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ORIGINAL ARTICLE

# Enhanced osteoblast adhesion on transglutaminase 2-crosslinked fibronectin

**J. Forsprecher · Z. Wang · V. Nelea · M. T. Kaartinen**

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**Abstract** Fibronectin (FN) is a cell adhesion protein that binds integrins in a process also involving the protein-crosslinking enzyme transglutaminase 2 (TG2) as a co-receptor. The cell-adhesive property of TG2 has been linked to a complex formation with FN and to its ability to crosslink and polymerize FN on the cell surface. We tested here the effects of extracellular FN, before and after in vitro crosslinking and polymerization by TG2, on MC3T3-E1 osteoblast adhesion. We show that TG2-mediated crosslinking creates large, compacted chain-like protein clusters that include both TG2 and FN molecules as analyzed by Western blotting and atomic force microscopy. Crosslinking of FN significantly promotes osteoblast adhesion as measured by crystal violet staining, and enhances $\beta_1$-integrin clustering on the cell surface as visualized by immunofluorescence microscopy. We hypothesize that TG2-mediated crosslinking enhances the cell-adhesive properties of FN by increasing the molecular rigidity of FN in the extracellular matrix.

**Keywords** Transglutaminase 2 · Protein crosslinking · Fibronectin · Osteoblast adhesion · Integrin clustering

J. Forsprecher · Z. Wang · V. Nelea · M. T. Kaartinen (✉)
Division of Biomedical Sciences, Faculty of Dentistry,
McGill University, 3640 University Street,
Strathcona Building, Room M-72, Montreal, QC, Canada
e-mail: mari.kaartinen@mcgill.ca

M. T. Kaartinen
Division of Experimental Medicine,
Department of Medicine, Faculty of Medicine,
McGill University, Montreal, QC, Canada

## Introduction

Bone formation is orchestrated by osteoblasts during intramembranous ossification. This involves cell condensation and osteoblast adhesion to extracellular matrix within connective tissues rich in type I collagen and cell adhesion proteins such as fibronectin (FN). FN is a ubiquitous, Arg–Gly–Asp (RGD)-containing cell adhesion protein which mediates cell-matrix adhesion and signaling in many biological processes via integrin receptors (Pankov and Yamada 2002). Transglutaminase 2 (TG2) has recently been reported to act as a $\beta_1$- and $\beta_3$-integrin-binding co-receptor for FN. TG2 functions on the cell surface, accumulates in focal adhesions and can mediate RGD-independent adhesion of FN to integrins (Akimov et al. 2000), and promotes integrin clustering and cell signaling in fibroblasts (Janiak et al. 2006). TG2 belongs to a group of protein-crosslinking enzymes which create large protein polymers of its substrate proteins by forming covalent, isopeptide crosslinks between protein chains (Lorand and Graham 2003). FN is polymerized by TG2 and Factor XIIIA, with both enzymes promoting FN fibrillogenesis and assembly (Barry and Mosher 1988; Zhang and Mosher 1996). Numerous reports have described the involvement of TG2 in cell attachment and cell spreading of fibroblasts and endothelial cells, and it is widely thought that these activities represent major functions for TG2 (Gentile et al. 1992; Martinez et al. 1994; Jones et al. 1997; Verderio et al. 1998; Gaudry et al. 1999). In these reports, TG2 was localized to the cell surface, where it was postulated to participate in cell-matrix interactions and in the organization of matrix molecules at the cell surface and in the adjacent pericellular matrix. Related to skeletal development, TG2 has been shown to be involved in cell adhesion and spreading of primary human osteoblasts and

