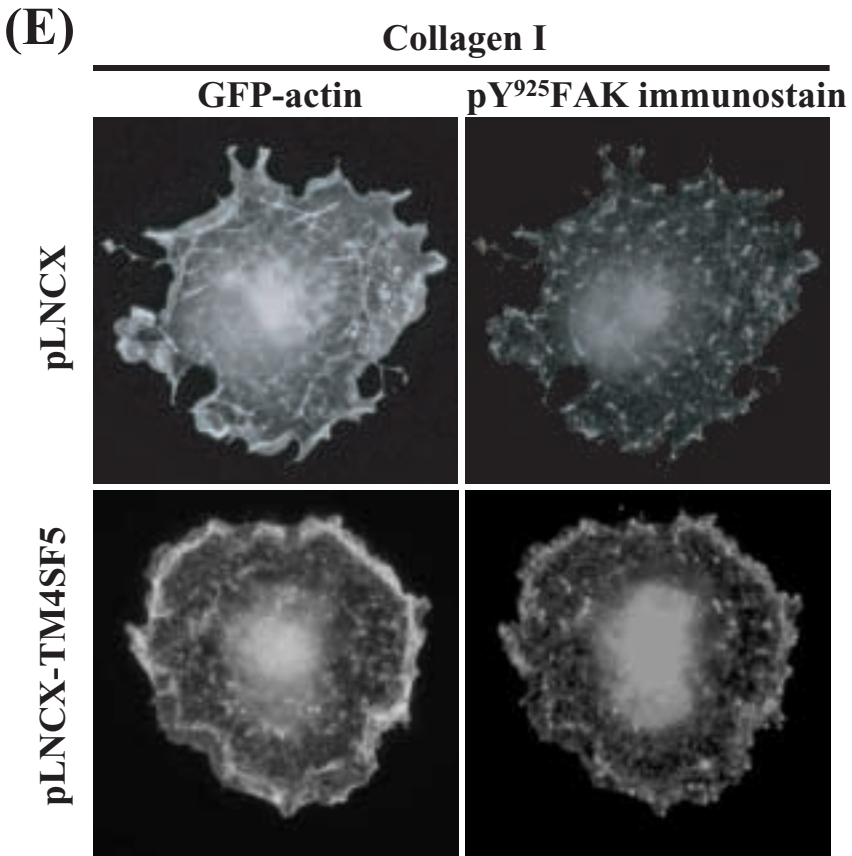


Figure 6:.ECR-06-138

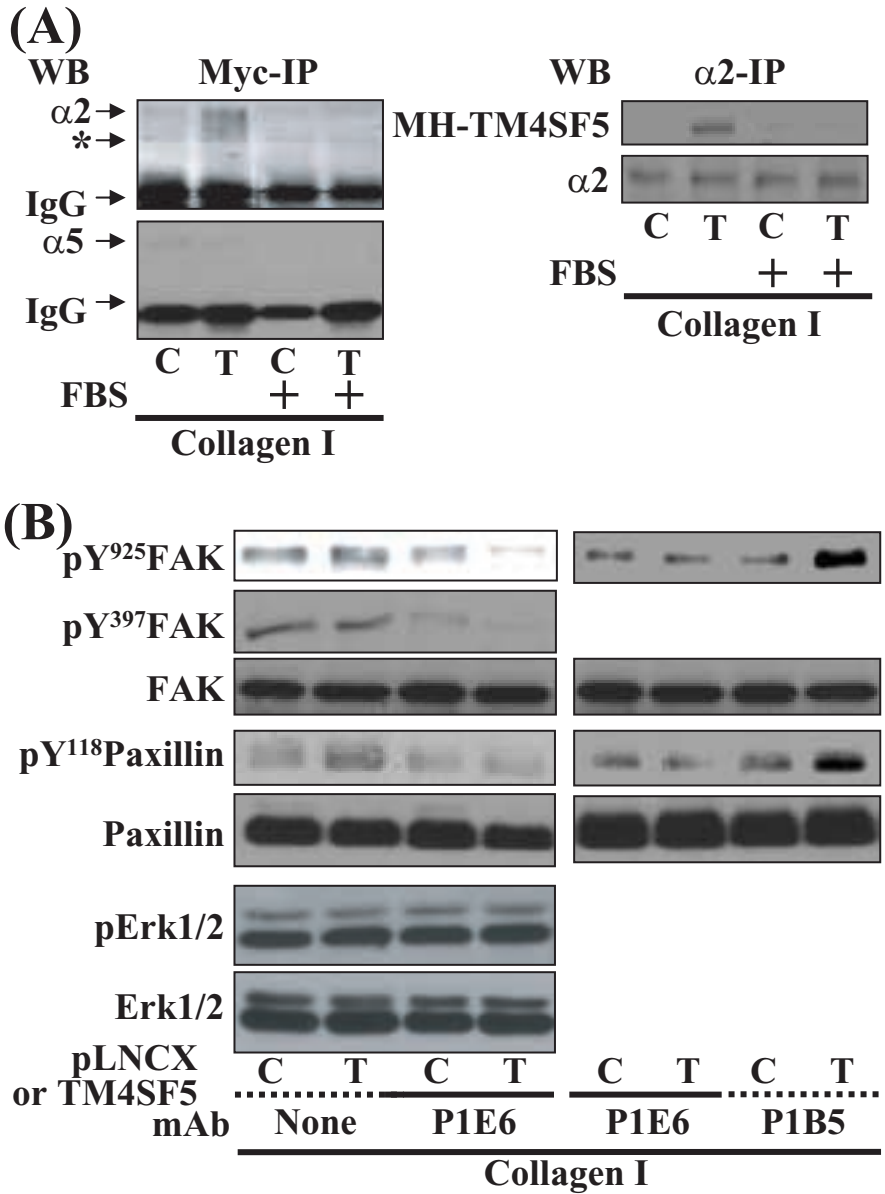


Figure 6C

Figure 6:.ECR-06-138

