# The role of type V collagen fibril as an ECM that induces the motility of glomerular endothelial cells

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*Abbreviations* : Ab, antibody; BSA, bovine serum albumin; Col I, type I collagen; Col V, type V collagen; ECM, extracellular matrix; FAK, focal adhesion kinase; FAKpY397, FAK phosphorylated at tyrosine 397; FAKpY861, FAK phosphorylated at tyrosine 861; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GEC, glomerular endothelial cells; NG2, neuroglycan2; PBS, phosphate-buffered saline; paxillin–pS178, paxillin phosphorylated at serine 178; paxillin– pY118, paxillin phosphorylated at tyrosine 118; TBS, Tris buffer sulfate

**Abstract**

Although type V collagen (Col V) is present in developing and mature connective tissues of glomeruli, its primary function has not been elucidated yet. The purpose of this study was to elucidate the role of Col V fibrils in glomerular cells. We isolated primary cells from porcine kidney and cultured them on Col V fibrils reconstructed from purified Col V molecules extracted from porcine cornea. Time-lapse observation showed that Col V fibrils induce dynamic movement of glomerular endothelial cells (GEC) by stimulating them to extend long filopodial protrusions and wide lamellipodia. Col V signaling mediated through β1 integrin activated phosphorylation of paxillin at tyrosine 118 (paxillin–pY118) and of focal adhesion kinase at tyrosine 861 (FAKpY861) at the cell periphery; a second Col V signal was mediated through neuroglycan 2 and activated FAKpY397. FAKpY861 was present in loose attachment points between Col V fibrils and GEC, allowing the cells to migrate easily. Activation of FAKpY397 induced incomplete focal adhesion at the centers of cells and caused cell movement. Therefore both signaling pathways facilitated cell motility, which was inhibited by the addition of antibodies to β1 integrin, NG2, and Col V. We suggest that Col V fibrils activate ‘outside-in’ signaling in GEC and induce their dynamic motility.

## Introduction

Type V collagen (Col V) occurs in the kidney interstitium, mesangium, and the endothelial aspect of the glomerular basement membrane [1, 2]. Because Col V also is present on the glomerular basement membrane during collagenofibrotic glomerulopathy [3] and is expressed in the glomerular mesangial matrix during progeria syndrome [4], this protein has been considered to be an extracellular matrix (ECM) component involved in kidney disease. Other pathogenesis such as diffused connective tissue disease also reported the increased Col V in the intracellular matrix of myocardium in dilated cardiomyopathy [5], and Col V has also been considered playing a role in the control of fibrillogenesis [6,7]. In contrast, Col V also occurs in the pericellular–intercellular matrix at the onset of morphologic differentiation, during the S-shaped body stage of kidney development [8] and is thought to be an ECM necessary for the developing kidney. Why Col V causes disease in adult glomeruli when it is necessary for the differentiation of the developing kidney is unclear.

Cell culture is a useful method to study the function of the ECM on cells *in vitro*. Several studies using cell culture methods have reported that Col V is a negative ECM component for cell attachment. Col V was first described as causing the disassembly of F-actin filaments in cultured human endothelial cells [9]. Disassembly was elucidated by the fact that Col V failed to form the ECM-integrin-cytoskeleton complex [10]. The inhibitory effect of Col V on cell adhesion was reported later [11]. Recently, Col V fibrils were shown to have an intermediate effect of Col I and Col IV collagen by which satellite cells and hepatocytes can be localized in liver separately [12]. Mizuno and Hayashi developed a new method for reconstituting Col V fibrils in vitro, thereby

enabling studies to distinguish between the functions of Col V fibrils and Col V molecules [13, 14]. Fibroblast cultures showed that cell detachment from the dish coated with Col V fibril is not due to incomplete cell adhesion but to the unstable cellular state induced by Col V fibrils despite strong cell adhesion [15].

Although the previously cited studies unveiled the effect of Col V on cells, all of the experiments were limited by their use of cell lines as cell sources. Because no glomerular cell lines are currently available, the relationship between Col V and glomerular cells has remained unclear. Moreover, the disadvantage of using cell lines in studying the ECM is that they have high proliferative ability as well as innate dynamic activity, and they are relatively insensitive to the cellular microenvironment and can scarcely be influenced by the surrounding environment. To clarify the relationship between glomerular cells and Col V and to characterize the response of glomerular cells to Col V fibrils and the effect of Col V fibrils on glomerular cells, we isolated glomerular endothelial cells (GEC) from porcine kidney and established primary cell cultures on Col V fibrils, which more closely mimic the in vivo environment than does culture on collagen molecules.

Among the various collagens, Col I is most commonly used as a scaffold in cell cultures. Col I supports the mechanical structure framework for bone tissue [16], and synthesized Col I polymers have been used as scaffolds on micropatterned surfaces for directed cell growth [17], and tissue engineering has made use of the association between Col I with growth factors [18]. Therefore, Col I is considered a typical ECM that can provide cells with a static environment. In the present study, we used Col I fibril as a control scaffold against which to compare the effect of Col V fibrils on GEC, especially in distinguishing the microenvironmental changes associated with the

dynamic versus static states. In addition, we performed a time-lapse investigation of the cellular morphology, which revealed that Col V fibrils induce GEC dynamics through 'outside-in' signaling via two ECM receptors, β1 integrin and neuroglycan 2 (NG2). These two receptors activated the phosphorylation of different adaptor proteins to induce cell attachment at the central site and detachment at the periphery of cells. Such mechanisms of the cellular response to Col V in the ECM may explain why Col V fibrils are unfavorable to adult renal glomeruli, which typically do not migrate in normal kidney tissue. On the other hand, such mechanism may also suggest Col V is an ECM initialize cell migration during the regeneration process of injured tissue.

## Materials and Methods

*Preparation of molecules and fibrils of Col I, Col IV, and Col V*

Col V was prepared from pig cornea according to methods described previously [12, 15, 19, 20, 21, 22] with modification. All procedures were conducted in clean bench. Briefly, the corneas isolated from 50 pig eyes (purchased from Tsuchiura slaughterhouse, Ibaraki, Japan) were homogenized in liquid nitrogen, suspended in 500 ml phosphate-buffered saline (PBS, pH 7.2) with stirring at 4$^{o}$C for 3 days, and then centrifuged at 9000 × *g* for 50 minutes. The precipitate was suspended in 500 ml PBS containing 4 M urea and 1 M NaCl at 4$^{o}$C for 2 weeks and centrifuged at 9000 × *g* for 50 minutes.

For separation of Col V from Col I, the precipitate was suspended in 500 ml PBS containing 0.01% 2-mercaptoethanol at 4$^{o}$C for 7 days, followed by centrifugation at

9000 × *g* for 50 minutes. The precipitate then was suspended in 500 ml 0.5 M acetic acid; 700 μg pepsin then was added under stirring. The suspension was stirred at 4°C for 1 day and centrifuged at 9000 × *g* for 50 minutes to obtain a supernatant enriched in Col V. Powdered NaCl was added to the supernatant under stirring to a final concentration of 0.7 M; the resulting solution was stirred at 4°C for 1 day. The solution was centrifuged at 9000 × *g* for 50 minutes to separate Col V molecules into the supernatant and Col I molecules into the pellet.