in the adhesion of osteosarcoma cells (cell lines MG-63 and HOS) (Heath et al. 2001; Verderio et al. 2001, 2003), and we have shown that TG activity is critical for osteoblast differentiation (Al-Jallad et al. 2006). In mineralized tissues, we have localized TG2 in bones and teeth to osteoblast and odontoblast cell-matrix adhesion sites (Kaartinen et al. 2002, 2005). Although the ability of TG2 to function as a cell surface co-receptor for integrins has been reported *not* to require its protein crosslinking activity (Akimov et al. 2000) but simply complex formation between the two proteins (Verderio et al. 2003), many other studies have documented that the involvement of TG2 in cell adhesion is tightly linked to its ability to polymerize, i.e., crosslink, FN (Martinez et al. 1994; Jones et al. 1997). It is therefore possible that cell-surface TG2 and secreted TG2 have different, but complementary, roles in promoting cell adhesion. In the present study, we tested the hypothesis that crosslinking and polymerization of FN by extracellular TG2 is an important part of the cell adhesion process. We report that FN crosslinking by TG2 outside the cell significantly promotes osteoblast adhesion and increases $\beta_1$-integrin clustering at focal adhesion sites. We hypothesize that these TG2-mediated effects on cells are likely related to changes in matrix stiffness and to the molecular rigidity of the FN molecule.

## Materials and methods

### Preparation and characterization of fibronectin polymers

Polymer FN was prepared by incubating 2 μg of FN (from bovine plasma, Sigma) with 2 mU of TG2 from guinea pig liver (Sigma) in 10 mM Tris–HCl, pH 8.3, 3 mM $CaCl_2$ and 1 mM dithiothreitol (DTT) for 2 h at 37°C. Controls for these samples were (1) FN monomer in buffer, and (2) FN + TG2 in buffer (but without enzyme activation by DTT and without the 37°C incubation, i.e., kept at 4°C) (inactive TG2, iTG2). The extent of protein polymerization was assessed by Western blotting using FN (Chemicon) and TG2 (Labvision) antibodies as described previously (Kaartinen et al. 2005). Protein samples (2 μg of FN) were adsorbed onto each of the plastic surfaces overnight at 4°C. Protein binding to all the plastic surfaces used in this study was assessed per unit area by adding equal amounts of water and bicinchonic acid, followed by a 2-h incubation at 60°C, after which optical density was read at 562 nm in a microplate reader. Protein polymerization was confirmed by Western blotting and visualized by atomic force microscopy (AFM) using an atomic force multi-mode scanning probe microscope (Digital Instruments NanoScope IIIa Multimode® SPM, Santa Barbara, CA, USA) in tapping mode in air at room temperature. AFM probes for imaging were PointProbe®Plus and SuperSharpSilicon (NANO*SENSORS*™, Neuchatel, Switzerland). Samples were injected into a droplet of water and left on fresh-cleaved Muscovite mica and incubated for 5 min in ambient temperature, and then washed and dried under a slow jet of compressed air. AFM scanning started no later than 10 min after the specimen was prepared.

### Cell adhesion assay

Serial dilutions of the protein samples described above, in concentrations ranging from 0.015 to 0.125 μg/well, were coated onto a 96-well microplate (High Protein Binding Type I, Costar, Corning) and left to adsorb overnight at 4°C. Wells were subsequently blocked with 2% BSA in PBS for 2 h at room temperature. MC3T3-E1 osteoblast cells (Wang et al. 1999) were serum-starved for 1 h, then trypsinized, counted and re-suspended in serum-free medium (complete modified alpha minimum essential medium (α-MEM, Gibco) containing L-glutamine and 2.2 g/l of sodium bicarbonate, but without ascorbic acid, deoxyribonucleosides and ribonucleosides. Approximately $4 \times 10^4$ cells were added per well. The plates were placed at 37°C into a 5% $CO_2$ incubator for 2 h and then washed with serum-free α-MEM five times or until the majority of cells were removed from the BSA-coated wells (negative control). Cells were fixed with 1% glutaraldehyde and stained with 0.5% crystal violet in 20% methanol in PBS for 20 min at room temperature on a shaker. The staining solution was removed; the wells were washed twice with water and then air-dried. Crystal violet was dissolved with 1% SDS for 1 h or overnight in the dark. The optical density was read at 595 nm with a microplate reader.