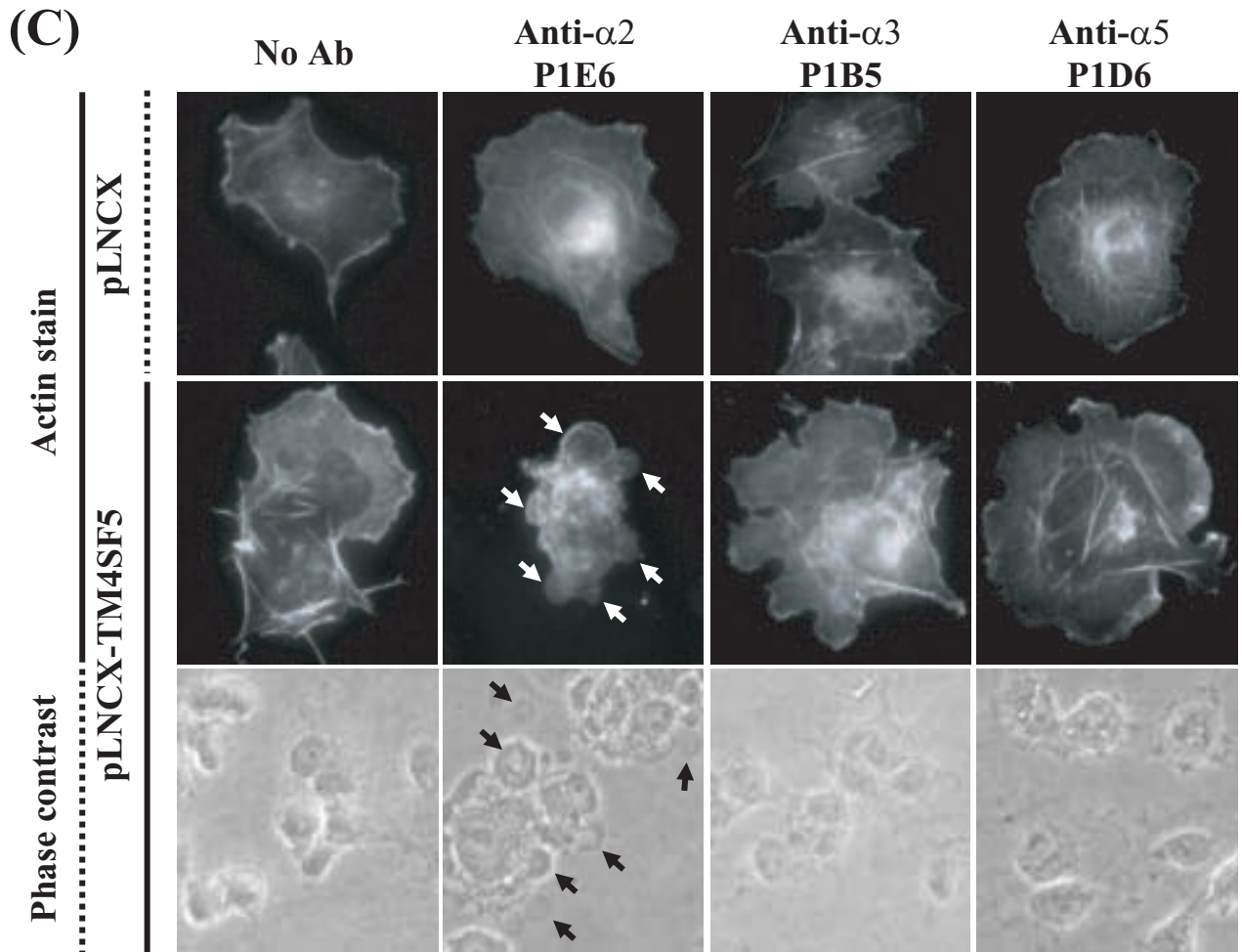


Figure 7A

Figure 7:.ECR-06-138

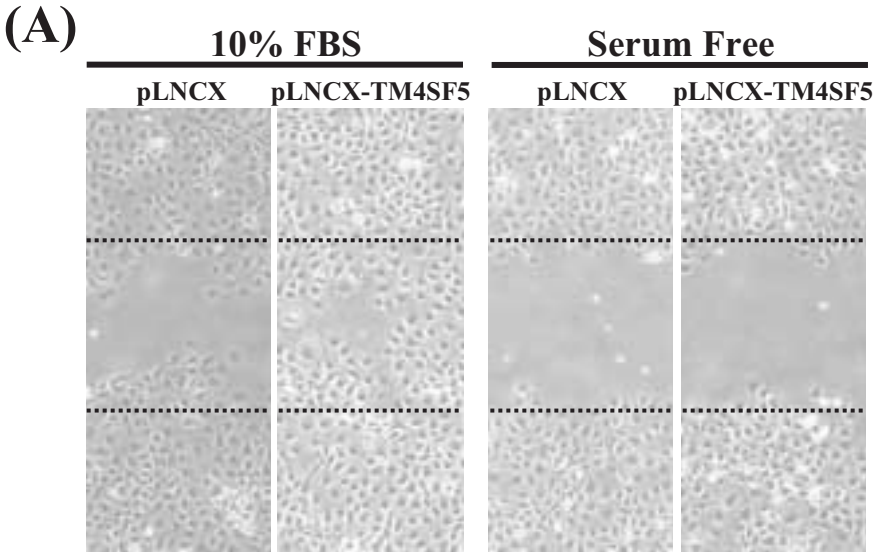


Figure 7B

Figure 7:.ECR-06-138