To purify the Col V solution, the supernatant was dialyzed against 0.05 M acetic acid and then PBS containing 2 M urea and 0.07 M NaCl. The eluate underwent heparin–Sepharose CL6B (Amersham Bioscience, Uppsala, Sweden) column chromatography; the bound resin was washed 3 times with PBS containing 2 M urea and 0.07 M NaCl. Bound protein was eluted twice with PBS containing 0.5 M NaCl and 2 M urea and once with PBS containing 1 M NaCl and 2 M urea. The 3 protein-containing eluates were combined and dialyzed twice against 10 volumes of 0.05 M acetic acid, and the purified Col V solution was stored in 0.05 M acetic acid at 4°C until reconstruction of collagen fibrils. Purified Col I solution was obtained by the same method, except that the precipitate containing Col I molecules was first suspended in 0.5 M acetic acid. The purity of all molecule solutions was confirmed by subjecting 0.1-mg/ml samples to 7.5% SDS-PAGE.

Col V fibrils were reconstituted through the following steps: urea and NaCl were added to 10 ml of purified Col V solution to final concentrations of 1 M each. The solution was neutralized by the addition of 250 μl 2 M Tris-HCl (pH 8.0) with stirring. After neutralization, the solution was dialyzed overnight against PBS containing 1 M urea and 1 M NaCl, against PBS twice during each dialysis, and against 10 volumes of

PBS in a 34°C water bath to form collagen fibrils. The solution containing collagen fibrils was twice dialyzed against 10 volumes of PBS (pH 7.2) at 4°C and then stored before use. Col I fibrils were prepared by the same method [14].

*Isolation of GEC*

Glomeruli were isolated from pig kidneys (purchased from Tsuchiura slaughterhouse, Ibaraki, Japan) according to the method described previously [23]. For isolation of GEC, DHSF medium (per liter: DMEM, 5 g; Ham F12, 5.3 g; $NaHCO_3$, 1.9 g; ITS, 6.25 mg, EGF, 1 μg; supplemented with 10% FBS) was used during the first 4 days of culture. Glomeruli were seeded on dishes and examined after outgrowth at 37°C in a 5% $CO_2$ incubator. When cell outgrowth reached 50%, each dish was gently treated with 0.025% trypsin for 30 seconds and rinsed twice with PBS to remove fibroblasts and glomerular debris, and then GEN medium (medium for glomeruli endothelium cells; per liter: RPMI, 10.4 g; heparin, 50 mg; βECGF (R&D Systems), 10 μg; penicillin, 5000 U; streptomycin, 5 mg; supplemented with 10% FBS) was added to the dish to continue cell culture for 2 days. After cell colonies appeared, stainless steel cloning rings (inner diameter, 0.8 cm) were placed to isolate individual colonies. Cell colonies were trypsinized, and the dispersed cells were rinsed twice with PBS and then re-seeded on dishes coated with Col I. Cells that stained positively for the endothelial marker Factor VIII (Chemicon; data not shown) were used for subsequent experiments.

*Preparation of collagen-coated cover slips and culture dishes*

To each 13-mm glass cover slip, we dropwise added 500 μl collagen fibril solution; the cover slip was incubated at 37 °C in a 5% $CO_2$ incubator for 6 hours and then washed with PBS.

For cell culture, we used glass-bottom culture dishes (Matsunami, Odaka, Japan) that each contained a collagen-fibril–coated culture in the center. GEC ($2.5 \times 10^5$ cells in 250 μl) were seeded on cover slips, which had been coated with either Col I or Col V fibrils. The cells were incubated at 37 °C in 5% $CO_2$ incubator for 24 hours, after which 2 ml of GEN medium was added. The culture medium was changed every 3 days.

*Immunofluorescent staining*

GEC cultured on cover slips were fixed with 10% formalin in PBS for 10 min at ambient temperature and then rinsed with PBS. Cells were incubated in blocking buffer (0.1% BSA in PBS) for 15 min, rinsed four times with PBS, and then incubated in primary antibody (Ab; 1 to 10 μg) for 50 minutes. Cells were washed four times in PBS, followed by incubation with a fluorescent secondary Ab for 50 minutes and four washes in PBS. To distinguish actin fibers from other cellular proteins, nonspecific binding was blocked by incubating the cells in 0.1% BSA in PBS; cells then were washed four times in PBS and incubated in rhodamine–phalloidin (Molecular Probes) for 50 minutes. After four final washes with PBS, cover slips were inverted on glass slides (Matsunami), mounted with Gel Mount (Biomeda), and sealed to the slide with Mount Quick (Daido Sangyo). Immunofluorescently stained cells were analyzed by microscopy and photographed with a charge-coupled-device (CCD) camera.

*Microscopy*

Fluorescent cells were observed on an inverted microscope (model DMRB/E, Leica) linked to a color cooled CCD camera (VB7000, Keyence) or on an inverted microscope (model IX81, Olympus) linked to a monochrome cooled CCD camera (model DP30-BW, Olympus). Focal adhesion proteins in cells were observed by using a TIRF (Total Internal Reflection Fluorescence) microscope (BX2WI-TIRFM, Olympus) with a 60× water-immersion objective lens (LUMPlanFl60) linked to an emissioned enhanced CCD (EMCCD) camera (model C9100-13, Hamamatsu Photonics). Single cross-section images were obtained from the cells with a scanning confocal microscope (Leica TCS SP2), and whole-cell view was observed with maximum projection. Time-lapse observation was conducted by fitting a small $CO_2$ incubator (model MI-IBC-IF, Tokai-Hit) onto the previously described inverted microscopy system (Leica DMRB/E, Keyence VB7000) with a 20× dry objective lens and photographing every 3 min for 12 hours.

*Electron microscopy*

Copper grids were coated with a formvar film. A drop of the sample solution, 100 $\mu$ l, was placed on a sheet of parafilm (American Can Company). A 100-mesh copper grid was floated on the surface of the drop for 30 s. The sample was then fixed with a drop of 2% glutaraldehyde in the collagen-incubated buffer for approximately 30 s. The grid was then washed twice with a drop of distilled water, then stained with a drop of 0.5% uranyl acetate solution on the parafilm sheet for 1 min. Glutaraldehyde solution and

distileed water were warmed to the temperature used for sample incubation. The specimens thus prepared were examined with a transmission electron microsope JEM 1200 EX (JEOL Ltd, Tokyo) operating at 100kV.

*Cell extracts and immunoblotting*

To prepare cell extracts, cells cultured on dishes coated with Col I or Col V fibrils were trypsinized and counted. $1x10^5$cells cultured on the two substrates, respectively were rinsed three times with PBS and then lysed in 500 µl lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 05% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 1× protease inhibitor cocktail [Roche Molecular Biochemicals], and 1 mM sodium vanadate) for 15 min on ice. The lysates were centrifuged (model 5415R, Eppendorf) at $4^oC$ to remove debris, and the supernatants were resolved by 7.5% SDS-PAGE, transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare Bio-Sciences), and stained with Ponceau S to visualize total protein. After the membrane was washed with 0.1% Tween-TBS (pH 7.5), proteins were probed with the appropriate primary and secondary antibodies, and the resulting bands were then scanned (Odyssey Infrared Imaging System, Li-Cor) for data analysis. In this system, green bands indicated phosphorylated proteins containing tyrosine and serine residues, red bands comprised the targeted protein, paxillin, and yellow bands corresponded to phosphorylated paxillin.