### Fluorescence microscopy

One μg of protein sample was coated overnight at 4°C onto a Labtek 8 chamber Permanox® plastic slide (Nalge Nunc International) and wells were blocked with 2% BSA for 2 h at room temperature. Cells were serum-starved for 1 h and plated at a density of $10^4$ cells/well in serum-free or in 1% serum-enriched medium. Cells were left to adhere and grown for 4 h (0% serum) and 24 h (1% serum). Cells were fixed with 3.7% formaldehyde, permeabilized with 0.25% Triton X-100, washed twice with PBS and blocked with 0.1% BSA in PBS for 25 min. $\beta_1$-integrin was detected with primary antibody (Chemicon) incubation (1 h) followed by Alexa Fluor 546 anti-rat secondary antibody (Invitrogen) for 1 h. Actin was detected using Alexa Fluor 488-phalloidin (Invitrogen). Multi-chamber slides were air-dried and mounted under glass coverslips with ProLong® Antifade (Invitrogen) and viewed and recorded

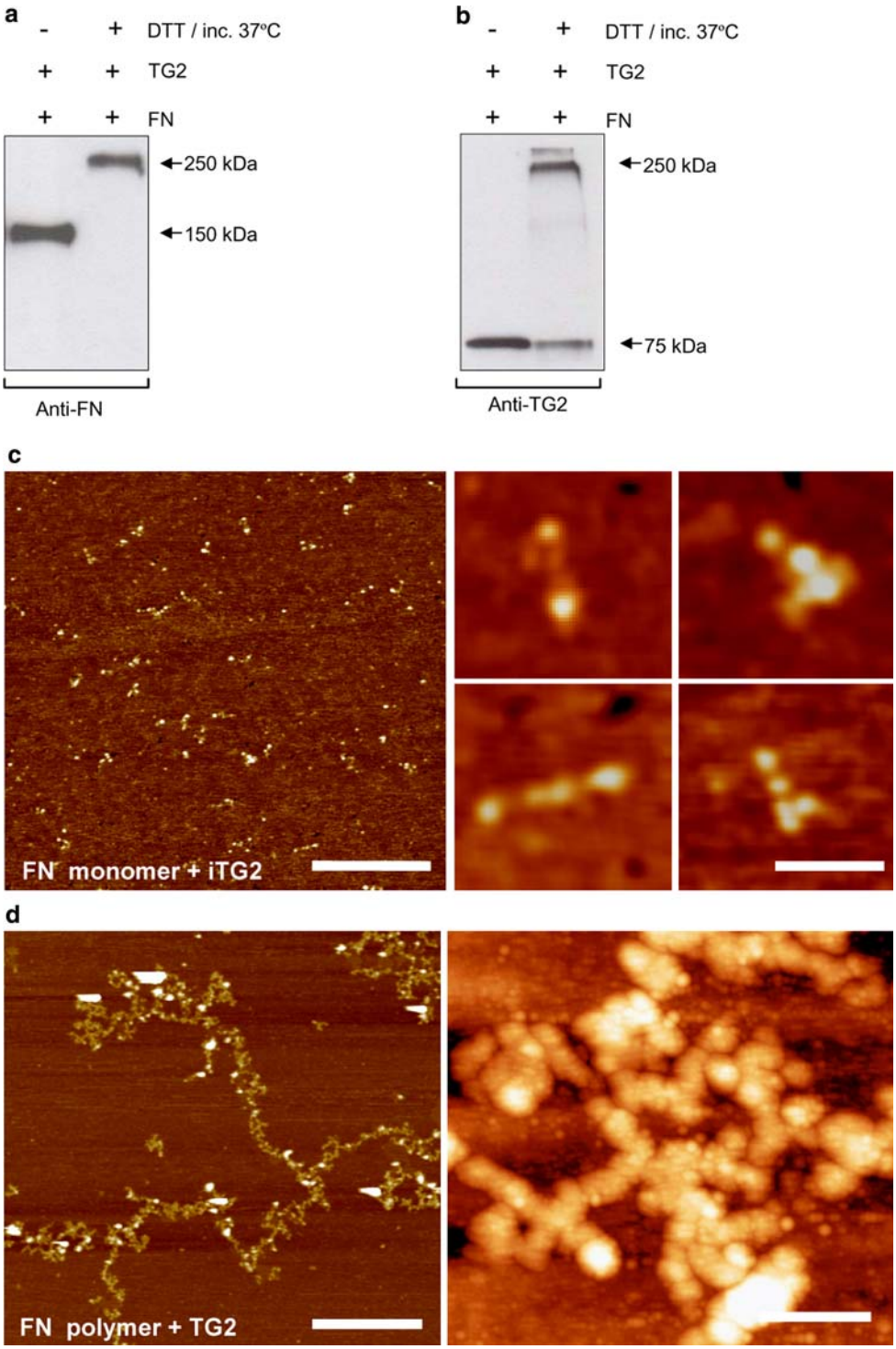


**Fig. 1** FN crosslinking and polymerization by TG2. **a** Western blot analysis of FN polymerization by TG2 detected by FN antibody and **b** by TG2 antibody. In addition to FN, TG2 is also incorporated into the high-molecular weight polymers (>250 kDa) during the catalysis. **c** Tapping mode AFM images of single FN molecules. *Left panel* $2 \times 2$ μm$^2$ scanned area (*bar* equals 500 nm). *Right panel* higher magnification of the same image ($200 \times 200$ nm$^2$ area) (*bar* equals 100 nm) showing elongated and semi-compact forms of FN monomers. **d** Tapping mode AFM images of crosslinked polymerized FN molecules. *Left panel* $5 \times 5$ μm$^2$ scanned area (*bar* equals 125 nm) showing a typical chain-like molecular formation of FN polymers. *Right panel* higher magnification of the same image ($400 \times 400$ nm$^2$) (bar equals 100 nm) detailing a tight and apparent dense structure of polymer FN

by fluorescence microscopy (Leica DM IL inverted fluorescent microscope and Leica DC 180/DC420 camera). Leica DCT v5.1.9 software was used to record fluorescent images and Adobe Photoshop® v6.0 was used to pseudocolor and merge images. Thresholding and pseudocoloring of fluorescence intensity to highlight focal adhesions (Fig. 3c) was performed using Northern Eclipse v5.0 software (Empix Imaging, Mississauga, ON).

## Statistical analysis

Standard error of the mean (SEM) was calculated from three independent experiments and is represented in each figure by error bars. Statistical significance was assessed using the Student's $t$ test, with asterisks in the figures indicating $P$ values as follows: $*P < 0.1$, $**P < 0.01$, $***P < 0.001$.

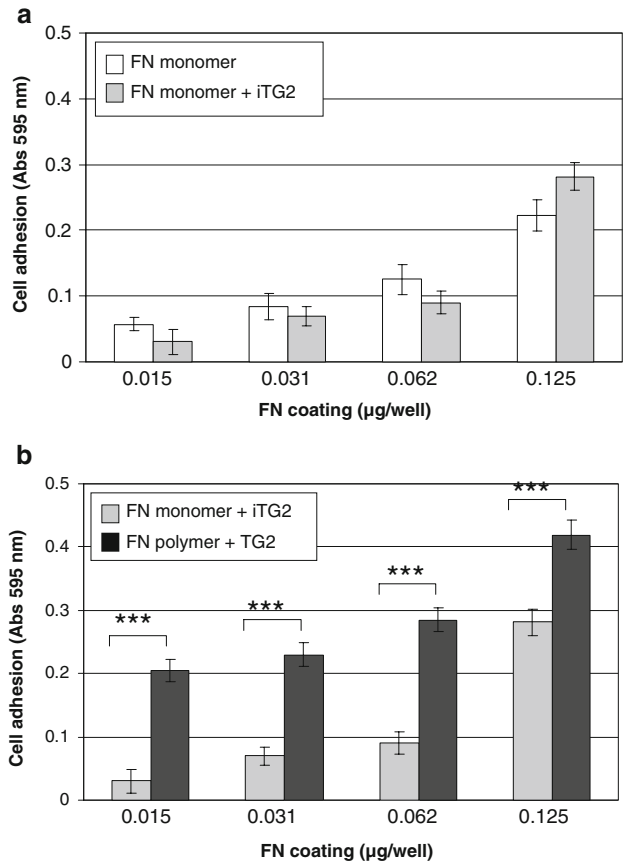


**Fig. 2** MC3T3-E1/C4 osteoblast cell adhesion on FN-coated surfaces as assessed by quantification of crystal violet staining. Prior to the adhesion assay, cells were starved of serum for 1 h, and the assay was conducted in the absence of serum. **a** Cell adhesion on FN monomer alone and FN monomer with inactive TG2. No significant change in cell adhesion was observed. **b** Cell adhesion on FN monomer with inactive TG2 compared to FN polymer with active TG2. Significant increases in cell adhesion were observed on the FN polymer-coated surface