Western blotting was used to detect phosphorlyation of focal adhesion kinase at tyrosines 861 and 397 (FAKpY861 and FAKpY397, respectively). Proteins on the transferred membrane were probed with the appropriate primary antibody, followed by incubation with an anti-rabbit IgG peroxidase-conjugated secondary antibody, and

finally visualized by chemiluminescence (ECL kit, GE Healthcare Bio-Sciences)

*Antibodies*

For cell staining, the primary antibodies used were rabbit monoclonal Abs against phosphopaxillin (paxillin–pY118; Santa Cruz Biotechnology), FAKpY861 (Sigma), FAKpY397 (Sigma), NG2 (Chemicon), and β1 integrin (Chemicon). Fluorochromes conjugated to primary antibodies were Alexa Fluor 350, Alexa Fluor 488, and Alexa Fluor 594 (all from Molecular Probes). For Western blotting, the primary antibodies used were mouse monoclonal Ab against GAPDH (Chemicon), mouse monoclonal Ab against phosphoserine (Biomedia), mouse monoclonal Ab against phosphotyrosine (Zymed) and rabbit monoclonal Ab against paxillin (H-114, Santa Cruz). The secondary antibodies were Alexa-680 anti-rabbit IgG (Molecular Probes) and IR-Dye800 anti-mouse IgG (Rockland).

**Results**

*Col V fibrils induce the dynamic motility of GEC*

Fig. 1 shows TEM of Col V molecules and reconstituted Col V fibrils. Super structure of fibrous form was detected only in the reconstituted fibrils of Col V (Fig. 1B), while such kind of structure was not detected in Col V molecules (Fig. 1A).

GEC adhere to the glomerular basement membrane, and dynamic motility of cultured adult glomerular cells has not been reported previously. Surprisingly, mature GEC

cultured on Col V fibrils showed continuous dynamic migration during the initial stage of their adhesion to the fibrils. In contrast, cells cultured on type I collagen (Col I) fibrils migrated slowly. Time-lapse observation showed that GEC continued to migrate for 12 hours after they were seeded on Col V fibrils (Fig. 2A, A1~A3). Arrows in Fig. 2A1, 2A2 and 2A3 indicated the same cell changed its shape and location during dynamic migration. The dramatic cellular migration was accompanied by the extension of long fillopodia at the edges of cells (Fig. 2B). Moreover, GEC on Col V fibrils also showed dynamic lamellipodia at the moving head (Fig. 2C, arrow head). However, GEC cultured on Col I fibrils moved slowly and in a limited area, with only ruffling of the membrane at the cell periphery (Fig. 2D).

Because α1β1 integrin and α2β1 integrin are the receptors for Col V [24], and because NG2, a membrane-spanning proteoglycan containing chondroitin sulfate chains, binds to the rod domain of Col V [25, 26, 27], we used fluorescent microscopy to examine the roles of β1 integrin and NG2 on GEC cultured on Col V fibrils after 24 h of culture. Cells cultured on Col I fibrils were used as controls. No NG2 was detected in control cells (Fig. 3A), whereas NG2 was localized near the center of cells and beneath the nucleus in cells cultured on Col V fibril (Fig. 3B). β1 integrin was detected along the cytoskeleton of control cells (Fig. 3C) but at the migration edges of cells cultured on Col V fibrils (arrows in Fig. 3D).

To investigate whether the various expression levels and locations of the receptors were mainly due to the fibrillar structure of Col V or whether Col V molecules can cause the same results, we compared cells cultured on Col V fibrils with those cultured on Col V molecules. Only Col V fibrils resulted in specific patterns of receptor expression. In contrast to the low expression of NG2 (visualized as blue signal) in cells

cultured on Col V molecules (Fig. 4A), those cultured on Col V fibrils showed high NG2 expression (Fig. 4B). Interestingly, β1 integrin was found throughout cells cultured on Col V molecules (Fig.4C), whereas it was mostly located at the edges of cells grown on Col V fibrils (Fig. 4D). The actin cytoskeleton (stained with rhodamine–phalloidin; red signal) formed stress fibers and stretched homogeneously throughout cells cultured on Col V molecules (Fig. 4E), whereas the actin cytoskeleton was located mainly at the periphery of cells cultured on Col V fibrils (Fig. 4F).

To further investigate the change of expression and location of β1 integrin, TIRF microscopy was used to compare GEC cultured for 24 h on Col I fibrils with those grown on Col V fibrils. The advantage of TIRF microscopy is its ability to precisely monitor proteins present in the narrow region between the cell surface and the collagen fibril by focusing light on the adhesion surface. Whereas β1 integrin was spread throughout control cells (Fig. 5A), it gathered at the dynamic migration edge of cells cultured on Col V fibrils (Fig. 5B arrow). Merging the image of β1 integrin with that of the rhodamine–phalloidin–labeled actin cytoskeleton suggested the co-localization (yellow signal) of β1 integrin and the actin cytoskeleton at the cellular migrating edge (Fig. 5D arrow). However, control cells lacked noteworthy overlapping of signals (Fig. 5C).

*Cell migration signals are mediated by ECM receptors of GEC cultured on Col V fibrils*

To determine whether ECM receptors mediate an ‘outside-in’ signal from Col V fibrils to induce the motility of GEC, we investigated the phosphorylation of two adaptor proteins, paxillin and FAK, and their intracellular localization. Paxillin phosphorylated

at tyrosine 118 (paxillin–pY118) was detected at the central bottom of control cells cultured for 24 h (Fig. 6A), whereas it was localized along the leading edge of cell periphery forming focal points in GEC cultured on Col V fibrils (Fig. 6B arrow). FAK phosphorylated at tyrosine 861 (FAKpY861) was located all along the bottom of control cells (Fig. 6C) but was limited to the protruding edge of fillopodia as small focal points when cells were cultured on Col V fibrils for 24 h (Fig. 6D, arrow). ECM signaling through integrin is known to induce both paxillin–pY118 and FAKpY861 and promote cell motility. The findings shown in Figure 5 indicate that paxillin–pY118 may induce the dynamism at the cell adhesion surface through the movement of short chains of actin. In comparison, FAKpY861 may be involved in the formation of focal adhesion appropriate to dynamic motility of cells, maintaining both the small focal points at the leading edges of fillopodia (arrow in Fig. 6D) and those in lamellipodia (arrowhead in Fig. 6D). Focal points reduce the assembly of focal adhesion and thus can prevent the formation of a large secondary cluster of focal complexes, which usually blocks dynamic cell movement [28]. We also investigated FAKpY397, a phosphoprotein that typically functions concurrently with FAKpY861 [27]. FAKpY397 was located centrally in basal area of GEC cultured on Col V fibrils (Fig. 6F), but could hardly detected throughout control cells (Fig. 6E).

To confirm that outside-in signaling from Col V fibrils to GEC exists and can be mediated by the receptors β1 integrin and NG2, antibody against Col V was added to cell culture to block Col V signaling outside of cells directly. Antibodies against β1 integrin and NG2 were also added to cell culture, respectively, to block receptors such that outside-in Col V signaling cannot be mediated to cells via receptors. All antibodies were added to cells, respectively, right after cells were seeded in separate cell cultures.