## Results

### Increased cell adhesion on fibronectin polymers

The aim of this study was to test if extracellular crosslinking and polymerization of FN by TG2 influences osteoblast cell adhesion. FN was crosslinked with TG2 in a 2-h incubation which resulted in complete polymerization of FN, demonstrated as a FN band shift from 150 to >250 kDa by Western blotting (Fig. 1a). Analysis of the samples for TG2 also shows that not only FN, but also the TG2 enzyme itself, incorporates into high-molecular weight polymers (>250 kDa) during the catalysis (Fig. 1b). The sample used as a negative control was FN together with TG2 in reaction buffer that did not contain DTT (used for activation of the enzyme) (iTG2; inactive TG2) incubated for 2 h at 4°C. As demonstrated in Fig. 1a, omission of DTT and keeping the sample cold prevented crosslinking of FN. We initially also used an additional control; FN monomer incubated at 37°C for 2 h, but results were identical (see, cell adhesion results) to FN with inactive TG2, and thus this monomer FN control was no longer used is subsequent studies. Figure 1c and d show AFM images of the two samples at both low and high magnification, revealing that TG2 treatment clusters FN molecules into lengthy, chain-like polymers (Fig. 1d). The polymers are large—several hundred nanometers long—and up to 20 nm high/thick ($z$-axis) whereas individual FN monomers are about 4 nm in the $z$-axis. After TG treatment it appears that individual FN molecules are more compact and are packed closer together to form larger units.

MC3T3-E1 osteoblast adhesion to protein surfaces was quantified by crystal violet staining. Both FN polymer and monomer samples used for the cell adhesion assays had similar coating efficiencies on both the microplate wells and the chamber slides (data not shown), hence, any differences observed would not be attributable to having different amounts of protein on the plate surfaces. FN monomer, or FN monomer together with inactivated TG2, supported cell adhesion in a similar manner as discussed above (Fig. 2a). When TG2 was activated and FN was crosslinked and polymerized prior to coating, cells showed significantly higher binding to the surface; 1.4–6.8 times higher cell counts were observed depending on the protein concentration used for coating (Fig. 2b). Hereafter, only surfaces coated with FN with TG2 (inactivated or activated) were compared.

### Fibronectin polymerization promotes $\beta_1$-integrin clustering

To gain insight into the mechanisms by which FN polymerization affects cell adhesion and proliferation, we followed cell adhesion for longer times by immunofluorescence microscopy, and we examined localization of $\beta_1$-integrin—a major adhesion receptor subunit for FN—to determine whether integrin clustering was occurring. MC3T3-E1 cells expressed $\beta_1$-integrin mRNA and protein (data not shown). Immunostaining for $\beta_1$-integrin (Fig. 3a) after 4 h of cell adhesion revealed that on both FN monomer (with iTG2)- and FN polymer-coated culture surfaces the cells formed similar, punctate $\beta_1$-integrin-containing 1–2 µm-sized focal contacts. After 24 h, cells grown on FN polymer showed mature, elongated adhesion contacts with $\beta_1$-integrin clustering aligned with the cytoskeletal actin stress fibers, whereas the focal contacts on FN monomer remained punctate in appearance. Higher magnification of integrin clustering observed by immunofluorescence microscopy (Fig. 3b), that was subsequently