To analyze whether outside-in signaling from Col V fibrils induced cell dynamics, we followed the localization of paxillin–pY118 for 24 h. Paxillin–pY118 was present at the periphery of GEC in the absence of inhibition by the anti–Col V antibody (Fig. 7A). In contrast, paxillin–pY118 disappeared from the periphery of cells and shifted centrally in cells when the signaling of Col V fibrils was blocked by the anti–Col V antibody (Fig.7B). In addition, paxillin–pY118 was localized peripherally in the absence of the anti–β1 integrin antibody (Fig. 7C), but it shifted to the center of cells when in the presence of anti–β1 integrin (Figs. 7D). Control cells cultured on Col I showed no distinct change in the localization of paxillin–pY118 regardless of whether anti-β1 integrin Ab or anti-Col V Ab was added or not added at the beginning of cell culture (data not shown). These results confirmed that Col V fibril signaling is mediated by β1 integrin, which is related to the phosphorylation of paxillin and the change of its localization from the periphery to the center of cells.

In addition to the outside-in signaling regulated through β1 integrin, we examined whether NG2 might also mediate signal from Col V fibrils to cells. Control GEC grown on Col V fibrils without inhibition of NG2 were elongated with fillopodia (Fig. 8A), whereas cells treated with the anti-NG2 antibody spread widely (Fig. 8B). In addition, paxillin–pY118 was distributed at the periphery or edges of control cells cultured on Col V fibrils (Figs 8A, 8C), whereas it was spread diffusely and evenly throughout cells when NG2 was inhibited (Figs 8B, 8D). Further, FAKpY397, which is generally understood to activate cell migration [29, 30] and which may mediate signal from Col V fibril through NG2, was localized centrally in control GEC (Fig. 8E). In contrast, this accumulation of FAKp397 disappeared when cells were cultured on Col V fibrils under NG2 inhibition (Fig. 8F).

The possible outside-in signaling from Col V fibrils through NG2 and FAKpY397 is summarized in Figure 9. When cell dynamism is induced on Col V fibrils (Fig. 2A), both receptors are involved. NG2, which is located centrally in cells (Fig.9A), mediates the outside-in signal and activates FAKpY397 at the same location (Fig. 9B). In contrast, β1 integrin, which is located at the leading edge of filopodia (Fig. 9C), also mediates outside-in signals from Col V fibrils but activates the phosphorylation of other site of FAK, FAKpY861, at the same location (Fig. 9D). In addition, β1 integrin also mediates signals at the migration front of lamellipodia (Fig. 9E) and activates paxillin–pY118 at the same location (Fig.9F). A model indicating the localizations of both cell receptors that respond to Col V signaling is shown in Figure 9G, in which β1 integrin localizes at the migration edge of cells to mediate the dynamic signals and trigger cell motility, whereas NG2 localizes in the center of cells to mediate the stabilizing signal and form focal adhesions. A model of the outside-in signaling of Col V fibril mediated by NG2 and β1 integrin to activate phosphorylation of FAK and paxillin is shown in Fig. 9H. NG2-mediated Col V signaling involves in the phosphorylation of the adaptor protein FAKY397, whereas the signals of Col V fibrils that are mediated by β1 integrin involve in the phosphorylation of FAKY861 at the leading edge of fillopodia and of paxillin–Y118 at the migration front of lamellipodia.

*Quantification of phosphorylated proteins in cells cultured on type V collagen fibrils*

The phosphorylation of tyrosine 118 and serine 178 of paxillin is known to involve in the induction of cell motility [31, 32]. We used Western blotting to investigate the amounts of tyrosine- and serine-phosphorylated paxillin in GEC cultured on Col V

fibrils. Cells cultured on Col I fibrils were used as control cells. The resulting bands were analyzed by Odyssey Infrared Imaging System. The imaging system comprises two channels for multi-assays. One channel is used to detect the bands of control proteins, and the other channel the bands of target proteins. Lysate collected from cells right before seeding at 0 hr was used as control protein because neither focal adhesion nor phosphorylation occurred at zero time. The data of band intensity of target proteins was calculated by comparing the scanned density of each band to the control band which is standardized as 1. The yellow band derived from GEC cultured on Col V fibrils for 3 d, indicating the phosphorylation of tyrosine in paxillin (Fig. 10 A), is more intense (3.02) than that of control cells cultured on Col I fibrils (1.80). Similarly, GEC cultured on Col V fibrils for 3 d contained more serine-phosphorylated paxillin (4.51) than did control cells (2.55; Fig. 10 B); total paxillin values were 5.50 and 3.23, respectively. In contrast, the amount of GAPDH in the cell lysate from GEC cultured on Col V (6.16) was similar to that of cells grown on Col I fibrils (6.22) (Fig. 10C).

Tyrosine phosphorylation of FAK is also involved in the induction of cell motility. We performed Western blotting to investigate the phosphorylation of FAK and paxillin in cells cultured on Col V or Col I fibrils for 4, 7, and 20 d. Cell lysate collected from cells right before seeding at zero time was used a control protein for each western blot and was scanned and standardized as 1. Tyrosine-phosphorylated paxillin (yellow band) was activated at 4 d, and non-phosphorylated paxillin (green band) was activated at 20 d (Fig. 10 D). In addition, FAK was present from day 4 through day 20 in cells cultured on Col V fibrils. The intensity of tyrosine-phosphorylated FAK peaked at 4 d, decreased at 7 d, and increased again at 20 d when GEC were cultured on Col V fibrils. However, control cells showed no tyrosine phosphorylation of FAK during the early period of

days 4 through 7. Tyrosine phosphorylation of paxillin followed the same pattern. These findings suggest that Col V initiates cellular dynamism early during cell growth on Col V fibrils, leading to the phosphorylation of adhesion adaptor proteins and the formation of focal adhesion to stabilize cells.

To clarify whether FAK phosphorylation induced by Col V fibrils continues in a long term, we examined the amounts of FAKpY861 and FAKpY397 at day intervals by Western blotting (Fig. 11). A band of 120 kD indicates FAK protein, and the phosphorylation of FAK causes a slight band shift on SDS-PAGE. Unlike control cells cultured on Col I, GEC cultured on Col V fibrils yielded a band larger than 120 kD (Fig. 11C), indicating that Col V fibrils continued and promoted phosphorylation of FAK at tyrosine 861 after 3, 10, and 30 d of culture. The amounts of FAKpY397 were similar between GEC cultured on Col V and Col I fibrils for 3 d (Fig. 11D), whereas cells cultured on Col I fibers had more FAKpY397 on days 10 and 30 than did those grown on Col V fibrils.

**Discussion**

Type V collagen (Col V) is present in kidney, especially in the glomerular mesangium and mesenchyme of adult tissue [1, 2]. It has been noted that Col V is present on the glomerulopathy [3] and other connective tissue diseases [4, 5]. Col V also is expressed in the developing kidney during the formation of the S-shaped body, which is a precursor tissue of glomeruli [8]. Although Col V has been reported to involve in the control of fibrillogenesis [6, 7], the real role that Col V fibrils play in glomerular cells has not yet been elucidated in detail.

Assembled matrices are a useful model of the *in vivo* environment that facilitates detailed studies of the behavior of cells in culture [13]. Here we used Col V fibrils as a scaffold to uncover their effect on adult GEC and elucidate the mechanism of the interactions between Col V fibrils and glomerular cells.

In time-lapse studies, Col V fibrils induced dynamic behavior by GEC, which formed various adhesion clusters. To elucidate why Col V fibrils induced GEC dynamics, we investigated the pattern of association between GEC and Col V fibrils and found that two receptors, β1 integrin and NG2, were localized differently in cells although they were expressed concurrently. NG2 was expressed in the central basal area of cells, whereas β1 integrin was found at the migration edge of cells.

We noted that small adhesion points formed at the migration edge and the tip of filopodia of GEC when they were cultured on Col V fibrils; we then investigated the relationship between the formation of these adhesion points and the phosphorylation of various adaptor proteins. Association between the ECM and integrin triggers the recruitment of adaptor proteins to form protein clusters that mediate focal adhesion at the basal site of cells [28]. Proteins form a small initial cluster at the initial stage of focal adhesion, and a large subsequent cluster containing more proteins is recruited after the activation of paxillin phosphorylation. The initial small cluster is known as a focal point, and the second cluster, called focal adhesion, is formed in a shape of large plaque [33]. The pattern of cluster formation affects cell attachment and detachment and is regulated by the phosphorylation of paxillin and FAK [28, 34].

Paxillin and FAK regulate the activity of small G protein exchange from GTP to GDP. In particular, the phosphorylation of paxillin is involved in the induction of cell migration, cell motility, and inactivation of Rho A [31, 35]. FAK is involved in the

signaling of focal adhesions. FAKpY397 stimulates the turnover of focal adhesions through the activation of Src and disassembly of α-actin [30, 36], whereas FAK pY861 induces cell detachment from the ECM and promotes cell motility through the activation of ERK signaling [30, 37]. Moreover, phosphorylation of the FAT domain leads to the dissociation of talin from the initial protein cluster through the activation of Grb2 and subsequent turnover in focal contact [29].

In our study, phosphorylated paxillin (paxillin–pY118) was localized at the migration edge of GEC cultured on Col V fibrils (arrow in Fig.6B); β1 integrin showed the same distribution (Fig. 3D, 5B). FAKpY861 was localized at the tips of fillopodia (arrow in Fig.6D) and the migration edge of lamellipodia (arrowhead in Fig.6D), whereas FAKpY397 was present at the center of cells (Figs. 6F, 8E, 9B). When the outside-in signaling from Col V fibrils was blocked by anti–Col V or anti–β1 integrin antibodies, paxillin–pY118 shifted from the migration edge (Figs. 7A, 7C) to the center (Figs. 7B, 7D) of cells. These facts support a mechanism in which β1 integrin recognizes and mediates the outside-in signaling of Col V fibrils, resulting in recruitment of paxillin and its phosphorylation to paxillin–pY118 at the migration edge of cells, thus leading to dynamic migration at the edges of cells.

Blocking of NG2 and its mediation of the outside-in signal of Col V led to morphologic changes from the elongated migrating configuration (Fig.8A) to the wide-spreading pattern (Fig.8B). Concurrently, paxillin–pY118 shifted from the periphery (Fig.8C) to a distribution throughout the cells (Fig.8D). The expression of FAKpY397, which had been distributed at the centers of cells before blocking of NG2 (Fig.8E). disappeared when NG2 was blocked (Fig.8F). These findings support a mechanism in which both NG2 and β1 integrin recognize the outside-in signaling of Col

V fibrils. Perhaps the Col V outside-in signal that is mediated through NG2 activates the recruitment of FAKpY397, after which activation of FAKpY397 induces cell attachment at the centers of cells to maintain paxillin–pY118 distribution at the migration edge, resulting in the formation of elongated fillopodia and promoting subsequent cell migration.

We also noted differences between the distribution patterns of FAKpY397 and FAKpY861. FAK pY397 was localized at the centers of cells, but FAKpY861 was limited to the cell periphery. These results suggest that only the loose focal points resulting from the initial cluster are formed and maintained in cells, whereas subsequent large clusters are not completed. These findings may explain why Col V fibril signaling can promote cell detachment and cell motility but not cell stabilization. Although we cannot exclude the possibility that the migration of GEC may result from co-induction of FAKpY397 and FAKpY861, because both of them have been reported to be involved in cell motility and have potential of cell invasion [38], other studies have shown that FAKpY861 facilitates epithelial cell migration during epithelial–mesenchymal transition [39] and promotes endothelial cell migration during vasculogenesis [40]. These observations are similar to our results. Our finding that FAKpY861, but not FAKpY397, is located at the migration edge of GEC may suggest that FAKpY861 plays a more important role in triggering cell detachment from Col V fibrils and leading to cell motility. Taking together all of these results, we suggest a model of the response of GEC to Col V fibrils (Fig.9H) in which the outside-in signal of Col V fibrils is mediated by β1 integrin to recruit the adaptor proteins paxillin and FAK and activate their phosphorylation to paxillin–pY118 and FAKpY861; β1 integrin, paxillin–pY118, and FAKpY861 are co-localized at the migration leading edge of cells (Figs. 8C, 8D, 8E,

and 8F). In our model, the activation of paxillin–pY118 induces disassembly of the short chain of actin, which results in dynamic cell movement along the adhesion surface, whereas the phosphorylation of FAK to FAKpY861 maintains loose focal points, allowing cells to migrate easily. Another outside-in signal from Col V fibrils is mediated by NG2 and recruits and activates FAKpY397 (Figs. 8E, 8F and Figs. 9A, 9B) to induce incomplete focal adhesion at the centers of cells; this process may be related to the maintenance of paxillin–pY118 at the migration edge and its subsequent role in cell movement (Figs. 8C, 8D). Overall, all of these signal pathways facilitate the dynamic motility of cells.

The biochemical data regarding the amounts of tyrosine- and serine-phosphorylated proteins in GEC addressed the timing and duration of phosphorylation. On Col V fibrils, tyrosine-phosphorylated paxillin peaked early during cell culture (4 d, yellow band, 65 to 70 kD, Fig. 10D) but decreased during the middle stage (7 d, yellow band, Fig. 10D) and disappeared during the late stage (20 d, yellow band, Fig. 10D). In contrast, non–tyrosine-phosphorylated paxillin increased markedly during the late stage (20 d, green band, Fig. 10D). Although cells cultured on Col I failed to show any total paxillin at 4 and 7 d (Fig. 10D), its presence was confirmed by chemical luminescence (data not shown). Total paxillin in the early- and middle-stage Col I culture samples was undetectable because a highly focused light was needed to reveal the paxillin band from cultures on Col V fibrils, thereby rendering the smaller amount of paxillin on Col I undetectable. Taken together, these findings suggest that phosphorylation of adaptor proteins for cell adhesion is activated predominantly during early stages of cell culture, decreases over 1 week, and disappears by 20 days. This pattern is consistent with the changes in the formation of focal points and focal adhesion. The loosely attached focal

point likely is formed first, thus initiating dynamic motility of cells, after which focal adhesions forms to replace the focal point, until only stable focal adhesion remains, which is mediated by non-tyrosine-phosphorylated paxillin (65 to 70 kD, green bands in Fig. 10D).