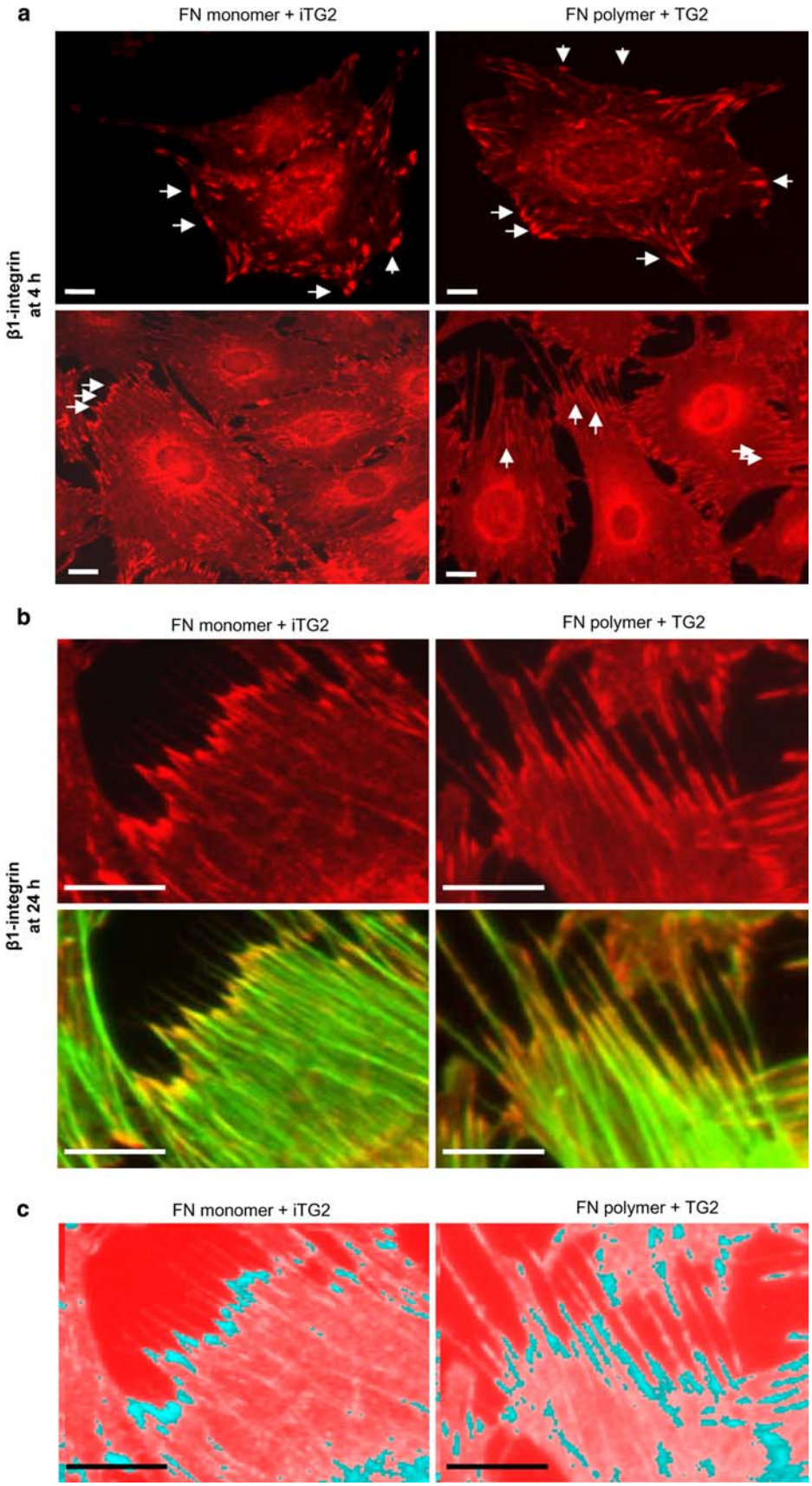


**Fig. 3** FN polymer-coated surfaces promote $\beta_1$-integrin clustering in MC3T3-E1/C4 osteoblasts. **a** Serum-starved MC3T3-E1/C4 cells were allowed to adhere to FN monomer- and FN polymer-coated surfaces for 4 h (in the absence of serum) or for 24 h (in 1% serum). Cells were fixed and stained using $\beta_1$-integrin primary antibody and Alexa Fluor 546 secondary antibody (*red*). **b** Enlarged and merged images of **a** after $\beta_1$-integrin staining (*red*) and actin cytoskeleton staining (*green*, Alexa Fluor 488-phalloidin) showing sites of co-localization (*orange*/*yellow*). **c** Computer-assisted thresholding and pseudocoloring of $\beta_1$-integrin immunostaining (*turquoise*) to highlight $\beta_1$-integrin clustering and to show its elongation along the actin stress fibers in the periphery of cells grown on FN polymer-coated surfaces (*right panel*) compared to the more punctate distribution on the FN monomer-coated surfaces (*left panel*). *Bars* equal 10 μm