In addition to paxillin–pY118, the total amount of tyrosine phosphorylation of FAK varied at 4, 7, and 20 d, indicating that FAK was activated continuously when GEC were cultured on Col V fibrils (120 kD, red bands, Fig. 10D). Western blotting showed the relative amount of two tyrosine-phosphorylated FAKs, FAKpY861 and FAKpY397, over time. The FAKpY861 band revealed prolonged, consistent activation for 30 days of culture on Col V fibrils (3, 10, and 30 d, 120 kD FAK, Fig. 11C). In contrast, FAKpY397 decreased dramatically within 10 d (3, 10, and 30 d, Fig. 11D). This result seems reasonable given that bright-field microscopy showed that cells stopped moving and static adhesion was maintained after 10 d of culture (data not shown). Activation of FAKpY861 alone likely is insufficient to maintain cell motility; maintenance of motility probably also requires the activation of FAKpY397. Moreover, the decreased activity of FAKpY397 after 10 d may induce cell adhesion and inhibit cell motility. Although the changes in FAK phosphorylation might merely reflect the microenvironment (niche) surrounding the cells at the various time points we examined, the induction of phosphorylation of adaptor proteins by the outside-in signaling from Col V during the initial stages of culture alters various features of cells, such as adhesion and the actin cytoskeleton, leading to changes in the properties of the ECM. It is possible that the synergetic reactions between cells and the ECM lead to the changes in FAK phosphorylation at the various time points, but this synergetic reaction needs further investigation. Table 1 summarizes the changes in the phosphorylation of the adaptor

proteins at the various time points. Taken together, the changes in the phosphorylation of paxillin and FAK were induced by the outside-in signaling of Col V and subsequently affect cell adhesion, morphology, and cell behavior and result in cell migration during early stages of culture on Col V fibrils.

In conclusion, we have revealed that Col V fibrils can induce cell dynamics. We also have elucidated that the mechanism underlying this cell dynamism involves outside-in ECM signaling pathways that are mediated by two types of ECM receptors, β1 integrin and NG2. Inhibition data further support the existence of outside-in signaling by Col V fibrils and its induction of cell dynamics. On the basis of the data we obtained, the existence of Col V in glomeruli may have two possible consequences. One is that Col V induces cell dynamics which is unfavorable to the stable cell tissue at basement membrane and causes glomerular disease. The other is that injured glomerular tissue requires Col V to induce cell dynmatics for the subsequent restoration. Therefore, Col V fibrils play an important role in cell migration in renal glomeruli and may be a promising ECM for generating a microenvironment at the initial moment when adult kidney tissue needs to be reconstituted.

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**References**


[1] A. Martinez-Hernandez, S. Gay, E.J. Miller, Ultrastructural localization of type V collagen in rat kidney, J. Cell Biol. 92 (1982) 343-349.

[2] J.I. Scheinman, J.M. Foidart, A.F. Michael, The immunohistology of glomerular antigens. V. The collagenous antigens of the glomerulus, J. Technical Methods and Pathol. 43 (1980) 373-381.

[3] H. Morita, A. Yoshimura, Collagenofibrotic glomerulopathy with a widespread expression of type V collagen, Virchows Arch. 442 (2003) 163-168.

[4] B. Delahunt, W.E. Stehbens, T. Shozawa, B.M. Ruger, Progeria kidney has abnormal mesangial collagen distribution, Pediatr. Nephrol. 15 (2000) 279-285.

[5] H. Yoshikane, M. Honda, Y. Goto, S. Morioka, A. Ooshima, K. Moriyama, Collagen in dilated cardiomyopathy- scanning electron microscopic and immunohistochemical observations, Jpn. Circ. J. 56 (1992) 899-910.

[6] A. Fichard, J.-P. Kleman, F. Ruggiero, Another look at collagen V and XI molecules, Matrix Biol. 14 (1994) 515-531.

[7] S. Franc, J.-C. Rousseau, R. Garrone, M. van der rest, M. Moradi-Ameli, Microfibrillar composition ofumbilical cord matrix: characterization of fibrillin, collagen VI and intact collagen V, Placenta 19 (1998) 95-104.

[8] J.F. Bonadio, H. Sage, F. Cheng, J. Bernstein, G.E. Striker, Localization of collagen types IV and V, laminin, and heparan sulfate proteoglycan to the basal lamina of kidney epithelial cells in transfilter metanephric culture, Am. J. Pathol. 116 (1984) 289-296.

[9] M. Hatai, H. Hashi, I. Kato, Y. Yaoi, Inhibition of cell adhesion by proteolytic

fragments of type V collagen, Cell Struc. Func. 18 (1993) 53-60.

[10] K. Yamamoto, M. Yamamoto, T. Noumura, Disassembly of F-actin filaments in human endothelial cells cultured on type V collagen, Exp. Cell Res. 201 (1992) 55-63.

[11] K. Yamamoto, M. Yamamoto, The failure to reconstitute the second extracellular matrix-integrin-cytoskeleton system in human endothelial cells on type V collagen, Artery. 21 (1994) 76-93.

[12] K. Takai, S. Hattori, S. Irie, Type V collagen distribution in liver is reconstructed in coculture system of hepatocytes and stellate cells; the possible functions of type V collagen in liver under normal and pathological conditions, Cell Struc. Func. 26 (2001) 289-302.

[13] T. Hayashi, K. Mizuno, M. Hirose, K. Nakazato, E. Adachi, Y. Imamura, K. Yoshikawa, Cell type specific recognition of the reconstituted aggregates from isolated type I Collagen, type IV collagen, and type V collagen, Indian Acad. Sci. 111 (1999) 171-177.

[14] K. Mizuno, T. Hayashi, The fibril structure of type V collagen triple-helical domain, Micron. 32 (2001) 317-323.

[15] T. Kihara, Y. Takemura, Y. Imamura, K. Mizuno, T. Hayashi, Reconstituted type V collagen fibrils as cementing materials in the formation of cell clumps in culture, Cell Tissue Res. 318 (2004) 343-352.

[16] Z. Ge, F. Yang, J. C. H. Coh, S. Ramakrishna, E. H. Lee, Biomaterial and scaffolds for ligament tissue engineering. J. Biomed. Mat. Res. 77A (2006) 639-652.

[17] B.J. Papenburg, L. Vogelaar, L.A.M. Bolhuis-Versteeg, R.G.H. Lammertink, D. Stamatialis, M. Wessling, One-step fabrication of porous micropatterned scaffolds

to control cell behavior, Biomaterials. 28 (2007) 1998-2009.

[18] C.S. Cho, T. Hoshiba, I. Harada, T. Akaike, Regulation of hepatocyte behaviors by galactose-carring polymers through receptor-mediated mechanism, Reactive and Functional Polymers. 67 (2007) 1301-1310.

[19] T. Matsumura, Collagen fibrils of the sea cucumber, Stichopus japonicus: purification and morphological study, Connect. Tissue Res. 2 (1974) 117-125.

[20] E.J. Miller, R.K. Rhodes, Preparation and characterization of the different types of collagen, Meth. Enzymol. 82 (1982) 33-64.