thresholded and pseudocolored to highlight these focal adhesion (Fig. 3c), reveals that these structures reach up to 9 μm in length on the FN polymer (vs. 2–3 μm for the FN monomer with iTG2). The actin stress fiber extremities appeared thicker and longer for the cells grown on FN polymer (Fig. 3b, lower right panel).

## Discussion

In this study we show that surfaces coated with FN covalently crosslinked and polymerized by TG2 promote MC3T3-E1 osteoblast adhesion and increase $\beta_1$-integrin clustering at the cell periphery. These results demonstrate that TG2-mediated post-translational modification of proteins in the extracellular compartment can significantly alter protein function. The significant role of FN in guiding cell adhesion, migration, cell cycle progression and cell differentiation is well documented (Sechler and Schwarzbauer 1998; Geiger et al. 2001; Pankov and Yamada 2002); however, many mechanistic details related to these functions remain to be determined. The role of TGs in these FN-mediated processes has become evident by findings describing the role of TG2 and FXIIIa in FN assembly (Pankov and Yamada 2002) and by the discovery of TG2 acting as an integrin-binding co-receptor for FN (Akimov et al. 2000). Indeed, it has also been shown previously by the work of Sechler and Schwarzbauer (1998) that FN–FN interactions and the micro-architecture of the FN matrix serve a regulatory role in controlling cell cycle progression. Similarly, FN fibrillogenesis, where TGs play an important role, promotes osteoblast differentiation (Moursi et al. 1996; Tang et al. 2004). While the molecular mechanisms underlying the changes in cellular response to the functional modification of FN by TG are unknown, it is reasonable to consider that TG2 can change the architecture and "quality" of the FN matrix.

We recognize that our TG-mediated crosslinking of FN in vitro does not replicate the events associated with FN assembly and fibrillogenesis in vivo since the cell- and matrix-derived mechanobiological forces (tension) at the cell surface—which stretch FN molecules and promote their assembly—is absent. Therefore, it is reasonable to think that the cellular response to the crosslinked FN polymer in vitro is mediated by factors other than the fibrillar properties of crosslinked FN. We have recently shown by nano-indentation AFM studies that crosslinking and polymerization of FN by TG2 significantly increases its molecular stiffness (Nelea et al. 2008, in press). It is known that cells not only respond to proteins within the matrix (via integrins), but also sense matrix rigidity (compliance) (Choquet et al. 1997; Engler et al. 2004, 2006) and migrate towards stiffer matrices (durotaxis) (Jiang et al. 2006; Kostic and Sheetz 2006). Cell adherence, integrin clustering and actin cytoskeletal organization is favored by stiff substrata that resist forces and create tension between cells and matrix (Choquet et al. 1997; Engler et al. 2004, 2006). Tension in the cells generated by the rigid matrix leads to conformational changes in integrins that bring phosphorylating enzymes into close physical proximity with their substrates leading to signaling and changes in cell behavior (Jiang et al. 2006; Kostic and Sheetz 2006). Although the molecular mechanisms effecting matrix rigidity are poorly understood, crosslinking is generally viewed as an event that increases molecular rigidity, and TGs have been speculated to partake in this process (Geiger et al. 2001). It is therefore possible, and even likely, that TG2 can regulate osteoblast behavior and further promote cell adhesion by increasing the stiffness of the FN-containing bone matrix. Furthermore, TGs could play an important role in establishing cell adhesion by "locking" the FN matrix into place and thus serving as a checkpoint between cell detachment versus attachment and differentiation. In support of this view, Verderio et al. (2003) have shown that cells lacking TG2 are more susceptible to anoikis and that collagen type I crosslinked by TG2 promotes osteoblast differentiation (Chau et al. 2005).

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