[21] K. Mizuno, T. Hayashi, Separation of the subtypes of type V collagen molecules, [alpha 1(V)]2 alpha 2(V) and alpha 1(V) alpha 2(V) alpha 3(V), by chain composition-dependent affinity for heparin: single alpha 1(V) chain shows intermediate heparin affinity between those of the type V collagen subtypes composed of [alpha 1(V)]2 alpha 2(V) and of alpha 1(V) alpha 2(V) alpha 3(V), J. Biochem. 120 (1996) 934-939.

[22] Y. Takemura, K. Mizuno, Y. Imamura, T. Hayashi, Preferential Liberation of Type V Collagen from Bovine Corneal Stroma by Limited Treatment with Proteases, Connective Tissue. 35 (2003) 133-140.

[23] P-C. Wang, T. Takezawa, Reconstruction of renal glomerular tissue using collagen vitrigel scaffold, J. Biosci. Bioeng. 99 (2005) 529-540.

[24] F. Ruggiero, J. Comte, C. Cabanas, R. Garrone, Structural requirements for α1β1andα2β1 integrin mediated cell adhesion to collagen V, J. Cell Sci. 109 (1996) 1865-1874.

[25] M.A. Burg, E. Tillet, R. Timpl, W.B. Stallcup, Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules, J. Biol. Chem. 271

(1996) 26110-26116.

[26] E. Tillet, F. Ruggiero, A. Nishiyama, W.B. Stallcup, The membrane-spanning proteoglycan NG2 binds to collagens V and VI through the central nonglobular domain of its core protein, J. Biol. Chem. 272 (1997) 10769-10776.

[27] E. Tillet, B. Gential, R. Garrone, NG2 proteoglycan mediates b1 integrin-independent cell adhesion and spreading on collagen VI, J. Cell. Biochem. 86 (2002) 726-736.

[28] V. Petit, J-P. Thiery, Focal adhesions: structure and dynamics, Biol. Cell 92 (2000) 477-494.

[29] D.D. Schlaepfer, S.K. Mitra, D. Ilic, Control of motile and invasive cell phenotypes by focal adhesion kinase, Biochimica et Biophysica Acta. 1692 (2004) 77-102.

[30] D.J. Webb, K. Donais, L.A. Whitmore, S.M. Thomas, C.E. Turner, J.T. Parsons, A.F. Horwitz, FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly, Nature Cell Biology. 6 (2004) 154-161.

[31] A. Tsubouchi, J. Sakakura, R. Yagi, Y. Mazaki, E. Schaefer, H. Yano, H. Sabe, Localized suppression of RhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration, J. Cell Biol. 159 (2002) 673-683.

[32] C. Huang, Z. Rajfur, C. Borchers, M. D. Schaller, K. Jacobson, JNK phosphorylates paxllin and regulates cell migration, Nature 424 (2003) 219-223.

[33] R.K. Vadlamudi, L. Adam, D. Nguyen, M. Santos, R. Kumar, Differential Regulation of Components of the Focal Adhesion Complex by Heregulin: Role of Phosphatase SHP-2, J. Cell. Physiol. 190 (2002) 189-199.

[34] S.K. Mitra, D.A. Hanson, D.D. Schlaepfer, Focal adhesion kinase: in command and control of cell motility, Nature Reviews. Mol. Cell Biol. 6 (2005) 56-68.

[35] L.Y. Romanova, S. Hashimoto, K-O. Chay, M.V. Blagosklonny, H. Sabe, J.F. Mushinski, Phosphorylation of paxillin tyrosines 31 and 118controls polarization and motility of lymphoid cells and is PMA-sensitive, J. Cell Sci. 117 (2004) 3759-3768.

[36] Z. Zhang, S-Y. Lin, B.G. Neel, B. Haimovich, Phosphorylated alpha-actinin and protein-tyrosine phosphatase 1B coregulate the disassembly of the focal adhesion kinase x Src complex and promote cell migration, J. Biol. Chem. 281 (2006) 1746-1754.

[37] R.S. Sawhney, M.M. Cookson, Y. Omar, J. Hauser, M.G. Brattain, Integrin 2-mediated ERK and calpain activation play a critical role in cell adhesion and motility via focal adhesion kinase signaling. Identification of a novel signaling pathway, J. Biol. Chem. 281 (2006) 8497-8510.

[38] D.D. Schlaepfer, S.K. Mitra, Multiple connections link FAK to cell motility and invasion, Curr. Opin. Gen. Dev. 14 (2004) 92-101.

[39] K. Nakamura, H. Yano, E. Schaefer, H. Sabe, Different modes and qualities of tyrosine phosphorylation of Fak and Pyk2 during epithelialmesenchymal transdifferentiation and cell migration: analysis of specific phosphorylation events using site-directed antibodies, Oncogene. 20 (2001) 2626-2635.

[40] R. Abu-Ghazaleh, J. Kabir, H. Jia, M. Lobo, I. Zachary, Src mediates stimulation by vascular endothelial growth factor of the phosphorylation of focal adhesion kinase at tyrosine 861, and migration and anti-apoptosis in endothelial cells, Biochem. J. 360 (2001) 255-264.

**Legends for Figures**

Fig. 1 TEM of type V collagen (Col V). (A) Col V molecules; (B) reconstituted fribrils of Col V. Scale bar, 96nm.

Fig. 2. Type V collagen fibrils induced dynamic motility of adult glomerular endothelial cells (GEC). (A) GEC cultured on Col V fibrils for 4h (panel A1), 8h (panel A2) and 12h (panel A3) were detected by time-lapse observation, GEC moved dynamically by extending multi-fillopodia and lamellipodia ; (B) GEC cultured on Col V fibrils moved by extending long fillopodia at the protruding edge; (C) GEC cultured on Col V fibrils migrated ahead by extending lamellipodia; (D) GEC cultured on type I collagen (Col I) fibrils migrated slowly and stably with membrane ruffling in a limited area. Scale bar, 25 $\mu$ m.

Fig. 3. NG2 and β1 integrin expressed at the different locations in GEC cultured on Col V fibrils for 24h. GEC cultured on Col I fibrils under the same culture conditions were used as control cells. Immunofluorescence analysis of neuroglycan (NG2) and β1 integrin were conducted by staining NG2 and β1 integrin with primary antibodies conjugated with secondary antibodies labeled by Alexa Fluor 488 (green), which were observed by fluorescent microscopy. Actin cytoskeleton was stained with Rhodamin-phalloidin (red). (A) NG2 on control cells; (B) NG2 on GEC cultured on type Col V fibrils; (C) β1 integrin on control cells; (D) β1 integrin on GEC cultured on Col V fibrils. Scale bar, 25 $\mu$ m.

Fig. 4. Col V fibrils and molecules induced different expression and localization of

neuroglycan (NG2), β1 integrin and actin cytoskeleton on GEC for 24h. GEC were cultured on Col V molecules and Col V fibrils, respectively. Immunofluorescence analysis of NG2 was conducted by staining NG2 with primary antibody conjugated with secondary antibody labeled by Alexa Fluor 350 (blue). β1 integrin was stained with primary antibody conjugated with secondary antibody labeled by Alexa Fluor 488 (green). Actin cytoskeleton was stained with Rhodamin-phalloidin (red). (A) NG2, (C) β1 integrin and (E) cytoskeleton actin were detected in GEC cultured on Col V molecules for 24 h; (B) NG2, (D) β1 integrin and (F) actin cytoskeleton were detected on GEC cultured on Col V fibrils for 24h. Scale bar, 25 $\mu$ m.

Fig. 5. Col V fibrils induced β1 integrin localized at the migration edge of GEC cultured on Col V fibrils for 24h. GEC cultured on Col I fibrils under the same culture condition were used as control cells. Immunofluorescence analysis of β1 integrin was conducted by staining β1 integrin with primary antibody conjugated with secondary antibody labeled by Alexa Fluor 488 (green), which was observed by TIRF microscopy. Cytoskeleton actin was stained with Rhodamin-phalloidin (red). (A) β1 integrin in control cells; (B) β1 integrin in GEC cultured on Col V fibrils; (C) β1 integrin merged with actin cytoskeleton in control cells; (D) β1 integrin merged with actin cytoskeleton in GEC cultured on Col V fibrils. Scale bar, 25 $\mu$ m.

Fig. 6. Col V fibrils induced paxillin pY118, FAK pY861 and FAK pY397 expressed at various locations of GEC for 24h. GEC cultured on Col I fibrils under the same culture conditions were used as control cells. Immunofluorescence analyses of pY1118, FAK pY861 and FAK pY397 were conducted by staining each protein with primary

antibody conjugated with secondary antibody labeled by Alexa Fluor 488 (green), which was then observed by TIRF microscopy. Actin cytoskeleton was stained with Rhodamin-phalloidin (red). (A) paxillin pY118, (C) FAK pY861 and (E) FAK pY397 were detected in control cells; (B) paxillin pY118, (D) FAK pY861 and (F) FAK pY397 were detected in GEC cultured on Col V fibrils. Scale bar, 25 $\mu$ m.

Fig. 7. Inhibition of the signaling of Col V fibrils by adding anti-β1 integrin antibody or anti-Col V antibody induced the location change of paxillin pY118 in GEC cultured on Col V fibrils for 24h. Immuno-fluorescence analyses of paxillin pY118 were performed by staining paxillin pY118 with primary antibody conjugated with secondary antibody labeled by Alexa Fluor 488 (green), which was observed by confocal microscopy. Actin cytoskeleton was stained with Rhodamin-phalloidin (red). (A) GEC cultured on Col V fibrils; (B) GEC cultured on Col V fibrils after addition of anti-Col V antibody ; (C) GEC cultured on Col V fibrils ; (D) GEC cultured on Col V fibrils after addition of anti-β1 integrin antibody. Scale bar, 25 $\mu$ m.

Fig. 8. Inhibition of the signaling of Col V fibrils by adding anti-NG2 antibody induced the location change of paxillin pY118 and FAK pY397 in GEC cultured on Col V fibrils for 24h. Actin cytoskeleton was stained with Rhodamin-phalloidin (red), paxillin pY118 was stained with anti paxillin pY118 antibody labeled by Alexa Fluor 488 (green), and FAK pY397 was stained with anti FAKPY397 antibody labeled by Alexa Fluor 633 (blue). (A)(C)(E) GEC cultured on Col V fibrils without addition of anti-NG2-antibody; (B)(D)(F) GEC cultured on Col V fibrils after addition of anti-NG2-antibody. (A)(B) the merged figures of cells stained by Rhodamin-phalloidin (red), paxillin pY118 (green)

and FAK pY397 (blue), respectively; (C)(D) Paxillin pY118 distribution in GEC without and with addition of anti-NG2-antibody, respectively; (E)(F) FAK pY397 distribution in GEC without and with addition of anti-NG2-antibody, respectively. Scale bar, 25 $\mu$ m.

Fig. 9. Relationship between ECM receptors (NG2 and β1 integrin) and focal adhesion proteins (FAK pY397, FAK pY861 and paxillin pY118) in GEC cultured on Col V fibrils for 24h. Immunofluorescence analyses of NG2, β1 integrin, FAK pY861 and FAK pY397 were conducted by staining each protein with primary antibody conjugated with secondary antibody labeled by Alexa Fluor 488 (green), which was then detected by TIRF microscopy. Paxillin pY118 was stained with first antibody conjugated with secondary antibody labeled by Alexa Fluor 350 (blue) and actin cytoskeleton was stained with Rhodamin-phalloidin (red). (A) NG2; (B) FAK pY397; (C) β1 integrin; (D) FAKpY861; (E) β1 integrin; (F) paxillin pY118. The dotted circle indicates strong expression of each protein; (G) NG2 localized at the central site and β1 integrin at the migration edge of GEC cultured on Col V fibrils. (H) A model showing signaling of Col V fibrils mediated through NG2 and β1 integrin induces phosphorylation of focal adhesion proteins which exert their effect on cell migration at the leading edge of fillopodia and lamellipodia. Scale bar, 12.5 $\mu$ m.

Fig. 10. Col V fibrils induced high levels of expression of paxillin pY118 and pS178 than Col I fibrils. Immunoblotting was conducted by the method described in experimental procedure. Phosphorylated tyrosine and serine proteins extracted from GEC cultured on Col V fibrils and control cells were labeled by first antibody of

phospho-tyrosine and phospho-serine, respectively and conjugated with secondary antibody labeled by Alexa Fluor 680 (green). Total paxillin was labeled with first antibody conjugated with secondary antibody of IR-Dye800 (red). PY118 and pS178 are indicated in yellow of the merged band of total paxillin with phosphorylated tyrosine and serine, respectively. Quantification was conducted by scanning protein bands and analyzed by *Odyssey* Infrared Imaging System (LI-COR). (A) pY118 in GEC cultured on Col V fibrils (left), pY118 in control cells (middle), and marker (right); (B) pS178 in GEC cultured on Col V fibrils (left), pY118 in control cells (middle), and marker (right); (C) GAPDH in GEC of Col V fibrils (left), GAPDH in control cells (middle), and marker (right); (D) Comparison of the change of total phosphorylated paxillin in GEC cultured on Col V fibrils and Col I fibrils for 4d (left), 7d (middle) and 20 d (right) culture. Marker is indicated in the most right lane. Phosphorylated tyrosine proteins were labeled by anti-phospho-tyrosine antibody conjugated with IR-Dye800 (red). Total paxillin was labeled with anti-paxillin antibody conjugated with Alexa Fluor 680 (green). Merged band of phosphorylated tyrosine and total paxillin shows phospho-paxillin (yellow). Another band showing 120KD protein, which was labeled with anti-phospho-tyrosine antibody conjugated with IR-Dye800 (red), merged with total FAK (red), was phospho-tyrosine-FAK (red).

Fig. 11. Comparison of FAK phosphorylation at tyrosine 861 and tyrosine 397 of GEN cultured on Col V fibril and Col I in a time course of 3, 10 and 30 days. Total proteins were collected from 1x $10^4$ GEC on V fibril and on I fibril, respectively, and were visualized by ponceau S staining after loading on SDS-PAGE (Fig. 11A, 11B). A band close to 120kD indicated FAK protein, and the phosphorylation of FAK revealed a

band slightly higher or lower than 120kD. As compared to FAK in the control cells cultured on Col I, FAK pY861 was activated constantly by Col V fibril, while FAK pY397 had no significant change on Col V fibril in the whole time course of 3d, 10d and 30d (Fig. 11C and 11D).